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Case study on the use of Integrated Approaches for Testing and Assessment for DNT to prioritize a class of Organophosphorus flame retardants

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NO. 364

Case study on the use of Integrated Approaches for Testing and Assessment for DNT to prioritize a class of Organophosphorus flame retardants

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Paris 2022

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Foreword

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by the United State (US) (Helena T Hogberg[1, 2)], Jui-Hua Hsieh[1)], Xiaoqing Chang[3)], Nisha Sipes[4)], Tim Shafer[4)], Mamta Behl[1, 5)]) for illustrating practical use of IATA and submitted to the 2021 review cycle of the IATA Case Studies Project.

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The case study was reviewed by the project team, and endorsed at the 6th meeting of the Working Party on Hazard Assessment in June 2022.

The case study is illustrative examples, and their publication as OECD monographs does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, the cases study should not be interpreted as official regulatory decisions made by the authoring member countries.

This document is published under the responsibility of the Chemicals and Biotechnology Committee of the OECD.

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Abbreviations and Acronyms

AOP	Adverse Outcome Pathway
AUC	Area under the curve
BDE-47	2,2'4,4'-brominated diphenyl ether
BDE	Diphenyl ether
BFR	Brominated flame retardant
BMC	Benchmark concentration
BrdU	Bromodeoxyuridine
CPSC	Consumer Product Safety Commission
CNN	Convolutional neural network
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity
DNT-DIVER	Developmental NeuroToxicity Data Integration and Visualization Enabling Resource dpf: Days post fertilization
EHDP	2-ethylhexyl diphenyl phosphate
ESC	Embryonic stem cell
FHSA	Federal Hazardous Substances Act
HTTK	High-throughput toxicokinetic model
IVIVC	*In vitro* to *in vivo* correlation
iPSC	Induced pluripotent stem cell
IATA	Integrated Approaches for Testing and Assessment
IDDP	Isodecyl diphenyl phosphate
IPP	Isopropylated phenyl phosphate
LUHMES	Lund human mesencephalic cell
MEA	Micro-electrode array
NAS	National Academy of Science
NTP	National Toxicology Program
NCC	Neural crest cell
NPC	Neural progenitor cell
NAM	New Approach Methodology
OECD	Organisation for Economic Co-operation and Development
OPFR	Organophosphorus flame retardant
PNS	Peripheral nervous system
POD	Point of departure
BPDP	Tert-butylated phenyl diphenyl phosphate
TMPP	Trimethyl phenyl phosphate
TPHP	Triphenyl phosphate
TDCIPP	Tris(1,3-dichloroisopropyl phosphate
TCEP	Tris(2-chloroethyl)phosphate
TBBPA	3,3',5,5'- tetrabromobisphenol A
WoE	Weight of evidence

Executive Summary

This Integrated Approaches for Testing and Assessment (IATA) case study was developed to show how a battery of *in vitro* and complementary non-mammalian animal models (e.g., zebrafish) can be used to prioritize a class of compounds for further testing. Herein, we compare the relative toxicity of organophosphorus flame retardants (OPFRs) that are currently being used as replacements of some of the phased-out brominated flame retardants (BFRs). This class of chemicals was selected as a case example because while the replacement OPFRs have been extensively used, their toxicity profile remains unknown. It is not feasible or practical to run guideline developmental neurotoxicity (DNT) studies on all members of the class and hence, there is a need to prioritize and rank the toxicity of these compounds for further *in vivo* testing.

This case study comprised of several *in vitro* assays and zebrafish as a complementary animal model. *In vivo* neurobehavioral data and human exposure data was collected wherever available. Open-source pharmacokinetic models were used to predict internal concentration from external exposure to facilitate contextualizing the *in vitro* finding in the battery to human exposure data.

Overall, this case study demonstrates applicability of the DNT battery in a prioritization and hazard characterization context. Furthermore, exposure data in humans helped with interpreting the findings in the battery thereby supporting the contextualization of these studies in potential future risk assessment.

1. Introduction

Due to their toxicity and persistence in the environment, brominated flame retardants (BFRs) are being phased-out of commercial use, leading to the increased use of alternative chemicals such as the organophosphorus flame retardants (OPFRs). There is, however, limited information on the potential health effects of OPFRs. Due to the structural similarity of the OPFRs to organophosphorus insecticides, there is concern regarding developmental and acute neurotoxicity. In 2019, the National Academy of Science (NAS) released a report on the need for a class approach of the hazard assessment of flame retardants (NAS 2019). In the report, they recognized that one of the biggest challenges for the risk-assessment community is how to move away from the traditional chemical-by-chemical approach to analyses that evaluate multiple chemicals together. Furthermore, they identified that some primary issues with the traditional approach are that chemicals on which data are insufficient are typically treated as not hazardous, that untested chemicals are often substituted for hazardous chemicals, and that cumulative exposure and risk are often ignored (NAS 2019).

Although it is challenging to evaluate chemical groups, the number of chemicals in use today demands a new approach to risk assessment, and, per the NAS, the class approach is a scientifically viable option. In light of the momentum to regulate by chemical class, a coalition of organizations and individuals petitioned the U.S. Consumer Product Safety Commission (CPSC) to initiate regulatory action under the U.S. Federal Hazardous Substances Act (FHSA) to ban several flame retardant products. To decide whether a ban should be enacted, U.S. CPSC must first conduct a hazard assessment to determine whether the chemical is toxic as defined in the U.S. FHSA. Consequently, OPFRs were nominated by U.S. CPSC to the National Toxicology Program (NTP) for testing based on projected increased exposure and lack of hazard characterization data.

In this IATA for developmental neurotoxicity (DNT), we evaluated a set of OPFRs (triphenyl phosphate [TPHP]), isopropylated phenyl phosphate [IPP], 2-ethylhexyl diphenyl phosphate [EHDP], tert-butylated phenyl diphenyl phosphate [BPDP], trimethyl phenyl phosphate [TMPP], isodecyl diphenyl phosphate [IDDP], (tris(1,3-dichloroisopropyl) phosphate [TDCIPP], and tris(2-chloroethyl)phosphate [TCEP]) in a battery of cell-based *in vitro* assays and alternative model organisms and compared the results to those obtained for two classical BFRs (3,3’,5,5′- tetrabromobisphenol A [TBBPA] and 2,2′4,4′-brominated diphenyl ether [BDE-47]) (Table 1). A single assay cannot reflect the complexity of the developing nervous system. A comprehensive coverage of most neurodevelopmental processes is necessary since compounds oftentimes act by different mechanisms. Hence, we utilized a combination of high throughput, high content functional assays as well as complementary models to evaluate neurobehavior. This guidance document provides a suggested framework for use of New Approach Methodologies (NAMs) for prioritization of classes of environmental chemicals with concern for DNT. It follows the general principles of OECD guidance as described elsewhere (OECD 2014; OECD 2016a; OECD 2016b; OECD 2016c).

An initial set of assays (Table 2) comprised neural proliferation (human), neurite outgrowth (human), neural crest migration (human), neuronal firing (rat), and behavior of zebrafish (Danio rerio) in addition to other assays on developmental toxicity (Behl et al. 2015). Later, the neuronal network formation (Frank et al. 2017), 3-D neurospheres (multiple end-points, human) (Klose J 2021), and additional studies using some similar assays (neurite outgrowth, zebrafish behavior), were added (Behl et al.

2019; Delp et al. 2018) to better understand variability in outcome (Table 2). All assays were performed in a concentration-response format, allowing for the determination of the benchmark concentration (BMC), the lowest concentration where a chemically-induced response exceeds background noise (Figure 1). Two more mechanistic studies using transcriptomics and metabolomics were considered for future use in the development of AOPs (Hogberg et al. 2021; Klose et al. 2020) together with additional *in vitro*, *in vivo* and epidemiology data obtained from the literature (Table 3). However, these studies will not be incorporated in the current case study which is mainly focused on prioritization.

2. Purpose

2.1. Purpose of use

This IATA case study was developed to be included in the OECD Guidance Document under development by the DNT expert group of the OECD, with the objective to inform on the testing battery, its usage and interpretation. Specifically, this case study is intended to provide a broad example for the use and application of the DNT battery for prioritization of a class of compounds. An IATA, such as this one can help inform organizations like the U.S. CPSC who are evaluating NAMs for use in prioritization and ultimately decision-making.

2.2. Target chemical(s)

The target class of chemicals for this case study was aromatic organophosphorus flame retardants (OPFRs), the most common replacements of the phased-out polybrominated diphenyl ethers (BDEs). These chemicals were nominated to the NTP by the U.S. CPSC based on projected increased exposure and lack of hazard characterization data. Herein, we compare relative activity of the aromatic OPFRs using a DNT battery. Furthermore, we compare the relative toxicity of the novel aromatic OPFRs to brominated flame retardants (BDE-47 and TBBPA), as well as representative halogenated OPFRs (TDCIPP and TCEP). Individual compounds in this IATA are shown in Table 1.

Table 1. Chemicals assessed in this case study

CAS	Chemical Name	Chemical.ID	Structure
5436-43-1	2,2'4,4'-Tetrabromodiphenyl ether	BDE-47*	
79-94-7	3,3',5,5'-Tetrabromobisphenol A	TBBPA	
115-86-6	Triphenyl phosphate	TPHP	
68937-41-7	Phenol, isopropylated, phosphate (3:1)	IPP**	
1241-94-7	2-Ethylhexyl diphenyl phosphate	EHDP**	
1330-78-5	Tricresyl phosphate	TMPP**	
29761-21-5	Isodecyl diphenyl phosphate	IDDP	
56803-37-3	tert-Butylphenyl diphenyl phosphate	BPDP**	
13674-87-8	Tris(1,3-dichloro-2-propyl)phosphate	TDCIPP	
115-96-8	Tris(2-chloroethyl) phosphate	TCEP	

*Phased out flame retardant

**These are not single compounds but instead, an isomeric mixture obtained as a result of the manufacturing process

2.3. Endpoints

This IATA comprises representative *in vitro* and non-mammalian model assays that cover domains of developmental neurotoxicity (DNT) and acute neurotoxicity (NT) (Table 2). Assays and the relevance of selected key processes/endpoint for DNT are briefly described. For more detailed information of the specific assays see EFSA report (Masjosthusmann et al. 2020) and references in Table 2. The critical contribution of this IATA is that a unified data analysis pipeline was applied across a common set of characterized chemicals that was provided to each investigator. Individual researchers tested the same set of chemicals in their respective cell-based or alternative animal assay that captured a unique aspect of neurodevelopment. The NTP then developed a data analysis pipeline that allowed for these assays to be combined to form a battery with improved biological coverage.

2.3.1. Proliferation

Proliferation of neural progenitor cells (NPCs) is an important key process that if perturbed can decrease the number of cells in the CNS and lead to e.g., microcephaly (Cugola et al. 2016; Ming et al. 2016). Two assays were used to assess proliferation.

i) proliferation@IUF

Human NPCs (hNPCs) of fetal origin (Lonza) from three different individuals (gestational week 16-19) were cultured as free floating neurospheres in proliferation medium (Klose et al. 2021). Proliferation of primary hNPC, grown as neurospheres in 3D, was studied by bromodeoxyuridine (BrdU), analyzed after 3 DIV of exposure via a luminescence based BrdU Assay (Roche).

ii) proliferation@USEPA

Cell proliferation was determined in human neural progenitor cells (hNP1) derived from embryonic stem cells by immunocytochemical assessment of BrdU incorporation into replicating DNA (Behl et al. 2015). Briefly, cells were exposed to chemicals for twenty hours before BrdU was added to the media for another four hours. Cells were then fixed and stained for BrdU. Total amount of cells and BrdU positive cells were counted using high-content imaging on a Cellomics Arrayscan VTI (Software: TargetActivation Bioapplication V4).

2.3.2. Oligodendrocyte differentiation

After proliferation the neural progenitor cells start to differentiate into neurons and glial cells. Oligodendrocytes is one type of glial cells with the function of myelinating axons in the CNS. Oligodendrocytes are operating near their metabolic capacity, and are therefore especially sensitive to chemical disturbances and alternations in oligodendrocyte proliferation and differentiation (Wiggins 1986). One assay was used to assess oligodendrocyte differentiation.

i) oligodendrocyte differentiation@IUF

Primary hNPCs of fetal origin (Lonza) grown as neurospheres in 3D were plated to attach in the absence of growth factors (Klose et al. 2021). This initiated cell migration out of the plated sphere and differentiation into radial glia, neurons, and oligodendrocytes. After 5 days of migration and differentiation during chemical exposure, human neurospheres were fixed and stained for the oligodendrocyte marker O4. The number of oligodendrocytes within the migration area were counted

manually or by using the Omnisphero platform and divided by the number of total nuclei (Hoechst 33258) to express the % of differentiated oligodendrocytes.

2.3.3. Migration

Recent proliferated cells will migrate away from their place of origin to their final position in the brain. The cell migration process occurs at different time points for different cell types. Since the migration process can continue for several months after birth, the brain is especially sensitive to injuries during a long period (Rice and Barone 2000). Migration was assessed for three different cell types, neural crest cells (NCC), neurons and oligodendrocytes.

i) NCC migration@UKonstanz

Human embryonic stem cells (H9 line) were differentiated into neural rosettes and FACS-sorted for positive expression of the NCC marker HNK-1 and negative for expression of Dll1 (Nyffeler et al. 2017). NCC were seeded in wells containing silicon stoppers (Platypus Technologies, Madison, WI, US) to create a cell free area. One day after seeding the stoppers were removed and the cells could migrate into the cell free area. After 24 hours of migration the cells were exposed to chemicals for additional 24 hours before the migration was assessed by imaging on a high content microscope (Cellomics ArrayScanVTI, Thermo Fischer). Quantification of the migration was performed using a software tool (freely accessible at http://invitrotox.unikonstanz.de/).

ii) neuronal migration@IUF
iii) oligo migration@IUF

Primary hNPCs of fetal origin (Lonza) grown as neurospheres in 3D were plated in the absence of growth factors (Klose et al. 2021). Once attached the cells started to migrate and differentiate. After 5 days of chemical exposure the cells were fixed and stained for neuronal (βIII tubulin) and oligodendrocyte (O4) markers. The migration from the sphere core of the different cell types were assessed automatically using two convolutional neural networks (CNN) based on the Keras architecture implemented in Python 3, trained to identify both cell types.

2.3.4. Neurite outgrowth

During neuronal differentiation and maturation, axons and dendrites form a neuronal network, initially by elongation and branching of the neurites (outgrowth). Disturbance to this process can lead to impaired synaptogenesis and neuronal function. The complexity of the network formation makes it a vulnerable process for chemical perturbation and any interference with e.g., gene expression, membrane receptors and ion channels or intracellular signaling, can affect the neurite elongation and growth (Audesirk and Audesirk 1998). Six different assays (rodent, human, 2D, 3D, PNS and CNS neurons) were applied to assess the neurite outgrowth.

i) neurite outgrowth@USEPA (rat)

Rat primary mixed cortical cultures were prepared from postnatal day 0 Long-Evans rat pups and seeded at low density (Behl et al. 2015). The cells were exposed 2h post-seeding to chemicals for 48h, then fixed and stained for the neuronal marker βIII tubulin. Neurite outgrowth was assessed by measuring total neurite length per neuron using high-content imaging on a Cellomics Arrayscan VTI (Software: NeuronalProliferation Bioapplication, V4).

ii) neurite outgrowth@MolDevices

Human iPSCs-derived neurons (iCell), consisting of a mixture of postmitotic GABAergic and glutamatergic neurons, were provided by Cellular Dynamics International (CDI, Madison, WI, USA) (Ryan et al. 2016). Cells were seeded at low density for 48 h prior to chemical treatment. After 72 h exposure the cells were stained with Calcein AM and Hoechst 33342. Images of the cells were acquired with ImageXpress Micro XLS system (Molecular Devices, LLC, Sunnyvale, CA, USA) and analysed with

MetaXpress5software (MolecularDevices, LLC, Sunnyvale,CA, USA). Neurite outgrowth was characterized by the extent of the outgrowth (e.g., length of total outgrowth, and mean outgrowth per cell), the number of neurite processes (e.g., total number of processes and mean number of processes per cell), and the extent of branching (e.g., total number of branches and mean number of branches per cell).

iii) neurite outgrowth@USEPA (human)

Human embryonic stem cell-derived neurons (hN2™) obtained from ArunA Biomedical (Athens, GA) were seeded at low density and exposed to chemicals 2h post-seeding ((Behl et al. 2015)). After 48 h, cells were fixed and stained for βIII tubulin (neuronal marker). Neurite outgrowth was assessed by measuring total neurite length per neuron using high-content imaging on a Cellomics Arrayscan VTI (Software: NeuronalProliferation Bioapplication, V4).

iv) iV) CNS neurite outgrowth@UKonstanz

Lund human mesencephalic cells (LUHMES) were differentiated into dopaminergic like neurons for 48 h (Delp et al. 2018). Cells were then replated at a low density and differentiation was continued for 24 h in the presence of chemicals. Cells were stained with Calcein AM and Hoechst 33342 before imaged with a high-content imaging ArrayScan VTI microscope (Cellomics, Waltham, MA, USA) to assess neurite area. For neurite area determination, an automated algorithm was used, which calculates the area of the cell soma and subtracts this area from all calcein-positive pixels imaged.

v) PNS neurite outgrowth@UKonstanz

Human sensory neurons derived from human embryonic stem cells (WA09 Line) were obtained from WiCell (Madison, WI, USA) (Delp et al. 2018). Cells were seeded at low density and exposed to chemicals for 24 h, subsequently stained with Calcein AM and Hoechst 33342 and imaged with a high-content imaging ArrayScan VTI microscope (Cellomics, Waltham, MA, USA) to assess neurite area. For neurite area determination, an automated algorithm was used, which calculates the area of the cell soma and subtracts this area from all calcein-positive pixels imaged.

vi) neurite outgrowth@IUF

Primary hNPCs of fetal origin (Lonza) grown as neurospheres in 3D were plated to attach in the absence of growth factors (Klose et al. 2021). After 5 days of chemical exposure the cells were fixed and stained for neuronal marker (βIII tubulin) and were analyzed for their morphology by characterizing the neurite length (in µm) and area (amount of pixel) calculated automatically by the high-content image analysis (HCA) tool Omnisphero (Schmuck et al. 2017).

2.3.5. Firing/Network formation

To achieve neuronal mature function, the neurons must form cell-cell connections through synapses. In spontaneously electrically active *in vitro* models, electrophysiological techniques can evaluate the neuronal network development with functional synapses. Any disturbance of the synaptogenesis process induced by chemical exposure would most likely alter the electrical activity (Bal-Price et al. 2010). Two assays were used to assess network formation and neuronal function.

i) acute neuronal firing@USEPA

Rat primary mixed cortical cultures were prepared from postnatal day 0 Long-Evans rat pups and plated into the center of each well of a 48 well micro-electrode array (MEA) plate (Behl et al. 2015). On day 13 or 14 *in vitro*, spontaneous network spiking activity (extracellularly recorded action potential “spikes”) was recorded using an Axion Biosystems Maestro 768 channel amplifier and Axion Integrated Studios (AxIS) v1.7 software. Activity was recorded prior to chemical exposure for 1 h (baseline), then each chemical was added and an additional 1 h of activity was recorded. Effects of chemicals were expressed as a percentage of the baseline activity in each well following exposure.

ii) network formation@USEPA

Rat primary mixed cortical cultures were prepared from postnatal day 0 Long-Evans rat pups and plated into the center of each well of a 48 well micro-electrode array (MEA) plate (Frank et al. 2017). Cells were exposed to chemicals 2h after seeding and for 12 days. The spontaneous electrical activity was recorded for 15 min using an Axion Biosystems Maestro 768 channel amplifier and Axion Integrated Studios (AxIS) v1.7 software on DIV 2, 5, 7, 9 and 12. Various parameters were analysed and potency values for chemicals that impacted network activity, including both decreased terminal DIV 12 activity and delayed development of activity over time, the temporal component of each well's recordings was reduced to a single area under the curve (AUC) value (Brown et al. 2016).

2.3.6. Behavior

Behavioral tests (e.g., locomotor activity) are considered critical for DNT risk assessment but are difficult to measure *in vitro*. Zebrafish at early developmental stages (0-5 days post fertilization (dpf), considered non-animal testing according to EU legislation) have shown their potential as a whole organism approach to predict human DNT and are included in this IATA. The fundamental principles of key cellular events during brain development are remarkably conserved across species (Howe et al. 2013; Khan et al. 2017), and the use of alternative species will allow direct testing of cause-effect relationships between toxicant effects on cellular and behavioral endpoints (Nishimura et al. 2015; Padilla et al. 2011). Similar locomotor assays using zebrafish were evaluated from three different laboratories.

i) behavior@Biobide

Adult zebrafish from wildtype AB strain were housed and maintained in accordance with standard 14 h light/10 h dark cycle (Quevedo et al. 2019). Embryos were exposed to chemicals at 3 dpf for 48 h, followed by placement in Daniovision automated tracking system powered by Ethovision (Noldus, The Netherlands). Several parameters were analyzed including velocity, movement duration, and frequency of activity, but the total distance moved was selected as representative of locomotor activity. The mean total distance moved by larvae in each group was measured in 2-min time bins in 2 rounds of 10 min light and 10 min dark phases.

ii) behavior@OregoneStateU

Tropical 5D wildtype adult zebrafish were housed at Oregon State University, Sinnhuber Aquatic Research Laboratory (SARL) in standard 14 h light/10 h dark cycle (Hagstrom et al. 2019). Embryos were collected and cleaned, at 4 hours post fertilization (hpf), before chorions were enzymatically removed at 6 hpf and exposed to chemicals followed by behavioral assessments at 5 dpf using Viewpoint LifeScience Zebraboxes (Truong et al., 2014; Zhang et al., 2017). The testing paradigm of the photomotor response assay consisted of 4 light cycles, each cycle consisting of 3 min of alternating light and dark.

iii) behavior@UCDavis

Tropical 5D wildtype adult zebrafish were obtained from the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University (Corvallis, Oregon) and subsequent generations were raised at UC Davis. Adult zebrafish were maintained under standard laboratory conditions in standard 14 h light/10 h dark cycle (Dach et al. 2019). Embryos were collected and chorions were enzymatically removed and exposed to chemicals at 6 hpf and photomotor behavior was assessed at 4 and 5 dpf using the DanioVision system (Noldus, Leesburg, Virginia). The testing paradigm consisted of 4 light cycles, each cycle consisting of 5 min of alternating light and dark (last dark cycle 15 min). Larval movement was recorded using a GigE camera (Noldus) with infrared filter and tracked using EthoVisionXT software (Noldus). The total distance swam in mm was measured in 1 min bins.

2.3.7. BMC approach

Herein, we re-evaluated datasets from individual assays in the battery using the BMC approach. The advantage of this approach is that the results from the individual assays can now be directly compared with each other using a unified data analysis pipeline to identify potential underlying brain development pathways that may be perturbed following chemical exposure.

The BMC data of neurite outgrowth@USEPA, proliferation@USEPA, and acute neuronal firing@USEPA were retrieved from Behl M. et al., 2015. All the other BMC data, except the BMC data of endpoints at IUF, were retrieved from the DNT-DIVER database (BMC model = "Curvep"). The BMC data of endpoints at IUF were derived based on the raw data published in Klose et al., 2021 using the same analysis method as in the DNT-DIVER.

2.4. Exposure information and estimation of plasma concentration

To relate toxicity data from *in vitro* and complementary animal models, as well as *in vivo* guideline animal studies to human exposure, a PK model included in the U.S. Environmental Protection Agency (EPA) high-throughput toxicokinetics (HTTK) R packaged (httk v1.9) was used to relate external exposure to internal concentration, thereby allowing comparisons across the various exposure scenarios (Blum et al. 2019). The PK model is a 3-compartment model including gut, liver and rest-of-body compartments. The model requires input parameters such as physico-chemical properties, fraction of the chemical unbound in plasma, and intrinsic metabolic clearance (Pearce et al. 2017; Wambaugh et al. 2019). Additional details about the httk three-compartment model can be found in the PBPK model reporting template in Annex 1: PBK Model Reporting and Annex 2: Physiological and pharmacokinetic parameters used in the httk.3comp model).

The internal concentrations (µM) of FRs were estimated from human biomonitoring data using several steps: 1) human biomonitoring data of breast milk, handwipes, and house dust (Cariou et al. 2008; Castorina et al. 2017; Kim et al. 2014; Klose et al. 2021; Stapleton et al. 2012; Stapleton et al. 2014; Sugeng et al. 2017) were converted to daily oral doses for an infant using information (e.g., infant weight/age) in the biomonitoring study, where available, and using standard hand-mouth equations and breastmilk intake amounts (Medicine 1991; Stapleton et al. 2008). Compared to previously used method (Blum et al. 2019), adjustment using lipid content were applied when converting breast milk biomonitoting data, given in ng/g lipid weight, to daily oral doses. Similar adjustment was also applied when converting plasma measurements, given in ng/g lipid weight, to chemical concenteration in total plasma. The following total lipid contents were used: 33 g/L for breast milk (Klose et al. 2021), 2 g/L for cord plasma (Cariou et al. 2008), 7.7 g/L for adult plasma (Cariou et al. 2008), and 1.8 g/L for child plasma (Pac-Kozuchowska et al. 2018). (Medicine 1991; Stapleton et al. 2008). Compared to previously used method (Blum et al. 2019), adjustment using lipid content were applied when converting breast milk biomonitoting data, given in ng/g lipid weight, to daily oral doses. Similar adjustment was also applied when converting plasma measurements, given in ng/g lipid weight, to chemical concenteration in total plasma. The following total lipid contents were used: 33 g/L for breast milk (Klose et al. 2021), 2 g/L for cord plasma (Cariou et al. 2008), 7.7 g/L for adult plasma (Cariou et al. 2008), and 1.8 g/L for child plasma (Pac-Kozuchowska et al. 2018). 2) The 3-compartment human HTTK model simulations were run over a year of exposure (365 days) and at intervals of 8 times a day for breastmilk exposure, and 18 contacts per hour over a 12 hour awake period for exposure via house dust or handwipe samples. When measured plasma data were available, serum density of 1.06 kg/L was used for unit conversion. 3) Plasma concentrations (µM) corresponding to rodent study points-of-departure (POD) and Minimum Risk Levels (MRLs) were estimated using a similar approach as step 2 (e.g., using the 3-compartment HTTK model, simulating over a year, etc.), and using the rodent POD/MRL as daily dose. For further details about the calculations, please see the supporting information of Blum et al. 2019.

On the other hand, the lowest concentration at which activity was noted in the *in vitro* assays was chosen as *in vitro* BMC. The *in vitro* BMCs are compared with the projected concentrations of human exposures or rodent POD/MRL (Blum et al. 2019) and the comparison is presented in Figure 2.

Table 2. Assays represented in the DNT Battery

Assay	Model	References
Proliferation		
proliferation@IUF	Human 3D neurosphere	(Klose et al. 2021)
proliferation@USEPA	Human hNP1	(Behl et al. 2015)
Oligo differentiation		
oligodendrocyte differentiation@IUF	Human 3D neurosphere	(Klose et al. 2021)
Migration		
NCC migration@UKonstanz	Human crest cells	(Nyffeler et al. 2017)
neuronal migration@IUF*	Human 3D neurosphere	(Klose et al. 2021)
oligo migration@IUF*	Human 3D neurosphere	(Klose et al. 2021)
Neurite outgrowth		
neurite outgrowth@USEPA	Rat primary cortical	(Behl et al. 2015)
neurite outgrowth@MolDevices	Human iPSC-derived	(Ryan et al. 2016)
neurite outgrowth@USEPA	Human hN2	(Behl et al. 2015)
CNS neurite outgrowth@UKonstanz	Human LUHMES	(Delp et al. 2018)
PNS neurite outgrowth@UKonstanz	Human ESC-derived	(Delp et al. 2018)
neurite outgrowth@IUF*	Human 3D neurosphere	(Klose et al. 2021)
Firing/Network formation		
acute neuronal firing@USEPA	Rat primary cortical	(Behl et al. 2015)
network formation@USEPA	Rat primary cortical	(Frank et al. 2017)
Behavior		
behavior@Biobide	Zebrafish	(Quevedo et al. 2019)
behavior@OregoneStateU	Zebrafish	(Hagstrom et al. 2019)
behavior@UCDavis	Zebrafish	(Dach et al. 2019)

*Performed together

3. Hypothesis for performing IATA

This IATA case study has been developed to test the hypothesis that the inclusion of data from the DNT battery will aid in the prioritization of a class of chemicals for further testing and would enhance the certainty for the DNT hazard identification and characterization of the novel flame retardants that are being used as replacements for some of the BDEs.

4. Approaches used (Potential Blocks for Inclusion)

We used a variety of *in vitro* and complementary animal models representing domains of acute and developmental neurotoxicity (Table 2) to compare the relative effects of OPFRs with BFRs and utilized point of departure/ benchmark concentration measurements to make activity comparisons across these multiple assays. We then compared the toxicity data from *in vitro* and alternative animal models, to guideline DNT *in vivo* studies and human exposure, by using a high-throughput toxicokinetic model (HTTK) to convert the data, thereby allowing comparisons across the various exposure scenarios. For further details on the HTTK modelling see (Blum et al. 2019).

5. Data/Information gathering

Data from the DNT battery, presented in Figure 1, was collected from the publications described in Table 2. Benchmark concentrations were calculated as described elsewhere (Behl et al. 2015; Hsieh et al. 2019). The data in Figure 1 was grouped based on key processes (proliferation, migration, differentiation, maturation, and function). To contextualize the *in vitro* findings with *in vivo* and exposure assessment, we evaluated studies in the literature and presented them in Figure 2. Citations of publication and estimation of plasma concentration from *in vivo* human and rodent exposure are summarized in Blum et al. 2019. Additional information gathered from the literature on OPFRs and DNT endpoints are summarized and categorized in Table 3. This table includes key processes that currently are not covered by the DNT battery such as microglia differentiation and function and ontogeny of neurotransmitters and receptors. These endpoints are not included as weight of evidence in this IATA but might be important to include in the future. Furthermore, several of these studies include transcriptomics and metabolomics approaches that will be useful for the development of potential DNT AOPs for these compounds and have been summarized elsewhere (Patisaul et al. 2021) and will not be discussed here. Currently, there are data gaps to develop complete AOPs for OPFRs as molecular initiating events (MIE) and key event relationships are missing. However, many effects of OPFRs exposure are key events in AOPs responsible for DNT Li et al. 2019).

Table 3. Preliminary list of endpoints defined during development

Model system	Key Process/ Endpoint Categories	Specific Endpoints/ Cell type	References
In vitro studies	Endocrine disruption	Hormone receptor agonist/antagonist	(Fang et al. 2015; Kojima et al. 2013; Liu et al. 2012)
		Altered levels of hormones and/or related genes	(Kim et al. 2015; Liu et al. 2012)
	Proliferation	Neural progenitor cells*	(Behl et al. 2015; Klose et al. 2021)
	Apoptosis/necrosis		(Pei et al. 2016)
	Migration	Neural crest cells*	(Nyffeler et al. 2017)
		Neurosphere*	(Klose et al. 2021)
	Glial differentiation and function	Astrocytes	(Hogberg et al. 2021; Slotkin et al. 2017)
		Oligodendrocytes*	(Hogberg et al. 2021; Klose et al. 2021; Klose et al. 2020)
		Microglia	(Hogberg et al. 2021)
	Neuronal differentiation and maturation	Ontogeny of neurotransmitters and receptors	(Dishaw et al. 2011; Hausherr et al. 2014; Hogberg et al. 2021)
		Neurite morphology and/or outgrowth*	Behl et al. 2015; Hausherr et al. 2014; Ryan et al. 2016)
		Synaptogenesis	(Hogberg et al. 2021)
	Neuronal function	Electrical activity*	(Behl et al. 2015; Frank et al. 2017; Shafer et al. 2019)
Zebrafish studies	Endocrine disruption	Altered levels of hormones and/or related genes	(Kim et al. 2015; Liu et al. 2012)
	Apoptosis/necrosis		(Wu et al. 2016)
	Glial differentiation and function	Astrocytes	(Sun et al. 2016)
		Microglia	(Shi et al. 2018)
	Neuronal	Ontogeny of neurotransmitters and	(Li et al. 2020; Shi et al. 2018)

	Differentiation and maturation	receptors	
		Neurite morphology and/or outgrowth	(Shi et al. 2018; Sun et al. 2016)
		Synaptogenesis	(Shi et al. 2018; Sun et al. 2016)
	Behavior	Hyperactivity	(Oliveri et al. 2015)
		Anxiety like behavior	(Li et al. 2020)
		Locomotor behavior*	(Dach et al. 2019; Dishaw et al. 2014; Glazer et al. 2018; Hagstrom et al. 2019; Quevedo et al. 2019; Shi et al. 2018; Sun et al. 2016)
In vivo studies	Endocrine disruption	Altered levels of hormones and/or related genes	(Adams et al. 2020)
	Glial differentiation and function	Microglia	(Liu et al. 2020)
	Neuronal differentiation and maturation	Ontogeny of neurotransmitters and receptors	(Liu et al. 2020; Yang et al. 2018)
	Behavior	Anxiety behavior	(Wiersielis et al. 2020)
		Impaired learning and memory	(Yang et al. 2018)
Human observational studies	Endocrine disruption	Altered levels of hormones	(Luo et al. 2020; Yao et al. 2021)
	Behavior	Cognitive development	(Doherty et al. 2019a)
		Adverse behavioral development including withdrawal, attention problems, depression, hyperactivity, and aggression	(Doherty et al. 2019b)
		Decrease in IQ and working memory	(Castorina et al. 2017)
		Social behavioral problems including less responsible behavior, and more externalizing behaviors	(Lipscomb et al. 2017)

*Assays included in the testing battery

6. Application of IATA

This IATA provides a case study that uses the aromatic OPFRs as an example of how the relative activity/toxicity of chemicals within a class can be prioritized using New Approach Methodologies (NAMs). Multiple approaches could be taken to determine activity and/or determine a significance level for chemicals tested in a concentration-response format. In this example, we placed a high emphasis on overall activity, and ranked the chemicals based on the lowest BMCs (Figure 1 & Figure 2). Alternatively, higher emphasis could be placed on the selectivity of effects, wherein those compounds, which have effects on development and on the nervous system with little or no effects on cytotoxicity would be of greater concern. Another prioritization method would be to use the "weight-of-evidence" approach (e.g., the total number of assays in which a chemical has an effect in combination with the degree of that effect).

Overall, it appears that the OPFRs as a class are active. In this case other factors such as chemical structure might be taken into consideration while prioritizing compounds for further testing. For example, the National Toxicology Program has selected TPHP and IPP as two representative compounds to test in *in vivo* DNT studies. The rationale for selection of these compounds was to compare toxicity in a branched (IPP) versus unbranched (TPHP) compound. Additionally, TPHP forms the building blocks for all other chemicals in this class, and hence, it is valuable to understand the toxicity due to the presence of TPHP. The goal is to use these two representative compounds (TPHP and IPP) as "anchors" for the remaining class. For example, understanding how the *in vitro* data compare to *in vivo* findings for these compounds, along with exposure assessment, will enable us to understand and contextualize the *in vitro* battery for this class similar to that noted for other flame retardants like BDE-47 (Blum et al. 2019) (Figure 2) without having to run guideline DNT studies on all members of the class. In Figure 2, in comparing the *in vitro* BMC to human plasma concentration we note that the BMCs is about two orders of magnitude above the range of plasma concentrations estimated from human exposure of the parent compound.

However, it should be noted that, even though the *in vitro* BMC appears to be higher for these latter compounds, there are limited exposure data for these OPFRs. This is important because, while we do not know the potential health effects of these OPFRs, they have patterns of *in vitro* activity at comparable concentrations similar to that of the phased-out flame retardant (e.g., BDE-47 and TBBPA). Furthermore, for TDCIPP, the plasma concentration estimated from *in vivo* MRL lies within the range of plasma concentrations estimated from human exposure data, which might raise a concern for the regulatory community.

While prioritizing compounds, there are some noteworthy limitations with the current DNT battery: 1) lack of metabolism in the *in vitro* models (although some of that is covered by zebrafish), 2) lack of presence of assays for some key processes such as myelination and the differentiation and proliferation of astrocytes, and microglia.

Nonetheless this approach appears to be a good starting point for prioritization. We are currently adding data that provide a better mechanistic understanding on these compounds (Hogberg et al. 2021; Klose et al. 2020; Patisaul et al. 2021) that may help towards creation of putative AOPs in the future.

Overall, while using an IATA for regulatory decision-making, information from the literature (hazard identification and *in silico*) along with that from the DNT testing battery can be combined to first evaluate

the quality of data that exists (Figure 3). From a pragmatic standpoint, the data may not always be sufficient and hence decision-making may be associated with some level of uncertainty. Any exposure information (when available) can then be converted into estimate plasma concentration by consideration of exposure scenario and pharmacokinetics. The estimated plasma concentration can then be compared to *in vitro* BMCs from the testing battery to inform correlation between the range of human exposure and *in vitro* DNT activity. If the *in vitro* BMC of a chemical is much higher than the plasma concentration estimated from human exposure, the chemical won't cause a big concern. *In vivo* animal studies should be included wherever possible for weight of evidence (WoE). All the available data is then taken into consideration to either prioritize compounds for further testing by running subsequent *in vivo* DNT studies which can be tailored to evaluate corresponding endpoints triggered by the *in vitro* battery or 2) the data may be used for regulatory decision-making (e.g., weight of evidence) in the absence of running further tests depending on the context and resources (Figure 3).

6.1. Uncertainty

1) **Lack of data across assays in the battery:** One of the sources of uncertainty is that some of the flame retardants (e.g., TDCIPP) have not been tested in all of the assays in the battery. Hence, it is a challenge to discuss what might be the most sensitive endpoint for this compound.

2) **Assay confidence:** Although assays like "neurite outgrowth" are generically used in the field, it is important to note that all neurite growth assays are not alike. For example, across the six neurite outgrowth assays tested in this battery, the OPFRs are active in 3/6 and inactive in the other 3. Similarly, for zebrafish behavior, TCEP is active in 1/3 assays. Some sources of this discrepancy include but are not limited to (i) dose/concentration-range tested (ii) models used (iPSC vs ESC for *in vitro* and zebrafish strain for complementary animals), (iii) other protocol parameters (type of media, chorion on/off, volume of media, exposure paradigm - static versus renewal).

3) **# of hits within the assay battery:** Another important factor to consider when prioritizing compounds for further testing is how many assays does a compound need to be active in. For example, TCEP is active in neuronal proliferation and 1/3 zebrafish assays. While TCEP may have a very specific mechanism (inhibit proliferation) compared to other flame retardants that affect multiple pathways, we have lower confidence in this data due to the discrepancy within zebrafish assays.

4) **Lack of directionality of outcome across assays:** While some compounds are active in multiple assays, at this point, we are not certain about the order in which these effects are noted. For example, for BDE-47, it appears that network formation is the most sensitive (lowest BMC); however, it is difficult to tell whether alterations in network formation affect neurite outgrowth or vice versa.

5) **Metabolism:** One of the large limitations and uncertainty associated with *in vitro* assays is relating findings to humans due to lack of metabolic capability. Hence, it is unknown whether a compound's metabolite might be bioactivated or deactivated *in vivo* based on *in vitro* findings.

6) **Underlying principles with IVIVC:** When contextualizing *in vitro* data to human exposure levels, the approach utilizes underlying PK model assumptions including absorption, clearance, and bioavailability (e.g., binding to plasma proteins, potential to cross the BBB and placenta). Moreover, there is limited information about the bioavailability *in vitro* as the chemicals can bind to plastic ware and components in the cell media. Inter-individual variability in physiology and pharmacokinetic parameters also exist. All these factors may cause some uncertainty when using the approach.

7) **Weighting assays:** In this example, all assays have been given equal weightage. However, something to consider might be as to whether assays with higher biological complexity (such as network formation and zebrafish) may be given more weightage relative to the less biologically complex assays.

However, it is important to note that despite the above uncertainties, using the battery is extremely valuable in prioritizing compounds for further testing because these are high content functional assays that represent critical neurodevelopmental milestones.

6.2. Strategy and integrated conclusion

The overall purpose of this IATA was to address the primary question: How can we prioritize the class of OPFRs for further testing. Additionally, the IATA also sheds light on the relative hazard characterization of compounds within the class that may be used as WoE.

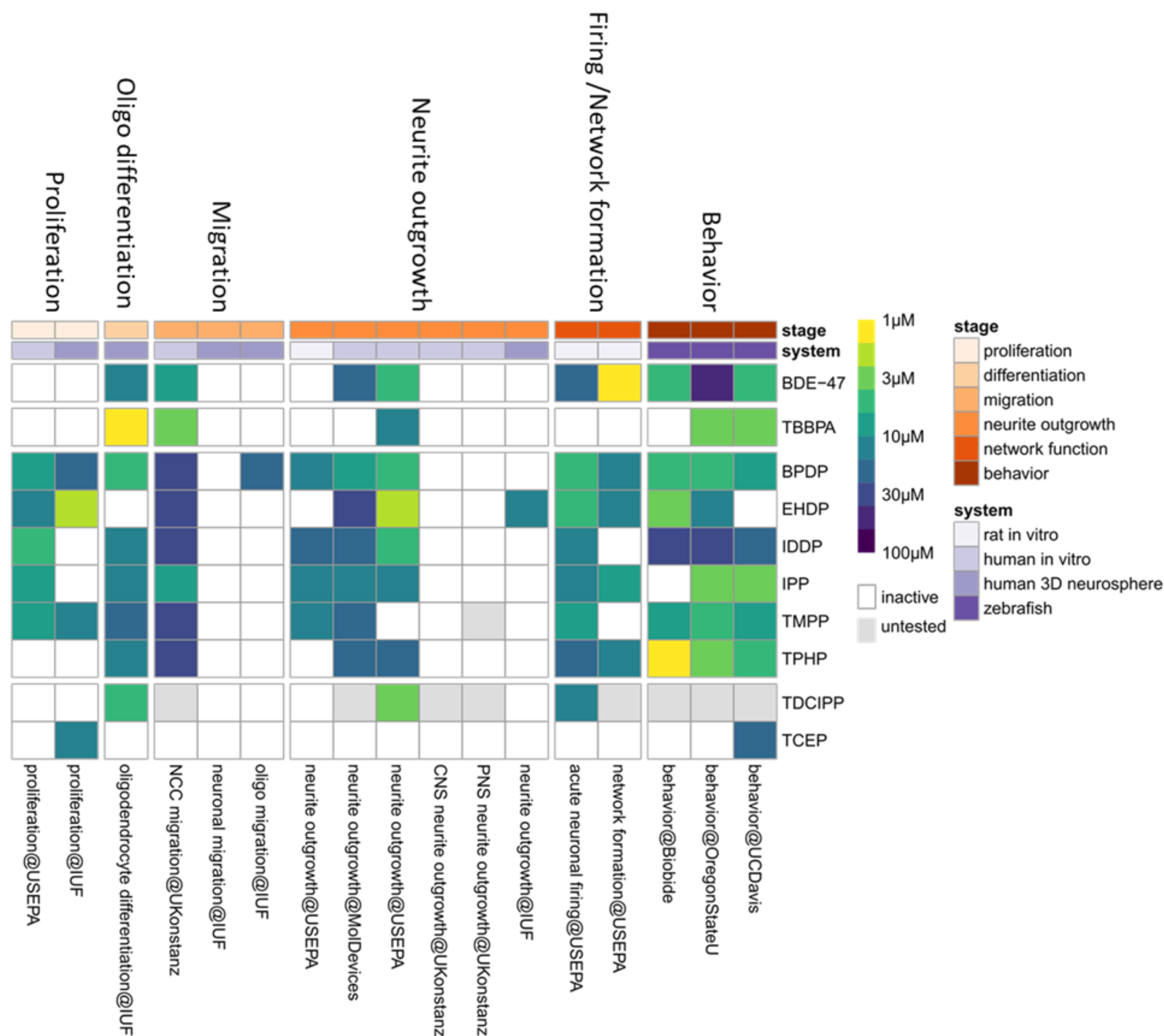
This question was addressed by an analysis of various lines of evidence including *in vitro* and exposure assessment. While there is no existing AOP for this class of compounds, some key events have been laid out which may form a putative AOP. Some of this has been described elsewhere (Patisaul et al. 2021).

We used a variety of *in vitro* and alternative species models representing domains of DNT, and acute neurotoxicity, to compare the relative toxicity of OPFRs with BFRs, and utilized BMC to make activity comparisons across these multiple assays. For all the potential key processes covered, it is appeared that the OPFRs as a group had activity in these assays with potencies comparable to that of the BFRs with the exception of TCEP (Behl et al. 2015). A similar pattern of relative toxicity of the compounds was noted *in vivo* (most of the OPFRs had similar potency to the phased-out BFRs) thereby underscoring the utility in using the *in vitro* assay battery for prioritizing compounds for further *in vivo* toxicity testing. Finally, high throughput toxicokinetic models were used to correlate levels in *in vitro* and rodent models to human exposure. This enables to contextualize and interpret *in vitro* findings for some of the brominated and halogenated flame retardants for which data is present (Blum et al., 2019). Measured plasma concentrations and external exposures were gathered from the literature and converted using a modified method of that previously described (Blum et al. 2019). Updated results suggested that for most of the tested flame retardants, the *in vitro* BMCs are approximately two orders of magnitude above the range of plasma concentrations estimated from human exposure of the parent compound.

However, it should be noted that, even though the *in vitro* BMC appears to be higher for these latter compounds, there are limited exposure data for most of the aromatic OPFRs. Herein, we evaluated only the parent compound, however, if the major metabolites where to be included the expected total exposure would be higher. Since major metabolites (e.g., DPHP, ip-DPHP) are not specific to one parent compound, it makes it challenging to associate one specific compound with a unique metabolite. With the phasing out of the BDEs and the proposal to ban halogenated flame retardants, the use of the aromatic flame retardants is expected to be on the continual rise (Robrock et al. 2018). Furthermore, it has been shown that children have a higher burden to flame retardants compared to mothers thereby raising concerns of DNT (Gibson et al., 2019). The current approach has not accounted for accumulating effect from multiple sources of exposure, exposure to sensitive populations, and exposure to mixture of OPFRs, which is typically how they exist in the environment. In the fact several flame retardants are present as a commercial mixture. This is important because, while we do not know the potential health effects of these OPFRs, they have patterns of *in vitro* activity at comparable concentrations to that of the phased-out flame retardants (e.g., BDE-47 and TBBPA). For TDCIPP, the plasma concentration estimated from *in vivo* MRL lies within the range of plasma concentrations estimated from human exposure data, which might raise a concern for the regulatory community.

Although there are uncertainties associated with this approach as described above, IATAs such as this one may help inform agencies such as the CPSC on prioritizing chemicals within a class as well as characterizing hazard of flame retardants.

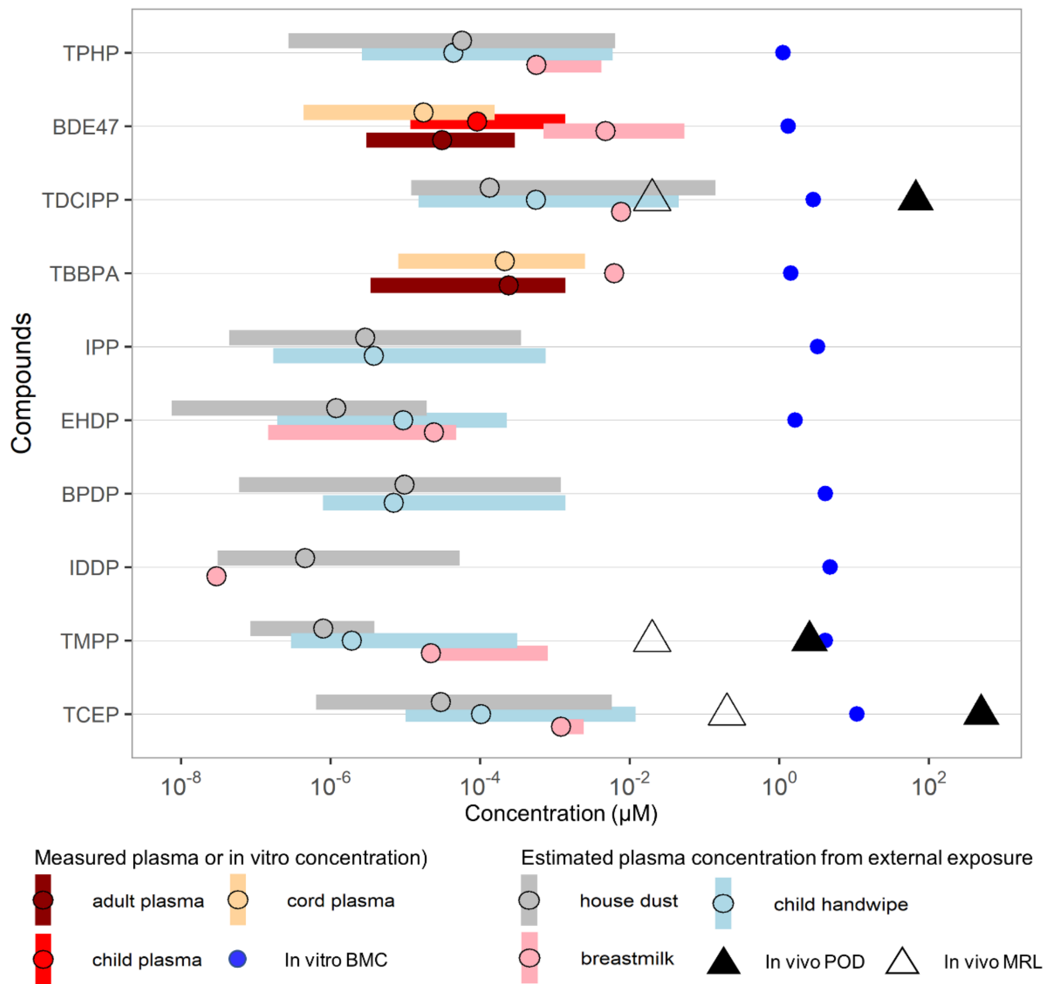
Figure 1. DNT Battery for Flame Retardants



The heatmap above shows benchmark concentration of different assays in our DNT battery. Since these assays are high content with multiple endpoints, the lowest BMC is presented for an assay where multiple BMCs are available. The BMCs were calculated based on primary findings in the literature (for data sources see Table 2). White indicates inactive and grey not tested.

Figure 2. Flame retardant plasma concentrations of parent compound (no metabolites evaluated) measured or estimated from ingestion using exposure data from house dust, breast milk, and/or handwipe samples (colored bars and circles) are compared to the most potent *in vitro* concentration (*in vitro* BMC, blue dots) from Figure 1, rat plasma concentrations based on *in vivo* POD values (solid triangles), and MRLs (open triangles).

The colored bars represent the range of measured or estimated plasma concentrations, and the circles represent the mean, median, or maximum median (for details on calculations, see Blum et al. 2019).

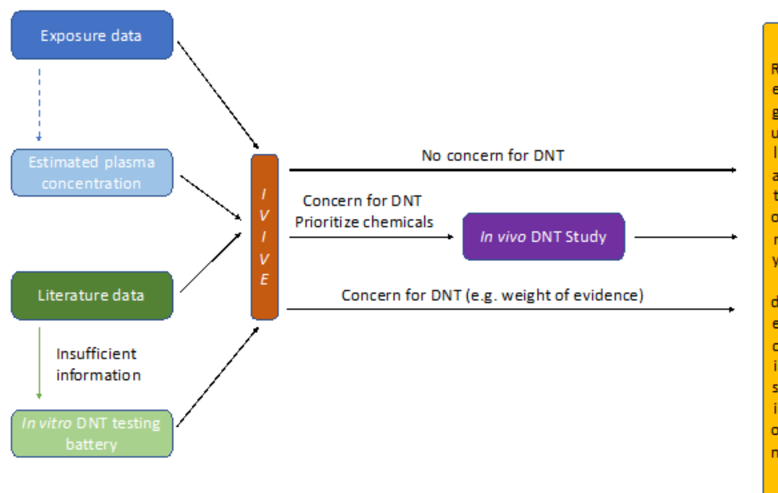


Some uncertainties such as different models resulting in different outcomes are not limited to this specific IATA, but rather are a more general issue. It is imperative to understand underlying protocol parameters and models that may result in differing outcomes and harmonize studies. One such effort on harmonizing zebrafish protocols is currently underway as an OECD DNT expert sub-group. This approach should be taken for more assays as we move towards using these batteries in lieu of animal studies. Additionally, data analysis is a huge component that may result in variability in outcome. While researchers don't need to necessarily change the way they are running studies or analyzing results, it is noteworthy to have vetted methods in the field to ensure an accurate data analysis and interpretation. This will be especially important if incorporating data from less standardized assays.

We are currently working on methods to distinguish between neuro-specific outcomes and general activity/ toxicity using a benchmark concentration approach. Example of this approach have been published (Harrill et al. 2018; Ryan et al. 2016). Methods to integrate across multiple data-streams using "specificity" cutoffs are currently underway at the NTP. We are also working with other researchers to identify knowledge gaps in the representative assays. We are also further refining HTTK models to better represent specific tissue compartments.

This is a case example of how a battery of *in vitro* and alternate animal model assays can be used to prioritize classes of compounds for further testing. Current studies are underway at the NTP on 2 representative FRs (TPHP and IPP) on which in-depth developmental exposures including DNT studies will be performed to anchor to some of these *in vitro* findings.

Figure 3. Rationale for performing IATA and use approach



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Annex 1: PBK Model Reporting

PBK Model Reporting Template sections	PBK Model Reporting Template sections
A. Name of model	Httk.3comp
B. Model developer and contact details	Xiaoqing Chang xiaoqing.chang@inotivco.com
C. Summary of model characterisation, development, validation, and regulatory applicability	We developed chemical-specific, three-compartment PK model using a generic 3-compartment model structure (httk.3comp) included in the high-throughput toxicokinetics model (HTTK) R package (httk v1.9). The HTTK R package is developed by U.S. Environmental Protection Agency (EPA). Please refer to Pearce et al. 2017 (<doi:10.1007/s10928-017-9548-7>) for details about the HTTK R package. The regulatory applicability of the model is for testing prioritization.
D. Model characterisation (modelling workflow) Step 1 – Scope and purpose of the model (problem formulation) Step 2 – Model conceptualisation (model structure, mathematical representation) Step 3 – Model parameterisation (parameter estimation and analysis) Step 4 – Computer implementation (solving the equations) Step 5 – Model Performance Step 6 – Model Documentation	**Step 1** – Scope and purpose: a model for oral exposure to relate external exposure and internal concentration in a high throughput way. **Step 2** – Model conceptualization: the model is a three-compartment model including gut, liver and rest-of-body compartments. Each compartment is described as perfusion rate limited. The model is expressed as a set of mass balance differential equations describing the rate of change of the amount of a chemical in each compartment. **Step 3** – Model parameterization: experimental data for fu and intrinsic clearance are used when available. In the absence of experimental values, *in silico* predictions from HTTK package are used. Please refer to Pearce et al. 2017 (<doi:10.1007/s10928-017-9548-7>) for details in model prediction for fu and intrinsic clearance. **Step 4** – Computer implementation (solving the equations): MCSim (Bois and Maszle 1997, doi:10.18637/jss.v002.i09) was used for converting the model equations into C code, which is used with R packaged: deSolve (Soetaert et al. 2016, URL https://CRAN. R-project.org/package='deSolve)' in solving each system of equations. **Step 5** – Model Performance: not performed because there is no *in vivo* PK data for tested chemicals. **Step 6** – Model Documentation: https://cran.r-project.org/web/packages/httk/httk.pdf
E. Identification of uncertainties model structure input parameters model output other uncertainties (e.g. model developed for different substance and/or purpose)	The uncertainty mainly arises from input parameters and model output. 1) Uncertainty analysis for fu and intrinsic clearance had been carried out (Wambaugh et al., 2019) The calibrated methods for predicting tissue:plasma partition coefficients and volume of distribution are also included in Pearce et al. 2017 (doi:10.1007/s10928-017-9548-7). 2) The model output is Cmax. Uncertainty of Cmax estimate may arise from population variability. In addition, because specific assumption is applied when converting human exposure data

	from various exposure sources to daily oral exposure (mg/kg/day), uncertainty may exist as different assumptions can be applied.
F. Model implementation details software (version no) availability of code software verification / qualification	The httk v 1.9 was used in the case study. The R code for the HTTK package and installation information can be found here, https://cran.r-project.org/web/packages/httk/index.html. The code for exposure estimation from various exposure source and apply httk.3comp model to OPFRs are written in R and is available upon request.
G. Peer engagement (input/review)	The initial release of the httk.3comp model is via the publication of Pearce et al. 2017 (doi:10.1007/s10928-017-9548-7), which has been undergone peer review for publishing.
H. Parameter table	Please see Annex 2: Physiological and pharmacokinetic parameters used in the httk.3comp model
References and background information publications links to other resources	Pearce et al. 2017, doi:10.1007/s10928-017-9548-7; https://cran.r-project.org/web/packages/httk/index.html;

Annex 2: Physiological and pharmacokinetic parameters used in the httk.3comp model

CAS	115-86-6	115-96-8	1241-94-7	1330-78-5	13674-87-8	29761-21-5	5436-43-1	79-94-7	56803-37-3	68937-41-7	5436-43-1	79-94-7	78-30-8	1330-78-5	13674-87-8	115-96-8	Note for abbreviation
Chem.name.ab	TPHP	TCEP	EHDP	TMP	TDCIPP	IDDP	BDE47	TBBPA	BPDP	IPP	BDE47	TBBPA	TOCP	TMPP	TDCIPP	TCEP	
Chem.name	Triphenyl phosphate	Tris(2-chloroethyl) phosphate	2-Ethylhexyl diphenyl phosphate	Tricresyl phosphate	Tris(1,3-dichloro-2-propyl) phosphate	Isodecyl diphenyl phosphate	2,2'4,4'-Tetrabromodiphenyl ether	3,3',5,5'-Tetrabromobisphenol A	tert-Butylphenyl diphenyl phosphate	Isopropylphenyl phosphate	2,2'4,4'-Tetrabromodiphenyl ether	3,3',5,5'-Tetrabromobisphenol A	Tri-o-cresyl phosphate	Tricresyl phosphate	Tris(1,3-dichloro-2-propyl)phosphate	Tris(2-chloroethyl) phosphate	
Species	Human	Human	Human	Human	Human	Human	Human	Human	Human	Human	Rat	Rat	Rat	Rat	Rat	Rat	
BW for house dust exposure	20.25	20.25	20.25	10.75	10.75	20.25			20.25	20.25	0.25	0.25	0.25	0.25	0.25	0.25	Body Weight, kg.

CAS	115-86-6	115-96-8	1241-94-7	1330-78-5	13674-87-8	29761-21-5	5436-43-1	79-94-7	56803-37-3	68937-41-7	5436-43-1	79-94-7	78-30-8	1330-78-5	13674-87-8	115-96-8	Note for abbreviation
BW for handwipe exposure	20.25	20.25	20.25	10.75	20.25				20.25	20.25							
BW for breast milk exposure	4	4	4	4	4	4	4	4									
Clint	26.44	0.00030998	98.1979798	74.02993131	5.092	313.3414141	0	0	225.7131313	558.7371313	0	0	93.63872323	74.02993131	0	0	*in vitro* intrinsic clearance, ul/ml/10^6 cells
Clmetabolismc	18.500272	5.76E-05	285.7015392	232.1131265	1.510850541	6129.096727	0	0	1000.824087	407247.1441	0	0	252.3382779	330.6427801	0	0	Hepatic Clearance, L/h/kg BW.
Fgutabs	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Fraction of the oral dose absorbed, i.e. the fraction of the dose that enters the gutlumen.
Fhep.assay.correction	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	To correct the fraction of chemical unbound in hepatocyte assay using the method of Kilford et al. (2008)
Funbound.plasma	0.001575711	0.163601877	0.000411806	0.000374582	0.007975697	5.27E-05	3.13E-05	0.0006624	0.000252739	1.93E-06	0.000130785	0.001384036	0.00203567	0.001272803	0.023806853	0.170366559	Fraction of plasma that is not bound.
Funbound.plasma.adjustment	0.552493382	0.947867189	0.071000989	0.073447497	0.739929242	0.011967289	0.001590449	0.315428636	0.053774213	0.000773395	0.006638832	0.659064659	0.370121786	0.249569303	0.926336689	0.98706002	Adjustment factor for Funbound.plasma
hemato	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.46	0.46	0.46	0.46	0.46	0.46	Percent volume of red blood cells in the

CAS	115-86-6	115-96-8	1241-94-7	1330-78-5	13674-87-8	29761-21-5	5436-43-1	79-94-7	56803-37-3	68937-41-7	5436-43-1	79-94-7	78-30-8	1330-78-5	13674-87-8	115-96-8	Note for abbreviation
crit																	blood.
Kgut2pu	1899.900039	6.503904931	12584.87942	13806.75996	254.6064443	103038.7319	174530.8614	6208.569596	20785.91985	2829545.644	124141.1403	5199.605286	5643.920423	10309.23181	99.33920156	6.432871672	Ratio of concentration of chemical in gut tissue to unbound concentration in plasma.
kgutabs	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	2.18	Rate that chemical enters the gut from gutlumen, 1/h.
Kliver2pu	3946.679786	15.89070297	23503.20627	25795.71535	570.4636282	190765.8148	322642.6901	12137.85124	38719.67001	5230418.154	97163.35835	4801.159436	4738.483596	8423.902384	126.3121862	14.50080597	Ratio of concentration of chemical in liver tissue to unbound concentration in plasma.
Krbc2pu	83.72714302	1.44539733	629.4863761	689.5040142	12.28203156	5169.844292	8769.712714	295.3807502	1039.543731	141923.0305	4999.033673	173.9654414	212.0895712	397.4939037	4.886888448	1.461758927	Ratio of concentration of chemical in red blood cells to unbound concentration in plasma.
Krest2pu	1222.902637	4.843273259	7236.373167	7942.349819	177.6813267	58705.34531	99280.47261	3741.832631	11919.52542	1609435.785	41608.36052	2021.922859	2013.323068	3589.797244	48.85668292	3.967976927	Ratio of concentration of chemical in rest of body tissue to unbound concentration in plasma.
liver.density	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	Density of liver tissue
million.cells.per.gliver	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	110	Millions cells per gram of liver tissue.
MW	326.2831	285.49	362.41	368.37	430.9048	390.46	485.82	543.9	382.4	452.53	485.82	543.9	368.37	368.37	430.91	285.49	Molecular Weight, g/mol.
Qcardiacc	13.8840366	13.8840366	13.8840366	13.8840366	13.8840366	13.8840366	13.8840366	13.8840366	13.8840366	13.8840366	12.5582166	12.5582166	12.5582166	12.5582166	12.5582166	12.5582166	Cardiac Output, L/h/kg BW^3/4.
Qgfrc	0.3099114	0.309	0.309	0.309	0.3099	0.309	0.3099	0.309	0.309	0.3099	0.222	0.2223	0.2223	0.2223	0.222	0.2223	Glomerular Filtration

CAS	115-86-6	115-96-8	1241-94-7	1330-78-5	13674-87-8	29761-21-5	5436-43-1	79-94-7	56803-37-3	68937-41-7	5436-43-1	79-94-7	78-30-8	1330-78-5	13674-87-8	115-96-8	Note for abbreviation
		9114	9114	9114	114	9114	114	9114	9114	114	3144	144	144	144	3144	144	Rate, L/h/kg BW^3/4, volume of fluid filtered from kidney and excreted.
Qgutf	0.205358145	0.205358145	0.205358145	0.205358145	0.205358145	0.205358145	0.205358145	0.205358145	0.205358145	0.205358145	0.132439187	0.132439187	0.132439187	0.132439187	0.132439187	0.132439187	Fraction of cardiac output flowing to the gut.
Qliverf	0.053586721	0.053586721	0.053586721	0.053586721	0.053586721	0.053586721	0.053586721	0.053586721	0.053586721	0.053586721	0.027042056	0.027042056	0.027042056	0.027042056	0.027042056	0.027042056	Fraction of cardiac output flowing to the liver.
Rblood2plasma	0.618049108	0.664046675	0.674059484	0.673641419	0.603101417	0.679778426	0.680899334	0.64609051	0.675602536	0.680738752	0.84074736	0.650756222	0.738602396	0.772728541	0.59351706	0.654556026	The ratio of the concentration of the chemical in the blood to the concentration in the plasma from available_rblood2plasma.
Vgutc	0.0158	0.0158	0.0158	0.0158	0.0158	0.0158	0.0158	0.0158	0.0158	0.0158	0.0259	0.0259	0.0259	0.0259	0.0259	0.0259	Volume of the gut per kg body weight, L/kg BW.
Vliverc	0.0245	0.0245	0.0245	0.0245	0.0245	0.0245	0.0245	0.0245	0.0245	0.0245	0.0349	0.0349	0.0349	0.0349	0.0349	0.0349	Volume of the liver per kg body weight, L/kg BW.
Vrestc	0.78805	0.78805	0.78805	0.78805	0.78805	0.78805	0.78805	0.78805	0.78805	0.78805	0.74221	0.74221	0.74221	0.74221	0.74221	0.74221	Volume of the rest of the body per kg body weight, L/kg BW.