Comparative Toxicity of Organophosphate Flame Retardants and Polybrominated Diphenyl Ethers to *Caenorhabditis elegans*

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ABSTRACT

With the phasing-out of the polybrominated diphenyl ether (PBDE) flame retardants due to concerns regarding their potential developmental toxicity, the use of replacement compounds such as organophosphate flame retardants (OPFRs) has increased. Limited toxicity data are currently available to estimate the potential adverse health effects of the OPFRs. The toxicological effects of 4 brominated flame retardants, including 3 PBDEs and 3,3',5,5'-tetrabromobisphenol A, were compared with 6 aromatic OPFRs and 2 aliphatic OPFRs. The effects of these chemicals were determined using 3 biological endpoints in the nematode *Caenorhabditis elegans* (feeding, larval development, and reproduction). Because *C. elegans* development was previously reported to be sensitive to mitochondrial function, results were compared with those from an *in vitro* mitochondrial membrane permeabilization (MMP) assay. Overall 11 of the 12 flame retardants were active in 1 or more *C. elegans* biological endpoints, with only tris(2-chloroethyl) phosphate inactive across all endpoints including the *in vitro* MMP assay. For 2 of the *C. elegans* endpoints, at least 1 OPFR had similar toxicity to the PBDEs: triphenyl phosphate (TPHP) inhibited larval development at levels comparable to the 3 PBDEs; whereas TPHP and isopropylated phenol phosphate (IPP) affected *C. elegans* reproduction at levels similar to the PBDE commercial mixture, DE-71. The PBDEs reduced *C. elegans* feeding at lower concentrations than any OPFR. In addition, 9 of the 11 chemicals that inhibited *C. elegans* larval development also caused significant mitochondrial toxicity. These results suggest that some of the replacement aromatic OPFRs may have levels of toxicity comparable to PBDEs.

Key words: *Caenorhabditis elegans*; flame retardants; mitochondrial toxicity.

To meet flammability standards, chemical flame retardants are extensively incorporated into commercial products including electronics, upholstered furniture, building materials, textiles, and baby products (Shaw *et al.*, 2010; Stapleton *et al.*, 2005). Prior to 2005, the polybrominated diphenyl ethers (PBDEs) were a major class of flame retardants used in the United States. These brominated flame retardants (BFRs) were produced as mixtures of penta-, octa-, and deca-brominated diphenyl ethers (BDEs) with 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and 2,2',4,4',5-penta-bromodiphenyl ether (BDE-99) being the major congeners in the penta-BDE mixture. A widely used commercial mixture marketed as DE-71 contained PBDEs (BDE-47 and BDE-99) along with 2,2',4,4',5,5'-hexabromodiphenyl ether (BDE-153), which were low molecular weight, lipophilic PBDEs that bioaccumulated in the environment and in human tissues (Birnbaum and Staskal, 2004; Hakk and Letcher, 2003).

The PBDEs were phased-out of use in 2005 due to concerns over their environmental persistence, bioaccumulation, and association with several adverse human health effects including altered circulating hormone levels, decreased fertility, and impaired neurodevelopment (Frederiksen et al., 2009; Harley et al., 2010; Herbstman et al., 2010; Meeker and Stapleton, 2010). They are increasingly being replaced by organophosphate flame retardants (OPFRs) (Stapleton et al., 2011, 2012). Use estimates of some OPFRs, which are also used in other manufacturing processes most commonly as PVC plasticizers (van der Veen and de Boer, 2012), have risen dramatically over the past 30 years from ∼14 000 to 38 000 tons per year (Schreder et al., 2016). Like PBDEs, OPFRs can leach out of treated materials and persist in the environment. They have been detected in indoor air, household dust, wastewater treatment plant effluent, drinking water, and wildlife samples (Barcelo et al., 1990; Meeker et al., 2013; Sundkvist et al., 2010; van der Veen and de Boer, 2012). In addition, they have been found in human tissues at levels similar to the PBDEs (Hoffman et al., 2014). Although OPFR usage is continually increasing, there is a paucity of information on the toxicity of this class of chemicals. Similar to PBDEs, exposures to OPFRs are associated with altered hormone levels, decreased reproductive function, and adverse developmental outcomes in humans and animal models (Farhat et al., 2013; Li et al., 2015; Liu et al., 2013, 2016; McGee et al., 2012; Meeker and Stapleton, 2010; Wang et al., 2013). Because structurally related compounds such as organophosphate pesticides (OPs) have been shown to adversely affect the development of the nervous system and alter reproductive parameters (Munoz-Quezada et al., 2013; Okamura et al., 2009; Roegge et al., 2008; Slotkin et al., 2006; Venerosi et al., 2012), the presence of a phosphate backbone in OPFRs suggests a potential for neurotoxicity and developmental toxicity (Crump et al., 2012; Dishaw et al., 2011; Egloff et al., 2014; Hendriks et al., 2014; McGee et al., 2012).

The nematode Caenorhabditis elegans was used as an alternative toxicological model because it offered a number of practical advantages similar to cell-based assays, but with the complexity of a whole organism (Leung et al., 2008). In addition, the effects of various environmental chemicals on a number of C. elegans outcomes have been well documented including development (Altun et al., 2002–2015; Anderson et al., 2001; Boyd et al., 2016), nervous system function (Boyd et al., 2003; Chen et al., 2015; Chisholm and Jin, 2005; Hobert, 2005; Leung et al., 2008; Mori et al., 2007; Silhankova and Korswagen, 2007), and reproduction (Harada et al., 2007; Hoss and Weltje, 2007). Of critical importance to this study was the ability to assess the effects of exposures across the lifespan of the nematode at multiple developmental stages within a few days.

The objective of the current study is to compare the developmental and neurotoxic potential of several phased-out PBDEs with representative replacement FRs. To do this, 3 medium-throughput C. elegans toxicological assays—larval development, feeding, and reproduction—were used to test the effects of 4 BFRs—including 3 phased-out PBDEs [2,2'4,4'-tetrabromodiphenyl ether (BDE-47), 2,2',4,4',5-pentabromodiphenyl ether (BDE-99), and the pentabromodiphenyl ether commercial mixture (DE-71)] and 3,3',5,5'-tetrabromobisphenol A (TBBPA)—to 6 aromatic OPFRs [triphenyl phosphate (TPHP), t-butylphenyl diphenyl phosphate (BPDP), isopropylated phenol phosphate (IPP), trimethyl phenyl phosphate (TMPP, aka tricresyl phosphate or TCP), isodecyl diphenyl phosphate (IDDP), and 2-ethylhexyl diphenyl phosphate (EHDP)], and 2 aliphatic OPFRs (tris(2-chloroethyl) phosphate (TCEP) and tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) (see Table 1). Two positive control compounds were also used for comparative purposes: the OP chlorpyrifos (CPF), which was previously tested using the 3 C. elegans assays (Boyd et al., 2010b), and tri-O-cresyl phosphate (TOCP), which was structurally similar to the aromatic OPFRs and was shown to cause delayed neurotoxicity in several species (reviewed in Weiner and Jortner, 1999). Because genetic mutations and exposures to environmental chemicals that affect mitochondrial function also alter development in C. elegans (Addo et al., 2010; Bess et al., 2012; Tsang and Lemire, 2002; Tsang et al., 2001), the effects observed in C. elegans were further compared with those from the Tox21 mitochondrial membrane permeabilizataion (MMP) dataset (Attene-Ramos et al., 2015).

MATERIALS AND METHODS

Flame Retardants

Full chemical names, IDs, CASRNs, and structures for all test compounds are presented in Table 1. Chemicals were purchased from the following vendors: BDE-47 and BDE-99 (Cerilliant Corp), BPDP (Ubichem Fine Chemicals), CPF (Toronto Research Chemicals), DE-71 (Great Lakes Chemical Company), EHDP (TCI America; Portland, OR), IDDP (Bayville Chemical Supply), IPP (Amfinecom Inc), TBBPA (Albemarle), DMSO, TPHP, TCEP and TDCIPP (Sigma Aldrich Chemical Co), TOCP and TMPP (Acros Organics).

Stock solutions were prepared in 100% DMSO at 100 mM (final concentration). Test solutions were then diluted from the stock solutions to final test concentrations in 1% DMSO. This allowed testing up to a maximum concentration of 1.0 mM. Chemical concentration ranges were adjusted for each in vivo assay to capture the full range of biological responses, from no observable to maximal sub-lethal effects. All concentration levels tested for each assay/chemical combination are provided in Supplementary Table 1.

Nematode Culture

Bristol N2 (wild-type) C. elegans (Caenorhabditis Genetic Center) were maintained at 20 °C on K-agar plates (2% bacto-agar, 0.25% bacto-peptone, 51 mM sodium chloride, 32 mM potassium chloride, 13 μM cholesterol) seeded with E. coli OP50 as the food source (Williams and Dusenbery, 1988). Age-synchronized cultures were prepared using a sodium hypochlorite solution (250 μM sodium hydroxide, 1% bleach) as previously described (Khanna et al., 1997). After a final rinse with K-medium (51 mM sodium chloride, 32 mM potassium chloride), embryos were transferred to a complete K-medium solution (K-medium plus 3 mM calcium chloride, 3 mM magnesium sulfate, 13 μM cholesterol) (Boyd et al., 2009) to hatch and arrest at the first larval stage (L1s) for the larval development assay, or were placed on K-agar plates with the OP50 strain of E. coli and allowed to develop to the appropriate life stage for the reproduction and feeding assays.
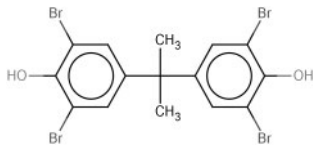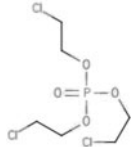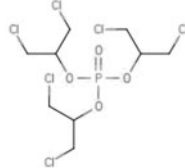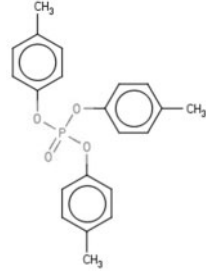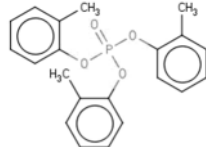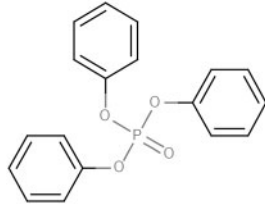
Larval Development, Feeding, and Reproduction Assays

Larval development, feeding and reproduction assays were performed as previously described using the COPAS Biosort (Union Biometrica Inc) (Boyd et al., 2007, 2010a; Rice et al., 2014). Age-synchronized nematodes were placed in 96-well plates containing the test compound, E. coli food source and either K-medium (reproduction and feeding) or complete K-medium (larval development). Following chemical exposure, 2 size characteristics for individual nematodes [length or "time of flight" (TOF) and optical density or "extinction" (EXT)] and fluorescence were

TABLE 1. Chemical Names, CASRNs, and Structures

Chemical ID	Chemical Name	CASRN	Structure	Determined Purity (%)
BDE-47	2,2'4,4'-Tetrabromodiphenyl ether	5436-43-1		100
BDE-99	2,2',4,4',5-Pentabromodiphenyl ether	60348-60-9		100
BPDP[a]	t-Butylphenyl diphenyl phosphate	56803-37-3		BPDP (64.5)TPHP (35.2)
CPF	Chlorpyrifos	2921-88-2		99.84
DE-71[b]	Pentabromodiphenyl ether	32534-81-9		BDE-47(42.27)BDE-99 (38.72)
EHDP	2-Ethylhexyl diphenyl phosphate	1241-94-7		98.48
IDDP[c]	Isodecyl diphenyl phosphate	29761-21-5		96.07
IPP[d]	Isopropylated phenol phosphate	68937-41-7		IPP (72.92)TPHP (26.01)

TABLE 1. (continued)

Chemical ID	Chemical Name	CASRN	Structure	Determined Purity (%)
TBBPA	3,3',5,5'-Tetrabromobisphenol A	79-94-7		98.93
TCEP	Tris(2-chloroethyl) phosphate	115-96-8		98.95
TDCIPP	Tris(1,3-dichloro-isopropyl) phosphate	13674-87-8		95.7
TMPP[e]	Trimethyl phenyl phosphate	1330-78-5		71.35
TOCP[f]	Tri-o-cresyl phosphate	78-30-8		97.1
TPHP	Triphenyl phosphate	115-86-6		99.35

[a]Mixture of 2-, 3-, and 4-tert-butylphenyl diphenyl phosphates; TPHP is a known byproduct of BPDP.
[b]Mixture of phenoxybenzene derivatives; see discussion for individual constituents.
[c]Mixture of isodecyl isomers.
[d]Isomeric mixture including TPHP.
[e]Mixture of isomeric tricresylphosphates; impurities consist of mixtures of methyl and ethyl tri-substituted compounds.
[f]Isomer of TMPP.

measured. Three independent biological replicates with 6 technical replicate wells per treatment group were conducted for each chemical-assay combination on separate days.

To determine the effects of the chemicals on larval development, 50 synchronized L1 larvae were placed in each well of a 96-well plate. The sizes of individual nematodes, which were linked to specific *C. elegans* larval stages (Boyd et al., 2016), were measured after 48-h exposures using the COPAS Biosort. In the absence of developmental inhibition, larvae will develop from the first larval stage (L1) to the fourth and final larval stage (L4) within 48 h. *C. elegans* maintained in the presence of chemicals may exhibit a range of developmental inhibition, from those that develop to L4s to those that do not develop and arrest as L1s.

To evaluate reproduction, 5 L4 larvae were placed into each well of a 96-well plate and allowed to grow and lay embryos in the presence of toxicant for 48 h at 20 °C (Boyd et al., 2010a). Total numbers of adults and offspring were counted at the end of the exposure using the COPAS Biosort.

For the feeding assay, 25 young adult nematodes were placed into each well of a 96-well plate and incubated for 24 h at 20 °C in the presence of toxicant. To measure feeding rates, 5 µl fluoresbrite polychromatic red 0.5 µm microspheres (Polysciences Inc) in deionized water were added to each well (Boyd et al., 2007). Nematodes were allowed to feed for 15 min, after which 5 µl of 170 mM sodium azide was added to arrest feeding. The levels of fluorescence in the nematode pharynx and intestine were measured using the COPAS Bisort.

Statistical Analyses

Incubation of nematodes for 48 h in the growth assay leads to the accumulation of detritus, such as shed cuticles and other waste products that are smaller than nematodes. These noise observations were removed using statistical methods described previously (Boyd et al. 2010b). Observations [log(EXT)] were indicative of nematode size. Each observation was subtracted from the mean plate control response to adjust for differences between plates. The feeding assay observations [log(RED fluorescence)] of adult nematodes did not contain detritus and therefore no observations were removed. Large fluorescence intensity values indicate healthy feeding in the nematodes. Fluorescence intensity observations were subtracted from the plate control mean to adjust for plate effects. For development and feeding, the plate-adjusted values [log(EXT) or log(RED)] resulted in the observed effect, which increased with decreasing nematode size, indicating toxicity. These effects were fit to a Hill function that provided EC50 estimates (effective concentration for effect equal to 50% of the maximum effect) with confidence intervals. When needed, an upper bound limit, determined from previous experiments, was used in the estimation. The lowest effective concentration (LEC) was estimated using a t test with the Bonferroni correction to compare each dose group with the plate controls. Analyses were performed twice: once individually on the 3 biological replicates and a second time using the 3 combined replicates.

For the reproduction assay, number of offspring (L1 and L2 larva) was used in the analyses. Three biological replicates were fit by a Hill function individually, as well as together. The LEC values could only be estimated after combining the replicates, because a single observation (nematode count) was measured within each replicate, and were determined using Dunnett's test to account for multiple testing of each dose group against the plate controls.

MMP Assay

The Tox21 MMP assay was used to identify chemically induced MMP disruption. This assay uses a water-soluble sensor, Mito-MPS (BD Biosciences), to detect chemically induced changes in MMP(Attene-Ramos et al., 2015). The dye produces red fluorescence when it accumulates and aggregates in the mitochondria of healthy cells. If the MMP is disrupted, then the dye does not accumulate and produces a green cytoplasmic fluorescence. To evaluate the specificity of chemical-induced mitochondrial toxicity, cell viability was also measured in the same compound well. The specificity of the MMP effect in relation to the cytotoxicity was quantified by the fold change of the weighted area under the curve (Hsieh et al., 2015). A threshold of 4 was chosen based on the activity variation.

Assay data were retrieved from PubChem [PubChem AID: 720637] and processed as previously described (Judson et al., 2010). Briefly, 3 types of activity calls (active, inactive, or inconclusive)

were generated. For those compounds that were active or inconclusive due to cytotoxicity, the point-of-departure (POD) (ie the concentration at which the response was equivalent to the assay-dependent noise threshold) was reported. The relative order of toxicity was compared using the POD values in the MMP with the LEC values and effect size from the C. elegans assays (Table 2).

RESULTS

Toxicity of Flame Retardants to Caenorhabditis elegans

Larval Development
All chemicals were tested at concentrations designed to capture effects ranging from no effect to a maximal effect, with the highest concentration tested (1 mM) being limited to 1% DMSO tolerated by C. elegans (Boyd et al., 2010a). TCEP, TMPP, TDCIPP, IDDP, EHDP, and TOCP required exposures to concentrations that included the maximum 1 mM concentration in an attempt to observe maximal decreases in development (Figure 1A; Supplementary Table 1). PBDEs were among the most potent flame retardants affecting C. elegans larval development at the lowest concentrations including: BDE-99, DE-71, and BDE-47 with LECs of 0.16, 0.16, and 0.4 µM, respectively (Table 2; Supplementary Table 1). The aromatic OPFR, TPHP, showed comparable potencies to the PBDEs with an LEC of 0.16 µM. Chlorpyrifos, an OP pesticide used as a positive control and reference compound, had similar potency with an LEC = 0.25 µM. Four additional aromatic OPFRs; BPDP, EDHP, IDDP, and IPP; and the BFR, TBBPA, had LECs < 5 µM (Table 2).

Figure 1B shows the potency or LEC against the effect size in the larval development assay, where larger effect sizes correspond to smaller nematodes. Untreated nematodes correspond to an effect size of zero. Visual observations of larval stages were also performed by microscopy. The maximum effect size, designated by a dashed horizontal line in Figure 1B, is based upon the size of the nematodes at the first larval stage (L1) when loaded into the exposure plates at $t = 0$ h and would correspond to no growth over the 48 h exposure period. Likewise, treatments with insignificant effects would have an effect size near zero and nematodes would have developed to the fourth larval stage (L4). PBDEs and TPHP activities clustered together with low LECs and large effects on growth and development at the highest concentrations tested (Figure 1B). Most of the other OPFRs had slightly lower potencies with observed maximum effects similar to the PBDEs and TPHP, whereas TBBPA and EHDP had smaller maximum effects. TCEP was the only compound that did not affect larval development within the tested concentration range. Representative concentration response curves are presented in Figure 1C–D for a PBDE (DE-71), and OPFR (BPDP). All concentration response curves are provided in Supplementary Figure 1.

Feeding
In general, the flame retardants had lesser effects on C. elegans feeding compared with larval development and growth (Figure 2A; Supplementary Table 1). Eight compounds (TCEP, EHDP, IDDP, TOCP, TMPP, BPDP, TDCIPP, and TPHP) were tested in the feeding assay up to the maximum testable concentration. TCEP, EHDP, and IDDP were inactive within the tested concentration ranges. PBDEs affected C. elegans feeding with LECs between 5 and 10 µM, whereas 4 aromatic OPFRs; BPDP, IPP, TDCIPP, and TPHP; and the BFR, TBBPA, had LECs ranging from 20 to 63 µM. Interestingly, the PBDEs, TDCIPP, and TBBPA produced effect sizes at least as large as CPF in the feeding assay but at much lower

Table 2. Comparisons of flame retardant toxicity to *C. elegans* with *in vitro* mitochondrial activity.

| | | Brominated | | | Aromatic OPFRs | | | | | | Aliphatic OPFRs | | Positive Controls | |
| | | PBDE | | | Bisphenol | tri-phenyl OPFRs | | | | di-phenyl OPFRs | | Chlorinated | | | |
Assay		BDE-99	DE-71	BDE-47	TBBPA	TPHP	IPP	BPDP	TMPP	EHDP	IDDP	TDCIPP	TCEP	CPF	TOCP
C. elegans (LEC in μM)	Reproduction	13[a]	1.60[b]	13[a]	13[a]	6.30[b]	10[b]	16[a]	63	-	32[a]	130	-	0.04[c]	250
	Larval Development	0.16[c]	0.16[c]	0.40[c]	3.20[b]	0.16[c]	1.60[b]	2.50[b]	100	1.60[b]	2.50[b]	13[a]	-	0.25[c]	10[b]
		(**1.72**)[c]	(**2.01**)[c]	(**1.75**)[c]	(0.9)[b]	(**1.62**)[c]	(**2.23**)[b]	(**2.17**)[b]	(0.78)	(1.0)[b]	(**2.02**)[b]	(**2.83**)[a]	-	(**2.38**)[c]	(**1.79**)[b]
	Feeding	7.90[b]	5[b]	6.30[b]	20[a]	40[a]	63	63	630	-	-	50[a]	-	0.12[c]	400
		(1.22)[b]	(**1.68**)[b]	(**2.08**)[b]	(**1.63**)[a]	(1.08)[a]	(0.69)	(1.30)	(0.2)	-	-	(**2.44**)[a]	-	(1.38)[c]	(0.65)
qHTS (POD in μM)	MMP inhibition	1.3[b]	6.7[b]	12.8[a]	6.7[b]	0.60[c]	5.7[b,c]	3.7[b]	11.7[a]	-	-	64.7[c]	-	67.4[c]	15.3[a,c]
	Cell Viability	-	-	-	6.7[b]	13.2[a]	5.9[b]	28.0[a]	19.0[a]	29.0[a]	16.4[a]	58.3	-	41.4[a]	7.4[b]

Lowest effective concentration (LEC) or point of departure (POD); $10 < a < 30\,\mu M$; $1 < b < 10\,\mu M$; $c < 1\,\mu m$; Dashes (-) indicate no toxicity at concentrations tested
[d]indicates cytotoxicity at concurrent concentrations; numbers in parentheses indicate effect sizes of *C. elegans* endpoints; bolded text indicates effect size >1.5.

potencies (Figure 2B); whereas the isomers, TMPP and TOCP, had similar effect sizes and potencies. Representative concentration effect plots are presented in Figure 2C–D and all concentration effect curves are provided in Supplementary Figure 2.

Reproduction

Similar to feeding, *C. elegans* reproduction was generally less sensitive than larval development. Five compounds (TCEP, EHDP, TOCP, TDCIPP, and TMPP) were tested up to the maximum testable concentration (Figure 3A). TCEP and EHDP also did not affect reproduction. Only DE-71 had an LEC less than 10 μM, whereas several flame retardants shared similar potencies ranging from 10 to 16 μM including BDE-47, BDE-99, TPHP, IPP, TBBPA, and BPDP (Table 2). IDDP, which was inactive in the feeding assay, was slightly less potent with an LEC of 32 μM compared with the other flame retardants. For the reproduction assay, the effect size of all active chemicals except TCEP and EHDP reached ~100% or no offspring; thus potency verses effect size plots are not presented. Representative concentration response plots are presented in Figure 3B–C and all concentration response curves are provided in Supplementary Figure 3.

Comparisons Between Caenorhabditis elegans and Tox21 Results

Approximately 8 300 unique compounds, including pharmaceuticals and environmental chemicals and the 14 compounds tested in *C. elegans* in this study, have been evaluated for toxicity in the Tox21 program using a suite of quantitative high-throughput screening (qHTS) assays (Huang *et al.*, 2016). To test what biological activities might be associated with this group of chemicals, Fisher's exact test was performed using all Tox21 assays. Only 3 types of activities were considered significantly enriched ($\alpha < 0.05$) in this group of chemicals after applying the Bonferroni correction: MMP, increase of constitutive androstane receptor transcriptional factor activity (data not shown) and decrease of androgen receptor transcriptional factor activity (data not shown). Because MMP was more potent than the others when activities co-occurred and mitochondrial toxicity has been linked to developmental arrest in *C. elegans*, the results from *C. elegans* assays were compared with Tox21 *in vitro* MMP data (Table 2).

Our findings indicate that all of the PBDEs show strong effects on MMP (ie, POD < 15 μM) in the absence of cytotoxicity, whereas all of the OPFRs, except the completely inactive TCEP, cause some degree of cytotoxicity. Three tri-phenyl OPFRs (TPHP, BDHP, and TMPP) showed comparable effects to the PBDEs in altering MMP but with cytotoxicity at higher concentrations. The exception was the tri-phenyl OPFR IPP, which showed nonspecific effects on MMP with observations of cytotoxicity at a similar concentration. In total, 11 of the 14 chemicals tested (except EHDP, IDDP, and TCEP) caused significant MMP disruption with various degrees of cytotoxicity (Table 2). Sample concentration–response curves of the MMP data are presented in Supplementary Figure 4 for BDE-99 (selective for MMP), TPHP (selective for MMP), and TDCIPP (nonselective MMP inhibitor).

Figure 4 shows the flame retardants ranked according to the relative toxicity to *C. elegans* endpoint (larval development, feeding and reproduction) and the mammalian cell MMP endpoint from Tox21 qHTS. As seen in the figure, the *C. elegans* larval development assay was the most sensitive of all endpoints tested; whereas the phased-out BDEs and the positive control CPF were the most potent (as indicated by the lowest POD). Of the 13 chemicals that affected *C. elegans* larval development, 11 chemicals (except the di-phenyl OPFRs, EHDP, and IDDP) also caused
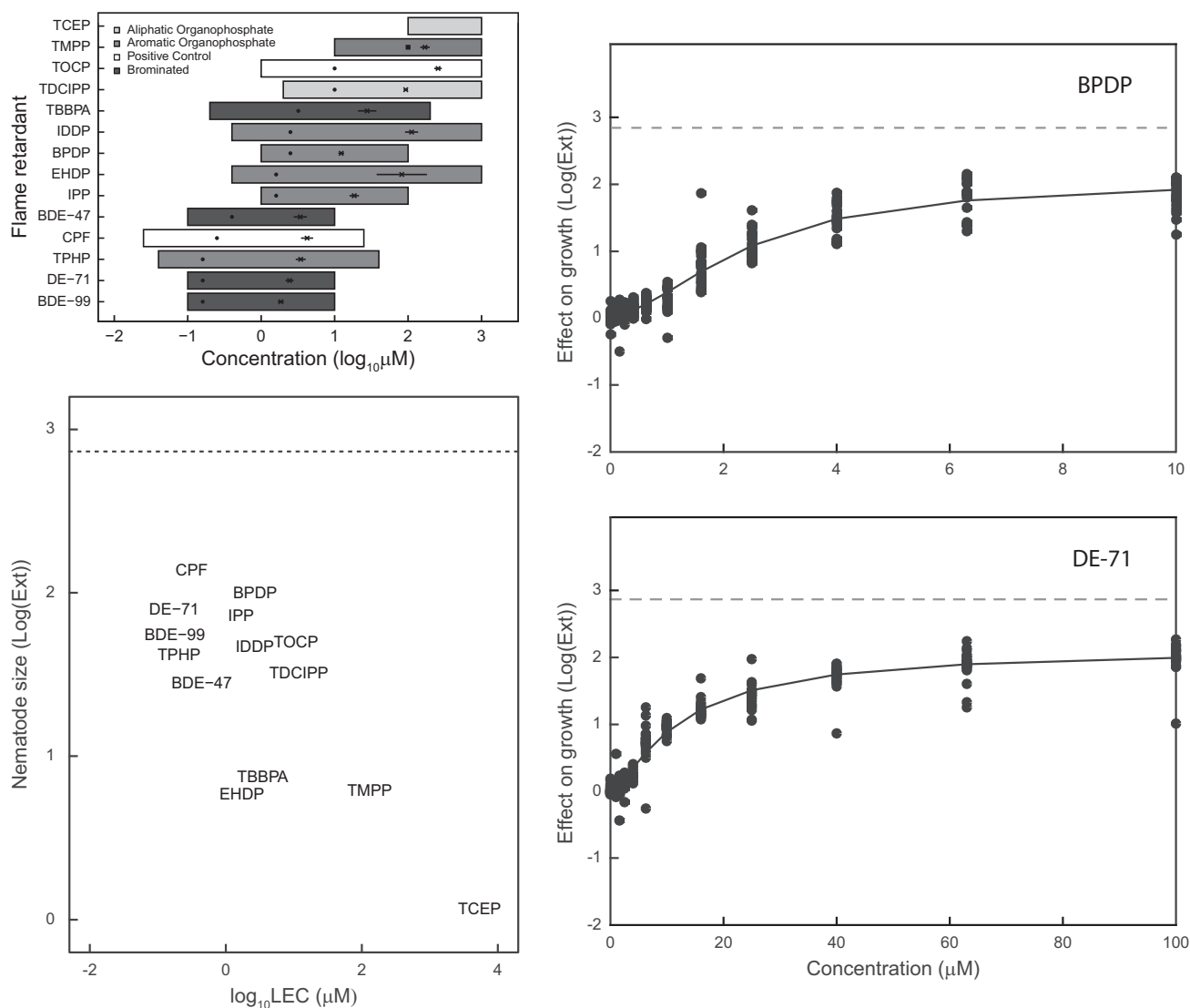
FIG. 1. Effects of flame retardants on *C. elegans* larval development and growth. A, *Top left:* Groups of 50 L1 nematodes were exposed for 48 h to various concentrations of flame retardants, illustrated by the horizontal bars. Flame retardants are grouped as: aliphatic organophospate flame retardants (OPFRs); aromatic OPFRs; brominated flame retardants including BDEs. The organophosphate pesticide chlorpyifos and aromatic OPFR TOCP were used as positive controls. Lowest effective concentrations (LECs) [*dots*] and EC50s [*crosses*] with 95% confidence intervals are also displayed. B, *Bottom left:* Scatterplots of observed maximum effect size (log(EXT)) and lowest effective concentration (LEC, in log scale). The horizontal dashed line refers to the maximum effect, no growth or development, for this assay. C, *Top right:* Sample concentration-effect plot for BPDP, a representative OPFR. D, *Bottom left:* Sample concentration-effect plot for DE-71, a representative PBDE.

significant MMP disruption; 7 of which showed specific effects on MMP (ie, in the absence of cytotoxicity or cytotoxicity observed at higher concentrations); whereas the remaining 4 non-specifically altered MMP in the presence of cytotoxicity (Table 2). The POD for MMP disruption (either specific or nonspecific) and the LEC for *C. elegans* larval development closely tracked with each other: the fold change between the LEC and POD is less than 10 for 8 of the 11 chemicals (except CPF, DE-71, and BDE-47). TCEP was the only compound that did not cause toxicity as measured by any *C. elegans* endpoint or by the MMP assay.

DISCUSSION

With the phasing out of the PBDEs, the usage of and exposure to alternative flame retardants is increasing. As a result, the levels of exposure to a number of OPFRs are approaching those

comparable to the PBDEs (Stapleton *et al.*, 2014). The potential health effects of these chemicals however, remain largely unknown. An additional concern has been raised due to the structural similarity to OP pesticides, which are associated with developmental impairments in infants and children (Gonzalez-Alzaga *et al.*, 2014).

In the current study, the relative toxicity of 8 OPFRs was compared with 4 BFRs and 2 positive controls using 3 *C. elegans* biological endpoints. These endpoints are designed to assess toxicity across the lifespan of the nematode and to monitor a range of adverse health effects including delayed larval development, decreased production of offspring or egg-laying, and neurotoxicity via the feeding. Our results indicated that several of the emerging OPFRs; namely TPHP, BPDP, and IPP; were consistently among the most active chemicals with comparable toxicity to the phased-out PBDEs and TBBPA (Table 2). TCEP was
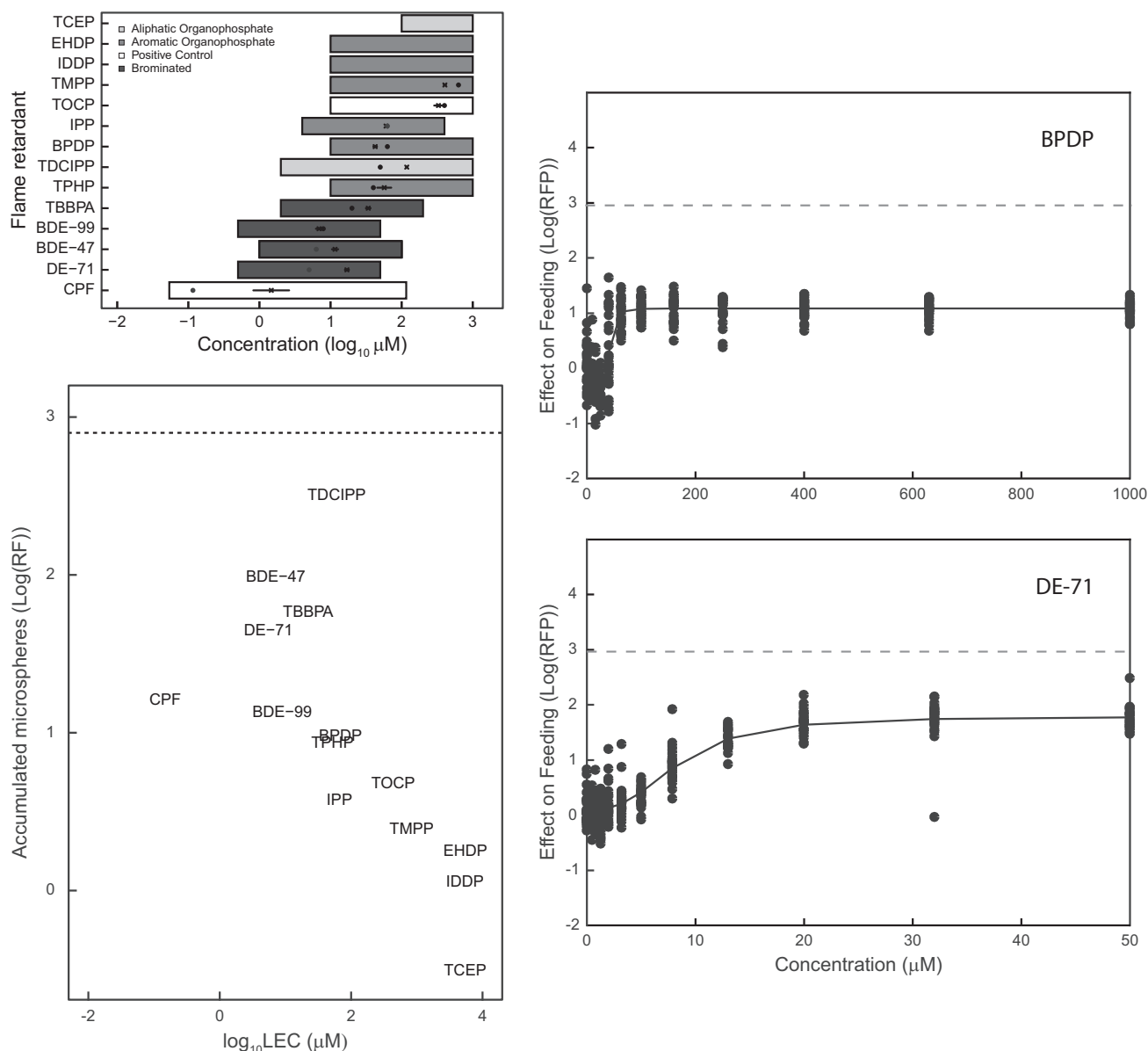
FIG. 2. Effects of flame retardants on *C. elegans* feeding. A, *Top left:* Groups of 25 adult nematodes were exposed for 24 h to various concentrations of flame retardants, illustrated by the horizontal bars. Flame retardants are grouped as: aliphatic organophospate flame retardants (OPFRs); aromatic OPFRs; brominated flame retardants including BDEs. The organophosphate pesticide chlorpyifos and aromatic OPFR TOCP were used as positive controls. Lowest effective concentrations (LECs) [*dots*] and EC50s [*crosses*] with 95% confidence intervals are also displayed. B, *Bottom left:* Scatterplots of observed maximum effect size (log(EXT)) and lowest effective concentration (LEC, in log scale). The horizontal dashed line refers to the maximum effect, no feeding, for this assay. C, *Top right:* Sample concentration-effect plot for BPDP, a representative OPFR. D, *Bottom right:* Sample concentration-effect plot for DE-71, a representative PBDE.

the only flame retardant that was inactive across all of the *C. elegans* endpoints (Table 2; Supplementary Figs. 1–3).

In all but 2 cases, larval development was the most sensitive endpoint in terms of potency; whereas reproduction was the most sensitive endpoint for TMPP and the positive control, CPF (Table 2; Figure 4). BDE 99, DE-71, and BDE-47 were the most active of the BFRs (LECs $<1\,\mu$M) with the aromatic OPFR, TPHP, showing comparable activity (Table 2; Figure 4). Other aromatic OPFRs such as BPDP, IPP, and IDDP and the aliphatic OPFR, TDCIPP, closely followed with LECs less than $5\,\mu$M. DE-71 was the most active reproductive toxicant (LEC <2 μM) closely followed by the OPFRs, TPHP, and IPP (LEC <10 μM). Many of these compounds affected more than one C. elegans endpoint

involving distinct biological processes, suggesting that these compound may alter multiple targets during development. Whereas the LEC determines the lowest concentration at which the compounds were active, the effect size determines the magnitude of the effect. For this class, the same compounds that had the lowest LEC also had the highest effect size thereby indicating that exposure to these compounds caused a significant arrest in the magnitude of response (Table 2; Figure 1B).

To determine the relationship between compound solubility and toxicity, correlations were calculated using Pearson's correlation coefficient (R) for the active chemicals between the log K_{OW} of each compound and the $\log_{10}$(LEC) for each C. elegans assay. No significant correlation was found between any of the
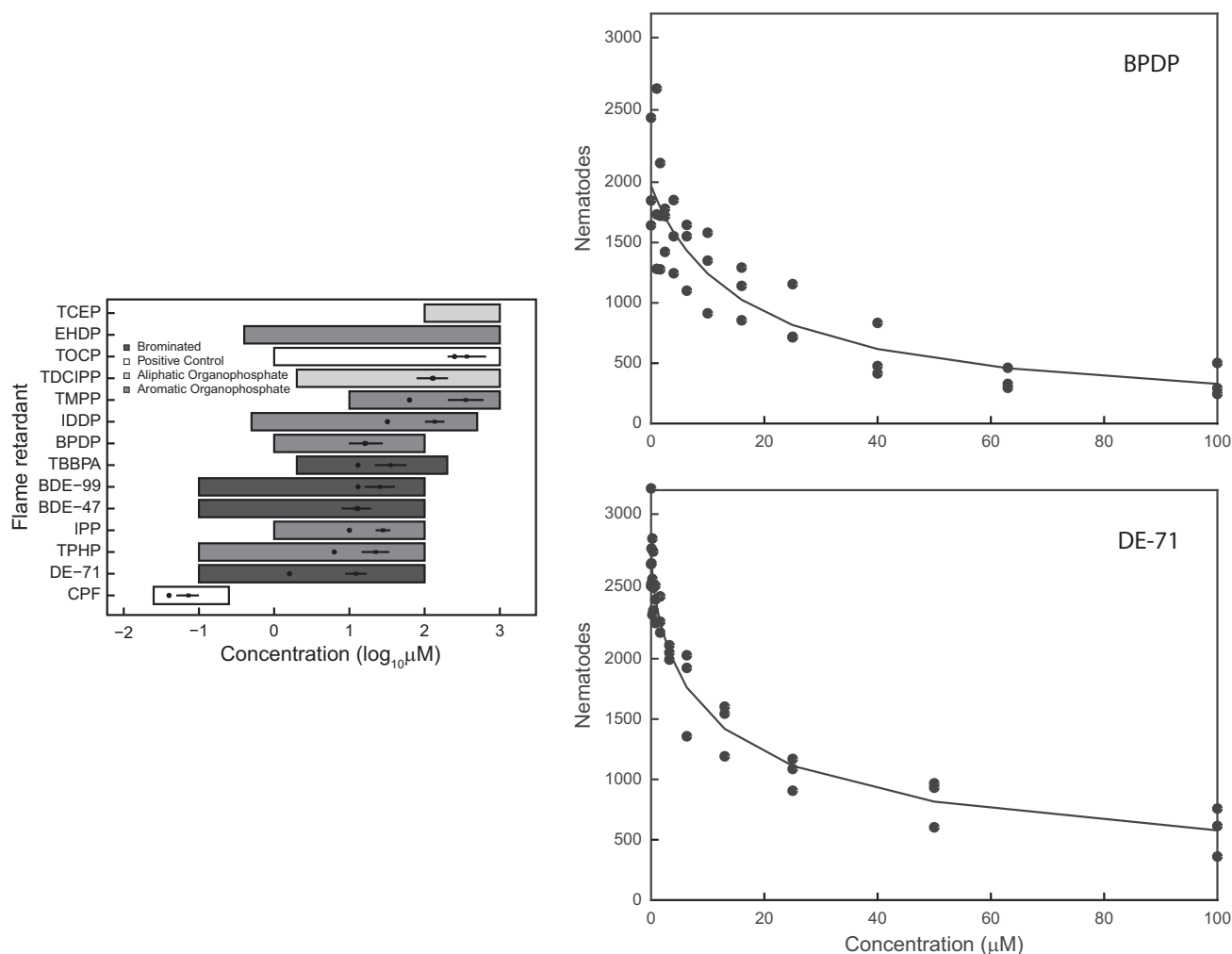
FIG. 3. Effects of flame retardants on *C. elegans* reproduction. A, *Left panel:* Groups of 5 L4 nematodes were exposed for 48 h to various concentrations of flame retardants, illustrated by the horizontal bars. Flame retardants are grouped as: aliphatic organophospate flame retardants (OPFRs); aromatic OPFRs; brominated flame retardants including BDEs. The organophosphate pesticide chlorpyifos and aromatic OPFR TOCP were used as positive controls. Lowest effective concentrations (LECs) [*dots*] and EC50s [*crosses*] with 95% confidence intervals are also displayed. B, *Top right:* Sample concentration-effect plot for BPDP, a representative OPFR. C, *Bottom right:* Sample concentration-effect plot for DE-71, a representative PBDE.

pairs [R = 0.06 (Log K_{OW}—Reproduction), 0.28 (Log K_{OW}—Larval development, and 0.06 (Log K_{OW}—Feeding)] suggesting that hydrophobicity alone did not drive the observed toxicities across these assays.

Although the OPFRs affected larval development and reproduction at concentrations similar to the PBDEs, feeding was affected by lower concentrations of the PBDEs than the OPFRs (Table 2). This suggests that neurotoxicity related to neuromuscular function may be of greater concern for PBDEs than for the OPFRs. These observations are consistent with previous findings that show associations between PBDE exposures and neurobehavioral deficits in rodents (Cheng *et al.*, 2009; Kodavanti *et al.*, 2010; Suvorov *et al.*, 2009). It is interesting to note that while the OPFRs may not affect neuromuscular function, they appear to selectively inhibit neuronal firing and result in neuronal cell death at comparable concentrations *in vitro* (Behl *et al.*, 2015). This highlights the need for a battery of assays to evaluate differences in activity between several models (rat and human *in vitro* models versus *C. elegans* whole organism models), differences in endpoints being assessed (neuromuscular function in *C. elegans* versus neuronal firing

and neuronal death *in vitro*), differences in underlying bioavailability between the assays, and potential differences in metabolism.

Because the objective was to compare the toxicity of compounds used as flame retardants, several of the compounds tested are commercial mixtures such as DE-71, whereas others are single, pure chemicals such as BDE-47 (see footnotes in Table 1). Although complete chemical analyses of all compounds were outside the scope of the current work, purity analysis was completed for the DE-71 mixture. The following PBDEs were observed in DE-71 by gas chromatography: BDE-47 (42.27%), BDE-99 (38.72%), BDE-100 (8.32%), BDE-85 (2.19%), BDE-154 (2.26%), and BDE-153 (2.61%) for a total purity of 96.37%. Eleven additional peaks making up less than 1% of the total area of the chromatogram were not able to be identified (data not shown). Because dioxins and furans are known contaminants of DE-71 (Hanari *et al.*, 2006), it cannot be completely ruled out that these compounds may be contributing to the observed toxicities. However, it is of note that the single chemicals BDE-47 and BDE-99, which are the major measured constituents, exhibited very similar toxicities to the DE-71 mixture across the 3
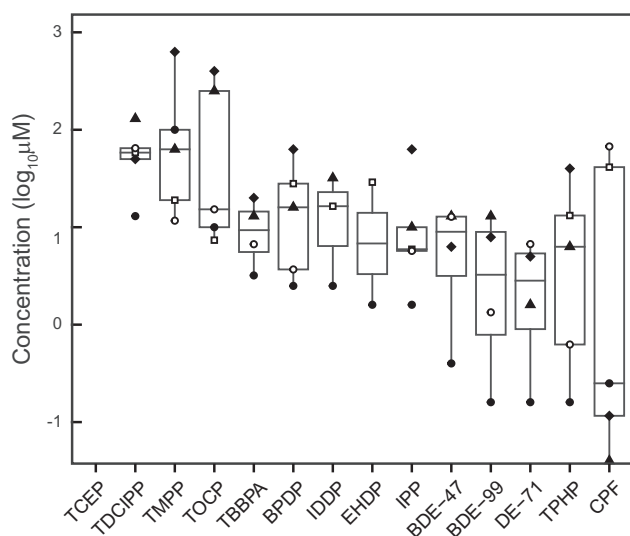
FIG. 4. Comparison of flame retardant activity in *C. elegans* and *in vitro* MMP assays. Box and whisker plot displaying the points of departure (PODs) for *C. elegans* larval development [*filled circles*], feeding [*filled diamonds*], and reproduction [*filled triangles*] as well as qHTS *in vitro* MMP assay [*open circles*] and cytotoxicity [*open squares*]. The x-axis displays chemicals in increasing order of activity from left to right.

C. elegans endpoints, indicating that they may be acting via similar modes of action.

To identify a potential mechanism by which exposure to flame retardants may affect larval development, we compared *C. elegans* development with mammalian cell mitochondrial toxicity *in vitro* because mitochondrial dysfunction was previously associated with disrupted *C. elegans* larval development (Bess *et al.*, 2012). Nine of the 11 flame retardants that affected *C. elegans* larval development also caused significant mitochondrial toxicity in mammalian cells (Figure 4; Table 2); TPHP, IPP, and BPDP were the most active OPFRs in the MMP assay with comparable activity to the phased-out PBDEs (POD < 10 µM) suggesting an association between disruption of mitochondrial function and altered larval development following FR exposure.

To understand the utility of *C. elegans* as a screening tool, we compared our findings with other *in vivo* studies. For DE-71, a highly active chemical in the *C. elegans* assays, a wide spectrum of effects were noted in rodents ranging from negative findings (Gonzalez-Alzaga *et al.*, 2014) to selective lesions in the thyroid gland and reproductive tissues (Stoker *et al.*, 2004) to complete reproductive failure in the mink model at comparable concentrations (Zhang *et al.*, 2009). Another compound, TBBPA showed selective activity in all 3 *C. elegans* biological endpoints, but was found in a previous study to nonselectively alter endpoints of developmental neurotoxicity and acute neurotoxicity in the range of 10–12 µM in the presence of concomitant cytotoxicity (Behl *et al.*, 2015), and resulted in mortality in developing zebrafish embryos (Jarema *et al.*, 2015; Wu *et al.*, 2015). Although the reasons for these differences remain unknown, these findings emphasize the importance of evaluating compounds in multiple species and models to better understand differences in species susceptibility, and ultimately impact on human health.

In conclusion, *C. elegans* was used as an *in vivo* phenotypic screening tool to evaluate the potential effects of a subset of flame retardants. The phased-out PBDEs and TBBPA were consistently among the most active chemicals in all 3 *C. elegans* biological endpoints and *in vitro* mitochondrial membrane potential assay. The replacement OPFRs TPHP, IPP, and BPDP

showed comparable activity to the PBDEs thereby warranting further hazard characterization of these replacement OPFRs.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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