



Published in final edited form as:

Environ Sci Technol. 2015 June 16; 49(12): 7400–7410. doi:10.1021/acs.est.5b00977.

Gene Transcription, Metabolite and Lipid Profiling in Eco-Indicator *Daphnia magna* Indicate Diverse Mechanisms of Toxicity by Legacy and Emerging Flame-Retardants

Leona D. Scanlan^{†,‡}, Alexandre V. Loguinov[†], Quincy Teng[‡], Philipp Antczak[§], Kathleen P. Dailey[†], Daniel T. Nowinski[†], Jonah Kornbluh[†], Xin Xin Lin[†], Erica Lachenauer[†], Audrey Arai[†], Nora K. Douglas^{||}, Francesco Falciani[§], Heather M. Stapleton^{||}, and Chris D. Vulpe^{†,*,#}

[†]University of California Berkeley, Department of Nutritional Sciences and Toxicology, Berkeley, California 94720, United States

[‡]U.S. EPA, National Exposure Research Laboratory, Athens, Georgia 30605, United States

[§]University of Liverpool Institute for Integrative Biology, Liverpool, L69 7ZB, United Kingdom

^{||}Nicholas School of the Environment, Duke University, Durham, North Carolina 27708, United States

Abstract

The use of chemical flame-retardants (FR) in consumer products has steadily increased over the last 30 years. Toxicity data exist for legacy FRs such as pentabromodiphenyl ether (pentaBDE), but less is known about effects of new formulations. To address this issue, the toxicity of seven FR chemicals and formulations was assessed on the freshwater crustacean *Daphnia magna*. Acute 48-h nominal LC₅₀ values for penta- and octabromodiphenyl ether (pentaBDE, octaBDE), Firemaster 550 (FM550), Firemaster BZ-54 (BZ54), bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP), triphenyl phosphate (TPhP), and nonbrominated BEH-TEBP analog bis(2-ethylhexyl) phthalate (BEHP) ranged from 0.058 mg/L (pentaBDE) to 3.96 mg/L (octaBDE). mRNA expression, ¹H NMR-based metabolomic and lipidomic profiling at 1/10 LC₅₀ revealed distinct patterns of molecular response for each exposure, suggesting pentaPBDE affects transcription and translation, octaBDE and BEH-TEBP affect glycosphingolipid biosynthesis and BZ54 affects Wnt and

*Corresponding Author. cvulpe@ufl.edu.

‡Present Addresses

National Institute of Standards and Technology, Biomolecular Measurement Division, Gaithersburg, MD 20899.

#University of Florida, Gainesville, Center for Environmental and Human Toxicology, Department of Physiological Sciences, Gainesville, FL 32611

ASSOCIATED CONTENT

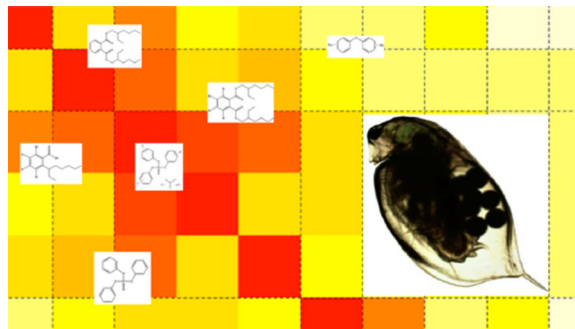
Supporting Information

Detailed methods on differential mRNA determination, qPCR, FM550 dose–response analysis, and metabolomic and lipidomic analyses; Table S1, chemical components of COMBO media; Table S2, raw acute toxicity data; Table S3, PentaBDE and OctaBDE congener profiles and weight; Table S4, primer sequences used in qPCR; Table S5, acute, 48-h LC₅₀ values of flame-retardants; Table S6, concentration data from absorption studies; Table S7a, numbers of differentially expressed genes in each 1/10 LC₅₀ FR exposure; Table S7b, numbers of differentially expressed genes in FM550 dose–response; Table S8, qPCR results; Table S9, KEGG pathway analysis on FM550 dose–response data; Table S10, gene ontology enrichment analysis on FM550 dose–response data; Figure S1, HOPACH clustering based on chemical profiles of FM550 dose–response data; and Figure S2, PLS-DA plots of FM550 and pentaBDE metabolomic data. The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.est.5b00977](https://doi.org/10.1021/acs.est.5b00977).

The authors declare no competing financial interest.

Hedgehog signal pathways as well as glycosaminoglycan degradation. Brominated components of FM550 (i.e., BZ54) were significantly higher in *Daphnia* after 48 h following 1/10 LC₅₀ exposure. FM550 elicited significant mRNA changes at five concentrations across a range from 1/10⁶ LC₅₀ to 1/2 LC₅₀. Analyses suggest FM550 impairs nutrient utilization or uptake in *Daphnia*.

Graphical Abstract



INTRODUCTION

The use of chemical flame-retardants (FR) in furniture became common in the United States and around the world in the 1970s. The state of California, for example, passed TB117, a mandate requiring the use of FR chemicals in all upholstered furniture. FRs such as polybrominated diphenyl ethers (PBDE) were used to meet this requirement.^{1,2} Recent laws amended decades-old flammability standards,³ but FR chemicals are still used in consumer products and are present in homes and the environment. Leaching of PBDEs from consumer products led to global contamination in soil,⁴ sewage sludge,⁵ coastal sediments,⁶ the atmosphere,⁷ and the arctic.⁸ PBDEs have been detected in biota including sea turtle eggs,⁹ lake trout, Chinook salmon,¹⁰ humpback dolphins,¹¹ and frogs.¹² Humans can be exposed to PBDEs through dietary sources and from inadvertent ingestion of contaminated house dust particles.^{13,14} Toxicity data for PBDEs and other FRs exist for humans and terrestrial animals (see Dishaw et al. 2014 for a recent review¹⁵).

PBDEs can accumulate in marine copepods,^{16,17} affect molting and cause toxicity in aquatic invertebrates,¹⁸ and biomagnify in aquatic food chains.¹⁹ Studies on *Daphnia magna* show that hexaBDE affects reproduction in the $\mu\text{g/L}$ range.²⁰ Tetra- and triBDE can delay molting in adult daphnids,²¹ while pentaBDE changes retinoid status in zebrafish.²² PBDEs induce many adverse effects, but specific molecular mechanisms of toxicity are not fully understood.²³

PBDEs are found globally and therefore have the potential to affect terrestrial and aquatic organisms. As persistent and bioaccumulative chemicals prone to long-range global transport,^{24,25} pentaBDE and octaBDE were banned from use in the European Union, added to the Stockholm Convention as Annex A chemicals slated for elimination, and voluntarily phased-out of use in the United States.^{24,26–28} As a result, levels of PBDEs are decreasing in homes, and there is a corresponding increase in a pentaBDE replacement chemical, Firemaster 550 (FM550).²⁹

FM550 was first used as a pentaBDE replacement in 2004.² It is a mixture of four different chemicals: two brominated components, bis (2-ethylhexyl) tetrabromophthalate (BEH-TEBP, 8%) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB, 30%), and two aryl phosphate ester compounds, triphenyl phosphate (TPhP, 17%) and isopropylated triaryl phosphates (ITP, 45%). ITP is a mixture of ortho-, meta-, and para-substituted isomers of mono-, di-, tri-, and tetra-ITPs.³⁰⁻³² Related mixture Firemaster BZ54 consists of BEH-TEBP (30%) and EH-TBB (70%).³³

FM550 is applied to polyurethane foam in furniture³⁴ and some infant products.³⁵ The brominated components BEH-TEBP and EH-TBB have been detected in house dust,^{31,36} marine biota,¹¹ mysid shrimp,³⁷ a bivalve, and a gastropod.³⁸ FM550 component triphenyl phosphate (TPhP, also called TPP) is a EU high-production volume plasticizer and flame-retardant that can enter the environment by diffusive volatilization, leaching, and abrasion.³⁹ It has been detected in sewage treatment plant influent and effluent,⁴⁰ air, water, house dust, and sediment.⁴¹

In fathead minnow liver cells, EH-TBB and BEH-TEBP caused a significant increase in DNA strand breaks during exposure but not after a recovery period.⁴² Fathead minnow and carp hepatic subcellular fractions metabolize EH-TBB and BEH-TEBP in vitro.³³ EH-TBB and BEH-TEBP affect fecundity, perhaps through an endocrine effect, in Japanese medaka.⁴³ TPhP and monoITP exposures affect zebra fish embryo heart development through AhR-independent (TPhP, monoITP) and AhR-dependent (monoITP) pathways.^{32,44} 96-h LC₅₀ values for TPhP on fish species range from 0.3 to 1.2 mg/L, but can be as high as 300 mg/L; LC₅₀ values for TPhP on *Daphnia* range from 1 to 1.35 mg/L.⁴⁵ The present study is the first to look at mechanistic effects of FM550 to the lower-level trophic species *Daphnia magna*.

Aquatic toxicants are commonly divided into categories based on chemical properties and presumed mechanism of action: nonpolar hydrophobic chemicals causing Type I narcosis, polar hydrophobic chemicals causing Type II narcosis, unselective reactive chemicals, and chemicals with specific modes of action.⁴⁶ Hydrophobic chemicals with a log K_{ow} 2.7 are often considered narcotics. Toxicity attributed to narcosis is postulated to result from chemical disruption of lipid-based cellular membranes.^{47,48} Type I molecules are believed to move three-dimensionally through membranes, while Type II molecules interact with charged phospholipid headgroups;⁴⁹ but toxicity is attributed to a shared mechanism.⁴⁸ Some hydrophobic chemicals cause greater toxicity than is predicted from K_{ow} ,⁴⁹ which cannot be attributed to narcosis.⁴⁶

Daphnia are parthenogenetic filter feeders used to evaluate invertebrate response to environmental pollutants.^{50,51} Exposure of biota to FRs may occur through disposal of consumer goods containing FRs and subsequent leaching, and it is therefore important to understand the effects of FRs on environmentally relevant *Daphnia magna*. This work represents the first comprehensive analysis of the acute toxicity of seven emerging and legacy chemical FR formulations: PentaBDE, octaBDE, FM550, BZ54, BEH-TEBP, TPhP, and BEHP. Our goals were to determine LC₅₀ values for each chemical and formulation and to investigate molecular mechanisms of toxicity by assessing effects on mRNA with

microarrays, metabolic changes by ^1H NMR-based metabolomics and lipidomic profiling. In addition, we determined if the bromine-containing FM550 components accumulate in *Daphnia*, and identified potential biomarkers of exposure for FM550.

MATERIALS AND METHODS

Daphnia Culture

Daphnia magna were cultured asexually in a growth chamber (Conviron) at 21 ± 1 °C with 16 h of light and 8 h of dark per day in COMBO⁵² media. They were fed 1 mL/L *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) from a 3.0×10^7 cells/mL stock and 1 mL/L yeast cereal-leaf and trout chow mix (YCT) three times per week following renewal of media. Media was aerated overnight to increase dissolved oxygen levels. pH was maintained at 7.4–7.8. Media chemical composition is described in Supporting Information (SI) Table S1. All chemical exposures were done in glass beakers (acute: 50 mL, for RNA extraction: 1 L, for absorption: 4 L). Animals and feed were obtained from Aquatic Research Organisms.

Toxicity Assays

Acute toxicity assays were similar to U.S. EPA Whole Effluent Toxicity guidelines.⁵³ Five first instar (<24 h old) daphnids were added to 35 mL COMBO media. Chemical FRs were dissolved in 0.05–0.1% dimethyl sulfoxide (DMSO) (EMD Chemicals, Inc.). Typically, four replicates of five daphnids were exposed to five different concentrations of FR or DMSO-control simultaneously. Concentrations tested can be found in SI Table S2. At least three sets of four replicates each were conducted per FR, with the exception of BZ54, for which a significant LC₅₀ was determined after fewer exposures. Animals were fed algae after 24 h, and lethality was measured after 48 h. Acute LC₅₀ values were determined using probit⁵³ or Spearman–Karber method.⁵⁴ Three statistical methods were used to investigate correlation between log K_{ow} and log LC₅₀ for each compound (SI). Briefly, log K_{ow} values were taken from published studies and, for PBDEs, log K_{ow} values were weighted by congener abundance (SI Table S3).^{32,55–58} Chemicals were manufactured by Chemtura (FM550 and BZ54), Unitex (BEH-TEBP), Aldrich (TPhP), Larodan Chemicals (pentaBDE), Bromine Compounds Ltd. (octaBDE), and Aldrich (BEHP). Chemical abbreviation nomenclature is from Bergman et. al 2012.⁵⁹ Chemical structures are shown in Figure 1. All subsequent assays were done at 1/10 LC₅₀ (1/10 of one toxic unit) or dilutions thereof.

Uptake of FM550

100 adult (14 day old) daphnids were exposed in 4 L COMBO media to 1/10 LC₅₀, 1/10 000 of the LC₅₀ (0.0486 mg/L, 0.0486 µg/L), or to a DMSO control. Exposure concentrations are based on LC₅₀ values and are not necessarily environmentally relevant. Animals were fed algae after 24 h, removed from exposure media at 48 h and put in chemical-free media with food for 24 h to clear the alimentary canal. Animals were removed and frozen at –80 °C for analytical analyses, or, for microarray analyses, RNA was extracted immediately with methods described below. Exposures were repeated until a total of four biological replicates of ~400 daphnids were collected per concentration. Samples were analyzed for EH-TBB and BEH-TEBP concentration using gas chromatography mass spectrometry operated in

negative chemical ionization mode (GC/ECNI-MS).³¹ Detailed methods are in the SI. A Welch two-sample *t* test was performed to determine significant differences in concentration of FRs between exposure groups. Samples with *p*-value <0.05 were considered significant. EH-TBB was not detected in laboratory blanks (<0.5 ng) but was detected in the control samples (83.6 ±22.8 ng). BEH-TEBP was not detected in laboratory blanks (1.2 ± 1.1 ng) and was above the method detection limit (MDL) in one control sample.

BEH-TEBP and EH-TBB were also measured in the YCT daphnia food. One L YCT was centrifuged in 250 mL aliquots at 3000 rpm for 20 min. The aqueous supernatant was removed and samples were dried at 80 °C for 4 h. Dried samples were then analyzed with the above analytical and computational methods.

mRNA Microarray

Effects of exposure to each chemical (pentaBDE, octaBDE, FM550, BZ54, BEH-TEBP, TPhP, or BEHP) was assayed via mRNA microarray. For each chemical, 15–20 adult (14 day old) daphnids were exposed to 1/10 LC₅₀ or to an equal-volume DMSO control in 800 mL COMBO media. Animals were fed algae after 24 h, and after 48 h RNA was extracted in Trizol reagent (Invitrogen) with a hand-held homogenizer (Biospec Products Inc.). RNA quality was assessed with spectrophotometry and agarose gel electrophoresis. 200 ng RNA was then reverse-transcribed, amplified and hybridized onto a custom Agilent Oligonucleotide DNA microarray (AMADID #023710) with the Agilent Low-Input Quick-Amp one-color array kit and protocol (Santa Clara, CA). Four exposed and three or four control arrays were done for each condition. Arrays were scanned with a 16-bit GenePix 4000B Microarray Scanner with 5-µm resolution. Features were edited and regression analysis was performed with GenePix Pro, and the resultant data were processed as in Loguinov et al.⁶⁰ Detailed statistical analysis methods are available in the SI. Array data are available at <http://www.ncbi.nlm.nih.gov/geo>.

Quantitative Reverse Transcription PCR

To independently verify microarray results, transcription of nine genes in ten conditions was analyzed with qPCR. Genes were chosen based on *q*-value, degree of differential transcription or potential mode of toxicity. One µg RNA (from independent biological replicates) was reverse transcribed and amplified as in Scanlan et al.⁶¹ Actin and GAPDH were used as housekeeping genes. Primer sequences are shown in SI Table S4. Detailed methods are available in the SI.

Gene Ontology, Pathway Enrichment and Cluster Analysis

The *Daphnia magna* array was annotated with a protein blast as in Antczak et al. 2013,⁶² which identified 4958 *Daphnia pulex* homologues with an expect (*E*) value less than or equal to 10⁻⁴. The list was matched with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database annotation (www.genome.jp/kegg) and genes were sorted into respective pathways. Of these, 1425 mapped onto 114 *Daphnia pulex* KEGG pathways. Pathways representing less than five genes in the array were removed, leaving 95 pathways and 1402 genes total in an unbiased sample of the original 371 KEGG pathways. Significance was calculated using a modified Fisher Exact Probability *p*-value.^{63,64}

The R package *hopach* (implementation of Hierarchical Ordered Partitioning And Collapsing Hybrid algorithm⁶⁵) from bioconductor.org was applied to data to compute ordered distance matrices using the cosangle-based distance metric⁶⁵ as a measure of similarity. HOPACH is considered superior to standard hierarchical clustering because it applies nonparametric bootstrap resampling to determine the probability of “cluster membership” for each gene or chemical. Nonparametric strapping (resampling with replacement) analyses were run 1000 times to determine cluster membership probability for each gene and each main cluster. Further methodological and statistical details are in the SI.

Blast2GO⁶⁶ (B2G) gene ontology enrichment analysis was performed on mRNA data to determine gene functions affected in each exposure. Microarray gene probes and EST sequences were annotated with B2G (default parameters) to create a reference gene set. Data for each chemical was compared to the reference set via Enrichment Analysis (default, two-tailed settings). Enrichment was performed with 0.01, 0.05, and 0.15 False Discovery Rates (FDR, measure of significance).

Metabolomics of FM550 and pentaBDE-exposed *Daphnia*

For instrument optimization, hemolymph from 80 unexposed adult (14 day old) daphnids was collected from daphnids incubated in 800 mL COMBO media with feeding after 24 h. After 48 h, hemolymph was extracted by piercing the carapace with a needle and aspirating hemolymph with a small pipet.⁵² For metabolomics analysis, animals were exposed to 1/10 LC₅₀ FM550 or pentaBDE or DMSO control (with equal volume chemical), fed after 24 h and frozen on dry ice after 48 h. Seven biological replicates of 40 animals each were collected for each condition and stored at -80 °C. Hemolymph was extracted using a dual phase extraction. Detailed extraction, analysis and statistical methods are available in the SI. Briefly, a mixture of methanol, chloroform and water in the volume ratio of 4:4:2.85 was used to generate a two-phase extract.⁶⁷ ¹H NMR spectra were acquired at 20 °C on an Agilent Inova 600 MHz NMR spectrometer with a cryogenic triple-resonance flow probe using direct-injection NMR analysis.^{68,69} Multivariate data analysis, principal components analysis and partial least-squares discriminant analysis were performed with SIMCA-P+ (Umetrics Inc., Umea, Sweden); univariate analysis of the binned spectra was conducted using Excel. Results were further analyzed with MetaboAnalyst 2.0 (<http://www.metaboanalyst.ca>)^{70,71} to determine overrepresentation of biological function groups and biological pathways.

Lipidomics of FM550 and pentaBDE-exposed *Daphnia*

Daphnids were exposed to 1/10 LC₅₀ FM550 or pentaBDE or DMSO control, as described above for mRNA assays. After 48 h, animals were removed from culture, flash-frozen on dry ice, ground in 1.6 mL ultrapure water with a hand-held homogenizer (Biospec Products Inc.) and frozen at -80 °C. Lipids were extracted and analyzed by the Kansas State University Lipidomics Research Center. Five replicates of 10–11 animals were collected for each condition. Data were log transformed and significance was determined with a standard two-sample *t* test and a Wilcoxon rank sum test for two sample data. Additional details on extraction methods and statistical analyses are available in SI.

RESULTS

Flame-Retardant Toxicity in *Daphnia magna*

LC₅₀ values were determined for each FR after a 48 h exposure (SI Table S5 and Figure 2) and are reported as milligrams chemical per liter COMBO media (mg/L). PentaBDE was significantly more toxic than any other FR (LC₅₀ = 0.058 mg/L); the second most toxic chemical was 1 order of magnitude less toxic (FM550, 0.0486 mg/L). Significance was determined by binary comparison of LC₅₀ values and corresponding confidence intervals.⁷² OctaBDE and BEHP were the least toxic (3.96 and 3.31 mg/L). Of note, BEHP was significantly less toxic to *Daphnia* than its brominated homologue BEH-TEBP. No correlation was found between the log K_{ow} and the log LC₅₀ for each compound (SI), although sample size precludes robust statistical confidence.

FM550 Detected in *Daphnia magna* after a 48-h Exposure to 1/10 LC₅₀

Daphnids exposed to the higher concentration (0.0486 mg/L) for 48 h showed a significant increase in both BEH-TEBP and EH-TBB as compared to control (Figure 3). Exposure at the lower concentration (0.0486 µg/L) was not significantly higher than control. Daphnid food YCT had an average of 0.625 ng/g (dry) EH-TBB and 0.629 ng/g BEH-TEBP ($n = 3$). Measured concentrations are reported in SI Table S6.

Each Flame-Retardant Caused Distinct Patterns of Change in mRNA levels at 1/10 LC₅₀

mRNA microarray, qPCR and computational analyses were used to investigate and compare biological effects of FR exposure. SI Table S7a lists the number of differentially expressed genes in each condition. qPCR independently verified microarray results for 25 out of 29 genes tested (SI Table S8). HOPACH cluster analysis of differential mRNA levels grouped BEH-TEBP and BZ54 together, while all other FRs clustered individually (Figure 4). KEGG pathway enrichment analysis found a total of 12 pathways overrepresented (Table 1), four in common to two conditions.

FM550 Dose Response: All Concentrations Changed mRNA

Microarrays analysis was performed with RNA from daphnids exposed to five FM550 concentrations ($N =$ four replicates of ~20 animals each). Concentrations were: 1/2 LC₅₀ (0.243 mg/L), 1/10 LC₅₀ (0.0486 mg/L), and three additional dilutions: 0.243 µg/L, 0.0486 µg/L, and 0.0486 ng/L. Exposure at 1/10,000 LC₅₀ (0.0486 µg/L) resulted in the largest number of differentially expressed genes when compared to the untreated control. All concentrations caused differential mRNA levels; the no-observed transcriptional effect level^{73,74} was not reached (SI Table S7b). Three genes (a trichohyalin-like protein, peroxidase and an unknown protein) were differentially expressed at each concentration (Table 2a). When compared to other FRs, HOPACH clustered all concentrations together, with three concentrations significantly similar to each other (SI Figure S1). KEGG pathway enrichment found significant enhancement of pathways (p -value < 0.1) for three concentrations (0.0486 mg/L, 0.0486 µg/L, and 0.0486 ng/L). A total of seven pathways were affected; each was unique to one concentration (SI Table S9). Analyses for linear trend⁷⁵⁻⁷⁷ found three genes significantly, negatively associated with concentration (Table

2b) and a large decrease in gene response at the highest concentrations (not shown). Clustering based on mRNA levels found 44 clusters (not shown). B2G⁶⁶ gene ontology analysis with GOSSIP⁷⁸ on the largest cluster indicated overexpression of functions related to oxygen binding, oxygen transporter activity and the hemoglobin complex (SI Table S10).

FM550 Metabolite Profiles Are Distinguishable from Control, while PentaBDE Profiles Are Not

¹H NMR was used to investigate changes in hemolymph metabolomic profiles between control and 1/10 LC₅₀-exposed *Daphnia* (Figure 5). PLS-DA scores plots (SI Figure S2) show considerable separation between control and FM550-exposed daphnids (validated with ANOVA, *p*-value = 0.04). FM550 exposure changed levels of 14 small-molecule metabolites (Table 3). Pathway analysis with MetaboAnalyst showed an increase in nitrogen metabolism, arginine and proline metabolism, a decrease in valine, leucine, and isoleucine degradation and changes in aminoacyl-tRNA biosynthesis (Table 4). Enrichment analysis with MetaboAnalyst showed an increase in ammonia recycling, a decrease in valine, leucine, and isoleucine degradation, and changes in protein biosynthesis (Table 5). There was no significant difference between pentaBDE-exposed and control groups (Figure 5 and SI Figure S2).

Changes in Lipidome Were Detected after Exposure to both FM550 and PentaBDE

To complement transcriptomic and metabolomic studies, changes in the lipidome of FM550 and pentaBDE-exposed animals were analyzed. Both pentaBDE and FM550 significantly changed the level of two fats out of 352 tested (*p*-value = 0.04, Table 6). FM550 increased a phosphatidylcholine with 42 carbons and seven double bonds (PC 42:7), while pentaBDE increased lysophosphatidylcholine (LPC 20:0). Both increased phosphatidylcholine (PC 44:9).

DISCUSSION

Flame-Retardants Are Toxic to *Daphnia magna*

LC₅₀ values show that FM550 and its components are highly toxic to aquatic organisms as defined by the U.S. EPA (LC₅₀ values of 0.1–1 mg/L). In situ toxicity to *Daphnia magna* could cause ecologically relevant physiological disruptions and results in wide-reaching effects in freshwater ecosystems.⁷⁹ PentaBDE was one to 2 orders of magnitude more toxic than any other FR; octaBDE was one of the least toxic. The phenomenon of increased toxicity with less-brominated PBDEs has been seen in other organisms,^{80–82} but the trend is not always true in *Daphnia magna*.²¹ BZ54, which contains BEH-TEBP and EH-TBB, was significantly more toxic than BEH-TEBP alone. TPhP, BEHP, and BZ54 LC₅₀ values agree with those previously derived.^{32,83–85} Actual dissolved chemical concentrations for each FR are unknown as quantification in aqueous media is difficult and inaccurate. This work establishes for the first time nominal LC₅₀ values for the FM550 mixture, BEH-TEBP, and octaBDE on *Daphnia magna*.

Molecular Effects May Be Unique for Each Chemical and Formulation

HOPACH analysis (Figure 4) showed that BEH-TEBP and BZ54 were the only exposures to cause similar effects, which is not surprising as BZ54 contains 30% BEH-TEBP and 70% BEH-TBB.³³ Three of the five FM550 concentrations grouped into one cluster, indicating similar but not identical effects. Analyses with KEGG suggest distinct molecular effects by each chemical (Table 1). For example, octaBDE and BEH-TEBP increased the glycosphingolipid biosynthesis pathway, which is involved in growth factor signaling and morphogenesis in arthropods.⁸⁶ Glycosphingolipid function is not comprehensively understood,⁸⁷ but changes in function or abundance could affect cell proliferation, apoptosis, necrosis, senescence, and differentiation, as well as inflammation and autophagy and the stress response.⁸⁸ BZ54, on the other hand, affected Wnt signal transduction (active during developmental processes⁸⁹), Hedgehog signal transduction and glycosaminoglycan degradation (the latter two are largely unstudied in *Daphnia*). Changes to Wnt signaling could affect embryonic patterning and morphogenesis, and result in population effects. Results for pentaBDE suggest an effect on transcription and translation. In mammals, pentaBDE affect thyroid hormone homeostasis.¹⁵ Thyroid hormone acts as a translation factor that promotes differentiation and maturation of various tissues in vertebrates.⁹⁰ In *Daphnia*, thyroid hormone has been shown to cause an increased growth rate.⁹⁰ If pentaBDE causes increased growth in *Daphnia*, natural daphnid populations could be affected (reduced food availability, overcrowding, etc.). It is important to note that the microarray does not represent the entire *Daphnia* transcriptome and *Daphnia* gene-functions are not well characterized, so additional mechanisms may be involved.

Hydrophobic chemicals with a $\log K_{ow} > 2.7$ are often considered “narcotic.” If chemicals are more toxic than predicted by K_{ow} , then effects cannot be attributed to narcosis alone.^{46,49} To investigate narcosis, we compared the $\log K_{ow}$ and $\log LC_{50}$ of each chemical,⁹¹ and did not find a relationship between the two variables (SI). This work is limited because we were not able to measure actual aquatic FR concentrations. Previous work by Vandebrouck et al. in *Daphnia* on two presumed narcotics (pyrene and fluoranthene) did not find clear separation between gene transcription profiles.⁹² However, in the present work, clear differences were seen at the mRNA, metabolomic, and lipidomic levels. Taken together, these results suggest that the traditional definitions of Type I and Type II narcosis^{48,49} may not sufficiently encompass the biological effects of hydrophobic chemicals.

FM550 May Cause Nutritional Dysregulation in *Daphnia magna*

Transcriptomic, metabolomic, and lipidomic data indicate general xenobiotic response and effects related to nutritional status in *Daphnia* after exposure to FM550 (Figure 6). Specific metabolites with the largest changes (Table 3) are histidine, glucose, and lysine. Histaminergic signaling in the *Daphnia* central nervous system controls phototactic behavior (response to UV light) and is moderated by food abundance and quality.⁹³ Changes to the system can effect *Daphnia* populations and ecosystems.⁹³

Glucose is used as fuel as well as conjugate for Phase II metabolism, to increase xenobiotic excretion.⁹⁴ *Daphnia* have genes that encode enzymes for gluconeogenesis and for

breakdown of glycogen, to maintain the minimal glucose levels for survival.⁹⁵ Increased glucose levels, detected by metabolomics, could be caused by reduced Phase II metabolism, or by glycogen breakdown. However, changes to the related enzymes and pathways were not detected with microarray analysis.

Enrichment analysis of ¹H NMR metabolomic data (Table 5) shows changes in ammonia recycling, protein biosynthesis, and amino acid degradation. *Daphnia* has two main forms of nitrogen: amino acids (AAs) and ammonia (NH₃).⁹⁶ Essential AAs are highly correlated to changes in nitrogen status and occur during fasting and growth.⁹⁷ During starvation, NH₃ excretion increases; NH₃ is primarily sourced from amino acids and secondarily from nucleic acids.⁹⁸ Ammonia cycling is related to protein catabolism in *Daphnia*⁹⁹ and can increase along with the rate of ammonia excretion when Cladocera are starving.^{100,101}

Daphnia growth is primarily associated with AA abundance.¹⁰² Pathway analysis (Table 4) detected effects on valine, leucine, and arginine metabolism and changes in aminoacyl-tRNA. Valine, leucine, proline, and arginine are three of the most common *Daphnia* AAs.⁹⁷ Proline is an antioxidant in *Daphnia*.⁹⁴ Aminoacyl-tRNA is used by the ribosome to make peptide chains, and aminoacyl-tRNA synthase activity is correlated with somatic growth in *Daphnia*.¹⁰² Together, the results indicate a clear effect on metabolites mediating growth.

KEGG analysis complements the metabolomics data. While the KEGG findings were not the same for each of the five FM550 concentrations tested, all results indicate changes to nutritional status and growth (ribosome, fatty acid metabolism, valine, leucine and isoleucine degradation, amino sugar, and nucleotide sugar metabolism) or xenobiotic metabolism (peroxisome and glutathione metabolism). Glycosphingolipid is involved in growth and in the stress response.⁸⁸ Transcriptomic data also show possible effects on oxygen transport (SI Table S10). One differentially expressed gene common to all FM550 exposure concentrations (translation initiation factor) decreased as the concentration of FM550 increased, again indicating effects on protein synthesis and growth. Lipidomic analysis showed an increase in one highly unsaturated fatty acid (HUFA) (42:7, Phosphatidyl choline). Growth in *Daphnia* is secondarily associated with HUFA availability.¹⁰² HUFA are important for cell membrane support and are conserved during starvation.⁹⁴

Three genes differentially expressed in all five concentrations may be useful biomarkers of exposure to FM550. Validation of biomarkers would require further studies to identify and verify gene products in *Daphnia* and in other organisms.

The brominated compounds in FM550 and BZ54, BEH-TBPH, and EH-TBB, are detected in marine mammals,¹¹ accumulate in fathead minnows,⁴² and have potential to accumulate in other aquatic organisms. We found the compounds in *D. magna* after 48 h (Figure 3), suggesting the possibility for trophic transfer. Low levels of the compounds were also detected in YCT daphnid food (BEH-TBB: 0.625 ± 0.198 ng/g; BEH-TEBP: 0.629 ± 0.018 ng/g), which is made from yeast, fermented trout chow and cereal leaves. YCT contamination is the probable source of detected FRs in control animals (SI Table S6) and illuminates the broader problem of global FR contamination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NSF CBET-1066358 to C.D.V., NIEHS R01ES016099 to H.M.S., and NERC Grant NE/1028246/2 to F.F. The lipid analyses performed at Kansas Lipidomics Research Center Analytical Laboratory were supported by NSF EPS 0236913, MCB 0455318, and DBI 0521587. The views expressed in this paper are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. TOC graphic image was taken at the U.C. Berkeley College of Natural Resources Biological Imaging Facility.

REFERENCES

1. Shaw SD, Blum A, Weber R, Kannan K, Rich D, Lucas D, Koshland CP, Dobraca D, Hanson S, Birnbaum LS. Halogenated flame retardants: do the fire safety benefits justify the risks? *Rev. Environ. Health.* 2010; 25(4):261–305. [PubMed: 21268442]
2. Blum A. The fire retardant dilemma. *Science (New York NY).* 2007; 318(5848):194–195.
3. Bureau of Electronic and Appliance Repair, Home Furnishings; and Thermal Insulation. , editor. State of California Department of Consumer Affairs, Technical Bulletin 117-2013. CA: 2013.
4. Gao S, Hong J, Yu Z, Wang J, Yang G, Sheng G, Fu J. Polybrominated diphenyl ethers in surface soils from e-waste recycling areas and industrial areas in South China: Concentration levels, congener profile, and inventory. *Environ. Toxicol. Chem./SETAC.* 2011; 30(12):2688–2696.
5. Daso AP, Fatoki OS, Odendaal JP, Olujimi OO. Occurrence of selected polybrominated diphenyl ethers and 2,2',4,4',5,5'-hexabromobiphenyl (BB-153) in sewage sludge and effluent samples of a wastewater-treatment plant in Cape Town, South Africa. *Arch. Environ. Contam. Toxicol.* 2011; 62(3):391–402. [PubMed: 22002787]
6. Kimbrough, KL.; Johnson, WE.; Lauenstein, GG.; Christensen, JD.; Apeti, DA. An Assessment of Polybrominated Diphenyl Ethers (PBDEs) in Sediments and Bivalves of the U.S. Coastal Zone. Memorandum, NT., editor. Silver Spring, MD: 2009. p. 87Vol. *NOS NCCOS*
7. Birgul A, Katsoyiannis A, Gioia R, Crosse J, Earnshaw M, Ratola N, Jones KC, Sweetman AJ. Atmospheric polybrominated diphenyl ethers (PBDEs) in the United Kingdom. *Environ. Pollut.* 2012; 169:105–111. [PubMed: 22705503]
8. Xiao H, Hung H, Wania F, Lao R, Sabljic E, Sverko E, Lei YD, Fellin P, Barresi E. Field evaluation of a flow-through sampler for measuring pesticides and brominated flame retardants in the arctic atmosphere. *Environ. Sci. Technol.* 2012; 46(14):7669–7676. [PubMed: 22702375]
9. Alava JJ, Keller JM, Wyneken J, Crowder L, Scott G, Kucklick JR. Geographical variation of persistent organic pollutants in eggs of threatened loggerhead sea turtles (*Caretta caretta*) from southeastern United States. *Environ. Toxicol. Chem./SETAC.* 2011; 30(7):1677–1688.
10. Kuo YM, Sepulveda MS, Hua I, Ochoa-Acuna HG, Sutton TM. Bioaccumulation and biomagnification of polybrominated diphenyl ethers in a food web of Lake Michigan. *Ecotoxicology (London).* 2010; 19(4):623–634.
11. Lam JC, Lau RK, Murphy MB, Lam PK. Temporal trends of hexabromocyclododecanes (HBCDs) and polybrominated diphenyl ethers (PBDEs) and detection of two novel flame retardants in marine mammals from Hong Kong, South China. *Environ. Sci. Technol.* 2009; 43(18):6944–6949. [PubMed: 19806725]
12. Liu PY, Du GD, Zhao YX, Mu YS, Zhang AQ, Qin ZF, Zhang XY, Yan SS, Li Y, Wei RG, Qin XF, Yang YJ. Bioaccumulation, maternal transfer and elimination of polybrominated diphenyl ethers in wild frogs. *Chemosphere.* 2011; 84(7):972–978. [PubMed: 21676430]
13. Zota AR, Rudel RA, Morello-Frosch RA, Brody JG. Elevated house dust and serum concentrations of PBDEs in California: unintended consequences of furniture flammability standards? *Environ. Sci. Technol.* 2008; 42(21):8158–8164. [PubMed: 19031918]

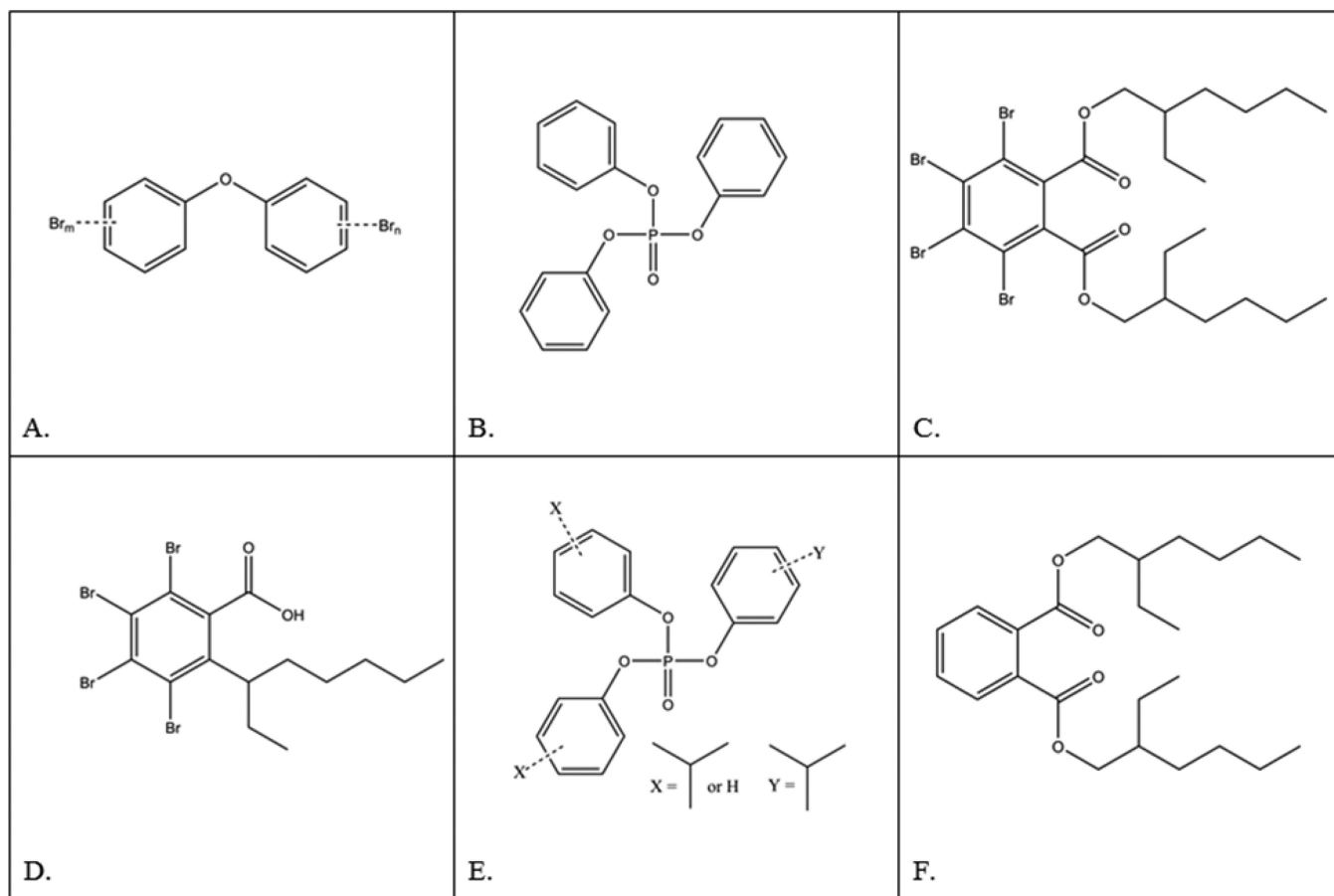
14. Huwe JK, West M. Polybrominated diphenyl ethers in U.S. meat and poultry from two statistically designed surveys showing trends and levels from 2002 to 2008. *J. Agric. Food Chem.* 2011; 59(10):5428–5434. [PubMed: 21491934]
15. Dishaw LV, L JM, Roberts SC, Stapleton HM. Exposures, mechanisms, and impacts of endocrine-active flame retardants. *Curr. Opin. Pharmacol.* 2014; 19:125–133. [PubMed: 25306433]
16. Magnusson K, Tiselius P. The importance of uptake from food for the bioaccumulation of PCB and PBDE in the marine planktonic copepod *Acartia clausi*. *Aquat. Toxicol. (Amsterdam)*. 2010; 98(4):374–380.
17. Magnusson K, Magnusson M, Ostberg P, Granberg M, Tiselius P. Bioaccumulation of 14C-PCB 101 and 14C-PBDE 99 in the marine planktonic copepod *Calanus finmarchicus* under different food regimes. *Mar. Environ. Res.* 2007; 63(1):67–81. [PubMed: 16949662]
18. Gismondi E, Thome JP. Effects of two PBDE congeners on the moulting enzymes of the freshwater amphipod *Gammarus pulex*. *Environ. Pollut.* 2014; 191:119–125. [PubMed: 24832922]
19. Losada S, Roach A, Roosens L, Santos FJ, Galceran MT, Vetter W, Neels H, Covaci A. Biomagnification of anthropogenic and naturally-produced organobrominated compounds in a marine food web from Sydney Harbour, Australia. *Environ. Int.* 2009; 35(8):1142–1149. [PubMed: 19665796]
20. Nakari T, Huhtala S. Comparison of toxicity of congener-153 of PCB, PBB, and PBDE to *Daphnia magna*. *Ecotoxicol. Environ. Safety.* 2008; 71(2):514–518. [PubMed: 18082264]
21. Davies R, Zou E. Polybrominated diphenyl ethers disrupt molting in neonatal *Daphnia magna*. *Ecotoxicology.* 2012; 21(5):1371–1380. [PubMed: 22476648]
22. Chen L, Hu C, Huang C, Wang Q, Wang X, Yang L, Zhou B. Alterations in retinoid status after long-term exposure to PBDEs in zebrafish (*Danio rerio*). *Aquat. Toxicol.* 2012; 120:11–18. [PubMed: 22580571]
23. Siddiqi MA, Laessig RH, Reed KD. Polybrominated diphenyl ethers (PBDEs): new pollutants-old diseases. *Clin. Med. Res.* 2003; 1(4):281–290. [PubMed: 15931321]
24. Stockholm Convention on persistent organic pollutants (POPs). , editor. Stockholm Convention, Decision POPRC-1/3: Pentabromodiphenyl ether. 2011.
25. Wania F, Dugani CB. Assessing the long-range transport potential of polybrominated diphenyl ethers: a comparison of four multimedia models. *Environ. Toxicol. Chem.* 2003; 22(6):1252–1261. [PubMed: 12785581]
26. The European Parliament, Directive 2002/95/EC of the European Parliament and of the Council of 27 January 2003 on the restriction of the use of certain hazardous substances in electrical and electrical equipment. 2003.
27. Stapleton HM, Sharma S, Getzinger G, Ferguson PL, Gabriel M, Webster TF, Blum A. Novel and high volume use flame retardants in US couches reflective of the 2005 PentaBDE phase out. *Environ. Sci. Technol.* 2012; 46(24):13432–13439. [PubMed: 23186002]
28. The European Parliament Directive 2003/11/EC of the European Parliament and of the Council of 6 February 2003 amending for the 24th time Council Directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations (pentabromodiphenyl ether, octabromodiphenyl ether), 2003.
29. Dodson RE, Perovich LJ, Covaci A, Van den Eede N, Ionas AC, Dirtu AC, Brody JG, Rudel RA. After the PBDE phase-out: a broad suite of flame retardants in repeat house dust samples from California. *Environ. Sci. Technol.* 2012; 46(24):13056–13066. [PubMed: 23185960]
30. Klosterhaus, S.; Konstantinov, A.; Davis, E.; Klein, J.; Stapleton, H. Characterization of the Brominated Chemicals in a PentaBDE Replacement Mixture and their Detection in Biosolids. Ottawa, Ontario, Canada: 2009.
31. Stapleton HM, Allen JG, Kelly SM, Konstantinov A, Klosterhaus S, Watkins D, McClean MD, Webster TF. Alternate and new brominated flame retardants detected in U.S. house dust. *Environ. Sci. Technol.* 2008; 42(18):6910–6916. [PubMed: 18853808]

32. McGee SP, Konstantinov A, Stapleton HM, Volz DC. Aryl phosphate esters within a major PentaBDE replacement product induce cardiotoxicity in developing zebrafish embryos: Potential role of the aryl hydrocarbon receptor. *Toxicol. Sci.: Off. J. Soc. Toxicol.* 2013; 133(1):144–156.
33. Berr JS, Mitchelmore CL, Roberts SC, Stapleton HM. Species specific differences in the in vitro metabolism of the flame retardant mixture, Firemaster(R) BZ-54. *Aquat. Toxicol. (Amsterdam)*. 2012; 124-125C:41–47.
34. Stapleton HM, Klosterhaus S, Eagle S, Fuh J, Meeker JD, Blum A, Webster TF. Detection of organophosphate flame retardants in furniture foam and U.S. house dust. *Environ. Sci. Technol.* 2009; 43(19):7490–7495. [PubMed: 19848166]
35. Stapleton HM, Klosterhaus S, Keller A, Ferguson PL, van Bergen S, Cooper E, Webster TF, Blum A. Identification of flame retardants in polyurethane foam collected from baby products. *Environ. Sci. Technol.* 2011; 45(12):5323–5331. [PubMed: 21591615]
36. Stapleton HM, Misenheimer J, Hoffman K, Webster TF. Flame retardant associations between children’s handwipes and house dust. *Chemosphere.* 2014; 116:54–60. [PubMed: 24485814]
37. Verslycke TA, Vethaak AD, Arijs K, Janssen CR. Flame retardants, surfactants and organotins in sediment and mysid shrimp of the Scheldt estuary (The Netherlands). *Environ. Pollut. (Barking)*. 2005; 136(1):19–31.
38. La Guardia MJ, Hale RC, Harvey E, Mainor TM, Ciparis S. In situ accumulation of HBCD, PBDEs, and several alternative flame-retardants in the bivalve (*Corbicula fluminea*) and gastropod (*Elimia proxima*). *Environ. Sci. Technol.* 2012; 46(11):5798–5805. [PubMed: 22571713]
39. Lin K. Joint acute toxicity of tributyl phosphate and triphenyl phosphate to *Daphnia magna*. *Environ. Chem. Lett.* 2009; 7(4):309–312.
40. Marklund A, Andersson B, Haglund P. Organophosphorus flame retardants and plasticizers in Swedish sewage treatment plants. *Environ. Sci. Technol.* 2005; 39(19):7423–7429. [PubMed: 16245811]
41. van der Veen I, de Boer J. Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis. *Chemosphere.* 2012; 88(10):1119–11153. [PubMed: 22537891]
42. Berr JS, Stapleton HM, Mitchelmore CL. Accumulation and DNA damage in fathead minnows (*Pimephales promelas*) exposed to 2 brominated flame-retardant mixtures, Firemaster 550 and Firemaster BZ-54. *Environ. Toxicol. Chem.* 2010; 29(3):722–729. [PubMed: 20821500]
43. Saunders DM, Podaima M, Codling G, Giesy JP, Wiseman S. A mixture of the novel brominated flame retardants TBPH and TBB affects fecundity and transcript profiles of the HPGL-axis in Japanese medaka. *Aquat. Toxicol. (Amsterdam)*. 2015; 158:14–21.
44. Gerlach CV, Das SR, Volz DC, Bisson WH, Kolluri SK, Tanguay RL. Mono-substituted isopropylated triaryl phosphate, a major component of Firemaster 550, is an AHR agonist that exhibits AHR-independent cardiotoxicity in zebrafish. *Aquat. Toxicol. (Amsterdam)*. 2014; 154:71–79.
45. United States Environmental Protection Agency. Furniture Flame Retardancy Partnership Environmental Profiles of Chemical Flame-Retardant Alternatives for Low-Density Polyurethane Foam. Vol. 2. Cincinnati, OH: 2005.
46. Verhaar HJ, Solbe J, Speksnijder J, van Leeuwen CJ, Hermens JL. Classifying environmental pollutants: Part 3. External validation of the classification system. *Chemosphere.* 2000; 40(8):875–883. [PubMed: 10718581]
47. Sanderson H, Thomsen M. Ecotoxicological quantitative structure-activity relationships for pharmaceuticals. *Bull. Environ. Contam. Toxicol.* 2007; 79(3):331–335. [PubMed: 17701090]
48. Veith GD, Broderius SJ. Rules for distinguishing toxicants that cause type I and type II narcosis syndromes. *Environ. Health Perspect.* 1990; 87:207–211. [PubMed: 2269227]
49. Roberts DW, Costello JF. Mechanisms of action for general and polar narcosis: A difference in dimension. *QSAR Comb. Sci.* 2003; 22(2):226–233.
50. Watanabe H, Kobayashi K, Kato Y, Oda S, Abe R, Tatarazako N, Iguchi T. Transcriptome profiling in crustaceans as a tool for ecotoxicogenomics: *Daphnia magna* DNA microarray. *Cell Biol. Toxicol.* 2008; 24(6):641–647. [PubMed: 18956242]
51. Seda J, Petrusek A, Sehnal F. *Daphnia* as a model organism in limnology and aquatic biology: some aspects of its reproduction and development PREFACE. *J. Limnol.* 2011; 70(2):336–336.

52. Kilham SS, Kreeger DA, Lynn SG, Goulden CE, Herrera L. COMBO: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologia*. 1998; 377:147–159.
53. Agency, U. S. E. P. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*. 5th. Washington DC: U.S. Environmental Protection Agency, Office of Water; 2002.
54. Hamilton MA, Russo RC, Thurston RV. Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.* 1977; 11(7):714–719.
55. Agency for Toxic Substances and Disease Registry (ATSDR). *Toxicological profile for Di(2-ethylhexyl)phthalate (DEHP)*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service; 2002.
56. Reemtsma T, Quintana JB, Rodil R, Garcia-Lopez M, Rodriguez I. Organophosphorus flame retardants and plasticizers in water and air I. Occurrence and fate. *Trac-Trends Anal. Chem.* 2008; 27(9):727–737.
57. La AGMJ, Hale RC, Harvey E. Detailed polybrominated diphenyl ether (PBDE) congener composition of the widely used penta-, octa-, and deca-PBDE technical flame-retardant mixtures. *Environ. Sci. Technol.* 2006; 40(20):6247–6254. [PubMed: 17120549]
58. Sjodin, A. *Occupational and Dietary Exposure to Organohalogen Substances, with Special Emphasis on Polybrominated Diphenyl Ethers*. Stockholm: Stockholm University; 2000.
59. Bergman A, Ryden A, Law RJ, de Boer J, Covaci A, Alaee M, Birnbaum L, Petreas M, Rose M, Sakai S, Van den Eede N, van der Veen I. A novel abbreviation standard for organobromine, organochlorine and organophosphorus flame retardants and some characteristics of the chemicals. *Environ. Int.* 2012; 49:57–82. [PubMed: 22982223]
60. Loguinov AV, Mian IS, Vulpe CD. Exploratory differential gene expression analysis in microarray experiments with no or limited replication. *Genome biology*. 2004; 5(3):R18. [PubMed: 15003121]
61. Scanlan LD, Reed RB, Loguinov AV, Antczak P, Tagmount A, Aloni S, Nowinski DT, Luong P, Tran C, Karunaratne N, Pham D, Lin XX, Falciani F, Higgins CP, Ranville JF, Vulpe CD, Gilbert B. Silver nanowire exposure results in internalization and toxicity to *Daphnia magna*. *ACS Nano*. 2013; 7(12):10681–10694. [PubMed: 24099093]
62. Antczak P, Jo HJ, Woo S, Scanlan L, Poynton H, Loguinov A, Chan S, Falciani F, Vulpe C. Molecular toxicity identification evaluation (mTIE) approach predicts chemical exposure in *Daphnia magna*. *Environ. Sci. Technol.* 2013; 47(20):11747–11756. [PubMed: 23875995]
63. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 2009; 4(1):44–57. [PubMed: 19131956]
64. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 2009; 37(1):1–13. [PubMed: 19033363]
65. van der Laan MJ, Pollard KS. A new algorithm for hybrid hierarchical clustering with visualization and the bootstrap. *J. Stat. Plan. Inf.* 2003; 117(2):275–303.
66. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics (Oxford)*. 2005; 21(18):3674–3676.
67. Viant MR. Revealing the metabolome of animal tissues using 1H nuclear magnetic resonance spectroscopy. *Methods Mol. Biol. (Clifton)*. 2007; 358:229–246.
68. Teng Q, Ekman DR, Huang W, Collette TW. Push-through direct injection NMR: an optimized automation method applied to metabolomics. *Analyst*. 2012; 137(9):2226–2232. [PubMed: 22434060]
69. Bax A, Davis DG. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* 1985; 65(2):355–360.
70. Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Res.* 2009; 37(Web Server issue):W652–W660. [PubMed: 19429898]

71. Xia J, Mandal R, Sinelnikov IV, Broadhurst D, Wishart DS. MetaboAnalyst 2.0—a comprehensive server for metabolomic data analysis. *Nucleic Acids Res.* 2012; 40(Web Server issue):W127–W133. [PubMed: 22553367]
72. Wheeler MW, Park RM, Bailer AJ. Comparing median lethal concentration values using confidence interval overlap or ratio tests. *Environ. Toxicol. Chem./SETAC.* 2006; 25(5):1441–1444.
73. Lobenhofer EK, Cui X, Bennett L, Cable PL, Merrick BA, Churchill GA, Afshari CA. Exploration of low-dose estrogen effects: identification of no observed transcriptional effect level (NOTEL). *Toxicol. Pathol.* 2004; 32(4):482–492. [PubMed: 15223774]
74. Zarbl H, Gallo MA, Glick J, Yeung KY, Vouros P. The vanishing zero revisited: Thresholds in the age of genomics. *Chem.-Biol. Interact.* 2010; 184(1–2):273–278. [PubMed: 20109442]
75. Smyth, GK. *Limma: Linear Models for Microarray Data.* New York: Springer; 2005. p. 397–420.
76. R Core Development Team. *R: A Language and Environment for Statistical Computing.* Vienna, Austria: R Foundation for Statistical Computing; 2008.
77. Pollard, KS.; Dudoit, S.; van der Laan, MJ. Multiple Testing Procedures: R Multtest Package and Applications to Genomics. In: Gentleman, R.; Carey, V.; Huber, W.; Irizarry, R.; Dudoit, S., editors. *Bioinformatics and Computational Biology Solutions Using R and Bioconductor.* New York: Springer; 2005. p. 251–272.
78. Bluthgen N, Brand K, Cajavec B, Swat M, Herzel H, Beule D. Biological profiling of gene groups utilizing Gene Ontology. *Genome informatics. International Conference on Genome Informatics.* 2005; 16(1):106–115. [PubMed: 16362912]
79. Poynton HC, Taylor NS, Hicks J, Colson K, Chan S, Clark C, Scanlan L, Loguinov AV, Vulpe C, Viant MR. Metabolomics of microliter hemolymph samples enables an improved understanding of the combined metabolic and transcriptional responses of *Daphnia magna* to cadmium. *Environ. Sci. Technol.* 2011; 45(8):3710–3717. [PubMed: 21417318]
80. Mhadhbi L, Fumega J, Beiras R. Toxicological effects of three polybromodiphenyl ethers (BDE-47, BDE-99 and BDE-154) on growth of marine algae *Isochrysis galbana*. *Water Air Soil Pollut.* 2012; 223(7):4007–4016.
81. Pellacani C, Buschini A, Galati S, Mussi F, Franzoni S, Costa LG. Evaluation of DNA damage induced by 2 polybrominated diphenyl ether flame retardants (BDE-47 and BDE-209) in SK-N-MC Cells. *Int. J. Toxicol.* 2012; 31(4):372–379. [PubMed: 22710639]
82. Usenko CY, Robinson EM, Usenko S, Brooks BW, Bruce ED. PBDE developmental effects on embryonic Zebrafish. *Environ. Toxicol. Chem.* 2011; 30(8):1865–1872. [PubMed: 21560146]
83. Waaijers S, Bleyenbergh TE, Dits A, Schoorl M, Schutt J, Kools SA, de Voogt P, Admiraal W, Parsons JR, Kraak M. Daphnid life cycle responses to new generation flame retardants. *Environ. Sci. Technol.* 2013; 47(23):13798–13803. [PubMed: 24180581]
84. Adams WJ, Biddinger GR, Robillard KA, Gorsuch JW. A summary of the acute toxicity of 14 phthalate-esters to representative aquatic organisms. *Environ. Toxicol. Chem.* 1995; 14(9):1569–1574.
85. Chemtura USA Corporation. Material Safety Data Sheet 00896: Firemaster® 550. Middlebury, Connecticut: 2006. p. 8
86. Tiemeyer, M.; Selleck, S.; Esko, J. Arthropoda. In: Varki, A.; Cummings, R.; Esko, J.; Freeze, H.; Stanley, P.; Bertozzi, C.; Hart, G.; Etzler, M., editors. *Essentials of Glycobiology.* 2nd. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2009.
87. Kojima H, Tohsato Y, Kabayama K, Itonori S, Ito M. Biochemical studies on sphingolipids of *Artemia f ranciscana*: complex neutral glycosphingolipids. *Glycoconjugate J.* 2013; 30(3):257–268.
88. Hannun YA, Obeid LM. The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J. Biol. Chem.* 2002; 277(29):25847–25850. [PubMed: 12011103]
89. Janssen R, Le Gouar M, Pechmann M, Poulin F, Bolognesi R, Schwager EE, Hopfen C, Colbourne JK, Budd GE, Brown SJ, Prpic NM, Kosiol C, Vervoort M, Damen WG, Balavoine G, McGregor AP. Conservation, loss, and redeployment of Wnt ligands in protostomes: implications for understanding the evolution of segment formation. *BMC Evol. Biol.* 2010; 10:374. [PubMed: 21122121]

90. Kashian DR, Dodson SI. Effects of vertebrate hormones on development and sex determination in *Daphnia magna*. Environ. Toxicol. Chem. 2004; 23(5):1282–1288. [PubMed: 15180381]
91. Cristale J, Garcia Vazquez A, Barata C, Lacorte S. Priority and emerging flame retardants in rivers: occurrence in water and sediment *Daphnia magna* toxicity and risk assessment. Environ. Int. 2013; 59:232–243. [PubMed: 23845937]
92. Vandenbrouck T, Jones OA, Dom N, Griffin JL, De Coen W. Mixtures of similarly acting compounds in *Daphnia magna*: from gene to metabolite and beyond. Environ. Int. 2010; 36(3): 254–268. [PubMed: 20117838]
93. McCooile MD, Baer KN, Christie AE. Histaminergic signaling in the central nervous system of *Daphnia* and a role for it in the control of phototactic behavior. J. Exp. Biol. 2011; 214(Pt 10): 1773–1782. [PubMed: 21525325]
94. Smirnov, NN. Physiology of the Cladocera. London: Academic Press; 2014.
95. Campos B, Garcia-Reyero N, Rivetti C, Escalon L, Habib T, Tauler R, Tsakovski S, Pina B, Barata C. Identification of metabolic pathways in *Daphnia magna* explaining hormetic effects of selective serotonin reuptake inhibitors and 4-nonylphenol using transcriptomic and phenotypic responses. Environ. Sci. Technol. 2013; 47(16):9434–9443. [PubMed: 23855649]
96. Gardner WS, Miller WH Iii. Intracellular composition and net release rates of free amino acids in *Daphnia magna*. Can. J. Fish. Aquat. Sci. 1981; 38(2):157–162.
97. Ventura M, Catalan J. Variability in amino acid composition of alpine crustacean zooplankton and its relationship with nitrogen-15 fractionation. J. Plankton Res. 2010; 32(11):1583–1597.
98. Vonk, HJ. Metabolism and Growth. In: Waterman, T., editor. The Physiology of Crustacea. Vol. 1. New York: Elsevier Science; 1960.
99. Scavia D, Gardner WS. Kinetics of nitrogen and phosphorous release in varying food supplies by *Daphnia magna*. Hydrobiologia. 1982; 96(2):105–111.
100. Hessen DO. Carbon, nitrogen and phosphorous status in *Daphnia* at varying food conditions. J. Plankton Res. 1990; 12(6):1239–1249.
101. D’Abramo, L.; Conklin, D.; Akiyama, D. Nutrition I. W. G. o. C. Crustacean Nutrition. Vol. 6. Baton Rouge, FL: World Aquaculture Society; 1997.
102. Yebra L, Hernández-León S. Aminoacyl-tRNA synthetases activity as a growth index in zooplankton. J. Plankton Res. 2004; 26(3):351–356.

**Figure 1.**

Molecular structures of chemical flame-retardants. A. Polybromodiphenyl ether. B. Triphenyl phosphate (TPhP). C. bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP). D. bis(2-ethylhexyl) tetrabromobenzoate (EH-TBB). E. Isopropylated triaryl phosphates (ITP). F. bis(2-ethylhexyl) phthalate (BEHP). B–E are components of Firemaster 550. C and D are components of Firemaster BZ-54. E contains a mixture of molecules with one, two or three isopropyl-substituted phenol rings.

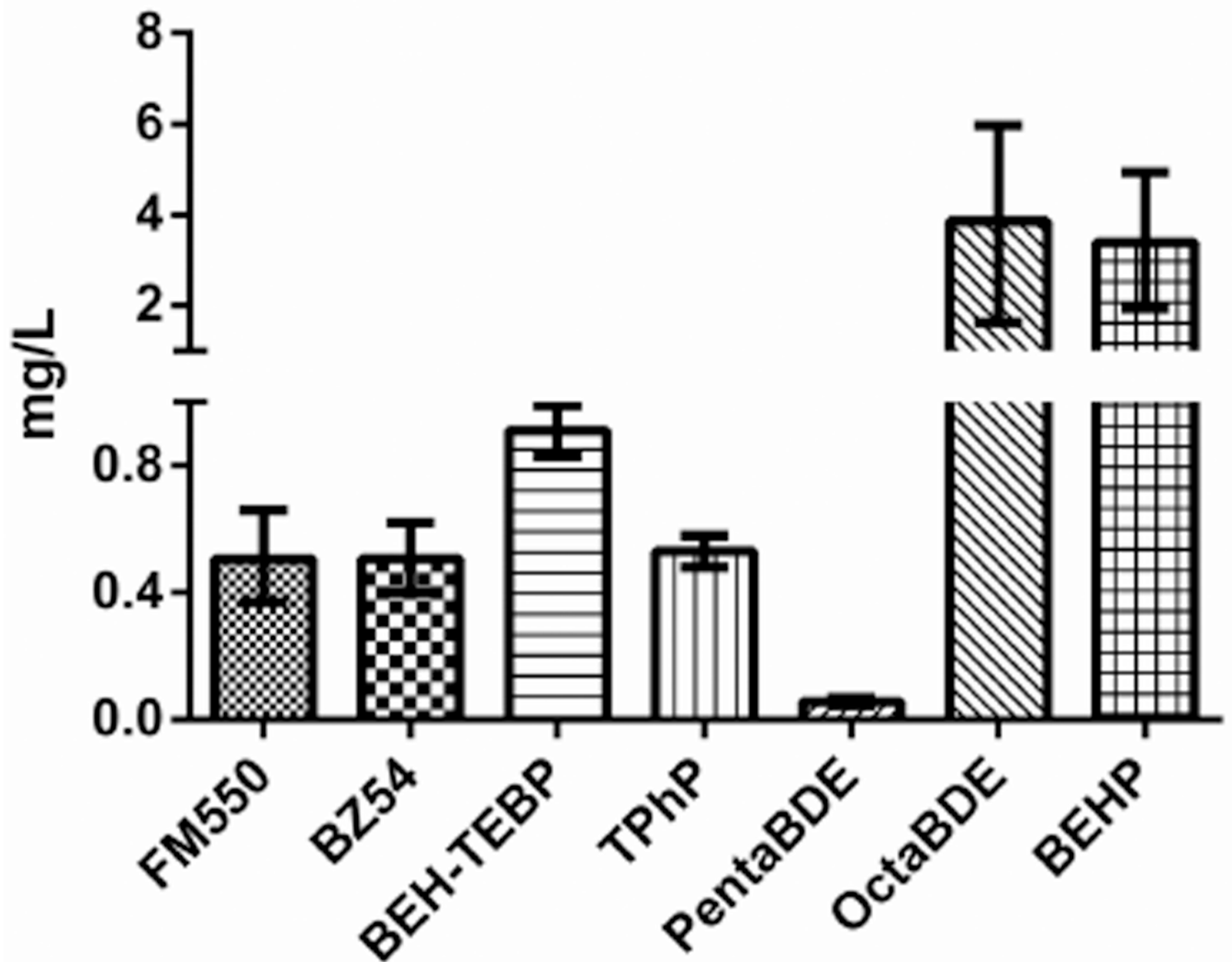


Figure 2. Acute, 48-h LC₅₀ values for flame-retardants on *Daphnia magna*. LC₅₀ values were determined with probit or Spearman–Kärber statistical programs. Error bars represent 95% confidence interval.

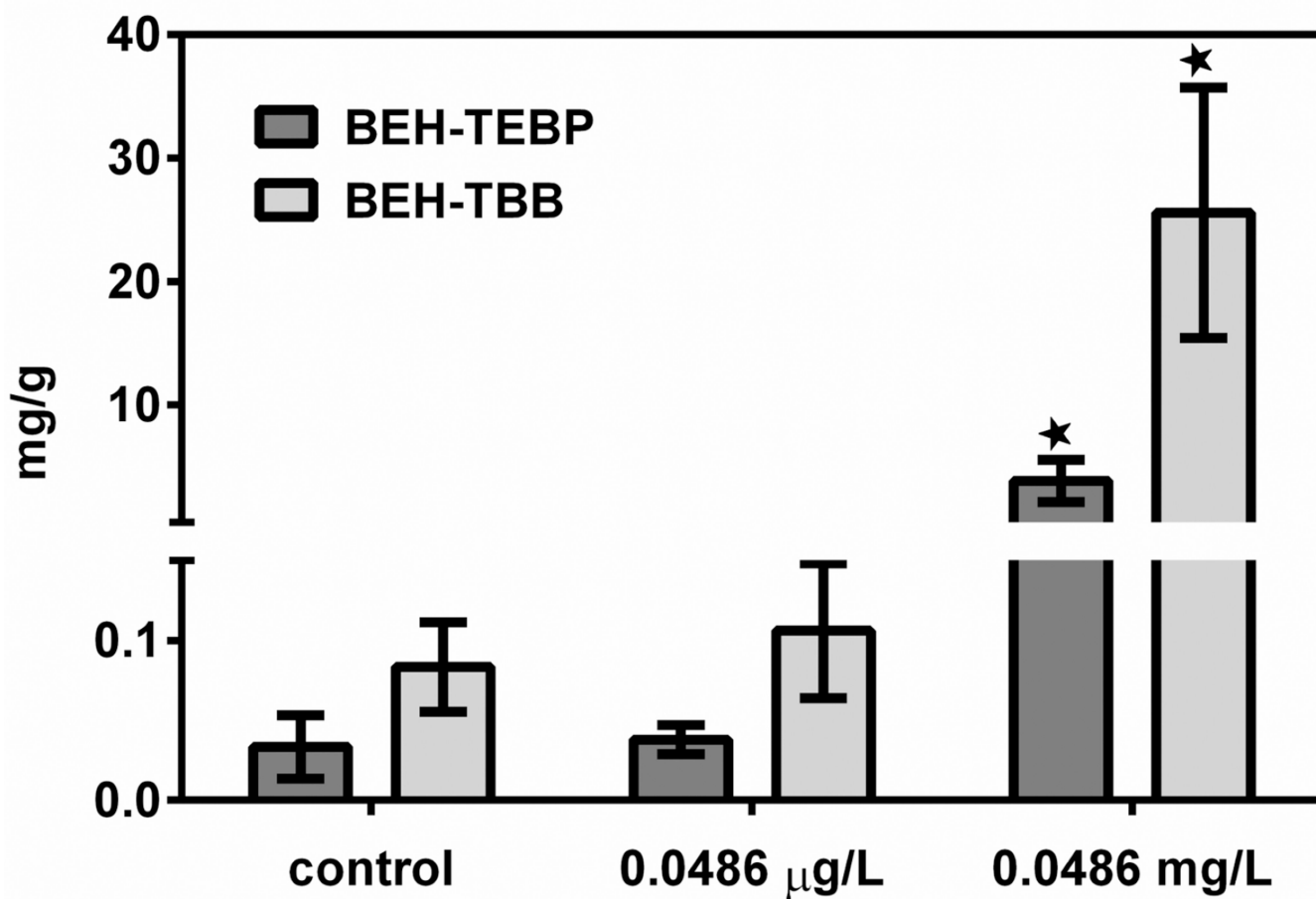


Figure 3. Absorption of BEH-TEBP and EH-TBB was measured after 48-h exposure to 0.0486 μg/L (1/10 LC₅₀) or 0.0486 mg/L FM550. Concentrations: nanograms chemical per gram daphnid, dry weight (ng/g).

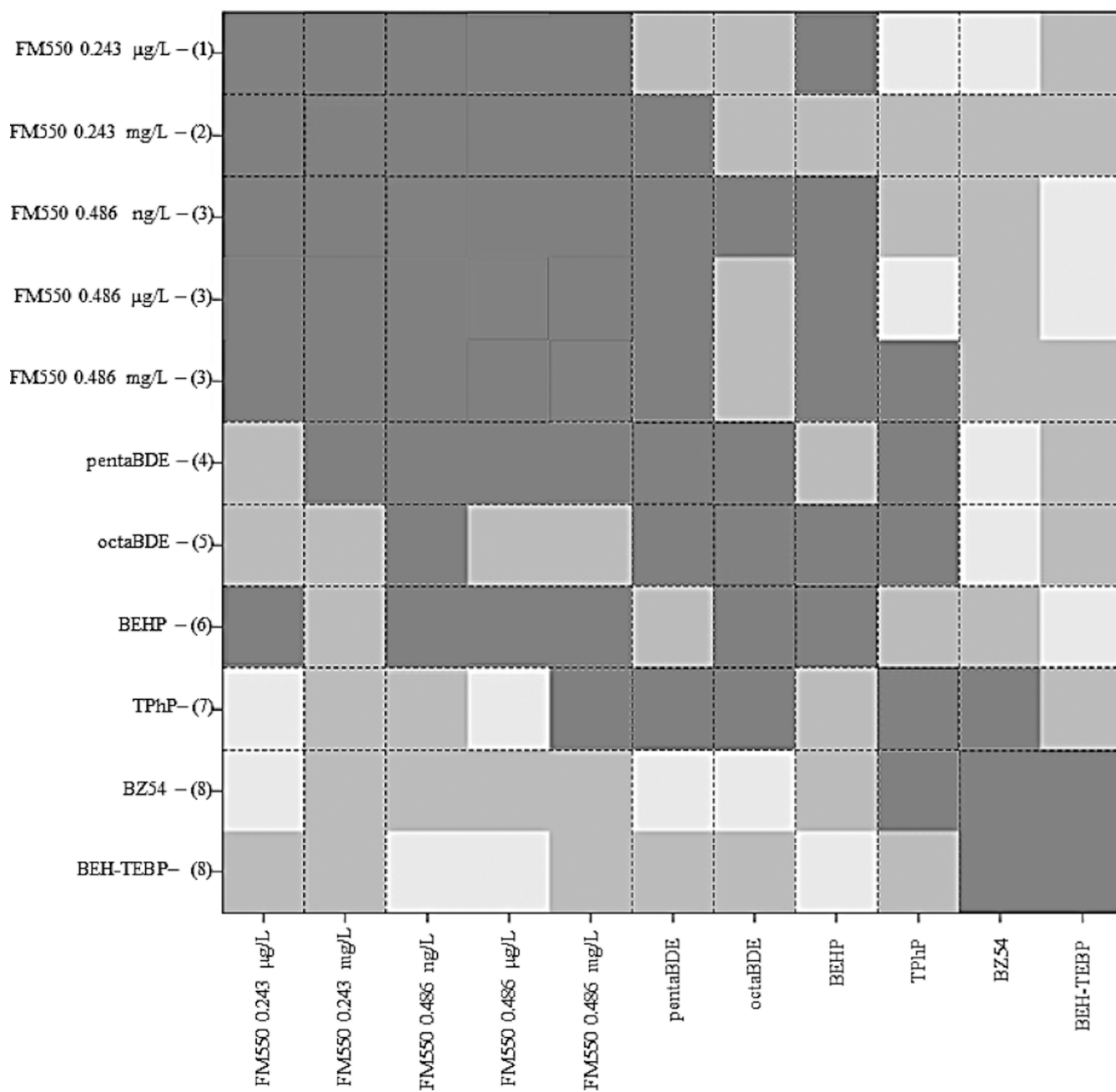


Figure 4. HOPACH cluster analysis of chemical profiles shows visual representation of the corresponding ordered distance matrix. Darker colored squares indicate more similarity. Clusters are numbered 1–8 on the y-axis. Three FM550 concentrations were significantly similar (cluster 3), as were BZ54 and BEH-TEBP (cluster 8).

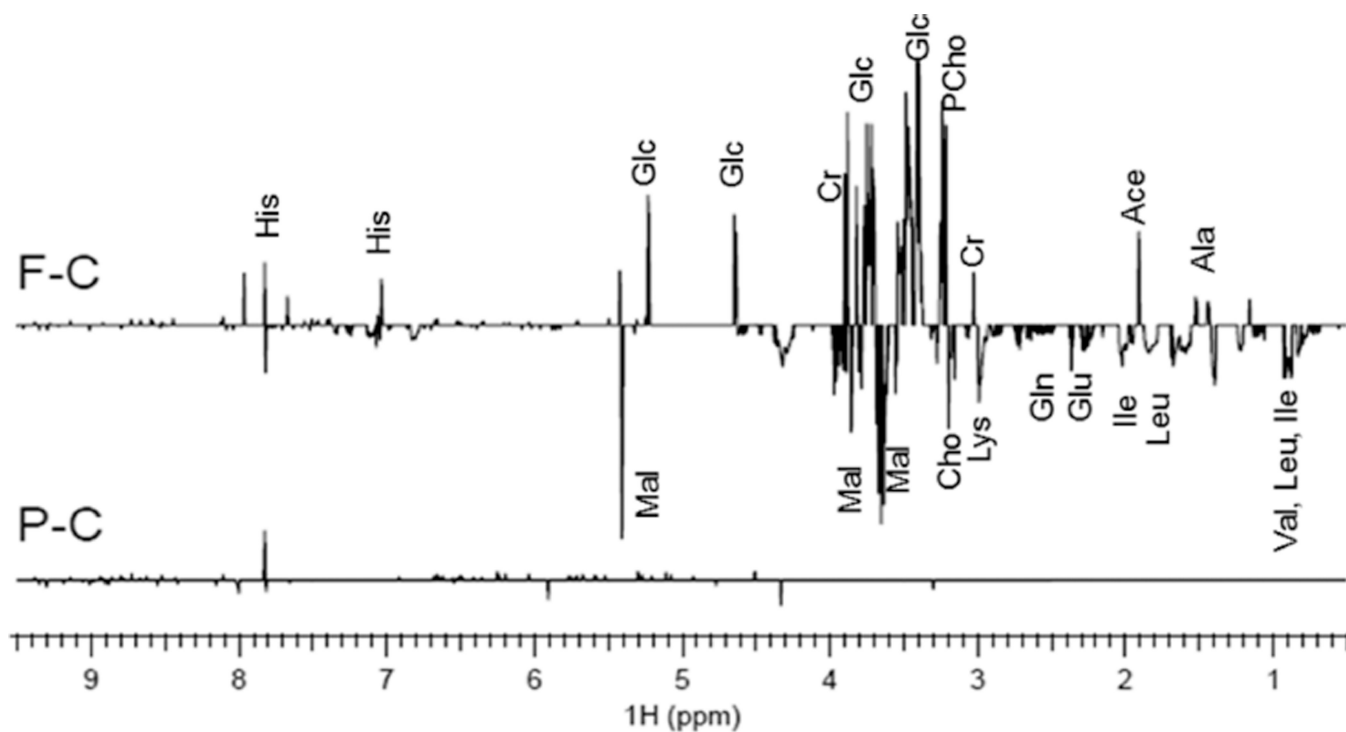


Figure 5. ^1H NMR t test filtered difference spectra showing metabolomics changes in *Daphnia* exposed to 1/10 LC_{50} FM550 (F-C) or pentaBDE (P-C) as compared to control. The t test filtered difference spectra were obtained using 7 replicates for each class. Abbreviations: acetate (Ace), alanine (Ala), choline (Cho), creatine (Cr), glucose (Glc), glutamate (Glu), glutamine (Gln), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), malate (Mal), phosphocholine (PCho), and valine (Val). Spectra were t test filtered difference and include the p -values (less than 0.05).

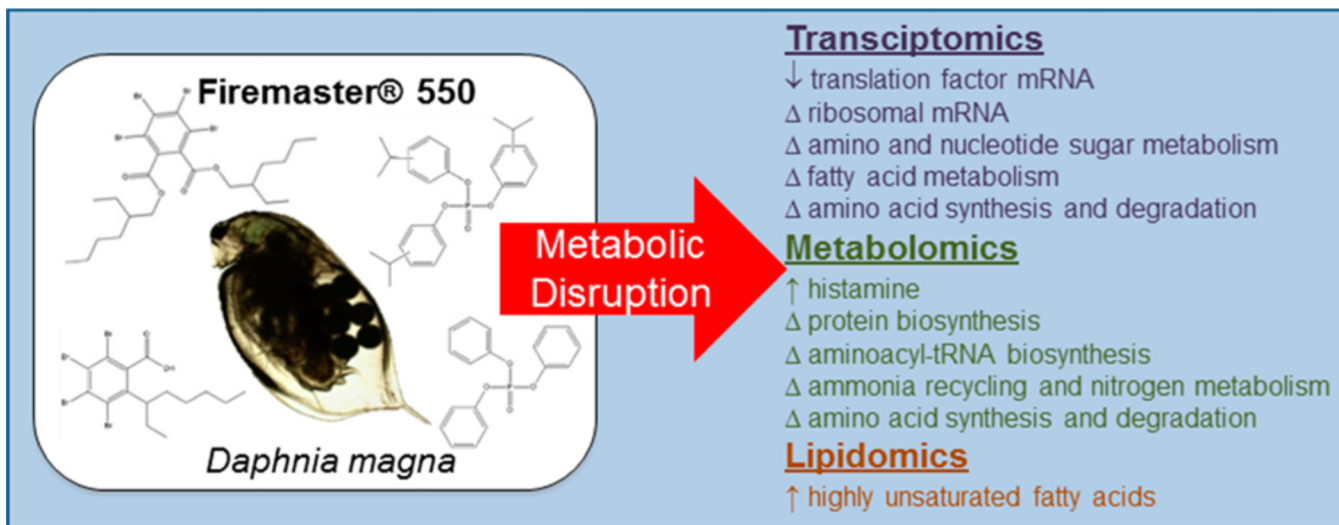


Figure 6. Schematic showing proposed toxic effects of chemical flame-retardant formulation FM550 on *Daphnia magna*. Omic techniques picked up changes related to nutritional status.

KEGG Pathway Analyses of Gene Transcription Data Show That Exposure to Flame-Retardants Largely Affected Different Biological Pathways^a

Table 1

biological pathways affected by exposure to different chemical flame-retardants						
KEGG biological pathway	FM550	octaBDE	pentaBDE	BZ54	BEH-TEBP	BEHP
ribosome	0.061		0.030			
glycosphingolipid biosynthesis		0.086				0.089
spliceosome			0.066			
pyrimidine metabolism			0.002			
proteasome			0.074			
WNT signaling pathway				0.089		
porphyrin and chlorophyll metabolism			0.020	0.026		
glycosaminoglycan degradation				0.052		
hedgehog signaling pathway				0.052		
arginine and proline metabolism					0.083	
tyrosine metabolism					0.048	0.042
phenylalanine metabolism						0.044

^aNumbers in this graph represent the *p*-value of each analysis. Because annotation of the array is limited, *p*-values up to 0.1 were included.

Table 2

Potential Gene Transcription Biomarkers of *Daphnia* Exposure to Firemaster 550^a

A. genes differentially expressed in all Firemaster 550 exposures						
gene ID	0.243 mg/L	0.0486 mg/L	0.0486 µg/L	0.243 µg/L	0.0486 ng/L	gene function
DM05947	-1.3	-1.3	-1.3	-1.3	-2	peroxidase
DM12670	-1.1	-1.6	-2.3	-2	-2.3	unknown
DM09101	-1.1	-1.6	-1.9	-1.8	-2.2	ABC transporter

B. genes associated linearly with FM550 concentration						
gene ID	trend	slope	rawp	Holm	BH	gene function
DM02757	negative	-0.0129	6.7×10^{-05}	0.0226	0.0226	eukaryotic translation initiation factor
DM12108	negative	-0.0328	1.9×10^{-04}	0.0646	0.0324	unknown
DM12547	negative	-0.0032	4.0×10^{-04}	0.1343	0.0450	unknown

^aRNA sequences from the microarray (Gene ID) were functionally annotated with Blast2Go. Part A shows log2 change of differential gene transcription in exposed *Daphnia magna* compared to control. Part B lists genes that showed a small but significant negative linear trend with increasing concentration. Holm: correction for multiple comparisons. BH (Benjamini–Hochberg procedure): controls false discovery rate.

Table 3Small Molecule Metabolomics Changes in FM550-Exposed Daphnids^a

metabolite	abbreviation	% change	<i>p</i> -value (<i>t</i> test)
Histidine	His	37	0.0140
Glucose	Glc	24	0.0010
Phosphocholine	PCho	17	0.0020
Glutamine	Gln	11	0.0005
Creatine	Cre	10	0.0110
Acetate	Ace	7	0.0470
Alanine	Ala	6	0.0043
Valine	Val	-12	0.0280
Glutamate	Glu	-14	0.0007
Choline	Cho	-16	0.0070
Leucine	Leu	-16	0.0120
Isoleucine	Ile	-17	0.0240
Malate	Mal	-23	0.0030
Lysine	Lys	-24	0.0004

^aAnalysis was done with ¹H NMR and subsequent statistical analyses. Data is from seven exposed and control biological replicates of 40 daphnids each.

Table 4

Molecular Pathway Analysis on Metabolomic Data from FM550-Exposed Daphnids^a

pathway analysis of Firemaster 550 metabolomic data							
increased metabolites							
	total ^b	expected ^c	hits ^d	raw p ^e	Holm adjust ^f	FDR ^g	organism ^h
nitrogen metabolism	9	0.047	2	8.29 × 10 ⁻⁰⁴	0.067	0.067	D.r. ⁱ
arginine and proline metabolism	43	0.225	2	0.019	1	0.699	D.r.
aminoacyl-tRNA biosynthesis	67	0.350	2	0.044	1	0.749	D.r.
decreased metabolites							
	total ^b	expected ^c	hits ^d	raw p ^e	Holm adjust ^f	FDR ^g	organism ^h
valine, leucine and isoleucine biosynthesis	13	0.090	3	5.62 × 10 ⁻⁰⁵	0.004	0.004	D.r. and D.m.
aminoacyl-tRNA biosynthesis	67	0.463	4	5.28 × 10 ⁻⁰⁴	0.041	0.021	D.r. and D.m.
valine, leucine and isoleucine degradation	35	0.242	3	0.001	0.093	0.032	D.r. and D.m.

^aAnalysis was performed with MetaboAnalyst 2.0 and included *D. rerio* (D.r.) and *D. melanogaster* (D.m.) pathways with more than one hit and a raw *p*-value <0.05.^bTotal genes in biological pathway.^cNumber of genes expected in a random data set.^dNumber of hits in FM550 data.^e*p*-Value calculated from raw data.^f*p*-Value adjusted for multiplicity.^gFalse discovery rate.^hOrganism metabolome that resulted in significant findings.ⁱAlso seen in *D. melanogaster*. If pathway was affected in both species, numbers represent data for the more significant species.

Enrichment Analysis on Metabolomic Data from FM550-Exposed Daphnids Compared to All Known Metabolites in the MetaboAnalyst Database^a

Table 5

enrichment analysis of Firemaster 550 metabolomic data						
increased metabolites						
	total ^b	expected ^c	hits ^d	raw p ^e	Holm p ^f	FDR ^g
ammonia recycling	18	0.153	2	0.009	0.709	0.394
protein biosynthesis	19	0.161	2	0.010	0.779	0.394
decreased metabolites						
	total ^b	expected ^c	hits ^d	raw p ^e	Holm p ^f	FDR ^g
protein biosynthesis	19	0.161	4	6.77×10^{-06}	0.001	0.001
valine, leucine and isoleucine degradation	36	0.305	3	0.002	0.187	0.095

^aBiological functions with more than one hit and with raw $p < 0.05$ are included as significant.^bTotal genes in biological pathway.^cNumber of genes expected in a random data set.^dNumber of hits in FM550 data.^e p -Value calculated from raw data.^f p -Value adjusted for multiplicity.^gFalse discovery rate.

Table 6Changes in *Daphnia magna* Lipidome^a

lipidome changes after flame-retardant exposure				
mass of lipid	formula	name	exposure	fold change
552.4	C ₂₈ H ₅₈ O ₇ PN	LPC(20:0)	pentaBDE	6.60
884.6	C ₅₂ H ₈₆ O ₈ PN	PC(44:9)	pentaBDE	2.18
860.6	C ₅₀ H ₈₆ O ₈ PN	PC(42:7)	FM550	1.36
884.6	C ₅₂ H ₈₆ O ₈ PN	PC(44:9)	FM550	2.91

^aEndogenous lipids were measured after 48 h exposure to 1/10 LC50 pentaBDE or 1/10 LC50 FM550 or to solvent-exposed controls.

p-value 0.04.

LPC: lysophosphatidylcholine, PC: phosphatidylcholine.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript