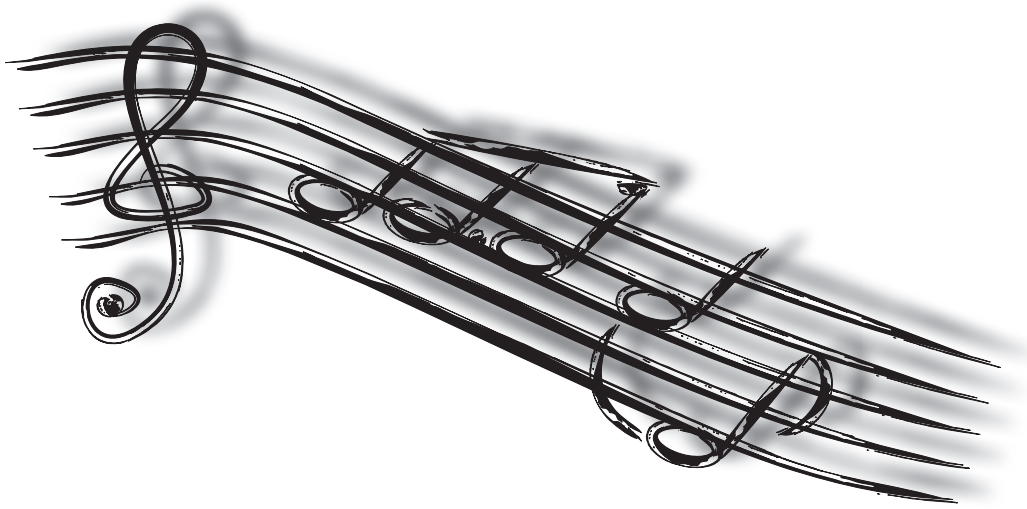
Neurotoxicity of past, present and future flame retardants

neurotoxic hazard characterization and risk assessment of (alternative) flame retardants



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Neurotoxicologie van vlamvertragers in het verleden, heden en de toekomst

karakterisering van neurotoxische effecten en
risicobeoordeling van (alternatieve) vlamvertragers

(met een samenvatting in het Nederlands)

Proefschrift

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Sola dosis facit venenum

"De dosis maakt het vergif", Paracelsus, 1493-1541

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Abbreviations

$[Ca^{2+}]_i$	intracellular calcium
6-OH-BDE-47	6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether
AB	alamar blue
ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
ADME	bsorption, distribution, metabolism, and excretion
Alpi	aluminium dietyhlphosphinate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP	ammonium polyphosphate
ATH	aluminium trihydroxide
ATO	antimony trioxide
B35 cells	B35 neuroblastoma cells
BCF	bioconcentration factor
BDE-47	2,2′,4,4′-tetrabromodiphenyl ether
BDE-209	decabromodiphenyl ether (decaBDE)
BDP	bisphenol-A bis(diphenylphosphate)
BFRs	brominated flame retardants
BPS	brominated polystyrene
CA1	*cornu Annon*
Ca^{2+}	calcium
CaMK-II	calcium/calmodulin-dependent protein kinase-II
cDNA	complementary DNA
Cl^-	chlorine
CNS	central nervous system
CYP	cytochrome P-450
Daphnia magna	water fleas
DCF	2′,7′-dichlorofluorescein
decaBDE	decabromodiphenyl ether (BDE-209)
DOPO	9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide
DT_{50}	half maximal dissipation time
EC_{50}	half maximal effective concentration
ECHA	European Chemicals Agency
EDTA	ethylenediamine tetraacetic acid
EPA	US Environmental Protection Agency
ER	endoplasmic reticulum
FCCP	4-(trifluoromethoxy)phenylhydrazone
fEPSP	field-excitatory postsynaptic potentials
FRs	flame retardants
GABA	γ-aminobutyric acid
GAP-43	growth associated protein-43
H	Henry's law constant
H_2-DCFDA	2′,7′-dichlororfluorescin diacetate
HBCD	hexabromocyclododecane
HFFRs	halogen-free flame retardants
HRICP-MS	High Resolution Inductively Coupled Plasma Mass Spectrometry
IC_{50}	half maximal inhibition concentration
ICP-MS	inductively coupled plasma mass spectrometry
IO	input-output
ISI	inter-stimulus intervals

K^+	potassium
LC_{50}	half maximal lethal concentration
LD_{50}	half maximal lethal dose
LOEC	lowest observed effect concentration
Log K_{AW}	air-water partition coefficient
Log K_{OW}	octanol-water partition coefficient
LTP	long-term potentiation
Mg^{2+}	magnesium
MHO	magnesium hydroxide
MMT	montmorillonite
MPP	melamine polyphosphate
MW	molecular weight
Na^+	sodium
nACh-R	nicotinic acetylcholine receptor
NMDA-R	*N*-methyl-D-aspartate receptor
NOEC	no observed effect concentration
NR	neutral red
OH	hydroxylate
oleum arachidis	peanut oil
PBDEs	polybrominated diphenyl ethers
PBT	persistence, bioaccumulation and toxicity
PC12 cells	pheochromocytoma cells
PCB-47	2,2′,4,4′-tetrachlorobiphenyl
PCBs	polychlorinated biphenyls
PER	pentaerythritol
PND	postnatal day
POPs	persistent organic pollutants
PPF	paired-pulse facilitation
PPR	paired-pulse ratio
PSD 95	postsynaptic density 95
PTP	post-tetanic potentiation
R	ratio
RDP	resorcinol bis(diphenylphosphate)
ROS	reactive oxygen species
SD	standard deviation
SEM	standard error of the mean
S_{max}	maximal water solubility
$t_{1/2}$	half-life
TBBPA	tetrabromobisphenol-A
TG	thapsigargin
TPP	triphenylphosphate
TR	treatment ratio
VGCCs	voltage-gated calcium channels
vPvB	very persistent and very bioaccumulative
Xenopus laevis	South-African clawed frog
ZB	zinc borate
ZHS	zinc hydroxystannate
ZS	zinc stannate

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Chapter 1

General introduction

1

1. History of flame retardants

Through the centuries, fire has been a major cause of property damage, injuries and death. In the Roman times, vinegar was used as a chemical flame retardant to prevent siege towers from catching fire. The first patent on a flame retardant was the British Patent 551 in 1735 by Obadiah Wilde. He developed a mixture of alum, ferrous sulfate and borax to reduce the flammability of canvas in theatres and public buildings. In 1820, the French chemist Joseph Louis Gay-Lussac composed a mixture of ammonium phosphate, ammonium chloride and borax which was used as flame retarding mixture on textiles of French theatres (BSEF, 2013; Charles Parsons, 2013).

Later on, the invention of polymers and modern materials stimulated modern technology to develop flame retardant chemicals to prevent or slow the onset and spread of fire. Polychlorinated biphenyls (PCBs; see Figure 1A) were commercially introduced in the 1920's. They were used as flame retardant, but also in numerous other applications, such as dielectric and coolant fluids in transformers, capacitors and electric motors. In 1966, the first detection of PCBs in the environment was reported in dead white-tailed sea eagles in Sweden (Jensen, 1966). Human adverse health effects of PCBs are generally a result of long-term low-level chronic exposure and include liver, thyroid, dermal and ocular changes, immunological alterations, neurodevelopmental changes, reduced birth weight, reproductive toxicity, and cancer (Agency for Toxic Substances and Disease Registry, 2013). As a result of the environmental presence and adverse health effects, most industrial countries prohibited the use of PCBs in the late 1970's. However, PCBs are still detectable in biota all over the world and regarded as a significant global environmental hazard.

The ban of PCBs resulted in the production and use of brominated flame retardants (BFRs). In many countries, including the United States (US) and United Kingdom (UK), legislation of high fire safety standards resulted in an increase of the use of flame retardant chemicals. In addition, due to the increasing use of flammable materials such as plastics and synthetic fibers, the use of chemical flame retardants increased as well. Typical applications to make products more fire-resistant are in polyurethane foam, plastics used in electric and electronic equipment, printed circuit boards, textile back-coating in furniture, various textiles, rubber for coating wire, etc. A major BFR is a commercial mixture of polybrominated diphenyl ethers (PBDEs; see Figure 1B) like pentabromodiphenyl ether (pentaBDE) and decabromodiphenyl ether (decaBDE or BDE-209). Other major

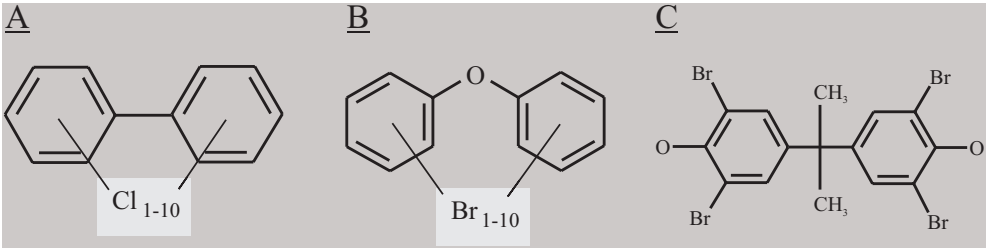

Figure 1. Schematic representation of the chemical structures and numbering system of PCBs (A) and PBDEs (B). Both PCBs and PBDEs are a group of synthetic organic compounds consisting of two benzene rings substituted with two to ten halogen atoms (chlorine for PCBs and bromine for PBDEs). Positions 2, 2', 6, and 6' are called *ortho* positions, positions 3, 3', 5, and 5' are called *meta* positions, and positions 4 and 4' are called *para* positions. The benzene rings can rotate around the bond between the positions 1 and 1' from which the two extreme configurations are coplanar (the two benzene rings in the same plane) and non-coplanar, where the benzene rings are at a 90° angle to each other. Chemical structure of TBBPA is also shown (C).

BFRs include tetrabromobisphenol-A (TBBPA; see Figure 1C), and hexabromocyclododecane (HBCD). The estimated annual worldwide market demands in 2010 were 7,500 tons for pentaBDE, >65,000 tons for BDE-209, >120,000 tons for TBBPA, and >17,000 tons for HBCD (de Wit *et al*., 2010). Most BFRs (i.e. PBDEs and HBCD) are additives that are mixed directly into the product during manufacture. Since these BFRs do not react with the material, they can easily leach out of the product and enter the environment. On the other hand, TBBPA is chemically bound to the product and was therefore not expected to leach into the environment in large amounts. However, several studies have shown that TBBPA can still leak into the environment from treated products (Sellstrom and Jansson, 1995) and is detectable in wildlife and human samples (Covaci *et al*., 2009; de Wit *et al*., 2010; Law *et al*., 2014). PBDEs and HBCD have also been found in human and environmental samples (Andersson and Blomkvist, 1981; Law *et al*., 2014; Zweidinger *et al*., 1966). In 2004, the use of pentaBDE was banned in the European Union (EU) and in eleven states of the US. Recently, it was announced that BDE-209 will be phased out on a voluntary basis by the producers in the US, and it is restricted in its use in Europe (Earnshaw *et al*., 2013).

2. Exposure and toxicity

2.1 Environmental contamination and human exposure

Widespread contamination of the environment by BFRs and their detection in wildlife and human tissues, raised concerns for the possible effects on wildlife and human health. BFRs are also globally spread via air and water, and can now be found in both abiotic and biotic samples from the polar regions (de Wit *et al*., 2010).

PBDEs are closely related to PCBs from a physicochemical point of view (see Figure 1). Both groups of chemicals are extremely persistent, non-combustible, thermostable, lipophilic and resistant to both biotic and abiotic degradation and are therefore called persistent organic pollutants (POPs). POPs are a group of organic carbon-based chemical compounds that bioaccumulate, biomagnify, persist in the environment, and demonstrate a capacity for long-range geographic transport (de Wit *et al*., 2010). The lipophilicity and persistence of PBDEs (which depends on the physicochemical properties like the molecular weight (MW) and octanol-water partition coefficient (Log K_{OW})) determine the degree of accumulation in fatty tissues. Bioaccumulation of these compounds in organisms can result in an increase of the internal concentration. Consequently, internal concentrations may increase at higher trophic levels, leading to high concentrations and adverse effects in top-predators. These processes are indicated as biomagnification and secondary poisoning. As a consequence, a major source for human exposure is the consumption of fatty fish from contaminated areas (Birnbaum and Staskal, 2004; Costa and Giordano, 2007; Sjödin *et al*., 2003). Besides exposure due to fish consumption, significant exposure to e.g. PBDEs is based on their presence in other major food groups, such as vegetables and animal products (Domingo, 2004; Domingo, 2012). Although TBBPA is not as persistent as PBDEs, it is lipophilic and may therefore also bioaccumulate in fatty tissues (Birnbaum and Staskal, 2004) though only low concentrations in seafood and other food items are observed (Colnot *et al*., 2013). Following the lower production volume of HBCD, it does not seem to be as persistent as PBDEs though due to its lipohphilicity, HBCD may bioaccumulate.

Due to the preferred accumulation of PBDEs in fatty tissues, human milk is an important source of exposure for breast-fed children (Abdallah and Harrad, 2011; Toms *et al*., 2012). Although the placenta can be an effective barrier against fetal exposure to toxicants circulating in maternal blood, studies have shown that PBDEs are able to transfer through the placenta easily, resulting in prenatal exposure (Jakobsson *et al*., 2012; Li *et*

al., 2013; Vizcaino *et al*., 2014). Moreover, multiple studies have detected the presence of BFRs in house dust in significant amounts. As a result, frequent hand to mouth exposure is the most significant route of exposure in toddlers and crawling infants (Abb *et al*., 2011; de Wit *et al*., 2012). The predominant route of exposure for TBBPA is supposed to be via direct exposure e.g. through particle inhalation or dust uptake. Due to this hand to mouth exposure, young children have a distinctly higher exposure to BFRs compared to adults (Abb *et al*., 2011; de Wit *et al*., 2012).

2.2 Environmental fate and toxicokinetics

Generally, BFRs have limited biodegradability, are persistent and tend to accumulate in the environment. However, in some environmental conditions, abiotic and biotic processes can change the environmental fate of BFRs. Abiotic processes are physicochemical processes that include photodegradation, decomposition at high temperature, chemical reactions with other compounds, and changes in the characteristics of the compound due to other environmental factors (temperature, pH, etc.). Photodegradation is a physical process that naturally occurs in the environment (Segev *et al*., 2009). BDE-209 for example, can be easily photochemically degraded resulting in the formation of less brominated PBDEs, which are more persistent and potentially more toxic (Eriksson *et al*., 2004; Rayne *et al*., 2003). TBBPA is also photolytically decomposed when exposed to UV light (de Wit, 2002). The environmental factor thermal stress (but also due to elevated temperatures in consumer products like hair dryers and television sets) can result in conversion of PBDEs and TBBPA into dioxin-like compounds (Janssen, 2005). Moreover, pH differences in the environment can change the solubility of a compound. TBBPA, for example, has a very low solubility at neutral pH, whereas at higher pH, the solubility of TBBPA increases, potentially resulting in a higher potential for e.g. contamination of groundwater (Hakk and Letcher, 2003).

The environmental fate of BFRs clearly depends on several physicochemical properties of these compounds and the abiotic and biotic processes occurring in the environment. However, the widespread and long term use of large quantities of BFRs in consumer and household products resulted in an environment contamination that will remain present for years (Earnshaw *et al*., 2013).

Toxicokinetics describe the disposition of a toxicant within an organism and are required to determine e.g. the potential for bioaccumulation. Toxicokinetics are frequently based on four criteria: absorption, distribution, metabolism, and excretion (ADME). Besides the mentioned bioaccumulation, another important biotic process that plays a key role in the toxicity of BFRs is biotransformation. Biotransformation processes are essential to metabolize toxicants in an organism into more hydrophilic compounds, which can easily be excreted via the urine or bile. The most well understood detoxification system is through the cytochrome P-450 (CYP) oxidase system. This Phase I metabolism results in hydroxylation (OH) of the toxicant to make it more hydrophilic. Hydroxylation by the CYP enzymes is followed by conjugation by Phase-II enzymes that convert the toxicant into more polar metabolites. OH-BDEs and MeO-BDE (MeO: methylation) metabolites are detected in human and wildlife samples (Hakk and Letcher, 2003; Sjödin *et al*., 2003) and as 'natural products' in marine algae, cyanobacteria and sponges (Agarwal *et al*., 2014; Malmvärn *et al*., 2008; Teuten *et al*., 2005). Moreover, human exposure studies have shown that the more polar OH-BDEs, which should be more easily excreted, are still present in serum at concentrations similar to or even higher than the parent compounds (Athanasiadou *et al*., 2008; Qiu *et al*., 2009).

In vivo in rats it was shown that TBBPA after oral administration undergoes first-pass metabolism in the gastro-intestinal tract and/or liver to form several conjugates that are subsequently eliminated from the liver with bile and excreted with feces (Colnot *et al*., 2013; Hakk and Letcher, 2003; Sjödin *et al*., 2003). The rapid excretion of polar conjugates (within 72 hours in oral exposed rats) supports the view that TBBPA is not bioaccumulative (Colnot *et al*., 2013). Comparable to TBBPA, the majority of e.g. BDE-209 cannot be absorbed from the intestines and will be excreted in the feces (Mörck and Klasson-Wehler, 2001). In rats, a small part of BDE-209 is metabolized to fecal metabolites via oxidative debromination, including debrominated mono-OH- and *ortho*-MeO-OH-BDEs (Hakk *et al*., 2002; Law *et al*., 2014; Mörck and Klasson-Wehler, 2001; Örn and Klasson-Wehler, 1998). Typically, biotransformation results in inactivation of the toxicant and subsequent elimination from the body. In some cases, biotransformation results in the formation of more biologically active metabolites compared to the parent compound (bioactivation), which is among others observed for PBDEs in relation to several endocrine and neurodevelopmental effects (Dingemans *et al*., 2011).

Depending on the rate of biotransformation and possible bioaccumulation, each chemical has a unique half-life ($t_{1/2}$) in an organism. For example, the $t_{1/2}$ of TBBPA in blood serum of occupationally exposed Swedish workers was estimated to be only 2.2 days, which is relatively short (Hagmar *et al*., 2000). Since BDE-209 is believed to be debrominated to lower brominated congeners, the $t_{1/2}$ of PBDEs in humans is still unclear, though the mean $t_{1/2}$ of BDE-47 and its metabolites was estimated on 1795 days (4.9 years; Geyer *et al*., 2004), which is substantial longer compared to TBBPA.

2.3 Toxicity

As exposure to BFRs appears to be highest in (young) children, a major current concern relates to the possible developmental effects. Epidemiological studies indeed describe human health effects associated with PCB and PBDE exposure. For example, prenatal PCB and PBDE exposure is associated with decreased total and free thyroxine levels (T4; a thyroid hormone that is responsible for regulation of metabolism) in infants born by spontaneous delivery (Herbstman *et al*., 2008). In adolescents, BFR exposure is also associated with changes in the serum levels of thyroid stimulating hormones (TSH) (Johnson *et al*., 2013; Kicinski *et al*., 2012). Another human study among subjects exposed to e-waste in the workplace also showed an increase of serum levels PBDEs and TSH (Yuan *et al*., 2008).

In vivo animal studies confirm the adverse effects on thyroid hormones (TH). Reduction in serum T_4 levels induced by TBBPA and/or PBDEs (either as parent compound or hydroxylated metabolite) can displace T_4 from serum binding proteins as transthyretin (TTR) resulting in free T_4 which will be eliminated (Hallgren *et al*., 2001; Hamers *et al*., 2006; Kodavanti *et al*., 2010; Meerts *et al*., 2002; Richardson *et al*., 2008). As a result, the hypothalamic-pituitary axis triggers the thyroid to synthesize and secrete more T_4 (Lema *et al*., 2008). PBDEs bound to TTR can now easily reach target organs including the liver and brain to elicit toxic effects. In the liver for example, PBDEs activate receptors that initiate transcription of metabolizing enzymes for T_4 elimination, which subsequently results in a further decrease of serum T_4 levels (Lema *et al*., 2008; Szabo *et al*., 2009). Consequently, as the developing brain is dependent on TH for normal development, abnormal TH levels will result in an impaired brain growth and differentiation, leading to e.g. mental retardation (Darnerud, 2008). TH disruption by BFRs (and other toxicants) thus involves multiple mechanisms.

Other studies observed strong inverse associations between PBDE concentrations in (house) dust and serum luteinizing and follicle stimulating hormone (Johnson *et al*., 2013; Meeker *et al*., 2009). In addition, PBDEs are associated with longer time to pregnancy (Harley *et al*., 2010). These adverse effects on male and female reproductive development and function is shown in several *in vivo* as well as *in vitro* studies. For example, a decrease in spermatogenesis, abnormally low concentrations T_4 in the blood, and delayed puberty in PBDE exposed male rats (Kuriyama *et al*., 2005; Stoker *et al*., 2005; Stoker *et al*., 2004). Other studies showed interactions of PBDEs or their hydroxylated metabolites on nuclear hormone receptors; e.g. antagonistic effects on the androgen receptor (AR), resulting in a disrupted androgen homeostasis and altered function of male reproductive organs (Hamers *et al*., 2006; Kojima *et al*., 2009; Stoker *et al*., 2005; van der Ven *et al*., 2008). The adverse effects of OH-BDEs may also outcompete T_4 for serum binding proteins (Szabo *et al*., 2009) resulting in endocrine disruption comparable with the effects caused by the parent compounds (Cantón *et al*., 2008; Hamers *et al*., 2006; Kojima *et al*., 2009).

The immune system is also affected by some BFRs. In adolescents, PBDEs were negatively associated with the number of lymphocytes (a type of white blood cell, leukocyte, that is of fundamental importance in the immune system) (Leijs *et al*., 2009). Rats exposed to large doses of BDE-209 showed inhibition of immune function, altered structure of immune organs, and suppression of lymphocyte proliferation and antibody productions (Liu *et al*., 2012). In rats perinatal exposed to the commercial DE-71 mixture (a mixture containing mainly tetra- and penta-brominated BDE congeners) altered splenic lymphocyte populations were observed, which has been linked to hypothyroidism (Bondy *et al*., 2013). The same mixture suppressed in mice the ability to mount an immune response (Fowles *et al*., 1994), and another PBDE mixture (Bromkal 70-DE) resulted in immunotoxicity in mice (Thuvander and Darnerud, 1999). Comparable, in mice PBDEs were transferred from the dam to the offspring during gestation and lactation, which affected their immune system (Hong *et al*., 2010).

3. Neurotoxicity

Several human epidemiological studies indicate the adverse effects of PCBs, which include correlations between prenatal exposure and impaired cognitive and motor functioning in children (Berghuis *et al*., 2013; Fonnum and Mariussen, 2009; Lonky *et al*., 1996; Tilson and Kodavanti, 1997; Winneke *et al*., 2013). In adults, consumption of PCB-contaminated fish is associated with impairments in certain aspects of memory and learning (Schantz and Widholm, 2001). Other studies indicate the vulnerability of the developing nervous system for adverse effects of a wide range of environmental contaminants, including PBDEs (Jurewicz *et al*., 2013). For example, PBDE concentrations in breast milk measured at three months of age is at 36 months associated with more anxious behavior, increased withdrawal and improved activity of daily living skills (Adgent *et al*., 2014). Other studies indicate that PBDE exposure of (school)children is associated with reduced psychomotor development index and full scale IQ performance (Chen *et al*., 2014; Herbstman *et al*., 2010; Roze *et al*., 2009). In adolescents, PBDE exposure is also associated with changes in motor function (Kicinski *et al*., 2012). These similarities may very well indicate that the brain is a highly vulnerable target for the neurotoxic actions of PCBs and BFRs.

3.1 Behavioral effects

During human perinatal development, the brain grows rapidly. This is referred to as the brain growth spurt, which starts during the third trimester of pregnancy and continues throughout the first two years of life. The brain growth spurt is associated with several

biochemical changes that transform the feto-neonatal brain into a mature adult brain by outgrowth of nerve fibers like axons and dendrites, and establishment of neural connections (Dobbing and Sands, 1979). In rodents (i.e. mouse and rat), this period occurs neonatally during the first three to four weeks after birth. During this period, animals also acquire many new motor and sensory abilities (Bolles and Woods, 1964) and their spontaneous motor behavior peaks (Campbell *et al*., 1969). In mammals, the lactation period coincides with the brain growth spurt. As mentioned, (human) milk is the main elimination route of highly lipophilic chemicals like PCBs and PBDEs, resulting in neonatal exposure during a critical period of brain development.

In vivo studies show that mice exposed on postnatal day (PND) 10 to PBDEs develop permanent aberrations in spontaneous behavior and habituation capability (Eriksson *et al*., 2001; Eriksson *et al*., 2002; Viberg *et al*., 2003a; Viberg *et al*., 2003b; Viberg *et al*., 2004), comparable with effects observed following PCB exposure during this critical period (Eriksson and Fredriksson, 1996). Exposure of mice to TBBPA on PND 10, however, did not cause any significant change in studied behavioral parameters (Eriksson *et al*., 2001). In contrast, behavioral effects in mice following acute TBBPA exposure were reported (Nakajima *et al*., 2009). Clearly, the discrepancy in (neurotoxic) effects of the different compounds argues for more research. To investigate and fully understand the mechanism of action on the brain and central nervous system (CNS) of PCBs, PBDEs and other BFRs like TBBPA, knowledge of neuronal function is essential.

3.2 Neuronal function

Neuronal communication

At the most basic level, the nervous system is responsible for sending, receiving, and interpreting information from all parts of the body. The brain is the center of the nervous system and has millions of neurons (nerve cells) that act as the functional units. Neurons have two types of extensions on their cell body: numerous dendrites and a single axon, which are all able to conduct and transmit signals by electrochemical impulses or action potentials (see Figure 2). Dendrites are highly branched extensions that receive signals,

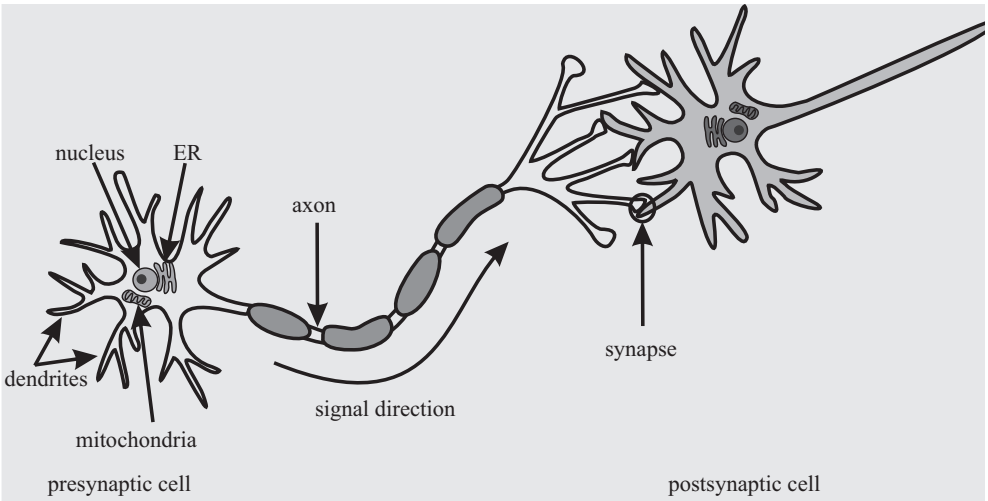

Figure 2. Image illustrating neuronal signaling: communication between neurons is via an action potential to the axon of the presynaptic cell, which makes synaptic contact with the postsynaptic cell at the synapse. Intracellular organelles nucleus, ER (endoplasmic reticulum) and mitochondria in the cell body are shown.

while an axon transmits signals to the synapse. Near its end and close to another cell, axons divide into several branches and ends in synaptic terminals. From this synaptic terminal, the presynaptic (sending) cell sends a signal to the postsynaptic (receiving) cell (see Figure 2).

In short, depolarizing pulses (action potentials) travel along the membrane of the presynaptic axon towards the synapse. The resulting depolarization of the presynaptic cell results in activation of voltage-gated calcium channels (VGCCs), and subsequent influx of Ca^{2+} through the cell membrane of the synaptic terminal. As a result, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases strongly and this activates a set of Ca^{2+}-sensitive proteins on the membrane of synaptic vesicles thereby triggering fusion of these vesicles with the cell membrane (see Figure 3). Synaptic vesicles contain neurotransmitter molecules: endogenous chemicals that transmit signals across the synapse. Due to fusion of the synaptic vesicles with the cell membrane at the synapse, the synaptic vesicles open and neurotransmitters are released into the synaptic cleft (exocytosis). The neurotransmitters diffuse through the synaptic cleft and bind to neurotransmitter receptors on the postsynaptic neuron to activate them. Postsynaptic receptor activation results in hyper- or depolarization (depending on the type of receptor) of the postsynaptic cell. In the case of activation of transmembrane G-protein-linked receptors, intracellular signal transduction pathways will be activated resulting in cellular responses.

Neurotransmitter systems

Neurotransmitters activate their corresponding neurotransmitter-receptors on the postsynaptic cell, which can result in excitation or inhibition of neuronal activity. Several neurotransmitter systems are critically involved in a variety of processes, including development and differentiation of the nervous system and synaptic plasticity (Collingridge *et al*., 1983; Dwyer *et al*., 2009; D'Hulst *et al*., 2009). Long-term potentiation (LTP) is a form of synaptic plasticity that is suggested to be the cellular mechanism underlying learning and memory, and occurs in several parts of the brain, including the hippocampus. LTP depends on the ability to change the strength of the synapses between two neurons, resulting in the increase of the rate, or frequency of synaptic transmission. To perform tasks, attention and rapid processing of information is required. This processing, work memory and new/reversal learning involves at least three major neurotransmitter systems of the CNS: the cholinergic, GABAergic and glutamatergic system.

The cholinergic system is a major neurotransmitter system that undergoes rapid development during e.g. the brain growth spurt. The neurotransmitter of the cholinergic system is acetylcholine (ACh) that binds to the nicotinic acetylcholine (nACh) receptor, an abundant excitatory receptor in the central and peripheral nervous system. Activation of the nACh receptor by ACh leads to an influx of cations, resulting in depolarization of the cell membrane and excitation of neuronal activity. The nACh receptor is involved in many behavioral and cognitive functions, including synaptic plasticity (Bartus *et al*., 1982; Karczmar, 1975).

γ-Aminobutyric acid (GABA) is the neurotransmitter of the GABAergic system. GABA receptors are divided into two main classes: $GABA_A$ receptors, which are members of the ligand-gated ion channel superfamily (ionotropic), and $GABA_B$ receptors, which are members of the G-protein-linked receptor superfamily (metabotropic). Binding of GABA on the binding sites of a $GABA_A$ receptor, results in opening of the receptor channel and influx of chlorine (Cl^-). Opposite to the above mentioned nACh receptors, activation of $GABA_A$ receptors by GABA in the mature brain causes a hyperpolarization of the cell membrane, which decreases the probability of firing (inhibitory receptors). However, in the developing brain the actions of $GABA_A$ receptors are primarily excitato-

ry. Immature neurons have a reversed Cl^- gradient that is higher than the resting membrane potential of the cell. Consequently, activation of $GABA_A$ receptors leads to efflux of Cl^- from the cell, thereby depolarizing the cell membrane, resulting in neuronal excitation (Kilb, 2011). $GABA_A$ receptors are the main inhibitory receptors in the mature CNS and play also an important role in early brain development and LTP (D'Hulst *et al*., 2009).

Glutamate is the most abundant excitatory neurotransmitter in the CNS. The receptors of the glutamatergic system can, similar to the GABA receptors, be divided into two groups: ionotropic glutamate receptors and metabotropic glutamate receptors. The ionotropic receptors are comparable to the ion channels of nACh- and $GABA_A$ receptors and can be divided in NMDA (*N*-methyl-D-aspartate), kainate and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors. At resting membrane potential, the NMDA cation channel is blocked by magnesium (Mg^{2+}). The ion channel will only open when the postsynaptic cell is depolarized, which results in removing Mg^{2+} that blocks the channel. NMDA receptors mediate a major part of the postsynaptic Ca^{2+} influx, though it are nonspecific cation channels and also permeable to sodium (Na^+) and potassium (K^+). The excitation of neuronal activity by NMDA receptors plays also important roles in LTP and synaptic plasticity (Collingridge *et al*., 1983; McEntee and Crook, 1993).

Thus, for proper neuronal functioning and related intercellular communication, intact and healthy cells with a normal cell metabolism, Ca^{2+} homeostasis, and neurotransmitter release are essential. However, several studies indicate the involvement of neurotransmitter systems in neurotoxicity. For instance, neonatal exposure of mice to PCBs, PBDEs and/or TBBPA is associated with disturbances in the cholinergic system as indicated by several behavioral experiments (Eriksson *et al*., 2001; Eriksson *et al*., 2006; Johansson *et al*., 2008; Viberg and Eriksson, 2011). Exposure to BDE-99 or TBBPA resulted in a decrease in binding sites of the nicotinic ligand cytisine in mice (Viberg and Eriksson, 2011),

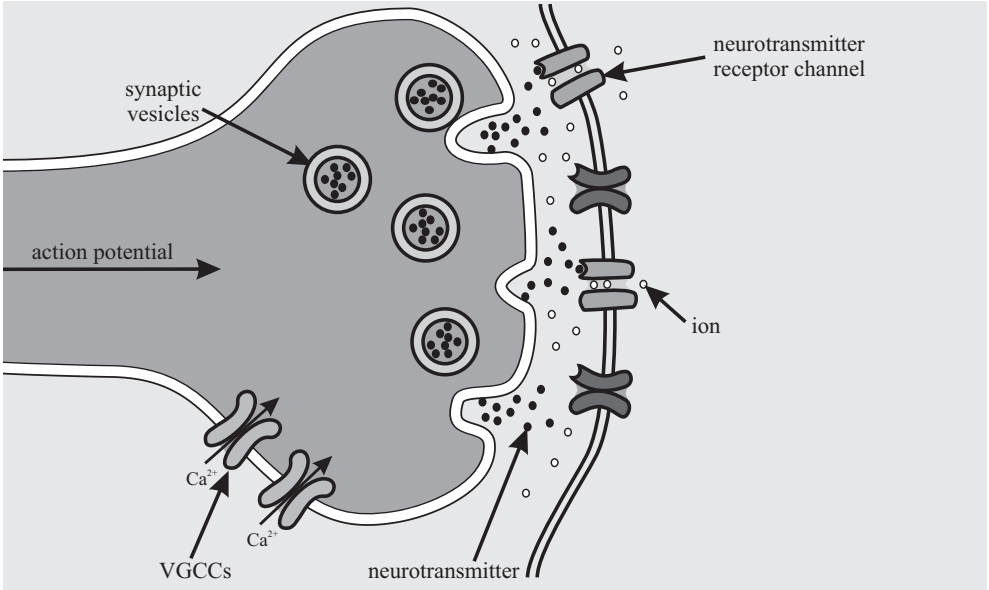

Figure 3. Simplified representation of neurotransmission. An action potential from the presynaptic cell (left) opens voltage-gated calcium-channels (VGCCs), allowing Ca^{2+} to enter the cell. The increase in $[Ca^{2+}]_i$ is a trigger for the release of neurotransmitters via exocytosis of the synaptic vesicles into the synaptic cleft. The released neurotransmitters bind to specific receptors of the postsynaptic cell (right) and allow an influx of anions or cations, depending on the receptor type.

whereas BDE-71 exposure affected protein levels of the GABAergic and glutamatergic neurotransmitter systems in mice frontal cortex (Bradner *et al.*, 2013). Furthermore, using *ex vivo* hippocampal slices, it was shown that mice neonatally exposed to BDE-47 have reduced LTP (Dingemans *et al*., 2007), probably in part as a result of a reduction in glutamate receptor subunits. BDE-209 also reduced LTP in rats exposed via the mother during different developmental periods (pregnancy, lactation via mother's milk, lactation via intragastric administration, after weaning, and prenatal to life; Xing *et al*., 2009).

Unlike experimental animals, humans and wildlife are generally not exposed to a single environmental pollutant, but to complex mixtures of potential toxic substances such as PCBs, BFRs, pesticides, and their (toxic) metabolites. Interactions between compounds is likely, and may result in synergism, additivity, antagonism, competition, etc., and depends also on their single or shared mode of actions. For instance, *in vivo* exposure of mice to a mixture of a PCB and PBDE resulted in an interaction between the compounds and enhanced the developmental neurotoxicity (Eriksson *et al*., 2006), for review see (Fonnum and Mariussen, 2009).

3.3 Cellular and molecular mechanisms

Neuronal cell viability and ROS production

To further unravel the mechanisms underlying the observed developmental and behavioral effects, *in vitro* studies using neuronal cells have been performed (for review see e.g. Costa *et al*., 2013). Cell viability assays are often used to screen chemicals for their possible acute effect on cell death (cytotoxicity). Several studies reported the cytotoxic effects of PCBs or BFRs, due to activation of apoptosis (programmed cell death) (Dingemans *et al*., 2011; Fonnum and Mariussen, 2009). This onset of apoptosis can be triggered by many different stimuli, including oxidative stress or an increase in $[Ca^{2+}]_i$.

Oxidative stress is the result of an excessive formation of reactive oxygen species (ROS). These ROS particles are formed in the mitochondria (the energy suppliers of the cell that also function as a Ca^{2+} store) as a natural byproduct of normal cell metabolism. ROS can usually be effectively removed by anti-oxidants such as glutathione. However, the toxicant-induced increase in ROS formation and/or the disturbance of scavenging function of anti-oxidants can result in oxidative stress. This may eventually lead to damage of mitochondrial DNA and other cellular macromolecules that consequently results in e.g. lipid peroxidation of lipids in the plasma membrane (Orrenius *et al*., 2011). *In vitro* studies indeed indicate that PCBs as well as BFRs are able to increase the formation of ROS and it may be (partly) due to disruption of the mitochondrial Ca^{2+} homeostasis (Costa and Giordano, 2007; Fonnum and Mariussen, 2009).

Calcium homeostasis

Ca^{2+} plays an essential role in many cellular functions, including those that are correlated with several stress responses in the cell. The earlier mentioned increase of $[Ca^{2+}]_i$ that triggers the onset of cell death can arise from several sources, including damage to the cell membrane, disturbed ion channels, and Ca^{2+} release from the endoplasmic reticulum (ER; produces proteins and also stores Ca^{2+}). In general, an increase in $[Ca^{2+}]_i$ can partly temporary be stored in the mitochondria. However, Ca^{2+} uptake into the mitochondria together with a strong increase of $[Ca^{2+}]_i$ in the cytoplasm results in cytochrome *c* release from the mitochondria. Cytochrome *c* is a protein that already in small amounts activates the inositol-1,4,5-trisphosphate (IP_3) receptors in the ER followed by a subsequent Ca^{2+} release. The released calcium triggers a massive release of cytochrome *c* from all mitochondria, followed by a positive feedback loop to maintain the ER Ca^{2+} release through IP_3 receptors. The release of cytochrome *c* in turn binds apoptotic protease activating

factor-1 (APAF-1) that recruits and activates caspase. The caspase (cysteine-dependent aspartate-specific proteases) family initiates a proteolytic cascade that ultimately results in cell death (Boehning *et al*., 2003; Orrenius *et al*., 2011). Several studies have described which parts in the apoptosis pathway of neuronal cells are activated by PCBs and some BFRs. For instance, PCBs and BFRs are able to activate the IP_3 receptor directly (Fonnum and Mariussen, 2009). Other studies report that PCBs and BFRs activate the ryanodine receptor (RyR), a Ca^{2+}-mediated Ca^{2+}-channel, localized in the ER (for review see Fonnum and Mariussen, 2009). Activation of the RyR causes Ca^{2+} release from the ER, which in turn increases the production of ROS. It is also reported that PCBs and BFRs induce caspase activity and consequently apoptosis (Howard *et al*., 2003; Yu *et al*., 2008a).

Obviously, Ca^{2+} is the intracellular messenger that mediates physiological responses to chemical and electrical stimulation in neuronal cells. For neurotransmission, an increase in $[Ca^{2+}]_i$ will trigger the protein synaptotagmin, located in the membrane of synaptic vesicles. The activation results in fusion of the synaptic vesicle with the presynaptic cell membrane and ultimately release of the neurotransmitters into the synaptic cleft (see Figure 3 for a simplified illustration). This process is regulated by a set of proteins of the SNARE-complex (Soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) Attachment protein Receptor). After diffusion of the released neurotransmitters through the synaptic cleft, they bind a receptor on the postsynaptic cell to continue the neuronal signaling (Garcia *et al*., 2006; Westerink, 2006).

In the postsynaptic cell, again Ca^{2+} plays an important intracellular role. For example, Ca^{2+} influx into the postsynaptic cell results from receptor activation at excitatory synapses and appears to be crucial for the induction of LTP. Ca^{2+} activates the second messenger system protein kinase C (PKC), a peripheral membrane protein, which serves as cellular receptor. Once activated, PKC phosphorylates specific substrates, resulting in several cellular responses like Ca^{2+} release from the ER (Mochly-Rosen *et al*., 2012). Both PKC and Ca^{2+} play a significant role in the modulation of mitogen-activated protein kinase (MAPK) pathways that also participates in various signaling pathways (Pearson *et al*., 2001). $[Ca^{2+}]_i$ is also involved in the activation of phospholipase A (PLA), an enzyme that cleaves arachidonic acid (AA) present in the phospholipids of the cell membrane. AA can subsequently be oxidized and modified into eicosanoids ('messenger molecules') or diffuse into the nucleus and interact with transcription factors to control DNA transcription (Striggow and Ehrlich, 1997).

Ca^{2+} thus plays an important role in a wide range of physiological and pathological processes, including cell death, gene expression, neuronal intra- and intercellular signaling pathways, and neurotransmitter release. Several *in vitro* neurotoxicity studies are therefore focused on the disturbance of this neuronal signaling. Indeed, reports describe that Ca^{2+} homeostasis in neuronal cells can be disturbed in several ways following acute PCB or BFR exposure and can be attributed to release from both extracellular and intracellular stores. For example, PCBs (Langeveld *et al*., 2012), PBDEs (Dingemans *et al*., 2008; Dingemans *et al*., 2010), and HBCD (Dingemans *et al*., 2009) inhibit VGCCs, which results in a reduced depolarization of the cell and consequently decreased Ca^{2+} influx into the cell, followed by e.g. a reduced neurotransmitter secretion. Increases in $[Ca^{2+}]_i$ following PCB or BFR exposure is frequently caused by release of Ca^{2+} from intracellular stores (e.g. from the ER and mitochondria) (Costa and Giordano, 2007; Dingemans *et al*., 2011; Fonnum and Mariussen, 2009; Langeveld *et al*., 2012). Other studies have shown that both PCBs and PBDEs are able to induce translocation of PKC, stimulate MAPKs and activate PLA resulting in AA release (Fan *et al*., 2010; Kodavanti and Tilson, 2000; Kodavanti and Derr-Yellin, 2002; Kodavanti and Ward, 2005; Kodavanti *et al*., 2010; Madia *et al*., 2004).

Neurotransmitter receptors
Despite the clear involvement of neurotransmitter systems in the toxic effects of PCBs and BFRs, only a few studies so far described the direct effects on (post)synaptic receptors. *In vitro*, PCBs were shown to act as full and partial agonist on postsynaptic human $GABA_A$ receptors (Antunes Fernandes *et al*., 2010a; Antunes Fernandes *et al*., 2010b). Thus, interruption of (neuronal) receptors and channels also affect neuronal cellular communications, resulting in neuronal damage and subsequent neurotoxicity. Considering the fact that PCBs and PBDEs frequently seem to have a common mode of action, it can be proposed that PBDEs may also interact with the $GABA_A$ receptor. Moreover, both PCBs and PBDEs may also affect other neurotransmitter receptors like the nACh receptor.

In summary, the above described processes in neuronal signaling indicate that for proper neuronal communication healthy and intact cells are essential, just as normal cell metabolism, Ca^{2+} homeostasis, and properly functioning neurotransmitter receptors. However, some BFRs (and PCBs) have been shown to affect these (cellular) processes, resulting in several neurotoxic effects.

4. Thesis outline

The use of flame retardant chemicals undoubtedly reduces deaths and injuries. However, it should be recognized that there are significant disadvantages that concern the human health. Thus, without doubt there is an actual need to replace many BFRs by less toxic and persistent alternatives. The EU funded project ENFIRO (A Life Cycle Assessment of Environment-Compatible Flame Retardants: Prototypical Case Study; 2009 - 2012) is a prototypical case study on the substitution options for specific BFRs. Within this project, three BFRs and 13 alternative halogen-free flame retardants (HFFRs) were investigated (see Table 1). As part of the ENFIRO project, the principal aim of this PhD study was to assess the neurotoxic potential of these HFFRs in relation to the (neuro) toxicity of selected BFRs. The (neuro)toxic endpoints used in this PhD study, as well as applied experimental methodologies, are discussed in **Chapter 2**.

Following the discovery of adverse effects on human health and in wildlife of chemicals like polychlorinated biphenyls (PCBs), a first regulation in the EU was introduced in 1976 resulting in restricted marketing or use of selected toxic substances. In 2001 a new and more extensive regulation was set up named REACH (Registration, Evaluation, Authorization, and Restriction of Chemical substances). REACH addresses the production and use of chemicals and their potential impacts on both human health and the environment. Despite regulations like REACH, some of the selected HFFRs used in our studies have already been marketed without having a full profile of their environmental behavior and toxicological properties. Therefore, the first aim of this PhD study was to make an inventory of the available data on the physical-chemical properties, production volumes, persistence, bioaccumulation, and toxicity of the selected HFFRs. In **Chapter 3** the available data of the selected flame retardants is reviewed.

Although the neurotoxicity of BFRs has been described, most *in vitro* studies so far focused on cytotoxicity or presynaptic effects on neurotransmission. Recently, it has been shown that several PCBs - which have a comparable chemical structure to polybrominated diphenyl ethers (PBDEs, see Figure 1) and elicit comparable neurotoxic effects - act as agonist on the postsynaptic human $GABA_A$ receptor. The effects of PBDEs and their (possible more toxic) metabolites on $GABA_A$ receptor functioning is, however, unknown.

Table 1. Different classes of flame retardants investigated in this research

Flame retardant *(product name)*	Abbreviation	Molecular formula	Chemical purity	CAS number	Company
Polychlorinated biphenyl					
2,2′,4,4′-tetrachlorobiphenyl	PCB-47	$C_{12}H_6C_{l4}$	>99.9%	2437-79-8	Neosync Inc.
Brominated flame retardants					
2,2′,4,4′-tetrabromodiphenyl ether	BDE-47	$C_{12}H_6Br_4O$	~99%	5436-43-1	Stockholm University
6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether	6-OH-BDE-47	$C_{12}H_6Br_4O_2$	~99%	79755-43-4	Stockholm University
brominated polystyrene *(UNFR-07)*	BPS	$(C_8H_5Br_3)_n$	>99%	88497-56-7	MPI Chemicals
decabromodiphenyl ether	BDE-209	$C_{12}Br_{10}O$	98%	1163-19-5	Sigma-Aldrich
tetrabromobiphenol-A	TBBPA	$C_{15}H_{12}Br_4O_2$	>99%	79-94-7	Sigma-Aldrich
Phosphorous flame retardants					
bisphenol A bis (diphenylphosphate) *(fyrolflex BDP)*	BDP	$C_{39}H_{34}O_8P_2$	>96%	5945-33-5	ICL
resorcinol bis (diphenylphosphate) *(fyrolflex RDP)*	RDP	$C_{30}H_{24}O_8P_2$	>95%	57583-54-7	ICL
triphenylphosphate	TPP	$C_{18}H_{15}O_4P$	>99%	115-86-6	Sigma-Aldrich
9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide	DOPO	$C_{12}H_9O_2P$	>99%	35948-25-5	Krems Chemie Chemical Services AG
aluminium diethylphosphinate *(Exolit OP 1230)*	Alpi	$[C_4H_{10}P0_2]_3Al$	>99%	225789-38-8	Clariant
Inorganic halogen-free flame retardants and synergists					
aluminium trihydroxide	ATH	$Al(OH)_3$	>99%	21645-51-2	Merck
ammonium polyphosphate *(Exolit AP 422)*	APP	$(NH_4PO_3)_n$	>99%	68333-79-9	Clariant
antimony trioxide *(Fireshield H)*	ATO	Sb_2O_3	>99%	1309-64-4	Chemtura
magnesium hydroxide	MHO	H_2MgO_2	>99%	1309-42-8	Sigma-Aldrich
zinc hydroxystannate *(Flamtard H)*	ZHS	$ZnSn(OH)_6$	94%	12027-96-2	William Blythe
zinc stannate *(Flamtard S)*	ZS	O_3SnZn	>98%	12036-37-2	William Blythe
Nanoclay					
cloisite 30B (nanoclay, montmorrillonite)	MMT	$C_{23}H_{50}O_2N$	n/a		NRC Nordmann Rassmann
Nitrogen-based organic flame retardant					
melamine polyphosphate *(Melapur 200)*	MPP	$C_3H_6N_6{\cdot}(H_3PO_4)_n$	>95%	218768-84-4	BTC

n/a, not applicable

Chapter 4 describes the first experimental research of this PhD study focusing on PCB-47, BDE-47, and its metabolite 6-OH-BDE-47. The different postsynaptic effects of these compounds were investigated on the human $GABA_A$ receptor using the two-electrode voltage-clamp technique (see Chapter 2). Since human $GABA_A$ receptors and $\alpha_4\beta_2$ nACh receptors are both critically involved in brain development as well as long-term potentiation (LTP), direct effects of the three mentioned compounds were also investigated on $\alpha_4\beta_2$ nACh receptor functioning. Finally, mixture studies with the three compounds were performed as humans are generally exposed to complex mixtures of pollutants and additive effects of different toxicants have been observed previously.

PBDEs are clearly associated with neurotoxicity, while information on the neurotoxicity and corresponding modes of action of tetrabromobisphenol-A (TBBPA) is limited. Therefore, following the observed effects described in Chapter 4, the effects of TBBPA on human $GABA_A$ and $\alpha_4\beta_2$ nACh receptors were investigated as shown in **Chapter 5**. To gain more insight in possible adverse effects on functional neurotransmission endpoints, dopaminergic pheochromocytoma (PC12) and neuroblastoma (B35) cells were used to investigate acute effects of TBBPA on Ca^{2+} homeostasis and ACh- and depolarization-evoked Ca^{2+} homeostasis using single-cell fluorescent Ca^{2+} imaging. To exclude that results would be confounded by acute TBBPA-induced cytotoxicity, effects on the cell viability of the neuronal cell lines were also determined using a combined Alamar Blue and Neutral Red assay. In addition, effects on ROS production using the fluorescent dye H_2-DCFDA, and TBBPA induced effects on apoptosis-related caspase activation were assessed. The used techniques are explained in Chapter 2.

As mentioned and described in Chapter 3, there is a general lack of data regarding the toxicity of the selected HFFRs. Since previous research identified the nervous system as a sensitive target organ for BFRs, it is essential to assess the (neuro)toxic potential of the HFFRs before large scale use. In Chapter 4 and 5 it is shown that one of the modes of action of BFRs is modulation of the $\alpha_4\beta_2$ nACh receptor. **Chapter 6** therefore describes effects of the HFFRs and three BFRs (BPS, BDE-209 and TBBPA) on $\alpha_4\beta_2$ nACh receptor functioning, measured using the two-electrode voltage-clamp technique. Based on these results, an initial rank-order potency was made, which serves as a first step in prioritization of viable HFFRs from a neurotoxic point of view.

In order to obtain a broader overview of the suitability of the HFFRs with respect to neurotoxicity, it is essential to collect data on a number of critical established toxicological and biological endpoints. Therefore, **Chapter 7** describes the effects of the selected HFFRs and BFRs on different *in vitro* neurotoxic endpoints. First, effects on cell viability and ROS production were determined using PC12 and B35 cells. These experiments were followed by investigating effects on changes in Ca^{2+}-homeostasis and depolarization-evoked Ca^{2+}-homeostasis by single-cell fluorescent Ca^{2+} imaging. Together with the data presented in Chapter 6, again a rank ordering from a neurotoxic point of view was made to prioritize the suitability of HFFRs to replace BFRs.

Since the *in vitro* studies described in this PhD thesis lack metabolism and the sophisticated interactions in the brain between different cell types, extrapolation from the obtained *in vitro* data to the *in vivo* situation can be difficult. Therefore, *ex vivo* validation experiments with three selected compounds were performed (**Chapter 8**). Mice were neonatal exposed (single oral exposure on postnatal day (PND) 10) to TBBPA, aluminium diethylphosphinate (Alpi) or zinc stannate (ZS), and potential neurodevelopmental

effects were investigated by examining effects on NMDA-dependent LTP (see Chapter 2). Since several specific neurotransmitter receptors and protein kinases are involved in LTP induction and maintenance, levels of critical synaptic proteins were measured. Moreover, to gain insight in the internal dose, bioavailability and distribution, levels of the compounds were measured in several tissues.

The research presented in this PhD thesis describes the neurotoxic potential of flame retardants that ranges from the past (PCBs) via the present (BFRs) to the future (HFFRs). In **Chapter 9**, the observed effects as presented in Chapter 3-8 are discussed and integrated with other *in vitro* and *in vivo* studies. First, the question "what have we learned from the PCBs and BFRs?" is discussed from a neurotoxicological point of view. Next, a risk assessment is made to compare the neurotoxicity of the 'old' flame retardants and halogen-free 'new' flame retardants by combining *in vitro* and *in vivo* data with available plasma concentrations, house dust concentrations, physical chemical properties, etc. Finally, recommendations for future research are described and the conclusive statements of this PhD study are summarized.

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Chapter 2

Toxicological endpoints and applied methodologies

2

1. Flame retardants

In this PhD study, neurotoxic effects of the 'old' flame retardant PCB-47, the 'present' flame retardants BDE-47, its metabolite 6-OH-BDE-47, BPS, BDE-209 and TBBPA, and the 'future' flame retarants as listed in Table 1 of Chapter 1 are investigated. Commercially available PCBs and PBDEs often contain impurities such as dioxin-like-PCBs, polybrominated dibenzofurans, or dibenzo-*p*-dioxins (Hanari *et al.*, 2006). These impurities were removed from the PCB, PBDE and metabolite used in this study by elution through a charcoal column (Danielsson *et al.*, 2008; Örn *et al.*, 1996). Purity and composition of the investigated compounds is often not reported, except for BDP and RDP, which may contain up to 5% TPP as impurity (Clean Production Action, 2007; Umwelt Bundes Amt, 2001). The flame retardants used in the studies were therefore purchased at the highest available purity from different companies (see Table 1, Chapter 1).

For *in vitro* experiments, the PCB, BFRs and phosphorous HFFRs were dissolved in purity-checked dimethyl sulfoxide (DMSO) and stock-solutions of 10 or 100 mM were further diluted to obtain final concentrations of 0.01 to 100 μM. The final concentration of DMSO in flame retardant-containing saline was always kept below 0.1% (v/v). The other HFFRs are poorly soluble in DMSO or other solvents and therefore, an excess of the compound was stirred for seven days in saline solution or culture medium. After filtration (Nalgene, Thermo Scientific, 0.45 μM filter with SFCA membrane), the maximal water solubility (S_{max}) was measured by inductively coupled plasma mass spectrometry (ICP-MS). For *ex vivo* experiments, TBBPA, Alpi and ZS were directly dissolved at the desired final concentration in a fat emulsion vehicle (a mixture of egg lecithin, peanut oil, and water).

2. *In vitro* models

In vitro test systems are frequently used for toxicity testing as they represent a simplification of the *in vivo* situation, thereby enabling a detailed study of the mechanism of action. In this PhD study, several *in vitro* neurotoxic endpoints were used to determine the neurotoxic potential of the selected flame retardants, including modulation of $GABA_A$ and nACh neurotransmitter receptors as well as cell viability, ROS production and Ca^{2+} homeostasis in neuronal cell lines.

2.1 Neurotransmitter receptor expression in *Xenopus* oocytes

As described in Chapter 1, human $GABA_A$ and nACh receptors are ligand-gated ion-channels that open upon binding of the (natural) agonist. Both receptors consist of a co-assembly of several different subunit isoforms that come together in various combinations to form a pentameric channel that is selectively permeable to ions. Opening of the channel results in an inflow of Cl^- ($GABA_A$ receptor) or cations (nACh receptor), resulting in respectively hyperpolarization or depolarization of the cell membrane. Functional responses of both $GABA_A$ and nACh receptors can be measured in biological systems, for example by using electrophysiological techniques.

In the human brain, the most common combination of subunits for the $GABA_A$ receptor is two α_1 subunits, two β_2 subunits and one γ_2 subunit ($\alpha_1\beta_2\gamma_2$), with each receptor containing two GABA binding sites located at the β/α subunit interface (Figure 1A) (McKernan and Whiting, 1996). In addition, $GABA_A$ receptors possess a variety of allosteric binding sites, which may modulate the receptor activity independent of the agonist binding sites (D'Hulst *et al.*, 2009; Johnston, 2005).

The predominant subtype of nACh receptors in the brain is the combination of two α_4 subunits and three β_2 subunits ($\alpha_4\beta_2$). Two ACh binding sites exist at the interface be-

tween the α and β subunits, and multiple allosteric binding sites are distributed throughout the extracellular domain of the subunits (Figure 1B) (Paterson and Nordberg, 2000).

Oocytes (precursors of mature egg cells) of the South-African clawed frog *Xenopus laevis* can express foreign proteins after microinjection of cDNA encoding the foreign protein into the nucleus. The oocyte efficiently transcribes and translates the injected genetic information, assembles the protein products into a functional protein, and inserts it in the cell membrane within 2-5 days of incubation. Since oocytes do not need to take up nutrients from the environment, the oocytes express only a small number of endogenous membrane transport proteins, i.e., have a low background expression of endogenous ion channels (Sigel and Minier, 2005; Wagner *et al*., 2000). As a result, oocytes are an efficient and commonly used model for electrophysiological recordings.

Oocytes are isolated from an ovarian lobe that is extracted from a mature, female frog during a small surgical procedure. The fully grown oocyte (stage V-VI) is a large cell with a diameter of 1-1.2 mm, which is surrounded by a non-cellular, fibrous vitelline layer and several layers of follicular cells. These follicular cells are removed by chemical (collagenase) treatment and/or manual defolliculation. cDNA encoding for the different subunits of either the human $\alpha_1\beta_2\gamma_2$ $GABA_A$ or $\alpha_4\beta_2$ nACh receptors are injected into the nucleus of each oocyte. Experiments are performed using naked oocytes that are still surrounded by the vitelline layer, which provides mechanical stability to the oocyte (Sigel and Minier, 2005; Wagner *et al*., 2000).

2.2 Neuronal cell lines

Cell lines are frequently used as *in vitro* models to study cellular mechanisms. Cell lines expressing neuronal properties are useful for studying the nervous system at the single cell and molecular levels. Clonal cell lines have been established from tumors, for example, rat central nervous system tumors (e.g. B35 neuroblastoma cells) (Schubert *et al*., 1974) and rat pheochromocytoma cells (cells from a neuroendocrine tumor of the adrenal gland, e.g. PC12 cells) (Greene and Tischler, 1976).

B35 neuroblastoma cells

B35 neuroblastoma cells display neuronal properties, such as membrane excitability and expression of enzymes for neurotransmitter metabolism including acetylcholinesterase and glutamic acid decarboxylase (Schubert *et al*., 1974). The B35 cell line has been characterized as a suitable model in the molecular analysis of endocytosis and intra- and intercellular signaling pathways (Croslan *et al*., 2008; Otey *et al*., 2003; Yang *et al*., 2011). In Chapter 1, the importance of Ca^{2+} in multiple (neuronal) physiological and pathological

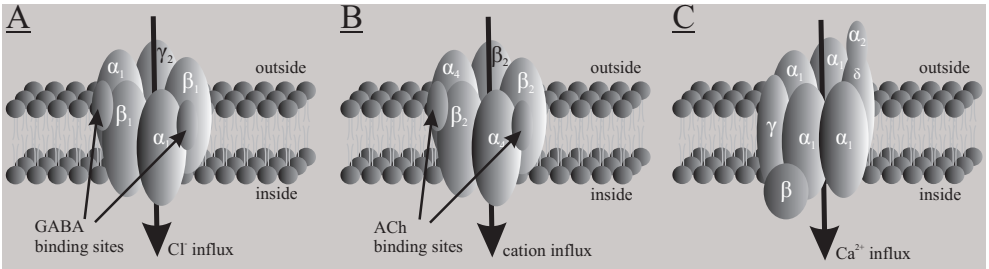

Figure 1. Schematic representation of the lipid bilayer of a cell membrane with a pentameric $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor (A), and a pentameric $\alpha_4\beta_2$ nACh receptor (B) and their natural agonist binding sites. Both receptors contain a receptor-integral ion channel permeable to anions ($GABA_A$ receptor) or cations (nACh receptor). In VGCCs (C), the α_1 subunits form the Ca^{2+} selective pore and contains the voltage-sensing machinery and binding sites for drugs and toxicants.

processes is described. Although B35 cells lack voltage-gated calcium channels (VGCCs), they have a high expression of calcium permeable nACh receptors. Stimulation of nACh receptors with ACh results in an influx of Ca^{2+}, which makes B35 cells useful for studying ACh-mediated signaling and intracellular Ca^{2+} homeostasis.

Pheochromocytoma (PC12) cells
The dopaminergic pheochromocytoma (PC12) cell line is very well characterized, easy to culture, and versatile for pharmacological manipulation. These cells are therefore widely used to assess neurotoxicity, neuronal function, and Ca^{2+} homeostasis *in vitro* (Westerink, 2013a). PC12 cells contain large dense core vesicles with catecholamines (primarily dopamine) and small, clear vesicles with acetylcholine. Following differentiation with nerve growth factor or other substances like dexamethasone, PC12 cells undergo a variety of changes in their phenotype, depending on the differentiation substance: they stop dividing, extend numerous neurites, and/or up-regulated several neural markers (Westerink and Ewing, 2008).

Although PC12 cells are chromaffin-derived cells, Ca^{2+} homeostasis in (undifferentiated) PC12 cells resembles that of sympathetic neurons (Duman *et al*., 2008). Comparable to neuronal cells, the main influx route for Ca^{2+} in PC12 cells is via VGCCs, which open upon depolarization with potassium (K^+). The rapid influx of Ca^{2+} through VGCCs can trigger various intracellular processes, including neurotransmitter release. Comparable to the mentioned $GABA_A$ and nACh receptors, VGCCs consists of different subunits (α_1, α_2, β, δ, see Figure 1C) that all have several isoforms, depending on the cell type. In the PC12 cells used in this study, the L-type VGCC is the most abundant type, although N- and P/Q-type VGCCs both also account for ~20% of total Ca^{2+} influx during depolarization (Dingemans *et al*., 2009; Heusinkveld *et al*., 2010).

3. *In vitro* techniques

3.1 Electrophysiological measurement of neurotransmitter receptor modulation

The two-electrode voltage-clamp technique is in oocytes the most widely used electrophysiological technique to measure whole cell currents through ion channels. This technique allows for the control of the membrane potential (clamping) to measure the current flowing through ion channels upon activation. As shown in Figure 2, the oocyte is placed in a (custom made) tube that is continuously perfused with saline and has two glass microelectrodes impaled: a membrane potential recording electrode (V), and a current-delivering electrode (I). The membrane potential recording electrode connects to a feedback amplifier where the signal is compared to the voltage clamp command given by a generator. The highly amplified difference of these signals is applied as a current through the current-delivering electrode, across the membrane of the oocyte, and to the bath-grounding electrode (Stühmer, 1992). All electrogenic ion or substrate fluxes across the membrane of the oocyte are now measured as a deflection from the baseline current. For example, when an oocyte expressing human $GABA_A$ receptors is superfused with GABA-containing saline, an inward membrane current will be measured due to opening of the ion channels and an influx of Cl^-.

Using this technique, the effects of flame retardant exposure on neurotransmitter receptor function can be determined. To investigate whether a flame retardant is able to activate $GABA_A$ or $\alpha_4\beta_2$ nACh receptors (agonistic effect), GABA- or ACh-responsive oocytes were superfused with saline containing different concentrations of the flame retardant. Possible partial agonistic effects on the $GABA_A$ receptor were determined

by applying the flame retardant together with a low concentration GABA. Similarly, possible antagonistic effects on the $\alpha_4\beta_2$ nACh receptor were determined by co-applying the flame retardant with ACh.

3.2 Screening methods using neuronal cell lines

Cell viability assays

Cell viability assays are often used to screen chemicals for their possible effect on cell death (cytotoxicity). In this PhD study, the cytotoxic effects of the selected flame retardants on B35 and PC12 cells were investigated following 24 h exposure. A combination of the Alamar Blue (AB) and Neutral Red (NR) assay is a fast and easy method to detect cytotoxic effects. The AB assay is based on the ability of the cells to reduce the non-fluorescent dye resazurin to fluorescent resofurin, which can be measured spectrophotometrically at 530/590 nm (excitation/emission). Viable cells reduce resazurin by mitochondrial enzymes (oxidoreductases). As such, the AB assay reflects the mitochondrial activity which can be used as a measure of cell viability (Magnani and Bettini, 2000). The disadvantage of using the AB assay as a measure for cell viability is that changes in mitochondrial activity not necessarily reflect changes in cell viability. For example, an increase in mitochondrial activity could mask reductions in cell viability. Therefore, the AB assay was followed by the NR assay, an independent second measure of cell viability based on membrane integrity and lysosomal (cellular organelles with break down enzymes) activity. NR - which is fluorescent - crosses the cell membrane and concentrates in lysosomes

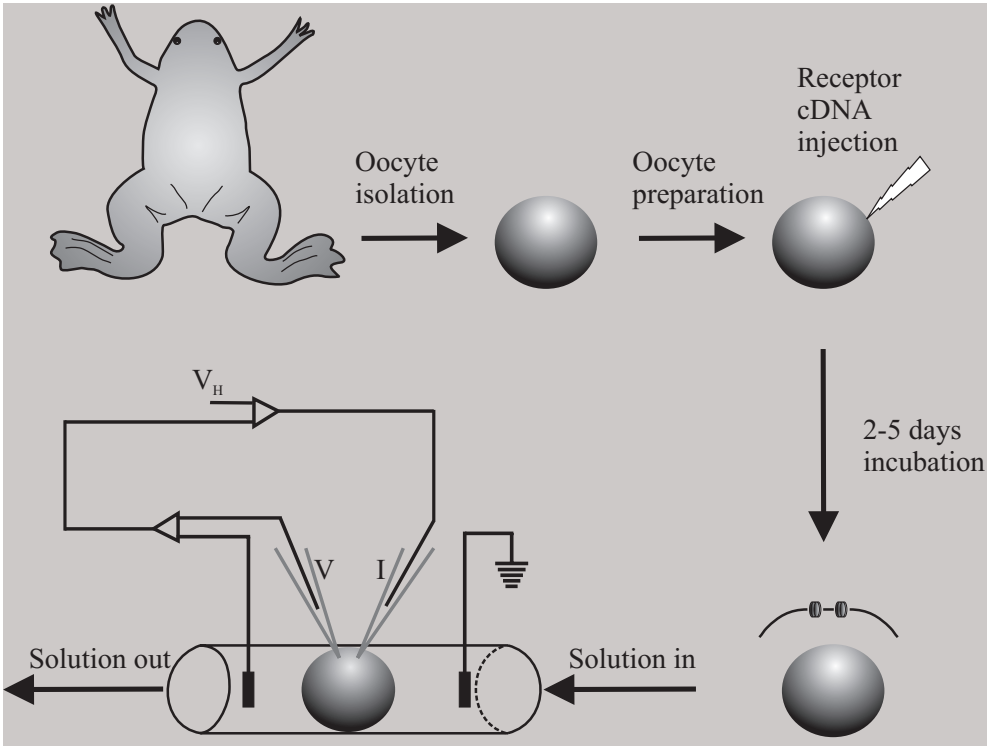

Figure 2. Schematic representation of oocyte isolation, preparation, injection with receptor cDNA, incubation and set-up for two-electrode voltage clamp. The electrode delivering current (I) is used to clamp the oocyte to the desired membrane potential (-60 mV). Changes in membrane potential are measured through the voltage electrode (V).

where it binds by electrostatic hydrophobic bonds to anionic and/or phosphate groups of the lysosomal matrix (Repetto *et al*., 2008). Alterations of the cell membrane or lysosomal membrane induced by a toxicant will result in a decreased uptake and binding of NR, which can then be quantified spectrophotometrically at 430/480 nm (excitation/emission) after washing and lysing the cells.

H_2-DCFDA assay
ROS is formed as a natural by-product of normal cell metabolism, though increased ROS levels (oxidative stress) can result in cellular damage. The H_2-DCFDA assay is a rapid method to measure ROS formation (Lebel and Bondy, 1990). The cell-permeable non-fluorescent probe 2′,7′-dichlorofluorescin diacetate (H_2-DCFDA) is intracellular de-esterified by cellular esterases and subsequently oxidized by ROS to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). As such, an increase in fluorescence, measured spectrophotometrically at 485/530 nm (excitation/emission), represents a toxicant-induced increase in intracellular ROS production. After loading B35 or PC12 cells with H_2-DCFDA, cells were exposed to different concentrations of the flame retardants and ROS production was subsequently measured at several time points up to 24 h.

Caspase activity
Caspases play an important role in the apoptosis pathways that can be triggered by Ca^{2+} signals or directly by a toxicant. Caspases, especially caspase-3, cleaves key components of the cellular signaling system, i.e., capase-3 cleaves the IP_3 receptor and a host of target proteins (Orrenius *et al*., 2011). The activity of caspase-3 in cells can be detected and measured using acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC). Capsase-3 hydrolyses Ac-DEVD-AMC into the fluorescent 7-amino-4-methylcoumarin (AMC), which can be measured spectrophotometrically at 360/460 nm (excitation/emission).

3.3 Ca^{2+} homeostasis in neuronal cell lines

As mentioned in Chapter 1, changes in $[Ca^{2+}]_i$ are not only an important trigger for apoptosis, but also play an essential role in intra- and intercellular signaling pathways. Using single-cell fluorescent microscopy, acute effects of the selected flame retardants on (basal) Ca^{2+}-homeostasis and stimulation-evoked changes in $[Ca^{2+}]_i$ in Fura-2-loaded PC12 and B35 cells were investigated. Both cell types have low basal $[Ca^{2+}]_i$, which rapidly increases upon depolarization with K^+ resulting in an influx of Ca^{2+} through VGCCs (PC12 cells) or activation of nACh receptors with ACh (B35 cells). During a recovery period, $[Ca^{2+}]_i$ returns to near basal levels and cells were exposed to saline-containing DMSO or flame retardant to determine the direct effects on Ca^{2+} homeostasis. Following this exposure, cells were stimulated with K^+ or ACh in combination with a flame retardant to derive a 'treatment ratio' to determine effects of flame retardants on VGCCs and nACh-receptors, respectively.

To investigate the mechanism of action of TBBPA on basal Ca^{2+} homeostasis in PC12 and B35 cells, the involvement of intracellular Ca^{2+} stores was determined. First, cells were exposed under Ca^{2+}-free conditions to TBBPA to identify the contribution of influx of extracellular Ca^{2+}. Next, the involvement of intracellular Ca^{2+} stores was determined by depleting mitochondria and ER (using thapsigargin and 4-(trifluoromethoxy)phenylhydrazone (FCCP)), and subsequent exposure to TBBPA.

Fura-2 AM is a high-affinity, intracellular Ca^{2+} indicator that consists of a carboxylic acid with acetoxymethyl (AM) ester. Due to the AM ester group, it is an uncharged molecule that can permeate cell membranes. Once inside the cell, the AM groups are hydrolysed by cellular esterases (de-esterification), which is essential for binding Ca^{2+} ions. Fura-2 bound to free intracellular Ca^{2+} is excited at 340 and 380 nm (F_{340} and F_{380}; emission at 510 nm); i.e., $[Ca^{2+}]_i$ is measured as a ratio (R) of F_{340}/F_{380}. Due to the spectral shift upon binding Ca^{2+}, the fluorescence evoked by F_{340} is positively correlated with free $[Ca^{2+}]_i$, while the fluorescence evoked by F_{380} is negatively correlated with free $[Ca^{2+}]_i$. Thus, at low $[Ca^{2+}]_i$, the emission at 340 nm is lower than at 380 nm (R<1), while at high $[Ca^{2+}]_i$, the emission at 340 nm increases, and decreases at 380 nm (R>1). Ratio dyes allow for accurate measurement of $[Ca^{2+}]_i$ as it reduces the effects of uneven dye loading, leakage of dye, photobleaching, and problems associated with measuring Ca^{2+} in cells of unequal thickness (Grynkiewicz *et al*., 1985; Molecular Probes, 2011). Free cytosolic $[Ca^{2+}]_i$ was calculated according to a modified Grynkiewicz's equation: $[Ca^{2+}]_i = K_d \times (R\text{-}R_{min})/(R_{max}\text{-}R)$ where K_d is the dissociation constant of Fura-2 AM determined in the experimental set-up. Maximum and minimum ratios (R_{max} and R_{min}) were determined at the end of each experiment by using ionomycin (increases cytosolic Ca^{2+} concentration by influx of extracellular Ca^{2+}) and ethylenediamine tetraacetic acid (EDTA, scavenges Ca^{2+}), respectively (Deitmer and Schild, 2000; Grynkiewicz *et al*., 1985).

4. *In vivo* models

Since *in vitro* cellular responses may be species-specific, lack metabolism (toxicokinetics) and cellular interactions, and the absence of a blood-brain barrier and the sophisticated cell-cell interactions that are all present in the intact brain of an organism, it is difficult to extrapolate *in vitro* results to the *in vivo* situation. Therefore, additional *ex vivo* validation experiments (measurement of synaptic plasticity in hippocampal slices of exposed and control mice) were performed to bridge the gap between experimental *in vitro* data to the *in vivo* situation. The used models and techniques are outlined below.

4.1 Mouse hippocampal slices

Ex vivo validation experiments with three selected flame retardants (TBBPA, aluminium diethylphosphinate (Alpi) and zinc stannate (ZS)) were performed. The potential neurodevelopmental effects of these three compounds were investigated in mice following a single neonatal exposure on postnatal day (PND) 10 by examining effects on *N*-methyl-D-aspartate (NMDA)-dependent LTP. On PND 17-19 (i.e. directly after the brain growth spurt), exposed pups were killed by decapitation after inhalation anesthesia (isoflurane). The brain was rapidly dissected on ice and the two hemispheres were separated. One hemisphere was used to prepare hippocampal slices as described previously (Dingemans *et al*., 2007; Notenboom *et al*., 2010). 250 μm coronal slices were cut in ice-cold carbogenated artificial cerebrospinal fluid (ACSF) using a vibratome. Slices were allowed to stabilize at room temperature in carbogenated ACSF for at least 1 h.

4.2 Cortex synaptic protein expression

Previous studies have shown that PBDEs (including BDE-209) affect several critical proteins involved in (mice) brain development (calcium/calmodulin-dependent protein kinase-II (CaMK-II), growth associated protein-43 (GAP-43) and synaptophysin) when exposed during the brain growth spurt (Viberg *et al*., 2008a; Viberg, 2009). CaMK-II is one of the most abundant protein kinases in the mammalian brain and increase in expression during brain development, and is involved in regulation of both synaptogenesis and syn-

aptic plasticity (Oestreicher *et al*., 1997). GAP-43 is frequently used as a marker for axonal sprouting and growth and is highly expressed during brain development (Rongo and Kaplan, 1999), whereas synaptophysin is involved in synapse formation and synaptic plasticity (Tarsa and Goda, 2002). Postsynaptic density protein 95 (PSD-95) is a synaptic protein which increases the number of functional channels at the cell surface and channel opening rate of NMDA-receptors and consequently also involved in development and LTP (Lin *et al*., 2004). Glutamate Receptor 1 (GluR1) is a subunit of glutamate receptors which also plays an important role in LTP (Boehm *et al*., 2006). In this PhD study, cortex tissue of exposed and control mice was used to investigate if neonatal exposure to one of the selected flame retardants affects the expression of CaMK-II, GAP-43, synaptophysin, PSD 95, and/or GluR1.

4.3 Internal dose analysis

Measuring levels of toxicants in tissues of exposed animals gives insight in the bioavailability and internal distribution of the toxicant. To relate possible flame retardant induced effects on LTP or protein expression to internal doses of the exposed mice, levels of TBBPA, Alpi and ZS were measured in non-cortex brain, muscle and liver tissue.

5. *Ex vivo* techniques

5.1 *Ex vivo* LTP

LTP is a form of synaptic plasticity which is suggested to be the cellular substrate for learning and memory (Malenka and Bear, 2004). LTP includes the action of the cholinergic, GABAergic and glutamatergic neurotransmitter system. NMDA-dependent LTP in hippocampal slices is an extensively studied form of synaptic plasticity (Blundon and Zakharenko, 2008). In the hippocampus (see Figure 3A), the Schaffer collateral-commissural pathway makes excitatory synaptic contact with the dendrites of CA1 pyramidal neurons (see Figure 3B). By placing extracellular electrodes in both regions, field-excitatory postsynaptic potentials (fEPSP) in the CA1 region can be measured following stimulation of the Schaffer collateral-commissural pathway: the stimulation results in a

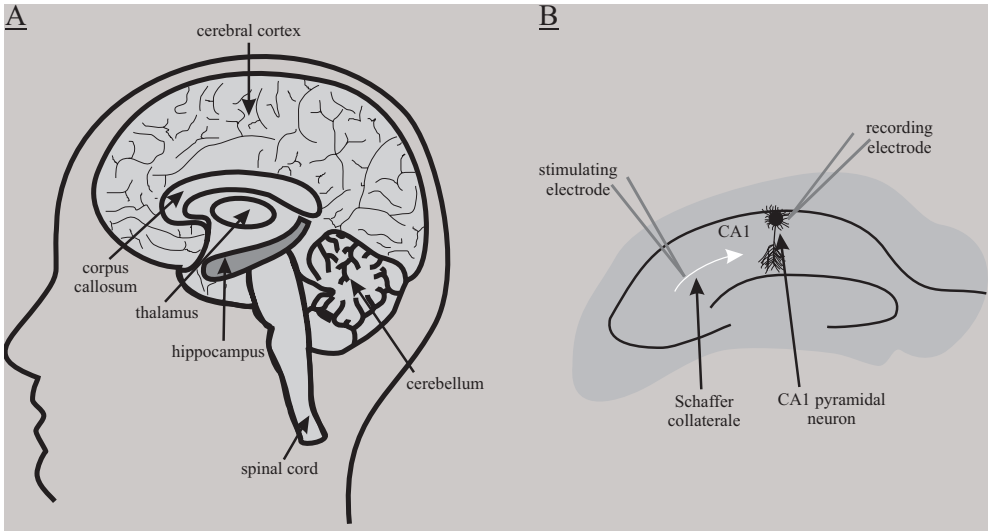

Figure 3. Illustration of human brain structures (A) and schematic representation of electrophysiological recording with electrodes in hippocampal mouse slice (B). Following stimulations of the Schaffer collateral-commissural pathway, field potentials of dendritic synapses in the CA1 region are recorded.

depolarization response of the postsynaptic neurons in the cornu Annon (CA1) region due to opening of ligand-gated ion channels and inflow of cations.

In this research, synaptic transmission and activity-dependent plasticity in slices of the hippocampal CA1 region of control and exposed mice was investigated using fEPSP recordings as described previously (Dingemans *et al*., 2007; Notenboom *et al*., 2010), with minor modifications. Synaptic responses in hippocampal slices were evoked by local extracellular stimulation of the Schaffer collateral-commissural pathway using a bipolar borosilicate electrode. fEPSPs were recorded with a second electrode, connected to an amplifier. Stimulation intensities for threshold and maximum fEPSP were determined for every slice using four single stimuli of increasing intensity. The obtained input-output curve gives an indication of possible treatment-induced changes in basal excitability and threshold for synaptic transmission. The stimulus intensity that evoked half-maximal fEPSP was used during the remainder of the experiment. To assess possible flame retardant-induced presynaptic effects, paired-pulse facilitation (PPF) was examined. PPF is a form of short-term plasticity (Xu-Friedman and Regehr, 2004) expressed as a paired-pulse ratio (PPR) showing an increased response to the second of two identical stimuli separated by an interstimulus interval of 50-200 ms. Finally, LTP was induced by a single tetanic stimulation (100 Hz, 1 s), which results in an immediate large increase of the fEPSP in control slices. The increase of fEPSP during the first 7.5 minutes post-tetanus is classified as post-tetanic potentiation (PTP). After PTP, fEPSP decreases but stabilizes at a higher level than baseline and is maintained for at least 60 minutes.

5.2 Slot-blot protein analysis

Slot-blot is a biochemical technique based on filtration of liquid samples, which is used to detect specific proteins in a sample of tissue homogenate or extract. The slot-blot analysis was performed as described previously (Viberg *et al*., 2008a; Viberg *et al*., 2008b). In short, supernatant of homogenized cortices were applied to a nitrocellulose membrane and stained overnight with antibodies specific to the target proteins. The intensity of the immunoreactive bands were analyzed and corrected for control mice.

5.3 Brain, muscle and liver concentrations

For determination of the internal dose of TBBPA-exposed mice, extracts of freeze dried liver, muscle and non-cortex brain tissue were measured with gas chromatography combined with mass spectrometry (GCMS). The gas chromatography separates the chemical mixture by volatility, the mass spectrometer subsequently identifies and quantifies the chemicals. Tissues of Alpi- and ZS-exposed mice contain possible metals and were therefore analyzed using another technique. Tissue samples were freeze dried, homogenized, and digested and analyzed by High Resolution Inductively Coupled Plasma Mass Spectrometry (HRICP-MS). HRICP-MS relies on a magnetic component to separate ions according to their mass/charge ratio.

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Chapter 3

Persistence, bioaccumulation, and toxicity of halogen-free flame retardants

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Summary

Polymers are synthetic organic materials having a high carbon and hydrogen content, which make them readily combustible. Polymers have many indoor uses and their flammability makes them a fire hazard. Therefore, flame retardants (FRs) are incorporated into these materials as a safety measure. Brominated flame retardants (BFRs), which accounted for about 21% of the total world market of FRs, have several unintended negative effects on the environment and human health. Hence, there is growing interest in finding appropriate alternative halogen-free flame retardants (HFFRs). Many of these HFFRs are marketed already, although their environmental behavior and toxicological properties are often only known to a limited extent, and their potential impact on the environment cannot yet be properly assessed. Therefore, we undertook this review to make an inventory of the available data that exists (up to September 2011) on the physical-chemical properties, production volumes, persistence, bioaccumulation, and toxicity (PBT) of a selection of HFFRs that are potential replacements for BFRs in polymers. Large data gaps were identified for the physical-chemical and the PBT properties of the reviewed HFFRs. Because these HFFRs are currently on the market, there is an urgent need to fill these data gaps. Enhanced transparency of methodology and data are needed to reevaluate certain test results that appear contradictory, and, if this does not provide new insights, further research should be performed. TPP has been studied quite extensively and it is clearly persistent, bioaccumulative, and toxic. So far, RDP and BDP have demonstrated low to high ecotoxicity and persistence. The compounds ATH and ZB exerted high toxicity to some species and Alpi appeared to be persistent and has low to moderate reported ecotoxicity. DOPO and MPP may be persistent, but this view is based merely on one or two studies, clearly indicating a lack of information. Many degradation studies have been performed on PER and show low persistence, with a few exceptions. Additionally, there is too little information on the bioaccumulation potential of PER. APP mostly has low PBT properties; however, moderate ecotoxicity was reported in two studies. $Mg(OH)_2$, ZHS, and ZS do not show such remarkably high bioaccumulation or toxicity, but large data gaps exist for these compounds also. Nevertheless, we consider the latter compounds to be the most promising among alternative HFFRs. To assess whether the presently reviewed HFFRs are truly suitable alternatives, each compound should be examined individually by comparing its PBT values with those of the relevant halogenated flame retardant. Until more data are available, it remains impossible to accurately evaluate the risk of each of these compounds, including the ones that are already extensively marketed.

1. Introduction

Polymers are synthetic organic materials that have a high carbon and hydrogen content, which renders them readily combustible. When used in buildings, electrical appliances, furniture, textiles, transportation, mining, and in many other applications, polymers have to fulfill flame retardancy regulatory requirements, primarily as mandatory specifications that often differ among countries. To achieve these requirements, chemical additives known as flame retardants (FRs) are incorporated into the polymers. In contrast to most additives, FRs can appreciably impair the material properties of polymers (UNEP, 2008). The key challenge is therefore to find a suitable compromise between the performance of the polymers and fulfilling flame retardancy requirements. Brominated flame retardants (BFRs) are rather widely used because they have a low impact on the polymer's characteristics, are very effective in relatively low amounts compared to other FRs (Alaee *et al.*, 2003), and are relatively cheap (Birnbaum and Staskal, 2004). In 2004, BFRs accounted for about 21% of the total world production of FRs (SRI Consulting, 2004). Many BFRs, however, have unintended negative effects on the environment and human health. Some are very persistent (Robrock *et al.*, 2008), some bioaccumulate in aquatic and terrestrial food chains (Boon *et al.*, 2002), and some show serious adverse effects such as endocrine disruption (Meerts *et al.*, 2001). Some BFRs (polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), and tetrabromobisphenol-A (TBBPA), in particular) 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). In 2000, exponentially increasing PBDE concentrations were measured in Swedish human milk (Norén and Meironyté, 2000), and this was later followed by reports of even higher PBDE concentrations in human milk from the USA (Schecter *et al.*, 2008).

Concerns about the persistence, bioaccumulation, and toxicity (PBT) of some BFRs have led to a ban on the production and use of many of these compounds, i.e., the hexa-, octa-, and deca-brominated biphenyls (polybrominated biphenyls or PBBs); the tetra-, penta-, hexa-, hepta-, octa-, and deca-BDEs; and HBCD (Albemarle corporation, 2009; Chemtura, 2009; European Parliament, 2002; ICL, 2009; OSPAR, 2001; UNEP 2009a; WHO, 1994). Hence, there is growing interest in substituting BFRs with alternative halogen-free flame retardants (HFFRs), and several furniture manufacturers have already voluntarily replaced BFRs with alternative HFFRs (Betts, 2007).

Many HFFRs are already marketed, although their environmental behavior and toxicological properties are only known to a limited extent and their potential impact on the environment cannot yet be properly assessed. Therefore, banning BFRs and replacing them with HHFRs introduces the dilemma that little is known about the environment and human health risks of the HFFRs. Consequently, there is urgent need for information on the PBT properties of HFFRs. Therefore, the aim of this review is to make an inventory of the data that are available on the physical-chemical properties, production volumes, PBT of a selection of HFFRs that are suitable replacements for BFRs in polymers.

2. Selected HFFRs

HFFRs can be divided into several categories (see Table 1), the most important ones being inorganic flame retardants and synergists (mostly used for electronics and electrical equipment), organophosphorus compounds and their salts (housings of consumer products), nitrogen-based organic flame retardants (electronics and electrical equipment), and intumescent systems (textile coatings). From these categories, 13 HFFRs were selected for inclusion in this review as potential replacements for BFRs in polymers:

aluminum trihydroxide, magnesium hydroxide, zinc borate, zinc hydroxystannate and zinc stannate (inorganic flame retardants and synergists); aluminum diethylphosphinate, bisphenol-A bis(diphenylphosphate), 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (or dihydrooxahosphaphenanthrene), resorcinol bis(diphenylphosphate) and triphenylphosphate (organophosphorus compounds and salts), melamine polyphosphate (nitrogen based organic flame retardant); ammonium polyphosphate, and pentaerythritol (intumescent systems).

3. Characteristics of the selected HFFR

In each of the following sections, a specific group of flame retardants and their intrinsic properties is addressed. We start with their physical-chemical properties and then we present PBT data. In the toxicity paragraphs, we report ecotoxicity data as well as effects on mammals and data on *in vitro* toxicity endpoints. The available data are classified based on the REACH system (*R*egistration, *E*valuation, *A*uthorisation and Restriction of *Ch*emical substances), i.e., European Union REACH legislation Regulation No. 1907/2006 Annex XIII and No. 1272/2008 Chaps. 3 and 4 (European Union, 2006; European Union, 2008). This means that we assigned the data categories as being "high", "moderate", and "low".

During our literature search, we preferred data published in peer-reviewed scientific papers over those in reports and other so-called grey literature. Whenever provided in the papers we found, the most relevant details are reported. The transparency of the experimental setup was of high importance; the more study detail that was provided on test conditions and results, the more reliable we considered the data to be. Although we

Table 1. Brominated flame retardant (BFR) applications and halogen-free flame retardant (HFFR) alternatives

Application	Main BFR	HFFR Alternatives
Printed circuit boards Electronic components encapsulations Technical laminate	Tetrabromobisphenol-A (TBBPA)	• Dihydrooxahosphaphenanthrene (DOPO) • Zinc hydroxystannate (ZHS) • Zinc stannate (ZS)ZHS/ZS coated with aluminum trihydroxide (ATH)
Housings of electronic products Wiring parts Housings for business machines, toys, telephones and others consumer electronics	TBBPA Decabrominated diphenyether (DecaBDE) and other BFR	• Resorcinol bis(diphenylphosphate) (RDP) • Bisphenol-A bis(diphenylphosphate) (BDP) • Triphenylphosphate (TPP)
Electrical and electronic equipment, connectors, switches etc. Encapsulated electronic components	Brominated polystyrenes and other BFR	• Aluminum diethylphosphinate (Alpi) • Melamine polyphosphate (MPP) • Anhydrous zinc borate (ZB) • ZS
Wire and cables	DecaBDE and other BFRs	• ATH • $Mg(OH)_2$ • ZHS • ZS • ZB
Textile coatings	Hexabromocyclododecane (HBCDD)	Intumescent systems consisting of ammonium polyphosphate (APP) + Pentaerythritol (PER) + MPP

preferred primary sources, in some cases we referred to secondary reports (trusted independent sources such as UNEP and US EPA). Therefore, when using such data reported in this review, we strongly recommend readers also consult the original reference.

Details about endpoints chosen and the classification system used are explained in the following paragraphs.

3.1 Physical-chemical properties

Physical-chemical properties are highly important in assessing the environmental fate and behavior of compounds. Properties of particular interest are: molecular weight (MW), melting point and temperature of decomposition, vapor pressure, water solubility, Henry's law constant (*H*), the air-water partition coefficient (K_{AW}, which is closely related to *H*), and the octanol-water partition coeffcient (K_{OW}). Specific approaches for checking the consistency between different reported values of solubility in water, vapor pressure, and Henry's law constant are available, such as the three solubility approach (Cole and Mackay, 2000; Schenker *et al.*, 2005). However, we have not attempted to differentiate methods for gathering such data in this review, since HFFR data are often scattered and fragmentary. Instead, when few reliable data points were available on a compound, estimation software or on-line calculators were used to estimate values for physical-chemical properties. When software estimators were needed for organic chemicals (or chemicals acting like organics from the provisional list), tools such as COSMOtherm® Vers. C2.1, EPI Suite 4.1 and SPARC On-Line Calculator 4.5 from the US EPA (Eckert and Klamt, 2010; Hilal *et al.*, 2003b; Hilal *et al.*, 2004b; US EPA, 2011) were used. To our knowledge, no tools are available for estimating the physical-chemical properties of inorganic substances. Nor, are some property descriptors relevant for describing the partitioning of inorganic substances.

3.2 Environmental presence and production volumes

The environmental occurrence of the selected HFFRs was surveyed by searching the published literature. The results of this survey revealed a lack of data on environmental presence; therefore, we thought it advisable to add information on production volumes to the review. The production volumes of the selected HFFRs can be categorized as low production, import volumes (LPV), or high production volume (HPV). The HFFRs having LPV had volumes varying between 10 and 1,000 t $year^{-1}$, whereas those with HPV exceeded 1,000 t $year^{-1}$ (European Union, 1993).

3.3 Persistence

The persistence of the selected compounds was evaluated by collecting data on ready biodegradability and/or dissipation times. Ready biodegradability is usually determined by performing biological degradation tests (often by microorganisms from waste water treatment plant sludge) in water, according to standard OECD guidelines (OECD, 1992). Dissipation times may be reported for air, water, sediment/soil, and sludge. Often, half-lives were given, which is the time required for 50% of the compound to be transformed. However, it was often unclear whether these half-lives represented full mineralization, oxidation, or merely primary degradation (removal of the parent compound only). Therefore, we chose to report these values as dissipation times (DT_{50}), in which the concentration is reduced to 50% of the initial concentration after a given period. If DT_{50} values were not available, DT_x values are reported, where x represents the converted percentage (e.g., DT_{30} means time for the concentration to dissipate to 30% of the initial concentration). Depletion processes not involving transformations, such as sorption, evaporation, and scavenging were not searched out.

It should be noted that primary degradation can lead to the production of substances that are more harmful than the parent compound. This subject is not addressed extensively in this review, although we do report whether the dissipation time includes full mineralization, and any information found on metabolites formed. The concept of biodegradation has little or no meaning for inorganic compounds and metals. Metals will not decompose, but complexation or changes in speciation for them may occur during transport through the different environmental compartments, and thus their intrinsic properties and availability also may be altered. Such potential behavior, however, was beyond the scope of this review.

3.4 Bioaccumulation

The potential of a compound to bioaccumulate is characterized herein, and is expressed by using the bioconcentration factor (BCF). The BCF is the concentration of the chemical in an organism divided by the concentration that exists in the surrounding environment, providing that uptake occurs only through absorption from water via the respiratory surface (e.g., gills). BCFs can only be derived under laboratory conditions, when dietary uptake is minimized, or by theoretical estimation. The Log K_{OW} (octanol-water partition coefficient) value is often used to estimate the BCF and to indicate what the probable bioaccumulation potential for organic compounds is. Generally, there is a good correlation between Log K_{OW} and BCF values (Shüürmann *et al.*, 2007), because compounds having a high Log K_{OW} also have a high tendency to partition to lipids, and therefore possess a high potential for bioaccumulation. The Log K_{OW} value for each HFFR compound addressed is given in the physical-chemical properties section. It should be noted that substances that are rapidly metabolized will have a low bioaccumulation potential, even if they have a high Log K_{OW} (Gobas *et al.*, 2003; Wu *et al.*, 2008). It is currently not possible to include a more refined assessment of bioaccumulation potential, because of the paucity of information that currently exists on the metabolism rates for the HFFRs.

3.5 Toxicity

The *in vivo* and *in vitro* toxicity data available for the HFFRs were addressed separately. *In vivo* toxicity was also addressed separately for ecotoxicity data (from now on merely referred to as ecotoxicity) and mammalian endpoints (often lethal dose studies on rodents).

Ecotoxicity

In vivo aquatic ecotoxicity data are usually reported as lethal concentrations (LC_{50}). In some studies, exposure did not produce an effect. In such cases, the no observed effect concentration (NOEC) is reported, being the highest concentration tested that did not cause an adverse effect compared to the control. This does imply, however, that higher concentrations might show an effect. Alternatively, the lowest observed effect concentration (LOEC) is reported, if available. Ideally, this value implies that lower concentrations will not show an effect and it is best used in combination with a well-defined NOEC. Nevertheless, often LOEC values were reported if the lowest concentration tested showed an effect and no lower concentrations were tested.

In vivo toxicity

In vivo toxicity data are reported as the lethal dose (LD_{50}) for feeding or for dermal or inhalation exposure. It should be noted that, in some studies, exposure was not high enough to reach an LD_{50} value. In such cases, the NOEC or LOEC value is reported.

In vitro toxicity

For *in vitro* toxicity we focused on a limited number of well-defined endpoints (as listed below), in which a cell's or organ's function is clearly affected. Mutagenicity and carcinogenicity are addressed as the genotoxic endpoints. The following endpoints were addressed for endocrine toxicity: the activation of the Ah-receptor (also called dioxin receptor, DR), the potency to displace thyroxin from its plasma carrier protein transthyretin (TTR), the formation of possibly active metabolites (bioactivation), and the activation of the estradiol receptor (ER) or the androgenic receptor (AR). Finally, we addressed the following neurotoxic endpoints: cytotoxicity, production of reactive oxygen species (ROS), disruption of calcium homeostasis and changes in neurotransmitter levels or neurotransmitter receptor activity. Results from the literature are expressed as either EC_{50}, IC_{50} (Inhibition Concentration), LOEC or NOEC values. The literature search revealed that, as for *in vivo* (eco)toxicity studies, many different *in vitro* test methods and systems as utilized. In contrast, mutagenic effects were often limited to results of AMES tests (Mortelmans and Zeiger, 2000) and are classified as being simply positive or negative.

3.6 Classification

For risk assessment purposes chemicals are often classified according to the different categories of potential harm that they may cause. In this review, we based our classification on the European Union REACH regulations (European Union, 2006; European Union, 2008). Therefore, where relevant, each of our tables that display intrinsic properties of HFFRs contain a column in which reported values are disclosed as being either "high", "moderate", or "low". Instead of referring to a disappearance "half-life", we prefer to use the term dissipation time, i.e., DT_{50}. Compounds that are classified as being "very persistent" and "very bioaccumulative" (vPvB) are based on an existing system that exists in the Regulation of the European Commission (European Union, 2006). Atmospheric dissipation times were reported but were not classified. In Table 2 , we show the threshold values for each classification level. Complete concentration-response curves were usually absent for *in vitro* toxicity tests. When toxicity data came from several different studies,

Table 2. Classification for persistence, bioaccumulation and toxicity (PBT)

Classification	Persistence	Bioaccumulation	Toxicity
High	Not "ready biodegradable" Or Soil/sediment or sludge DT_{60+}>28 days Or water (pH 7) DT_{70+}>28 days	BCF >500 Log K_{OW}≥4	LD_{50} ≤1mg L^{-1} EC_{50} ≤1mg L^{-1} LC_{50} ≤1mg L^{-1}
Moderate	-	-	1 mg L^{-1} <LD_{50} ≤10 mg L^{-1} 1 mg L^{-1} <EC_{50} ≤10 mg L^{-1} 1 mg L^{-1} <LC_{50} ≤10 mg L^{-1}
Low	"Ready biodegradable" Or soil/sediment or sludge DT_{60+} ≤28 days Or water (pH 7) DT_{70+} ≤28 days	BCF <500 Log K_{OW}<4	LD_{50} >10 mg L^{-1} EC_{50} >10 mg L^{-1} LC_{50} >10 mg L^{-1}
vPvB	DT_{50}>60 days (marine, fresh or estuarine water) *or* DT_{50}>180 days (soil, marine, fresh or estuarine water sediment) AND a BCF >5,000		

BCF bioconcentration factor, DT_x dissipation time of x% of the compound, EC_{50} the concentration that causes 50% effect to the test species population, LD_{50} the concentration that causes 50% mortality to the test species population, Log K_{OW} logarithmic octanol-water partitioning coefficient

it was generally difficult to classify the risk of a compound, according to our preferred classification scheme, i.e., from "no potency" to "very high potency". Therefore, data are presented as "low toxicity" when no effects were observed, "toxic" when effects were observed, and "not enough data to classify" when data were incompatible with the predefined risk assessment criteria or too few details were provided.

4. Inorganic flame retardants and synergists

In this section, we address the compounds aluminum trihydroxide (ATH), magnesium hydroxide ($Mg(OH)_2$), ammonium polyphosphate (APP), zinc borate, zinc hydroxystannate (ZHS), and zinc stannate (ZS).

4.1 Aluminum trihydroxide

Aluminum trihydroxide (ATH, CAS nr 21645-51-2) is a weak inorganic acid. It is a hydrate, which means that in its solid form it contains water ($Al(OH)_3 \cdot H_2O$). ATH is commonly used as a smoke suppressor and as a flame retardant synergist, together with other FRs such as organophosphorus compounds (ENFIRO, 2009). ATH was classified as an HPV chemical in the EU (European Chemicals Bureau, 2011). For the USA, the total annual production was given as <450,000 t in 2006 (US EPA, 2006).

Physical-chemical properties

ATH is solid at environmentally relevant temperatures (-40 to +40°C), since most reported melting points range from 150 to 300°C (European Chemicals Bureau, 2000a; Lewis, 2000; Walter and Wajer, 2010).

Our literature search revealed that ATH had a wide range of water solubility, with values ranging from 0.015 to 1.5 mg L^{-1} (1.92E-4 or 1.90E-2 mol m^{-3}) (European Chemicals Bureau, 2000a). Two other studies simply refer to ATH as "insoluble" (European Chemicals Bureau, 2000a; Rio Tinto Alcan (RTA), 2008a). Clearly, ATH has low water solubility, although its reported solubility values vary by a factor of one hundred. The properties of ATH are listed in Table 3.

Bioaccumulation

In a draft EPA report, it was estimated that the BCF value for ATH is <500 (US EPA, 2008), and it was stated in another study that its bioaccumulation potential is low (German Federal Environmental Agency *et al.*, 2001), but neither study gave further details (Table 3).

Toxicity

1. Ecotoxicity
   Reported effect concentrations cover a wide range, and consequently, the classification of ecotoxicity varies between low and high (Table 3). It is not expected that ATH will easily decompose to produce freely dissolved Al^{3+} ions, unless conditions such as a low pH favor Al^{3+} dissociation. The toxicity of aluminum has been extensively discussed elsewhere (Berthon, 2002; Kucera *et al.*, 2008) and is not repeated here.
2. *In vivo* toxicity
   The acute toxicity of ATH to rats is very low, with LD_{50} values higher than 5,000 mg kg^{-1} bwt (Table 3).
3. *In vitro* toxicity
   Data on ATH were limited (Subcommittee on Flame-Retardant Chemicals, 2000). As shown in Table 3, ATH is not carcinogenic in animal tests (German Federal Environmental Agency *et al.*, 2001; O'Connell *et al.*, 2004; Subcommittee on Flame-Retardant

Table 3. Aluminum trihydroxide (ATH, CAS nr 21645-51-2)

	Data	Details	References
Physical-Chemical Properties			
Molecular weight	78.01 g mol^{-1}		
Melting point	150-220°C (Decomposition)		European Chemicals Bureau, 2000a
Melting point	200°C		European Chemicals Bureau, 2000a
Melting point	230°C		Walter and Wajer, 2010
Melting point	300°C		Lewis, 2000
Melting point	2,030°C		Rio Tinto Alcan (RTA), 2008a
Water solubility	0.015 mg L^{-1}	[at 20°C]	European Chemicals Bureau, 2000a
Water solubility	1.5 mg L^{-1}	[at 20°C]	European Chemicals Bureau, 2000a
Water solubility	insoluble		European Chemicals Bureau, 2000a; Rio Tinto Alcan (RTA), 2008a
Bioaccumulation			
Low	*BCF <500*[c,e]	Fish, estimated	US EPA, 2008
Low	Not specified		German Federal Environmental Agency *et al.*, 2001
Ecotoxicity			
High; aquatic	EC_{50} = 0.8240[e] mg L^{-1}	*Daphnia magna*, 48h	TSCATS, DuPont Central Research, 1996 (not found) from draft US EPA, 2008
High; aquatic	EC_{50} = 0.6560[e] mg L^{-1}	*Selenastrum capricornutum*, 96h	TSCATS, DuPont Central Research, 1996 (not found) from draft US EPA, 2008
Moderate; aquatic	LC_{50} = 2.6-3.5 mg L^{-1}	Daphnids	Illinois Environmental Protection Agency, 2007
Low; aquatic	Not specified	Fish, lethality only at low pH	Illinois Environmental Protection Agency, 2007
Low; aquatic	NOEC >100 mg L^{-1}	Fish (*Salmo trutta*), 96h; Crustacean (*Daphnia magna*), 48h; Algae (*Selenastrum capricornutum*), 72h	European Chemicals Bureau, 2000a

(continued)

Table 3. (continued)	Data	Details	References
Low; aquatic	Not specified		German Federal Environmental Agency *et al.*, 2001; Stevens and Mann, 1999
In vivo toxicity			
Low	LD_{50} >5,000 mg kg^{-1} bwt	Rats	European Chemicals Bureau, 2000a; Illinois Environmental Protection Agency, 2007
Low	Not specified		Stevens and Mann, 1999; UNEP, 2008
In vitro toxicity			
Low	Genotoxicity; Carcinogenicity	Rats	German Federal Environmental Agency *et al.*, 2001; O'Connell *et al.*, 2004; Subcommittee on Flame-Retardant Chemicals, 2000

Italic values are predicted: [a]Modeled, [b]Calculated, [c]Expert judgment
[e]Not all primary sources are found from (US EPA, 2008), also this reference is a draft report, so reported values may be not final

Chemicals, 2000). In one report, it was stated that ATH was mutagenic and cytotoxic, although the concentrations or test conditions were not mentioned (German Federal Environmental Agency *et al.*, 2001). Therefore, the genotoxicity is classified as being low.

No *in vitro* endocrine toxicity or neurotoxicity data were reported for ATH. However, it was shown that ATH causes cytostatic activity with induction of neurites at >200 mM in neuroblastoma cells (Zatta *et al.*, 1992). Moreover, at a concentration of >10 mM, ATH did bind to the *N*-methyl-d-aspartate receptor (NMDA-R) in human cerebral cortex (Hubbard *et al.*, 1989). In another study, it was reported that there were adverse effects of ATH on the learning ability in rats and that cholinergic activity was diminished (Bilkei-Gorzo, 1993). Despite these adverse neurotoxic effects, there are insufficient data available to classify the overall *in vitro* toxicity of ATH.

In summary, ATH is a solid at room temperature, and has a low, but uncertain water solubility. Its bioaccumulation potential is estimated to be low and the *in vivo* and *in vitro* toxicity of ATH is also low. However, ATH may pose a risk to aquatic communities, with EC_{50} values varying from low to high.

4.2 Magnesium hydroxide, $Mg(OH)_2$

Magnesium hydroxide (CAS 1309-42-8) is an inorganic salt that consists of hydroxide and magnesium ions. Magnesium hydroxide, $Mg(OH)_2$, used as a flame retardant or flame retardant additive, is very effective in reducing smoke emissions from burning plastics (Walter and Wajer, 2010). This compound is classified as an HPV chemical in the EU (European Chemicals Bureau, 2011). For the USA, total annual production was 45,000 to <227,000 t in 2006 (US EPA, 2006).

Physical-chemical properties

Magnesium hydroxide is a solid at room temperature, since its melting point is approximately 350°C; oddly, an excessively high melting point of 2,800°C was reported in one study, which seems unlikely (Table 4). This compound has no real boiling point as it will undergo endothermic decomposition at 330 or 340°C, with release of water (AluChem, 2003; Walter and Wajer, 2010). Magnesium hydroxide is insoluble in water (Albemarle corporation, 2003a; Albemarle corporation, 2003b; Albemarle corporation,

2003c; AluChem, 2003; Fisher Scientific, 1999). An overview of its physical-chemical properties is shown in Table 4.

Bioaccumulation
There are no data available on the bioaccumulation of magnesium hydroxide.

Toxicity
1. Ecotoxicity
   There are no data available on the ecotoxicity of magnesium hydroxide. Magnesium is an essential metal and it is a major component of natural waters (European Chemicals Bureau, 2000c). Therefore, it is not expected that this compound has a high aquatic toxicity.
2. *In vivo* toxicity
   Data on the *in vivo* toxicity of this compound are quite sparse, with only two acute LD_{50} values (each >5,800 mg kg^{-1} bwt) for rats being reported (Table 4).

Table 4. Magnesium hydroxide ($Mg(OH)_2$, CAS nr 1309-42-8)

	Data	Details	Reference
Physical-Chemical Properties			
Molecular weight	58.32 g mol^{-1}		
Melting point	330°C (Decomposition)		Walter and Wajer, 2010
Melting point	340°C (Decomposition)		AluChem, 2003
Melting point	350°C		Fisher Scientific, 1999
Melting point	2800°C		Merck Chemicals - Product Information
Water solubility	Insoluble mg L^{-1}	[at 25°C]	Albemarle corporation, 2003a; Albemarle corporation, 2003b; Albemarle corporation, 2003c; Albemarle corporation, 2009; AluChem, 2003; Fisher Scientific, 1999
In vivo toxicity			
Low	LD_{50} = 8,500 mg kg^{-1}	Rats	Merck Chemicals - Product Information
Low	LD_{50} = 5,800 mg kg^{-1} bwt	No details provided	Nabaltec, 2009

Italic values are predicted: [a]Modeled, [b]Calculated, [c]Expert judgment

3. *In vitro* toxicity

In vitro toxicity data on magnesium hydroxide ($Mg(OH)_2$) are scarce (Table 4). It is expected that magnesium hydroxide dissociates in the acid environment of the stomach to Mg^{2+}. Therefore, the toxic effects of Mg^{2+} should be included in the risk assessment. For other magnesium salts several toxic effects were described (Subcommittee on Flame-Retardant Chemicals, 2000). There are not enough data to classify the *in vitro* toxicity of magnesium hydroxide.

In summary, magnesium hydroxide is a solid at room temperature and has low water solubility. Hardly any data are available on the PBT properties of this compound. $Mg(OH)_2$ displayed a low *in vivo* toxicity in two studies.

4.3 Ammonium polyphosphate

Ammonium polyphosphate (APP, CAS 68333-79-9) is an ionic inorganic polymeric compound that, due to the polymerization process, consists of a mixture of polymers of different chain lengths and degrees of branching. It is an intumescent flame retardant, which means that the compounds swells when exposed to heat, and thereby reduces heat transfer (ENFIRO, 2009). In soil and sewage sludge, APP was reported to break down rapidly into ammonia and phosphate (no reported half-life) (German Federal Environmental Agency *et al.*, 2001). When in contact with water APP undergoes slow hydrolysis with the release of ammonium phosphate (Clariant Flame Retardants, pers. comm.). The indicative production volume for the APP market in Europe is >1,500 t $year^{-1}$ in 1995 (WHO, 1997). APP is currently classified as an HPV chemical in the EU (European Chemicals Bureau, 2011). For the USA, total annual production was given as 45,000 to <227,000 t in 2006 (US EPA, 2006).

Physical-chemical properties

The physical-chemical properties of polymers strongly depend on the size or length of the polymeric chain. For example, it is usually observed that as chain length increases, melting and boiling temperatures also increase. A common means of expressing the length of a polymer chain is the degree of polymerization, in which the number of monomers incorporated into the chain is quantified. As with other molecules, a polymer's size may also be expressed in terms of molecular weight. Since synthetic polymerization techniques typically yield a polymeric product including a range of molecular weights, the weight is often expressed statistically to describe the distribution of chain lengths present (e.g., average molecular weight). According to a manufacturer (Clariant Flame Retardants, pers. comm.), APP polymers typically have a molecular weight of ca. 100,000 g mol^{-1} (based on an average chain length of 1,000; Figure 1). We assumed that all measures of physical-chemical properties involve the testing of the technical product (MW ca. 100,000 g mol^{-1}), because to our knowledge, no purified monomeric APP is currently available on the market. The reported melting point of APP was ≥275°C (European Chemicals Bureau, 2000d; German Federal Environmental Agency *et al.*, 2001) and indicates that these polymers are solids at environmentally relevant temperatures. The water solubility of APP is high and was reported as being 10 g L^{-1} or miscible with water (European Chemicals Bureau, 2000d; German Federal Environmental Agency *et al.*, 2001). The vapor pressure of this compound is <10 Pa (German Federal Environmental Agency *et al.*, 2001) or <100 Pa (European Chemicals Bureau, 2000d). However, these values are misleading (and could wrongly be interpreted as indicating high volatility); we are instead inclined to believe the manufacturer's statement (Clariant Flame Retardants, pers. comm.) that the substance is non-volatile. An overview of the physical-chemical properties of APP is shown in Table 5.

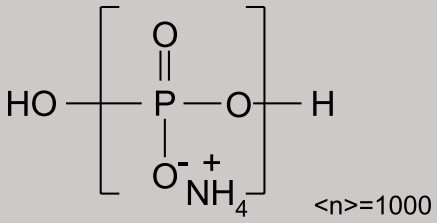

Figure 1. Schematic representation of the chemical structure of the polymer APP. The product typically consist of a mixture of polymers with an average chain length of 1000.

Bioaccumulation
APP has a low bioaccumulation potential (German Federal Environmental Agency *et al.*, 2001; Table 5), although no specific BCF values or other details were given. We estimated the Log K_{OW} as -2.15 (ACD/Labs, 2011), showing the compound to have high hydrophilicity. Because of the high aqueous solubility of the polymer and the large molecular size, APP is not expected to bioaccumulate (MW ca. 100,000 g mol^{-1}) (Dimitrov *et al.*, 2002).

Toxicity
1. Ecotoxicity
   Data from several aquatic toxicity studies indicate low to moderate toxicity to several algal species, crustaceans, and fish (Table 5).
2. *In vivo* toxicity
   Two studies on APP were reported in which the toxicity to rats was low (European Chemicals Bureau, 2000d; UNEP, 2007; Table 5).
3. *In vitro* toxicity
   Limited *in vitro* data are available for APP. No carcinogenic, endocrine, or neurotoxic data were found, although AMES test results showed no response for mutagenic activity (Table 5) (European Chemicals Bureau, 2000d). This suggests that the chemical has a low genotoxicity. APP is probably hydrolyzed by stomach acids into phosphate and ammonium ions, and various effects could be expected based on the structural similarities with other compounds, e.g., inositol polyphosphates or adenosine polyphosphates. Therefore, there were insufficient data to classify either endocrine- or neurotoxicity. Data on developmental toxicity were not available.

In summary, APP is a solid that has high water solubility and is expected to degrade in natural environments. It predominantly exerts a low toxicity on the aquatic community, although two authors reported moderate toxicity to daphnids or algae. Toxicity to rats is low, as is the reported *in vitro* toxicity, although the number of available studies is limited.

Table 5. Ammonium polyphosphate (APP, CAS nr 68333-79-9)

	Data	Details	References
Physical-Chemical Properties			
Molecular weight	≈ 100,000 g mol^{-1}		Clariant pers. comm.
Melting point	275°C		German Federal Environmental Agency *et al.*, 2001
Melting point	>275°C		European Chemicals Bureau, 2000d
Melting point	300°C (Decomposition)		German Federal Environmental Agency *et al.*, 2001
Water solubility	10000 mg L^{-1}	[at 25°C]	European Chemicals Bureau, 2000d; German Federal Environmental Agency *et al.*, 2001
Water solubility	<1000 mg L^{-1}	[at 25°C]	Budenheim, 2010
Water solubility	<5000 mg L^{-1}	[at 25°C]	Clariant, 2010
Vapor pressure	<10 Pa	[at 20°C]	German Federal Environmental Agency *et al.*, 2001
Vapor pressure	<100 Pa	[at 20°C]	European Chemicals Bureau, 2000d
Log K_{OW}	-2.15[a] Pa	[at 20°C]	ACD/Labs, 2011
Bioaccumulation			
Low	not specified		German Federal Environmental Agency *et al.*, 2001
Ecotoxicity			
Low; aquatic	NOEC = 87.6 mg L^{-1}	Algae	UNEP, 2007
Low; aquatic	EC_{50} = 813-848 mg L^{-1}	Crustacean, *Daphnia magna*, 48h[d]	McDonald *et al.*, 1996
Low; aquatic	NOEC >500 mg L^{-1}	Fish, *Danio rerio,* 96h,	Budenheim, 2010
Low; aquatic	LC_{50} >500 mg L^{-1}	Fish, *Danio rerio,* 96h	Clariant, 2010
Low; aquatic	LC_{50} = 1,326.0 mg L^{-1}	Fish, *Oncorhynchus mykiss,* 96h, pH 7	Blahm 1978 (not found) from US EPA, 2012
Low; aquatic	LC_{50} = 123.0 mg L^{-1}	Fish, *Oncorhynchus mykiss*, 96h, pH 8	Blahm 1978 (not found) from US EPA, 2012
Low; aquatic	LC_{50} >101 mg L^{-1}	Fish, fresh water	UNEP, 2007
Low; aquatic	not specified	NOEC exceeds solubility, low acute aquatic toxicity	European Chemicals Bureau, 2000d
Low; aquatic	LD_{50} >500 mg L^{-1}	Fish, fresh water	European Chemicals Bureau, 2000d
Moderate; aquatic	EC_{50} = 1.790 mg L^{-1}	Crustaceans (*Daphnia carinata*), 72 hours	UNEP, 2007

(continued)

Table 5. (continued)	Data	Details	References
Moderate; aquatic	IC_{50} = 10 mg L^{-1}	Algae, *Selenastrum capricornutum*, 96h[d]	McDonald *et al.*, 1996
In vivo toxicity			
Low	LD_{50} >2000 mg kg^{-1} bwt	Rats	UNEP, 2007
Low	LD_{50} >4740 mg kg^{-1} bwt	Rats	European Chemicals Bureau, 2000[d]
In vitro toxicity			
Low	Genotoxicity; Mutagenicity	Salmonella and E. coli, AMES test	European Chemicals Bureau, 2000[d]

Italic values are predicted: [a]Modeled, [b]Calculated, [c]Expert judgment
[d]APP has the technical name Fire-Trol LCG-R (McDonald *et al.*, 1996)

4.4 Zinc borate (ZB)

Zinc borates (ZBs) exist in different mineral compositions and have different zinc oxide and borate ratios. Additionally, some borates contain structurally bound water (hydrates). The specific compound discussed in this review is $2ZnO{\cdot}3B_2O_3$ (CAS 138265-88-0 or 12767-90-7), a non-hydrate. It is being used as a flame retardant synergist and smoke suppressor (EFRA and Cefic, 2006). ZB breaks down to zinc hydroxide and boric acid under natural conditions (EFRA and Cefic, 2006). ZB is currently classified as an LPV chemical in the EU (European Chemicals Bureau, 2011). No information is available on production volumes in the USA.

Physical-chemical properties

There is very little information on the physical-chemical properties of ZB, or even for other mineral compositions of this compound. It is solid at room temperature and decomposes at 650°C (synthetic ZB) (Borax, 2004). ZB has a solubility in water of 2.8 g L^{-1} (6.44 mol m^{-3}) (Borax, 2004).

Bioaccumulation

No information on bioaccumulation of ZB is available.

Toxicity

Zinc is an essential element for animals (Maret and Sandstead, 2006) and plants (EFRA and Cefic, 2006). However, intake of more than 100-300 mg zinc per day results in adverse health effects (Fosmire, 1990; Rout and Das, 2003). Depending on the prevailing conditions in the environment, ZB can decompose to produce freely dissolved zinc ions. The toxicity of zinc has been studied extensively (Barceloux, 1999; Cummings and Kovacic, 2009; Nagajyoti *et al.*, 2010) and will not be repeated here. The same holds true for the toxicity of boric acid (European Chemicals Bureau, 2000b).

1. Ecotoxicity
   According to the few reports available, zinc borate has a high aquatic toxicity to daphnids, algae, and several fish species (Table 6).
2. *In vivo* toxicity
   There are only a few studies on ZB toxicity (Table 6). An LOEC value of 0.91 mg L^{-1} day^{-1} was reported for humans.

3. *In vitro* toxicity

   Limited information is available in the literature on the *in vitro* toxicity of ZB. There are no data on carcinogenicity, endocrine disruption, or neurotoxicity. Zinc borate is not mutagenic (Illinois Environmental Protection Agency, 2007; Table 6). As ZB probably readily breaks down in the stomach to zinc oxide (ZnO) and boric acid (H_3BO_3), these compounds should also be included in any risk assessment. Although there are extensive databases on the toxicity of zinc oxide and boric acid (Subcommittee on Flame-Retardant Chemicals, 2000), there are not enough data to classify the *in vitro* toxicity of ZB.

In summary, zinc borate is a solid with moderate aqueous solubility. It has high aquatic toxicity, whereas reported values for *in vivo* toxicity vary from low to high. According to the results of one study, zinc borate has low mutagenicity; however, there is a lack of information on the *in vitro* toxicity of the compound.

Table 6. Zinc borate (ZB, CAS nr 138265-88-0 or 12767-90-7)

	Data	Details	References
Physical-Chemical Properties			
Molecular weight	434.66 g mol^{-1}		
Melting point	650°C (Decomposition)		Borax, 2004
Water solubility	2,800 mg l^{-1}	[at 25°C]	Borax, 2004
Ecotoxicity			
High; aquatic	EC_{50} = 0.015-0.178 mg L^{-1}	Algal inhibition	Illinois Environmental Protection Agency, 2007
High; aquatic	EC_{50} = 0.068-1.59 mg L^{-1}	Daphnia	Illinois Environmental Protection Agency, 2007
High; aquatic	LC_{50} = 0.59-5.9 mg L^{-1}	Fish	Illinois Environmental Protection Agency, 2007
High; aquatic	not specified	Aquatic species	EFRA and Cefic, 2006; UNEP, 2009b
In vivo toxicity			
Low	LD_{50} >10,000 mg kg^{-1}	Rats & rabbits, oral and dermal exposure	EFRA and Cefic, 2006
Low	LD_{50} >2,000 mg kg^{-1}	Rat, mice, dog	Illinois Environmental Protection Agency, 2007
High	LOEC = 0.91 mg L^{-1} day^{-1}	Humans, zinc blood effects	Illinois Environmental Protection Agency, 2007
High	Can be harmful to the unborn	Not specified	McPherson *et al.*, 2004
In vitro toxicity			
Low	Genotoxicity; Mutagenicity	-	Illinois Environmental Protection Agency, 2007

-, No effects observed

4.5 Zinc hydroxystannate

Zinc hydroxystannate (ZHS; $ZnSn(OH)_6$, CAS 12027-96-2) is an inorganic, bimetallic hydroxide used as a smoke suppressant (William Blythe, 2010a). No information is available on production volumes in the EU (European Chemicals Bureau, 2011). For the USA, total annual production was <227 t in 2006 (US EPA, 2006).

Physical-chemical properties

ZHS decomposes at 180-200°C (Australian Government Regulator of Industrial Chemicals, 1994; ITRI, 2009; Rio Tinto Alcan (RTA), 2008b), at these temperatures dehydroxylation occurs, releasing water from the crystal (William Blythe Ltd., pers. comm.). It has a low water solubility of 1 mg L^{-1} (0.0035 mol m^{-3}) (Australian Government Regulator of Industrial Chemicals, 1994; Rio Tinto Alcan (RTA), 2008b) (primary source not stated). The vapor pressure and Log K_{OW} of ZHS were reported to be low (Table 7).

Table 7. Zinc Hydroxystannate (ZHS, CAS nr 12027-96-2)

	Data	Details	References
Physical-Chemical Properties			
Molecular weight	286.11 g mol^{-1}		
Melting point	>180°C (Decomposition)		ITRI, 2009
Melting point	200°C (Decomposition)		Australian Government Regulator of Industrial Chemicals, 1994; Rio Tinto Alcan (RTA), 2008b, William Blythe Ltd. (pers. comm.)
Water solubility	1 mg L^{-1}	[at 20°C]	Australian Government Regulator of Industrial Chemicals, 1994; Rio Tinto Alcan (RTA), 2008b
Water solubility	Insoluble		William Blythe, 2010a
Vapor pressure	<10 Pa	[at 20°C]	Australian Government Regulator of Industrial Chemicals, 1994; William Blythe, 2010a
Log K_{OW}	<-1.05		Australian Government Regulator of Industrial Chemicals, 1994
Log K_{OW}	<-1		William Blythe Ltd. (pers. comm.)
Log K_{OW}	<0.09		Rio Tinto Alcan (RTA), 2008b
Bioavailability			
Low	*Low potential estimated*[c]	Based on low K_{OW} value, no specified data	William Blythe Ltd. (pers. comm.)
Ecotoxicity			
Low; aquatic	LD_{50} >3.3 mg L^{-1}	Fish, NOEC>water solubility	Joseph Storey & Co. Ltd., 1994
Low; aquatic	EC_{50} >3.3 mg L^{-1}	Crustaceans, NOEC> water solubility	Joseph Storey & Co. Ltd., 1994

(continued)

Table 7. (continued)	Data	Details	References
Low; aquatic	LC_{50} >0.079 mg L^{-1}	Rainbow Trout, LC_{50}> water solubility, acute, no details provided	William Blythe, 2010a
Low; aquatic	EC_{50} >0.023 mg L^{-1}	Daphnia magna, EC_{50}>water solubility, 48 hours, no details provided	William Blythe, 2010a
In vivo toxicity			
Low	LD_{50} >5,000 mg kg^{-1} bwt	Rats	Gardner, 1988a; Joseph Storey & Co. Ltd., 1994
Low	LD_{50} >2,466 mg kg^{-1}	Rats, dermal exposure	Joseph Storey & Co. Ltd., 1994
Low - Moderate	LD_{50} >4.3 mg L^{-1}	Rats, inhalation exposure	Joseph Storey & Co. Ltd., 1994
In vitro toxicity			
Low	Genotoxicity; mutagenicity	- Salmonella, with and without metabolic activation (S9), AMES test	Australian Government Regulator of Industrial Chemicals, 1994, William Blythe Ltf. (pers. comm.)

Italic values are predicted: [a]Modeled, [b]Calculated, [c]Expert judgment
-, no effects observed

Bioaccumulation
According to one producer of ZHS, the bioaccumulation potential is estimated to be low, based on its low water solubility and low K_{OW} value (Table 7). There is no further information on the bioaccumulation of ZHS.

Toxicity
As stated previously for ZB, zinc is an essential element for animals (Maret and Sandstead, 2006) and plants (EFRA and Cefic, 2006). However, intake of more than 100-300 mg zinc per day produces adverse health effects (Fosmire, 1990; Rout and Das, 2003). Zinc hydroxide can decompose to produce freely dissolved Zn^{2+} ions, depending on the prevailing conditions in the environment. The toxicity of zinc has been discussed extensively elsewhere (Barceloux, 1999; Cummings and Kovacic, 2009; Nagajyoti *et al.*, 2010) and is not repeated here.

1. Ecotoxicity
   Low ecotoxicity of ZHS for fish and crustaceans was reported in two studies, in which the NOECs and EC_{50}s exceeded the water solubility (Joseph Storey & Co. Ltd., 1994; William Blythe, 2010a). In the latter study, the EC_{50} and LC_{50} values exceeded 0.02 mg L^{-1}. However, this is still a very low concentration and the NOEC or LOEC values from this study are inconclusive.
2. *In vivo* toxicity
   There is very limited information on the *in vivo* toxicity of ZHS. A few studies provided data to show low acute toxicity to orally exposed rats (Table 7). Low to moderate toxicity was reported for ZHS in an inhalation exposure study (Joseph Storey & Co.

Ltd., 1994). However, because this LD_{50} value was reported as being "greater than" a moderate value, it is not clear whether this value represented just the highest concentration tested, or whether there was an effect observed at this level.

3. *In vitro* toxicity
   In vitro toxicity data for ZHS are also scarce, and no carcinogenic, endocrine disrupting, or neurotoxic data were available. One author reported no mutagenic activity in an AMES test performed with and without metabolic activation (Australian Government Regulator of Industrial Chemicals, 1994).

In summary, ZHS is a solid that has low water solubility. It presumably has a low aquatic toxicity. With only two studies available, the effects on rats vary from low to moderate. There are not enough data to fully classify the *in vitro* toxicity of the compound; only one study existed, and no mutagenic effects in an AMES test was reported therein.

4.6 Zinc stannate

Zinc stannate (ZS; ZnSnO 3 , CAS 12036-37-2) is an inorganic, bimetallic oxide used as a smoke suppressant (William Blythe, 2010b). No information is available on production volumes in the EU (European Chemicals Bureau, 2011). For the USA, total annual production was <227 t in 2006 (US EPA, 2006).

Physical-chemical properties
ZS decomposes at 397-570°C (Gelest, 2008; ITRI, 2009; Rio Tinto Alcan (RTA), 2008c) (Table 8). It has low solubility in water that varies between 1 and 13 mg L^{-1} (0.0043 and 0.056 mol m^{-3}) (Gelest, 2008; Rio Tinto Alcan (RTA), 2008c) (primary sources are not stated).

Bioaccumulation
According to one producer, ZS is estimated to have a low bioaccumulation potential, based on low water solubility and low K_{OW} values (Table 8). There is no further information on the bioaccumulation of ZS.

Toxicity
The toxicity of the zinc ion is discussed in the section of ZB and ZHS and is not repeated here. The toxicity of ZS is discussed below.

1. Ecotoxicity
   Low ecotoxicity of ZS for fish and crustaceans was reported in one study (Table 8), in which LC_{50} and EC_{50} values probably exceeded the water solubility (William Blythe, 2010b). In this study, the EC_{50} and LC_{50} values were >0.02 mg L^{-1}. However, this is still a very low concentration and it is not clear what the definitive NOEC or LOEC values from this study were.
2. *In vivo* toxicity
   There is very limited information about the *in vivo* toxicity of ZS. A low acute toxicity to orally exposed rats was reported in one study (Gardner, 1988b; Table 8).
3. *In vitro* toxicity
   There is no information on carcinogenic, endocrine disrupting, or neurotoxic effects of ZS.

In summary, zinc stannate is a solid with low water solubility. One author reported low toxic effect on rats. There is an obvious lack of data on other PB&T properties.

Table 8. Zinc stannate (ZS, CAS nr 12036-37-2)

	Data	Details	References
Physical-Chemical Properties			
Molecular weight	232.10 g mol^{-1}		
Melting point	>397°C (Decomposition)		Rio Tinto Alcan (RTA), 2008c
Melting point	>570°C		Gelest, 2008; ITRI, 2009
Water solubility	Insoluble		William Blythe, 2010b
Water solubility	1 mg L^{-1}	[at 20°C]	Gelest, 2008
Water solubility	13 mg L^{-1}	[at 25°C]	Rio Tinto Alcan (RTA), 2008c
Vapor pressure	<0.13 Pa	[at 25°C]	Gelest, 2008
Vapor pressure	<10 Pa	[at 20°C]	William Blythe, 2010b
Bioavailability			
Low	*Low potential estimated*[c]	Based on low K_{OW} value, no specified data	pers. comm. William Blythe Ltd
Ecotoxicity			
Low; aquatic	LC_{50}>0.079 mg L^{-1}	Rainbow Trout, LC_{50}> water solubility, acute, no details provided	William Blythe, 2010b
Low; aquatic	EC_{50}>0.023 mg L^{-1}	*Daphnia magna*, EC_{50}> water solubility, 48 hours, no details provided	William Blythe, 2010b
In vivo toxicity			
Low	LD_{50}>5 g kg^{-1} bwt	Rats	Gardner, 1988b

Italic values are predicted: [a]Modeled, [b]Calculated, [c]Expert judgment

5. Organophosphorus flame retardant compounds and their salts

The following organophosphorus HFFRs are discussed in this section: triphenylphosphate (TPP), resorcinol bis(diphenylphosphate) (RDP), bisphenol-A bis(diphenylphosphate) (BDP), dihydrooxaphosphaphenanthrene (DOPO), and aluminum diethylphosphinate (Alpi).

5.1 Triphenylphosphate

Triphenylphosphate (TPP, CAS 115-86-6) is an aryl phosphate, mainly being used as a flame retardant in polymers (European Chemicals Bureau, 2002), and is the best studied compound of the selected HFFRs (Bergh *et al.*, 2011; Hoenicke *et al.*, 2007). It is present in all environmental compartments, ranging from, e.g., air (23.2 ng m^{-3}) (Danish EPA *et al.*, 1999) to fish (21-180 ng g^{-1}) (Sundkvist *et al.*, 2010). The global production (excluding East Europe) was estimated to be 20,000-30,000 t in one study (UNEP, 2002). Of this production estimate, approximately 25% was produced in Western Europe, 40% in the USA and 35% in Asia by 15 producers (UNEP, 2002). TPP is classified as an HPV chemical in the EU (European Chemicals Bureau, 2011). For the USA, the total annual production was given as 4,500 to <22,700 t in 2006 (US EPA, 2006).

Physical-chemical properties
TPP is solid at environmentally relevant temperatures and has a relatively low melting point of approximately 50°C (European Chemicals Bureau, 2000e; Hilal *et al.*, 2003b; Merck & Co. Inc., 2006; US EPA, 2011). Its water solubility is low, showing a wide range from 55 μg/L to 5 mg L^{-1} (1.70E-4 to 1.43E-2 mol m^{-3}) and thereby varies by a factor of about 100 (Eckert and Klamt, 2010; European Chemicals Bureau, 2000e; Hilal *et al.*, 2004a; Saeger *et al.*, 1979; US EPA, 2011). In Table 9, we present an overview of the physical-chemical properties of TPP. The low water solubility, Henry's law constant, and K_{AW} vs the high Log K_{OW} indicate that, once released into the environment, TPP probably partitions mainly into organic and lipid-rich compartments such as soil and biota.

Persistence
Degradation data on TPP in the atmosphere, water and soil/sediment, are also presented in Table 9. Atmospheric half-lives are predominantly determined by photolysis (break-down of the compound by light), and therefore the half-life of TPP is often measured by testing the photolysis rate. In water, degradation can occur by abiotic, e.g., hydrolysis (reaction of the compound with water), or biotic (mediated by microorganisms) mechanisms. The persistence of this compound is classified as varying from high to low (Table 9). The fastest atmospheric degradation rate of TPP was reported to be a few hours (European Chemicals Bureau, 2000e), whereas the longest degradation time was reported to be 406 days in water (European Chemicals Bureau *et al.*, 2007; US EPA, 2005). Given the large variation in the persistence data, we additionally estimated TPP degradation by using EPI Suite 4.1. The resulting degradation half-lives from these estimates were 24 h in air, 900 h in water, and 1,800 h in soil; these estimated values are within the ranges of experimental values. On the basis of these data, this substance is expected to be stable and persistent in the environment.

Bioaccumulation
The bioaccumulation of TPP has mainly been studied in fish, and results vary from low to high for different species (Table 9). A BCF as low as 0.06 was reported for *Phoxinus phoxinus*, a fresh water minnow (Bengtsson *et al.*, 1986; European Chemicals Bureau, 2000e), and a BCF as high as 1,743 for *Pimephales promelas*, the fathead minnow (US EPA, 2005). Our literature research revealed remarkably diverging opinions on what is considered to be a high bioconcentration factor. The Clean Production Action reported a high BCF for TPP of >100 (Clean Production Action, 2007), whereas the Illinois EPA reported a low potential, with a BCF as high as 2,590 (Illinois Environmental Protection Agency, 2007). In comparison, the REACH criterion states that a compound with a BCF larger than 500 is classified as bioaccumulative (European Union, 2008); Table 2). We believe that the order of magnitude in the REACH guidelines (i.e., BCF >500) is more realistic concerning what should be considered as potentially bioaccumulative. On the basis of this classification, most experimental data indicate that this compound is bioaccumulative.

Toxicity
1. Ecotoxicity
   The aquatic toxicity of TPP is, in many cases, high to many types of fish, algae, and crustaceans, since the LC_{50}s recorded for these species is about or lower than 1 mg L^{-1} (Table 9).
2. *In vivo* toxicity
   Many low effect concentrations were reported for higher organisms (e.g., rodents), as shown in Table 9. Hence, the toxicity to these species is considered to be low.

3. *In vitro* toxicity
An overview of the *in vitro* toxicity data is shown in Table 9. TPP is not considered to be a potent anticholinesterase agent, however, exposure to 150 to 300 mg kg^{-1} bwt^{-1} TPP does inhibit cholinesterase in rats, *in vitro* as well as *in vivo* (Bingham *et al.*, 2001). TPP may be metabolized into diphenylhydroxyphenolphosphate and diphenylphosphate (Eto *et al.*, 1975; Snyder, 1990). Several *in vitro* effects were reported in rats exposed to a commercial cresyldiphenylphosphate product that contains TPP (Vainiotalo *et al.*, 1987). Several neurotoxic effects were observed in *in vitro* studies, e.g., cytotoxicity in PC12 cells and inhibition of the GABA-regulated chloride channel (Flaskos *et al.*, 1994; ant *et al.*, 1987; Padilla *et al.*, 1987; Vainiotalo *et al.*, 1987). However, the neurotoxicity of TPP has been debated since the early studies of Smith *et al.* (Smith *et al.*, 1930; Smith *et al.*, 1932), because neurotoxic changes in animals after short-term exposure were not identified in other studies (Wills *et al.*, 1979). Since TPP exposure does result in several toxic effects, the *in vitro* endocrine and neurotoxicity is classified as being low to high.

In summary, TPP is a solid with low, but uncertain water solubility and a high potential to partition into lipids. There are many studies available on the PBT properties of TPP. The degradation rate of TPP in air is fast. In water, as well as in sediment and soil, contradictory results on persistence were found. Both long and short dissipation times were reported. Estimations with EPI Suite con firm high persistence in water and soil. The bioaccumulation potential of TPP depends on the species exposed and ranges from low to high values. The ecotoxicity of this HFFR is predominantly moderate to high, whereas other *in vivo* toxicity is low. For *in vitro* toxicity, a low genotoxicity and high neurotoxicity were reported. Based on all the observed adverse effects, TPP is labeled as a compound with dangerous effects for the environment by the (ECHA Database, Accessed 2011).

5.2 Resorcinol bis(diphenylphosphate)

Resorcinol bis(diphenylphosphate) (RDP, CAS 57583-54-7) is a polymeric aryl phosphate and a flame retardant (ICL, 2011), typically consisting of a mixture of oligomers having chain lengths between 1 and 7 (Figure 2). The influence of chain length on the properties of the compound is discussed in the section on APP. Little information is available on the occurrence of RDP in the environment. RDP has been measured in the indoor environment and was present at around 1 ng m^{-3} in domestic air, and at a level of 1,700 ng g^{-1} in house dust (Matsukami *et al.*, 2010). RDP is currently classified as an LPV chemical in the EU (European Chemicals Bureau, 2011). For the USA, total annual production was <227 t in 2006 (US EPA, 2006).

Physical-chemical properties

We assume that all physical-chemical properties reported are based on tests with the technical product (CAS 57583-54-7), because to our knowledge, no purified monomeric RDP is currently available on the market. The monomer of RDP weighs 574.47 g mol^{-1} and for $n = 7$, the polymer weighs 2,063.50 g mol^{-1}. RDP is a liquid at room temperature. In Table 10, we show an overview of the physical-chemical properties of this compound. The solubility of RDP in water was measured as being low (1.5 mg L^{-1}, ICL, pers. comm.). Since there are no experimental data for Log K_{AW} or Log K_{OW}, we estimated values for these properties (Table 10). The vapor pressure, Henry's law constant, and K_{AW} value were all estimated to be low. The Log K_{OW} value was predicted to be high, i.e., up to 11.09 (Hilal *et al.*, 2004a), showing a preference for partitioning into organic and lipid-rich phases such as soil and biota.

Table 9. Triphenylphosphate (TPP, CAS nr 115-86-6)

	Data	Details	References
Physical-chemical properties			
Molecular weight	326.29 g mol^{-1}		
Melting point	48-50°C		ICL, pers. comm.
Melting point	49-50°C		European Chemicals Bureau, 2002; Merck & Co. Inc., 2006
Melting point	50.5°C		Hilal *et al.*, 2003a; US EPA, 2011
Melting point	*86.5*[a]°C		US EPA, 2011
Water solubility	*5.55E-2*[a] mg L^{-1}	[at 25°C]	Hilal *et al.*, 2003b
Water solubility	2.5E-2 mg L^{-1}	[at 25°C]	European Chemicals Bureau, 2002
Water solubility	7.5E-1 mg L^{-1}	[at 25°C]	European Chemicals Bureau, 2002
Water solubility	<1 mg L^{-1}	[at 25°C]	ICL (pers. comm.)
Water solubility	*1.03*[a] mg L^{-1}	[at 25°C]	US EPA, 2011
Water solubility	1.9 mg L^{-1}	[at 25°C]	European Chemicals Bureau, 2002; Saeger *et al.*, 1979
Water solubility	*2.66*[a] mg L^{-1}	[at 25°C]	Eckert and Klamt, 2010
Water solubility	*4.67*[a] mg L^{-1}	[at 25°C]	US EPA, 2011
Vapor pressure	1.5E-6 Pa	[at 25°C]	European Chemicals Bureau, 2002
Vapor pressure	*4E-6*[a] Pa	[at 25°C]	Eckert and Klamt, 2010
Vapor pressure	*6.29E-5*[a] Pa	[at 25°C]	US EPA, 2011
Vapor pressure	*1.65E-4*[a] Pa	[at 25°C]	ACD/Labs, 2011
Vapor pressure	8.35E-4 Pa	[at 25°C]	ICL, (pers. comm.)
Vapor pressure	8.37E-4 Pa	[at 25°C]	Dobry and Keller, 1957
Vapor pressure	*3.82E-2*[a] Pa	[at 25°C]	Hilal *et al.*, 2003b
Henry's Law constant	*4.03E-3*[a] Pa m^3 mol^{-1}		US EPA, 2011
Henry's Law constant	3.35E-1 Pa m^3 mol^{-1}		US EPA, 2011

(continued)

Table 9. (continued)	Data	Details	References
Henry's Law constant	1.22 Pa m^3 mol^{-1}		Syracuse Research Corporation, 2006
Log K_{OW}	4.59		Syracuse Research Corporation, 2006
Log K_{OW}	4.6		Saeger *et al.*, 1979
Log K_{OW}	<4.77		European Chemicals Bureau *et al.*, 2007
Log K_{OW}	*4.9*[a]		Eckert and Klamt, 2010
Log K_{OW}	*6.78*[a]		Hilal *et al.*, 2003b
Log K_{AW}	-3.87		US EPA, 2011
Log K_{AW}	*-6.66*[a]		Eckert and Klamt, 2010
Persistence			
Low	-not specified; primary source not found-		CEPA, 2007; Clean Production Action , 2007
Low; water	DT_{50} = 1.2-2 days	pH 8.8, natural water	UNEP, 2002; WHO, 1997
Low; water	DT_{50}=1.3 days	21°C, pH 9.5, hydrolysis	UNEP, 2002
Low; water	DT_{50} = 1.3 days	21°C, pH 9.5, hydrolysis	Howard and Deo 1979 from UNEP, 2002
Low; water	DT_{50} = 3 days	pH 9, 25°C	European Chemicals Bureau, 2000e
Low; water	DT_{50} = 3 days	pH 9, hydrolysis	Mayer *et al.*, 1981
Low; water	DT_{100} <7 days	River water	WHO, 1991
Low; water	$DT_{50\text{-}100}$ <8 days	River die-away test (presumably primary degradation)	US EPA, 2005
Low; water	DT_{50} <5 days	Hydrolysis, 20°C, pH 9	European Chemicals Bureau, 2000e; US EPA, 2005
Low; water	DT_{50} = 7.5 days	21°C, pH 8.2, hydrolysis	UNEP, 2002
Low; water	DT_{50} = 19 days	pH 7, hydrolysis	Mayer *et al.*, 1981
Low; water	DT_{50} = 19 days	pH 7, 25°C	European Chemicals Bureau, 2000e
Low; water	DT_{50} >28 days	pH 5, 25°C	European Chemicals Bureau, 2000e

(continued)

Table 9. (continued)	Data	Details	References
Low; water	DT_{50}>28 days	pH 5, 25°C	European Chemicals Bureau, 2000e
Low; water	DT_{50}>28 days	pH 5, hydrolysis	Mayer *et al.*, 1981
Low; soil & sediment	DT_{50} = 3-12 days	(An)aerobic, river water/sediment and pond sediment	European Chemicals Bureau *et al.*, 2007; European Chemicals Bureau, 2000e
Low; soil & sediment	DT_{50} = 21 days	Anaerobic	UNEP, 2002
Low; sludge	$DT_{83\text{-}94}$<28 days (Ready biodegradable)		US EPA, 2005; UNEP, 2002
Low; sludge	Inherently biodegradable, degrades rapidly in pond and river sediment (not specified)	Aerobic	Danish EPA *et al.*, 2007
High; water	*DT_{50} = 37.5[a] days (900 hours)*	Primary degradation	US EPA, 2011
High; soil & sediment	DT_{50} = 32 days	Anaerobic	European Chemicals Bureau, 2000e
High; soil & sediment	DT_{50} = 37 days	5mg kg^{-1}, 20°C, BBA standard soil 2.2 1993, loamy sand	European Chemicals Bureau, 2000e; UNEP, 2002
High; soil & sediment	DT_{22} = 40 days	Aerobic	UNEP, 2002
High; soil & sediment	DT_{10} = 40 days (river sediment)	Aerobic mineralization, 90% primary degradation, river sediment	US EPA, 2005
High; soil & sediment	DT_{50} = 50-60 days (pond hydrosoil)	Anaerobic	European Chemicals Bureau *et al.*, 2007; Muir *et al.*, 1989
High; soil & sediment	*DT_{50} = 75[a] days (1800 hours)*	(An)aerobic	US EPA, 2011
High; soil & sediment	*DT_{50} = 377.5[a] days (8100 hours)*	Soil, primary degradation	US EPA, 2011

(continued)

Table 9. (continued)	Data	Details	References
High; soil & sediment	Partially degradable in river sediment and soil (not specified)	Sediment, primary degradation	Danish EPA *et al.*, 2007
n.c.; atmospheric	DT_{50} <1-12 hours	Anaerobic	European Chemicals Bureau, 2000e
n.c.; atmospheric	DT_{50} <1-12 hours	Anaerobic	European Chemicals Bureau, 2000e
High; soil & sediment	*DT_{50} = 377.5[a] days (8100 hours)*	Soil, primary degradation	US EPA, 2011
High; soil & sediment	Partially degradable in river sediment and soil (not specified)	Sediment, primary degradation	Danish EPA *et al.*, 2007
n.c.; atmospheric	DT_{50} <1-12 hours	Anaerobic	European Chemicals Bureau, 2000e
n.c.; atmospheric	DT_{50} = 12 hours	Photolysis	European Chemicals Bureau *et al.*, 2007; US EPA, 2005
n.c.; atmospheric	DT_{50} = 12 hours	Primary degradation	European Chemicals Bureau *et al.*, 2007
n.c.; atmospheric	*DT_{50} = 23.7[a] hours*	Primary degradation, modeled	US EPA, 2011
n.c.; atmospheric	DT_{50} = 36[e] hours	Photolysis	Draft UK Environment Agency *et al.*, 2009b
Bioaccumulation			
Low	BCF = 110-144	Fish species, fresh water	UNEP, 2002
	BCF <50	*Lemna minor* (duck weed) and *Typha sp.* (cat tail)	
Low	BCF = 420[e] kg L^{-1}	Fish	Draft UK Environment Agency *et al.*, 2009b; UK Environment Agency *et al.*, 2009a
Low	*BCF = 73.18[a] and BCF = 74.23[a] L kg^{-1} wet-wt*	-not specified-	CEPA, 2007
Low to high	BCF = 0.06-271, (*1,800*[b] calculated)	Fish species, fresh water	Danish EPA *et al.*, 2000; European Chemicals Bureau, 2000e; McPherson *et al.*, 2004
Low to high	BCF = 110-500	Fish species, *Carassius auratus* and *Oryzias latipes*	Sasaki *et al.*, 1981

(continued)

Table 9. (continued)	Data	Details	References
Low to high	BCF = 6 -18,900	Fish species	Value range from WHO, 1991[d]
Low to high	BCF = 18-2,590	Species not specified	Illinois Environmental Protection Agency, 2007
Low to high	BCF = 132 - 1,743	Fish; 132-264 rainbow trout and 218-1743 fathead minnow	US EPA, 2005
Low to high	LC_{50} = 0.24 - 290 mg L^{-1}	Various fresh water species	European Chemicals Bureau, 2000e
Low to high	LC_{50} = 0.36-290 mg L^{-1}	Fish	Illinois Environmental Protection Agency, 2007; WHO, 1991
Moderate	EC_{50} = 5 mg L^{-1}	Algae	WHO, 1991
Moderate	EC_{50} = 2.0 mg L^{-1}	Algae	US EPA, 2005
Moderate	EC_{50} = 2 mg L^{-1}	Algae, growth inhibition	Danish EPA *et al.*, 2007
Moderate	LC_{50} = 1.2 mg L^{-1}	Daphnids	US EPA, 2005
Moderate	LC_{50} = 1.0-1.2 mg L^{-1}	Daphnids	Illinois Environmental Protection Agency, 2007
Moderate	LC_{50} = 1.0 mg L^{-1}	Daphnids	WHO, 1991
Moderate to high	LC_{50}=1-1.35 mg L^{-1}	Crustaceans	Danish EPA *et al.*, 2007
Moderate to high	LC_{50}=0.36-1.2 mg L^{-1}	Fish	Danish EPA *et al.*, 2007
Moderate to high	LC_{50} >0.32-1.2 mg L^{-1}	Various fresh water species, with solvent	European Chemicals Bureau, 2000e
Moderate to high	LD_{50} = 0.290 -290 mg L^{-1}	Fish	Danish EPA *et al.*, 2000
Moderate	EC_{50} = 5 mg L^{-1}	Algae	WHO, 1991
Moderate to high	EC_{50} = 0.26-2.0 mg L^{-1}	Algae, growth inhibition	Danish EPA *et al.*, 2000; Illinois Environmental Protection Agency, 2007
Moderate to high	EC_{50} >0.18-1.00 mg L^{-1}	Invertebrates	European Chemicals Bureau, 2000e; UNEP, 2002
High	LC_{50} = 0.870 mg L^{-1}	Fish	US EPA, 2005
High	LC_{50} = 0.4 mg L^{-1}	Fish	Mayer *et al.*, 1981
High	LC_{50} = 0.31 mg L^{-1}	Trout	CEPA, 2007

(continued)

Table 9. (continued)	Data	Details	References
High	EC_{50} = 0.26-0.5 mgL^{-1}	Algae, growth inhibition	European Chemicals Bureau, 2000e; WHO, 1991
High	LC_{50} = 0.140-0.600 mg L^{-1}	Algae, growth inhibition, chronic	US EPA, 2005
High	LC_{50} = 0.1 mg L^{-1}	Daphnids, chronic exposure	US EPA, 2005
High	LC_{50} = 0.09-0.140 mg L^{-1}	Fish, chronic exposure	US EPA, 2005
High	-not specified (primary source not found)-		Clean Production Action, 2007; European Chemicals Bureau *et al.*, 2007
n.c.	NOEC = 0.0014 mg L^{-1}	Fish, chronic exposure	Illinois Environmental Protection Agency, 2007; WHO, 1991
n.c.	EC_{10} = 0.037 mg L^{-1}	Fish (*Oncorhynchus mykissi*), 30 days, since EC_{10} is very low, might expect high ecotoxicity	ICL pers. comm.
n.c.	Lowest chronic NOEC <0.01 mg L^{-1}		UK Environment Agency *et al.*, 2003
n.c.	NOEC = 0.1 mg L^{-1}	Algae, growth inhibition, chronic exposure	Danish EPA *et al.*, 2007
n.c.	NOEC = 0.1 mg L^{-1}	Daphnids, chronic exposure	Illinois Environmental Protection Agency, 2007
n.c.	NOEC = 0.25-2.5 mg L^{-1}	Algae (*Selenastrum capricornutum, Scenedesmus subspicatus, Chlorella vulgaris*), 96 h	ICL pers. comm.
In vivo toxicity			
Low	LD_{50} = 1,300 mg kg^{-1} bwt	Mice	Danish EPA *et al.*, 2007
Low	LD_{50} = 1,300- 10,800 mg kg^{-1} bwt	Mice, Rats, Cats	European Chemicals Bureau, 2000e; UNEP, 2002; WHO, 1991
Low	LD_{50} >3,000 mg kg^{-1} bwt	Mice, Rats, Cats	Danish EPA *et al.*, 2007
Low	LD_{50} >3,000 mg kg^{-1} bwt	Guinea pigs, Hen	European Chemicals Bureau, 2000e; UNEP, 2002; WHO, 1991
Low	LD_{50} = 3,500-20,000 mg kg^{-1} bwt	Rats	Danish EPA *et al.*, 2007; European Chemicals Bureau *et al.*, 2007; Illinois Environmental Protection Agency, 2007; Merck Chemicals - Product Information

(continued)

Tabel 9. (continued)	**Data**	**Details**	**References**
Low	LD_{50} >5,000 mg kg^{-1} bwt	Mice, Rats, Rabbits	US EPA, 2005
Low	LD_{50} >7,900 mg kg^{-1}	Rabbit, dermal exposure	US EPA, 2005
Low	LD_{50} >7,900 mg kg^{-1}	Rabbit, dermal exposure	Danish EPA *et al.*, 2007; Merck Chemicals - Product Information
Low	LD_{50} >8,000 mg kg^{-1}	Mammal (not specified), dermal exposure	US EPA, 2005
In vitro toxicity			
Low	Genotoxicity; Mutagenicity	Salmonella, AMES test	Danish EPA *et al.*, 1999; Illinois Environmental Protection Agency, 2007; Zeiger *et al.*, 1988
Low	Genotoxicity; Carcinogenicity	Expected low for human and animals	US Department of Health and Human Services *et al.*, 2009; Washington State Department of Ecology and Department of Health, 2006
Low	Neurotoxicity; Cytotoxicity	IC_{50} = 800 μM PC12 cells	Flaskos *et al.*, 1994
High	Neurotoxicity; Cholinesterase inhibitor -not further specified-		Bingham *et al.*, 2001
n.c.	Endocrinetoxicity; Metabolization	Diphenylphosphate (rat liver microsomes) and diphenyl p-hydroxyphenol phosphate (houseflies) houseflies, rats and goldfish	Eto *et al.*, 1975; Sasaki *et al.*, 1981; Snyder, 1990

Italic values are predicted : [a]Modeled, [b]calculated; [c]expert judgement; n.c., not enough data to classify; [d]For BCFs we could not find all primary; references from/or reported values were not corresponding to the references stated in (WHO, 1991); [e]These references (UK Environment Agency *et al.*, 2009b; US Department of Health and Human Services *et al.*, 2009), are draft reports, so reported values may be not final

Persistence

Data on RDP degradation in water were contradictory, with examples of low as well as high persistence reported. However, the degradation rate in the atmosphere is fast (see Table 10). One author reported high persistency in sludge (UK Environment Agency *et al.*, 2009a). In agreement, the estimated degradation half-lives using EPI Suite 4.0 (US EPA, 2011) indicted a high persistence; i.e., 12.1 h in air, 38 days in water, and more than 75 days in soil or sediment.

Bioaccumulation

There are very few data available on the bioaccumulation of RDP (Table 10). The available references give highly variable data with both high and low bioaccumulation values potential reported. There were little or no details given concerning the test species used or test conditions.

Toxicity

1. Ecotoxicity
   The ecotoxicity of RDP varies between low for some fish, algae, and bacteria (Illinois Environmental Protection Agency, 2007; Washington State Department of Ecology and Department of Health, 2006) and high for daphnids (Illinois Environmental Protection Agency, 2007; Washington State Department of Ecology and Department of Health, 2006) (Table 10).
2. *In vivo* toxicity
   The toxicity of RDP for rodents is mostly very low, as can be seen in Table 10, with the exception of two studies, in which moderate toxicity was reported Two reports mention the presence of TPP as an impurity in RDP (Clean Production Action, 2007; German Federal Environmental Agency *et al.*, 2001). The latter quantified the TPP content to be less than 5%. The presence of TPP, as well as the potentially formed toxic TPP-like products, may have an impact on the toxicity of technical RDP, particularly if the exposure is extended.
3. *In vitro* toxicity
   Toxicity data on RDP are scarce. However, as shown in Table 10, no mutagenic effects have been observed. Major fecal metabolites reported are also presented in Table 10. Animal studies do not show adverse biological effects for teratogenic and developmental endpoints at concentrations up to 20,000 mg kg^{-1} diet (Henrich *et al.*, 2000; Ryan *et al.*, 2000). Toxic effects at higher doses could be expected, since commercial RDP contains up to 5% TPP.

In summary, RDP has a low water solubility and high potential for partitioning into organic matter and lipid phases. Considering these characteristics, the high hydrophobicity of this compound and propensity to partition into soil and sediments, RDP might be persistent in the environment; however high as well as low dissipation times were reported. Bioaccumulation is poorly studied and RDP is classified as being low to highly bioaccumulative, although details of the studies cited were not reported. The *in vivo* (eco) toxicity varies from low to high, with special concern for the TPP impurities present in the commercial product. TPP-like products might also be formed as toxic breakdown products. There is, however, a lack of studies in which this phenomenon has been examined. There are very little data on the *in vitro* toxicity of RDP, although no mutagenic effects in an AMES test were reported in one study.

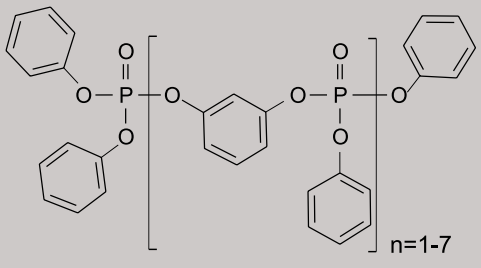

Figure 2. Schematic representation of the chemical structure of the polymer RDP. The product typically consists of a mixture of oligomers with a chain length varying from 1 to 7.

Table 10. Resorcinol bis(diphenylphosphate) (RDP, CAS nr 57583-54-7)

	Data	Details	References
Physical-chemical properties			
Molecular weight	574.47 g mol^{-1}		
Melting point	*90.27*[a]°C		US EPA, 2011
Water solubility	*1.65E-6*[a] mg L^{-1}	[at 25°C]	Hilal *et al.*, 2004a
Water solubility	*1.11E-4*[a] mg L^{-1}	[at 25°C]	Meylan *et al.*, 1996; US EPA, 2011
Water solubility	*6.88E-3*[a] mg L^{-1}	[at 25°C]	US EPA, 2011
Water solubility	1.05 mg L^{-1}	[at 25°C]	ICL (pers. comm.)
Vapor pressure	*5.01E-11*[a] Pa	[at 25°C]	ACD/Labs, 2011
Vapor pressure	*5.29E-7*[a] Pa	[at 25°C]	Hilal *et al.*, 2003a
Vapor pressure	*2.74E-6*[a] Pa	[at 25°C]	US EPA, 2011
Vapor pressure	*2.75E-6*[a] Pa	[at 25°C]	Neely and Blau, 1985
Vapor pressure	*2.59E-3* Pa	[at 20°C]	ICL (pers. comm.)
Henry's Law constant	*2.98E-8*[a] Pa m^3 mol^{-1}		Neely and Blau, 1985; US EPA, 2011
Henry's Law constant	*181.7*[a] Pa m^3 mol^{-1}		Hilal *et al.*, 2003a
Log K_{OW}	*4.93*		ICL (pers. comm.)
Log K_{OW}	*5.98*[a]		ACD/Labs, 2011
Log K_{OW}	*7.41*[a]		Meylan and Howard, 1991; US EPA, 2011
Log K_{OW}	*11.09*[a]		Hilal *et al.*, 2004a
Log K_{AW}	*-10.92*[a]		US EPA, 2011
Low; water	DT_{50} = 7-17 days	20°C, pH 7	European Chemicals Bureau *et al.*, 2007
Low; water	DT_{50} = 11 days	20°C, pH 4	European Chemicals Bureau *et al.*, 2007; UK Environment Agency *et al.*, 2009a
Low; water	DT_{50} = 17 days	20°C, pH 7	European Chemicals Bureau *et al.*, 2007; UK Environment Agency *et al.*, 2009a

(continued)

Table 10. (continued)	Data	Details	References
Low; water	DT_{50} = 20 days	10°C, pH 7, hydrolysis	European Chemicals Bureau *et al.*, 2007; UK Environment Agency *et al.*, 2009a
Low; water	DT_{50} = 21 days	20°C, pH 9, hydrolysis	European Chemicals Bureau *et al.*, 2007; UK Environment Agency *et al.*, 2009a
Low; water	DT_{50} = 21 days	20°C, pH 9	European Chemicals Bureau *et al.*, 2007
Low	not specified		Clean Production Action ., 2007
Low; sludge	Ready biodegradable ; DT_{60} < 28 days		ICL (pers. comm.)
High; water	DT_{50} = 32 days	10°C, pH 9, hydrolysis	UK Environment Agency *et al.*, 2009a
High; water	DT_{50} = 55 days	10°C, pH 4, hydrolysis	UK Environment Agency *et al.*, 2009a
High; water	*DT_{50} = 37.5*[a] days (900 hours)	Primary degradation	US EPA, 2011
High; sludge	*DT_{37} = 28* days		UK Environment Agency *et al.*, 2009a
High; soil & sediment	*DT_{50} = 75*[a] days (1800 hours)	Soil, primary degradation	US EPA, 2011
High; soil & sediment	*DT_{50} = 337.5*[a] days (8100 hours)	Sediment, primary degradation	US EPA, 2011
n.c.; atmospheric	*DT_{50} = 12.1*[a] hours	Primary degradation	US EPA, 2011
n.c.; atmospheric	DT_{50} = 36[e] hours		UK Environment Agency *et al.*, 2009b
Bioaccumulation			
Low	not specified		German Federal Environmental Agency *et al.*, 2001
Low-high	*BCF = 100-1,000*[b]	Based on Log K_{OW} = 4.93	ICL (pers. comm.)
Moderate	*BCF = 969*[b] *kg* L^{-1}		UK Environment Agency *et al.*, 2009a
High	not specified		Clean Production Action, 2007
High	*BCF = 316*[b]*-3,000*[a]		European Chemicals Bureau *et al.*, 2007; Washington State Department of Ecology and Department of Health, 2006

(continued)

Table 10. (continued)	Data	Details	References
Ecotoxicity			
Low	NOEC >1 mg L^{-1}	Daphnia, NOEC exceeds water solubility	ICL (pers. comm.)
Low	LC_{50} = 12.4 mg L^{-1}	Fish	Illinois Environmental Protection Agency, 2007; Washington State Department of Ecology and Department of Health, 2006
Low	LOEC = 48.64 mg L^{-1}	Algae, growth inhibition	Washington State Department of Ecology and Department of Health, 2006
Low	LC_{50} >100 mg L^{-1}	Daphnia, Fish, Algae	ICL (pers. comm.)
Low	EC_{10} >121.6 mg L^{-1}	Bacteria	Washington State Department of Ecology and Department of Health, 2006
Moderate	not specified		Washington State Department of Ecology and Department of Health, 2006
Moderate	not specified	Chronic exposure (may cause long term effects)	European Chemicals Bureau *et al.*, 2007; UK Environment Agency *et al.*, 2003
High	EC_{50} = 0.76 mg L^{-1}	Daphnids	Illinois Environmental Protection Agency, 2007; Washington State Department of Ecology and Department of Health, 2006
High	NOEC = 0.021 mg L^{-1}	Daphnia, 21 days, EC_{50} (immobility) estimated at 0.037 mg L^{-1} , co-solvent used	UK Environment Agency *et al.*, 2009a
In vivo toxicity			
Low	LD_{50} >2000 mg kg^{-1}	Rats, dermal	Washington State Department of Ecology and Department of Health, 2006
Low	LD_{50} >5000 mg kg^{-1}	Rats	Illinois Environmental Protection Agency, 2007
Low	LD_{50} >5000 mg kg^{-1} bwt	Rats	Washington State Department of Ecology and Department of Health, 2006

(continued)

Table 10. (continued)	Data	Details	References
Low	EC_{50} >20,000 mg kg^{-1}	Rats	Henrich *et al.*, 2000
Low	EC_{50} >1,000 mg kg^{-1}	Rabbits	Ryan *et al.*, 2000
Low	not specified		European Chemicals Bureau *et al.*, 2007
Moderate	not specified		Clean Production Action, 2007; UK Environment Agency *et al.*, 2003
Moderate	LC_{50} = 4.14 mg L^{-1}	Rats, inhalation	ICL (pers. comm.)
n.c.	*NOEC = 0.1*[a] mg L^{-1}	Rats, inhalation, (predicts high because of presence TPP, however *in vivo* toxicity TPP not high)	German Federal Environmental Agency *et al.*, 2001
In vitro toxicity			
Low	Genotoxicity; Mutagenicity	- , *Salmonella* and *E. coli*, AMES test	Washington State Department of Ecology and Department of Health, 2006
n.c.	Endocrinetoxicity; Metabolization	Metabolites formed: resorcinol diphenylphosphate, hydroxylresorcinol diphenylphosphate, dihydroxyresorcinol diphenylphosphate and hydroxylated parent compounds	Washington State Department of Ecology and Department of Health, 2006

Italic values are predicted : aModeled, bcalculated, cexpert judgement
n.c., not enough data to classify; -, no effects observed

5.3 Bisphenol-A bis(diphenylphosphate)

Bisphenol-A bis(diphenylphosphate) (BDP) is a polymeric aryl phosphate, commonly used as flame retardant (Supresta, 2006) and has the CAS registration number 181028-79-5. The technical product (CAS # 5945-33-5) consists of BDP itself (>85%), but its remaining ingredients are largely unknown. According to the Australian Department of Health and Ageing about 0.07% phenol (108-95-2) and <0.01% 4,4'-(1-methylethylidene)bisphenol (80-05-7) are present (Australian Government Regulator of Industrial Chemicals, 2000) in the product. These components were not mentioned in another report, in which it was stated that 11% of another phosphoric acid is present (bis[4-[1-[4-[(diphenoxyphosphinyl)oxy] phenyl]-1-methylethyl]phenyl] phenyl ester, CAS #3029-72-5) and <3% of triphenylphosphate (CAS #115-86-6) (Clean Production Action, 2007). The bisphenol-A bis(diphenylphosphate) product (CAS number 5945-33-5) will be discussed here to avoid this uncertainty. This is a polymeric compound and typically consists of a mixture of different chain lengths (Figure 3). As discussed previously, chain length strongly influences the properties of the substance. Environmental data on BDP are scarce. BDP has been measured in air samples at levels of about 1 ng m^{-3} in domestic indoor sites and at approximately 100 ng g^{-1} in dust (Matsukami *et al.*, 2010). No information is available on production volumes in the EU (European Chemicals Bureau, 2011) (European Chemicals

Bureau *et al.*, 2007). For the USA, total annual production in 2006 was given as 4,500 to <22,700 t for CAS number 181028-79-5 and an additional 450 to <4,500 t of BDP under the CAS number 5945-33-5 was produced (US EPA, 2006).

Physical-chemical properties
We assume that all physical-chemical properties published for this product are based on tests with the technical product (CAS # 5945-33-5), because to our knowledge no purified monomeric BDP is currently available on the market. The monomer of BDP weighs 693.25 g mol^{-1}, and for $n = 10$ it weighs 3,989.80 g mol^{-1}. An overview of its physical-chemical properties is shown in Table 11. BDP is a liquid at room temperature. Mainly low vapor pressure values were reported for the compound (Table 11). However, one author gave a value of 0.18 Torr (approximates to 24 Pa) (Supresta, 2006). This latter reference actually reported vapor pressures for a mixture containing >95% BDP and <5% triphenylphosphate. It is therefore expected that this higher reported vapor pressure is less reliable. Additionally, based on the reported low solubility, Henry's law constant and its high molecular weight, it is assumed that the vapor pressure of BDP is also low. Overall, BDP is likely to favor hydrophobic compartments such as soil and biota more than air and water.

Persistence
Data on this chemical are scarce and those available are contradictory, showing high as well as low persistence, with dissipation times ranging from 1 day to 1 year (European Chemicals Bureau *et al.*, 2007). Table 11 provides an overview of the reported data.

Bioaccumulation
There are no experimental studies available on the bioaccumulation of BDP (see Table 11). The few available theoretical studies provided estimates that varied between low and high bioaccumulation. However, it is noteworthy that the study in which a low BCF value was reported, also reported a high Log K_{OW} (Table 11). The Australian Department of Health predicted that the BCF for this substance would be high, because of its relatively low molecular weight and water solubility and high Log K_{OW} (Australian Government Regulator of Industrial Chemicals, 2000). Based on the contradictory persistence data given, we are not fully convinced of the validity of this conclusion. Clearly, there is a need for experimental data to confirm such statements.

Toxicity
1. Ecotoxicity
   The aquatic toxicity of BDP appears to be moderate, although there are only a few poorly described studies available (Table 11). The authors of one study reported the formation of bisphenol-A during testing and the presence of TPP as an impurity. Therefore, the authors of this study concluded that the ecotoxicity of BDP is high, although no experimental details were provided (Clean Production Action, 2007).

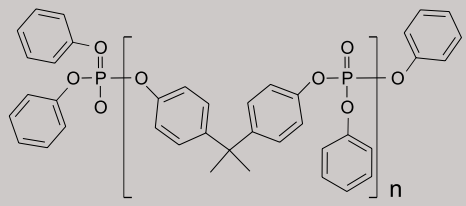

Figure 3. Schematic representation of the chemical structure of the polymer BDP. the product consists of a mixture of polymers with different chain lengths. The typical composition was unknown.

2. *In vivo* toxicity
   The *in vivo* toxicity to rats was described in several studies and was regarded to be low, with a minimum LD_{50} of 2,000 mg kg^{-1} bwt (e.g., Australian Government Regulator of Industrial Chemicals, 2000) (Table 11).
3. *In vitro* toxicity
   An overview of the *in vitro* toxicity is shown in Table 11. In one study (Maine, 2007), it was stated that one of the degradation products of BDP is bisphenol-A, which is an endocrine disrupting compound, but this should be verified as no further details were given. Such incidents make it important to study breakdown products and metabolites.

In summary, BDP has low water solubility. Considering the high hydrophobicity of this compound, it is likely that BDP will accumulate in soil and sediments once released into the environment. Reported persistence ranges from low to high. Bioaccumulation is poorly studied and is estimated to be low to high, although study details were not reported. Ecotoxicity is generally moderate. However high concerns were expressed about the toxicity of the TPP impurity that is present in the commercial product; there was also concern for the potential formation of the toxic breakdown product bisphenol-A (Clean Production Action, 2007). However, this phenomenon was not examined in any study. Low *in vivo* toxicity of BDP was reported in one study. Very limited data exist on the *in vitro* toxicity of BDP; in the single study available, no mutagenic effects were reported in an AMES test.

5.4 9,10-Dihydro-9-oxa-10-phosphaphenanthrene-10-oxide

The compound 9,10-Dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO) is currently used as a flame retardant in polymers (MaKuang Chemical Co. Ltd., 2009). There is no information available on production volumes of DOPO (9,10-dihydro- 9-oxa-10-phosphaphenanthrene-10-oxide or dihydrooxaphosphaphenantreneoxide, CAS 35948-25-5) from the EU (European Chemicals Bureau, 2011) or the USA (US EPA, 2006).

Physical-chemical properties

DOPO is a solid at room temperature with a melting point between 84.3 and 122°C (Chang *et al.*, 1998; Chernysh *et al.*, 1972; Kuo Ching Chemical Co Ltd., 2009; MaKuang Chemical Co. Ltd., 2009; US EPA, 2011). It has a molecular weight of 216.18 g mol^{-1}, and the reported solubility in water varies from moderate and high (0.009-28.97 g L^{-1} , 0.04-134 mol m^{-3} , respectively) (Eckert and Klamt, 2010; Hilal *et al.*, 2004b; US EPA, 2011). An overview of DOPO physical-chemical properties is shown in Table 12. DOPO has a low vapor pressure, Henry's law constant and Log K_{AW} , and a moderate Log K_{OW} value. It is clear that, once released into the environment, DOPO will not appreciably partition to air. Its properties suggest mostly partitioning to water and a low propensity to partition into soil and biota.

Persistence

In one draft report, it was stated that the chemical is non-persistent (US EPA, 2008). The DT_{50} in water is estimated to be shorter than 60 days and in air less than 2 days. However, when using EPI-Suite, the persistence was estimated to be high (US EPA, 2011), with a dissipation time (DT_{50}) in water, soil, and sediment of more than 37 days each (Table 12). More data are needed to make firm conclusions concerning environmental persistence of this compound.

Table 11. Bisphenol-A bis(diphenylphosphate) (BDP, CAS nr 5945-33-5)

	Data	Details	References
Physical-chemical properties			
Molecular Weight	693.25 g mol^{-1}		
Melting point	41.3-68.6°C		Australian Government Regulator of Industrial Chemicals, 2000
Melting point	40.85-68.85°C		ICL (pers. comm.)
Melting point	90°C		Syracuse Research Corporation, 2006
Melting point	*90.27*[a]°C		US EPA, 2011
Water solubility	*5.26E-10*[a] mg L^{-1}	[at 25°C]	Hilal *et al.*, 2004b
Water solubility	*1.92E-7*[a] mg L^{-1}	[at 25°C]	US EPA, 2011
Water solubility	*1.88E-6*[a] mg L^{-1}	[at 25°C]	US EPA, 2011
Water solubility	*2.27E-4*[a] mg L^{-1}	[at 25°C]	Eckert and Klamt, 2010
Water solubility	<1E-3 mg L^{-1}	[at 25°C]	Syracuse Research Corporation, 2006
Water solubility	4.15E-1 mg L^{-1}	[at 20°C]	Australian Government Regulator of Industrial Chemicals, 2000, ICL (pers. comm.) (latter stated no temperature)
Vapor pressure	*9.14E-18*[a] Pa	[at 25°C]	Eckert and Klamt, 2010
Vapor pressure	*2.74E-6*[a] Pa	[at 25°C]	US EPA, 2011
Vapor pressure	<1.33E-4 Pa	[at 25°C]	Syracuse Research Corporation, 2006
Vapor pressure	1.2E-3 Pa	[at 20°C]	ICL (pers. comm.)
Vapor pressure	<1.2E-3 Pa	[at 25°C]	Australian Government Regulator of Industrial Chemicals, 2000
Vapor pressure	*0.12*[a] Pa	[at 25°C]	Hilal *et al.*, 2003b
Vapor pressure	24 Pa	[at 25°C]	Supresta, 2006
Henry's Law Constant	*4.68E-9*[a] Pa m^3 mol^{-1}		US EPA, 2011
Henry's Law Constant	5.07E-9 Pa m^3 mol^{-1}		Syracuse Research Corporation, 2006

(continued)

Table 11. (continued)	Data	Details	References
Henry's Law Constant	*5.38*[a] Pa m^3 mol^{-1}		Eckert and Klamt, 2010
Log K_{OW}	4		Syracuse Research Corporation 2006
Log K_{OW}	≥6		Australian Government Regulator of Industrial Chemicals, 2000
Log K_{OW}	>6		Supresta, 2006
Log K_{OW}	*8.79*[a]		Eckert and Klamt, 2010
Log K_{OW}	*10.02*[a]		US EPA, 2011
Log K_{OW}	*10*[a]		Syracuse Research Corporation, 2006
Log K_{OW}	*14.4*[a]		Hilal *et al.*, 2004b
Log K_{AW}	*-13.95*[a]		Eckert and Klamt, 2010
Log K_{AW}	*-11.72*[a]		US EPA, 2011
Persistence			
Low	Not specified, primary source not found-		Clean Production Action, 2007
Low to high	DT_{50} = 1 day to 1 year		European Chemicals Bureau *et al.*, 2007
Low; water	*DT_{50}=60 days*[a] *(1,440 hours)*		US EPA, 2011
High; water	DT_{50}>1 year	pH 4.0, 7.0 and 9.0, 25°C	European Chemicals Bureau *et al.*, 2007
High; soil & sediment	*DT_{50} = 120 days*[a] *(2,880 hours)*	Soil	US EPA, 2011
High; soil & sediment	*DT_{50} = 542 days*[a] *(13,000 hours)*	Sediment	US EPA, 2011
High; sludge	Not ready biodegradable (DT_6 = 28 days)		Australian Government Regulator of Industrial Chemicals, 2000
n.c.; atmospheric	*DT_{50}=0.5 day*[a] *(12.1 hours)*		US EPA, 2011
Bioaccumulation			
Low to high?	*BCF = 3.16*[b]	This value is reported with high Log K_{OW}, so presumably it should be log 3.16 and thus a high value?	Washington State Department of Ecology and Department of Health, 2006

(continued)

Table 11. (continued)	Data	Details	References
High	Estimated high	high Log K_{OW} and relatively low S_W and M_W	Australian Government Regulator of Industrial Chemicals, 2000
Ecotoxicity			
Low-Moderate; aquatic	EC_{50} >1 mg L^{-1}	Algae, growth inhibition	Washington State Department of Ecology and Department of Health, 2006
Low-Moderate; aquatic	NOEC = 5 mg L^{-1}	Fish	Washington State Department of Ecology and Department of Health, 2006
Low-Moderate; aquatic	NOEC >1mg L^{-1}	Fish, daphnids & algae (EC_{50} exceeds solubility)	Australian Government Regulator of Industrial Chemicals, 2000
High; aquatic	Not specified	Acute & chronic exposure (bisphenol-A breakdown product is potentially developmentally- and, reproductive toxic, also an endocrine disruptor)	Clean Production Action, 2007
In vivo toxicity			
Low	LD_{50} >2000 mg kg^{-1} bwt	Rats	Australian Government Regulator of Industrial Chemicals, 2000; European Chemicals Bureau *et al.*, 2007; Washington State Department of Ecology and Department of Health, 2006
In vitro toxicity			
Low	Genotoxicity ; Mutagenicity	- *Salmonella* and *E. Coli*, AMES test	Washington State Department of Ecology and Department of Health, 2006
n.c.	Endocrinetoxicity ; Metabolization	Metabolite: Bisphenol-A	Maine, 2007

Italic values are predicted : [a]Modeled, [b]calculated, [c]expert judgement
n.c., not enough data to classify; -, no effects observed

Bioaccumulation

The estimated BCF for fish is 5.4, and therefore DOPO has been evaluated in this draft reference to be non-bioaccumulative (US EPA, 2008).

Toxicity

1. Ecotoxicity
   The aquatic toxicity is estimated to be moderate, since the acute LC_{50} value for algae, considered the most sensitive aquatic species, is estimated at 3 mg L^{-1} , with a reported (draft report) chronic EC_{50} value of 2.4 mg L^{-1} (US EPA, 2008). An LC_{50} value for fish of 370 mg L^{-1} was reported by Wetton (1999) in an unpublished draft report (US EPA, 2008).
2. *In vivo* toxicity
   No data are available on the *in vivo* toxicity of DOPO.

3. *In vitro* toxicity
   Limited information is available for DOPO. There are no data on carcinogenic, endocrine disruption, or neurotoxicity, although the AMES test showed a negative response for mutagenic activity (Hachiya, 1987) (Table 12).

In summary, DOPO is a solid at room temperature with a moderate to high water solubility. There is a lack of experimental persistence data. Modeled values of the persistence in water, which is probably the most relevant environmental compartment for DOPO, are contradictory and range from high to low; obviously more research is needed. Bioaccumulation of DOPO was estimated to be low in one study. Ecotoxicity was reported to range from moderate to low in the same study. There are not enough data to classify DOPO's *in vivo* toxicity. Only one study showed a negative response for mutagenic activity.

Table 12. Dihydrooxahosphaphenanthrene (DOPO, CAS nr 35948-25-5)

	Data	Details	References
Physical-Chemical Properties			
Molecular Weight	216.18 g mol^{-1}		
Melting point	*84.3*[a]°C		US EPA, 2011
Melting point	114-119°C		MaKuang Chemical Co. Ltd., 2009
Melting point	116-119°C		Kuo Ching Chemical Co Ltd., 2009
Melting point	117°C		Chernysh *et al.*, 1972
Melting point	122°C		Chang *et al.*, 1998
Water solubility	*9.01*[a] mg L^{-1}	[at 25°C]	Hilal *et al.*, 2004b
Water solubility	*71.14*[a] mg L^{-1}	[at 25°C]	US EPA, 2011
Water solubility	*2767.1*[a] mg L^{-1}	[at 25°C]	US EPA, 2011
Vapor pressure	*8.41E-4*[a] Pa	[at 25°C]	Eckert and Klamt, 2010
Vapor pressure	*1.3E-3*[a] Pa	[at 25°C]	Hilal *et al.*, 2003b
Vapor pressure	2.93E-3 Pa	[at 25°C]	McEntee, 1987
Vapor pressure	*3.84E-3*[a] Pa	[at 25°C]	US EPA, 2011
Henry's Law Constant	*5.50E-3*[a] Pa m^3 mol^{-1}		US EPA, 2011
Henry's Law Constant	*3.13E-2*[a] Pa m^3 mol^{-1}		Hilal *et al.*, 2003b
Log K_{OW}	*1.18*[a]		Eckert and Klamt, 2010
Log K_{OW}	*1.87*[a]		US EPA, 2011
Log K_{OW}	*3.32*[a]		Hilal *et al.*, 2004b
Log K_{AW}	*-5.65*[a]		US EPA, 2011
Log K_{AW}	*-8.6*[a]		Eckert and Klamt, 2010
Persistence			
Low; water	DT_{50} *<60*[a,e] *days*		US EPA, 2008
High; water	DT_{50} *= 37.5*[a] *days*	primary degradation, modeled	US EPA, 2011

(continued)

Table 12. (continued)	Data	Details	References
High; soil & sediment	DT_{50} = 75[a] *days*	soil, primary degradation, modeled	US EPA, 2011
High; soil & sediment	DT_{50} = 337.5[a] *days*	sediment, primary degradation, modeled	US EPA, 2011
n.c.; atmospheric	DT_{50} <2[a,e] *days*		US EPA, 2008
n.c.; atmospheric	DT_{50} = 43[a] *hours*	primary degradation, modeled	US EPA, 2011
Bioaccumulation			
Low	*BCF = 5.4*[a,e]	Fish	US EPA, 2008
Ecotoxicity			
Low	LC_{50} = 370[e] mg L^{-1}	Fish, 48h	Wetton 1999 unpublished from US EPA, 2008
Low	LC_{50} = 230[a,e] *mg* L^{-1}	Daphnids, 48h	US EPA, 2008
Low	EC_{50} = 23[a,e] *mg* L^{-1}	Daphnids, chronic	US EPA, 2008
Low	LC_{50} = 20[a,e] *mg* L^{-1}	Fish, 96h	US EPA, 2008
Low	EC_{50} = 16[a,e] *mg* L^{-1}	Fish, chronic	US EPA, 2008
Moderate	EC_{50} = 3[a,e] *mg* L^{-1}	Algae, 96h	US EPA, 2008
Moderate	EC_{50} = 2.4[a,e] *mg* L^{-1}	Algae, chronic	US EPA, 2008
In vitro toxicity			
Low	Genotoxicity ; Mutagenicity	-	Hachiya, 1987

Italic values are predicted: [a]Modeled, [b]Calculated, [c]Expert judgment
n.c., not enough data to classify; -, no effects observed
[e]Not all primary sources are found from (US EPA, 2008), also this reference is a draft report, so reportedvalues may be not final.

5.5 Aluminum diethylphosphinate

Aluminum diethylphosphinate (Alpi, CAS # 225789-38-8) is a metal phosphinate salt and used as a flame retardant in epoxies and polymers (Clariant, 2007). No information is available on production volumes in the EU (European Chemicals Bureau, 2011) or the USA (US EPA, 2006).

Physical-chemical properties

Alpi is solid at room temperature. A melting point has not been reported, probably because Alpi is reported to decompose at temperatures ranging from 400°C (Australian Government Regulator of Industrial Chemicals, 2005). It has a reported solubility in water of 2.5 g L^{-1} (2.56E-3 to 6.41 mol m^{-3}) (Clariant, 2007). Alpi has a low vapor pressure and K_{OW}, presumably favoring the water phase over other compartments once released into the environment. An overview of its properties is shown in Table 13.

Persistence

As mentioned previously in the introduction, persistence expressed as dissipation times is not considered to be very relevant for metals, in this case aluminum. However, the counter ion diethylphosphinate is organic. Three references were found in which a moderate to high persistence of Alpi was claimed (Table 13).

Bioaccumulation
In a draft report, the US EPA predicted that the BCF value for Alpi is <1,000, meaning that it has a low bioaccumulation potential (US EPA, 2008). A low bioaccumulation potential was reported in three other studies, although no detailed information or specific data were provided (Danish EPA *et al.*, 2007; Dekant, 2009; European Chemicals Bureau *et al.*, 2007) (Table 13).

Toxicity
When Alpi is dissolved in water, the complexation of the ions will depend on the prevailing conditions. A low pH, for example, Al^{3+} dissolution will be favored. Complexation and speciation are not treated in detail in this review. Freely dissolved aluminum (Al^{3+}(aq)) can be highly toxic. The toxicity of aluminum has been studied extensively (Berthon, 2002; Kucera *et al.*, 2008) and is not addressed here.

1. Ecotoxicity
   A few studies are available on the aquatic toxicity of Alpi, on algae, daphnids, crustaceans, and fish, and these generally show a low to moderate toxicity (Table 13).
2. *In vivo* toxicity
   Several low NOEC and LC_{50} values that were >1 g kg^{-1} day^{-1} were reported for *in vivo* rodent toxicity for Alpi in two studies (Table 13).
3. *In vitro* toxicity
   Although toxicity data on aluminum and several aluminum compounds are available, data on Alpi were limited (for a review see Subcommittee on Flame-Retardant Chemicals, 2000). No mutagenic activity was observed in the AMES test, with or without metabolic activation (European Chemicals Bureau *et al.*, 2007). *In vitro* toxicity of Alpi is classified as low.

In summary, Alpi is a solid at room temperature with moderate water solubility. Persistence was reported as being moderate to high; however, this was based on three studies in which few details were provided. The bioaccumulation of Alpi was estimated to be low, although there was also a scarcity of studies. The ecotoxicity of Alpi is low, with the exception of one study in which moderate toxicity for algea was reported. For *in vivo* toxicity, two studies are available and indicate low toxicity to rodents. The *in vitro* mutagenicity of Alpi was classified as low; no other *in vitro* toxicity data were available.

6. A nitrogen-Based organic flame retardant: melamine polyphosphate

Melamine polyphosphate (MPP, CAS 218768-84-4) is a nitrogen-based organic salt and is used as a flame retardant (Budenheim, 2010; Ciba, 2010). The compound dissociates into melamine and polyphosphoric acid in water. There is no information on the environmental presence available for MPP, nor is there information available on production volumes in the EU (European Chemicals Bureau, 2011) or the USA (US EPA, 2006).

6.1 Physical-chemical properties

As is the case for APP, MPP is chemically synthesized as an ionic polymer. It is thus a mixture of polymers having different chain lengths or degrees of branching, and accordingly the physical-chemical properties change with those factors as well (as discussed in section Ammonium polyphosphate (APP)). MPP was reported to decompose at 400°C or higher (Australian Government Regulator of Industrial Chemicals, 2006). The solubility in water was reported to be high, i.e., <100 g L^{-1}, i.e., miscible (Eckert and Klamt, 2010; Nordin, 2007). Its physical-chemical properties are shown in Table 14.

Table 13. Aluminum diethylphosphinate (Alpi, CAS nr 225789-38-8)

	Data	Details	References
Physical-Chemical Properties			
Molecular Weight	390.3 g mol^{-1}		
Melting point	>400°C (Decomposition)		Australian Government Regulator of Industrial Chemicals, 2005
Water solubility	2,500 mg L^{-1}	[at 25°C]	Clariant, 2007
Log K_{OW}	-0.44		Clariant, 2007
Persistence			
Moderate	*Days to weeks*[a,c,e]		US EPA, 2008
High; Sludge	Not inherently biodegradable	OECD Guideline 302 C (Inherent Biodegradability: Modified MITI Test (II)), aerobic, 28 days	ECHA Database, original study 2009c
High; Sludge	Not ready biodegradable	OECD Guideline 301 F (Ready Biodegradability: Manometric Respirometry Test), aerobic 28 days	Clariant, 2007; Danish EPA *et al.*, 2007; ECHA Database, original study 2009b; European Chemicals Bureau *et al.*, 2007[d]
Bioaccumulation			
Low	*BCF <1,000* [c,e]		US EPA, 2008
Low	-not specified-		Danish EPA *et al.*, 2007; Dekant, 2009; European Chemicals Bureau *et al.*, 2007
Ecotoxicity			
Low	LC_{50} >9.2[e] mg L^{-1} LC_{50} >11[e] mg L^{-1}	Zebra fish, 96h	US EPA, 2008
Low	EC_{50} = 60-76 mg L^{-1}	Algae, *Scenedesmus subspicatus*, 72h, pH not adjusted	Clariant, 2007[d]
Low	EC_{50} = 50-76[e] mg L^{-1}	Algae, 72h	US EPA, 2008
Low	LC_{50} >33[e] mg L^{-1}	Daphnids, 48h	US EPA, 2008

(continued)

Table 13. (continued)	Data	Details	References
Low	EC_{50} = ca. 46.2 mg L^{-1}	*Daphnia magna*, chronic, reproduction, LOEC = ca.32 mg L^{-1} ; NOEC= 10 mg L^{-1}	ECHA Database, original study 2005-Apr-13; US EPA, 2008
Low	*EC_{50} = 48[a,e] mg L^{-1}*	Fish, chronic	US EPA, 2008
Low	EC_{50}>100 mg L^{-1}	*Daphnia magna*, 48h, EC_{50} exceeds solubility	ECHA Database, original study 1998c
Low	EC_{50}>100 mg L^{-1}	Zebrafish, EC_{50} exceeds solubility	ECHA Database, original study 1998d
Low	EC_{50}>100 mg L^{-1}	Zebrafish, chronic, 28 days (nominal concentration)	ECHA Database, original study 2009a
Low	NOEC >180 mg L^{-1}	Algae	ECHA Database, original study 1998e[d]
Low	EC_{50}=1968 mg L^{-1}	OECD Guideline 209 (Activated Sludge, Respiration Inhibition Test), 3 hours, NOEC = 483 mg L^{-1}, nominal concentrations	ECHA Database, original study 1998f
Moderate	ChV = 1.8[e] mg L^{-1}	Algae, chronic exposure, ChV = chronic value, no further details provided	US EPA, 2008
Moderate	*ChV = 1.4[a,b,c,e] mg L^{-1}*	Algae, chronic exposure, ChV = chronic value, no further details provided	US EPA, 2008
In vivo toxicity			
Low	NOEC >1 g kg^{-1} bwt day^{-1}	Rats, repeated dose study, 28 days	ECHA Database, original study 1998b
Low	NOEC = 1 g kg^{-1} bwt day^{-1}	Rats, reproduction toxicity, minor changes in both sexes at the highest dosage of 1 g kg^{-1} bwt day^{-1}, ~48 days	ECHA Database, original study 2008a; ECHA Database, original study 2008b
Low	LD_{50} >2 g kg^{-1} bwt	Acute oral toxicity	ECHA Database, original study 1998a; US EPA, 2008[d], first is draft[e]

(continued)

Table 13. (continued)	Data	Details	References
Low	LD_{50} >2 g kg^{-1} bwt	Acute dermal toxicity	ECHA Database, original study 1998b
Low	non-mutagenic *in vivo*	No mutagenic effect up to 2 g kg^{-1} bwt, OECD Guideline 474	ECHA Database, original study 2008a
In vitro toxicity			
Low	Genotoxicity; Mutagenicity	- , Salmonella, AMES test	European Chemicals Bureau *et al.*, 2007
Low	Genotoxicity; Mutagenicity	-, OECD 471 (Bacterial Reverse Mutation Assay)	ECHA Database, original study 1998g[d]
Low	Genotoxicity; Mutagenicity	-, OECD Guideline 473 (*in vitro* Mammalian Chromosome)	ECHA Database, original study 1998h

Italic values are predicted: [a]Modeled, [b]calculated, [c]expert judgement
n.c., not enough data to classify; -, no effects observed
[d]A recent publication (Danish EPA, *et al.*, 2007) also discusses the PBT of Alpi, this is predominantly based on the same reports from Clariant (producer) as reffered to in this review
[e]Not all primary sources are found from (US EPA 2008), also this reference is a draft report, so reported values may be not final

6.2 Persistence

In one draft report, it was stated that MPP has a high persistence, namely DT_{50} >1 year for polyphosphoric acid, and a DT_{50} of weeks to months for melamine (US EPA, 2008).

6.3 Bioaccumulation

In a draft report, the EPA predicted that the BCF of MPP to be less than 1,000, meaning that it has a low bioaccumulation potential (US EPA, 2008). Another author reported a low bioaccumulation potential, but gave no data (Australian Government Regulator of Industrial Chemicals, 2006) (Table 14).

6.4 Toxicity

1. Ecotoxicity
   In the few studies available, low ecotoxicity of MPP to algae, daphnids, and fish were reported, with EC_{50} values of 3.0 mg L^{-1} or higher (algae) (Australian Government Regulator of Industrial Chemicals, 2006; European Chemicals Bureau *et al.*, 2007) as can be seen in Table 14.
2. *In vivo* toxicity
   The *in vivo* toxicity of MPP appears to be low as well, only showing effects to rodents when they are exposed to the compound at >1,000, and up to 4,000 mg kg^{-1} bwt (Table 14).
3. *In vitro* toxicity
   Information on *in vitro* toxicity of MPP is limited but, based on the toxicity of melamine, it is expected that MPP has low hazard for carcinogenicity (Illinois Environmental Protection Agency, 2007). In contrast, studies with MPP showed effects for *in vivo* chromosomal aberration tests and *in vivo* sister chromatic assays with mice in a draft report (US EPA, 2008). There are not enough data to classify the *in vitro* toxicity of MPP.

In summary, MPP is a solid at room temperature with high solubility in water. Once dissolved in water, it will dissociate into melamine and polyphosphoric acid. A high persistence of phosphoric acid was reported once. The bioaccumulation potential of MPP is low, although no details were provided. *In vivo* (eco)toxicity is low. There are not enough data to classify the *in vitro* toxicity.

Table 14. Melamine polyphosphate (MPP, CAS nr 218768-84-4)

	Data	Details	References
Physical-Chemical Properties			
Molecular weight	>10,000 g mol^{-1}		Australian Government Regulator of Industrial Chemicals, 2006
Melting point	>400°C		Australian Government Regulator of Industrial Chemicals, 2006
Water solubility	<100 mg L^{-1}	[at 22°C]	Nordin, 2007
Water solubility	*Miscible*[a]	[at 25°C]	Eckert and Klamt, 2010
Vapor pressure	6.65E-3 Pa	[at 25°C]	Eckert and Klamt, 2010
Vapor pressure	<<8 Pa	[at 25°C]	Australian Government Regulator of Industrial Chemicals, 2006
Vapor pressure	<8 Pa	[at 25°C]	Nordin, 2007
Log K_{OW}	<-2.3	[at 20°C]	Australian Government Regulator of Industrial Chemicals, 2006
Log K_{OW}	-2.3	[at 25°C]	Nordin, 2007
Log K_{OW}	*-2.15*[a]	[at 25°C]	Eckert and Klamt, 2010
Log K_{AW}	*-10.9*[a]	[at 25°C]	Eckert and Klamt, 2010
Persistence			
High	DT_{50} = weeks-months[e] for melamine		US EPA, 2009
Bioaccumulation			
Low	BCF <3.8[e]	*Cyprinus carpio*, Melamine	US EPA, 2009
Low	*BCF <1000*[c,e]		US EPA, 2009
Low	-not specified-		Australian Government Regulator of Industrial Chemicals, 2006
Ecotoxicity			
Low	EC_{50} >3.0[e] mg L^{-1}	Algae, Melamine	US EPA, 2008
Low	EC_{50} >3.0 mg L^{-1}	Algae	Australian Government Regulator of Industrial Chemicals, 2006; European Chemicals Bureau *et al.*, 2007

(continued)

Table 14. (continued)	Data	Details	References
Low	EC_{50} = 940[e] mg L^{-1} NOEC = 320.0[e] mg L^{-1}	Algae, Melamine, 96h	US EPA, 2008
Low	EC_{50} = 32-56[e] mg L^{-1}	Daphnids, Melamine, chronic exposure	US EPA, 2008
Low	EC_{50} >2,000[e] mg L^{-1}	Dapnnids, Melamine, 48h	US EPA, 2008
Low	LC_{50} >500[e] mg L^{-1}	Fish, Melamine	US EPA, 2008
Low	EC_{50} = 1,000-3,000[e] mg L^{-1}	Fish, Melamine, chronic	US EPA, 2008
In vivo toxicity			
Low	LD_{50} = 4,000[e] mg kg^{-1} bwt	Polyphosphoric acid, rats	US EPA, 2008
Low	LD_{50} = 3,160-7,014[e] mg kg^{-1} bwt	Melamine: Rats, mice	US EPA, 2008
Low	LD_{50} >1,000[e] mg L^{-1}	Rabbits, dermal	US EPA, 2008
Low	LD_{50} = 3,248[e] mg L^{-1}	Rats, inhalation	US EPA, 2008
Low	LD_{50} >2,000 mg kg^{-1} bwt	Rats, melamine polyphosphate (incl. different technical products tested)	Australian Government Regulator of Industrial Chemicals, 2006; Budenheim, 2010; Ciba, 2010; Nordin, 2007
In vitro toxicity			
n.c.	Genotoxicity; Carcinogenicity	±[e]	US EPA, 2008

Italic values are predicted: [a]Modeled, [b]Calculated, [c]Expert judgment
n.c., not enough data to classify; ±, toxicity effects observed
[d]The report refers to an OECD SIDS
[e]Not all primary sources are found from (US EPA, 2008), also this reference is a draft report, so reported values may be not final.

7. An intumescent system: pentaerythritol

Almost all intumescent systems consist of three basic components, a dehydrating component, such as ammonium polyphosphate, a charring component, such as pentaerythritol (PER) and a gas source or blowing agent, often a chemical containing nitrogen such as melamine polyphosphate or ammonium polyphosphate (ENFIRO, 2009). The latter two are reviewed in the sections Melamine polyphosphate and Ammonium polyphosphate, respectively. 1996 and 27,513 t in 1997 (European Chemicals Bureau, 1998; UNEP, 2005). PER is classified as an HPV chemical in the EU (European Chemicals Bureau, 2011). Total annual production was given for the USA as 45,000 to <227,000 t in 2006 (US EPA, 2006).

7.1 Physical-chemical properties

Pentaerythritol is a white crystalline solid at environmental temperatures and its melting point is 260°C (Hilal *et al.*, 2003b; Syracuse Research Corporation, 2009). It has a high solubility in water (Table 15). PER is a highly water soluble compound with a relatively low vapor pressure, low Henry's Law constant, low Log K_{OW}, and low Log K_{AW}. Its properties are reported in Table 15.

7.2 Persistence

PER has shown a high as well as low persistence in different degradation experiments (Table 15). Since PER is hydrophilic, the dissipation times in water (and sludge) seem most relevant. In a few studies, it was stated that this compound degrades very slowly in sludge and water. However, more studies show that PER degrades quickly, for example, a ring test from 1985 showed a DT 60 of 28 days (ECHA Database, original study 1985).

7.3 Bioaccumulation

PER has a low bioaccumulation factor of 0.3-0.6 for the fish species *Cyprinus carpio* (European Chemicals Bureau, 1998). Since it has a very low log K_{OW} value, its bioaccumulation potential is also likely to be low (Table 15).

7.4 Toxicity

1. Ecotoxicity
   The ecotoxicity of PER has been studied with several species, i.e., fish, algae, daphnia, and bacteria. PER has low toxicity to all species, with EC_{50} values over 100 mg L^{-1} (European Chemicals Bureau, 1998) (Table 15).
2. *In vivo* toxicity
   A low toxicity of PER to several rodent species was reported in two studies, with an LD_{50} >2,000 mg kg^{-1} bwt (ECHA Database, original study 1996-Jul-25) (Table 15).
3. *In vitro* toxicity
   Information on the *in vitro* toxicity of PER is limited (Table 15). However, in several studies no mutagenicity was observed. One author reported that reproduction and developmental studies did not show any toxicity up to 1,000 mg kg^{-1} day^{-1} (European Chemicals Bureau, 1998; UNEP, 2005).

In summary, PER is a solid at room temperature and is highly water soluble. In most studies, a low persistence was reported, although examples of high values were also reported. A low BCF value was reported for fish. The *in vivo* (eco)toxicity of PER appears to be low. There are not enough data to classify the *in vitro* toxicity of PER.

8. Discussion

8.1 Data availability

Ideally, the HFFRs that replace the existing halogenated flame retardants should pose lower risks to the environment and to human health. Yet, our review revealed that, apart from TPP, all potentially replacement compounds have large data gaps concerning their published PBT properties. Indeed, for some of these compounds, even the most basic physical-chemical properties have not yet been disclosed. Because these compounds are currently produced and distributed on a global scale, in some cases even as HPV chemicals, it is crucial to fill these data gaps. It is conceivable that, with the implementation of REACH, more data may become or are already available on these compounds. Dossiers with information on PBT properties may exist, for instance in the US EPA and the ECHA archives. If so, then these were not publically accessible. Data for some compounds have recently become available on the ECHA website (Registered substances http://echa.europa.eu/web/guest/information-on-chemicals/registered-substances), but we only noted this after we completed our literature review at the end of August 2011. The availability of such information could substantially contribute to filling the presently identified data gaps and would greatly accelerate the risk evaluation of the compounds addressed in this review; these data are also needed because these compounds are currently being marketed.

Table 15. Pentaerythritol (PER, CAS nr 115-77-5)

	Data	Details	References
Physical-Chemical Properties			
Molecular Weight	136.15 g mol^{-1}		
Melting point	*74*[a]°C		US EPA, 2011
Melting point	*119*[a]°C		US EPA, 2011
Melting point	258°C		ECHA Database, original study 2009e
Melting point	*258*[a]°C		US EPA, 2011
Melting point	260°C		Hilal *et al.*, 2003b; Syracuse Research Corporation, 2009
Water solubility	62,000 mg L^{-1}	[at 20°C]	ECHA Database, original study 2009f; Perstorp Specialty Chemicals AB, 2008
Water solubility	72,300 mg L^{-1}	[at 25°C]	Meylan and Howard, 1995
Water solubility	*3.93e+5*[a] mg L^{-1}	[at 25°C]	Hilal *et al.*, 2004b
Water solubility	*1e+6*[a] mg L^{-1}	[at 25°C]	US EPA, 2011
Water solubility	*3.65e+6*[a] mg L^{-1}	[at 25°C]	Eckert and Klamt, 2010
Vapor pressure	*3.37E-6*[a] Pa	[at 25°C]	Neely and Blau, 1985
Vapor pressure	*3.38E-6*[a] Pa	[at 25°C]	US EPA, 2011
Vapor pressure	*3.4E-6*[a] Pa	[at 20°C]	ECHA Database, original study 2010-Jan-31
Vapor pressure	*15E-6*[a] Pa	[at 20°C]	ECHA Database, original study 2010-Jan-31
Vapor pressure	*1.62E-5*[a] Pa	[at 25°C]	Hilal *et al.*, 2003b
Vapor pressure	<1E-3 Pa	[at 20°C]	Perstorp Specialty Chemicals AB, 2008
Vapor pressure	*2.95E-2*[a] Pa	[at 25°C]	Eckert and Klamt, 2010
Henry's Law Constant	*1.13E-9*[a] Pa m^3 mol^{-1}		Hilal *et al.*, 2003b
Henry's Law Constant	*4.15E-5*[a] Pa m^3 mol^{-1}		Meylan and Howard, 1991
Log K_{OW}	*-4.15*[a]		Hilal *et al.*, 2004b

(continued)

Table 15. (continued)	Data	Details	References
Log K_{OW}	*-1.77*[a]		US EPA, 2011
Log K_{OW}	-1.7		Perstorp Specialty Chemicals AB, 2008
Log K_{OW}	*-1.70*[a]		ECHA Database, original study 2009d
Log K_{OW}	*-1.69*		Syracuse Research Corporation, 2011
Log K_{OW}	*-1.4*[a]		Eckert and Klamt, 2010
Log K_{AW}	*-9.35*[a]		Eckert and Klamt, 2010
Log K_{AW}	*-7.78*[a]		US EPA, 2011
Persistence			
Low; Water	DT_{50} = *208*[a] hours (8.6 days)	Primary degradation, modeled	US EPA, 2011
Low; Water	DT_{60} <28 days	Ring test, 25 studies	ECHA Database, original study 1985
Low; Water	biodegradable	biodegradable under some test conditions, no details provided	ECHA Database, original study 1980-Feb-29 & 1981
Low; Sludge	DT_{84} = 28 days (3.87 S.D.), Ready biodegradable		ECHA Database, original study 1991-Sep-16
Low; Sludge	Ready biodegradable	99% DOC removal in 28 days	ECHA Database, original study 1990-Sep-24
Low; Sludge	Inherent biodegradable	99% DOC removal in 28 days	ECHA Database, original study 1994-Jan-25
Low; Sludge	Inherent biodegradable	>90% DOC removal, few details provided	ECHA Database, original study 1979-Feb-08
High; Water	no hydrolysis	At 20°C after 5 days for pH 5, 7, 9	ECHA Database, original study 2010-Aug-2
High; Water	DT_{13} = 25 days		European Chemicals Bureau, 1998
High; Soil & Sediment	DT_{50} = *416*[a] hours	Soil, primary degradation, modeled	US EPA, 2011
High; Soil & Sediment	DT_{50} = *1870*[a] hours	Sediment, primary degradation, modeled	US EPA, 2011
High; Sludge	DT_{50} = 14 to more than 28 days	Aerobic sewage sludge	European Chemicals Bureau, 1998

(continued)

Table 15. (continued)	Data	Details	References
High; Sludge	Not ready biodegradable		European Chemicals Bureau, 1998
n.c.; Atmospheric	DT_{50} = *15.4*[a] hours	Primary degradation, modeled	US EPA, 2011
Bioaccumulation			
Low	BCF = 0.3-0.6	Fish, *Cyprinus carpio*	European Chemicals Bureau, 1998
Ecotoxicity			
Low	LC_{50} >100mg L^{-1}	(Nominal concentration) *Oryzias latipes*, fresh water, 96h	ECHA Database, original study 1993-Jan-03
Low	EC_{50} >1,000 mg L^{-1}	(Nominal concentration) *Daphnia magna*, 24 hours	ECHA Database, original study 1993-March-01b
Low	NOEC = 1,000 mg L^{-1}	(nominal concentration) *Daphnia magna*, 21 days	ECHA Database, original study 1993-March-01a
Low	NOEC = 1,000 mg L^{-1}	(nominal concentration) Microorganisms (activated sludge from domestic sewage)	ECHA Database, original study 2010-April-29
Low	EC_{10} >500 mg L^{-1}	*Pseudomonas putida* (nominal concentration)	Knie *et al.* 1983 (not found) from ECHA Database, original study 1983
Low	LD_{50} >5,000 mg L^{-1}	Fresh water fish	European Chemicals Bureau, 1998
Low	EC_{50} >500 to >5000 mg L^{-1}	Crustaceans, algae & bacteria	European Chemicals Bureau, 1998
Low	IC_{50} = 600 mg L^{-1}	Daphnids	European Chemicals Bureau, 1998
In vivo toxicity			
Low	NOEC = 100 mg kg bwt^{-1} day^{-1}	Rats, repeated dose study, ~46 days	ECHA Database, original study 1996-Jul-28
Low	NOEC = 1,000 mg kg bwt^{-1} day^{-1}	Rats, repeated dose study, 28 days	ECHA Database, original study 1992-May-30

(continued)

Table 15. (continued)	Data	Details	References
Low	NOEC = 1000 mg kg bwt^{-1} day^{-1}	Rats, repeated dose study, ~46 days, Developmental and Reproductive toxicity	ECHA Database, original study 1996-Jul-28
Low	LD_{50} = low	Rats survived doses as high as 16000 mg kg^{-1} bwt, few details	ECHA Database, original study 1964b
Low	LD_{50} >2,000 mg kg^{-1} bwt	Rats	ECHA Database, original study 1996-Jul-25
Low	LD_{50} >5,110 mg kg^{-1} bwt	Rats	ECHA Database, original study 1990-Feb-15
Low	LC_{50} >10,000 mg kg^{-1}	Rabbits	ECHA Database, original study 1963-May-19
Low	LC_0= 11,000 mg m^{-3} air	Rats, inhalation, 6 hours exposure (LC_0, no effect)	ECHA Database, original study 1964a; ECHA Database, original study 1964c
Low	LD_{50} = 11,300 mg kg^{-1} bwt	Guinea pigs	ECHA Database, original study 1964b
Low	LD_{50} =25,500 mg kg^{-1} bwt	Mice	ECHA Database, original study 1964b
Low	LD_{50} >10,000 mg kg^{-1} bwt	Rats, mice, rabbits & guinea pigs	Merck Chemicals - Product Information; European Chemicals Bureau, 1998
In vitro toxicity			
Low	Genotoxicity ; Mutagenicity	-	UNEP, 2005
Low	Genotoxicity ; Mutagenicity	-, Ames test negative, tested up to 5000 ug plate^{-1}, OECD Guideline 471 (Bacterial Reverse Mutation Assay)	ECHA Database, original study 1994-Dec-21; Shimizu *et al.*, 1985
Low	Genotoxicity ; Mutagenicity	-, OECD Guideline 473 (*in vitro* Mammalian Chromosome Aberration Test)	ECHA Database, original study 1994-Dec-21
Low	Genotoxicity ; Mutagenicity	-, OECD Guideline 476 (*in vitro* Mammalian Cell Gene Mutation Test)	ECHA Database, original study 2010-Okt-2
Low	Genotoxicity ; Mutagenicity	-, OECD Guideline 471 (Bacterial Reverse Mutation Assay), tested up to 5000 ug plate^{-1}	Shimizu *et al.*, 1985

Italic values are predicted: [a]Modeled, [b]calculated, [c]expert judgement
n.c., not enough data to classify; -, no effects observed

Despite the REACH regulations, characterization of compounds often lacks important in-depth studies, such as the identification and characterization of potentially toxic metabolites or decomposition products. This is one of the reasons that the European Commission has funded a research project on HFFRs, called ENFIRO (A Life Cycle Assessment of Environment-Compatible Flame Retardants (Prototypical Case Study)). ENFIRO aims to fill a large part of the existing data gaps identified in the present review. ENFIRO is studying several aspects, including environmental and toxicological risks, fire safety, product application, and viability of industrial implementation (ENFIRO, 2009). If successful, a solid basis will be formed for assessing the suitability of the HFFRs as safe and environmentally friendly alternatives.

8.2 Inconsistency of data

We demonstrated in the present review that for many of the HFFRs, widely different values for the same properties have been published in literature. When assessing data quality, we considered the experimental values to be more reliable than the modeled ones; modeled values were, in turn, considered more reliable than the so-called expert judgments. Consequently, we preferred data published in peer-reviewed scientific papers over those in reports and other so-called grey literature. Perhaps the most important issue was the transparency of the experimental setup; the more detail that researchers provide on the test conditions and results, the better. Differences in prevailing conditions and methods may explain the observed differences between test results. For example, a low pH may favor degradation by hydrolysis or, in the case of a metal salt, the toxicity may change dramatically (Martinez and Motto, 2000; Peterson *et al.*, 1984; Spurgeon *et al.*, 2006). Additionally, the purity and composition of the products tested is often not reported. Possibly, the technical products used for the experiments vary in polymer formulations, e.g., coated vs uncoated forms, leading to different results in reported PBT properties.

8.3 Persistence, bioaccumulation, and toxicity of the selected HFFR

An overview of the classification of the selected compounds that is based on the REACH criteria for PBT and vPvB chemicals is given in Table 16. It is important to realize though, that these assessments are truncated, and data presented in the relevant sections should be consulted for the detailed data. In particular, bioaccumulation and toxicity are species dependent, and even variations among individuals within the same species are not uncommon (Baird *et al.*, 1989). Therefore, it is not surprising that high as well as low classifications sometimes were reported for the same parameter. Furthermore, bioaccumulation was a more difficult parameter to assess, because many studies did not consider depuration times of the chemical. In some toxicity experiments, carrier solvents were used for poorly water soluble organic compounds. Such solvents may enhance exposure concentrations of the tested compounds that exceed their water solubility, which then undermines environmental relevance of the data. Water solubility is often difficult to assess anyway, because reported water solubility values can range over several orders of magnitude. Nevertheless, we based our assessments on the reported effect concentrations.

Three HFFRs immediately drew our attention (Table 16): TPP, RDP, and BDP. TPP has been studied quite extensively and is clearly persistent, bioaccumulative, and toxic. Because they have high to low reported ecotoxicity and persistence, neither RDP nor BDP seem to have proven to be promising alternative flame retardants yet, but this view is based on a limited number of studies. Details on the bioaccumulation potential of RDP

and BDP were not provided, although both were classified as potentially highly bioaccumulative. Clearly, there is a need for research to clarify these uncertainties. As can be seen in Table 16, the compounds ATH, ZB, Alpi, PER, and DOPO scored high in at least one of the PBT categories. ATH and ZB exerted high toxicity to some species, while Alpi appeared to be persistent and may have moderate ecotoxicity, making them less suitable as alternative FRs. DOPO and MPP may be persistent, but this conclusion was based on fewer than two studies each, clearly indicating a lack of information. Most studies performed on PER showed that it had low persistence. Unfortunately, there is a lack of data on the bioaccumulation and *in vitro* toxicity of this compound. If future studies show that Alpi, DOPO, MPP, and PER are not bioaccumulative and toxic, they may still be considered as suitable FRs. Since two studies showed a moderate ecotoxicity for APP, it would not be a first choice alternative, although it scored low in all other PB&T categories. $Mg(OH)_2$, ZHS, and ZS did not show high bioaccumulation or toxicity, and so far, appear to be the most suitable HFFRs, but they also exhibited large data gaps, since none of the HFFRs were studied as elaborately as TPP. In Table 16, the HFFR are ranked according to suitability, with the highest PBT values on top. Future research is obviously necessary, to allow the PBT properties of each compound to be compared with those of the relevant halogenated flame retardant that it would replace. The different properties should be weighed and prioritized in a more extensive risk assessment, leading to a well-balanced trade-off between functionality and negative effects on humans and the environment.

Table 16. Overview of PBT properties for selected HFFRS

Compound	Persistence	Bioaccumulation	vPvB?	Toxicity			Overview
				Ecotoxicity	*In vivo* toxicity	*In vitro* toxicity	Table nr
TPP	Low to high	Low to high	(Yes)	Low to high	Low	Low to high	Table 9
RDP	Low to high	Low to high	No	Low to high	Low (to moderate)	(Low)	Table 10
BDP	Low to high	(Low to high)	(No)	Low to high	(Low)	(Low)	Table 11
ATH	-	(Low)	(No)	Low to high	(Low)	(Low)	Table 3
ZB	-	n.d.	n.d.	High	Low to high	(Low)	Table 6
Alpi	Moderate to high	(Low, not specified)	(No)	Low to moderate	Low	Low	Table 13
PER	Low to high	(Low)	(No)	Low	Low	Low	Table 15
DOPO	(Low to high)	(Low)	(No)	Low to moderate	n.d.	(Low)	Table 12
MPP	(High)	(Low)	(No)	Low	Low	n.d.	Table 14
APP	-	(Low, not specified)	(No)	Low (to moderate)	(Low)	(Low)	Table 5
ZHS	-	(Low, not specified)	(No)	Low	Low (to moderate)	(Low)	Table 7
$Mg(OH)_2$	-	n.d.	n.d.	n.d.	(Low)	n.d.	Table 4
ZS	-	(Low, not specified)	Low)	(Low)	(Low)	n.d.	Table 8

Please note that this table gives an overview of the data found in the literature and it is not an assessment. For a more detailed clarification refer to the corresponding paragraphs.
(Bracketed) = based on two or less studies; n.d. = no data.

4

Chapter 4

4

PCB-47, BDE-47, and 6-OH-BDE-47 differentially modulate human $GABA_A$ and $\alpha_4\beta_2$ nicotinic acetylcholine receptors

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Abstract

Polychlorinated biphenyls (PCBs) and the structurally related polybrominated diphenyl ethers (PBDEs) are abundant persistent organic pollutants that exert several comparable neurotoxic effects. Importantly, hydroxylated metabolites of PCBs and PBDEs have an increased neurotoxic potency. Recently, we demonstrated that PCBs can act as (partial) agonist on $GABA_A$ neurotransmitter receptors, with PCB-47 being the most potent congener. It is, however, unknown whether BDE-47 and its metabolite 6-OH-BDE-47 exert similar effects and if these effects are limited to $GABA_A$ receptors only. We therefore investigated effects of PCB-47, BDE-47, and 6-OH-BDE-47 on the inhibitory $GABA_A$ and excitatory $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptor expressed in *Xenopus* oocytes using the two-electrode voltage-clamp technique. Since human exposure is generally not limited to individual compounds, experiments with binary mixtures were also performed. The results demonstrate that PCB-47 and 6-OH-BDE-47 act as full and partial agonist on the $GABA_A$ receptor. However, both congeners act as antagonist on the nACh receptor. BDE-47 does not affect either type of receptor. Binary mixtures of PCB-47 and 6-OH-BDE-47 induced an additive activation as well as potentiation of $GABA_A$ receptors, whereas this mixture resulted in an additive inhibition of nACh receptors. Binary mixtures of BDE-47 and 6-OH-BDE-47 yielded similar effects as 6-OH-BDE-47 alone. These findings demonstrate that $GABA_A$ and nACh receptors are affected differently by PCB-47 and 6-OH-BDE-47, with inhibitory $GABA_A$-mediated signaling being potentiated and excitatory $\alpha_4\beta_2$ nACh-mediated signaling being inhibited. Considering these opposite actions and the additive interaction of the congeners, these effects are likely to be augmented *in vivo*.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) represent an important group of brominated flame retardants. A considerable dispersion has been found worldwide throughout the environment, similar to the environmental dispersion of polychlorinated biphenyls (PCBs) (Fonnum and Mariussen, 2009). PBDEs and PCBs have a structural similarity and for both groups of compounds, 209 different possible congeners exist. Since 2003, voluntary phase out of manufacturing PBDEs and use of safer substitutes have been taken place in North America and Europe. Although commercial PBDE production strongly declined, the levels of some PBDEs in the environment may still be increasing (Darnerud *et al.*, 2001; Hites, 2004), whereas the levels of PCBs are slowly diminishing (Darnerud *et al.*, 2001). Sources of human PBDE and PCB exposure can mainly be found in house dust, the diet, and in occupational settings (Carpenter, 2006; Frederiksen *et al.*, 2009).

Several *in vivo* and *in vitro* studies have shown that PCBs act on a range of different neurophysiological targets, including neurotransmitter transporters and receptors, calcium homeostasis, and oxidative stress (for review, see Fonnum and Mariussen, 2009). Because of their structural similarity, several comparable neurotoxic effects are observed for PBDEs (Costa and Giordano, 2007; Fonnum and Mariussen, 2009). Although most of these studies focused on cytotoxicity or presynaptic effects on neurotransmission, recent reports indicated that PCBs can also act as (partial) agonist on the postsynaptic human $GABA_A$ receptor (Antunes Fernandes *et al.*, 2010a; Antunes Fernandes *et al.*, 2010b).

As neurotransmission depends on the sum of inhibitory and excitatory input, possible effects of PCBs and PBDEs on inhibitory and excitatory neurotransmitter receptors are of considerable interest. The $GABA_A$ receptor is the main inhibitory neurotransmitter receptor in the central nervous system. Activation of the $GABA_A$ receptor results in influx of Cl^-, causing a hyperpolarization and subsequent inhibition of neuronal activity (D'Hulst *et al.*, 2009). The $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptor is an abundant excitatory neurotransmitter receptor in the central and peripheral nervous system. Activation of the nACh receptor by acetylcholine (ACh) leads to an influx of cations, resulting in depolarization of the cell membrane and excitation of neuronal activity. Both $GABA_A$ and $\alpha_4\beta_2$ nACh receptors are critically involved in brain development (Dwyer *et al.*, 2009; Represa and Ben-Ari, 2005) as well as in long-term potentiation and synaptic plasticity (Fujii *et al.*, 2000). Importantly, PCBs and PBDEs mainly target the developing nervous system (Eriksson *et al.*, 2002; Johansson *et al.*, 2008; Sugawara *et al.*, 2006; Viberg *et al.*, 2003a; Viberg *et al.*, 2003b) and reduce long-term potentiation and synaptic plasticity (Dingemans *et al.*, 2007; Gilbert, 2003; Xing *et al.*, 2009), possibly by changing the cholinergic system (Eriksson *et al.*, 2002; Viberg *et al.*, 2003a; Viberg *et al.*, 2003b). Considering the resemblance between (the effects of) PCBs and PBDEs, it is possible that, just as PCBs, PBDEs also interact with the $GABA_A$ receptor and that both PCBs and PBDEs can interact also with the excitatory nACh receptor.

Previous studies demonstrated that several PCBs, depending on their chlorination pattern, act as (partial) agonists on the human $GABA_A$ receptor (Antunes Fernandes *et al.*, 2010a; Antunes Fernandes *et al.*, 2010b). Of the 22 PCBs investigated, PCB-47 acted both as full and partial agonist on the human $GABA_A$ receptor and was the most potent congener. Importantly, as humans are exposed to complex mixtures of environmental pollutants, including PCBs and PBDEs, it is highly relevant to consider the possibility of synergistic, additive, or antagonistic interactions. For example, PCBs applied as binary mixtures resulted in competitive activation and potentiation of the human $GABA_A$ receptor (Antunes Fernandes *et al.*, 2010a; Antunes Fernandes *et al.*, 2010b), which is in

line with several other reports demonstrating additive neurotoxic effects of simple PCB and/or PBDE mixtures (Eriksson *et al.*, 2006; Fonnum and Mariussen, 2009; Gao *et al.*, 2009). In relation to this, it should be noted that the structurally similar BDE-47 is the most abundant PBDE congener found in the environment (Hites, 2004). Furthermore, hydroxylated metabolites of PBDEs, formed either by *in vivo* metabolism (Qiu *et al.*, 2009) or as natural product produced by marine organisms (Malmvärn *et al.*, 2008), have been detected in human blood (Athanasiadou *et al.*, 2008; Qiu *et al.*, 2009). Importantly, recent reports indicated that hydroxylated metabolites of PBDEs, including 6-OH-BDE-47, are more potent than their parent compounds with respect to neurotoxicity and endocrine disruption (Cantón *et al.*, 2008; Dingemans *et al.*, 2008; Kojima *et al.*, 2009). We therefore investigated the possible effects of PCB-47, BDE-47, and its metabolite 6-OH-PBDE-47 alone and as binary mixtures on the inhibitory human $GABA_A$ receptor and the excitatory human $\alpha_4\beta_2$ nACh receptor.

2. Material and methods

2.1 Animals

All experiments were conducted in accordance with Dutch law and approved by the Ethical Committee for Animal Experiments of Utrecht University. Adult female specimen of *Xenopus laevis* frogs (provided by Dr. Wim Scheenen, Radboud University, Nijmegen, The Netherlands) were kept in copper-free water (pH 6.5, 23°C) in standard aquaria (0.5 × 0.4 × 1 m; 1-10 per aquarium) with a 12 h light/dark cycle. The animals were fed earthworms three times a week (Hagens, Nijkerkerveen, The Netherlands).

2.2 Chemicals

ACh, gamma-aminobutyric acid (GABA), dimethyl sulfoxide (DMSO), neomycin solution (10 mg neomycin/ml in 0.9% NaCl), collagenase type I, NaCl, and 3-aminobenzoic acid ethyl ester (MS-222) were obtained from Sigma Chemical (St Louis, MO). $CaCl_2$ (1 M solution), $MgCl_2$ (1 M solution), $MgSO_4$, $NaHCO_3$, NaOH, $Ca(NO_3)_2$, KCl, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Merck (Darmstadt, Germany). 2,2',4,4'-Tetrachlorobiphenyl (PCB-47) was purchased from Neosync Inc. and purified (>99.9% purity) as described previously (Danielsson *et al.*, 2008). 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) and 6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (6-OH-BDE-47) were synthesized and purified (~99% purity) at the Department of Environmental Chemistry of Stockholm University as described previously (Marsh *et al.*, 1999; Örn *et al.*, 1996).

PCB-47, BDE-47, and 6-OH-BDE-47 were dissolved in purity-checked DMSO, and stock solutions of 25 mM (PCB-47) or 20 mM (BDE-47 and 6-OH-BDE-47) were further diluted to obtain final concentrations of 0.1-10 μM. DMSO at concentrations up to 0.5% (vol/vol) had no effect on $GABA_A$ or nACh receptor-mediated currents. The final concentration of DMSO in congener-containing saline was always kept below 0.1% (vol/vol).

2.3 Expression of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ and $\alpha_4\beta_2$ nACh receptors in *Xenopus laevis* oocytes

All procedures have been described previously (Antunes Fernandes *et al.*, 2010b). Briefly, female *Xenopus laevis* were anesthetized by submersion in 0.1% MS-222, and ovarian lobes were surgically removed. Oocytes were treated with collagenase type I (1.5 mg/ml Ca^{2+}-free Barth's solution) for 90 min at room temperature before manual defolliculation.

Complementary DNA (cDNA) coding for the human α_1, β_2, and γ_{2L} subunits of human $GABA_A$ receptors (kindly provided by Dr Paul J. Whiting, Merck Sharp & Dohme

Research Laboratories, Neuroscience Research Center, Harlow, Essex, UK) was dissolved in distilled water at a 1:1:1 molar ratio and injected (23 nl/oocyte, ~1 ng of each subunit) into the nuclei of stage V or VI oocytes using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA). cDNA coding for the human α_4 and β_2 subunits of the human neuronal nACh receptors (kindly provided by Janssen Pharmaceutica N.V., Beerse, Belgium) was dissolved in distilled water at a 1:1 molar ratio and injected in a total injection volume of 18.4 nl/oocyte (~0.2 ng of each subunit).

Sham-injected oocytes were injected only with 18.4 or 23 nl of distilled water, i.e., without cDNA. Following injection of the cDNA or only distilled water, oocytes were incubated at 21°C in modified Barth's solution containing (in millimolar) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES, and 10 lg/ml neomycin (pH 7.6 with NaOH). Experiments were performed on oocytes after 2-5 days of incubation.

2.4 Electrophysiological recordings

Following translation of injected cDNA, the oocytes expressed functional $GABA_A$ or $\alpha_4\beta_2$ nACh receptors in the membrane. Ion currents associated with $GABA_A$ or $\alpha_4\beta_2$ nACh receptor activity were measured with the two-electrode voltage-clamp technique using a Gene Clamp 500B amplifier (Axon Instruments) with high-voltage output stage as described previously (Antunes Fernandes *et al.*, 2010b). Recording microelectrodes (0.5-2.5 MΩ) were filled with KCl (3 M). Oocytes, placed in a custom-built Teflon oocyte recording chamber, were voltage clamped at -60 mV and continuously superfused (~6 ml/min) with saline solution containing (in millimolar) 115 NaCl, 2.5 KCl, 1 $CaCl_2$, 10 HEPES (pH 7.2 with NaOH). Membrane currents were low-pass filtered (8-pole Bessel; 3 dB at 0.3 kHz), digitized (12 bits; 1024 samples per record), and stored on disk for computer analysis.

Aliquots of freshly thawed stock solutions of GABA or ACh in distilled water and the different PCB or PBDE congeners in DMSO were added to the saline immediately before the experiments. Oocytes were exposed to compounds by switching the perfusate from saline to congener- and/or agonist containing saline using a ValveLink8.2 perfusion system (AutoMate Scientific, Berkeley, CA). For specific experiments, oocytes were exposed to an equimolar mixture of BDE-47 and 6-OH-BDE-47 or an approximately equipotent mixture of PCB-47 and 6-OH-BDE-47. To minimize adsorption of PCB and PBDE congeners to the perfusion system, glass reservoirs and Teflon tubes (polytetrafluoroethylene; 1.57 × 2.17 mm, Rubber, Hilversum, The Netherlands) were used.

Oocytes were exposed repeatedly to different GABA-, ACh-, and/or congener-containing solutions for 20-40 s. A washout period of 2-5 min between each application was introduced, allowing receptors to recover from desensitization. Sham-injected oocytes did not show any ion current upon superfusion with GABA, ACh, PCB-47, BDE-47, or 6-OH-BDE-47, either alone, in mixtures, or during co-application (data not shown).

2.5 Data analysis and statistics

Peak amplitudes of agonist-induced ion currents were measured and normalized to the maximal amplitude (at 1 mM) of agonist-induced control responses to adjust for differences in receptor expression levels among oocytes and for small variations in response amplitudes over time. Normalized ion currents were plotted against agonist concentration in each experiment. Agonist concentration-effect curves were fitted to the data obtained in separate experiments using Prism (Graphpad Software, La Jolla, CA). The percentage of congener-induced potentiation or inhibition of the agonist-induced

ion current was calculated from the quotient of the maximum amplitude of the agonist-congener co-application response and the maximum amplitude of the control (agonist) response. Data represent mean ± SEM of *n* oocytes. Each experiment was repeated on oocytes obtained from at least two different animals.

The concentration dependence of the potentiating or inhibiting effects of the congeners was determined by a two-tailed Student's *t*-test. One-way ANOVA and post hoc Bonferroni test were used to determine the effects of binary mixtures. *P* values below 0.05 were considered statistically significant.

3. Results

3.1 Functional properties of $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ and $\alpha_4\beta_2$ nACh receptors in *Xenopus* oocytes

Voltage-clamped (-60 mV) oocytes expressing human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ or $\alpha_4\beta_2$ nACh receptors were superfused with various concentrations of GABA or ACh, resulting in concentration-dependent ion currents. Agonist-induced ion currents were normalized to the response obtained with 1 mM and plotted against the agonist concentration to obtain a concentration-effect curve according to the Hill equation. This curve was used to determine effective concentration $(EC)_{20}$ and EC_{50} values, i.e., the concentrations producing 20 and 50% of the maximal response. In line with previous reports (e.g., Antunes Fernandes *et al.*, 2010b), the Hill slope for the fitted GABA concentration-effect curve was 1.30 ± 0.04 and mean values for EC_{20} and EC_{50} amounted to 22 ± 1 and 63 ± 1 μM, respectively (n = 9; Figure 1A). For ACh, the concentration-effect curve was best fitted with a Hill slope of 1.13 ± 0.01 and EC_{20} and EC_{50} amounted to 42 ± 9 and 145 ± 10 μM (n = 5; Figure 1B), respectively, comparable with previous reports (e.g., Smulders *et al.*, 2005).

3.2 Agonistic effects of congeners on human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptor

As also shown previously (Antunes Fernandes *et al.*, 2010a), PCB-47 acted as full and partial agonist on the inhibitory $GABA_A$ receptor. We therefore investigated possible agonistic effects of the structurally related BDE-47 and its hydroxylated metabolite, 6- OH-BDE-47. Oocytes expressing human $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors were superfused with 1 or

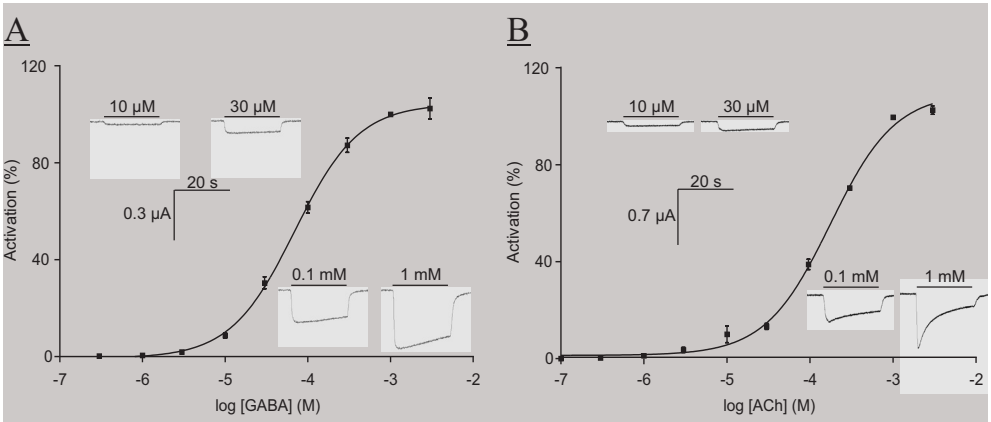

Figure 1. GABA (A) and ACh (B) concentration-response curves of human $GABA_A$ and $\alpha_4\beta_2$ nACh receptors expressed in *Xenopus* oocytes. Insets show example recordings evoked by superfusion with saline-containing different GABA or ACh concentrations, as indicated above the recordings. Scale bar applies to all traces. Fitted lines are Hill curves with mean values (± SEM) obtained from 5 to 9 separate experiments.

10 μM PCB-47, BDE-47, or 6-OH-BDE-47. As shown in Figure 2A, BDE-47 was not able to activate the $GABA_A$ receptor. However, superfusion with PCB-47 or 6-OH-BDE-47 resulted in a concentration-dependent activation of the $GABA_A$ receptor (Figure 2B, Table 1). At the low concentration (1 μM), superfusion with PCB-47 resulted in a larger activation of the $GABA_A$ receptor compared with 6-OH-BDE-47. At the high concentration (10 μM), however, 6-OH-BDE-47 is more potent (>50% of the maximum response evoked by GABA).

PCB-47, BDE-47, and 6-OH-BDE-47 were co-applied with a low concentration (EC_{20}) GABA to determine possible partial agonistic effects. Co-application of BDE-47 (at 1 and 10 μM) with GABA at EC_{20} did not affect the GABA-induced ion current. On the other hand, co-application of PCB-47 or 6-OH-BDE-47 with GABA (at EC_{20}) resulted in a concentration-dependent potentiation of the GABA-induced ion current (Figure 2C, Table 1). When co-applied with GABA (EC_{20}), PCB-47 showed a larger potentiation at the low concentration (1 μM), whereas 6-OH-BDE-47 was more potent at the high concentration (10 μM). This indicates that although BDE-47 is not able to activate the $GABA_A$ receptor, the hydroxylated metabolite 6-OH-BDE-47 acts, like PCB-47, as full and partial agonist on the $GABA_A$ receptor.

To investigate possible effects of combinations of these compounds, oocytes were exposed to an equimolar (1 μM) mixture of BDE-47 and 6-OH-BDE-47. As shown in Table 1, this binary mixture did not induce a change in response compared with oocytes exposed to 6-OH-BDE-47 alone. In addition, oocytes co-exposed to a low concentration of GABA (EC_{20}) and an equimolar (1 μM) mixture of BDE-47 and 6-OH-BDE-47 showed no difference in the potentiating effect of the GABA-induced ion current compared with 6-OH-BDE-47 only. Both types of experiments again indicate that PBDE- 47 has no ability to change the GABA-evoked ion current, neither alone nor in mixture (Figures 2 and 3).

Table 1. Overview of the effects of PCB-47, PBDE-47, 6-OH-PBDE-47, and binary mixtures on $GABA_A$ receptor activation (full agonist; left) and potentiation (partial agonist; right)

		Full agonist		Partial agonist		
Congener	**Concentration (μM)**	***n***	**% activation**	**[GABA]**	***n***	**% potentiation**
PCB-47	0.1	8	8.1 ± 2.6	EC_{20}	10	85.5 ± 24.7
	1	14	14.8 ± 4.6	EC_{20}	10	169.9 ± 14.9
	10	4	25.0 ± 11.8	EC_{20}	12	198.3 ± 13.2
BDE-47	1	13	NE	EC_{20}	12	NE
	10	6	NE	EC_{20}	5	NE
6-OH-BDE-47	1	18	3.1 ± 0.8	EC_{20}	27	71.6 ± 10.3
	10	8	55.3 ± 16.1[a]	EC_{20}	7	843.1 ± 215.0[c]
BDE-47 + 6-OH-BDE-47	1 + 1	10	1.7 ± 0.5	EC_{20}	7	63.9 ± 9.5
PCB-47 + 6-OH-BDE-47	0.1 + 1	7	22.1 ± 5.9[b]	EC_{20}	8	205.9 ± 70.2[d]

Values are presented as mean ± SEM. NE, no effect.
[a] Significantly different from 1 μM 6-OH-BDE-47 (p<0.001).
[b] Significantly different from 0.1 μM PCB-47 (p<0.01) and 1 μM 6-OH-BDE-47 (p<0.001).
[c] Significantly different from 1 μM 6-OH-BDE-47 (p<0.0001).
[d] Significantly different from 0.1 μM PCB-47 (p<0.01) and 1 μM 6-OH-BDE-47 (p<0.001).

The effect of binary mixtures on the $GABA_A$ receptor was further tested by determining the effect of PCB-47 and 6-OHBDE-47 in an approximately equipotent mixture (0.1 μM PCB-47 and 1 μM 6-OH-BDE-47). As shown in Table 1, a significant larger activation, compared with the effect of the congeners alone, was observed when oocytes were superfused with the equipotent mixture. Oocytes were also co-exposed to a low concentration of GABA (EC_{20}) and the approximately equipotent mixture of PCB-47 (0.1 μM) and 6-OH-BDE-47 (1 μM). Co-application of GABA with the equipotent mixture resulted in a significant larger potentiation (Table 1). These findings demonstrate that exposure to a mixture of PCB-47 and 6-OH-BDE-47 results in an additive activation as well as potentiation (with GABA at EC_{20}) of the $GABA_A$ receptor.

3.3 Antagonistic effects of congeners on human $\alpha_4\beta_2$ nACh receptor

Oocytes expressing human $\alpha_4\beta_2$ nACh receptors were superfused with 1 or 10 μM PCB-47, BDE-47, or 6-OHBDE-47. None of the congeners induced any ion currents demonstrating that the congeners by themselves are not agonists of the nACh receptor (data not shown).

To determine possible partial agonistic effects of the congeners, the congeners were co-applied with a low-concentration ACh (EC_{20}). No effects were observed for BDE-47. However, instead of potentiation of the agonist-induced ion current as observed for GABA-induced ion currents, 6-OH-BDE-47 and PCB-47 produced small but concentration-dependent inhibition of the ACh-induced ion current (Figure 3B), indicating that these two congeners act as weak antagonists on the nACh receptor. At the low concentrations (1 μM), the inhibitory effects of both congeners are roughly equipotent, whereas at the high concentrations (10 μM), 6-OH-BDE-47 is more effective.

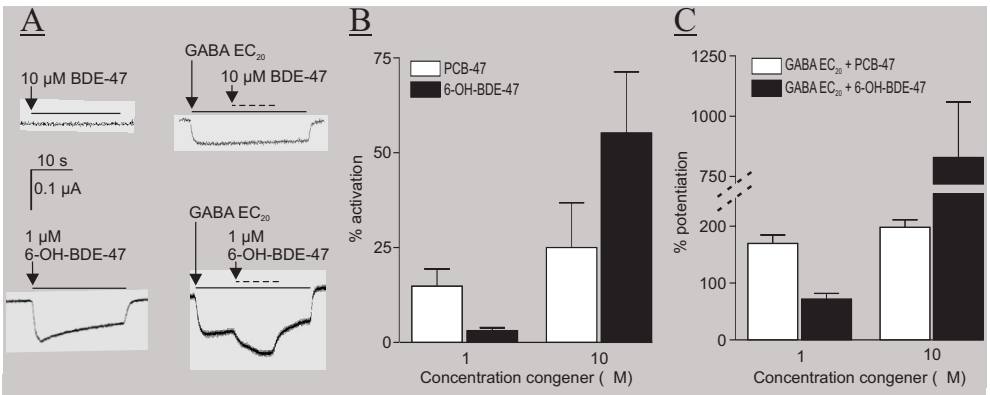

Figure 2. Full and partial agonistic effects of PCB-47 and 6-OH-BDE-47 on the $GABA_A$ receptor. (A) Example recordings of currents evoked by different congeners or of different congeners co-applied with GABA at EC_{20}. BDE-47 does not affect the $GABA_A$ receptor (upper left) or GABA-evoked (EC_{20}) ion current (upper right). Application of 6-OH-BDE-47 (lower left) or PCB-47 (not shown) resulted in activation of the $GABA_A$ receptor. When co-applied with GABA at EC_{20}, 6-OH-BDE-47 (lower right) and PCB-47 (not shown) potentiate the $GABA_A$ receptor. Scale bar applies to all traces. (B) Bar graph demonstrating the concentration-dependent activation of the $GABA_A$ receptor evoked by PCB-47 and 6-OH-BDE-47. Activation is presented as percentage of the maximum GABA-evoked response (1 mM). (C) Bar graph demonstrating the concentration-dependent potentiation of GABA-induced responses by PCB-47 and 6-OH-BDE-47. Potentiation is presented as percentage of the GABA-evoked response at EC_{20}. Bars represent mean ± SEM (n = 4-18).

Table 2. Overview of the effects of PCB-47, BDE-47, 6-OH-BDE-47 and binary mixtures on $\alpha_4\beta_2$ nACh receptor inhibition

Congener	Concentration (μM)	[ACh]	*n*	% inhibition
PCB-47	1	EC_{20}	14	-1.6 ± 0.3
	10	EC_{20}	8	-3.3 ± 0.4[a]
BDE-47	1	EC_{20}	6	NE
	10	EC_{20}	12	NE
6-OH-BDE-47	1	EC_{20}	12	-1.2 ± 0.4
	10	EC_{20}	11	-9.3 ± 1.3[b]
BDE-47 + 6-OH-BDE-47	10 + 10	EC_{20}	5	-8.4 ± 1.0
PCB-47 + 6-OH-BDE-47	1 + 1	EC_{20}	11	-3.9 ± 0.5[c]

Values are presented as mean ± SEM. NE, no effect.
[a] Significantly different from 1 μM PCB-47 (p<0.01).
[b] Significantly different from 1 μM 6-OH-BDE-47 (p<0.0001).
[c] Significantly different from 1 μM PCB-47 (p<0.001) and 1 μM 6-OH-BDE-47 (p<0.001).

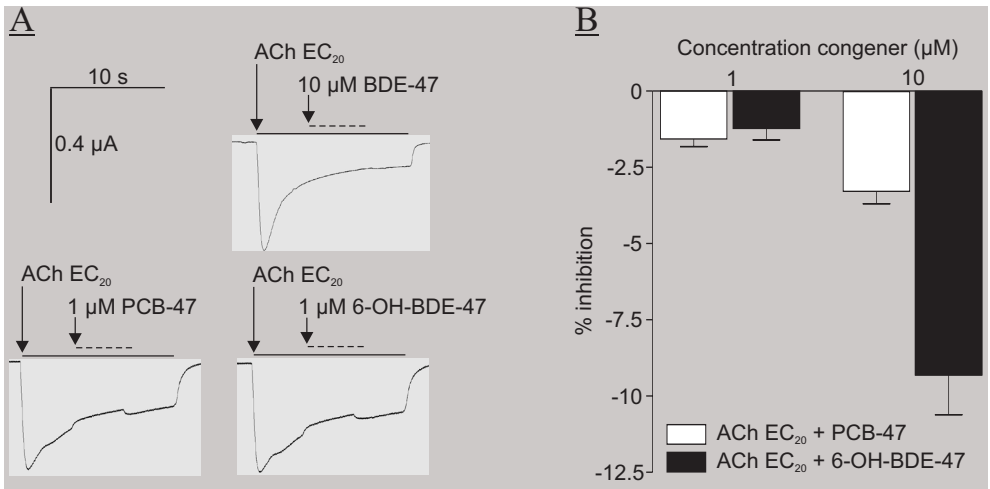

Figure 3. Antagonistic effects of PCB-47 and 6-OH-BDE-47 on the $\alpha_4\beta_2$ nACh receptor. (A) Example recordings of inhibition of ACh-evoked currents by co-application of PCB-47 (lower left) or 6-OH-BDE-47 (lower right) with ACh (EC_{20}). PBDE-47 does not affect the ACh-evoked ion current (upper right). Scale bar applies to all traces. (B) Bar graph demonstrating the concentration-dependent inhibition of ACh-induced responses by PCB-47 and 6-OH-BDE-47. Inhibition is presented as percentage of the ACh-evoked response at EC_{20}. Bars represent mean ± SEM (n = 8-14).

Although BDE-47 did not induce a change in the ACh-evoked ion current, it was tested for possible effects when mixed with another congener. Oocytes were co-exposed to ACh (at EC_{20}) with an equimolar (10 μM) mixture of BDE-47 and 6-OH-BDE-47. However, no change in the inhibitory effect of 6-OH-BDE-47 on the ACh-induced ion current was observed (Table 2), again indicating that BDE-47 does not have the ability to change the ACh-induced ion current, neither alone nor in mixture. Co-exposure to ACh (at EC_{20}) and the approximately equipotent mixture of 1 μM PCB-47 and 1 μM 6-OH-BDE-47 resulted in a significant larger inhibition compared with the inhibition of the congeners alone (Table 2), again suggesting an additive effect of a mixture of PCB-47 and 6-OH-BDE-47.

4. Discussion

In this study, we investigated the effects of three abundant congeners, alone and as binary mixtures, on the postsynaptic inhibitory human $GABA_A$ and excitatory human $\alpha_4\beta_2$ nACh receptor. The results demonstrate that PCB-47 and 6-OHBDE-47 both act as full and partial agonist on the $GABA_A$ receptor, resulting in concentration-dependent activation and potentiation of the $GABA_A$ receptor (Figure 2). On the contrary, both congeners act as antagonists on the $\alpha_4\beta_2$ nACh receptor (Figure 3). BDE-47 has no effects on either type of receptor (Tables 1 and 2), underlining the notion that oxidative metabolism can increase the neurotoxic potency (Cantón *et al.*, 2008; Dingemans *et al.*, 2008; Kojima *et al.*, 2009). At the highest concentration investigated (10 μM), 6-OH-BDE-47 is more effective in activating and potentiating the $GABA_A$ receptor and inhibiting the nACh receptor than PCB-47. Binary mixtures of PCB-47 and 6-OH-BDE-47 enhanced the effects on the $GABA_A$ and $\alpha_4\beta_2$ nACh receptor in an additive or possibly even synergistic manner, whereas co-exposure with BDE-47 did not change the effects induced by PCB-47 or 6-OH-BDE-47 (Tables 1 and 2). Though additive and synergistic interactions between organohalogen compounds were described earlier (Antunes Fernandes *et al.*, 2010b; Eriksson *et al.*, 2006; Fonnum and Mariussen, 2009; Gao *et al.*, 2009), further mixture studies will have to be performed to classify these effects as synergistic.

Although PBDEs are structurally similar to PCBs, no effect of BDE-47 on the human $GABA_A$ receptor or $\alpha_4\beta_2$ nACh receptor was observed in this study. The differences seen in receptor activity between PCB-47 and BDE-47 might be due to a difference in the planarity of the molecules, which is largely determined by halogen substitutions at the *ortho* positions of the ring structures. Because of the connecting oxygen atom between these rings in PBDEs, the planarity will be rather different compared with a PCB molecule with similar *ortho* substitutions. In addition to planarity, the difference in size between bromine-substituted BDE-47 and chlorine-substituted PCB-47 may also play a role. Taking these differences into account, it is possible that BDE-47 has a more non-coplanar, steroid-like structure than PCB-47 and is therefore less able to interact with a binding site. Conversely, the hydroxylated metabolite 6-OH-BDE-47, which clearly showed an effect on both the $GABA_A$ and $\alpha_4\beta_2$ nACh receptor, has an altered planarity because of the *ortho*-substituted hydroxy group that may explain the observed effects on the receptors. Noteworthy, the bromination pattern and the degree of shielding affect the potency of hydroxylated metabolites of BDE-47 to disturb intracellular calcium homeostasis (Dingemans *et al.*, 2010). However, it remains to be determined whether a similar structure-activity relation applies for interactions with $GABA_A$ and $\alpha_4\beta_2$ nACh receptors.

Since both the $GABA_A$ and the $\alpha_4\beta_2$ nACh receptors have several binding sites via which organic pollutants can exert their effects (D'Hulst *et al.*, 2009; Johnston, 2005; Paterson

and Nordberg, 2000), the different potencies of PCB-47 and 6-OH-BDE-47 suggest that they could bind to different binding sites. As it was recently suggested that binary mixtures of PCBs activate and potentiate the human $GABA_A$ receptor in a competitive manner (Antunes Fernandes *et al.*, 2010a; Antunes Fernandes *et al.*, 2010b), association with different binding sites can also explain the additive effect observed when PCB-47 and 6-OH-BDE-47 were applied as mixture. The observed activation and potentiation of the $GABA_A$ receptor is likely mediated via one of the agonist binding sites, e.g., the benzodiazepine binding site or the zinc binding site (Barberis *et al.*, 2000). As the antagonistic effects observed on the $\alpha_4\beta_2$ nACh receptor are very modest, the observed inhibition could be due to partitioning of PCB-47 and 6-OH-BDE-47 into the membrane, resulting in a small and nonspecific inhibition. However, it is unlikely that membrane partitioning can explain the observed inhibition as no effect is observed for the structurally related parent compound BDE-47. It is therefore more likely that the inhibition of the $\alpha_4\beta_2$ nACh receptor is due to competitive binding of PCB-47 and 6-OH-BDE-47 to the ACh binding site or one of the (negative) allosteric binding sites. Future research, possibly using mutated subunits, will have to elucidate which binding sites are involved in the effects found in this study.

The observed opposite effects, with the inhibitory GABA-mediated signaling being activated or potentiated and the excitatory ACh-mediated signaling being inhibited, are of concern, especially for the developing brain. Both the $GABA_A$ receptor and $\alpha_4\beta_2$ nACh receptor play an important role in long-term potentiation and synaptic plasticity, whereas PCBs and PBDEs have been shown to affect these processes (Dingemans *et al.*, 2007; Gilbert, 2003; Xing *et al.*, 2009). Interestingly, during early brain development (but not at other developmental stages), the $GABA_A$ receptor acts as an excitatory receptor (D'Hulst *et al.*, 2009). Consequently, the results of this study possibly underlie the neurobehavioral and neurodevelopmental effects seen after PCB and/or PBDE exposure (Eriksson *et al.*, 2002; Eriksson *et al.*, 2006; Viberg *et al.*, 2003a; Viberg *et al.*, 2003b), which may differ for the adult compared with the developing brain.

Plasma concentrations of PCB-47, BDE-47, and 6-OH-BDE-47 for the general human population are in the low nanomolar range (Qiu *et al.*, 2009; Wingfors *et al.*, 2000), though levels of PCBs and PBDEs in occupationally exposed people are one order of magnitude higher (Kontsas *et al.*, 2004; Schecter *et al.*, 2009). Even though the effects observed in this study occur in the low micromolar range, they should be taken into account for human risk assessment as humans are exposed to mixtures of persistent organic pollutants and these effects appear to be additive. Furthermore, it has been shown that congeners and their hydroxylated metabolites can accumulate in humans (Athanasiadou *et al.*, 2008; Qiu *et al.*, 2009). Moreover, PBDE metabolites not only have an increased neurotoxic potency but recent reports also indicated that the total concentration of hydroxylated PBDE metabolites in human blood is close to those of PBDEs (Qiu *et al.*, 2009). The observed additive interactions between congeners, together with the increasing levels of hydroxylated PBDE metabolites in human tissues and the background levels of PCBs, are therefore concerning and demonstrates that additivity and hydroxylated metabolites should be included in human risk assessment.

In summary, this is the first study demonstrating differential postsynaptic effects of persistent organic pollutants. Besides an agonistic effect on the $GABA_A$ receptor, we also demonstrated a concentration-dependent antagonistic effect of PCB-47 on the $\alpha_4\beta_2$ ACh receptor. These opposite effects, with the inhibitory $GABA_A$ receptor being activated or potentiated and the excitatory $\alpha_4\beta_2$ nACh receptor being inhibited, were also observed

for 6-OH-BDE-47. Importantly, additive agonistic and antagonistic effects were observed when a binary mixture of PCB-47 and 6-OH-BDE-47 was applied. Taking into account human exposure to complex mixtures, additive effects on both the $GABA_A$ and the $\alpha_4\beta_2$ nACh receptor, and the opposite actions of GABA and ACh in the nervous system, the adverse effects may very well be augmented *in vivo*.

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4

5

Chapter 5

Multiple novel modes of action involved in the *in vitro* neurotoxic effects of tetrabromobisphenol-A

5

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Abstract

Neurotoxicological data on the widely used brominated flame retardant tetrabromobisphenol-A (TBBPA) is limited. Since recent studies indicated that inhibitory $GABA_A$ and excitatory $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptors are sensitive targets for persistent organic pollutants, we investigated the effects of TBBPA on these receptors, expressed in *Xenopus* oocytes, using the two-electrode voltage-clamp technique. Our results demonstrate that TBBPA acts as full (≥10 μM) and partial (≥0.1 μM) agonist on human $GABA_A$ receptors, whereas it acts as antagonist (≥10 μM) on human $\alpha_4\beta_2$ nACh receptors. Next, neuronal B35 cells were used to further study the effects of TBBPA on calcium-permeable nACh receptors using single-cell fluorescent calcium imaging. These results demonstrate that TBBPA (≥1 μM) inhibits acetylcholine (ACh) receptors as evidenced by a reduction in the ACh-evoked increases in the intracellular calcium concentration ($[Ca^{2+}]_i$). Additionally, TBBPA (>1 μM) induced a strong and concentration-dependent increase in basal $[Ca^{2+}]_i$ in B35 cells. Similarly, TBBPA (>1 μM) increases basal $[Ca^{2+}]_i$ in dopaminergic PC12 cells. This increase is also evident under calcium-free conditions, indicating it originates from intracellular calcium stores. Moreover, depolarization-evoked increases in $[Ca^{2+}]_i$ are strongly reduced by TBBPA (≥1 μM), indicating TBBPA-induced inhibition of voltage-gated calcium channels. Our *in vitro* studies thus demonstrate that TBBPA exerts several adverse effects on functional neurotransmission endpoints with effect concentrations that are only two orders of magnitude below the highest cord serum concentrations. Although epidemiological proof for adverse TBBPA effects is lacking, our data justify the quest for flame retardants with reduced neurotoxic potential.

1. Introduction

Tetrabromobisphenol-A (TBBPA) is a widely used brominated flame retardant (BFR) in electrical and electronic equipment such as TVs, computers, printers, cell phones, etc. with an estimated annual worldwide market demand of >120,000 tons (de Wit *et al.*, 2010). It has been used as a replacement for polybrominated diphenyl ethers (PBDEs), whose persistence in the environment and potential for adverse health effects have prompted concerns (for review, see Dingemans *et al.*, 2011). As TBBPA is a phenolic compound primarily used as a chemically bound flame retardant, it was not expected to reach the environment in large amounts. However, several studies have shown that TBBPA can leak into the environment from treated products (Sellstrom and Jansson, 1995), and recent reports have shown its presence in wildlife and human samples (Covaci *et al.*, 2009; de Wit *et al.*, 2010). The presence of TBBPA in seafood (WHO, 1995) as well as in commercial drinking water that was stored in polycarbonate containers (Peterman *et al.*, 2000) may also contribute to the levels seen in human serum. TBBPA levels have been reported in human serum samples (0.40-0.71 ng/g lw; Thomsen *et al.*, 2002) and adipose tissue samples (0.048 ng/g lw; Johnson-Restrepo *et al.*, 2008) of non-occupationally exposed adults. Following analysis of house dust samples in Germany and the United States, the daily exposure for toddlers was estimated to be 0.2 ng/kg bw/d for TBBPA (Abb *et al.*, 2011). The daily exposure of a 1-month-old infant via breast milk was estimated to be as high as 1 ng/kg bw/d (Abdallah and Harrad, 2011). Because the usage of TBBPA in Asia is about eight times higher than in Europe (Law *et al.*, 2006), values for human serum samples as well as dust samples are at least a factor of three higher in Asia (Nagayama *et al.*, 2000; Takigami *et al.*, 2009).

Although PBDEs are clearly associated with neurotoxicity (for review, see Dingemans *et al.*, 2011), information on the neurotoxicity of TBBPA is limited. It has been demonstrated that neonatal TBBPA exposure does not result in behavioral effects in adult mice (Eriksson *et al.*, 2001) but can affect the cholinergic system in neonates (Viberg and Eriksson, 2011). Moreover, behavioral effects in mice following acute exposure to TBBPA have been reported (Nakajima *et al.*, 2009). Additionally, TBBPA was shown to concentration dependently increase the production of reactive oxygen species (ROS) *in vitro* (Reistad *et al.*, 2005; Reistad *et al.*, 2007). Furthermore, at low micromolar concentrations TBBPA increases the basal intracellular calcium concentration ($[Ca^{2+}]_i$), extracellular glutamate levels, and cell death in human neutrophil granulocytes and cerebellar granule cells (Reistad *et al.*, 2005; Reistad *et al.*, 2007). In Sertoli cells, TBBPA induces apoptosis via increases in $[Ca^{2+}]_i$ involving Ca^{2+}-dependent mitochondrial depolarization, inhibition of sarcoplasmic/endoplasmic reticulum (ER) Ca^{2+}-ATPases and activation of ryanodine receptor Ca^{2+}-channels (Ogunbayo *et al.*, 2008). These findings are of importance as Ca^{2+} plays an essential role in multiple physiological and pathological processes, including neurotransmission (Garcia *et al.*, 2006; Westerink, 2006) and cell viability (Orrenius *et al.*, 2011).

So far, most studies on the *in vitro* neurotoxic potential of TBBPA focused on cytotoxicity or presynaptic effects of neurotransmission, although recent studies indicate that persistent organic pollutants (POPs), such as PBDEs and polychlorinated biphenyls (PCBs), can also affect postsynaptic human $GABA_A$ and $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptors (Antunes Fernandes *et al.*, 2010b; Hendriks *et al.*, 2010). The $GABA_A$ receptor is the main inhibitory neurotransmitter receptor in the central nervous system, whereas the $\alpha_4\beta_2$ nACh receptor is an abundant excitatory neurotransmitter receptor in the central and peripheral nervous system. Both receptors are also critically involved in brain development as well as in long-term potentiation and synaptic plasticity (Dwyer

et al., 2009; D'Hulst *et al.*, 2009). Possible effects of TBBPA on these receptors are thus of considerable interest.

In the present study, we investigated the acute effects of TBBPA on human $GABA_A$ and $\alpha_4\beta_2$ nACh receptors, expressed in *Xenopus* oocytes, using the two-electrode voltage-clamp technique. To further unravel the mechanisms underlying the observed changes in neuronal signaling, B35 neuroblastoma cells were used to investigate the acute effects of TBBPA on Ca^{2+}-homeostasis and acetylcholine (ACh)- evoked increases in $[Ca^{2+}]_i$. Although B35 cells lack functional voltage-gated calcium channels (VGCCs), these cells have proven to be useful in the molecular analysis of endocytosis and intra- and intercellular signaling pathways (Otey *et al.*, 2003), including ACh-mediated signaling (Heusinkveld and Westerink, 2011), due to their high expression of calcium-permeable nACh receptors. Dopaminergic pheochromocytoma (PC12) cells, which are widely used to assess neurotoxicity, neuronal function, and calcium homeostasis (for review, see Westerink and Ewing, 2008), were used to study in more detail the observed effects of this BFR on basal and depolarization-evoked calcium homeostasis.

2. Material and methods

2.1 Chemicals

$CaCl_2$ (1 M solution), $MgCl_2$ (1 M solution), $MgSO_4$, $NaHCO_3$, NaOH, $Ca(NO_3)_2$, KCl, glucose, sucrose, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Merck (Darmstadt, Germany). RPMI 1640, DMEM, PenStrep, PBS, Fura-2 AM, and 2',7'-dichlororfluorescin 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 deionized water (Milli-Q; resistivity >10 MΩ × cm) and contained (in millimolar) 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). Saline solutions for oocyte electrophysiology contained (in millimolar) 115 NaCl, 2.5 KCl, 1 $CaCl_2$, and 10 HEPES (pH 7.2 with NaOH). Stock solutions of 2 mM ionomycin in dimethyl sulfoxide (DMSO) were kept at -20°C. Gabazine (SR-95531) stock solution of 100 mM in DMSO was further diluted to obtain an experimental concentration of 25 μM. Stock solutions of 0.1-100 mM TBBPA (>99% pure, Sigma-Aldrich) were prepared in DMSO and diluted in saline to obtain the desired concentrations just prior to the experiments (all solutions used in experiments, including control experiments, contained 0.1% DMSO). All experiments were performed at room temperature (20°C-22°C).

2.2 Electrophysiological recordings of $\alpha_1\beta_2\gamma_2$ $GABA_A$ or $\alpha_4\beta_2$ nicotinic ACh receptors function in *Xeonpus laevis* oocytes

All procedures have been described previously (Hendriks *et al.*, 2010) and were conducted in accordance with Dutch law and approved by the Ethical Committee for Animal Experiments of Utrecht University. Briefly, adult female *Xenopus laevis* (provided by Dr. Wim Scheenen, Radboud University, Nijmegen, The Netherlands) were anesthetized by submersion in 0.1% MS-222, and ovarian lobes were surgically removed. Oocytes were treated with collagenase type I (1.5 mg/ml Ca^{2+}-free Barth's solution) for 90 min at room temperature before manual defolliculation. Complementary DNA (cDNA) coding for the human α_1, β_2, and γ_2 subunits of human $GABA_A$ receptors (Origene, Rockville, MD) was dissolved in distilled water at a 1:1:1 molar ratio and injected (23 nl/oocyte, ~1 ng of each subunit) into the nuclei of stage V or VI oocytes using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA). cDNA coding for the human α_4 and β_2 subunits of

the human neuronal nicotinic acetylcholine receptors (kindly provided by Janssen Pharmaceutica N.V., Beerse, Belgium) was dissolved in distilled water at a 1:1 molar ratio and injected in a total injection volume of 18.4 nl/oocyte (~0.2 ng of each subunit). Sham-injected oocytes were injected with 23 or 18.4 nl distilled water, i.e., without cDNA. Following injection with cDNA or distilled water, oocytes were incubated at 21°C in modified Barth's solution containing (in millimolar) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES, and 10 μg/ml neomycin (pH 7.6 with NaOH).

Electrophysiological experiments were performed on oocytes after 2-5 days of incubation to ensure sufficient translation of injected cDNA and functional expression of $\alpha_1\beta_2\gamma_2$ $GABA_A$ or $\alpha_4\beta_2$ nACh receptors in the membrane. Ion currents associated with $GABA_A$ or $\alpha_4\beta_2$ nACh receptor activity were measured with the two-electrode voltage-clamp technique using a Gene Clamp 500B amplifier (Axon Instruments, Union City, CA) with high-voltage output stage as described previously (Hendriks *et al.*, 2010). Recording microelectrodes (0.5-2.5 MΩ) were filled with KCl (3 M). Oocytes, placed in a custom-built Teflon oocyte recording chamber, were voltage-clamped at -60 mV and continuously superfused (~30 ml/min) with saline. Membrane currents were low-pass filtered (8-pole Bessel; 3 dB at 0.3 kHz), digitized (12 bits; 1024 samples per record), and stored on disk for computer analysis.

Aliquots of freshly thawed stock solutions of GABA or ACh in distilled water and TBBPA and gabazine in DMSO were added to the saline immediately before the experiments. Oocytes were exposed by switching the perfusate from saline to TBBPA- and/or GABA or ACh-containing saline using a servomotor-operated valve. Oocytes were repeatedly exposed to different GABA or ACh- and/or TBBPA-containing solutions for 20-40 s. For specific experiments, $GABA_A$ receptor expressing oocytes were exposed to a mixture of TBBPA (10 μM) and gabazine (25 μM). A washout period of 2-5 min between each application was introduced, allowing receptors to recover from desensitization. Sham-injected oocytes did not show any ion current upon superfusion with saline containing 1mM GABA, 1 mM ACh, and/or 100 μM TBBPA (data not shown). To minimize adsorption of TBBPA to the perfusion system, glass reservoirs and Teflon tubes (polytetrafluoroethylene; 4 × 6 mm, Rubber, Hilversum, The Netherlands) were used.

2.3 Cell culture

B35 rat neuroblastoma cells (Otey *et al.*, 2003) were grown for maximal 10 passages in DMEM medium supplemented with 10% fetal calf serum (ICN Biomedicals, Zoetermeer, The Netherlands), 1% additional amino acids (stock solution containing 40 mM of L-Cys, L-Ala, L-Asp, L-Pro, L-Glu, and L-Asx), 100 U/ml penicillin, and 100 mg/ml streptomycin as described previously (Heusinkveld and Westerink, 2011).

PC12 rat pheochromocytoma cells (Greene and Tischler, 1976) were grown for a maximum of 10 passages in RPMI 1640 medium supplemented with 5% fetal calf serum, 10% horse serum (ICN Biomedicals,), 100 U/ml penicillin, and 100 mg/ml streptomycin as described previously (Hondebrink *et al.*, 2011a).

Cells were grown in a humidified incubator at 37°C and 5% CO_2 and subcultured 1 day prior to measurements of $[Ca^{2+}]_i$, cell viability, or ROS production. For fluorescent microscopy Ca^{2+} imaging experiments, undifferentiated B35 or PC12 (both 1.4×10^6 cells per dish) cells were subcultured in glass-bottom dishes (MatTek, Ashland, MA). For measurements of cell viability or ROS production, undifferentiated B35 or PC12 cells were seeded in 96-well plates (Greiner Bio-one, Solingen, Germany) at a density of 2×10^5 and 1.5×10^5 cells per well, respectively. All culture flasks, dishes, and plates were coated with poly-L-lysine (50 μg/ml).

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

$[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 AM as described previously (Heusinkveld *et al.*, 2010; Hondebrink *et al.*, 2011a). Briefly, B35 or PC12 cells were loaded with 5 μM Fura-2 AM for 20 min at room temperature, followed by 15-min de-esterification. After de-esterification, the cells were placed on the stage of an inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmBH, Gräfelfing, Germany). Fluorescence, evoked by 340 and 380 nm excitation wavelengths (F_{340} and F_{380}), was collected every 6 s at 510 nm with an Image SensiCam digital camera (TILL Photonics GmBH). Changes in F_{340}/F_{380} ratio (R), reflecting changes in $[Ca^{2+}]_i$, were analyzed using custom-made MS-Excel macros.

Cells were continuously superfused (~0.6 ml/min) with saline using a valvelink 8.2 (Automate Scientific, CA). Each experiment consisted of a 5-min baseline recording to measure basal $[Ca^{2+}]_i$, after which an increase in $[Ca^{2+}]_i$ was triggered by switching superfusion for 15 s to saline containing 100 μM ACh (B35 cells) or saline containing 100 mM K^+ (PC12 cells) to measure stimulation-evoked $[Ca^{2+}]_i$. Following this first stimulation and a 5- to 10-min recovery period, cells were exposed to saline-containing DMSO (0.1%) or TBBPA (0.1-10 μM) for 20 min prior to a second stimulation. For specific experiments, the exposure duration was increased from 20 to 40 min. A subset of experiments was performed without superfusion; after 5 min of baseline recording the cells were exposed for 20 min to TBBPA via bath application and subsequently stimulated with ACh or K^+. For mechanistic experiments, cells were washed with Ca^{2+}-free saline (containing 10 μM EDTA to remove residual extracellular Ca^{2+}) just prior to the imaging experiments. Where applicable, intracellular Ca^{2+} stores were emptied by incubation for 10 min with 1 μM thapsigargin (TG) and 1 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) in Ca^{2+}-free saline as described previously (Dingemans *et al.*, 2008).

Maximum and minimum ratios (R_{max} and R_{min}) were determined at the end of the recording by addition of ionomycin (5 μM) and ethylenediamine tetraacetic acid (EDTA; 17 mM). Free cytosolic $[Ca^{2+}]_i$ was calculated using a modified Grynkiewicz's equation: $[Ca^{2+}]_i = K_d \times (R\text{-}R_{min})/(R_{max}\text{-}R)$, where K_d is the dissociation constant of Fura-2 AM determined in the experimental setup (Deitmer and Schild, 2000). The amplitude of the TBBPA-induced increase in basal $[Ca^{2+}]_i$ was determined to quantify the effects of TBBPA on basal $[Ca^{2+}]_i$. The amplitude of the second K^+- or ACh-evoked increase in $[Ca^{2+}]_i$ (after 20 min of exposure to DMSO or TBBPA) was expressed as a percentage of the amplitude of the first stimulation-evoked increase in $[Ca^{2+}]_i$ per cell to obtain a "treatment ratio" (TR) as indicated in Figure 3A and Figure 4A. As persistent changes in basal $[Ca^{2+}]_i$ can influence the amplitude of the stimulation-evoked increase in $[Ca^{2+}]_i$, a net stimulation-evoked increase in $[Ca^{2+}]_i$ was calculated by subtracting the amplitude of $[Ca^{2+}]_i$ just prior to stimulation from the amplitude of the stimulation-evoked increase in $[Ca^{2+}]_i$. Net increases in stimulation-evoked increases in $[Ca^{2+}]_i$ were used to derive a "net TR" as described previously (Hondebrink *et al.*, 2011a; Langeveld *et al.*, 2012).

2.5 Cell viability, ROS, and caspase activation assay

To exclude that results are confounded by acute TBBPA-induced cytotoxicity, effects of TBBPA on cell viability in B35 and PC12 cells were determined using a combined Alamar Blue (AB) and Neutral Red (NR) assay as described previously (Heusinkveld *et al.*, 2010). The AB assay, which is based on the ability of the cells to reduce resazurin to resorufin, records mitochondrial activity of the cells as a measure of cell viability. Membrane integrity and lysosomal activity were subsequent determined in the NR assay as an independent measure of cell viability. Briefly, following 24 h exposure to TBBPA (1-100 μM)

in serum-free medium, cells were incubated for 30 min with 200 µl resazurin solution (12 µM in PBS) after which resorufin was measured spectrophotometrically at 530/590 nm (excitation/emission; FLUOstar Galaxy V4.30-0, BMG Labtechnologies, Offenburg, Germany). After removal of the AB solution, cells were incubated for 1 h with 200 µl NR solution (12 µM in PBS). Following the incubation, cells were rinsed with warm (37°C) PBS, and 100 µl extraction solution (1% glacial acetic acid, 50% ethanol, and 49% H_2O) was added to the wells. After 30-min extraction, fluorescence was measured spectrophotometrically at 430/480 nm (excitation/emission).

ROS production was assessed using the fluorescent dye H_2-DCFDA as described previously (Heusinkveld *et al.*, 2010). Briefly, B35 or PC12 cells seeded in black glass-bottom 96-well plates (Greiner Bio-one) were loaded with 1.5 µM H_2-DCFDA for 30 min at 37°C. Subsequently, cells were exposed for up to 24 h to TBBPA-containing saline (1-100 µM). ROS production was measured spectrophotometrically as an increase in fluorescence at 485/530 nm (excitation/emission; FLUOstar Galaxy V4.30-0, BMG Labtechnologies).

Effects of TBBPA on activation of caspase-3 were determined using the Casp3F kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, PC12 cells were seeded in 24-wells plates at a density of ~4.5×10^5 cells per well and exposed to TBBPA (0.1-100 µM) for 24 h in serum-free medium. Staurosporine (STS) exposure (1 µM) was included as a positive control for caspase-3 induction. Following exposure, cells were exposed to lysis buffer for 15 min on ice, and subsequently, a triplicate of the cell lysate was transferred to a black clear-bottom 96-wells plate. The samples were incubated with 16 µM caspase-3 substrate (acetyl Asp-Glu-Cal-Asp 7-amido-4- methylcoumarin) for 45 min at 37°C, and after hydrolysis, cleaved substrate was measured spectrophotometrically at 360/460 nm. To correct the measured fluorescence for cell number, the protein content of the sample was measured using a fluorescamine-based assay (Udenfriend *et al.*, 1972).

2.6 Data analysis and statistics

All data are presented as mean ± SEM from the number of wells, cells, or oocytes (*n*) indicated, derived from 3 to 11 independent experiments (*N*).

The percentage of TBBPA-induced potentiation of inhibition of the GABA- or ACh-evoked ion current was calculated from the quotient of the maximum amplitude of the GABA- or ACh-TBBPA co-application response and the maximum amplitude of the control (GABA or ACh) response.

Cells exposed to only DMSO were used as control (set at 100%) and effects of TBBPA on $[Ca^{2+}]_i$ concentrations, cell viability or ROS formation are expressed as percentage of control. Effects on cell viability <15% were considered irrelevant. For calcium-imaging experiments, the variation within the dish (i.e., between cells) is larger than the variation between the dishes. The individual cells (*n*) rather than the different dishes (*N*) are thus 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. We therefore used the cells (*n*) rather than the dish (*N*) as statistical unit for experiments with single-cell resolution (see also Heusinkveld and Westerink, 2012).

Cells or wells that showed effects two times SD above or below average were considered outliers and excluded from further analysis of calcium homeostasis, cell viability, or ROS production. As the SD for the TR in control cells amounted to ~21%, effects on basal $[Ca^{2+}]_i$ and stimulation-evoked TR <25% were considered irrelevant. Because control cells show basal ROS production over time, these data are expressed as average percentage

compared with the time-matched control values. All relevant effects are statistically significant (p <0.05; Student's t-test, paired or unpaired where applicable).

The concentration-depended effects of TBBPA were determined by one-way ANOVA and post hoc Bonferroni tests. A p value of <0.05 was considered statistically significant. Concentration-response curves were calculated using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA).

3. Results

3.1 Antagonistic effects of TBBPA on human $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor

Voltage-clamped (-60 mV) oocytes expressing $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were exposed to different GABA concentrations (0.1 μM-3 mM; see Supplementary Figure S1 for example recordings). GABA-evoked ion currents were normalized to the response obtained with 1 mM GABA and plotted against the agonist concentration to obtain a concentration-effect curve according to the Hill equation. This curve was used to determine the concentrations producing 20 and 50% of the maximal response (EC_{20} and EC_{50}). EC_{20}, EC_{50}, and Hill slope amounted 14 μM, 40 μM, and 1.20 ± 0.16, respectively (n = 9; Supplementary Figure S1), which is in line with previous results (Hendriks *et al.*, 2010; Hondebrink *et al.*, 2011b).

To investigate whether TBBPA is able to activate the human $GABA_A$ receptor, GABA responsive oocytes were superfused with saline containing 0.1-10 μM TBBPA (n = 4-5 oocytes/concentration). Superfusion with 1 μM TBBPA resulted in a very small, not significant, activation of the $GABA_A$ receptor, whereas superfusion with 10 μM TBBPA resulted in a significant activation of the $GABA_A$ receptor (8 ± 1%; p <0.001; normalized to the current observed at 1 mM GABA; Figures 1A and B). The kinetics of the observed effect

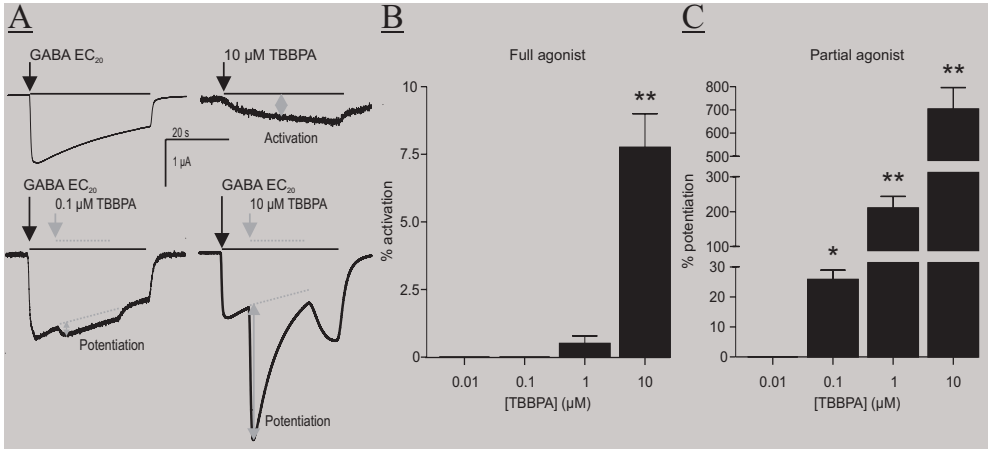

Figure 1. Full and partial agonistic effects of TBBPA on human $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor. (A) Example recordings of ion currents evoked by TBBPA alone or co-applied with GABA at EC_{20}. Application of TBBPA at 10 μM resulted in activation of the $GABA_A$ receptor (upper right). When co-applied with GABA at EC_{20}, TBBPA concentration-dependently potentiate the $GABA_A$ receptor as is shown for 0.1 μM (lower left) and 10 μM TBBPA (lower right). Scale bar applies to all traces. (B) Bar graph demonstrating activation of the $GABA_A$ receptor evoked by TBBPA (LOEC = 10 μM; p <0.001). Activation is expressed as percentage of the maximum GABA-evoked response (1 mM). (C) Bar graph demonstrating the concentration-dependent potentiation of GABA-induced responses by TBBPA (LOEC = 0.1 μM; p <0.05). Potentiation is expressed as percentage of the GABA-evoked response at EC_{20}. Bars represent mean ± SEM; n = 4-5 oocytes; *p <0.05 vs control; **p <0.001 vs control. The GABA concentration-response curve is shown in Supplementary Figure S1.

are slow and not completely saturated during the application time (40 s).

Next, TBBPA was co-applied with a low-concentration GABA (EC_{20}) to determine possible partial agonistic effects, as demonstrated previously for PCBs and PBDEs (Hendriks *et al.*, 2010). At this low receptor occupancy, TBBPA induced a concentration-dependent potentiation of the GABA-induced ion current (Figures 1A and C). Although a modest potentiation (26 ± 3%; p <0.05; normalized to the current observed at GABA EC_{20}) was observed for 0.1 μM TBBPA, a very strong potentiation of the $GABA_A$ receptor was observed after co-application with 1 or 10 μM TBBPA (210 ± 33%; p <0.001 and 703 ± 93%; p <0.001, respectively). These results indicate that TBBPA acts both as partial and full agonist of the $GABA_A$ receptor with lowest observed effect concentrations (LOECs) of 0.1 and 10 μM, respectively.

Pharmacologically blocking the GABA-binding sites of the $GABA_A$ receptor using gabazine (25 μM) completely abolished the GABA-evoked current (Supplementary Figure S2A), whereas gabazine only partly blocked the TBBPA-induced effect on the $GABA_A$ receptor (Supplementary Figure S2B). Because no response was observed in sham-injected oocytes, these combined results indicate that the effect of TBBPA is a direct $GABA_A$ receptor-mediated effect that at least partly involves the GABA-binding sites of the $GABA_A$ receptor.

3.2 Agonistic effects of TBBPA on human $\alpha_4\beta_2$ nicotinic acetylcholine receptor

Oocytes expressing human $\alpha_4\beta_2$ nACh receptors were voltage-clamped (-60 mV) and superfused with various concentrations of ACh (0.1 μM-3 mM; see Supplementary Figure S3 for example recordings). ACh-evoked ion currents were normalized to the response obtained with 1 mM ACh and plotted against the agonist concentration to obtain a concentration-effect curve according to the Hill equation (see Supplementary Figure S3). The Hill slope for the fitted ACh concentration-effect curve was 1.0 ± 0.1, and mean values for

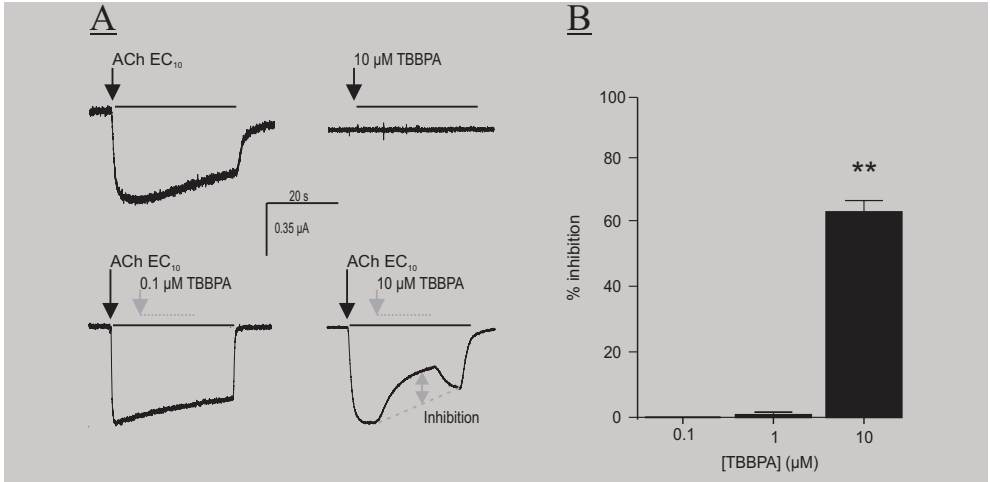

Figure 2. Inhibitory effects of TBBPA on human $\alpha_4\beta_2$ nACh receptor activation. (A) Example recordings demonstrating TBBPA has no agonistic properties on the nACh receptor (upper right). During co-application with ACh, no effect was observed for 0.1 μM TBBPA (lower left), though 10 μM TBBPA inhibited the ACh-evoked current (lower right). Scale bar applies to all traces. (B) Bar graph demonstrating the TBBPA-induced inhibition of ACh-evoked (EC_{10}) responses. Bars represent mean inhibition ± SEM; LOEC = 10 μM (p <0.001); n = 3-6 oocytes; **p <0.001 vs control. The ACh concentration-response curve is shown in Supplementary Figure S3.

EC_{10} and EC_{50} amounted to 14 ± 2 and 139 ± 17 μM (n = 5), which is in line with previous results (Hendriks *et al.*, 2010).

When human $\alpha_4\beta_2$ nACh receptor expressing oocytes were superfused with 10 or 100 μM TBBPA, no change in ion current was detected, demonstrating that TBBPA is not an agonist of the nACh receptor (Figure 2A). However, as previously reported (Hendriks *et al.*, 2010), at low receptor occupancy, PCBs and PBDEs can affect the ACh-evoked current in a concentration-dependent manner. We therefore co-applied TBBPA with a low concentration of ACh (10 μM; ~EC_{10}). Although 0.1 and 1 μM TBBPA were without effect, exposure to 10 μM TBBPA resulted in a strong inhibition of the ACh-evoked ion current (63 ± 4%; p <0.001; Figures 2A and B). Comparable inhibitory effects of 10 μM TBBPA were observed when co-exposed with ACh at 1 mM (see Supplementary Figure S4). These measurements thus demonstrate that TBBPA acts as an antagonist of the human $\alpha_4\beta_2$ nACh receptor at a wide range of ACh concentrations.

3.3 TBBPA dose dependently affects basal and ACh-evoked $[Ca^{2+}]_i$ in B35 cells

To further investigate the mechanisms underlying the observed changes in cholinergic signaling, we used single-cell fluorescent Ca^{2+}-imaging. Fura-2 AM-loaded B35 neuroblastoma cells were used to determine acute effects of TBBPA exposure on Ca^{2+}-homeostasis and ACh-evoked increases in $[Ca^{2+}]_i$ as B35 cells have a high expression of calci-

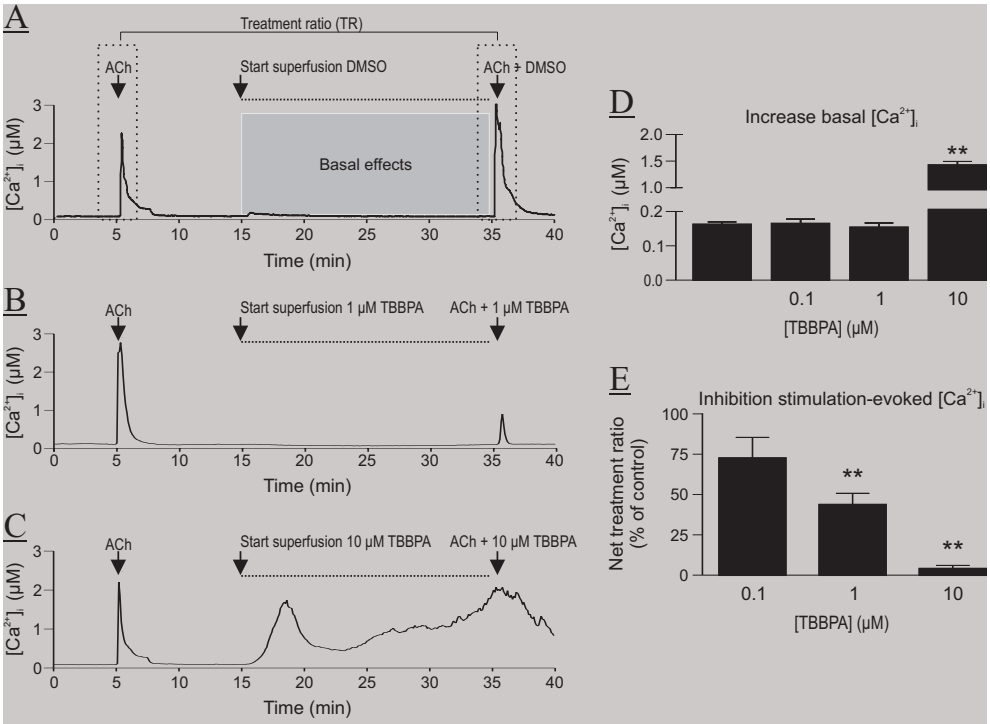

Figure 3. TBBPA-induced effects on $[Ca^{2+}]_i$ in B35 cells. Example recordings of single-cell $[Ca^{2+}]_i$ imaging from individual B35 cells. In between two 15 s stimulations (100 μM ACh), cells were exposed for 20 min to 0.1% DMSO (A) or different concentrations TBBPA (B and C), resulting in an increase in basal $[Ca^{2+}]_i$ and inhibition of the second ACh-evoked stimulation. Bar graphs illustrate the concentration-dependent TBBPA-induced increase in basal $[Ca^{2+}]_i$ (D) and inhibition of the stimulation-evoked increase in $[Ca^{2+}]_i$ expressed as a net TR normalized to DMSO-exposed control cells (E). n = 29-88 cells, **p <0.001 vs control.

um-permeable nACh receptors. B35 cells have a low basal $[Ca^{2+}]_i$ of 101 ± 2 nM (n = 88), which rapidly and transiently increases to 1.9 ± 0.8 μM upon activation of nACh receptors for 15 s with 100 μM ACh (Figure 3A). During a subsequent 5 min recovery period, $[Ca^{2+}]_i$ returned to near basal levels. Next, $[Ca^{2+}]_i$ remained low and comparable to control (0.1% DMSO) cells during a 20-min exposure to 0.1 and 1 μM TBBPA (0.1% DMSO: 163 ± 7 nM; 0.1 μM TBBPA: 190 ± 17 nM; 1 μM TBBPA: 154 ± 12 nM; Figures 3A, B, and D). However, exposure to 10 μM TBBPA resulted in a strong and rather persistent increase in basal $[Ca^{2+}]_i$ (1.4 ± 0.1 μM) after a delay of 1.0 ± 0.1 min (Figures 3C and D).

Following the 20-min exposure to DMSO or TBBPA (0.1-10 μM), cells were challenged for a second time with 100 μM ACh (with or without TBBPA) to derive a net TR (see Materials and Methods section). For DMSO-exposed control cells, the second stimulation resulted in an increase in $[Ca^{2+}]_i$ up to 2.4 ± 0.2 μM, i.e., 135 ± 16% of the first stimulation (net TR; Figure 3A). Although exposure to 0.1 μM TBBPA did not affect net TR compared with control cells, exposure to 1 μM TBBPA resulted in a significant reduction of the net TR (44 ± 7% of control cells, p <0.001; Figures 3B and E). When cells were exposed to 10 μM TBBPA, the second ACh-evoked increase in $[Ca^{2+}]_i$ was virtually absent (Figures 3C and E).

Because cells were continuously superfused with TBBPA-containing medium, possible accumulative effects of TBBPA in the cells were investigated in two separate sets of experiments. First, instead of 20-min exposure to 1 μM TBBPA, cells were superfused for 40 min prior to a second stimulation with 100 μM ACh. No significant change in TR was observed between the 20 and 40 min exposed cells (44 ± 7% vs 53 ± 7% of control cells; p >0.05; data not shown).

Second, cells were exposed for 20 min to 10 μM TBBPA without superfusion, i.e., after 5 min of baseline recording, saline-containing 10 μM TBBPA was bath-applied to the cells. A significant increase in basal $[Ca^{2+}]_i$ was observed compared with control cells. However, no significant differences in basal $[Ca^{2+}]_i$ were observed between superfusion with 10 μM TBBPA (1.4 ± 0.1 μM) or bath application of 10 μM TBBPA (1.8 ± 0.2 μM; p >0.05; data not shown).

Subsequently, cells were stimulated with 100 μM ACh following 20 min superfusion or bath application. Again, no significant differences in $[Ca^{2+}]_i$ were observed between superfusion experiments (0.9 ± 0.1 μM) and bath application experiments (1.2 ± 0.3 μM; p >0.05; data not shown). The TBBPA-induced inhibition of the ACh-evoked increase in $[Ca^{2+}]_i$ as well as the TBBPA-induced increase in basal $[Ca^{2+}]_i$ are thus rather independent of the exposure duration.

3.4 TBBPA dose-dependently affects basal and depolarization-evoked $[Ca^{2+}]_i$ in PC12 cells

Because B35 cells lack functional VGCCs, single-cell fluorescent Ca^{2+}-imaging of Fura-2 AM-loaded dopaminergic PC12 cells was used to study in more detail the effects of TBBPA on basal and depolarization-evoked calcium homeostasis. Comparable to B35 cells, PC12 cells have low basal $[Ca^{2+}]_i$ (112 ± 5 nM; n = 77). Upon depolarization with 100 mM K^+ for 15 s, $[Ca^{2+}]_i$ rapidly and transiently increases to 2.0 ± 0.1 μM due to Ca^{2+} influx through VGCCs. During a 5-min recovery period, $[Ca^{2+}]_i$ returned to near basal levels and the cells were subsequently exposed to 0.1% DMSO (control) or TBBPA (0.1-10 μM) for 20 min to measure effects on basal $[Ca^{2+}]_i$ (Figure 4). Cells exposed to 0.1 and 1 μM TBBPA have low basal $[Ca^{2+}]_i$ that is comparable to control cells (0.1% DMSO: 146 ± 3 nM; 0.1 μM TBBPA: 126 ± 9 nM; 1 μM TBBPA: 172 ± 11 nM; Figures 4A, B, and D). However,

cells exposed to 10 μM TBBPA display a strong transient increase in basal $[Ca^{2+}]_i$ up to 2.2 ± 0.4 μM after a delay of 1.0 ± 0.1 min, that returns to near basal levels within minutes (Figures 4C and D).

Following the 20-min exposure to DMSO or TBBPA (0.1-10 μM), cells were challenged for a second time with 100 mM K^+ to derive a net TR. In DMSO-exposed control cells, $[Ca^{2+}]_i$ increased to 1.2 ± 0.1 μM during the second depolarization, i.e., 65 ± 3% of the first depolarization (net TR; Figure 4A). Compared with control cells, 0.1 μM TBBPA did not affect the net TR, whereas the net TR was significantly reduced in cells exposed to 1 μM TBBPA (28 ± 4% of control cells, p <0.001; Figures 4B and E). The second depolarization-evoked increase in $[Ca^{2+}]_i$ was virtually absent in cells exposed to 10 μM TBBPA (Figures 4C and E).

To investigate the possibility of accumulation of TBBPA in the cells due to continuously superfusion, the cells were superfused for 40 min with 1 μM TBBPA. Prolonged exposure did not result in a significant change of the TR compared with 20 min exposure (24 ± 3% after 40 min exposure vs 28 ± 4% after 20 min exposure; p >0.05; data not shown). Also when the cells were exposed for 20 min to 10 μM TBBPA via bath application, no significant difference in basal $[Ca^{2+}]_i$ was observed compared with cells exposed during 20 min with superfusion (1.8 ± 0.3 μM without superfusion vs 2.2 ± 0.4 μM with superfusion; p >0.05; data not shown). The degree of inhibition of the depolarization-evoked increase in $[Ca^{2+}]_i$ was also comparable between superfusion experiments (0.3 ± 0.1 μM)

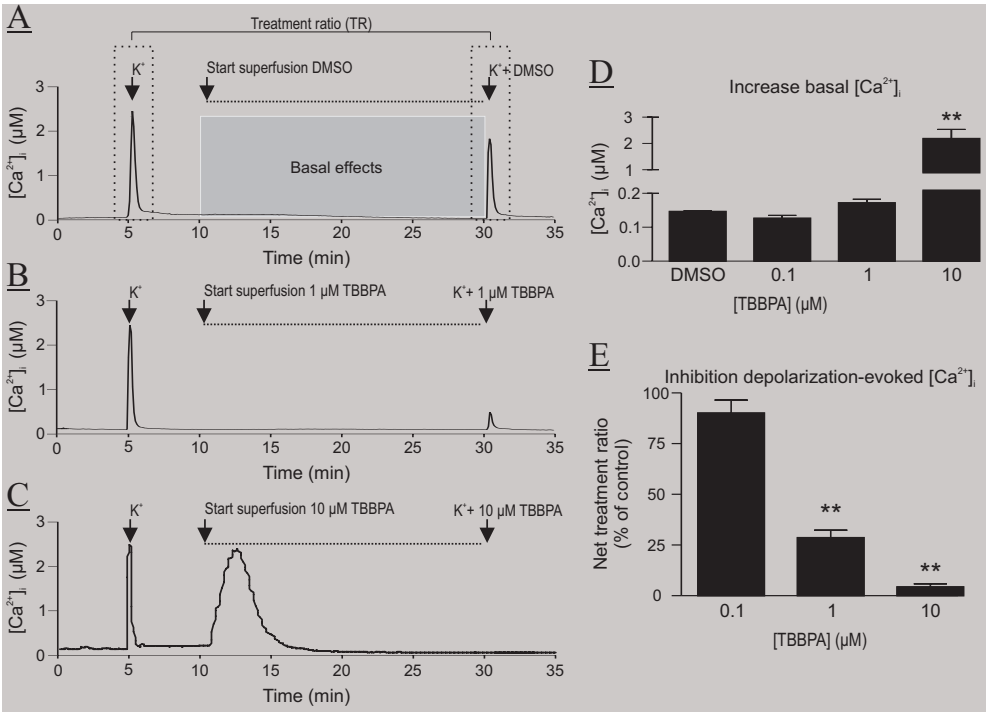

Figure 4. TBBPA-induced effects on $[Ca^{2+}]_i$ in PC12 cells. Example recordings of single-cell $[Ca^{2+}]_i$ imaging from individual PC12 cells. In between two 15 s depolarizations (100 mM K^+), cells were exposed for 20 min to 0.1% DMSO (A) or different concentrations TBBPA (B and C), resulting in a concentration-dependent increase of basal $[Ca^{2+}]_i$ and inhibition of the second-evoked depolarization. Bar graphs illustrate the TBBPA-induced increase in basal $[Ca^{2+}]_i$ (D) and inhibition of the depolarization-evoked increase in $[Ca^{2+}]_i$ expressed as a net TR normalized to DMSO-exposed control cells (E). n = 29-77 cells, **p <0.001 vs control.

and bath application experiments (0.4 ± 0.1 μM; p >0.05; data not shown). The TBBPA-induced inhibition of VGCCs as well as the TBBPA-induced increase in basal $[Ca^{2+}]_i$ are thus rather independent of the exposure duration, in line with the results obtained in B35 cells.

3.5 TBBPA-induced increase in $[Ca^{2+}]_i$ mainly originates from intracellular stores

To investigate the mechanisms underlying the observed increases in basal $[Ca^{2+}]_i$, additional experiments were performed with PC12 cells under Ca^{2+}-free conditions. In the absence of extracellular Ca^{2+}, TBBPA (10 μM) still induced an increase in $[Ca^{2+}]_i$, although the amplitude was significantly lower under these Ca^{2+}-free conditions (312 ± 18% of DMSO control under Ca^{2+}-free conditions, n = 71, vs 1835 ± 548% of DMSO control under physiological Ca^{2+} conditions, n = 47, p <0.001; Figure 5A). The TBBPA-induced increase in $[Ca^{2+}]_i$ thus depends to a large degree on influx of extracellular Ca^{2+} but likely originates from the release of Ca^{2+} from intracellular stores, such as ER, mitochondria, nucleus, and secretory vesicles. We therefore depleted the ER and mitochondria of PC12 cells by pretreatment with 1 μM TG and 1 μM FCCP as described previously (Dingemans *et al.*, 2008). After depletion of ER with TG under Ca^{2+}-free conditions, 10 μM TBBPA was still able to evoke an increase in basal $[Ca^{2+}]_i$, although the amplitude was significantly lower compared with normal and Ca^{2+}-free conditions (191 ± 11% of control, n = 29,

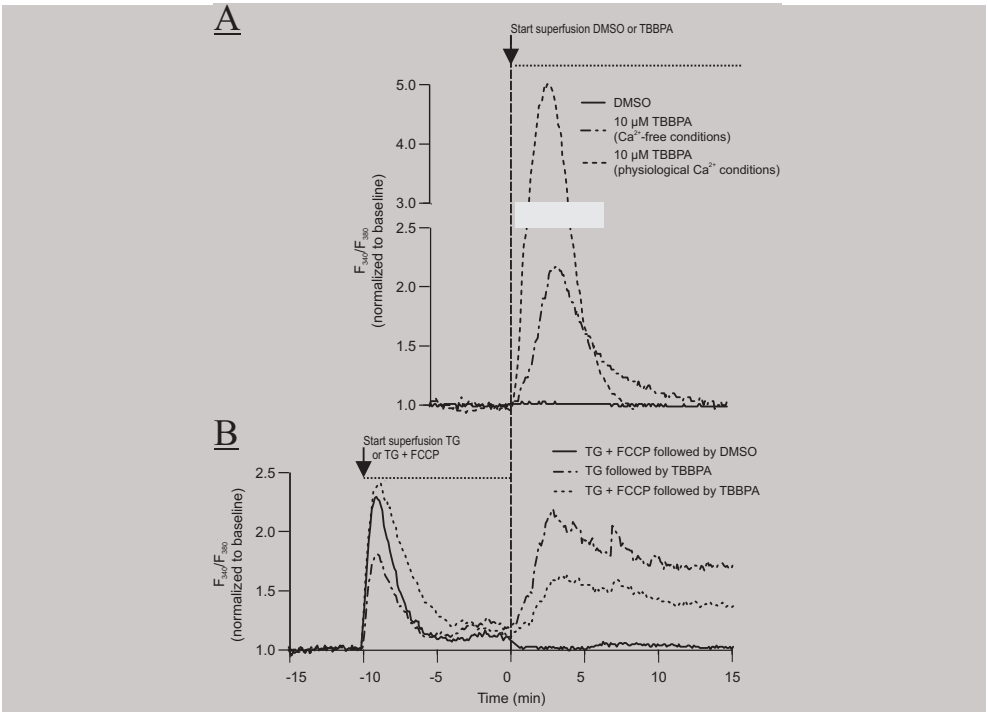

Figure 5. TBBPA-induced increase of $[Ca^{2+}]_i$ is store-mediated. Example recordings of single-cell $[Ca^{2+}]_i$ imaging from individual PC12 cells under Ca^{2+}-free conditions illustrating that influx of extracellular calcium significantly contributes to the observed TBBPA-induced increase in basal $[Ca^{2+}]_i$ (A). The TBBPA-induced increase in basal $[Ca^{2+}]_i$ apparently originates from intracellular calcium stores as the increase in basal $[Ca^{2+}]_i$ is strongly reduced after depletion of mitochondria and ER by TG and FCCP (B). Comparable effects are observed in B35 cells, see Supplementary Figure S5.

p <0.001; Figure 5B). Following depletion of both the ER and mitochondria by combined exposure to TG and FCCP prior to exposure to 10 μM TBBPA, the increase in basal $[Ca^{2+}]_i$ was further attenuated and only slightly higher than in control cells (125 ± 2% of control, n = 44, p <0.001; Figure 5B). The small remaining increase in basal $[Ca^{2+}]_i$ is probably due to release of Ca^{2+} from other intracellular stores, such as the nucleus and secretory vesicles.

Comparable effects were observed in B35 cells; in the absence of extracellular Ca^{2+}, 10 μM TBBPA still induced a transient increase in $[Ca^{2+}]_i$ (416 ± 14% of DMSO control under Ca^{2+}-free conditions, n = 41, vs 635 ± 26% of DMSO control under physiological Ca^{2+} conditions, n = 45, p <0.001; Supplementary Figure S5A). However, the TBBPA-induced fluctuations in $[Ca^{2+}]_i$ are absent under these Ca^{2+}-free conditions, indicating these are likely due to store-operated Ca^{2+} influx. After depletion of both ER and mitochondria under Ca^{2+}-free conditions prior to TBBPA exposure, the increase in basal $[Ca^{2+}]_i$ was decreased to only slightly higher than control cells (148 ± 5% of control, n = 39, p <0.001; Supplementary Figure S5B), again indicating that the transient increase in $[Ca^{2+}]_i$ mainly originates from intracellular calcium stores.

3.6 Effects of TBBPA on cell viability, caspase-3 activity, and oxidative stress

To ensure that the observed effects are not confounded by acute cytotoxicity, B35 and PC12 cells were exposed to 1-100 μM TBBPA for 24 h, and cell viability was determined using a combined AB and NR assay. Exposure of B35 and PC12 cells to 1 and 10 μM TBBPA did not affect cell viability. However, at 100 μM TBBPA, a significant decrease in cell viability compared with control cells was observed in B35 cells with the NR assay (70 ± 9%, p <0.001; Figure 6A) and in PC12 cells with both assays (AB: 50 ± 5%, p <0.001; NR: 29 ± 5%, p <0.001; Figure 6B). Exposure to TBBPA for 24 h did already induce a clear increase in apoptosis-related caspase-3 activity at ≥10 μM in PC12 cells (Supplementary Figure S6), although lower concentrations were without effect.

Similarly, exposure to 1 μM TBBPA did not increase ROS production in B35 or PC12 cells. In B35 cells, an increase in ROS production was observed at 10 μM TBBPA only after 24 h (206 ± 6%, p <0.001), whereas at 100 μM, TBBPA increased ROS production already after 2 h compared with time-matched controls (148 ± 1%, p <0.001), eventually

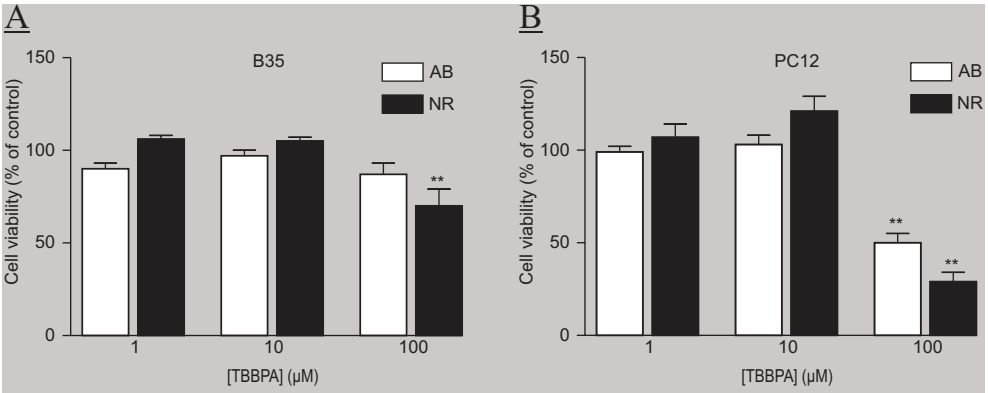

Figure 6. Effects of 24 h exposure to TBBPA on cell viability in B35 and PC12 cells. Bar graphs, representing cell viability determined using a combined Alamar Blue (AB; white bars) and Neutral Red (NR; black bars), demonstrate that TBBPA at 100 μM decreases cell viability in B35 (A) and PC12 (B) cells. Bars represent mean cell viability compared with controls (set at 100%) ± SEM (n = 27-35 wells per concentration). **p <0.001 vs control.

amounting to 346 ± 5% following 24 h exposure (Figure 7A). In PC12 cells, 10 μM TBBPA also induced a modest increase in ROS production only after 24 h (123 ± 6%, p <0.001). At 100 μM TBBPA, ROS production was already significantly higher compared with time-matched controls following 1 h exposure (124 ± 1%, p <0.001). ROS production increased further during the 24 h of exposure, eventually amounting to 167 ± 2% (Figure 7B). The combined results thus indicate that the observed acute TBBPA-induced neurotoxic effects *in vitro* at concentrations up to 10 μM are not confounded by acute cytotoxicty.

4. Discussion

Our results demonstrate that already a low concentration (0.1 μM) TBBPA is able to act as a strong partial agonist of the human $GABA_A$ receptor (Figure 1C). Furthermore, at higher concentrations TBBPA can also act as full agonist of the $GABA_A$ receptor (LOEC 10 μM; Figure 1B), whereas it acts as antagonist of the human $\alpha_4\beta_2$ nACh receptor (LOEC 10 μM; Figure 2B). These opposite effects of TBBPA, i.e., activation or potentiation of inhibitory GABA-mediated signaling and reduced excitatory ACh-mediated signaling, are of concern as they may add up *in vivo*. Moreover, comparable additive effects have been observed previously for PCB47 and 6-OH-BDE47 (Hendriks *et al.*, 2010). Interestingly, other studies also indicated the involvement of the cholinergic system in the neurotoxicity of chlorinated and brominated POPs (Eriksson *et al.*, 2001; Eriksson *et al.*, 2006; Johansson *et al.*, 2008), suggesting that the cholinergic system may be a more general target for these compounds and that its modulation possibly underlies the neurobehavioral and neurodevelopmental effects induced by these compounds. Importantly, both $GABA_A$ and nACh receptors play an important role in long-term potentiation, synaptic plasticity, and brain development (Dwyer *et al.*, 2009; D'Hulst *et al.*, 2009). It should be noted that the $GABA_A$ receptor acts as an excitatory receptor during early brain development (D'Hulst *et al.*, 2009), which may explain some of the differences observed between behavioral studies following adult and neonatal exposure to TBBPA (Eriksson *et al.*, 2001; Nakajima *et al.*, 2009). Additionally, the discrepancy in behavioral effects after TBBPA exposure may be caused by the differences in animal species but probably also by differences in the timing of TBBPA treatment and the rather low retention time of TBBPA (Viberg and Eriksson, 2011).

The inhibitory effects of TBBPA on nACh receptors were confirmed in B35 cells expressing calcium-permeable nACh receptors (LOEC 1 μM; Figure 3E) using single-cell

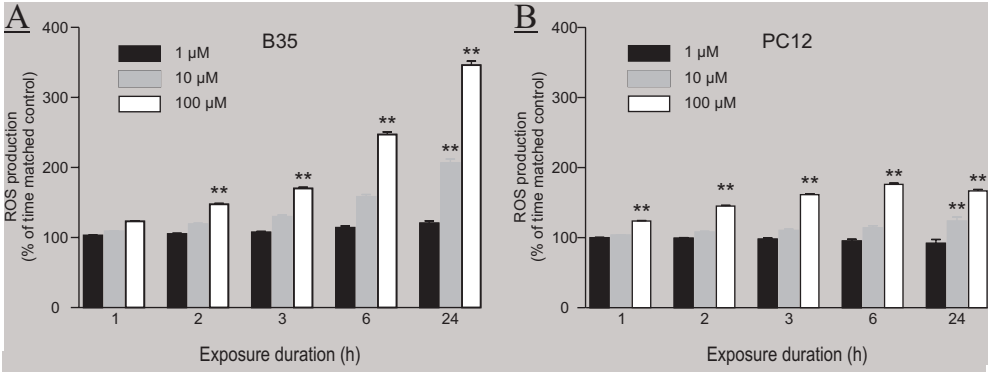

Figure 7. Exposure to TBBPA increases ROS production over time in a concentration-dependent manner in B35 and PC12 cells. Bar graphs demonstrate that 10 (gray bars) and 100 μM (white bars) TBBPA, but not 1 μM (black bars), significantly increases ROS production in B35 (A) and PC12 (B) cells. Bars represent mean ROS production compared with time-matched controls (set at 100%) ± SEM (n = 32-111 wells per concentration). **p <0.001 vs control.

fluorescent $[Ca^{2+}]_i$ imaging. Importantly, our data also show that TBBPA (≥1 μM) reduced the depolarization-evoked increase in $[Ca^{2+}]_i$ in PC12 cells (Figure 4E). This indicates that TBBPA is also a strong inhibitor of VGCCs, comparable with PBDEs (Dingemans *et al.*, 2011) and PCBs (Langeveld *et al.*, 2012). In neuronal cells, the main influx route of Ca^{2+} is via VGCCs. The rapid influx of Ca^{2+} can trigger various intracellular processes, including neurotransmitter release. In our PC12 cells, the L-type VGCC is the most abundant type, although N- and P/Q-type VGCCs both also account for ~20% of total calcium influx during depolarization (see also Dingemans *et al.*, 2009; Heusinkveld *et al.*, 2010). Since the depolarization-evoked increase in $[Ca^{2+}]_i$ was virtually absent in cells exposed to 10 μM TBBPA, the block of VGCCs is clearly not specific for a subtype of VGCCs, e.g., L-, N-, or P/Q-type. Moreover, the differences in LOECs between the increase in basal $[Ca^{2+}]_i$ (LOEC 10 μM) and the decrease in ACh- and depolarization-evoked $[Ca^{2+}]_i$ (LOECs 1 μM) indicate that the inhibition of nACh receptors and VGCCs is specific and independent of the foregoing increase of basal $[Ca^{2+}]_i$.

The observed robust TBBPA-induced (≥10 μM) increase in basal $[Ca^{2+}]_i$ in both B35 and PC12 cells (Figures 3D and 4D) is in line with previous studies on cerebellar granule cells, granulocytes, and Sertoli cells (Ogunbayo and Michelangeli, 2007; Ogunbayo *et al.*, 2008; Reistad *et al.*, 2005; Reistad *et al.*, 2007) and indicates this is a general mechanism of action of TBBPA. In a separate set of experiments, we confirmed that influx of extracellular calcium significantly contributes to the observed TBBPA-induced increase in basal $[Ca^{2+}]_i$. However, the increase in basal $[Ca^{2+}]_i$ apparently originates from intracellular calcium stores because the increase in basal $[Ca^{2+}]_i$ is strongly reduced after depletion of ER and mitochondria (Figure 5 and Supplementary Figure S5). Noteworthy, comparable store-mediated Ca^{2+} release was observed following exposure to PCBs and PBDEs (for reviews see Dingemans *et al.*, 2011; Fonnum and Mariussen, 2009).

Interestingly, the observed ER-mediated increase in basal $[Ca^{2+}]_i$, as was observed following exposure to ≥10 μM TBBPA, is an important trigger for induction of caspase activity and subsequent cell death (Orrenius *et al.*, 2011). Contrary, Reistad *et al.* (Reistad *et al.*, 2007) showed that TBBPA (5 μM) induces apoptotic cell death in cerebellar granule cells by a caspase-independent mechanism. It was suggested that caspase activity was suppressed by a TBBPA-induced increase in ROS production and that TBBPA-induced cell death was thus ROS-dependent. Our data (Figure 7) confirm the TBBPA-induced increase in ROS production but also revealed a TBBPA-induced (≥10 μM) increase in caspase-3 activity in PC12 cells (Supplementary Figure S6). Nonetheless, TBBPA-induced cell death was observed only at the highest concentration tested (100 μM; Figure 6) indicating that the specific neurotoxic effects of TBBPA at ≤10 μM we report in this study are not confounded by acute cytotoxicity.

Neuronal circuits of mammals contain excitatory and inhibitory systems that should be in balance for normal (neuronal) development and functioning. In order to compare and integrate the observed adverse effects of TBBPA in this *in vitro* study, the effects on the different endpoints and corresponding LOECs were ordered according to an effect (target)-concentration ranking ranging from a high impact to a very low impact on normal neuronal development and function.

The observed strong partial agonistic effect of 0.1 μM TBBPA on the inhibitory $GABA_A$ receptor is of major concern and suspected to potentially disturb normal neuronal development and function and is therefore ranked with a high impact. The observed inhibition of VGCCs and nACh receptors occurs only at higher concentrations (1 and 10 μM, respectively). Inhibition of VGCCs results in a reduced depolarization of the cell and less Ca^{2+} influx into the cell, thereby probably reducing, e.g., neurotransmitters secretion.

Importantly, TBBPA has opposite effects on $GABA_A$ and nACh receptors, which may add up *in vivo* resulting in disequilibrium between excitatory and inhibitory systems. However, since these effects *in vitro* only occur at concentrations ≥1 μM, it is ranked with a moderate impact. The observed store-mediated disturbances in basal Ca^{2+} homeostasis observed in B35 and PC12 cells occur only at acute exposure to 10 μM, indicative for a low impact on normal neuronal development and function. The observed increases in ROS production and cytotoxicity only occur at high concentrations (≥10 μM) and are therefore ranked as having a very low impact on normal neuronal development and function.

Clear epidemiological data on adverse effects of human (perinatal) TBBPA exposure are currently lacking, although TBBPA has been detected in human plasma (Thomsen *et al.*, 2002). Additionally, there are indications that acute exposure to TBBPA may cause behavioral disturbances (Nakajima *et al.*, 2009) and that developmental exposure can affect the cholinergic system in the neonatal mouse brain (Viberg and Eriksson, 2011). The margin of safety between the LOECs derived from our *in vitro* study and concentrations found in human serum of non-occupational exposed adults are at least three orders of magnitude lower. However, of particular concern is the presence of high-concentrations TBBPA in breast milk (up to 4.11 ng/g lw) and cord serum (up to 103.52 ng/g lw) (Abdallah and Harrad, 2011; Cariou *et al.*, 2008; Shi *et al.*, 2009), which corresponds with ~2 nM in blood (calculated using average human blood values). These cord serum values are not even two orders of magnitude below the LOECs of the most sensitive endpoint observed in this study (partial agonistic effect on the $GABA_A$ receptor, LOEC 100 nM), indicating a possible risk for newborns and their developing brain. Although TBBPA is not a very stable compound (Birnbaum and Staskal, 2004), it is lipophilic and has a reported bioconcentration factor ranging from 20 to 230, which can explain the increasing TBBPA levels in humans despite its relatively short half-life (two days in human adults; Sjödin *et al.*, 2003). Moreover, our *in vitro* experiments focused on acute exposure, whereas human exposure is in general chronic with concentrations that could increase over time. Consequently, these findings should be taken into account for human risk assessment purposes, especially because several studies suggested that POPs, including PCBs and PBDEs, may exert additive neurotoxic effects (Antunes Fernandes *et al.*, 2010b; Eriksson *et al.*, 2006; Gao *et al.*, 2009; Hendriks *et al.*, 2010).

In summary, this is the first study demonstrating differential effects of TBBPA at sub- and low-micromolar concentrations on human $GABA_A$ and nACh receptors. Importantly, TBBPA also inhibits VGCCs at ≥1 μM, resulting in decreased Ca^{2+} influx during neuronal activity and thus further inhibition of neuronal excitation. The observed increases in basal $[Ca^{2+}]_i$, ROS production, caspase-3 activity, and cell death were observed only at a higher TBBPA concentration (≥10 μM) and may thus be less relevant for human risk assessment. TBBPA has several modes of action (potentiation and activation of $GABA_A$ receptors, inhibition of nACh receptors and VGCCs, ER-mediated Ca^{2+} release) that are shared by PCBs and PBDEs. TBBPA and other POPs may consequently interact at these targets, potentially causing additive or synergistic effects *in vivo*. Considering these effects and the wide application of BFRs, our findings underline the need to replace BFRs by safe(r) and less persistent alternatives.

Acknowledgments

We gratefully acknowledge Aart de Groot, Harm Heusinkveld, and Mirthe Muilwijk (Neurotoxicology Research Group, Institute for Risk Assessment Sciences) for excellent technical assistance, Janssen Pharmaceutica N.V. (Beerse, Belgium) for providing the cDNA encoding the human $\alpha_4\beta_2$ nACh receptor subunits and Dr. Wim Scheenen (Radboud University, Nijmegen, The Netherlands) for providing the *Xenopus leavis* frogs.

Supplementary data

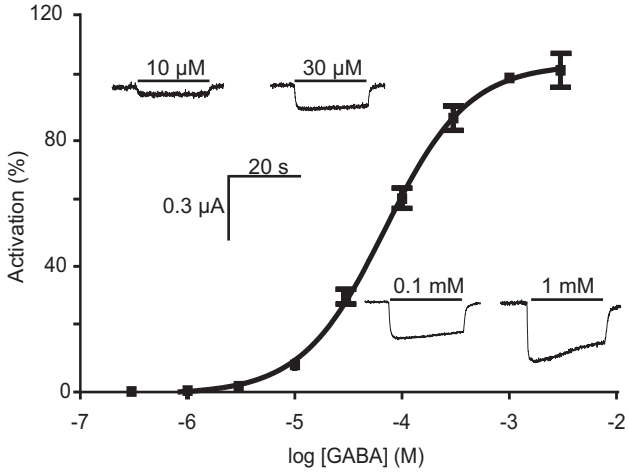

Figure S1. GABA concentration-response curve of human $GABA_A$ receptors expressed in *Xenopus* oocytes. Insets show example recordings evoked by superfusion with saline containing different GABA concentrations, as indicated above the recordings. Scale bar applies to all traces. Fitted line is Hill curve with mean values (± SEM) obtained from 4-7 separate experiments. For details see *Material and Methods* and *Results* section.

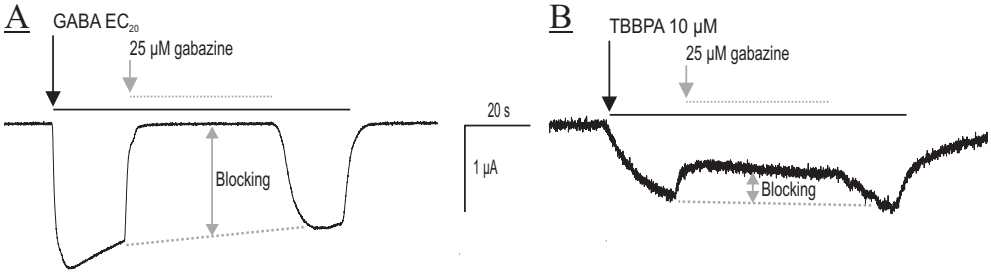

Figure S2. Pharmacologically blocking of the GABA-binding sites of the human $GABA_A$ receptor using gabazine (25 µM) completely abolished the GABA-evoked current (A). Gabazine only partly blocks the TBBPA-induced effect on the $GABA_A$ receptor (57 ± 1%; n = 6 oocytes; B), indicating that the TBBPA effect is at least partly mediated by the GABA-binding sites of the $GABA_A$ receptor. Scale bar applies to both traces. For details see *Material and Methods* and *Results* section.

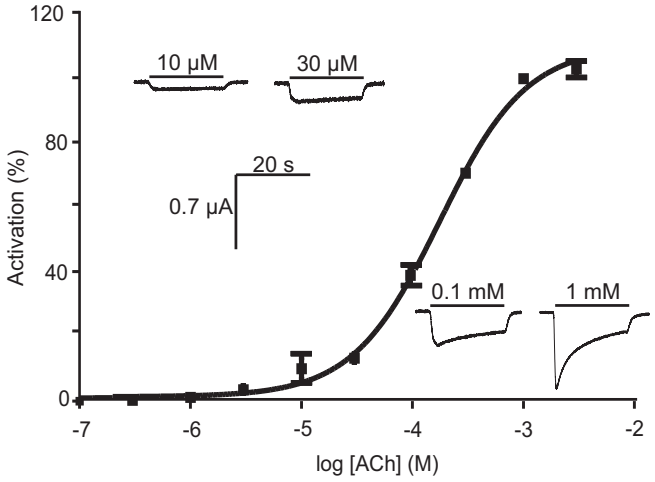

Figure S3. ACh concentration-response curve of human $\alpha_4\beta_2$ nACh receptors expressed in *Xenopus* oocytes. Insets show example recordings evoked by superfusion with saline containing ACh concentrations, as indicated above to the recordings. Scale bar applies to all traces. Fitted line is Hill curve with mean values (± SEM) obtained from 3-6 separate experiments. For details see *Material and Methods* and *Results* section.

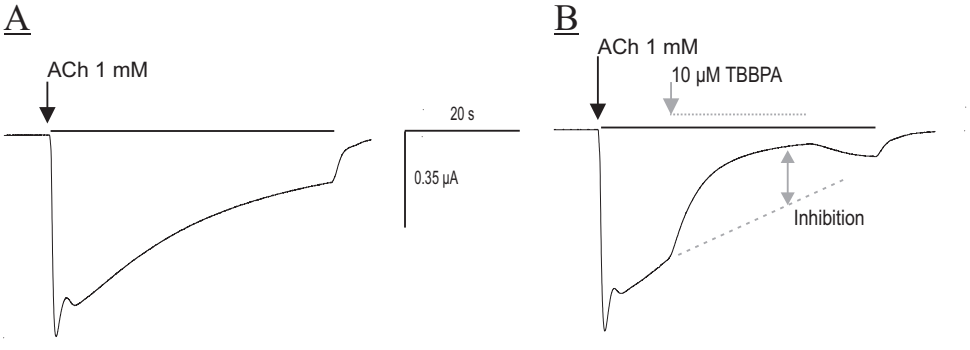

Figure S4. TBBPA inhibits nACh receptors when co-applied with a high concentration ACh (1 mM). Example recordings show the current evoked by 1 mM ACh (A) and the inhibition of the evoked current when co-applied with 10 µM TBBPA (B). Scale bar applies to both traces. Co-exposure resulted in a mean inhibition of the ACh-evoked current by TBBPA (10 µM) of 88 ± 4% (n = 5 oocytes).

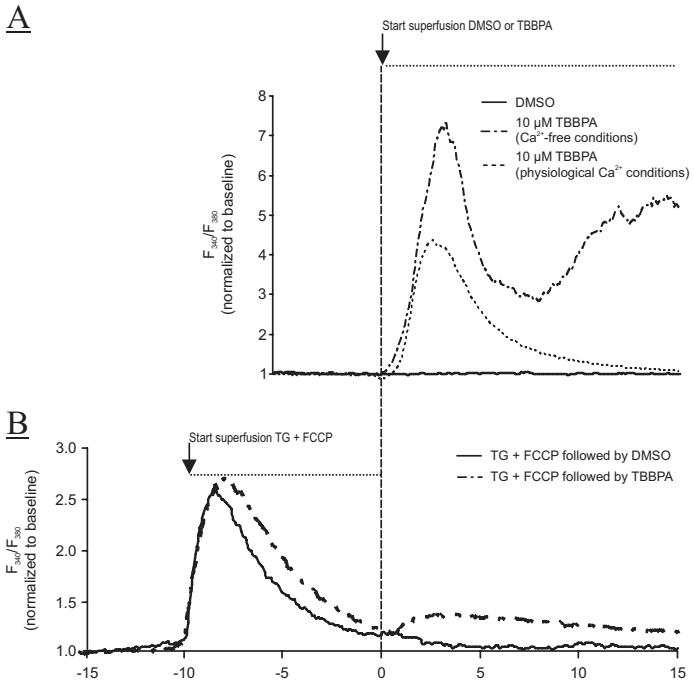

Figure S5. TBBPA-induced increase in basal $[Ca^{2+}]_i$ is store-mediated. (A) Example recordings of single cell $[Ca^{2+}]_i$ imaging from individual B35 cells under physiological Ca^{2+} conditions and Ca^{2+}-free conditions. Under Ca^{2+}-free conditions, 10 µM TBBPA still induced a first transient increase in $[Ca^{2+}]_i$, though to a lesser extent than under physiological Ca^{2+} conditions. The absence of the TBBPA-induced fluctuations in $[Ca^{2+}]_i$ under Ca^{2+}-free conditions indicate that these fluctuations are likely due to store-operated Ca^{2+} influx. (B) When TBBPA is applied under Ca^{2+}-free conditions following depletion of mitochondria and endoplasmic reticulum by TG and FCCP, the initial transient increase in $[Ca^{2+}]_i$ is almost completely diminished, indicating that the first peak observed under physiological Ca^{2+} conditions largely originates from intracellular stores. For details see *Material and Methods* and *Results* section.

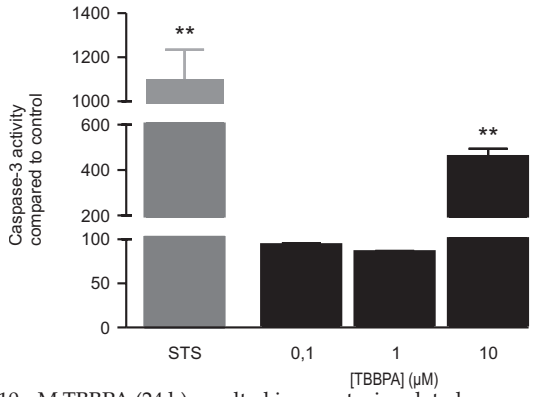

Figure S6. Exposure to 10 µM TBBPA (24 h) resulted in apoptosis-related caspase-3 activity in PC12 cells. Bars demonstrate mean caspase-3 activity compared to control (set as 100%) ± SEM. 1 µM staurosporine (STS, 6 h) was included as positive control. $n = 7$ wells/concentration of two independent plates; $^{**}p < 0.001$ vs control. For details see *Material and Methods* and *Results* section.

6

Chapter 6

Modulation of human $\alpha_4\beta_2$ nicotinic acetylcholine receptors by brominated and halogen-free flame retardants as a measure for *in vitro* neurotoxicity

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Abstract

Brominated flame retardants (BFRs) are abundant persistent organic pollutants with well-studied toxicity. The toxicological and ecological concern associated with BFRs argues for replacement by safer alternatives. However, the (neuro)toxic potential of alternative halogen-free flame retardants (HFFRs) is unknown. Previous research identified the nervous system as a sensitive target organ for BFRs, with modulation of excitatory nicotinic acetylcholine (nACh) receptors as one of the modes of action. Since it is essential to assess the (neuro)toxic potential of HFFRs before large scale use, we measured the effects of three BFRs and 13 HFFRs on the function of human $\alpha_4\beta_2$ nACh receptors, expressed in *Xenopus* oocytes, using the two-electrode voltage-clamp technique. The results demonstrate that some BFRs (TBBPA and to a lesser extent BDE-209) and HFFRs (TPP, Alpi, APP, MMT and to a lesser extent ATH, ATO, MHO, MPP, RDP and ZHS) act as nACh receptor antagonists. Contrary, BPS, BDP, DOPO and ZS were unable to modulate nACh receptors. Despite the lack of toxicological data on HFFRs and the need for additional studies to perform a full (neuro)toxic risk assessment, the current data on antagonistic effects on nACh receptors could be an important step in prioritizing viable HFFRs for substitution of BFRs.

1. Introduction

Fire has been a major cause of property damage, injuries and death throughout recorded history. Modern technology has responded to this threat by introducing flame retardant chemicals to prevent or slow the onset and spreading of fire. Following the discovery of the adverse effects of polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs) were introduced as the major chemical flame retardants. BFRs primarily include polybrominated diphenylethers (PBDEs) and tetrabromobisphenol-A (TBBPA). Unfortunately, BFRs can have unintended adverse effects on the environment and human health (Covaci *et al.*, 2011; Shaw *et al.*, 2010) and recent studies identified the nervous system as being among the most vulnerable target for the toxic actions of BFRs (for reviews see Costa and Giordano, 2007; Dingemans *et al.*, 2011; Fonnum and Mariussen, 2009).

As the wide application of BFRs raises toxicological and ecological concerns, there is a clear need to replace BFRs by safe(r) and less persistent alternatives. Suggested halogen-free substitutions by the industry include phosphorous flame retardant compounds such as triphenylphosphate (TPP), resorcinol bis(diphenylphosphate) (RDP), bisphenol-A bis(diphenylphosphate) (BDP), 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO) and aluminium diethylphosphinate (Alpi); inorganic halogen-free flame retardants and synergists such as 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). However, the (neuro)toxic potential of these proposed halogen-free flame retardants (HFFRs) is largely unknown (for review see Waaijers *et al.*, 2013b).

Previous studies have shown that environmental toxicants such as organophosphorous compounds, PCBs and PBDEs can interfere with the cholinergic system and contribute to neurodevelopmental abnormalities (Coccini *et al.*, 2007; Eriksson *et al.*, 2001; Johansson *et al.*, 2008; Slotkin, 2004). The $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptor is an abundant excitatory neurotransmitter receptor in the human central and peripheral nervous system. Activation of this receptor by acetylcholine (ACh) results in an influx of cations and subsequent depolarization of the cell membrane and increased neuronal activity. Earlier *in vitro* research demonstrated that nACh receptors are a direct target for e.g., organophosphates (Smulders *et al.*, 2004), PCBs, PBDEs (Hendriks *et al.*, 2010) and TBBPA (Hendriks *et al.*, 2012a). We therefore measured the effects of the 13 above-mentioned HFFRs and three BFRs (brominated polystyrene (BPS), decabromodiphenyl ether (BDE-209) and tetrabromobisphenol-A (TBBPA)) on the excitatory human $\alpha_4\beta_2$ nACh receptor for an initial assessment of the (neuro)toxic potential of HFFRs. This assessment can be an important step in prioritizing viable halogen-free alternatives for the replacement of BFRs prior to large scale and global use of these HFFRs.

2. Material and methods

2.1 Animals

All experiments were conducted in accordance with Dutch law and approved by the Ethical Committee for Animal Experiments of Utrecht University. Adult female specimen of *Xenopus laevis* frogs (provided by Dr. Wim Scheenen, Radboud University, Nijmegen, The Netherlands) were kept in copper-free water (pH 6.5, 21-23°C) in standard aquaria (0.5 m × 0.4 m × 1 m; 1-10 per aquarium) with a 12 h light/dark cycle. The animals were fed earthworms three times a week (Lasebo, Nijkerkerveen, The Netherlands).

2.2 Chemicals

Acetylcholine (ACh), dimethyl sulfoxide (DMSO), neomycin solution (10 mg neomycin/ml in 0.9% NaCl), collagenase type I, NaCl and 3-aminobenzoic acid ethyl ester (MS-222) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). $CaCl_2$ (1 M solution), $MgCl_2$ (1 M solution), $MgSO_4$, $NaHCO_3$, NaOH, $Ca(NO_3)_2$, KCl, and HEPES were purchased from Merck (Darmstadt, Germany).

The flame retardants (at the highest purity available, Table 1) were purchased from different companies (see Figures 2-4). The BFRs (BPS, BDE-209 and TBBPA) and phosphorous flame retardants (BDP, RDP, TPP and DOPO) 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 in saline to obtain final concentrations of 0.3-100 μM. The final concentration of DMSO in congener-containing saline was always kept below 0.1% (v/v) and DMSO had no effect on nACh receptor-mediated currents at this concentration (data not shown). The other HFFRs (Alpi, ATH, APP, ATO, MMT, MHO, MPP, ZHS and ZS) are poorly soluble in DMSO (or other solvents) and were directly dissolved in saline solution at the maximal water solubility as presented in Table 1 (S_{max}, measured by inductively coupled plasma mass spectrometry (ICP-MS), details not shown) and dilutions thereof.

2.3 Expression of $\alpha_4\beta_2$ nACh receptors in *X. laevis* oocytes

All procedures have been described previously (Antunes Fernandes *et al.*, 2010b; Hendriks *et al.*, 2010; Hondebrink *et al.*, 2011b). Briefly, female *X. laevis* were anesthetized by submersion in 0.1% MS-222 and ovarian lobes were surgically removed. Before manual defolliculation, the oocytes were treated with collagenase type I (1.5 mg/ml Ca^{2+} free Barth's solution) for 90 min at room temperature.

cDNA coding for the human α_4 and β_2 subunits of the human neuronal nACh receptors was kindly provided by Janssen Pharmaceutica N.V. (Beerse, Belgium). cDNA was dissolved in distilled water at a 1:1 molar ratio and injected in a total injection volume of 18.4 nl/oocyte (~0.2 ng of each subunit) into the nuclei of stage V or VI oocytes by use of a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA, USA). Oocytes were incubated for 2-5 days at 21°C in modified Barth's solution containing (in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 15 HEPES, and 10 μg/ml neomycin (pH 7.6 with NaOH).

2.4 Electrophysiological recordings

Following translation of injected cDNA, the oocytes expressed functional $\alpha_4\beta_2$ nACh receptors in the membrane. Ion currents associated with $\alpha_4\beta_2$ nACh receptor activity were measured with the two-electrode voltage-clamp technique using a Gene Clamp 500B amplifier (Axon Instruments) with high-voltage output stage as described previously (Antunes Fernandes *et al.*, 2010b; Hendriks *et al.*, 2010; Hondebrink *et al.*, 2011b). Recording microelectrodes (0.5-2.5 MΩ) were filled with KCl (3 M). Oocytes, placed in a custom-built Teflon oocyte recording chamber, were voltage-clamped at -60 mV and continuously superfused (~30 ml/min) with saline solution, containing (in mM): 115 NaCl, 2.5 KCl, 1 $CaCl_2$, 10 HEPES (pH 7.2 with NaOH). Membrane currents were low-pass filtered (8-pole Bessel; 3 dB at 0.3 kHz), digitized (12 bits; 1024 samples/record), and stored on disk for computer analysis.

Aliquots of freshly thawed stock solutions of ACh in distilled water and the BFRs and phosphorous flame retardants in DMSO were diluted in the saline solution immediately before the experiments. Oocytes were exposed for 20-40 s by switching the perfusate

from saline to flame retardant- and/or ACh-containing saline using a servomotor-operated valve. To minimize adsorption of the flame retardants to the perfusion system, glass reservoirs and Teflon tubes (polytetrafluoroethylene; 4 mm × 6 mm, Rubber, Hilversum, The Netherlands) were used. A washout period of 2-5 min between each application was introduced, allowing receptors to recover from desensitization.

2.5 Data analysis and statistics

As described previously, peak amplitudes of ACh-evoked ion currents were measured and normalized to the maximal amplitude (at 1 mM) of agonist-evoked control responses to adjust for differences in receptor expression levels among oocytes and for small variations in response amplitudes over time (Antunes Fernandes *et al.*, 2010b; Hendriks *et al.*, 2010; Hondebrink *et al.*, 2011b). Normalized ion currents were plotted against agonist concentration in each experiment. ACh concentration-effect curves were fitted to the data obtained in separate experiments using Prism (Graphpad Software, La Jolla, CA, USA). The percentage of flame retardant-induced inhibition of the ACh-evoked ion current was calculated from the quotient of the maximum amplitude of the ACh-congener co-application response and the maximum amplitude of the ACh response. Flame retardants were initially tested at two concentrations and complete concentration-response curves were measured where appropriate. Data represent mean ± standard error of the mean (SEM) of n oocytes.

Statistical differences (p <0.05) were calculated using unpaired two-tailed Student's t-test. The concentration-dependence of the inhibiting effects was determined by one-way ANOVA (p <0.05) and posthoc Bonferroni testing. Observed changes in ion current <5% were considered irrelevant.

MDL ISISTM/Draw version 2.5 was used to draw the chemical structures.

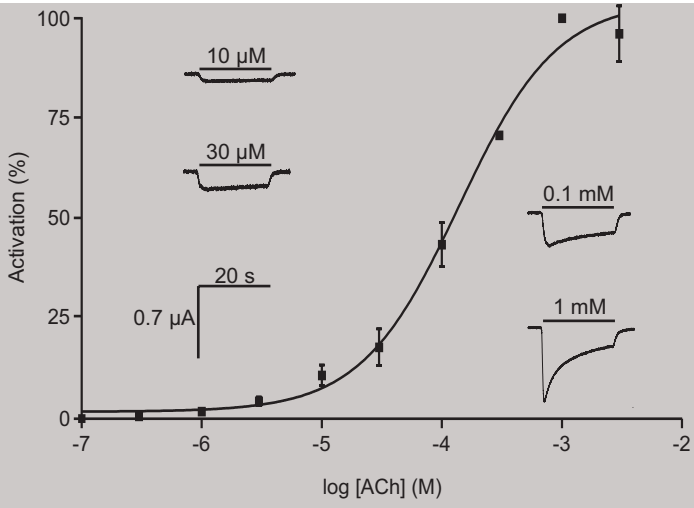


Figure 1. Concentration-response curve of the activation of human $\alpha_4\beta_2$ nACh receptors, expressed in *Xenopus* oocytes, by ACh. Insets show example recordings of ion currents evoked by superfusion of oocytes with saline containing different concentrations of ACh, as indicated above to the recordings. Scale bar applies to all traces. Fitted line is Hill curve with mean values (± SEM) obtained from 5 independent experiments.

3. Results

3.1 Functional properties of human $\alpha_4\beta_2$ nACh receptors in *Xenopus* oocytes

Voltage-clamped (-60 mV) oocytes expressing human $\alpha_4\beta_2$ nACh receptors were superfused with various concentrations of ACh (0.1 μM to 3 mM), resulting in concentration-dependent ion currents. These agonist-evoked ion currents were normalized to the maximal response obtained with 1 mM ACh and plotted against the agonist concentration to obtain a concentration-effect curve according to the Hill equation. This curve was used to determine EC_{10} and EC_{50} values, i.e., the concentrations producing 10% and 50% of the maximal response. In line with previous reports (Hendriks *et al.*, 2010; Smulders *et al.*, 2004), the Hill slope for the fitted ACh concentration-effect curve was 1.0 ± 0.1 and mean values for EC_{10} and EC_{50} amounted to 14 ± 2 and 139 ± 17 μM (n = 5, Figure 1).

3.2 Agonistic effects of BFRs on the human $\alpha_4\beta_2$ nACh receptor

Superfusion of ACh-responsive oocytes with saline containing BPS, BDE-209 or TBBPA (1 and 10 μM) did not result in a detectable ion current, clearly indicating that none of the tested BFRs can act as full agonist of the $\alpha_4\beta_2$ nACh receptor (Figure 2).

Since organophosphates, PCBs and PBDEs and TBBPA are able to modulate the ACh-evoked current in a concentration-dependent manner at low receptor occupancy (Hendriks *et al.*, 2010; Smulders *et al.*, 2004), we co-applied the BFRs with a low concentration

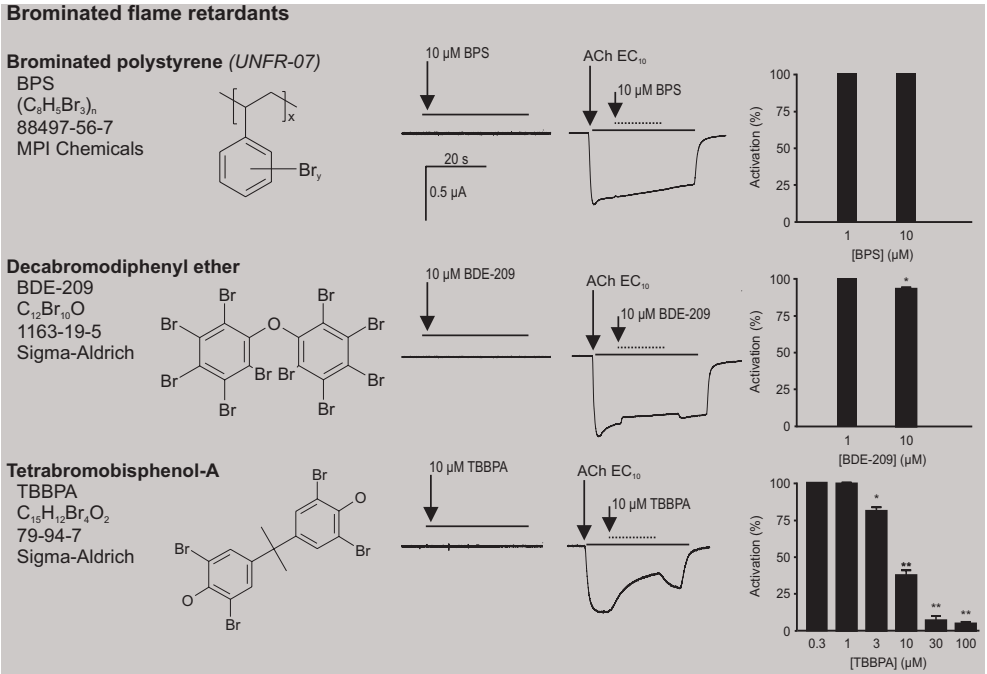


Figure 2. Overview of the tested brominated flame retardants. Full and product name (in italic between brackets), abbreviation, molecular formula, CAS-number, company and chemical structure of each BFR are shown. Example recordings demonstrate that none of the tested BFRs can activate the $\alpha_4\beta_2$ nACh receptor, whereas co-application of BDE-209 or TBBPA with ACh (EC_{10}) inhibits the ACh-evoked currents. Scale bar applies to all traces. Bar graphs represent (concentration-dependent) inhibition of ACh-induced currents (mean activation ± SEM, 100% activation demonstrates the absence of antagonistic effects of the tested flame retardant). n = 3-6 oocytes. *p <0.05 vs control; **p <0.001 vs control.

ACh (EC_{10}). Co-application of BPS (up to 10 μM) with ACh (EC_{10}) did not modulate the ACh-evoked ion current. However, co-application of the widely used BDE-209 (10 μM) with ACh (EC_{10}) resulted in a small inhibition (8 ± 1%; p <0.01) of the ACh-evoked response. As described previously (Hendriks *et al.*, 2012a), co-application of TBBPA with ACh (EC_{10}) induced a concentration-dependent inhibition of the ACh-evoked ion current

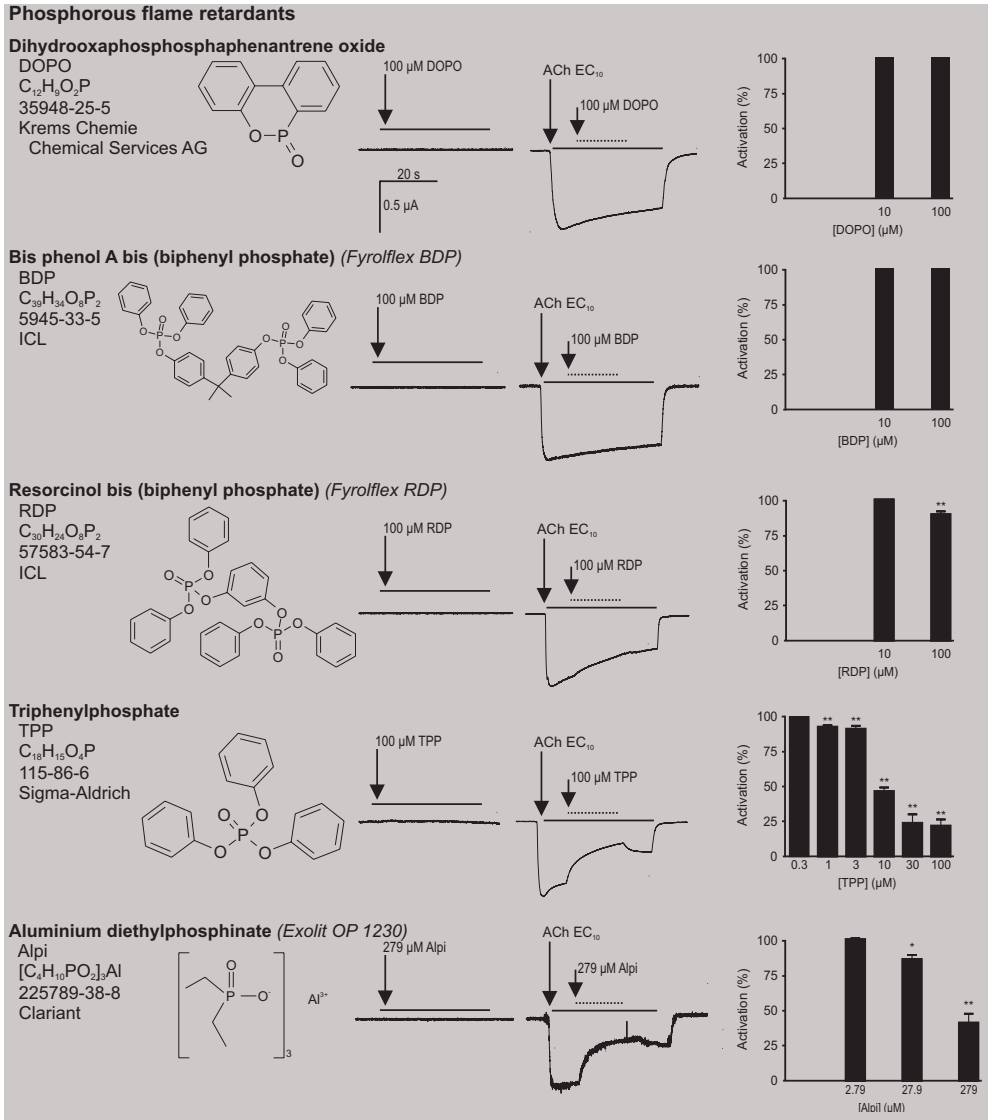
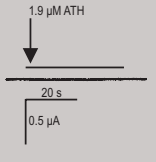


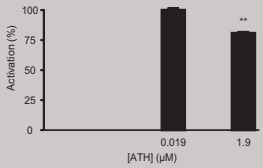
Figure 3. Overview of the tested phosphorous flame retardants. Full and product name (in italic between brackets), abbreviation, molecular formula, CAS-number, company and chemical structure of each compound are shown. Example recordings demonstrate that phosphorous flame retardants cannot activate the $\alpha_4\beta_2$ nACh receptor. Co-application of TPP, Alpi and to a lesser extent RDP with ACh (EC_{10}) resulted in inhibition of the ACh-evoked. Scale bar applies to all traces. Bar graphs represent (concentration-dependent) inhibition of ACh-induced currents (mean activation ± SEM, 100% activation demonstrates the absence of antagonistic effects of the tested flame retardant). n = 3-7 oocytes; *p <0.05; **p <0.001 vs control.

Inorganic halogen-free flame retardants

Aluminium trihydroxide

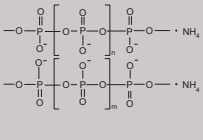
ATH
$Al(OH)_3$
21645-51-2
Merck

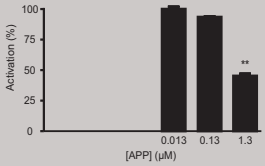




Ammonium polyphosphate *(Exolit AP 422)*

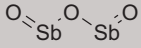
APP
$(NH_4PO_3)_n$
68333-79-9
Clariant

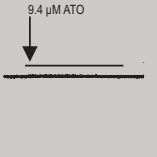


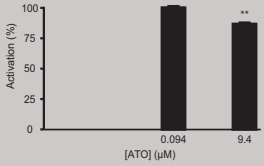


Antimony trioxide *(Fireshield H)*

ATO
Sb_2O_3
1309-64-4
Chemtura

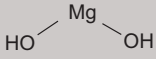


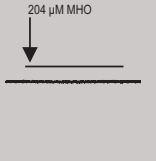


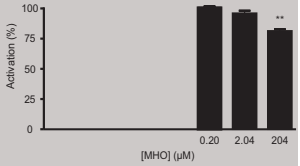


Magnesium hydroxide

MHO
H_2MgO_2
1309-42-8
Sigma-Aldrich

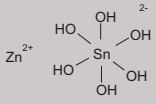


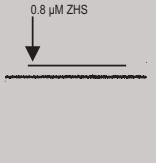


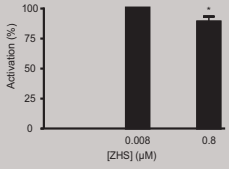


Zinc hydroxystannate *(Flamtard H)*

ZHS
$ZnSn(OH)_6$
12027-96-2
William Blythe

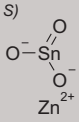


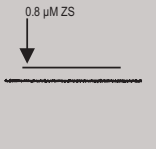


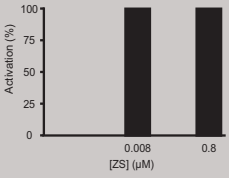


Zinc stannate *(Flamtard S)*

ZS
O_3SnZn
12036-37-2
William Blythe





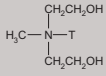


Nanoclay

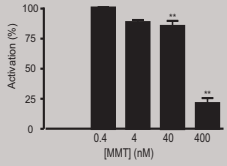
Montmorillonite *(Cloisite 30B)*

MMT
$C_{23}H_{50}O_2N$

NRC Nordmann
Rassmann



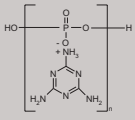
Where T is tallow (`65% C18)

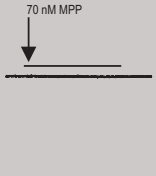


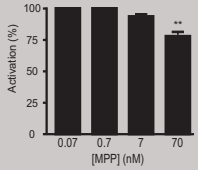
Nitrogen based organic flame retardant

Melamine polyphosphate *(Melapur 200)*

MPP
$C_3H_6N_4{\bullet}(H_3PO_4)_n$
218768-84-4
BTC







with a lowest observed effect concentration (LOEC) of 3 μM (p <0.01) and a calculated effective concentration producing 50% inhibition of the maximal response (IC_{50}) of 7 ± 1 μM. At ≥30 μM, TBBPA almost completely abolished the ACh-evoked ion current, indicating TBBPA is a strong antagonist of the $\alpha_4\beta_2$ nACh receptor (Figure 2).

3.3 Antagonistic effects of phosphorous flame retardants on the human $\alpha_4\beta_2$ nACh receptor

Superfusion of ACh-responsive oocytes with saline containing any of the phosphorous flame retardants (up to 100 μM) did not result in a detectable ion current, clearly indicating that none of these compounds can act as full agonist of the $\alpha_4\beta_2$ nACh receptor (Figure 3).

Co-application of DOPO (100 μM) or BDP (100 μM) with ACh (EC_{10}) did not affect the ACh-evoked ion current, whereas co-application of RDP (100 μM) with ACh (EC_{10}) resulted in a small inhibition of the ACh-evoked ion current (10 ± 2%; p <0.001). Co-application of TPP with ACh (EC_{10}) resulted in a concentration-dependent inhibition of the ACh-evoked ion current, with a LOEC of 1 μM (p <0.001; calculated IC_{50} 7.9 ± 1.4 μM; Figure 3) and a maximum inhibition at ≥30 μM (76 ± 6%; Figure 3). The metal-phosphinate Alpi also induced a concentration-dependent inhibition of the ACh-evoked ion current. The LOEC for the inhibition of the $\alpha_4\beta_2$ nACh receptor amounted to 27.9 μM, i.e., 10% of the maximum water solubility (p <0.05). At the highest concentration Alpi tested (279 μM, i.e., maximum water solubility), the inhibition of the ACh-evoked ion current amounted to 60 ± 6%, thus precluding reliable calculation of the IC_{50}.

3.4 Antagonistic effects of inorganic, nitrogen-based organic and nanoclay flame retardants on the human $\alpha_4\beta_2$ nACh receptor

Comparable to the other flame retardants, superfusion of ACh-responsive oocytes with saline containing inorganic halogen-free flame retardants, nitrogen-based organic flame retardant or nanoclay (up to S_{max}) did not result in a detectable ion current, demonstrating that none of these compounds can act as full agonist of the $\alpha_4\beta_2$ nACh receptor (Figure 4).

Co-application of ZS at S_{max} (0.8 μM) with ACh (EC_{10}) did not affect the ACh-evoked ion current. On the other hand, ATO, ATH, MHO and ZHS all induced a small inhibition at their S_{max} (Figure 4). The inorganic flame retardant APP induced a stronger inhibition that amounted to 55 ± 2% at S_{max} (1.3 μM, p <0.001; LOEC 0.13 μM, p <0.001), thus precluding reliable calculation of the IC_{50}. The nitrogen-based organic flame retardant MPP, which has a very poor water solubility, also induced an inhibition of the ACh-evoked ion current only at the S_{max} (70 nM), amounting to 23 ± 3% (p <0.001). Similarly, the poorly soluble nanoclay MMT induced an inhibition of the $\alpha_4\beta_2$ nACh receptor in the nanomolar range, with a LOEC of 40 nM (p <0.001) and a maximum inhibition amounting to 79 ± 4% at its S_{max} (400 nM; calculated IC_{50} 140 ± 1.2 nM; Figure 4).

Figure 4 (left). Overview of the tested inorganic halogen-free flame retardants, nitrogen-based organic flame retardant and nanoclay. Full and product name (in italic between brackets), abbreviation, molecular formula, CAS-number, company and chemical structure of each compound are shown. Example recordings show that none of the tested flame retardants can activate the $\alpha_4\beta_2$ nACh receptor. When co-applied with ACh (EC_{10}), ATH, APP, ATO, MHO, ZHS, MMT and MPP inhibit the ACh-evoked current. Scale bar applies to all traces. Bar graphs represent inhibition of ACh-induced currents evoked by the different compounds (mean activation ± SEM, demonstrates the absence of antagonistic effects of the tested flame retardant); n = 3-7 oocytes; *p <0.05; **p <0.001 vs control.

In Table 1, an overview is given of all tested flame retardants and their effects on $\alpha_4\beta_2$ nACh receptor function when co-exposed with 10 μM ACh (~EC_{10}). An initial rank order potency was derived based on the determined no observed effect concentration (NOEC, 'potency') and maximal effect size. Based on these criteria, TBBPA, TPP, Alpi, APP and MMT are classified as 'highly potent', whereas BDE-209, ATH, ATO, MHO, ZHS and MPP are classified as 'moderately potent'. On the other hand, the brominated flame retardant BPS and the alternative flame retardants BDP, RDP, DOPO and ZS were classified as 'not potent' with respect to modulation of $\alpha_4\beta_2$ nACh receptors function *in vitro*.

4. Discussion

In this study we determined the *in vitro* neurotoxic potential of 13 proposed HFFRs and three frequently used BFRs on human $\alpha_4\beta_2$ nACh receptors. Our results demonstrate that some BFRs and HFFRs were able to inhibit the ACh-evoked current, which is in line with previously observed antagonistic effects of organophosphates, PCBs, PBDEs and TBBPA (Hendriks *et al.*, 2010; Hendriks *et al.*, 2012a; Smulders *et al.*, 2004). Based on the present results, a rank-order potency was derived that can be used for an initial prioritization of viable halogen-free alternatives for substitution of BFRs.

Of the BFRs, only TBBPA was classified as highly potent based on the ranking as presented in Table 1. The observed inhibition of the ACh-evoked ion-current (Figure 2) is in line with previous results (Hendriks *et al.*, 2012a), and subsequent inhibition of the cholinergic system possibly underlies previously observed neurobehavioral and neurodevelopment effects (Nakajima *et al.*, 2009; Viberg and Eriksson, 2011).

Developmental exposure of rodents to BDE-209 has been reported to cause neurobehavioral defects (Costa and Giordano, 2011), though it was suggested these defects were caused by metabolites, such as PBDE-47 that can be further metabolized into e.g., 6-OH-PBDE-47. In our study, BDE-209 was able to inhibit the nACh receptor (Figure 2), comparable with the previously reported antagonistic effects of 6-OH-PBDE-47 (Hendriks *et al.*, 2010), indicating that BDE-209 itself also has some neurotoxic potential. Although BDE-209 was classified as moderately potent based on this study, other (neuro)toxic endpoints, congeners and metabolites should be included for a full risk assessment.

The other BFR investigated in this study, BPS, is chemically not well-defined. BPS has a NOEC ≥10 μM in this study (Figure 2) and the high molecular weight of BPS indicates a low potential for transport into the systemic circulation. Consequently, the toxicity is considered low and BPS was classified as not potent, though there is a lack of sufficient toxicological data.

Of the phosphorous HFFRs, TPP is labeled as a compound with dangerous effects for the environment by the European Chemicals Agency (ECHA) (ECHA Database, Accessed 2012b). The neurotoxicity of TPP has been debated since the studies of Smith (Smith *et al.*, 1930; Smith *et al.*, 1932) as other studies could not confirm neurotoxic changes in animals (Wills *et al.*, 1979). Nonetheless, several neurotoxic effects were observed *in vitro*, e.g., cytotoxicity in PC12 cells (Flaskos *et al.*, 1994). In the present study, antagonistic effects were observed on the nACh receptor (Figure 3) in the same micromolar range as previously observed for inhibition of the GABA-regulated chloride channel (Gant *et al.*, 1987), underlining the neurotoxic potential of TPP. Based on our results, TPP is classified as highly potent and is therefore not recommended as a viable alternative.

Commercially available BDP and RDP may contain up to 5% TPP as impurity (Clean

Production Action, 2007; Umwelt Bundes Amt, 2001), which could explain the observed small antagonistic effects of RDP (Figure 3). BDP and RDP are classified as not potent (Table 1), although it should be mentioned that TPP-like products may be formed as breakdown products of BDP and RDP. Moreover, one study identified the endocrine disruptor bisphenol-A as a degradation product of BDP (Maine, 2007). Toxicological data on BDP and RDP is limited (Clean Production Action, 2007; Washington State Depart-

Table 1. Overview of the achieved maximal solubility of BFRs and HFFRs and their effects on $\alpha_4\beta_2$ nACh receptor inhibition when co-exposed with 10 μM ACh.

Flame retardant	Chemical purity	Maximal concentration stock solution	NOEC (μM)	Potency	Maximal effect size	Rank ordering
Brominated flame retardants						
BPS	>99%	10 mM	≥10	-/+	-	Not potent
BDE-209	98%	1 mM	1	++	+	Moderately potent
TBBPA	>99%	100 mM	1	++	+++	Highly potent
Phosphorous flame retardants						
BDP	>96%; 3.5% TPP	100 mM	≥100	-	-	Not potent
RDP	>95%; 4.5% TPP	100 mM	10	+	+	Not potent
TPP	>99%	100 mM	0.3	+++	+++	Highly potent
DOPO	>99%	100 mM	≥100	-	-	Not potent
Alpi	>99%	279 μM	2.79	++	+++	Highly potent
Inorganic halogen free flame retardants and synergists						
ATH	>99%	1.9 μM	0.019	+++	+	Moderately potent
APP	>99%	1.3 μM[a]	0.013	+++	+++	Highly potent
ATO	>99%	9.4 μM	0.094	+++	+	Moderately potent
MHO	>99%	204 μM	2.04	++	+	Moderately potent
ZHS	94%	0.8 μM	0.008	+++	+	Moderately potent
ZS	>98%	0.8 μM	≥0.8	-/+++	-	Not potent
Nanoclay						
MMT	n/a	400 nM[b]	0.004	+++	+++	Highly potent
Nitrogen-based organic flame retardant						
MPP	>95%	70 nM[a]	0.007	+++	+	Moderately potent

No observed effect concentrations (NOEC). n/a not applicable. *Potency criteria*: -, no potency, NOEC ≥100 μM; +, low potency, 10 μM ≤NOEC <100 μM; ++, moderate potency, 1 μM ≤NOEC <10 μM; +++, high potency, NOEC <1 μM; *Effect size criteria*: -, no inhibition, ≤5% inhibition; +, small inhibition, <25% inhibition; ++, moderate inhibition, ≥25 - 50% inhibition; +++, strong inhibition, ≥50% inhibition.

[a] based on an average chain length of 1000.

[b] calculated assuming 0.1-1% silicon in the clay.

ment of Ecology and Department of Health, 2006; for review see Waaijers *et al.*, 2013b) and more information is needed before BDP and/or RDP can be designated as viable alternatives for current BFRs.

Similarly, DOPO was not able to affect the nACh receptor (Figure 3) and is therefore classified as not potent. Nevertheless, there is a general lack of toxicological data for DOPO (for review see Waaijers *et al.*, 2013b), which hampers proper risk assessment.

Toxicological data on Alpi is also limited (Clariant, 2007; US EPA, 2008; for reviews see Gardner and Walker, 2000; Waaijers *et al.*, 2013b). Based on the effects observed in our study (Figure 3), Alpi is classified as highly potent, although it should be noted that Alpi was tested at concentrations as high as 279 μM, which logically affected the maximal effect size. Moreover, aluminum could dissolute from the molecule and several reports describe adverse effects of aluminum on cation-channels (Hu *et al.*, 2007; Vijverberg *et al.*, 1994; Wakui *et al.*, 1990). The observed effects of Alpi in the present study could thus be (partially) due to dissipated aluminum and more research is needed to exclude Alpi as viable alternative.

Toxicity data on the tested inorganic halogen-free flame retardants and synergists are scarce (for review see Waaijers *et al.*, 2013b). Several studies report *in vitro* (neuro)toxicity of ATH, e.g., cytostatic activity with induction of neurites in neuroblastoma cells (Zatta *et al.*, 1992) and *N*-methyl-D-aspartate (NMDA) receptor binding in human cerebral cortex (Hubbard *et al.*, 1989). Previously observed diminished cholinergic activity in rats (Bilkei-Gorzo, 1993) could be (partly) explained by the direct inhibition of the nACh receptor as observed in the present study (Figure 4). ATH is classified as moderately potent and not expected to decompose under physiological conditions.

In our study, clear antagonistic effects of APP on the nACh receptor are observed (Figure 4), which is classified as highly potent. It is, however, unknown if this effect is caused by the polymer itself, which has a suspected low bioavailability, or monomeric ammonium phosphate since APP slowly hydrolysis in contact with water (Clariant, 2010). Assuming considerable hydrolysis occurs, our test solution would primarily contain monomers, which would significantly change the classification of APP from highly potent (assuming an average chain length of 1000) to moderately potent (assuming the monomer).

ATO is stated by the ECHA (ECHA Database, Accessed 2012a) as 'suspected of causing cancer but not sufficient for classification'. In the present study, antagonistic effects on the nACh receptor were observed (Figure 4) and ATO is classified as moderately potent. However, additional studies are needed to confirm the neurotoxic potential of ATO.

Small antagonistic effects of MHO on the nACh receptor were observed in this study (Figure 4) and MHO is therefore classified as moderately potent. Noteworthy, this classification is also based on the effect size at the highest concentration tested (204 μM). Additionally, it is expected that MHO will dissociate in the stomach to magnesium ions. The (neuro)toxic effects of (high concentrations) magnesium, including NMDA receptor activation (Domijan *et al.*, 2012) and Ca^{2+}-activated K^{+}-channels inhibition (Leinders *et al.*, 1992), should therefore be included in the assessment of MHO as viable alternative.

No effects were observed for ZS, which is therefore classified as not potent, whereas ZHS only induced a small inhibition of the ACh evoked current (Figure 4) and is classified as moderately potent. Noteworthy, this classification is based on the effect size at the maximal water solubility (0.8 μM), which is far below previous observed toxic effects of the single metals. However, metallic tin and its inorganic salts are generally considered to be of low toxicity due to their poor alimentary absorption (Cima, 2011). For a full risk assessment, the likeliness of dissolution of ZS and/or ZHS into zinc and tin ions and

subsequent metal toxicity should be incorporated, especially since both organotin compounds and excess zinc are known for their neurotoxic and neurodegenerative effects (for reviews see Boyer, 1989; Wright and Baccarelli, 2007; Zatta *et al.*, 2009).

The nanoclay used in this study, Cloisite 30B exists of quartz (0.1-1%) and alkyl quaternary ammonium bentonite (95-99%). No effects for quartz were observed up to 4000 μg/ml in neuroblastoma cells, though montmorillonite (the main constituent of bentonite) induced an acute impairment of cellular excitable function (studied concentration-range 100-1000 μg/ml) (Banin and Meiri, 1990). Although further toxicological studies are lacking, we found a clear antagonistic effect on the nACh receptor (Figure 4) and classified it as highly potent.

The antagonistic effect of MPP on the nACh receptor observed in our study (Figure 4) is classified as moderately potent, assuming an average chain length of 1000. In contrast to APP, calculations made assuming that our solution contains mainly monomeric MPP did not change the rank ordering. The bioavailability of MPP is suspected to be low, and in water MPP will dissociate into the monomers melamine and phosphoric acid. Melamine is known to affect e.g., voltage-gated Na^+ channels (Yang *et al.*, 2010) and should thus also be taken into account when assessing the viability of MPP as alternative.

5. Conclusion

Ideally, HFFRs that replace existing BFRs should pose lower risks to the environment and human health. However, there is a general lack of toxicological information regarding the suggested HFFRs, which makes it hard to assess the toxic potential of these compounds. Consequently, there is an urgent need for more research on the (eco)toxicological effects of these compounds and (expected) exposure levels before they are globally used on large scale. Our initial rank-order potency (Table 1) based on the *in vitro* inhibition of nACh receptors clearly indicates the neurotoxic potential of TBBPA, TPP, Alpi, APP and MMT. Noteworthy, additional studies, also focusing on expected concentrations in humans and the environment, are required before these compounds can be excluded as viable alternatives for the replacement of current BFRs. Importantly, five out of the sixteen tested compounds (BPS, BDP, RDP, DOPO and ZS) are classified as not potent. Based on this specific neurotoxic endpoint, these five compounds could therefore be selected for additional testing to further assess the viability of these HFFRs as alternatives to replace current BFRs.

Acknowledgments

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7

Chapter 7

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.

1. 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 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 behavior 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.*, 2012b; 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, 2013b). 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, 2013b). Though some neurotoxic effects of HFFRs have been described, e.g. TPP-induced cytotoxicity in PC12 cells (Flaskos *et al.*, 1994), ATH-induced neurites 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.

2. Material and methods

2.1 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 deionized 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, Figure 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, Figure 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.

2.2 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.

2.3 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.

2.4 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 Figure 3A for an example recording) to determine effects of flame retardants on

basal and depolarization-evoked $[Ca^{2+}]_i$, respectively (see Figure 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.

2.4 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

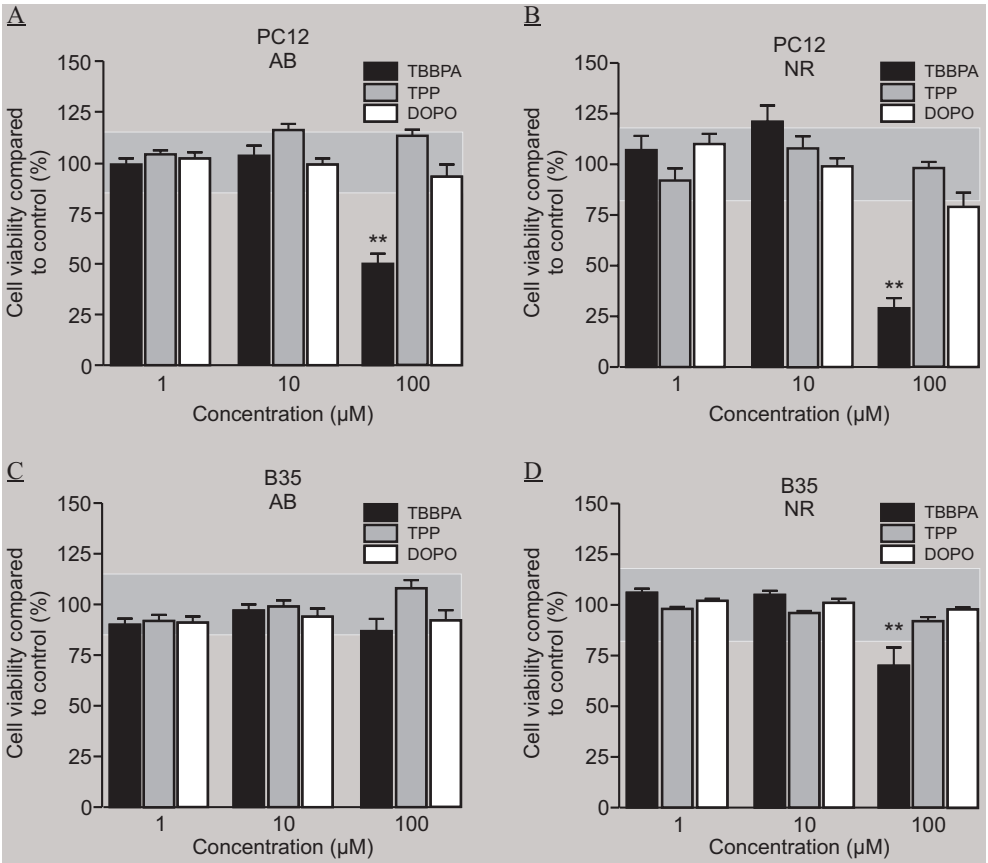

Figure 1. Effects of 24 h exposure to TBBPA, TPP and DOPO on cell viability in PC12 and B35 cells. Bar graphs, representing cell viability determined using a combined Alamar Blue (AB; graph A and C) and Neutral Red (NR; graph B and D), demonstrate that TBBPA at 100 μM decreases cell viability in PC12 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 vs control.

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).

3. Results

3.1 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 Figure 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, Figure 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.

3.2 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 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 Figure 2 and Figure 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 (Figure 2 and Figure 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.

3.3 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 Figure 3). During a subsequent 5-min recovery period, $[Ca^{2+}]_i$ returned to near basal levels. Next,

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 Figure 1); ROS effect size represents mean ROS production after 24 h (% of time-matched controls; see also Figure 2). Basal Ca^{2+} effects size: mean $[Ca^{2+}]_i$ in μM (see also Figure 3); depolarization-evoked Ca^{2+}: net treatment ratio (% of control; see also Figure 3). TBBPA data was previously published by (Hendriks *et al.*, 2012a). nACh-R effect size: inhibition of the ACh-evoked response (%). nACh receptor data was 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 Figure 1); ROS effect size represents mean ROS production after 24 h (% of time-matched controls; see also Figure 2). Basal Ca^{2+} effects size: mean $[Ca^{2+}]_i$ in μM (see also Figure 3); depolarization-evoked Ca^{2+}: net treatment ratio (% of control; see also Figure 3). nACh-R effect size: inhibition of the ACh-evoked response (%). nACh receptor data was previously published by (Hendriks *et al.*, 2012b). All data represent mean ± SEM. [a] Effects may be confounded due to dye-compound interaction.

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	4±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 Figure 1); ROS effect size represents mean ROS production after 24 h (% of time-matched controls; see also Figure 2). Basal Ca^{2+} effects size: mean $[Ca^{2+}]_i$ in μM (see also Figure 3); depolarization-evoked Ca^{2+}: net treatment ratio (% of control; see also Figure 3). nACh-R effect size: inhibition of the ACh-evoked response (%). nACh receptor data was previously published by (Hendriks *et al.*, 2012b). All data represent mean ± SEM. [a] Effects may be confounded due to dye-compound interaction.

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 Figure 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 $[Ca^{2+}]_i$ (Figure 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 Figure 3 and Table 2). No effects on basal $[Ca^{2+}]_i$ 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.

3.4 HHFR-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 Figure 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 (Figure 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 Figure 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

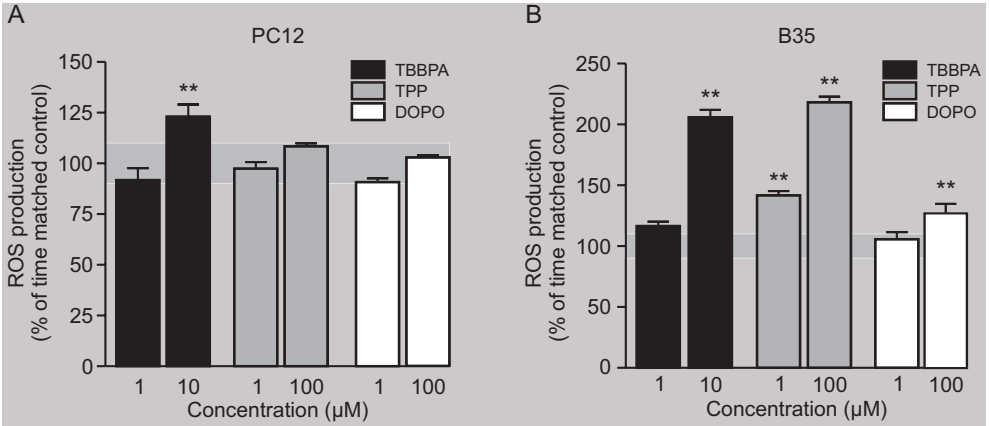

Figure 2. ROS production induced by TBBPA, TPP and DOPO. Exposure to TBBPA and TPP at non-cytotoxic concentrations increases ROS production in PC12 (A) and B35 cells (B) over time (graphs show results after 24 h exposure, see Supplemental Material Figure 2 for complete curves), while DOPO was able to 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 vs control.

$[Ca^{2+}]_i$, 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* (neuro)toxicological screening, since eight of the 16 compounds were able to reduce the net TR (see Table 5 for an overview).

3.5 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

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 Figure 1); ROS effect size represents mean ROS production after 24 h (% of time-matched controls; see also Figure 2). Basal Ca^{2+} effects size: mean $[Ca^{2+}]_i$ in μM (see also Figure 3); depolarization-evoked Ca^{2+}: net treatment ratio (% of control; see also Figure 3). nACh-R effect size: inhibition of the ACh-evoked response (%). nACh receptor data was previously published by (Hendriks *et al.*, 2012b). All data represent mean ± SEM.

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, 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).

4. 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 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)

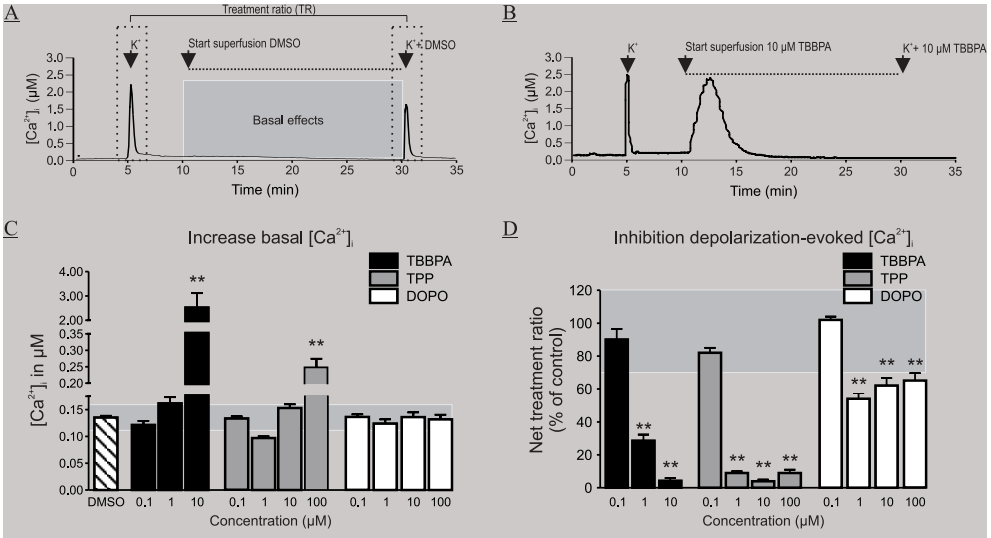

Figure 3. Flame retardant-induced effect on $[Ca^{2+}]_i$ in PC12 cells. Example recording of single-cell $[Ca^{2+}]_i$ imaging from individual PC12 cells. In between two 15 s depolarizations (100 mM K^+), cells were exposed for 20 min to 0.1% DMSO (A) or external saline as control or different non-cytotoxic 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 vs control.

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 (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, TPP 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.*, 2009). TPP is labelled by the European Chemicals Agency (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;

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						Nanoclay	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 1-4). 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 end points, despite the total number of points.

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.*, 2013b; 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 *et al.*, 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 (Covaci *et al.*, 2011; Stapleton *et al.*, 2009). 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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Supplementary data

Supplemental Material, Material and Methods

Cell culture

PC12 rat pheochromocytoma cells (Greene and Tischler, 1976) are widely used to assess neurotoxicity and neuronal function (for review see Westerink and Ewing, 2008). Cells were grown for maximal 10 passages in RPMI 1640 medium supplemented with 5% fetal calf serum, 10% horse serum (ICN Biomedicals, Zoetermeer, The Netherlands), 100 U/ml penicillin and 100 mg/ml streptomycin as described previously (Hendriks *et al.*, 2012a). B35 rat neuroblastoma cells are commonly used for molecular analysis of cellular signaling pathways (Otey *et al.*, 2003) and neurotoxicity studies (Hendriks *et al.*, 2012a). B35 neuroblastoma cells were grown for maximal 10 passages in DMEM medium supplemented with 10% fetal calf serum (ICN Biomedicals, Zoetermeer, The Netherlands), 1% additional amino acids (stock solution containing 40 mM of L-Cys, L-Ala, L-Asp, L-Pro, L-Glu and L-Asx), 100 U/ml penicillin and 100 mg/ml streptomycin as described previously (Hendriks *et al.*, 2012a).

Cells were grown in a humidified incubator at 37°C and 5% CO_2 and subcultured one day prior to measurements of cell viability, ROS production or $[Ca^{2+}]_i$. Undifferentiated PC12 or B35 cells were seeded in 24-wells plates (Greiner Bio-one, Solingen, Germany) at a density of respectively 6×10^5 cells/well and 3×10^5 cells/well for cell viability experiments. For measurements of ROS production, undifferentiated PC12 or B35 cells were seeded in black, glass-bottom 96-wells plates (Greiner Bio-one, Solingen, Germany) at a density of respectively 2×10^5 cells/well and 1.5×10^5 cells/well. For fluorescent microscopy Ca^{2+} imaging experiments, undifferentiated PC12 cells (1.4×10^6 cells/dish) were subcultured in glass-bottom dishes (MatTek, Ashland, MA). All culture flasks, dishes and plates were coated with poly-L-lysine (50 μg/ml).

Cell viability and ROS

Effects of the flame retardants on cell viability in PC12 and B35 cells were determined using a combined Alamar Blue (AB) and Neutral Red (NR) assay as described previously (Hendriks *et al.*, 2012a). The AB assay, which is based on the ability of the cells to reduce resazurin to resorufin, records mitochondrial activity of the cells as a measure of cell viability. Lysosomal integrity was subsequently determined in the NR assay as an independent second measure of cell viability. Briefly, following 24 h exposure to the flame retardant in serum-free medium, cells were incubated for 30 min with 200 μl resazurin solution (12 μM in PBS) after which resorufin was measured spectrophotometrically at 530/590 nm (excitation/emission; FLUOstar Galaxy V4.30-0, BMG Labtechnologies, Offenburg, Germany). After removal of the AB solution, cells were incubated for 1 h with 200 μl NR solution (12 μM in PBS). Following the incubation, cells were rinsed with warm (37°C) PBS and 100 μl extraction solution (1% glacial acetic acid, 50% ethanol and 49% H_2O) was added to the wells. After 30 min extraction, fluorescence was measured spectrophotometrically at 430/480 nm (excitation/emission).

ROS is formed as a natural by-product of the normal metabolism in a cell and increased ROS levels can result in cellular damage. ROS production was assessed using the fluorescent dye H_2-DCFDA as described previously (Hendriks *et al.*, 2012a). Briefly, PC12 or B35 cells seeded in black, glass-bottom 96-wells plates (Greiner Bio-one, Solingen, Germany) were loaded with 1.5 μM H_2-DCFDA for 30 min at 37°C. Subsequently, cells were exposed for up to 24h to flame retardant-containing saline. To exclude artifacts due to flame retardant mediated ROS production, cell-free interaction-experiments be-

tween H_2-DCFDA and flame retardants were performed. ROS production was measured spectrophotometrically as an increase in fluorescence at 485/530 nm (excitation/emission; FLUOstar Galaxy V4.30-0, BMG Labtechnologies, Offenburg, Germany).

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

$[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 AM as described previously (Hendriks *et al.*, 2012a). PC12 cells were loaded with 5 μM Fura-2 AM for 20 min at room temperature, followed by 15 min de-esterification. After de-esterification, the cells were placed on the stage of an inverted microscope (Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (TILL Photonics GmBH, Gräfelfing, Germany).

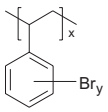
Cells were continuously superfused (~0.6 ml/min) with saline using a valvelink 8.2 (Automate Scientific, California, USA). Each experiment consisted of a 5 min baseline recording to measure basal $[Ca^{2+}]_i$, after which an increase in $[Ca^{2+}]_i$ was triggered by switching superfusion for 15 s to saline containing 100 mM K^+ to measure depolarization-evoked $[Ca^{2+}]_i$. Following this first stimulation and a 5-10 min recovery period, cells were exposed to saline containing DMSO (0.1%) or brominated or halogen-free flame retardant for 20 min prior to a second stimulation with 100 mM K^+ in the presence of the test compound (see Figure 3A for an example recording).

Fluorescence, evoked by 340 and 380 nm excitation wavelengths (F_{340} and F_{380}), was collected every 6 s at 510 nm with an Image SensiCam digital camera (TILL Photonics GmBH). Maximum and minimum ratios (R_{max} and R_{min}) were determined at the end of the recording by addition of ionomycin (5 μM) and ethylenediamine tetraacetic acid (EDTA; 17 mM). Changes in F_{340}/F_{380} ratio (R), reflecting changes in $[Ca^{2+}]_i$, were analyzed using custom-made MS-Excel macros.

Free cytosolic $[Ca^{2+}]_i$ was calculated as described previously (Hendriks *et al.*, 2012a), using a modified Grynkiewicz's equation: $[Ca^{2+}]_i = K_d * (R-R_{min})/(R_{max}-R)$, where K_d is the dissociation constant of Fura-2 AM determined in the experimental set-up. The amplitude of the flame retardant-induced increase in basal $[Ca^{2+}]_i$ was determined to quantify the effects of the flame retardant on basal $[Ca^{2+}]_i$. The amplitude of the second K^+-evoked increase in $[Ca^{2+}]_i$ (after 20 min of exposure to DMSO or flame retardant) was expressed as a percentage of the amplitude of the first stimulation-evoked increase in $[Ca^{2+}]_i$ per cell to obtain a 'treatment ratio' (TR), as indicated in Figure 3A. As persistent changes in basal $[Ca^{2+}]_i$ can influence the amplitude of the stimulation-evoked increase in $[Ca^{2+}]_i$, the net stimulation-evoked increase in $[Ca^{2+}]_i$ was calculated by subtracting the amplitude of $[Ca^{2+}]_i$ just prior to stimulation from the amplitude of the stimulation-evoked increase in $[Ca^{2+}]_i$ and used to derive a 'net TR' as described previously (Hendriks *et al.*, 2012a).

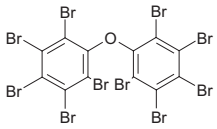
Brominated flame retardants

Brominated polystyrene *(UNFR-07)*
BPS
$(C_8H_5Br_3)_n$
88497-56-7
MPI Chemicals
>99%



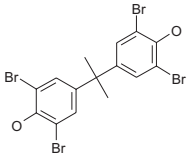
RPMI medium: 10
DMEM medium: 10
Saline ROS: 10
Saline Ca^{2+}: 10
Log K_{ow} n/a

Decabromodiphenyl ether
BDE-209
$C_{12}Br_{10}O$
1163-19-5
Sigma-Aldrich
98%



RPMI medium: 10
DMEM medium: 10
Saline ROS: 10
Saline Ca^{2+}: 10
Log K_{ow} 6.3

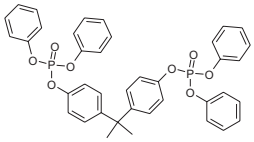
Tetrabromobisphenol-A
TBBPA
$C_{15}H_{12}Br_4O_2$
79-94-7
Sigma-Aldrich
>99%



RPMI medium: 100
DMEM medium: 100
Saline ROS: 100
Saline Ca^{2+}: 100
Log K_{ow} 4.5

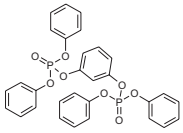
Phosphorous flame retardants

Bis phenol A bis (biphenyl phosphate) *(Fyrolflex BDP)*
BDP
$C_{39}H_{34}O_8P_2$
5945-33-5
ICL
>96%; 3.5% TPP



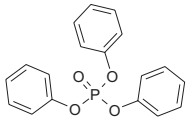
RPMI medium: 100
DMEM medium: 100
Saline ROS: 100
Saline Ca^{2+}: 100
Log K_{ow} 6

Resorcinol bis (biphenyl phosphate) *(Fyrolflex RDP)*
RDP
$C_{30}H_{24}O_8P_2$
57583-54-7
ICL
>95%; 4.5% TPP



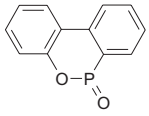
RPMI medium: 100
DMEM medium: 100
Saline ROS: 100
Saline Ca^{2+}: 100
Log K_{ow} 4.93

Triphenylphosphate
TPP
$C_{18}H_{15}O_4P$
115-86-6
Sigma-Aldrich
>99%



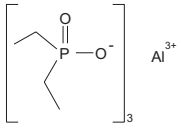
RPMI medium: 100
DMEM medium: 100
Saline ROS: 100
Saline Ca^{2+}: 100
Log K_{ow} 5

Dihydrooxaphosphosphaphenantrene oxide
DOPO
$C_{12}H_9O_2P$
35948-25-5
Krems Chemie
Chemical Services AG
>99%



RPMI medium: 100
DMEM medium: 100
Saline ROS: 100
Saline Ca^{2+}: 100
Log K_{ow} ~2 (modelled)

Aluminium diethylphosphinate *(Exolit OP 1230)*
Alpi
$[C_4H_{10}PO_2]_3Al$
225789-38-8
Clariant
>99%

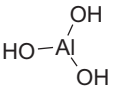


RPMI medium: 279
DMEM medium: 279
Saline ROS: 140
Saline Ca^{2+}: 279
Log K_{ow} -0.44

Figure S1. Continued

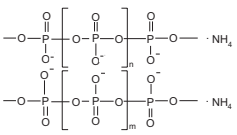
Inorganic halogen-free flame retardants

Aluminium trihydroxide
ATH
$Al(OH)_3$
21645-51-2
Merck
>99%



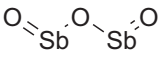
RPMI medium: 50
DMEM medium: 50
Saline ROS: 1
Saline Ca^{2+}: 1.9
Log K_{ow} n/a

Ammonium polyphosphate *(Exolit AP 422)*
APP
$(NH_4PO_3)_n$
68333-79-9
Clariant
>99%



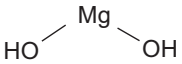
RPMI medium: 1300[a]
DMEM medium: 1300[a]
Saline ROS: 700[a]
Saline Ca^{2+}: 1300[a]
Log K_{ow} -2.15

Antimony trioxide *(Fireshield H)*
ATO
Sb_2O_3
1309-64-4
Chemtura
>99%



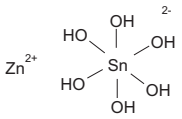
RPMI medium: 1.6
DMEM medium: 1.6
Saline ROS: 15
Saline Ca^{2+}: 29
Log K_{ow} n/a

Magnesium hydroxide
MHO
H_2MgO_2
1309-42-8
Sigma-Aldrich
>99%



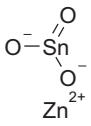
RPMI medium: 0.3
DMEM medium: 3100
Saline ROS: 412
Saline Ca^{2+}: 823
Log K_{ow} n/a

Zinc hydroxystannate *(Flamtard H)*
ZHS
$ZnSn(OH)_6$
12027-96-2
William Blythe
94%



RPMI medium: 0.5
DMEM medium: 0.5
Saline ROS: 0.4
Saline Ca^{2+}: 0.8
Log K_{ow} <-1

Zinc stannate *(Flamtard S)*
ZS
O_3SnZn
12036-37-2
William Blythe
>98%

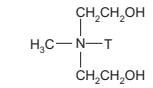


RPMI medium: 0.6
DMEM medium: 0.6
Saline ROS: 0.9
Saline Ca^{2+}: 1.8
Log K_{ow} <-1

Nanoclay

Montmorillonite *(Cloisite 30B)*
MMT
$C_{23}H_{50}O_2N$

NRC Nordmann
Rassmann
n/a

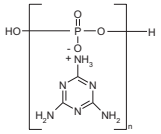


Where T is tallow (~65% C18)

RPMI medium: 0.4[b]
DMEM medium: 0.4[b]
Saline ROS: 0.2[b]
Saline Ca^{2+}: 0.4[b]
Log K_{ow} n/a

Nitrogen based organic flame retardant

Melamine polyphosphate *(Melapur 200)*
MPP
$C_3H_6N_6 \bullet (H_3PO_4)_n$
218768-84-4
BTC
>95%



RPMI medium: 70[a]
DMEM medium: 70[a]
Saline ROS: 35[a]
Saline Ca^{2+}: 70[a]
Log K_{ow} -2.3

Figure S1. Overview of the tested compounds. Full and product name (in italic between brackets), abbreviation, molecular formula, CAS-number, company and chemical purity of each compound are shown in the first column together with the respective chemical structures (second column). An overview of the achieved maximal solubility (in μM) of the flame retardants in DMSO, or RPMI cell culture medium, DMEM cell culture medium, external saline for ROS experiments and external saline for calcium imaging experiments and Log K_{OW} are shown in the third column. n/a, not applicable; a, based on the monomer; b, calculated assuming 0.1-1% silicon in the clay.

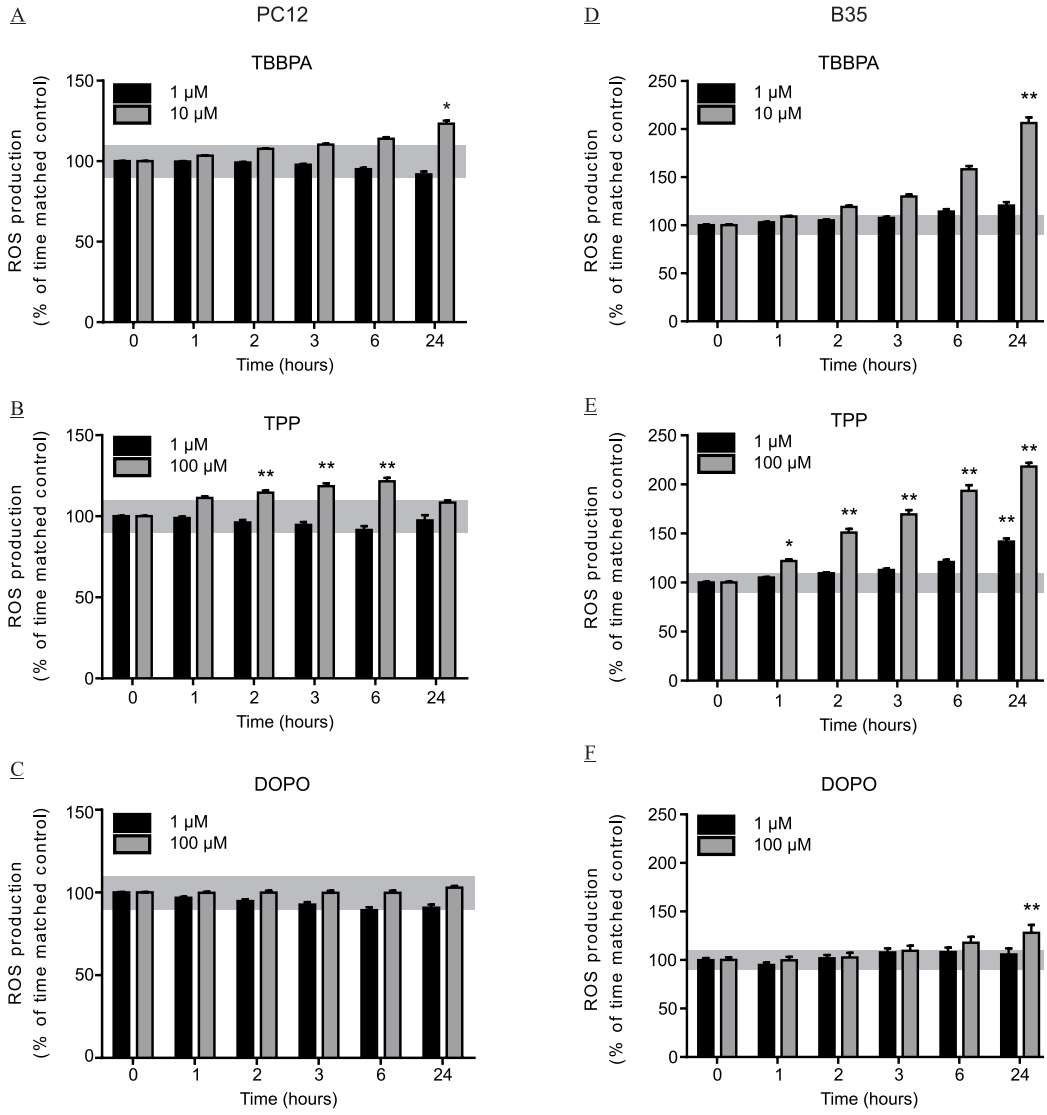


Figure S2. ROS production during 24h in PC12 and B35 cell induced by TBBPA, TPP and DOPO. Exposure to TBBPA and TPP increases ROS production in PC12 (A and B) and B35 cells (D and E) over time, while DOPO (C and F) was only able to affect ROS production in B35 cells. Graphs represent mean ROS production compared to time-matched controls (set at 100%) (n = 32-111 wells/concentration). * p <0.05 vs control; ** p <0.001 vs control.

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Chapter 8

Effects of neonatal exposure to the flame retardant tetrabromobisphenol-A, aluminium dietyhlphosphinate or zinc stannate on long-term potentiation and synaptic protein levels in mice

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Abstract

Brominated flame retardants like tetrabromobisphenol-A (TBBPA) may exert (developmental) neurotoxic effects. However, data on (neuro)toxicity of halogen-free flame retardants (HFFRs) is scarce. Recent *in vitro* studies indicated a high neurotoxic potential for some HFFRs, e.g., zinc stannate (ZS), whereas the neurotoxic potential of other HFFRs, like aluminium diethylphosphinate (Alpi), appears low. However, the *in vivo* (neuro)toxicity of these compounds is largely unknown. We therefore investigated effects of neonatal exposure to TBBPA, Alpi or ZS on synaptic plasticity in mouse hippocampus. Male C57bl/6 mice received a single oral dose of 211 μmol/kg bw TBBPA, Alpi, or ZS on postnatal day (PND) 10. On PND 17-19, effects on hippocampal synaptic plasticity were investigated using *ex vivo* extracellular field recordings. Additionally, we measured levels of postsynaptic proteins involved in long-term potentiation (LTP) as well as flame retardant concentrations in brain, muscle and liver tissue. All three flame retardants induced minor, but insignificant, effects on LTP. Additionally, TBBPA induced a minor decrease in post-tetanic potentiation. Despite these minor effects, expression of selected synaptic proteins involved in LTP was not affected. The flame retardants could not be measured in significant amounts in the brains, suggesting low bioavailability and/or rapid elimination/metabolism. We therefore conclude that a single neonatal exposure on PND 10 to TBBPA, Alpi or ZS does affect neurodevelopment and synaptic plasticity only to a small extent in mice. Additional data, in particular on persistence, bioaccumulation and (*in vivo*) toxicity following prolonged (developmental) exposure is required for further (human) risk assessment.

1. Introduction

Flame retardants are widely used to prevent or slow the onset and spread of fire, thereby reducing death, injuries and property damage. Tetrabromobisphenol-A (TBBPA) is a commonly used brominated flame retardant (BFR). As a consequence of its abundant use and environmental persistence, it can nowadays be found in house dust (Abb *et al.*, 2011) as well as in wildlife and human serum and adipose tissue samples (Covaci *et al.*, 2009; de Wit *et al.*, 2010; Johnson-Restrepo *et al.*, 2008). Earlier studies have shown that BFRs, such as polybrominated diphenyl ethers (PBDEs), are clearly associated with (developmental) neurotoxicity (Dingemans *et al.*, 2011). However, the potentially adverse effects of TBBPA on the (developing) nervous system are less extensively studied. Recently, it was shown that TBBPA exposure decreases the expression of $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptors in the frontal cortex of neonatally exposed mice (Viberg and Eriksson, 2011), whereas TBBPA also exerts acute modulatory effects on human $GABA_A$ and nACh receptors *in vitro* (Hendriks *et al.*, 2012a). $GABA_A$ and nACh receptors are widely distributed throughout the central nervous system and are critically involved in a variety of processes, including development and differentiation of the nervous system, learning, memory, and synaptic plasticity (D'Hulst *et al.*, 2009; Dwyer *et al.*, 2009). Fetal and neonatal exposure to BFRs such as TBBPA may thus have adverse effects on neurodevelopment, especially during the brain growth spurt (as has been previously shown for e.g. PBDEs; Eriksson *et al.*, 2001; Eriksson *et al.*, 2002; Viberg *et al.*, 2003a; Viberg *et al.*, 2003b; Viberg *et al.*, 2004), which in humans starts during the third trimester of pregnancy, peaks just before birth and continues throughout the first two years of life (Dobbing and Sands, 1979).

Considering the concerns associated with exposure to TBBPA and PBDEs (Dingemans *et al.*, 2011), there is a clear need to replace some BFRs by safer and less persistent alternative flame retardants, such as halogen-free flame retardants (HFFRs). However, the (neuro)toxic potential of such alternative flame retardants is largely unknown (for review see Waaijers *et al.*, 2013b). The acute environmental toxicity of aluminium diethylphosphinate (Alpi; a phosphorous flame retardant) and zinc stannate (ZS; an inorganic flame retardant) investigated in water fleas (*Daphnia magna)* indicated that both substances could be suitable candidates for BFR replacement (Waaijers *et al.*, 2013a). Recently, we investigated the *in vitro* neurotoxic potential of a number of suggested HFFRs on several neurotoxic endpoints *in vitro*, including cytotoxicity, production of reactive oxygen species (ROS), calcium homeostasis and nACh receptor function (Hendriks *et al.*, 2014; Hendriks *et al.*, 2012b), with the purpose of prioritizing HFFRs for substitution of BFRs. In these studies, Alpi was classified as having negligible neurotoxic potency *in vitro* and thus as a potentially suitable replacement of some BFRs. In the same studies, ZS was classified as having a high neurotoxic potency *in vitro* and thus likely unsuitable as replacement of BFRs (Hendriks *et al.*, 2014). However, as *in vitro* models generally lack metabolism and some of the more sophisticated interactions that are present in the intact brain, extrapolation of experimental *in vitro* data to the *in vivo* toxicity of HFFRs is difficult. Such extrapolation is further hampered by the general absence of *in vivo* toxicity data of HFFRs, including data on toxicokinetics, such as the possibility to pass the blood-brain barrier.

To bridge the gap between our experimental *in vitro* data and the *in vivo* situation, we performed *ex vivo* validation experiments to determine the neurophysiological effects of a single neonatal exposure to TBBPA, Alpi, or ZS. Long-term potentiation (LTP) is an extensively studied form of synaptic plasticity (Blundon and Zakharenko, 2008) and was previously shown to be affected by neonatal exposure to BDE-47 (Dingemans *et al.*, 2007) and BDE-209 (Xing *et al.*, 2009). Using comparable dosing protocols as used in these studies (mice exposed on PND 10, LTP measurements on PND 17-19), the neurodevelopmental hazard and risk of the (alternative) flame retardants can

be compared with existing data on PBDEs. Since induction and maintenance of LTP requires activation of specific neurotransmitter receptors and protein kinases, including calcium/calmodulin-dependent protein kinase-II (CaMK-II; Lisman *et al.*, 2012), levels of several critical synaptic proteins in brain tissue were also measured. Furthermore, we investigated paired-pulse facilitation (PPF), a form of short lasting plasticity that reflects presynaptic function (Schulz *et al.*, 1994), to reveal possible presynaptic effects. Finally, levels of TBBPA, Alpi and ZS were measured in brain, muscle and liver tissue of the exposed mice to relate possible effects to internal doses and gain insight in the bioavailability and distribution of these compounds.

2. Material and Methods

2.1 Chemicals

The flame retardants were purchased from different companies at the highest purity available: TBBPA (>99%, Sigma-Aldrich, Zwijndrecht, The Netherlands), Alpi (>99%, Clariant, Knapsack, Germany) and ZS (>98%, William Blythe, Acrington, England, United Kingdom). The vehicle components egg lecithin (L-α-phosphatidylcholine) and peanut oil (*oleum arachidis*) and all other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless otherwise indicated.

2.2 Animals

Male C57Bl/6 mice pups (litter culled to 5 pups each) with dam (Harlan, Venray, The Netherlands) were housed in a standard animal facility on a 12 h light/dark cycle with food and water ad libitum. All experiments were conducted in accordance with Dutch law and approved by the Ethical Committee for Animal Experimentation of Utrecht University.

Male C57Bl/6 pups received a single oral dose of vehicle (control), TBBPA (115 mg (211 μmol)/kg bw), Alpi (82.4 mg (211 μmol)/kg bw), or ZS (48.9 mg (211 μmol)/kg bw ZS) (all 10 ml/kg bw, i.e. 10 μl/g bw) via a metal feeding needle on PND 10, i.e. during the peak of the brain growth spurt. This dose is 10-fold higher than those used in earlier studies on TBBPA (Eriksson *et al.*, 2001; Viberg and Eriksson, 2011) that exerted only minor effects. To allow for comparison of TBBPA with both halogen-free flame retardants, equimolair concentrations were used for Alpi and ZS. The vehicle consisted of a 1:10 (wt/wt) mixture of egg lecithin and peanut oil, which was sonicated with water to obtain a 20% (wt/wt) fat:water emulsion. Experimental groups consisted of mice from at least four different litters. To exclude confounding effects induced by e.g. time and season, exposures were randomized (different experimental conditions within a litter) and effects were measured on different weekdays.

2.3 Hippocampal slice preparation and tissue collection

On PND 17-19 (just after the peak of the brain growth spurt), exposed pups were sacrificed by decapitation under inhalation anesthesia (isoflurane). The brain was rapidly dissected on ice and the two hemispheres were separated. One hemisphere was used to prepare hippocampal slices as described previously (Dingemans *et al.*, 2007; Notenboom *et al.*, 2010). Briefly, 250 μm coronal slices were cut in ice-cold carbogenated (95% O_2 and 5% CO_2) artificial cerebrospinal fluid (ACSF, containing in mM: 124 NaCl, 3.3 KCl, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 20 $NaHCO_3$, 10 glucose) using a Leica VT1200s vibratome (Leica Microsystems, Wetzlar, Germany). The slices were allowed to stabilize at room temperature in carbogenated ACSF for at least 1 h.

Non-cortex tissue (hippocampus and midbrain) of unused hemispheres, cortex tissues, hind limb muscles and livers were collected and stored at -80°C until further chemical and protein analysis (per treatment, tissue samples of 10-15 mice from at least four different litters were collected).

2.4 Extracellular recording of field potentials

Hippocampal slices were individually transferred to a recording chamber and superfused with carbogenated ACFS (~32°C, ~2 ml/min). Synaptic transmission and activity-dependent plasticity in the hippocampal CA1 region was investigated by measuring field-excitatory postsynaptic potentials (fEPSPs) as described previously (Dingemans *et al.*, 2007; Notenboom *et al.*, 2010), with minor modifications. Synaptic responses were evoked by local extracellular stimulation of the Schaffer collateral-commissural pathway using bipolar borosilicate electrodes (see Figure 1A). Data was recorded with an EPC10 amplifier (Heka, Lambrecht, Germany), digitized at 20 kHz and analyzed off-line using Igor Pro 5.05 A (Wavemetrics Inc., USA).

Input-output (IO) curves were made to determine possible treatment-induced changes in basal excitability and thresholds for synaptic transmission. Stimulation intensities for threshold and maximum fEPSP were determined for every slice using four single stimuli with increasing intensity. After obtaining the IO curve, the stimulus intensity that evoked half-maximal fEPSPs was determined and used during the remainder of the experiment. To determine the degree of paired-pulse facilitation (PPF, a form of short-term plasticity that reflects presynaptic function (Schulz *et al.*, 1994)), paired pulses were delivered at inter-stimulus intervals (ISI) of 50, 100 and 200 ms, and the ratio of the second and first response amplitude was calculated for each interval (paired-pulse ratio, PPR). Slices with a maximum fEPSP amplitude of ≥1 mV and with stable baseline fEPSP (half-maximal fEPSP was evoked every 30 s) for 30 min were included. LTP was induced with a single tetanic stimulation (100 Hz, 1 s) and post-tetanic fEPSPs were recorded for 60 min.

For data analysis, amplitudes of the fEPSPs (measured every 30 s) were determined (see Figure 1B). Each recording was normalized to the average amplitude of the last 10 baseline fEPSPs. Potential flame retardant-induced effects on synaptic plasticity were assessed by measuring the average fEPSP amplitude per animal (n = 6-9) in different time windows: 0-7.5 min after tetanic stimulation (post-tetanic potentiation; PTP) and 20-30 min after tetanic stimulation (long-term potentiation; LTP, comparable to Dingemans *et al.*, 2007) as well as 50-60 min after tetanic stimulation (sustained LTP). Per treatment group six to nine animals from at least four different litters were used.

2.5 Slot-blot analysis for CaMK-II, GAP-43, GluR1, PSD 95, and synaptophysin protein expression

To minimize the number of required experimental animals, we chose to analyze protein expression in cortex of the animals used for hippocampal LTP studies. Cortices were homogenized in a RIPA cell lysis buffer (containing in mM: 50 tris HCl (pH 7.4), 150 NaCl, 1 EDTA, 1 EGTA, 20 sodium pyrophosphate, 2 sodium orthovanadate, 1% Triton X-100 and 1% sodium deoxycholate; AssayDesign) with the addition of 0.5% Protease Inhibitor Cocktail Set III (Calbiochem). The homogenate was then centrifuged at 14,000 × g for 15 min at 4°C, and the protein content of the supernatant was measured using the BCA method (Pierce). Subsequently, the supernatant was stored at -80°C until use.

The slot-blot analysis was performed as described previously (Viberg *et al.*, 2008a; Viberg *et al.*, 2008b). Briefly, for CaMK-II and growth associated protein-43 (GAP-43) 4 μg of protein was optimal, for GluR1 and synaptophysin 3 μg, and for postsynaptic density 95 (PSD 95) 5 μg protein. The protein amount was diluted to a final volume of 200 μl with sample buffer (in mM: 120 KCl, 20 NaCl, 2 $NaHCO_3$, 2 $MgCl_2$, 5 HEPES (pH 7.4), 0.05% Tween 20, 0.2% NaN_3) and applied in duplicate to a nitrocellulose membrane (0.45 μm, Bio-Rad, Sundbyberg, Sweden) using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membranes were fixed in 25% isopropanol and 10% acetic acid solution, washed, and blocked for 1 h at room temperature in 5% non-fat dry milk containing 0.03% Tween-20.

The membranes were then incubated overnight at 4°C with either mouse monoclonal CaMK-II antibody (1:5,000; MAB8699, Chemicon), rabbit polyclonal GAP-43 antibody (1:10,000; AB5220, Chemicon), rabbit polyclonal GluR1 antibody (1:1,000; AB1504, Millipore), mouse monoclonal PSD 95 antibody (1:10,000; MABN68, Millipore), or mouse monoclonal synaptophysin antibody (1:10,000; 573822, Calbiochem). Immunoreactivity was detected using a horseradish peroxidase-conjugated secondary antibody against mouse (1:20,000; 074-1806, KPL, VWR, Stockholm, Sweden) or rabbit (1:20,000; 074-1506, KPL, VWR). Immunoreactive bands were detected using an enhanced chemiluminescent substrate (Pierce, Super Signal West Dura, Bio-Rad, Sundbyberg, Sweden) with imaging on a LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of bands was quantified using IR-LAS 1000 Pro (Fuji Film). For each exposure condition six animals were used originating from at least four different litters.

2.6 Internal dose analysis

For determination of the internal dose of TBBPA-exposed mice, small amounts of liver, muscle and non-cortex brain tissue (about 30-200 mg) were freeze dried (n = 4-5 samples per tissue from at least four different litters). The freeze dried samples were transferred to glass tubes, and 1 ml of hexane:acetone (3:1, v/v) and 100 µl internal standard (^{13}C labeled TBBPA, Cambridge Isotope Laboratories, Apeldoorn, The Netherlands) were added. The samples were agitated (Vortex) for 30 s followed by an ultrasonic extraction (5 min). The extracts were collected and the extraction step was repeated two times with hexane:acetone, agitation and ultrasonic extraction. The recombined extracts were evaporated to 1 ml with a gentle stream of nitrogen. These extracts were cleaned using a Pasteur pipette filled with 1 g 40% sulphuric acid silica (Silica gel 60, Merck, Darmstadt, Germany). TBBPA was eluted from the extracts with 5 ml dichloromethane (Picograde, Promochem GmbH, Wesel, Germany), and was subsequently evaporated to 0.5 ml, followed by adding one ml iso-octane and evaporation to 0.5 ml. The final extracts were measured with gas chromatography (GC6890N, Agilent, Joint Analytical Systems Benelux B.V., Eindhoven, The Netherlands) combined with mass spectrometry in the electron capture negative ionization mode (5975 MSD, Agilent). A DB-5 column (15 m × I.D 0.25 mm × film 0.25 µm, J&W Scientific, Agilent), splitless injection, and helium as carrier gas were used. The m/z 79, 81 ions and specific ions were used for the detection and quantification of TBBPA and ^{13}C-TBBPA.

Liver, muscle and non-cortex brain samples (n = 4-5 samples per tissue per compound from at least three different litters) of Alpi- or ZS-exposed mice were freeze dried and homogenized. A maximum of 100 mg of dried material was added to microwave digestion vessels, and 5 ml of ultrapure water (milliQ, Millipore) and 5 ml 65% nitric acid (Ultrex II, Baker, VWR, Amsterdam, The Netherlands) were added. After microwave digestion (MDS 2000, MW1, Beun-de Ronde, Abcoude, The Netherlands), 10 ml ultrapure water was added to the vessels. The digests were diluted and analyzed by High Resolution Inductively Coupled Plasma Mass Spectrometry (HRICP-MS; (Giessmann and Greb, 1994; Krystek and Ritsema, 2009). The elements of Zn and Al were measured in the medium resolution mode. For the quantification, external calibrations with internal standard correction were applied and the internal standard (rhodium) was added on-line.

2.7 Statistical analyses

LTP was defined as >15% increase above baseline, with all changes in amplitude >15% being significant (p <0.05; t-test). Normalized data were averaged across each selected time window (PTP, LTP and sustained LTP) for each individual animal in each group. The mean of the average fEPSP (per window) of the animals per group was then used for statistical analysis. Internal dose analysis of exposed animals was background corrected

using values from control animals. All data are presented as mean ± standard error of the mean (SEM) unless otherwise noted. The four experimental groups were statistically compared by one-way ANOVA with post hoc Newman-Keuls test (GraphPad Prism 6.01). Unpaired Student's *t*-tests were used for all other data. A p value of <0.05 indicates statistical significance.

3. Results

3.1 General toxicity

Pups exposed to TBBPA, Alpi or ZS did not differ in body weight compared to the pups exposed to the vehicle control (data not shown), indicating the absence of general toxicity or treatment-dependent food competition.

3.2 Basal excitability and synaptic transmission

None of the flame retardants affected the IO relationship (data not shown), the magnitude of the baseline fEPSP amplitude at half-maximal (control: -0.52 ± 0.03 mV; TBBPA: -0.56 ± 0.03 mV; Alpi: -0.51 ± 0.05 mV; ZS: -0.48 ± 0.05 mV; see Figure 1C) or maximal stimulation (≥1 mV; data not shown). To assess possible flame retardant-induced presynaptic effects, paired-pulse facilitation (PPF) was examined and expressed as a paired-pulse ratio (PPR) showing an increased response to the second of two identical stimuli separated by an inter-stimulus interval (ISI) of 50-200 ms. In controls, PPR decreased from 1.67 ± 0.04 (ISI 50 ms) to 1.45 ± 0.02 (ISI 200 ms), and none of the treatments affected PPR compared to control animals (Figure 1D).

3.3 Long-Term Potentiation

LTP was induced by tetanic stimulation (100 Hz, 1 s), which resulted in an immediate increase of the fEPSP amplitude (see Figure 2A). During PTP (i.e. first 7.5 min post-tetanus), the potentiation of the fEPSP amplitude in slices from TBBPA exposed animals was slightly but not significant lower compared to control animals (control: 140.9 ± 5.3%; TBBPA: 128.7 ± 3.0%), while effects comparable to control were observed for Alpi and ZS (Alpi: 143.3 ± 4.0%; ZS: 136.7 ± 5.7%). After PTP, the fEPSP amplitude decreased but remained well above the baseline level for at least 60 min in all experimental groups (see also Figure 2A and 2B). During LTP (i.e. 20-30 min post-tetanus), the potentiation of the fEPSP amplitude for TBBPA was again slightly but not significant lower compared to control animals (control: 130.7 ± 4.9%; TBBPA: 117.3 ± 4.5%), while the potentiation of the fEPSP amplitude was slightly but not significantly increased in the Alpi group (138.1 ± 8.8%). The degree of potentiation in the ZS group (132.7 ± 6.1%) was comparable to control. During sustained LTP (50-60 min post-tetanus), the potentiation of the fEPSP amplitude in the TBBPA group was slightly, but not significant, increased compared to control (control: 129.7 ± 10.6%; TBBPA: 136.9 ± 15.1%;), whereas the potentiation of the fEPSP amplitude was comparable to control in both the Alpi and ZS group (Alpi: 125.2 ± 10.5%; ZS: 135.8 ± 9.6%).

3.4 Cortex synaptic protein expression

To investigate if neonatal exposure affected the expression of specific neurotransmitter receptors and protein kinases essential for induction and maintenance of LTP, protein levels of CaMK-II, GAP-43, GluR1, PSD 95, and synaptophysin were measured in the cortex of control, TBBPA-exposed, Alpi-exposed and ZS-exposed mice. No significant effect of TBBPA, Alpi or ZS exposure on the different protein levels were observed ($n = 6$ animals/group, Figure 2C).

3.5 Brain, muscle and liver concentrations

In order to determine internal doses in flame retardant-exposed animals, non-cortex brain, muscle and liver tissues were collected, analyzed and corrected for background (values obtained from control animals). In TBBPA-exposed mice, high concentrations TBBPA were observed in the liver (823 ± 460 ng/g ww; p <0.01 compared to control (not detected)). Muscle (6 ± 2 ng/g ww, not significant) and brain (3 ± 1 ng/g ww, not significant, see Figure 2D) contained only low levels of TBBPA. In the tissues of both Alpi- or ZS-exposed mice, concentrations Al as proxy for Alpi and Zn as proxy for ZS were comparable to the control group (below the limit of detection).

4. Discussion

Previous studies demonstrated numerous neurotoxic effects *in vitro* following acute exposure to BFRs and HFFRs, including changes in cell viability, production of ROS, calcium homeostasis and nACh receptor function (Hendriks *et al*., 2014; Hendriks *et al*, 2012a; Hendriks *et al*., 2012b). However, the present study demonstrates that neonatal exposure of mice on PND 10 to the flame retardants TBBPA, Alpi or ZS induces only minor, insignificant, changes in LTP compared to control animals, whereas basal excitability and

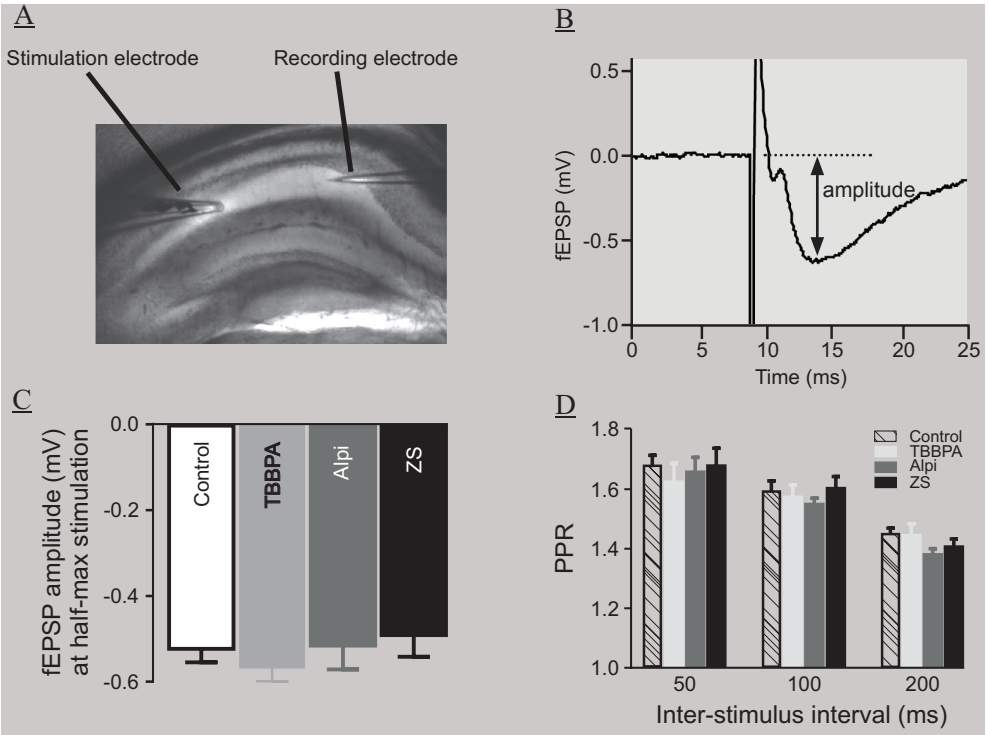

Figure 1. Example of a coronal hippocampal slice with stimulation and recording electrodes placed along the Schaffer collateral-commissural fiber trajectory in CA1 of mouse hippocampus for measurement of fEPSPs (A). For data analysis, the amplitude was determined as shown in the representative fEPSP recording (B). Baseline fEPSP amplitude at half-maximal stimulation as a measure of synaptic transmission did not show any effect of neonatal exposure to TBBPA, Alpi or ZS compared to control (C). Short-term plasticity, expressed as paired pulse ratio (PPR) at different inter-stimulus intervals, following exposure to control, TBBPA, Alpi or ZS did not show any effects of neonatal exposure on PND 10, indicating that the tested flame retardants do not affect short-term plasticity in the CA1 region of the hippocampus (D). Bar graphs represent mean values ± SEM, n = 6-9.

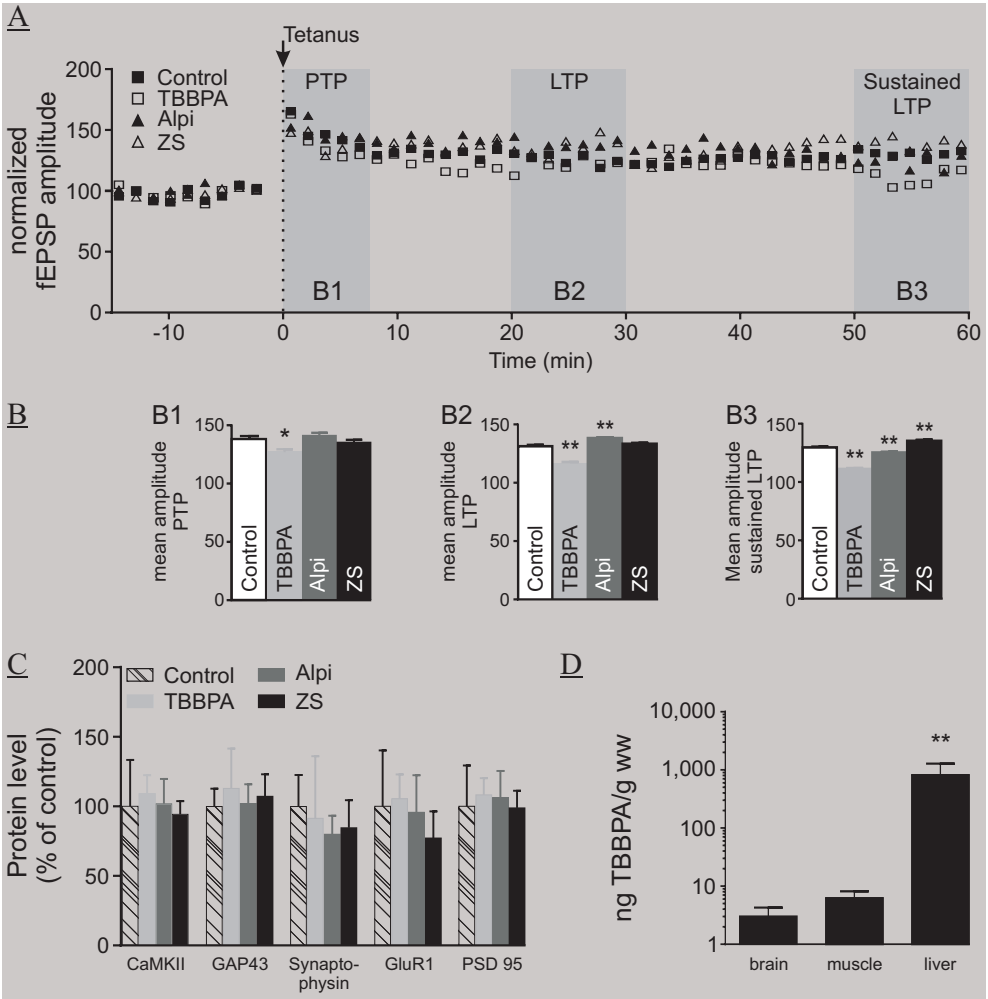

Figure 2. Normalized fEPSP amplitude data (± SEM) representing baseline, tetanic stimulation (arrow), PTP, LTP, and sustained LTP in hippocampal slices of control and neonatal exposed mice (A) (note: for better graphical representation only every third point in the timeline is shown). Bar graphs show mean fEPSP amplitudes ± SEM (n = 6-9) of control and exposed mice in the selected windows (B; PTP in B1, LTP in B2, and sustained LTP in B3). No differences in levels of postsynaptic proteins essential for LTP induction and maintenance were observed in cortices of control and neonatally exposed animals. Bar graphs represent mean values ± SD; n = 6 (C). Significant levels of TBBPA were measured in livers of TBBPA exposed mice. Bars represent mean concentration (ng/g ww) ± SEM; n = 4-5 animals (D). ** p <0.001 vs control.

paired-pulse facilitation are not affected (Figure 1 and 2). To confirm the validity of this conclusion, we performed additional measurements of protein expression. Though we cannot exclude transient changes in protein expression shortly after dosing and/or induction of LTP, our measurements demonstrate that basal expression levels of several synaptic proteins essential for neurotransmission and (long-term) synaptic plasticity are also unaltered (Figure 2). Similarly, in the absence of extensive studies on kinetics we cannot exclude that tissue concentrations have been higher at earlier time points after dosing, but we could not detect significant amounts of flame retardants in (non-cortex) brain tissue of the exposed mice. We therefore conclude that the minor flame retardant-induced effects on LTP following a single oral exposure on PND 10 may not be sufficient to affect normal brain development, function, or plasticity to an adverse degree.

Using a comparable dosing paradigm, i.e. a single oral exposure on PND 10, it was previously shown that BDE-47 induced clear and permanent alterations in spontaneous behavior and learning and memory functions (Eriksson *et al.*, 2001), expression of synaptic proteins (CaMK-II and GluR1) and synaptic plasticity (PTP and LTP; Dingemans *et al.*, 2007). Using this dosing paradigm (albeit at a 10-fold lower dose (21 μmol/kg bw) than in the present study (211 μmol/kg bw)), it was shown that TBBPA reduced the expression of $\alpha_4\beta_2$ nACh receptors in mice frontal cortex 24 hours after exposure (Viberg and Eriksson, 2011). However, in line with the present study, TBBPA did not affect expression levels of CaMK-II, GAP-43 and synaptophysin in mice frontal cortex (Viberg and Eriksson, 2011). Moreover, using this dosing protocol, it has also been shown that TBBPA (21 μmol/kg bw) did not induce permanent aberrations in spontaneous behavior or learning and memory functions (Eriksson *et al.*, 2001), indicating the absence of neurodevelopmental effects. The uncertainty whether or not TBBPA induces developmental neurotoxicity is further underlined by gestational/lactational exposure studies which indicate that developmental exposure of rats resulted in alterations of neuronal migration in the dentate gyrus (Saegusa *et al.*, 2012), while several critical neurochemical parameters were not affected (Saegusa *et al.*, 2009). Additionally, an one-generation reproduction study in rats showed that fear memory in adult rats is not affected, although effects on auditory neuron function were observed (Lilienthal *et al.*, 2008) and a comparable reproduction study indicated that thyroid hormones, which are also involved in neuronal development, are affected by TBBPA (Van der Ven *et al.*, 2008). Although several endocrine effects were observed in rats following chronic exposure (Van der Ven *et al.*, 2008), it remains debatable whether the observed disturbances of (thyroid) hormones affect the developing brain to such a degree that (developmental) neurotoxicity occurs. Nonetheless, acute exposure (three hours before the behavioral tests) of adult mice to TBBPA (≥0.1 mg/kg bw) has been reported to induce behavioral effects (e.g. increase in horizontal movement activities and more freezing behavior (Nakajima *et al.*, 2009)) that were accompanied by a rapid increase in brain TBBPA levels.

Upon oral exposure, TBBPA may thus reach the brain (Nakajima *et al.*, 2009; Viberg and Eriksson, 2011) to exert (transient) effects on neuronal development and function in rodents. However, TBBPA has a short half-live (<3 days in rats (Brady, 1979); 6.6 days in humans (Geyer *et al.*, 2004)), low systemic bioavailability (Knudsen *et al.*, 2014) and elimination from the brain appears to be efficient (Figure 2C; Viberg and Eriksson, 2011). Consequently, the likelihood for TBBPA-induced neurodevelopmental effects appears limited, unless exposure is both chronic and to high doses. Nevertheless, in mice on PND 10, the blood-brain-barrier is not yet fully developed, which may result in higher TBBPA levels in the developing brain compared to a mature brain.

Though TBBPA is found in human breast milk (up to several ng/g lw; Abdallah and Harrad, 2011; Shi *et al.*, 2009) and cord serum (up to 100 ng/g lw; Cariou *et al.*, 2008), and the estimated daily exposure of toddlers via dust amounts to 0.2 ng/kg bw/d (Abb *et al.*, 2011), the current levels of human TBBPA exposure are relatively low and, taking into

account the rapid excretion, may be of limited concern (see also Colnot *et al.*, 2014). In line with this notion, epidemiological studies have so far failed to consistently find an association between human TBBPA levels and adverse neurological/behavioral effects (Kicinski *et al.*, 2012; Williams and DeSesso, 2010). However, it should be noted that the absence of an association between human levels of a toxicant and human adverse effects does not imply that a compound is non-toxic and safe to use.

In contrast to TBBPA, *in vivo* data regarding the toxicity of Alpi and ZS is minimal (for review see Waaijers *et al.*, 2013b). The present study is thus the first to demonstrate limited, but insignificant effects of these HFFRs on synaptic (long-term and paired-pulse) plasticity. During LTP, the potentiation of the fEPSP amplitude was only slightly increased in the Alpi mice, while the fEPSP amplitude of ZS mice was close to controls. During sustained LTP, the fEPSP amplitude in both the Alpi and ZS group was comparable to controls. In addition to these minor and insignificant effects, no effects on expression of synaptic proteins were observed. Despite the use of relatively high doses (211 μmol/kg bw), ZS and Alpi were undetectable in liver, muscle and brain tissue at PND 17-19. The hydrophilicity of both Alpi and ZS (with Log K_{ow} values of -0.4 and <-1, respectively (Waaijers *et al.*, 2013b), compared to 4.5 for TBBPA (Birnbaum and Staskal, 2004)) may hamper absorption in the gut as well as passage across the blood-brain barrier. Alpi and ZS thus theoretically have a very low bioavailability, which is in line with the few available *in vivo* studies that report low toxicity (according to REACH regulations of the European Union) based on lethal doses (LD) for feeding, dermal or inhalation exposure (oral LD_{50} >2 g/kg for Alpi (rat) and an oral LD_{50} of >5 g/kg for ZS (rat) (U.S. Environmental Protection Agency (EPA), 2008; William Blythe, 2010). Our findings are also consistent with a toxicokinetic study of Alpi (ECHA Database, 2014) reporting that only a small amount of the Alpi dose was absorbed by the gastro-intestinal tract, and that the major part was excreted via feces. However, additional data, in particular regarding human exposure levels, are required to determine the human risk associated with the use of these HFFRs. Of importance in this respect, *in vitro* studies indicate a high neurotoxic potential of ZS (Hendriks *et al.*, 2014; Hendriks *et al.*, 2012b). It is not unlikely that conditions that in some way increase the bioavailability of ZS (e.g. pH) and/or affect the barriers of the gut and/or brain (e.g. disease), may increase the risk associated with ZS. The low observed toxicity in both *in vitro* and *in vivo* experiments suggests that Alpi may be a suitable alternative flame retardant, though additional data is needed to obtain a full toxicological profile for proper risk assessment.

Despite their shortcomings (absence of toxicokinetics, bioavailability, barrier functions and complex cell-cell interactions), *in vitro* studies are efficient and useful for prioritization, revealing mechanisms of action, and establishing effect concentrations. Although previous *in vitro* studies revealed HFFR-induced neurotoxicity, the present *in vivo/ex vivo* study indicates the absence of (developmental) neurotoxicity following one single oral exposure. The experimental setup with a single oral exposure on PND 10 (during the peak of the brain growth spurt) is an efficient and sensitive way to demonstrate the developmental neurotoxic effects of e.g. flame retardants. This has been demonstrated previously in *in vivo* and *ex vivo* studies in which mice exposed on PND 10 to BFRs developed permanent aberrations in spontaneous behavior and habituation capability, protein expression and LTP (Dingemans *et al.*, 2007; Eriksson *et al.*, 2001; Eriksson *et al.*, 2002; Gee and Moser, 2008; Viberg *et al.*, 2003a; Viberg *et al.*, 2003b; Viberg *et al.*, 2004). Similarly, effect assessment at PND 17-19 (just after the peak of the brain growth spurt) following the exposure on PND 10 has previously been shown to be efficient and sensitive (Dingemans *et al.*, 2007; Viberg and Eriksson, 2011). Accordingly, using the same dosing paradigm, possible (developmental) neurotoxic effects of TBBPA, Alpi and ZS can be compared with existing (developmental) neurotoxicity data of BFRs. While we

did not observe developmental neurotoxicity following a single oral TBBPA exposure, chronic exposure (which appears more realistic for the human situation) may affect e.g. auditory neuron function as a consequence of disturbance of thyroid hormones (Lilienthal *et al.*, 2008; Van der Ven *et al.*, 2008). Nonetheless, the present single dose study suggests a low concern for the developing brain following exposure to TBBPA, Alpi or ZS.

In conclusion, despite the *in vitro* neurotoxic potential of TBBPA and ZS, a single oral exposure to a high dose of TBBPA, Alpi or ZS on PND 10 induced only limited and insignificant effects on synaptic function and plasticity in mice. This is corroborated by the absence of effects on expression levels of synaptic proteins. The overall absence of adverse effects appears due to poor bioavailability of Alpi and ZS and/or rapid elimination of all three flame retardants. However, further risk assessment of HFFRs should also include the physicochemical properties (e.g. molecular weight, Log K_{ow}, and water solubility) of the compound, production volumes, presence in the environment, persistence, bioaccumulation, ecotoxicity, and chronic and/or developmental toxicity *in vitro* as well as *in vivo*. Moreover, humans are generally not acutely exposed to a single environmental pollutant, but chronically to (low dose) mixtures of potentially toxic substances. Since interactions with other compounds may result in synergism, additivity or antagonism, mixture studies should also be included for human risk assessments.

Acknowledgements

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Chapter 9

General discussion

9

1. The past and the present - what have we learned from the PCBs and BFRs?

1.1 Neurotoxicity of PCBs and BFRs

The 'old' flame retardants PCB-47, BDE-47 and 6-OH-BDE-47

Though the use of flame retardants reduces death and injuries, these chemicals pose serious disadvantages for human health and the environment. Studies identified the nervous system as being among the most vulnerable targets for the toxic actions of brominated flame retardants (BFRs) and polychlorinated biphenyls (PCBs) (for reviews see Costa and Giordano, 2007; Dingemans *et al.*, 2011; Fonnum and Mariussen, 2009). It has for example been shown that non-dioxin-like-PCBs (NDL-PCBs) affect the postsynaptic $GABA_A$ receptor, with 2,2',4,4'-tetrachlorobiphenyl (PCB-47) being the most potent NDL-PCB (Antunes Fernandes *et al.*, 2010a). As polybrominated diphenyl ethers (PBDEs) share structural similarities and (neuro)toxic effects with *ortho*-substituted PCBs (Figure 1) (Costa and Giordano, 2007; Fonnum and Mariussen, 2009), postsynaptic effects of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) on $GABA_A$ and $\alpha_4\beta_2$ nACh receptor functioning were compared to the effects of PCB-47. As shown in Chapter 4, PCB-47 acts as full and partial agonist on the $GABA_A$ receptor, and as antagonist on the $\alpha_4\beta_2$ nACh receptor, while BDE-47 does not affect either type of receptor. This is, despite the structural similarities, probably due to a difference in the planarity of the molecules and/or due to the differences in size between chlorine-substituted PCB-47 and bromine-substituted BDE-47. The observed effects of PCB-47 may (partly) underlie neurobehavioral and neurodevelopmental effects: both the $GABA_A$ and $\alpha_4\beta_2$ nACh receptors play an important role in long-term potentiation (LTP) and other forms of synaptic plasticity. Importantly, during early brain development, the $GABA_A$ receptor acts as an excitatory receptor (D'Hulst *et al.*, 2009).

Several *in vitro* studies indicated the neurotoxic potential of BDE-47, which includes apoptosis induced by oxidative stress and consequently damage of DNA, proteins and membrane lipids (He *et al.*, 2008; He *et al.*, 2009; Huang *et al.*, 2010; Jiang *et al.*, 2012; Zhang *et al.*, 2013), accumulation of BDE-47 in mitochondria (Huang *et al.*, 2010; Kodavanti *et al.*, 2005; Mundy *et al.*, 2004), increase in vesicular neurotransmitter release and simultaneous increase in the basal intracellular calcium ($[Ca^{2+}]_i$), and inhibition of the depolarization-evoked increase in $[Ca^{2+}]_i$ (Dingemans *et al.*, 2007; Dingemans *et al.*, 2008; Dingemans *et al.*, 2010; Gassmann *et al.*, 2014). Notably, several studies indicate that oxidative metabolism can increase the neurotoxic potential of PBDEs (Dingemans *et al.*, 2011). For example, PC12 and human progenitor cells exposed to hydroxylated PBDE metabolites, such as 6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (6-OH-BDE-47), show alterations in Ca^{2+} homeostasis and neurotransmitter release at much lower concentration than the parent compound (Dingemans *et al.*, 2008; Dingemans *et al.*, 2010; Gassmann *et al.*, 2014). The increased neurotoxic potential of OH-PBDEs is also evident for neurotransmitter receptors; 6-OH-BDE-47 was able to act as full and partial agonist on the $GABA_A$ receptor and as antagonist on the $\alpha_4\beta_2$ nACh receptor, whereas BDE-47 is not effective. From these

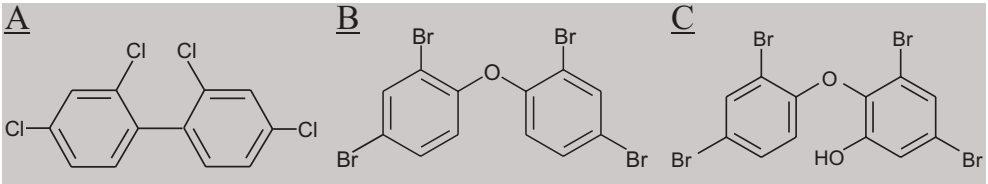


Figure 1. Schematic representation of the chemical structures of PCB-47 (A), BDE-47 (B) and 6-OH-BDE-47 (C).

studies it is clear that bioactivation should be included in (human) risk assessment of (alternative) flame retardants. The behavioral effects and accompanying neurochemical changes following exposure to BDE-47 has been extensively studied in rodents. Postnatal exposure to BDE-47 caused permanent aberrations in spontaneous behavior and relearning abilities (Eriksson *et al.*, 2001), changes in development of neuromotor systems (Gee and Moser, 2008), and impairment of LTP (Dingemans *et al.*, 2007). Perinatal exposure of mice to BDE-47 caused growth retardation, altered locomotor activity and relearning abilities (Koenig *et al.*, 2012; Ta *et al.*, 2011). Moreover, accumulation of BDE-47 was observed in several organs, including the brain, in both dams and their offspring (Ta *et al.*, 2011). The observed higher potency of 6-OH-BDE-47 *in vitro* emphasizes the increase in neurotoxic potential of metabolites that may (partly) underlie the observed *in vivo* effects.

Humans and wildlife are rarely exposed to only one single environmental pollutant, but exposure usually occurs to mixtures of (potential) toxic substances as PCBs, BFRs, and their (toxic) metabolites. Evaluation of the potential toxic effect of such combined exposures is therefore important. Additive and synergistic interactions between organohalogen compounds have been shown in some *in vitro* studies (Antunes Fernandes *et al.*, 2010b; Gao *et al.*, 2009; Pellacani *et al.*, 2012a; Tagliaferri *et al.*, 2010). In this PhD thesis it is shown that a mixture of PCB-47 and 6-OH-BDE-47 exerts additive agonistic and antagonistic effects on the $GABA_A$ and $\alpha_4\beta_2$ nACh receptor, respectively. These results emphasize the notion that (neuro)toxicity is affected by possible mixture interactions, which may be of significance given the common co-exposures to multiple toxicants.

The 'present' flame retardants BDE-209, TBBPA, and BPS

Although BDE-47 and several other lower brominated congeners have been banned in most countries, materials and consumer products treated with PBDE-containing flame retardants are still in use. Consequently, continuation of environmental contamination and human exposure is still expected for the coming decades. As mentioned in Chapter 1, evidence for the neurotoxic potential of decabromodiphenyl ether (BDE-209) or its metabolites is increasing. The *in vitro* neurotoxic potency of BDE-209 has been shown in Chapter 6 and 7. Comparable with PCB-47 and 6-OH-BDE-47, BDE-209 acts as antagonist on the $\alpha_4\beta_2$ nACh receptor. No cytotoxicity or disturbance of Ca^{2+}-homeostasis was observed in two neuronal cell lines (PC12 and B35), though in B35 cells a clear increase in the production of reactive oxygen species (ROS) was observed. Several other *in vitro* studies also demonstrated the neurotoxic potential of BDE-209, though most studies used a relative high and thus less realistic concentration range (≥10 μM) (Al-Mousa and Michelangeli, 2012; Chen *et al.*, 2010; Pellacani *et al.*, 2012b; Zhang *et al.*, 2010). At lower concentrations, comparable to those that exerted effects on $\alpha_4\beta_2$ nACh receptors (Chapter 6), BDE-209 was able to affect voltage-gated sodium channels (VGSCs) in primary cultured rat hippocampal neurons (Xing *et al.*, 2010). VGSCs play a pivotal role in depolarization of the membrane and disturbance of VGSCs may thus contribute to attenuation of the generation of action potentials in neurons. *In vivo* studies also indicate that BDE-209 is a developmental neurotoxicant although effects on locomotor activity are contradictory (Johansson *et al.*, 2008; Rice *et al.*, 2009; Rice *et al.*, 2007; Viberg *et al.*, 2003b; Viberg *et al.*, 2007; Viberg, 2009). The occurrence of developmental neurotoxicity is supported by the observed impairment of synaptic plasticity (Xing *et al.*, 2009) and altered expression of several important proteins involved in the formation of proper connectivity in the brain (Viberg *et al.*, 2008a). In addition, neonatal exposure alters the susceptibility of the cholinergic system in adult animals (Johansson *et al.*, 2008). The observed antagonistic

effects on $\alpha_4\beta_2$ nACh receptors confirm the adverse effects on the cholinergic system and contribute to unraveling the mechanisms of action of PBDE 209.

Since data on the neurotoxicity and modes of action of tetrabromobisphenol-A (TBBPA) was limited, the effects of TBBPA on $GABA_A$ and $\alpha_4\beta_2$ nACh receptor functioning were investigated. As shown in Chapter 5, also TBBPA induces opposite effects: agonistic effects on the $GABA_A$ receptor and antagonistic effects on the $\alpha_4\beta_2$ nACh receptor. Besides these novel modes of action, previous studies demonstrated TBBPA-induced inhibition of plasma membrane uptake of the neurotransmitters dopamine, glutamate and GABA in synaptosomes (Mariussen and Fonnum, 2003). It was also suggested that TBBPA can induce the release of neurotransmitters by a Ca^{2+}-dependent mechanism or by an effect on neurotransmitter transport and uptake mechanisms (Reistad *et al.*, 2007). The neurotoxic modes of action were further unraveled by investigating acute effects on basal as well as ACh- and depolarization-evoked $[Ca^{2+}]_i$ using single-cell fluorescent Ca^{2+} imaging. B35 cells express Ca^{2+}-permeable nACh receptors, which were inhibited by TBBPA. Comparable to PCBs (Langeveld *et al.*, 2012) and PBDEs (Dingemans *et al.*, 2011), TBBPA was able to inhibit voltage-gated calcium channels (VGCCs) as observed in experiments using PC12 cells. The effects of TBBPA on Ca^{2+}-homeostasis have been described in several cell lines. In B35 and PC12 cells, rat cerebellar granule cells (Ogunbayo *et al.*, 2007), human neutrophil granulocytes (Reistad *et al.*, 2005), human cerebellar granulocytes (Reistad *et al.*, 2005), and human SH-SY5Y neuroblastoma cells (Al-Mousa and Michelangeli, 2012), a TBBPA-induced increase in $[Ca^{2+}]_i$ was observed. This increase is caused by an influx of extracellular Ca^{2+}, but also originates from intracellular Ca^{2+} stores like the endoplasmic reticulum (ER) and mitochondria (Chapter 5). The ER-mediated increase in basal $[Ca^{2+}]_i$ is an important trigger for the induction of caspase activity (Orrenius *et al.*, 2011). Caspase-3-dependent apoptosis induced by TBBPA was observed in PC12 cells, in mouse neocortical cells (Wojtowicz *et al.*, 2013) and SH-SY5Y cells (Al-Mousa and Michelangeli, 2012). However, one study showed that TBBPA induces apoptotic cell death in cerebellar granulocytes by a caspase-independent mechanism (Reistad *et al.*, 2007). It was suggested that caspase activity was suppressed by a TBBPA-induced increase in ROS production and that TBBPA-induced cell death was thus ROS-dependent. Release of cytochrome *c* and induction of mitochondrial depolarization was also shown to result in an increase of ROS levels (Reistad *et al.*, 2005). Although TBBPA exerts numerous cellular effects, discrepancies are observed in *in vivo* studies. The observed minor TBBPA-induced effects on LTP (Chapter 8) may not be sufficient to affect normal brain development, function, or plasticity. Despite the clear adverse effects in *in vitro* studies and some (contradictory) effects in *in vivo* studies, the neurodevelopmental effects of TBBPA remain questionable.

With respect to other BFRs, no effects of brominated polystyrene (BPS) on several *in vitro* neurotoxic endpoints were observed. BPS is a chemically not well-defined substance and sufficient additional (neuro)toxicological data is lacking, apart from some scarce acute LD_{50} (lethal dose for 50% of the subjects) toxicity data in rats (US EPA, 2012).

1.2 Risk assessment of the 'old' and 'present' flame retardants

Clearly, PCBs, PBDEs and other BFRs share some modes of action on functional neurotransmission endpoints. From these studies it can be concluded that metabolites and/or oxidative metabolism of compounds (together: biotransformation) can increase their toxicity. In addition, it was shown that mixtures of these toxicants can result in additive, antagonistic and even synergistic effects (Westerink, 2013b). However, whether these compounds are of concern for human health and the environment depends also

on other factors, including the abundance in the environment, the physicochemical properties of the compound, human plasma concentrations, toxicokinetics, etc. In addition, extrapolation of experimental *in vitro* and *in vivo* data to the human situation should also be included in risk assessment (see also Figure 2).

Bioavailability

Fatty fish is a major source for human exposure to PCBs and BFRs. Physicochemical properties like the molecular weight and octanol-water partition coefficient (Log K_{OW}) determine the ability of a toxicant to enter the animal or human blood circulation following ingestion (deBruyn and Gobas, 2007; Gulden *et al.*, 2002; Hestermann *et al.*, 2000). For the risk assessment of the flame retardants in this PhD thesis, Log K_{OW} values of the compounds were taken into account (see Table 1). Based on Log K_{OW} values, the flame retardants are classified into two categories: hydrophilic molecules (Log K_{OW} ≤2) and lipophilic molecules (Log K_{OW} >2). The PCB and BFRs, probably with the exception of BPS, are (highly) lipophilic and are therefore expected to bind significantly to serum proteins (e.g. albumin), resulting in (very) low free available concentrations. Large molecules (i.e. the polymer BPS) have due to their high molecular weight a lower bioavailability. Besides, estimations should be made for smaller and/or the single molecule since these compounds comprise a mixture of polymers having different chain length or degree of

Table 1. Some physicochemical properties of the investigated flame retardants

Flame retardant		Molecular weight (g/mol)	Log K_{ow}	Hydrophilic/ Lipophilic
PCB-47	2,2′,4,4′-tetrachlorobiphenyl	292	5.9[a]	Lipophilic
BDE-47	2,2′,4,4′-tetrabromodiphenyl ether	4856	6.8[b]	Lipophilic
6-OH-BDE-47	6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether	502	~5.8[c]	Lipophilic
BPS	brominated polystyrene	1,000-200,000	n/a	
BDE-209	decabromodiphenyl ether	959	6.3[d]	Lipophilic
TBBPA	tetrabromobiphenol-A	5449	4.5[e]	Lipophilic
BDP	bisphenol A bis (diphenylphosphate)	693	<6[f]	Lipophilic
RDP	resorcinol bis (diphenylphosphate)	575	4.9[f]	Lipophilic
TPP	triphenylphosphate	326	~4.6[f]	Lipophilic
DOPO	9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide	216	~2[f,g]	Hydrophilic
Alpi	aluminium diethylphosphinate	390	-0.4[f]	Hydrophilic
ATH	aluminium trihydroxide	78	n/a	
APP	ammonium polyphosphate	~100,000	-2.2[f,g]	Hydrophilic
ATO	antimony trioxide	292	n/a	
MHO	magnesium hydroxide	58	n/a	
ZHS	zinc hydroxystannate	286	<0.1[f]	Hydrophilic
ZS	zinc stannate	232	<-1[f]	Hydrophilic
MMT	cloisite 30B	n/a	n/a	
MPP	melamine polyphosphate	>10,000	-2.3[f]	Hydrophilic

n/a, not applicable; [a] (Geyer *et al.*, 2000) ; [b] (Braekevelt *et al.*, 2003) ; [c] (Yu *et al.*, 2008b) ; [d] (Hardy, 2002); [e](Birnbaum and Staskal, 2004); [f] see Chapter 3; [g] modeled, see Chapter 3

branching, and accordingly the physicochemical properties change with those factors. Due to the high molecular weight of plasma proteins, they cannot cross capillary walls. Consequently, the fraction of monomers or short polymers of BPS bound to plasma proteins is not immediately available for distribution into the extracellular space or filtration and excretion by the kidneys. As a result, it is expected that these chemicals will not readily cause toxic effects. However, it should be mentioned that although the majority of the lipophilic chemicals is bound to plasma proteins, they will dissociate from the protein until equilibrium between the vascular space and extravascular space is reached. Also, active transport processes are not limited by the binding of chemicals to plasma proteins, and since the kidneys are not able to filtrate these large molecules the toxicants may remain in the body for a long period.

Toxicokinetics

Since PCBs and BFRs are potentially toxic during development, it is essential to understand maternal kinetic parameters (i.e. absorption, distribution, metabolism and excretion) in order to describe the dose available to target tissues (e.g. placenta) after exposure. Due to the lipophilicty of PCBs and BFRs, transfer from the aqueous environment of the

Figure 2. Factors mentioned in this PhD thesis necessary for proper risk assessment. The classical risk assessment involves four components: hazard identification, dose-response assessment, exposure assessment, and risk characterization. This Figure illustrates the complex and multiple factors involved in the risk assessment of alternative flame retardants. Note that this Figure only presents the factors mentioned in this PhD thesis.

intestine across cell membranes is generally a passive process. The compounds have a low affinity for blood, which frequently results in accumulation in lipid-rich tissues like brain, adipose tissue and liver. Nevertheless, differences in absorption rate and ability to enter cells are observed among congeners, mainly as a consequence of the number and position of the halogen molecules on the PCBs and PBDEs. Half-lives ($t_{1/2}$) of PCB-47 in rodents are shown in Table 2. Mobilization of PCB-47-containing body fat during e.g. lactation results in high PCB-47 concentrations in milk and exposure of pups with their susceptible developing brain may occur. PCBs and PBDEs are primarily hydroxylated and dehalogenated to increase the water solubility, which facilitates excretion from the body. Hydroxylated products are the major metabolites, with hydroxylation occurring primarily at the *para* or *meta* positions if these sites are not substituted with halogens (Safe, 1984). However, it was shown that PCB-47 is relatively slowly metabolized in mammalian species and consequently, high concentrations of the parent PCB-47 were found in *in vivo* studies (Saghir *et al.*, 1999; Soontornchat *et al.*, 1994). Furthermore, difference in toxicokinetics of BDE-47 were observed between developing and adult mice (see Table 2), which has been attributed to differences in urinary elimination, the major excretion route in mice for this BFR (Staskal *et al.*, 2006). Consequently, higher BDE-47 concentrations at target tissues like the brain during critical periods of the development are expected. In addition, it was shown that tissue levels of BDE-47 in pups of chronically exposed dams decreased between parturition and weaning, indicating mobilization of BDE-47 during lactation (Ta *et al.*, 2011). The adverse effects of bioactivation of BDE-47 have already been discussed earlier. In contrast, BDE-209 with its bulky configuration is poorly absorbed compared to the lower brominated BDEs (Costa and Giordano, 2007; Costa and Giordano, 2011; Hakk and Letcher, 2003) and once taken up from the gastro-intestinal tract it has a remarkable and much shorter $t_{1/2}$ than e.g. BDE-47. Metabolism of BDE-209 is suggested to involve rapid debromination to lower brominated congeners, but the relevance for toxicity of this metabolic process in mammals, including humans, is still unclear (Costa and Giordano, 2011; Stapleton *et al.*, 2009). Oral administration of TBBPA results in rapid absorption from the gastro-intestinal tract, followed by a fast elimination primarily as the unchanged parent compound (Brady, 1979; Hakk and Letcher, 2003), though another study indicated that the very low systemic bioavailability of TBBPA after oral administration is due to efficient hepatic metabolism and biliary excretion of formed conjugates (Schauer *et al.*, 2006). The fast elimination of TBBPA from the mammalian body clearly prevents bioaccumulation, and therefore - based on toxicokinetics - TBBPA

Table 2. Half-lives of PCB-47, BDE-47, BDE-209 and TBBPA

Compound	Species	$t_{1/2}$ (days)	Accumulation	Reference
PCB-47	Mice	9.2	Preferable fatty tissues	Hany *et al.*, 1999
	Prepubertal rats	14.6	Preferable fatty tissues	Saghir *et al.*, 1999
BDE-47	Developing mice	50	Fatty tissues	Staskal *et al.*, 2006
	Adult mice	22	Fatty tissues	Staskal *et al.*, 2006
	Human	664	Fatty tissues and breast milk	Geyer *et al.*, 2004
BDE-47 + metabolites	Human	1795	Fatty tissues and breast milk	Geyer *et al.*, 2004
BDE-209	Rats	8.6		Huwe and Smith, 2007
TBBPA	Rats	<3		Brady, 1979
	Human	6.6		Geyer *et al.*, 2000; Geyer *et al.*, 2004

has a relatively low (neuro)toxic potential compared to other, more persistent, BFRs such as PBDEs.

Routes of exposure and internal concentrations
Human exposure to PCBs and BFRs occurs predominantly via food and house dust. PCBs and BFRs are measured in various human tissues, including serum, placenta, liver, adipose tissue and breast milk (Cui *et al.*, 2012; Frederiksen *et al.*, 2009; Wingfors *et al.*, 2000). While PCB levels are significantly decreasing in the environment, PBDE levels in human serum and breast milk were still increasing up to 2000 (Norén and Meironyté, 2000), but fortunately showed a decrease over the years 2003-2008 (Sjödin *et al.*, 2014). Table 3 presents some serum and breast milk concentrations of both groups of compounds from the general population (background levels), pregnant women, and (newborn) children. For comparison it should be noted that cord serum (indicated as "fetal" under population) has a significant lower lipid content than maternal serum. Due to accumulation in fatty tissues and excretion via milk, breast-fed children with a developing nervous system are (highly) exposed via human milk. In breast milk of Texas mothers (Schecter *et al.*, 2008), BDE-209 was only measured in 6 of the 23 samples, which indicates that despite a lower bioavailability compared to lower brominated congeners (e.g. BDE-47), a part is still bioavailable and present in human milk. In spite of the relative low lipophilic properties, short $t_{1/2}$, and fast excretion of TBBPA, still a significant maternal transfer has been observed. The large differences in PBDE levels in house dust that have been found between countries reflect a much higher usage of these BFRs in the US than in Europe (BSEF, 2013). Consequently, average levels of the lower brominated PBDEs in the US population are significantly higher than those e.g. in Europe, and are obviously associated with exposure to house dust (Johnson *et al.*, 2010). Clearly, this higher usage of PBDEs is of particular concern in view of the high concentrations in breast milk and cord serum.

Blood-brain- and placental barrier
To protect the brain from injury by toxicants, the blood-brain barrier (BBB) separates the circulating blood from the brain extracellular fluid. The blood vessels in the brain tightly regulate the movement of molecules, ions, and cells between blood and brain tissue, as the brain needs highly specific ionic concentrations for proper neuronal function. Most of the properties of the BBB are manifested within the endothelial cells that form the walls of the blood vessels (Daneman, 2012). These cells are highly polarized and held together by tight junctions that greatly limit the movement of molecules and ions between blood and brain cells. Consequently, large and hydrophilic molecules are hardly able to enter the neuronal tissue, whereas small and lipophilic molecules are much more likely to pass this barrier. Additionally, many molecules will be excluded from passing the BBB by efflux transporters (Löscher and Potschka, 2005). Also, the endothelial cells are covered by glycocalyx, a complex mixture of carbohydrates, which acts as a primary barrier providing a charge/size-selective sieve that limits the interactions of molecules and cells with the endothelium (Van Teeffelen *et al.*, 2007). Only few data exist on PCB and BFR levels in animal brains, though these compounds have been detected in brains of birds (Naert *et al.*, 2007), polar bears and seals (Letcher *et al.*, 2009; Recke and Vetter, 2007), suggesting limitations in the protective function of the BBB for these compounds. The scarce studies performed on humans (autopsy samples) also indicate the presence of PCBs in brain tissue with a mean total PCB value of 52 μg/kg lipid weight (lw), which is about eight times lower than the accompanying mean adipose tissue extracts (403 μg/kg lw) (Dewailly *et al.*, 1999; Hirakawa *et al.*, 1992). Only one recent study describes the

Table 3. Concentrations in human serum, breast milk and dust

Compound	Population	Concentration (ng/g lipids)	Country	Reference
Serum				
PCB-47	General	1.4	Mexico City	Orta-García *et al.*, 2014
		0.48	Sweden and Spain	Wingfors *et al.*, 2000
BDE-47	General	23	Canada	Rawn *et al.*, 2014
		2	Spain	Garí and Grimalt, 2013
		0.8	UK	Thomas *et al.*, 2006
		20.5	US	Sjödin *et al.*, 2008
	Fetal	70	US	Qiu *et al.*, 2009
	Children	0.87	Faroe Islands	Fängström *et al.*, 2005
		1.4	Sweden	Sahlstrom *et al.*, 2014
	Maternal	1.3	Faroe Islands	Fängström *et al.*, 2005
		16.5	North Carolina	Stapleton *et al.*, 2011
		0.58	Sweden	Sahlstrom *et al.*, 2014
		17	US	Qiu *et al.*, 2009
		20.1	US	Chen *et al.*, 2014
6-OH-BDE-47	Fetal blood	9.9	US	Qiu *et al.*, 2009
	Maternal	0.17	North Carolina	Stapleton *et al.*, 2011
		0.39	US	Qiu *et al.*, 2009
BDE-209	General	1.9	Canada	Rawn *et al.*, 2014
		3.5	Spain	Garí and Grimalt, 2013
		0.24	UK	Thomas *et al.*, 2006
	Children	1	Faroe Islands	Fängström *et al.*, 2005
		2.1	Sweden	Sahlstrom *et al.*, 2014
	Maternal	0.7	Faroe Islands	Fängström *et al.*, 2005
		0.86	Sweden	Sahlstrom *et al.*, 2014
TBBPA	General	20	France	Cariou *et al.*, 2008
		0.7	Norway	Thomsen *et al.*, 2002
	Fetal	104	France	Cariou *et al.*, 2008
		78	Korea	Kim and Oh, 2014
	Maternal	20	France	Cariou *et al.*, 2008
		11	Korea	Kim and Oh, 2014
Breast milk				
BDE-47	Toddlers	2	Sweden	Meironyte *et al.*, 1999
		3.3	UK	Abdallah and Harrad, 2014
		50	US	Johnson-Restrepo *et al.*, 2007; Schecter *et al.*, 2008; She *et al.*, 2007
		73	US	Park *et al.*, 2011

(continued)

Table 3 (continued)				
Compound	**Population**	**Concentration (ng/g lipids)**	**Country**	**Reference**
		23	Canada, 2005	Ryan and Rawn, 2014
		39.5	Canada, 2002	Ryan and Rawn, 2014
		4.6	Canada, 1992	Ryan and Rawn, 2014
BDE-209	Toddlers	8	Texas	Schecter *et al.*, 2008
		0.31	UK	Abdallah and Harrad, 2014
		3.8	US	Park *et al.*, 2011; She *et al.*, 2007
TBBPA	Toddlers	4.1	France	Cariou *et al.*, 2008
		0.067	Norway	Thomsen *et al.*, 2002
		0.06	UK	Abdallah and Harrad, 2011
Dust		**(ng/g)**		
BDE-47	General	11.7	Germany	Fromme *et al.*, 2014
PBDEs	General	14	Germany	Sjödin *et al.*, 2008
		340	US	Sjödin *et al.*, 2008
		132	Germany	Fromme *et al.*, 2014
BDE-209	General	63	Germany	Sjödin *et al.*, 2008
		10,000	UK	Sjödin *et al.*, 2008
		2,000	US	Sjödin *et al.*, 2008
		1233	Germany	Fromme *et al.*, 2014
TBBPA	General	44	Germany	Fromme *et al.*, 2014
TBBPA		89	Germany, UK and US	Abb *et al.*, 2011; Abdallah and Harrad, 2011

"Fetal" indicates measurement in serum of cord blood

possible mechanism of action of these compounds on the BBB function. ROS produced by PCBs may alter the BBB integrity in rats, which is paralleled by cytoskeletal rearrangements and disappearance of tight junction proteins (Selvakumar *et al.*, 2013). In conclusion, toxicants may thus affect directly or indirectly the BBB, resulting in entering of toxicants in the brain.

The placental barrier acts as a membrane between the maternal and fetal blood circulation. The transfer of any compound from the maternal to the fetal circulation involves transport across a brush border membrane, followed by transport across a basal membrane. As mentioned before, a toxicant bound to plasma proteins decreases the placental transport since generally only unbounded toxicants can pass the placental barrier. The transport of toxicants may either be passive or active via one of the transporters in the placenta. Clearly, toxicants with low molecular weight and high lipophilicity are more likely to pass the placental barrier by passive diffusion. In addition, the human placenta is also able to metabolize toxicants e.g. via cytochrome P-450 enzymes. Several studies indicate this placental transfer of PCBs and PBDEs, resulting in prenatal exposure (Koenig *et al.*, 2012; Lanting *et al.*, 1998; Meijer *et al.*, 2008; Schecter *et al.*, 2006). As mentioned, in the postnatal period, these compounds are transferred from the nursing mother to the child via human milk. Clearly, (unborn) children are exposed during their critical brain growth spurt, which argues for additional research and risk assessments.

2. The future - (neuro)toxicity and risk assessment of alternative flame retardants

2.1 Neurotoxicity of halogen-free flame retardants

In the *in vitro* studies presented in Chapters 6 and 7, it was shown that bisphenol A bis (diphenylphosphate) (BDP) induces minor cytotoxicity in PC12 and B35 cells, and only minor effects on the depolarization-evoked increase in $[Ca^{2+}]_i$ in PC12 cells. At high concentrations, resorcinol bis (diphenylphosphate) (RDP) is able to inhibit $\alpha_4\beta_2$ nACh receptors, whereas inhibition of depolarization-evoked Ca^{2+} in PC12 cells was observed at lower concentrations. *In vivo* studies describe however that RDP induces no adverse teratogenic and developmental effects (at concentrations up to 20,000 mg/kg diet; Henrich *et al.*, 2000; Ryan *et al.*, 2000).

Triphenylphosphate (TPP) is better studied than most other phosphorous flame retardants. Previous *in vitro* studies reported cytotoxicity in PC12 cells and inhibition of GABA-receptors (Flaskos *et al.*, 1994; Gant *et al.*, 1987; Padilla *et al.*, 1987; Vainiotalo *et al.*, 1987). In the same micro-molar range as the inhibition of GABA-receptors, it is now shown that TPP also inhibits $\alpha_4\beta_2$ nACh receptors. Clear effects on ROS production as well as on basal and depolarization-evoked changes in $[Ca^{2+}]_i$ were also observed. However, the *in vivo* neurotoxicity of TPP has been debated since the early studies of Smith *et al*. (Smith *et al.*, 1930; Smith *et al.*, 1932), which reported delayed neuropathy in cats and monkeys exposed to TPP in acute and short-term studies, while Wills *et al*. (Wills *et al.*, 1979) could not demonstrate ataxia or neuropathic damage in cats. The commercial cresyldiphenylphosphate product contains about 35% TPP and was shown to induce several effects in rats, including acute neurotoxic effects (Vainiotalo *et al.*, 1987). In another rat study, a no observed adverse effect level (NOAEL) of 161 mg/kg/d for decreased weight gain and neurological effects was determined (Illinois Environmental Protection Agency, 2007). Even human exposure studies have been performed with TPP, though no evidence of neurological disease or other abnormalities in workers exposed to TPP vapor or dust were observed (Sutton *et al.*, 1960). In the case of 9,10-dihydro-9-oxa-10-phosphaphenanthrene-10-oxide (DOPO), at concentrations ≥1 μM, ROS production was increased and the depolarization-evoked increase in $[Ca^{2+}]_i$ was inhibited. Aluminium diethylphosphinate (Alpi) was shown to inhibit $\alpha_4\beta_2$ nACh receptors and increases basal $[Ca^{2+}]_i$ as well as ROS production, though only at concentrations ≥140 μM. In the *ex vivo* study, no significant adverse effects of Alpi on LTP and synaptic protein levels in mouse hippocampus were observed in mice that received a single exposure of 211 μmol/kg bw Alpi on postnatal day 10 (PND 10).

Some studies report the *in vitro* neurotoxicity of aluminium trihydroxide (ATH), such as induction of neurites in neuroblastoma cells (Zatta *et al.*, 1992) and NMDA receptor binding in human cerebral cortex (Hubbard *et al.*, 1989). More *in vitro* neurotoxic effects of ATH were observed and described in Chapter 6 an 7. The general acute toxicity for ATH in rats is very low, though adverse effects on the learning ability and diminished cholinergic activity have been observed in rats (Bilkei-Gorzo, 1993). Despite clear neurotoxic *in vitro* effects at low concentrations (≥0.019 μM), it is questionable if the amounts used in the mentioned *in vivo* study represent background levels of the general population. Studying the (neuro)toxicity of ammonium polyphosphate (APP) is hampered by the hydrolysis of APP into monomeric ammonium phosphate in contact with water (Clariant, 2010). Nevertheless, clear cytotoxic effects - possibly of the monomer - were observed, as well as an increase in ROS levels, disturbance of depolarization-evoked Ca^{2+} homeostasis and inhibition of $\alpha_4\beta_2$ nACh receptors (all at concentrations ≥7 μM). At low concentrations (9 μM), antimony trioxide (ATO) showed clear antagonistic effects

on the $\alpha_4\beta_2$ nACh receptor. At concentrations ≥4.1 μM, magnesium hydroxide (MHO) was shown to increase ROS levels, disturb depolarization-evoked Ca^{2+} homeostasis and inhibit $\alpha_4\beta_2$ nACh receptors. In the case of zinc hydroxystannate (ZHS) and zinc stannate (ZS), clear *in vitro* neurotoxic effects are observed in this PhD study. Both compounds reduce in the same micro-molar concentration range cell viability, increase ROS production, and strongly reduce depolarization-evoked Ca^{2+} homeostasis. As can be concluded from the *ex vivo* study, no significant effects on LTP and synaptic protein levels following a single exposure on PND 10 to 211 μmol/kg bw ZS were observed.

The used nanoclay consist of quartz and alkyl quaternary ammonium bentonite. It has been reported that montmorillonite (the main constituent of bentonite) induces an acute impairment of cellular excitable function (Banin and Meiri, 1990; Murphy *et al.*, 1993), while quartz up to 4000 μg/ml did not affect neuroblastoma cells. The *in vitro* neurotoxic effects at low concentrations (≥0.004 μM) are confirmed in this PhD study. In water, melamine polyphosphate (MPP) will dissociate into the monomers melamine and phosphoric acid. It is therefore a subject of discussion whether the observed effects are realistic and useful for human exposure, and wether they are induced by the polymer or by melamine and/or phosphoric acid since it is known that at least melamine affects VGSCs (Yang *et al.*, 2010).

2.2 Risk assessment of the 'future' flame retardants

Bioavailability and toxicokinetics

BDP, RDP and TPP are classified as lipophilic and are consequently expected to bind to serum proteins. Although the expected free concentration of these compounds is low, they are hardly excreted from the human body. In addition, in the case of BDP, bisphenol-A is mentioned to be a degradation product, which is a low level endocrine disruptor that affects multiple reproductive endpoints as well as (indirectly) immune function and brain development (Maine, 2007). This observation again indicates the importance of studying metabolites or other breakdown products, comparable to the increased neurotoxicity of metabolites of e.g. BDE-47. Only one study reported the metabolism and toxicokinetics of RDP in rats, mice and monkeys, resulting in a $t_{1/2}$ of 2.7 days in rats following oral exposure (Freudenthal *et al.*, 2000). Diphenylphosphate (DPP) was identified as the major metabolite of TPP in rats (Sasaki *et al.*, 1981) and humans (Hoffman *et al.*, 2014; Van den Eede *et al.*, 2013), which is rapidly eliminated from the body. Due to this rapid biotransformation, the half-lives of parent TPP in blood and its metabolites in urine are thought to be very short, most likely in the order of several hours.

The remaining halogen-free flame retardants (HFFRs) are classified as hydrophilic and less likely to bind plasma proteins. As a consequence, these chemicals are theoretically freely available and have a high bioavailability, but systemic concentrations and associated bioaccumulation and toxic effects obviously depend on the exposure (dose, single/chronic, etc.). However, it should be noted that for inorganic substances or substances that ionize in e.g. the stomach, the Log K_{OW} is less relevant. The metallic-based flame retardants will dissociate into their ion constituents and the partitioning of the free metal ions will have a complex chemistry with their surrounding physiological environment, depending on their ionic state and an additional long list of physic-chemical parameters (acidity, hardness, redox status, etc.). Alpi is a metal-based phosphorous flame retardant that may dissociate into its ion constituents under some conditions. In rats orally exposed to Alpi, phosphonic acid was observed as metabolite in the feces and urine (ECHA Database, 2014a). No half-lives were reported, but after 36 h, no more test substance was detected in feces and/or urine. Inhalation of ATH by rabbits and rats

resulted in transfer of Al^{3+} to the brain (Röllin *et al.*, 1991; Schlesinger *et al.*, 2000). As an inorganic substance, ATO is not metabolized, but excreted via the feces and (to a lesser extent) urine. Half-lives in orally exposed rats for 14 days were estimated to be 9.3 days and had increasing brain concentrations following 24 weeks of exposure (ECHA Database). In the case of MHO, Mg^{2+} will dissociate from the molecule and about 40% of the Mg^{2+} will bind to proteins to the same degree as endogenous Mg^{2+}. However, a Mg^{2+} intoxication is rare due to its key role in a wide range of physiological cellular processes, including modulation of NMDA receptors (Domijan *et al.*, 2012) and Ca^{2+}-activated K^{+}-channels inhibition (Leinders *et al.*, 1992). Both ZHS and ZS are not expected to ionize in the test solutions of the performed *in vitro* studies, and metallic tin and its inorganic salts are generally considered to be of low toxicity due to their poor alimentary absorption (Cima, 2011). Excess zinc is a known neurotoxicant (for reviews see Boyer, 1989; Wright and Baccarelli, 2007; Zatta *et al.*, 2009), but an excess of zinc is not expected in the case of exposure to these flame retardants. The polymers APP and MPP are not expected to have a high bioavailability and it is not likely that they reach the extracellular space. Moreover, APP and MPP will dissociate in contact with water, which will affect bioavailability and thus toxicity. For example, as shown in Chapter 6, the classification of APP changes drastically from highly potent assuming the polymer (with a chain length of 1000) to a moderate potency assuming the monomer. Due to its composition of quartz and alkyl quaternary ammonium bentonite, the nanoclay MMT (cloisite 30B) is very poorly soluble and its bioavailability is expected to be low.

Routes of exposure and internal concentrations

Only a few studies describe concentrations of HFFRs in human milk, dust or transfer through the placental barrier, etc. BDP and RDP are relatively ubiquitous in house dust samples, as well as in cars and electronic stores (Ballesteros-Gómez *et al.* 2014; Brandsma *et al.*, 2013; Fan *et al.*; Yang *et al.*, 2014). In general, studies have observed high levels of organophosphorous flame retardants, which are comparable to or even higher than those of PBDEs in house dust (Brommer *et al.*, 2012; Dirtu *et al.*, 2012; Kim *et al.*, 2013; Stapleton *et al.*, 2009). Since no correlation between TPP in house dust and DPP in urine was observed in adult men, these results suggest that TPP in house dust may not be a primary source of exposure (Meeker *et al.*, 2013). Due to the high vapor pressure, inhalation of TPP in indoor air may play a more significant role for human exposure (Meeker *et al.*, 2013). For example, aircraft cabin air is contaminated with traces of hydraulic fluid, which may explain elevated TPP metabolites levels in air crew (Schindler *et al.*, 2013). TPP was also reported to be present in human milk up to 19 ng/g lw (Kim and Oh, 2014; Sundkvist *et al.*, 2010). In addition, it was shown that TPP levels were up to 10,000 times higher than those of the PBDEs measured in toys, which may be a potential health hazard of children following the observed effects *in vitro* and the adverse effects in the mentioned *in vivo* studies (Ionas *et al.*, 2014).

From the 14th century on, antimony was in use as medicine for the treatment of parasitic diseases, and is naturally released into the environment by natural discharges. In occupational settings, exposure to the possibly carcinogenic antimony compound antimony trioxide (ATO) is via for example uniforms of fire fighters that contain ATO as a flame retardant and consequently, also workers in the textile industry may be exposed. Clearly, toxicity may arise during occupational exposure or when used as therapy. Levels of exposure are limited (de Perio *et al.*, 2010; Sundar and Chakravarty, 2010), although one study indicates that no correlation was found between personal ATO exposure and textile industry workers, following measurement in urine (Iavicoli *et al.*, 2002). Another study reported the transfer of ATO over the placental barrier after injection and oral ad-

ministration in mice, and transfer into milk following oral and intravenous application in cattle (ECHA Database, 2014). In the case of MHO, Mg^{2+} was shown to cross the placental membrane and distribute in the fetus while distribution to breast milk is very limited (ECHA Database, 2014b). As mentioned, for ZHS and ZS a low acute toxicity is observed. No information on exposure was found for ATH, APP, MMT and MPP.

In general, trends observed in studies suggest that exposure via dust to HFFRs will be comparable as exposure via dust to BFRs, but their bioaccumulation potential appears to be much more limited. Consequently, levels detected so far in the human body most likely reflect the daily exposure to these compounds, in which the significant role of house dust is indicated.

Lack of data

Dossiers with information on the toxicity of chemicals are publically available at the US EPA (United States Environmental Protection Agency; www.epa.gov) and ECHA (European Chemicals Agency; www.echa.europa.eu) archives. In addition, in 2001, the EU introduced REACH (Registration, Evaluation, and Authorization of Chemicals), a regulation on the production and use of chemicals and their potential impact on human health and environment. Ideally, HFFRs that replace existing BFRs should pose lower risks to the environment and human health. However, some of the selected HFFRs investigated in this PhD thesis are already marketed without having a full profile of their physicochemical and toxicological properties or environmental properties. Despite the REACH regulations, characterization of chemicals often lacks in-depth studies like identification and characterization of potentially toxic metabolites. EU-funded research projects like ENFIRO aimed to fill large parts of the existing data gaps. By studying several aspects, including environmental and toxicological risks, a first step to a solid base for assessing the suitability of HFFRs as safe alternative(s) for BFRs is formed.

Rank-ordering

Several criteria for classification of chemicals with a similar mode of action exist. For example the Toxic Equivalency Factor (TEF) concept for PCBs, polychlorinated dibenzo-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Van den Berg *et al.*, 1998), which is based on the binding of these compounds to the aryl hydrocarbon receptor. A Neurotoxic Equivalence (NEQ) scheme was developed in an attempt to assess the risk of PCBs using relative potency values for a range of cellular and biochemical effects from *in vitro* studies (Simon *et al.*, 2007). For *in vitro* profiling of the endocrine-disrupting potencies of BFRs, classification criteria were based on dose-response curves and calculated as EC_{50} or IC_{50} values (half maximal response or inhibition) (Hamers *et al.*, 2006). As mentioned earlier in Chapter 7, the flame retardant-induced effects on different *in vitro* neurotoxicity endpoints cannot be classified using one of the existing classification schemes. In addition, the lack of complete dose-response curves hampered the use of e.g. EC_{50} or IC_{50} values to determine relative potencies for a given (*in vitro*) neurotoxic effect. In order to still provide relative potencies for BFRs and HFFRs, calculations were based on the lowest observed effect concentrations (LOECs). In Table 4, an overview of these *in vitro* neurotoxic potencies is given.

3. Recommendations and conclusions

3.1 Summary and recommendations for future research

The main objective of this PhD study was to assess the neurotoxic potential of halogen-free flame retardants (HFFRs) in relation to the (well-)known neurotoxicity of brominated flame retardants (BFRs). The studies described in this thesis illustrate the big data gaps in the physicochemical properties, production volume, persistence, bioaccumulation and toxicity of most HFFRs (Chapter 3). Chapter 4 and 5 demonstrate the *in vitro* neurotoxic modes of action of BFRs on postsynaptic $GABA_A$ and $\alpha_4\beta_2$ nACh receptors, intracellular Ca^{2+} homeostasis in neuronal cell lines, cytotoxicity and ROS production. These results indicate the potentially adverse effects of some BFRs on humans and wildlife, justifying the quest for flame retardants with reduced neurotoxic potential. Using the same *in vitro* techniques and neurotoxicological endpoints, selected HFFRs were investigated for their potentially adverse health effects, resulting in classification tables to prioritize the most suitable alternatives (Chapter 6 and 7). Finally, the gap between the *in vitro* experiments and *in vivo* situation was bridged with three selected compounds (TBBPA, ZS and Alpi) using *ex vivo* experiments (Chapter 8).

PCBs as well as BFRs have several neurochemical targets including decrease in cell viability, increase in ROS formation, disturbed Ca^{2+} homeostasis and differential effects on neurotransmitter receptor functioning (see also Figure 3). PCBs and BFRs can inhibit excitatory $\alpha_4\beta_2$ nACh receptors and VGCCs, whereas the inhibitory $GABA_A$ receptors can be activated or potentiated. A possible net result is reduced neuronal activity and reduced neurotransmitter secretion. On the other hand, intracellularly, Ca^{2+} can be released from intracellular stores, which may result in a temporarily increase of neurotransmitter secretion. Additionally, store-mediated Ca^{2+}-release can trigger activation of apoptosis. The observed increases in ROS formation can result in oxidative stress with consequently damage to mitochondrial DNA and cellular macromolecules.

Since both the $GABA_A$ and $\alpha_4\beta_2$ nACh receptors play an important role in LTP and synaptic plasticity, *in vivo* effects of PCB-47 (see for example Seegal *et al.*, 1997) may be (partly) explained by the adverse effects on these receptors. The observed higher *in vitro*

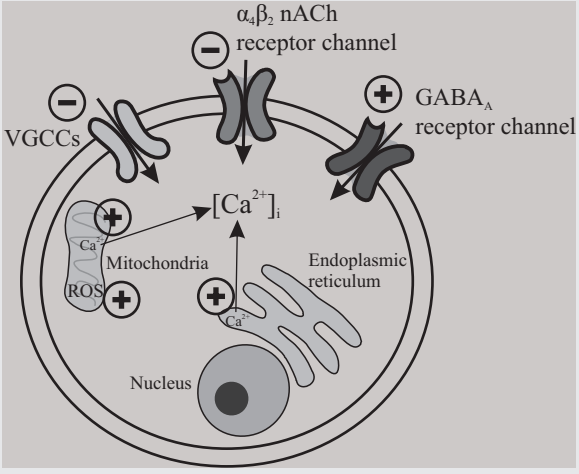


Figure 3. Summary of the general *in vitro* neurochemical targets of PCBs and BFRs as observed in this PhD study. Voltage-gated calcium channels (VGCCs) and $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptors are inhibited, while $GABA_A$ receptors are activated or potentiated. Ca^{2+} is released from intracellular stores, and ROS formation is increased.

potency of 6-OH-BDE-47 compared to the parent BDE-47 on the mentioned neurotransmitter receptors possibly also underlies the observed effect *in vivo* (see e.g. Dingemans *et al.*, 2007; Eriksson *et al.*, 2001). Also the antagonistic effects of BDE-209 on the $\alpha_4\beta_2$ nACh receptor contribute to the unraveling of the mechanism of action of these BFRs. Despite the newly obtained data on the *in vitro* and *ex vivo* neurotoxicity of TBBPA, the impact of TBBPA on neurodevelopmental effects remains - as discussed above - debatable.

From all mentioned experimental and human epidemiological studies, the adverse effects of PCBs and PBDEs are most clear. Besides effects on the (developing) nervous system, several other adverse effects on human health have been reported and toxicological relevant concentrations of PBDEs are observed in the environment. In the case of TBBPA, epidemiological studies have - so far - failed to find an unequivocal association between human levels and adverse neurological of behavioral effects, despite the observed (neuro)toxicity in experimental models (Colnot *et al.*, 2013; Kicinski *et al.*, 2012; Williams and DeSesso, 2010). No adverse effects of BPS on the endpoints investigated were observed, but additional toxicity data is also lacking. Taken together, experimental and epidemiological studies on PCBs and BFRs clearly underline the need for safer alternatives. Also, the lessons learned from the adverse health effects of PCBs and BFRs clearly show the need for a full (eco)toxicological risk assessment before new flame retardants are commercially introduced on the market. To protect the human health and environment, it is of the highest importance to first thoroughly investigate the persistence, bioaccumulation and toxicity of such new flame retardants. From a human health point

Table 4. Overview of the (neuro)toxicity of the HFFRs

Compound	*In vitro* toxicity[1]	*In vivo* toxicity[2]	Additional information	Suitable?
BDP	Negligible	Low	Contains TPP as impurity, high estimated dust ingestion for toddlers, and has bisphenol-A as metabolite	Unlikely
RDP	Low	Low	Oxidative metabolism	Likely
TPP	Low	Low	Present in human milk, labeled as "warning" and "a compound with dangerous effects for the environment" by the ECHA, $t_{1/2}$ of several hours in human	Unlikely
DOPO	Low	Moderate	Labeled as "warning" by the ECHA	Unlikely
Alpi	Negligible	Low	Dissociation is possible, no effects observed in *ex vivo* study[3]	Likely
ATH	High	Low	Transfer of aluminium to the brain in rodents	Maybe
APP	Low		Hydrolysis into the monomer	Likely
ATO	Low		Transfer over the placental barrier and into milk, $t_{1/2}$ 9.3 days in rats	Likely
MHO	Low	Low	Dissociation is possible	Likely
ZHS	High	Low	Low absorption	Likely
ZS	High	Low	No effects observed in *ex vivo* study[3]	Maybe
MMT	High		Chemical composition largely unknown	Maybe
MPP	Moderate	Low	Hydrolysis into the monomer	Likely

Underlined phrases indicate a more important factor than other mentioned factors for the decision of suitability. [1] Based on Chapter 6 and 7; [2] based on Chapter 3 and discussions in other chapters; [3] Based on Chapter 8

of view it is important to emphasize that a wide variety of environmental and toxicological properties should be assessed. These factors should at least include acute versus chronic low-dose exposure, bioactivation, bioconcentration factors, biodistribution, differences in susceptibility between adults and developing children, interaction with other pollutants (mixtures) and quantitative exposure pathways via multiple source like dust and food (see also Figure 2).

For neurotoxicity, a first step in the search for safer alternatives is made by investigating several *in vitro* neurotoxic endpoints and by an *ex vivo* case study with selected compounds. Reduction of depolarization-evoked increases in $[Ca^{2+}]_i$ as a result of inhibition of VGCCs by HFFRs seems to be the most sensitive endpoint since 8 of the 16 investigated compounds were able to affect this endpoint at concentrations from 0.004 μM on. Table 4 gives an overview of the hazardous effects of some HFFRs and their suitability to substitute BFRs, based on the described literature, including labels and statements of the ECHA (European Chemicals Agency) if available. It should be noted that their suitability is mainly based on *in vitro* and some *ex vivo* endpoints as described in this thesis, together with limited other *in vitro* and *in vivo* neurotoxicity studies and some factors necessary for risk assessment.

Following the results of these *in vitro* tests, Alpi and ZS were selected for a more in-depth study. The negligible neurotoxic potency of Alpi *in vitro* was confirmed in an *ex vivo* study as no (developmental) neurotoxicity (synaptic function and plasticity) was observed following one single oral exposure on PND 10. ZS evoked several adverse effects on the *in vitro* endpoints, while *in vivo* no effects were observed on neuronal development. Further *in vivo* research using a chronic exposure scenario can be used to establish the (absence of) developmental neurotoxicity of ZS.

In general, a lack of exposure and toxicological data hampers proper risk assessment and clearly more research is needed to obtain a full toxicological profile of the HFFRs studied in this thesis. The safety of these HFFRs as mentioned in Table 4 is based on the data described in this PhD thesis while for a complete risk assessment, a broader set of data (e.g. including data on other vulnerable targets like the (neuro)endocrine system) is essential. In addition, effects on the environment (distribution, effects on ecosystems, etc.) should be taken into account. From a consumer safety perspective, the application (e.g. plastic housings of electronic equipment, textile coatings and polymers), flame retardant capacity, and financial benefits of the different HFFRs should not be ignored either. Besides, this PhD study demonstrates the additivity of the *in vitro* adverse effects of a mixture of toxicants (PCB-47 and 6-OH-BDE-47, see Chapter 4). Although studying effects of mixtures is complex and often hard to predict, the combination of a wide range of chemicals in the environment and subsequent human and wildlife exposure argues for more research on mixture toxicity. The results presented in this PhD thesis clearly emphasize the need for strict regulations of chemicals on registration of environmental behavior and toxicological properties before they are allowed to enter the market.

3.2 Conclusions

- ✓ Inhibitory $GABA_A$ and excitatory $\alpha_4\beta_2$ nACh neurotransmitter receptors are differentially affected by PCB-47 and 6-OH-BDE-47: the $GABA_A$-mediated signaling is potentiated while the $\alpha_4\beta_2$ nACh-mediated signaling is inhibited. These opposite effects may underline the neurobehavioral and neurodevelopmental effects observed in animals following PCB and/or PBDE exposure, also considering the excitatory behavior of the $GABA_A$ receptor during early brain development.
- ✓ TBBPA exerts several *in vitro* effects on functional neurotransmission endpoints that are shared by PCBs and PBDEs and may have the same potential for adverse health effects. However, in line with some other studies, no TBBPA-induced adverse effects on the developing brain (i.e. synaptic function and plasticity) following a single oral exposure were observed, which contributes to the debate on the neurotoxic potential of TBBPA.
- ✓ BPS did not show any toxic effect in this PhD study, but caution should be taken because there is a significant lack of data on other toxicological aspects.
- ✓ Some HFFRs that are already on the market lack data on even the most basic physicochemical properties. In addition, no or few data on persistence, bioavailability, bioaccumulation, toxicokinetics and toxicity is available, though this is essential for complete (toxicological) risk assessment.
- ✓ From an *in vitro* neurotoxic point of view, effects on depolarization-evoked increases in $[Ca^{2+}]_i$ appear to be the most sensitive endpoint.
- ✓ Despite the clear *in vitro* neurotoxic effects of ZS, no neurotoxic effects were observed *ex vivo* with the used dosing paradigm.
- ✓ The (very) low observed *in vitro* and *in vivo* toxicity suggests Alpi may be a suitable alternative flame retardant. In addition, RDP, APP, MHO, ZHS and MPP are also selected as potentially suitable alternatives.
- ✓ For complete (toxic) risk assessment, additional data such as bioavailability and toxicokinetics are required.
- ✓ An initial rank ordering of the HFFRs based on the mentioned *in vitro* (neuro)toxic endpoints was made. BPS, BDP and Alpi were classified as having negligible *in vitro* neurotoxic potency, BDE-209, TBBPA, RDP, TPP, DOPO, APP, ATO and MHO as having low *in vitro* neurotoxic potency, MPP as having moderate *in vitro* neurotoxic potency, and ATH, ZHS, ZS and MMT as having high *in vitro* neurotoxic potency. However, the *in vivo* relevance of this classification depends on actual human systemic levels of these HFFRs, which is - so far - unknown for most compounds studied in this thesis.

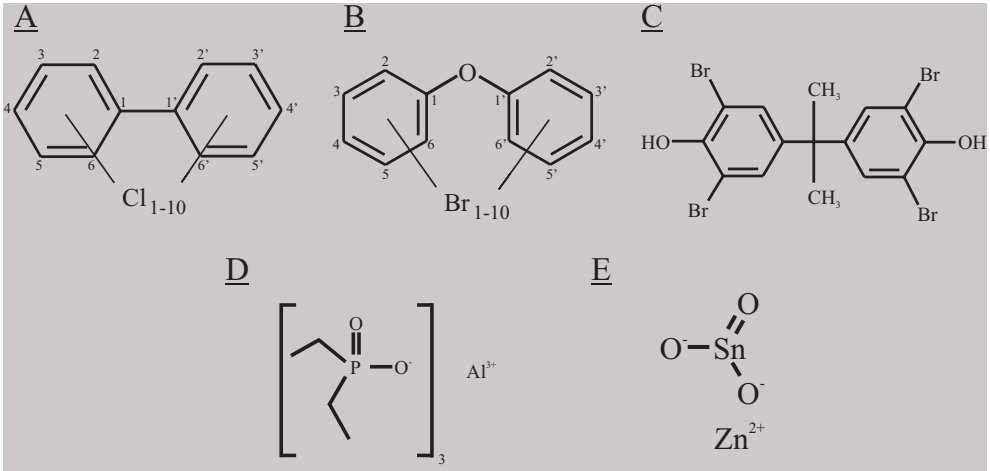
With respect to the final conclusion about "unknown" HFFRs, it would be wise to quote Paracelsus[1] who already stated in the 16th century: "alle Dinge sind Gift und nichts ist ohne Gift, allein die Dosis macht's, dass ein Ding kein Gift sei" ("all things are poison and nothing is without poison, only the dose makes a thing not a poison"). Thus, the observed *in vitro* or *ex vivo* neurotoxic effects of the investigated compounds should always be interpreted in combination with the actual human exposure situation.

[1] Paracelsus is the pseudonym of Philippus Aureolus Theophrastus Bombastus von Hohenheim, the father of toxicology, 1493-1541.

Samenvatting voor niet-ingewijden

Brand zorgde alleen in 2012 al voor meer dan 70 dodelijke slachtoffers, zo'n 800 gewonden en voor miljoenen euro's aan materiële schade in Nederland (bron: Centraal Bureau voor de Statistiek). Om het ontstaan en verspreiden van brand te vertragen wordt plastic van bijvoorbeeld televisiebehuizingen en schuimrubber van meubels tijdens het productieproces gemengd met vlamvertragende chemicaliën. Ondanks dat hierdoor het aantal dodelijk slachtoffers, gewonden en de materiële schade afneemt, heeft het één groot nadeel: veel van deze chemische stoffen blijken het milieu te vervuilen en schadelijk (giftig, toxisch) te zijn voor mensen en dieren.

In de jaren '20 van de vorige eeuw werden polychloorbifenylen (PCBs, zie figuur 1) op de markt gebracht als vlamvertragers. Jaren later werden ze echter terug gevonden in het milieu en sinds de jaren '70 van de vorige eeuw zijn ze dan ook verboden om nog toe te passen. Men kwam tot ontdekking dat mensen via het eten van 'vervuilde' vis werden blootgesteld aan PCBs. PCBs bleken hardnekkige stoffen te zijn die bij voorkeur in vetweefsel gaan zitten en zich hierin ophopen. Hierdoor kan het jaren duren totdat ze het menselijk lichaam weer verlaten. Ondanks deze ongewenste effecten kwamen er nieuwe regelgevingen met steeds strengere eisen voor wat betreft brandveiligheid, bijvoorbeeld 'veilige' kerstversiering. Brandrisico's nemen echter ook toe door het toenemend aantal elektronische apparaten per huishouden die door oververhitting of kortsluiting brand kunnen veroorzaken. Al snel werden dan ook nieuwe vlamvertragende chemicaliën ontwikkeld: gebromeerde vlamvertragers (BFRs; brominated flame retardants), organische verbindingen met broom, zie figuur 1. Een belangrijke groep binnen de BFRs zijn de polygebromeerde difenylethers (PBDEs). Daarnaast is tetrabroombisfenol-A (TBBPA; tetrabromobisphenol-A) een veel gebruikte gebromeerde vlamvertrager. Helaas werden ook deze vlamvertragers al snel terug gevonden in het milieu en in mensen en dieren. Net als met PCBs vindt blootstelling aan BFRs plaats door bijvoorbeeld het eten van vis. Ook werd duidelijk dat zowel de PCBs als een aantal BFRs het menselijk lichaam wel makkelijk verlaten via moedermelk (een substantie die vet bevat). Het gevolg is dat zuigelingen aan hoge concentraties vlamvertragers blootgesteld kunnen worden.

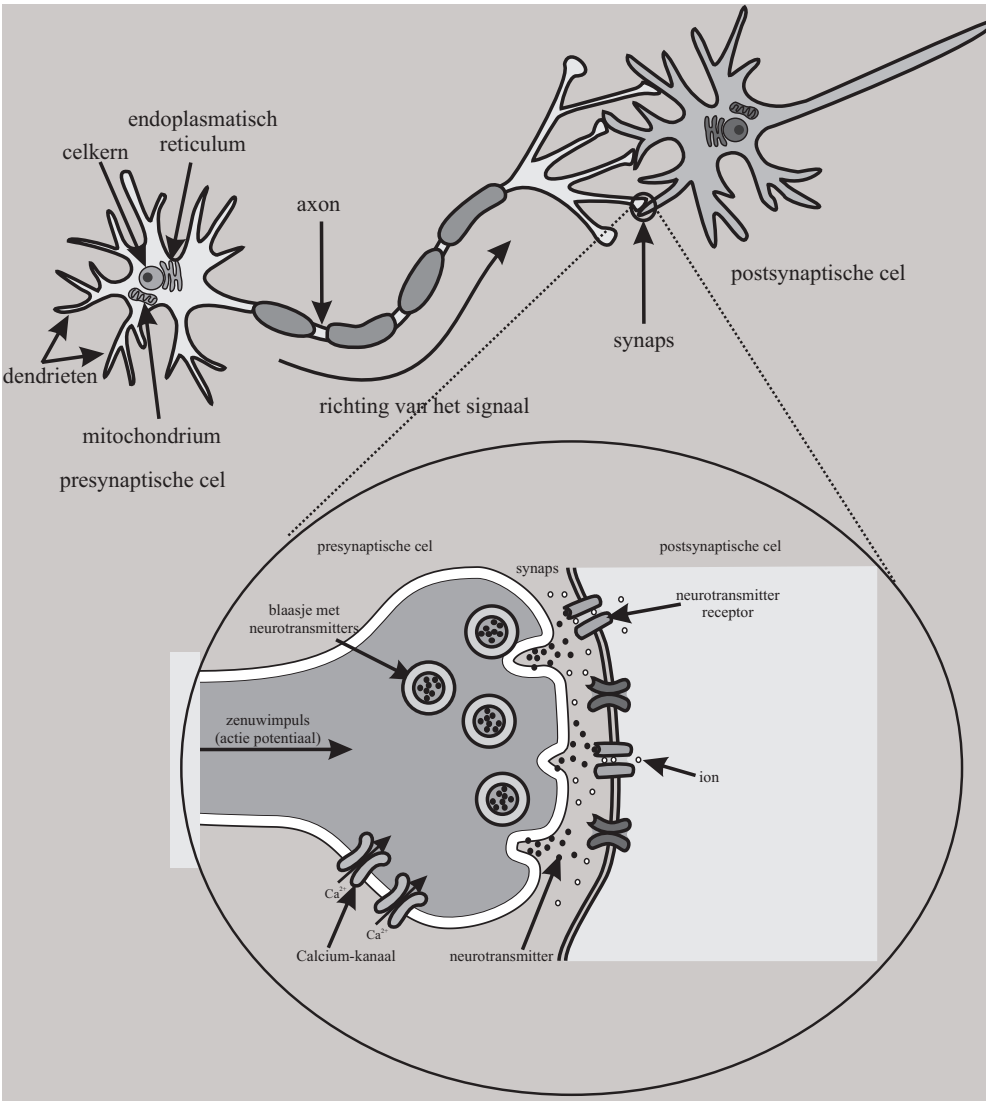


Figuur 1. Schematische weergave van de chemische structuren van polychloorbifenylen (PCBs; A), polygebromeerde difenylethers (PBDEs; B), tetrabroombisfenol-A (TBBPA; C), aluminium diethylfosinaat (Alpi; D) en zink stannate (ZS; E). Zowel PCBs als PBDEs zijn synthetisch-organische stoffen die bestaan uit twee benzeenringen met twee tot tien halogeenatomen (chloor voor de PCBs en broom voor de PBDEs). Hierdoor zijn 209 verschillende verbindingen (congeneren) mogelijk. TBBPA is ook een gebromeerde organische verbinding. De AFRs Alpi en ZS zijn respectievelijk een metaal (aluminium) fosfinaat zout en anorganisch bimetaal (zink en tin) zout.

Daarnaast werd ontdekt dat BFRs in de loop der jaren uit het plastic van bijvoorbeeld een televisie kunnen 'lekken' en vervolgens in huisstof terecht komen. Baby's en kinderen die rondkruipen en van alles in hun mond stoppen worden op die manier extra blootgesteld. Uit voorgaande onderzoeken naar de gevolgen van blootstelling aan PCBs en BFRs blijkt dat met name het (ontwikkelende) zenuwstelsel erg gevoelig is voor de ongewenste effecten van deze chemicaliën. Zo wordt blootstelling aan PCBs en/of PBDEs bij kinderen geassocieerd met verminderde psychomotorische ontwikkeling en IQ. Doordat (jonge) kinderen relatief meer worden blootgesteld dan volwassenen (via moedermelk en kruipen op de grond) en de hersenen van kinderen nog volop in ontwikkeling zijn, worden steeds meer vraagtekens gezet bij de veiligheid van vlamvertragers. Ze voorkomen of verminderen dan wel schade, gewonden en dodelijke slachtoffers door brand, maar weegt dat op tegen de ongewenste effecten bij mensen, dieren en in het milieu? In de Europese Unie (EU) en verschillende staten in de Verenigde Staten zijn dan ook steeds meer BFRs verboden. Om toch aan de huidige veiligheidseisen te kunnen voldoen is er dus vraag naar nieuwe chemicaliën die brand kunnen voorkomen en/of vertragen én die veilig zijn voor milieu, mens en dier.

Om te onderzoeken en te begrijpen hoe PCBs en BFRs schade kunnen veroorzaken aan het zenuwstelsel, is kennis over het functioneren van het zenuwstelsel essentieel (een gedetailleerdere beschrijving staat in hoofdstuk 1). De belangrijkste taak van het zenuwstelsel is het versturen, ontvangen en interpreteren van informatie uit alle delen van het lichaam. De hersenen zijn hierin het belangrijkst (het 'controlecentrum') en bevatten dan ook zo'n honderd miljard zenuwcellen (neuronen). In het bovenste deel van figuur 2 zijn twee neuronen met een aantal cel-onderdelen afgebeeld: een celkern waar DNA opgeslagen ligt, het endoplasmatisch reticulum waar o.a. eiwitvorming plaatsvindt en de mitochondria, de energiefabriekjes van de cel. Neuronen hebben veel uitlopers: dendrieten die informatie van andere cellen ontvangen en één axon wat signalen doorgeeft naar de synaps voor de voortgang van de communicatie. Deze communicatie vindt plaats in de vorm van het doorgeven van stroompjes. De zenuwimpuls (actiepotentiaal) gaat door het axon van de zogenaamde presynaptische cel naar de synaps, de contactplaats tussen twee cellen. Het onderste deel van figuur 2 zoomt in op de synaps. De synaps van de presynaptische cel bevat blaasjes met neurotransmitters, chemische stofjes die signalen via de synaps doorgeven. Als een actiepotentiaal bij de synaps aankomt, wordt de celmembraan gedepolariseerd waardoor calciumionen (Ca^{2+}) de cel in stromen. De verhoogde concentratie Ca^{2+} prikkelt de blaasjes met neurotransmitters die vervolgens in de synaps worden losgelaten. De neurotransmitters binden vervolgens aan de receptoren (eiwitten waar de neurotransmitter aan bindt waarna een signaal wordt doorgegeven) van de postsynaptische cel waardoor anionen of kationen (iedere neurotransmitter geeft een andere reactie) de cel binnen kunnen en het signaal in de vorm van een elektrisch stroompje voort wordt gezet. De belangrijkste neurotransmitters zijn acetylcholine (ACh; vooral betrokken bij gedrag en cognitieve functies), gamma-aminoboterzuur (GABA, γ-aminobutyric acid; speelt een belangrijke rol met de ontwikkeling van de hersenen) en glutamaat (betrokken bij synaptische plasticiteit: leren en het geheugen). Al deze processen kunnen al binnen slechts een aantal milliseconden gebeuren. Het functioneren van het zenuwstelsel is dus zeer complex waardoor onderzoek naar de ongewenste effecten van vlamvertragers op het zenuwstelsel vanuit verschillende oogpunten plaats moet vinden. In hoofdstuk 4 en 5 van dit proefschrift worden onderzoeken beschreven waarin het werkingsmechanisme van de toxische effecten van PCBs, PBDEs en TBBPA op het zenuwstelsel (neurotoxiciteit) zijn ontrafeld. De neurotoxiciteit is onderzocht met behulp van *in vitro* ("in glas") experimenten: het onderzoek vond plaats in een laboratorium met cellen die als representatief worden beschouwd voor zenuwcellen

in het lichaam (de gebruikte technieken staan beschreven in hoofdstuk 2). Hierdoor kunnen een aantal van de belangrijkste neuronale processen onderzocht worden zonder gebruik te moeten maken van intacte organismen, dieren of mensen. Allereerst is gekeken bij welke dosis van de vlamvertragers de cellen dood gaan. Daarna is meer specifiek onderzocht welk mechanisme (zoals de genoemde Ca^{2+}-huishouding) in de cel hoofdzakelijk zorgt voor de celdood. Met weer andere technieken is gekeken naar het effect van de vlamvertragers op receptoren voor de neurotransmitters (de ACh- en GABA-receptoren). Uit deze onderzoeken blijkt dat een deel van de onderzochte vlamvertragers verschillende aangrijpingspunten in de zenuwcellen en communicatie



Figuur 2. Het bovenste deel van dit figuur geeft twee sterk uitvergrote neuronen weer met een aantal cel-compartimenten, de dendrieten en een enkele axon per cel. Via de axon gaat een actiepotentiaal naar de synaps waar de cellen contact met elkaar maken. Het onderste deel van de figuur zoomt in op de synaps. Door de depolarisatie van de actiepotentiaal stroomt Ca^{2+} de cel in wat zorgt voor het loslaten van neurotransmitters in de synaps. Door binding aan receptoren wordt de communicatie voortgezet.

tussen cellen hebben, deze stoffen zijn duidelijk neurotoxisch. Zo binden PCBs en BFRs bijvoorbeeld aan ACh-en GABA-receptoren waardoor de neurotransmitters er niet of minder goed aan kunnen binden en de signaaloverdracht verstoord wordt. De resultaten van de onderzoeken beschreven in dit proefschrift dragen bij aan de theorieën hoe op celniveau de ontwikkeling van hersenen bij blootgestelde kinderen verstoord wordt wat kan resulteren in de eerder genoemde afwijkingen (verminderde psychomotorische ontwikkeling en IQ). Ook bevestigen de gevonden effecten dat er inderdaad alternatieve, minder toxische, vlamvertragers nodig zijn. Het is echter van groot belang dat deze alternatieve vlamvertragers (AFRs; alternative flame retardants) eerst grondig worden onderzocht op hun veiligheid voor mens en milieu.

In 2009 startte een project van de Europese Unie genaamd ENFIRO waarbij zowel universiteiten als de industrie betrokken werden om te zoeken naar veiligere AFRs (zie hoofdstuk 1, tabel 1 voor een overzicht van de onderzochte vlamvertragers). Het doel van dit promotieonderzoek was daar een steentje aan bij te dragen door te onderzoeken of de geselecteerde AFRs qua neurotoxiciteit veiliger zijn dan de genoemde PCBs en BFRs.

Allereerst werd geïnventariseerd welke informatie al beschikbaar is over de AFRs door te zoeken in wetenschappelijke literatuur en databases van overheden. De gevonden informatie is beschreven in hoofdstuk 3 en omvat de fysisch-chemische eigenschappen van de AFRs, hoe veel er nu al geproduceerd en gebruikt wordt door de industrie, hoe makkelijk het zich door het milieu verspreid, of het in een dieren en/of mensen wordt 'opgeslagen' zoals de hardnekkige PCBs en PBDEs en de toxiciteit. Ondanks dat er uit veiligheidsoverwegingen steeds strengere regels komen voor het op de markt brengen van chemische stoffen, blijkt uit deze literatuurstudie dat soms erg weinig informatie beschikbaar is over de AFRs terwijl ze al wel toegepast worden.

Om een vergelijking te kunnen maken tussen de neurotoxiciteit van de 'oude' en de alternatieve vlamvertragers, is de neurotoxiciteit van de AFRs met dezelfde *in vitro* experimenten onderzocht als de oude vlamvertragers (zie hoofdstuk 6 en 7). Met behulp van een classificatiesysteem (zie hoofdstuk 7, tabel 5) zijn de geteste AFRs op basis van de resultaten ingedeeld als "verwaarloosbaar neurotoxisch", "weinig neurotoxisch", "gemiddeld neurotoxisch" of "zeer neurotoxisch". Voor een volledig neurotoxische risicoanalyse is het echter noodzakelijk te weten hoe de AFRs zich gedragen in de hersenen van een intact organisme. Met behulp van een *in vivo* ("in het levende organisme") experiment kan ook nagegaan worden of de uitgevoerde *in vitro* experimenten inderdaad representatief zijn voor de *in vivo* situatie en zullen er in de toekomst steeds minder *in vivo* experimenten nodig zijn. Voor *in vivo* onderzoek met dieren zijn in Nederland strenge richtlijnen en is toestemming van de dierexperimenten-commissie nodig. Er is dan ook alles aan gedaan het aantal dieren zo laag mogelijk te houden en veel aandacht te besteden aan het welzijn van de dieren. Omdat van de oude vlamvertragers bekend is dat ze vooral negatieve effecten hebben op hersenen die nog in ontwikkeling zijn, werd gekozen om 10 dagen oude muizen eenmalig bloot te stellen aan een niet-dodelijke dosis van de oude vlamvertrager TBBPA, of de AFRs aluminium diethylfosinaat (Alpi; aluminium diethylphosphinate; zie figuur 1) of zink stannaat (ZS; zinc stannate; zie figuur 1). Zoals uitgebreider beschreven in hoofdstuk 8 werden de dieren een week na de blootstelling geofferd en vonden er direct metingen plaats aan plakjes van de hersenen. Ook werd gekeken of in de hersenen, lever en een stukje spier vlamvertragers terug te meten waren. Uit dit *in vivo* experiment bleek dat alle drie de stoffen geen ongewenste effecten hadden op de ontwikkelende hersenen. Daarnaast liet Alpi ook *in vitro* een weinig tot verwaarloosbare neurotoxiciteit zien (zie voor een volledig

overzicht hoofdstuk 9, tabel 4). Uit de resultaten van de onderzoeken beschreven in dit proefschrift blijkt dan ook dat er - vanuit een neurotoxicologisch oogpunt - geschikte AFRs zijn die de huidige vlamvertragers kunnen vervangen.
De belangrijkste conclusies van dit promotieonderzoek zijn:

- ✓ PCBs en PBDEs kunnen GABA en ACh receptoren beïnvloeden wat mogelijk de in andere studies gevonden afwijkingen in gedrag en hersenontwikkeling verklaard.
- ✓ TBBPA laat bij bijna alle *in vitro* onderzochte aspecten toxische effecten zien terwijl de toxiciteit *in vivo* nog altijd onduidelijk blijft.
- ✓ De gevonden toxische effecten van PCBs, PBDEs en TBBPA ondersteunen de noodzaak voor ontwikkeling van alternatieve, minder toxische vlamvertragers.
- ✓ Het is zorgwekkend dat sommige AFRs al gebruikt worden terwijl er nog nauwelijks informatie is over of ze veilig zijn voor milieu, mens en dier.
- ✓ De onderzochte AFRs zijn ingedeeld met behulp van een classificatiesysteem waaruit blijkt dat er (uit een neurotoxicologisch oogpunt) geschikte alternatieven zijn.
- ✓ Voor een volledige risicoanalyse is aanvullende informatie noodzakelijk zoals of de AFRs toxisch zijn voor andere belangrijke lichaamsfuncties en de gedraging in het milieu.

Ten slotte, ondanks de nog openliggende vragen over veiligheid van vlamvertragers gelden ook in dit geval de wijze woorden van Paracelsus[1] die in de 16^{e} eeuw al stelde: "alle Dinge sind Gift und nichts ist ohne Gift, allein die Dosis macht's, dass ein Ding kein Gift sei" ("alles is giftig en niets is zonder gif, alleen de dosis maakt dat een stof niet giftig is").

Gebruikte afkortingen:

ACh	acetylcholine; een neurotransmitter, boodschapper molecuul
AFRs	alternative flame retardants; afkorting voor de onderzochte alternatieve vlamvertragers
Alpi	aluminium diethylfosinaat; aluminium diethylphosphinate, een onderzochte AFR
BFRs	brominated flame retardants; overkoepelende term voor alle broomhoudende vlamvertragers
Ca^{2+}	calciumionen, spelen een belangrijke rol bij de communicatie tussen neuronen en bij cel sterfte
GABA	gamma-aminoboterzuur; γ-aminobutyric acid, een neurotransmitter
In vitro	in glas; experimenten met behulp van cellen
In vivo	in het levende organisme; experiment met behulp van proefdieren
PBDEs	polygebromeerde difenylethers; een groep gebromeerde vlamvertragers
PCBs	polychloorbifenylen, een groep oude vlamvertragers
TBBPA	tetrabroombisfenol-A; tetrabromobisphenol-A, een gebromeerde vlamvertrager
ZS	zink stannaat; zinc stannate, een onderzochte AFR

[1] Paracelsus, pseudoniem van Philippus Aureolus Theophrastus Bombastus von Hohenheim, de vader van de toxicologie, 1493-1541.

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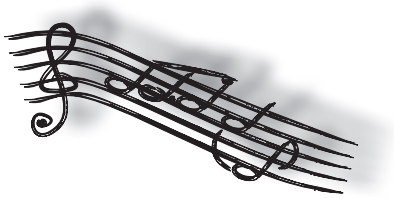

muziek is het hart van de mens

Richard Wagner
(componist, 1813-1883)

Curriculum Vitae, publications and conferences

Curriculum Vitae

Hester Hendriks (1984) grow up in Oosterbeek, the Netherlands, and graduated high school at the Thorbecke Scholengemeenschap in Arnhem in 2001. After obtaining a propaedeutics in Life Sciences at the HAN University of Applied Sciences in 2002, she started her study Biomedical Sciences at the Radboud University Nijmegen. In 2005, she obtained her bachelor's degree and continued her study with a master in Toxicology and minors in Drug Research, and Reproduction and Epidemiology. During her master, she completed three internships in different specialties of Toxicology. After successfully completing her final internship at the Neurotoxicology Research Group of the Institute of Risk Assessment Sciences of the Utrecht University, she started her PhD-research at the same department in 2009. Under supervision of Dr. Remco H.S. Westerink and Prof.dr. Martin van den Berg, she assessed the neurotoxic potential of alternative, halogen-free flame retardants in relation to the (neuro)toxicity of brominated flame retardants as part of the EU funded project ENFIRO (A Life Cycle Assessment of Environment-Compatible Flame Retardants: Prototypical Case Study). During her PhD-research, she followed the Postgraduate Education in Toxicology program to obtain a registration as toxicologist. Besides science, arts and music plays an important role in her life, e.g. she plays as concertmaster and tutti-violinist in several (amateur) orchestras and ensembles.

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- Hendriks, H.S., Koolen, L.A., Dingemans, M.M., Viberg, H., Lee, I., Leonards, P.E., Ramakers, G.M. and Westerink, R.H. (2014). ***Developmental neurotoxicity of brominated and halogen-free flame retardants in neonatal exposed mice.*** 35th Anniversary Meeting of The Netherlands Society of Toxicology (NVT) - Veldhoven, The Netherlands. Abstract, poster and oral presentation.
- Hendriks, H.S. and Westerink, R.H. (2013). ***In vitro neurotoxic hazard characterization of brominated and halogen-free flame retardants.*** 14th Meeting of the International Neurotoxicology Association (INA) - Egmond aan Zee, The Netherlands. Abstract, poster and oral presentation.
- Hendriks, H.S. and Westerink, R.H. (2013). ***In vitro neurotoxic hazard characterization of brominated and halogen-free flame retardants.*** Annual meeting of the Netherlands Society of Toxicology (NVT) - Zeist, The Netherlands. Abstract, poster and oral presentation.
- Hendriks, H.S. and Westerink, R.H. (2012). ***In vitro neurotoxic hazard characterization of brominated and halogen-free flame retardants.*** ENFIRO workshop "A workshop on alternative flame retardants looking at flammability, applications, toxicity, exposure to life cycle assessment" - Brussels, Belgium. Abstract and oral presentation.
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