SUPPORTING INFORMATION

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

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Supporting Methods
Differential gene transcription
Quantitative Polymerase Chain Reaction (qPCR)
FM550 dose-response analysis
Metabolomics
Lipidomics

Supporting Tables

Supporting Figures.

METHODS

Quantification of EH-TBB and BEH-TBBP uptake. Samples were extracted in a 50:50 mixture of dichloromethane:hexane. Extracts were further cleaned using Florisil solid phase extraction cartridges. GC/ECNI-MS conditions are reported in Stapleton *et al*. 2008.[1] Raw concentration data from pooled biological replicates (four replicates per exposure) were normalized for dry weight.

For statistical analyses, values were set equal to ½ MDL when chemical concentrations were below detection limits. Statistical outliers were determined with Thompson's tau outlier test and removed. A Welch two-sample t-test was to determine significant differences between exposure groups.

Determination of differentially expressed gene candidates. Microarray data were analyzed as in Scanlan *et al* 2013.[2] Briefly, a "Treatment vs. Control" design was used. Foreground intensities in each array were subtracted with local background. All negatives or flagged spots (labeled with GenePix Pro) were labeled as "NA" and treated as missing values. All positive values were log (base-2)-transformed. Relative intensity ratios were calculated (ratio= treatment sample /control) for the log-transformed values for each gene and were corrected for non-linear trends (if any) with loess global normalization.[3] Differential gene transcription was determined with an algorithm based on α-outlier detection procedures.[4] A local variance estimator based on loess was used to take heteroscedasticity (if any) into consideration.[4] Each gene in a given "Treatment vs. Control" pair was characterized by the normalized log-transformed ratio (fold change value) and the corresponding q-value (derived from p-values, adjusted for multiplicity of comparisons).[5] The multiple slide procedure method used to detect candidate genes was based on a number of biological replicates and treated the gene transcription outcomes as Bernoulli trials (independent binary outcomes). Fisher's method of meta-analysis was applied to combine p-values. Fisher's method-based p-values were adjusted with Bonferroni correction using effective number of genes (candidates are usually about 1% of all genes on a slide). A list of candidate genes was created for each treatment. All treatments were consolidated in one summary table: a gene is present in the table if it was detected as a candidate in at least one "Treatment vs. Control" group. Candidate genes were annotated using Blast2Go service.[6]

Quantitative polymerase chain reaction. To independently verify microarray results, separate biological replicates were analyzed with qPCR. RNA used for qPCR was extracted as detailed in the manuscript methods and cleaned on an RNeasy column (Qiagen). 1 μg RNA was reverse transcribed with iScript cDNA synthesis kit (BioRad, CA) on a Mycycler (Biorad). Primer sets were tested with the SsoFast (BioRad) amplification kit with a melt curve from 55 to 65C on a BioRad C1000 Thermal Cycler with CFX96 R-T System. Primers with only one qPCR product were used for subsequent analysis with SsoFast. Probes were designed on the NCBI online primer-designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and ordered from Elim Biopharm. Each gene amplification was performed in duplicate or triplicate in both the control and treated RNA samples. Actin and GAPDH were used as housekeeping genes. Housekeeping Ct was subtracted from gene of interest Ct and values were $\log_2$

transformed. Significance between control and exposed was determined by Student's T-test with $p<0.05$. Primer sequences are shown in **Table S4**.

Methods for dose-response analysis. To find linear trends in the gene transcription data for five different FM550 concentrations, a wrapper was written in R,[7] which applied the following functions for pre-processing and statistical inference: 1) lm() from built-in package STATS, 2) normalizeBetweenArrays() from package LIMMA,[8] and 3) mt.rawp2adjp() from package MULTTEST.[9]

Clustering was done with HOPACH (Hierarchical Ordered Partitioning And Collapsing Hybrid),[10] which builds a dendrogram (hierarchical tree of clusters) and combines both partitioning and agglomerative clustering methods. It uses the non-parametric bootstrap resampling to estimate the probability of cluster membership (fuzzy clustering). Data was clustered based on both chemical profile and gene profile using the cosangle metric.

The largest HOPACH cluster (cluster 0) was analyzed with Blast2Go enrichment analysis to determine if any gene ontology terms were enriched in a test group when compared to a reference group. Fisher's Exact Test with Multiple Testing Correction of FDR (Benjamini and Hochberg) and FDR cutoff of < 0.05 were used.

Metabolomics Analysis of PentaBDE and FM550 treated *Daphnia*. Samples of 20 pooled *Daphnia* were extracted using a dual phase extraction procedure.[11] 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. Only the aqueous phases were used for the present study. Each polar sample was then reconstituted in 220 μL of 0.1M sodium phosphate buffered deuterium oxide (pH 7.4) containing 20 μM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS). All NMR spectra were acquired at 20 ºC on an Agilent Inova 600 MHz NMR spectrometer with a cryogenic triple-resonance flow probe using direct-injection NMR analysis.[12] ^{1}H chemical shifts were referenced internally to DSS. One-dimensional (1D) NOESY spectra were collected using 1024 transients, 7,200-Hz spectral width, 50-ms mixing time, 2-s acquisition time and a 2-s presaturation pulse. Two dimensional (2D) TOCSY[13] experiment was done with a 30 ms mixing time, 16 transients and 200 increments. 1D ^{1}H NOESY spectra were processed with 0.3-Hz apodization followed by zero-filling to 128 k points, aligned and normalized to unit total intensity. Spectra with a range of 0.50 – 9.50 ppm were segmented into 0.005 ppm bins. Two-dimensional spectra were processed using line broadening of 0.5 Hz and 60º-shifted squared sine bell functions for both F1 and F2 dimensions.

For data analysis, a text file of binned spectra was imported into SIMCA-P+ (Umetrics Inc., Umea, Sweden) for multivariate data analysis. Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted for the entire dataset using mean-centered and Pareto-scaled bins. All samples (N = 7) were observed to fall inside of the Hotelling's T^2 ellipse at the 95% confidence interval in scores plots. The relative impact of a given chemical exposure was assessed, in part, by comparing score values for different treatment classes within a given PLS-DA model. All PLS-DA models are validated with 100 permutations. In addition, univariate analysis of the binned spectra was conducted using Excel. First, an "average class spectrum" was calculated by averaging the binned spectra across all class members, where class was

defined by exposure level (including controls) and duration. Next, a difference spectrum was generated by subtracting the averaged bins of the relevant control class from those of each exposed class. Then, a Student's t-test was conducted on each bin using a p-value < 0.05. As described previously,[14] to greatly reduce the rate of false positives, any single isolated bin that passed the t-test (without an adjacent bin also passing) was replaced with a zero (i.e., it was rejected), because legitimate metabolite peaks span more than one bin at this bin size. The result was a "t-test filtered difference spectrum" for each exposed class. Positive peaks in these difference spectra correspond to metabolites that increase (with statistical significance) upon treatment, whereas negative peaks represent metabolites that decrease.

Data were further analyzed with MetaboAnalyst 2.0 (http://www.metaboanalyst.ca).[15, 16] For enrichment analysis, a list of increased or decreased metabolite names were compared to all known metabolites to determine overrepresentation of biological function groups. For pathway analysis, a list of compound names was compared to both the *Drosophila melanogaster* and *Danio rerio* metabolomes using a hypogeometric test to determine "relative betweenness" of biological pathways.[15, 16]

Lipidomics Analysis of PentaBDE and FM550 exposed *Daphnia*. Data acquisition, analysis, and lipid quantification in comparison to internal standards was done with an automated electrospray ionization-tandem mass spectrometry approach, previously described by Sparkes *et al*., 2010,[17] except that an aliquot of 80 µL of extract in chloroform was used for analysis, and free fatty acids and acyl product ions were not analyzed. The molar results were normalized to the sample analyzed and the number of daphnids to produce data in nano-moles of each lipid per daphnid (nanomol/daphnid). More details on methodology can be found at http://www.k-state.edu/lipid/lipidomics/profiling.htm. To determine if any lipids were detected at statistically different levels in exposed compared to control samples, raw data were multiplied by 1000 (i.e. expressed in picomol lipid/daphnid) adding 1 (see below) and sample distributions were checked with diagnostic plots for closeness to normality and for heteroscedasticity (residuals versus fit plot).

A method for heteroscedasticity was applied, reducing transformation for two-factor experiments with replicates to determine any lipids that had different abundance in exposed versus control population.[18] The limit of lipid detection was 2 picograms, which allowed us to convert any "0" (zeros) in the data table to 1 pg, to allow for log-transformation. Significance was determined with a standard two-sample t-test and a Wilcox rank sum test for two sample data (equivalent to the Mann-Whitney test).

Log LC_{50} versus Log K_{ow}. The log LC_{50} versus Log K_{ow} was plotted to see if there was a correlation between LC_{50} values and chemical hydrophobicity. Log K_{ow} values were found in literature and, for PBDEs, values were weighted by congener abundance.[19 20 21 22 23] Three statistical analyses, below, do not support the hypothesis that LC_{50} is dependent on hydrophobicity. It is important to note that the sample size is small so statistical confidence is lowered.

1) Kendall's rank correlation: tau, T = 7, p-value = 0.4833, alternative hypothesis: true tau is not equal to 0, sample estimates: tau = 0.4

2) Spearman's rank correlation: rho, S = 8, p-value = 0.35, alternative hypothesis: true rho is not equal to 0, sample estimates: rho = 0.6

3) Pearson's product-moment correlation
t = 0.5287, df = 3, p-value = 0.6336, alternative hypothesis: true correlation is not equal to 0, 95 percent confidence interval: -0.7951152 0.9337153, sample estimates: cor = 0.2919673

TABLES

COMBO Media	
Chemical	Concentration (mg/L)
$CaCl_2 \cdot 2H_2O$	55.0
$MgSO_4 \cdot 7H_2O$	55.5
K_2HPO_4	4.4
$NaNO_3$	42.5
$NaHCO_3$	50.5
$Na_2SiO_3 \cdot 9H_2O$	14.2
H_3BO_3	12.0

Table S1. Chemical constituents of *Daphnia magna* COMBO growth media. Media was prepared in 20 L carboys, aerated overnight and maintained at pH 7.4 – 7.8.

TPhP		
mg/L	# responding	#total
4.95	20	20
4.05	20	20
3.15	20	20
2.25	37	40
1.35	33	40
1.05	20	20
0.75	20	20
0.45	27	50
0.3	17	30
0.15	0	10
0.075	0	10
0.0375	0	10
0.01875	0	10
0	10	50

BEH-TEBP		
mg/L	# responding	#total
1.35	34	40
1.2	25	30
1.05	23	30
0.9	23	40
0.75	17	30
0.6	8	30
0.45	0	10
0.225	0	10
0.113	0	10
0.056	0	10
0	7	40

BZ54		
mg/L	# responding	#total
0.9	30	33
0.6	30	36
0.45	12	30
0.3	6	30
0.15	6	30
0.075	3	33
0	0	33

FM550		
mg/L	# responding	#total
1	60	70
0.6	42	95
0.4	68	170
0.3	11	40
0.2	40	170
0.1	21	190
0.05	3	190
0.025	1	55
0.01	2	95
0.005	0	20
0.001	0	20
0	6	190

octaBDE		
mg/L	# responding	#total
10	36	40
12	38	40
9	11	20
8	55	60
7	15	20
6	51	60
5	13	20
4.2	3	20
4	46	60
3.4	0	20
2.6	0	20
2	25	40
1.8	0	20
1.4	0	20
1.2	8	15
1	7	35
0.8	5	55
0.64	0	40
0.6	3	15
0.48	1	40
0.4	0	15
0.32	0	40
0.24	0	40
0.2	1	15
0.16	0	40
0	13	140

pentaBDE		
mg/L	# responding	#total
0.3	19	20
0.2	34	40
0.16	30	35
0.15	34	40
0.12	16	20
0.1	45	60
0.08	36	55
0.06	9	20
0.05	16	40
0.04	13	35
0.025	9	40
0.02	8	35
0.01	7	95
0.005	3	35
0	2	95

BEHP		
mg/L	# responding	#total
40	20	20
30	12	15
20	108	135
10	83	120
5	69	124
2.5	70	120
1.25	31	99
0.625	3	15
0.3125	0	15
0	5	135

Table S2. Raw acute toxicity data for *Daphnia manga* exposed to chemical flame-retardants. Original chemical exposure concentrations were based on values from literature, if available. Typically, five animals were exposed per concentration to five different concentrations and to a DMSO control at one time. Exposure concentrations were subsequently adjusted.

		Bromkal 70-5 (pentaBDE)			GL DE-79 (octaBDE)		
PBDE congener	logKow	percent[a]	normalized[c]	contribution[b]	percent[a]	normalized[c]	contribution[b]
47b/71*	6.81	42.8	40.54	2.76			
100*	7.24	7.82	7.41	0.54			
99*	7.32	44.8	42.43	3.11			
85*	7.37	2.16	2.05	0.15			
154*	7.82	2.68	2.54	0.20	1.07	1.04	0.08
153*	7.90	5.32	5.04	0.40	8.66	8.41	0.66
175/183b*	8.27	0.33			42.00	40.77	3.37
197#	10.33	0			22.20	21.55	2.23
203*	8.71	0			4.40	4.27	0.37
196#	10.33	0			10.50	10.19	1.05
207#	11.22	0			11.50	11.16	1.25
206#	11.22	0			1.38	1.34	0.15
209#	12.11	0			1.31	1.27	0.15
	total	105.91	100.00	7.15	103.02	100	9.32
	correction (100/total)	0.94		correction (100/total)	0.97		

Table S3. Relative contribution of PBDE congeners to mixtures and log K_{ow}. *Measured or # EPISuite estimated log K_{ow} values derived from La Guardia *et al* 2006 and 2012[22 24] were used to estimate hydrophobicity of pentaBDE and octaBDE. Percent congener[a] was multiplied by log K_{ow} to determine contribution[b] of congener to log K_{ow}. The sum of all congener contributions is the estimated total log K_{ow} for each mixture. Congener percentage was normalized[c] to a total of 100 percent.

Gene	Forward	Reverse	Notes	Condition
DM00174	GCACGGAAGCAACCAAAGTT	GCCACTCCAGTAACGGTTGA	low fold change	BEHP
DM00871	GATTGTGGCAACTGGTGTCTG	TCCACCACCTTCATGACCAAG	low fold change	BEHP
DM00141	ATTTTTCGCCATCGTCCAGC	GTGTGGCTTCCCAAGTCAGT	less significant q-value	BZ54
DM06382	TTCAACTCCGTGACGCACAT	CCTTGGTTGAGGGAAACCCA	highly significant q-value	TPhP
DM00376	CGTTCGGGCAAAATGTGTCA	TCCATCCGAAGTGGAGGGAT	mode of toxicity TBPH	BEH-TEBP
DM05899	TTGCTCCAGCTCCCGTTATC	GAAACCTGGAACACCGCTGA	mode of toxicity pentaBDE	pentaBDE
DM01631	AAGTCGTTGAGGGCATGGAG	GTCGACGATCTTAACGGGCT	only PBDE-affected	penta and octaBDE
DM00800	GGAATCCCAGCATAGGGAGC	TGCAATTACGACCGTGGACA	highly significant KEGG	FM550
DM09101	CCTTCGCTCCAGCTCCATAC	TGAGCAACAGGGAACGAGTG	all FM550 affected	FM550

Table S4. Primer sequences for qPCR amplification of candidate differentially expressed genes. “Gene” corresponds to the microarray probe identification number. Genes were chosen due to degree of differential transcription, significance of transcription or potential mode of toxicity.

Chemical	LC_{50} (mg/L)	Statistical Method	95% Confidence Interval
FM550	0.486	Spearman-Karber	0.357-0.661
BEH-TEBP	0.91	Spearman-Karber	0.830-0.990
BZ54	0.5	Spearman-Karber	0.400 - 0.620
TPhP	0.53	Spearman-Karber	0.480 - 0.580
PentaBDE	0.058	probit	0.046 - 0.070
OctaBDE	3.96	probit	1.629-5.963
BEHP	3.31	probit	1.928-4.930

Table S5. Acute, 48-hour LC_{50} values of flame-retardants and related chemicals on freshwater crustacean *Daphnia magna*.

Dry Weight Concentration (ng/g)				
	control 1	control 2	control 3	control 4
BEH-TBB	112.74	~~1731.94~~	80.98	56.98
BEH-TEBP	<33	62.02	<49	<62
	low dose 1	low dose 2	low dose 3	low dose 4
BEH-TBB	125.25	57.78	~~5547.31~~	135.37
BEH-TEBP	<61	<65	3246.61	<97
	high dose 1	high dose 2	high dose 3	high dose 4
BEH-TBB	16581.01	17310.79	36414.51	32003.78
BEH-TEBP	2046.8	3042.7	4377.21	6015.26
	YCT 1	YCT 2	YCT 3	
BEH-TBB	0.766	0.345	0.766	
BEH-TEBP	0.615	0.654	0.619	

Table S6. FM550 components in *Daphnia magna* and in *Daphnia* food YCT (yeast, cereal, trout chow). Each daphnid sample (control, ng/L and µg/L) represents one biological replicate of ~400 daphnids exposed to DMSO control, low dose (0.4486 µg/L) or high dose (0.0486 mg/L, 1/10 LC_{50}) FM550 for 48 hours. Each YCT sample was derived from ~ 250 mL YCT mixture.

Numbers of Differentially Expressed Genes in Flame-Retardant Exposures							
Chemical	octaBDE	pentaBDE	FM550	BZ54	TPHP	BEH-TEBP	BEHP
# DEG	107	292	252	415	165	1187	153

Table S7a. Differential gene transcription at 1/10 LC_{50}. Number of genes differentially expressed in *Daphnia magna* after 48-hour exposure to 1/10 LC_{50} of chemical flame-retardants.

Numbers of Differentially Expressed Genes in Firemaster550 Exposures					
Concentration	0.0486 ng/L	0.243 µg/L	0.0486 µg/L	0.0486 mg/L	0.243 mg/L
# DEG	539	297	585	252	74

Table S7b. FM550 transcriptomic dose response. Exposure to each of five different FM550 concentrations caused differential gene transcription in *Daphnia magna*.

BZ54	housekeeper	fold change	array direction
DM00141 - 1	actin	0.817442863	down
DM00141 - 2	actin	0.752200827	down
DM00141 - 3	actin	0.747004997	down
TPhP	housekeeper	fold change	array direction
DM06382 -1	actin	0.463917674	down
DM06382 - 2	actin	0.346743493	down
DM06382 - 3	actin	0.69831057	down
BEH-TEBP	housekeeper	fold change	array direction
DM00376 - 1	actin	0.761194446	down
DM00376 - 2	actin	1.004402094	down
DM00376 - 3	actin	0.322268915	down
OctaBDE	housekeeper	fold change	array direction
DM01631 -1	GAPDH	0.054623896	down
DM01631 -2	GAPDH	0.035542181	down
PentaBDE	housekeeper	fold change	array direction
DM01631 -1	GAPDH	0.634859552	down
DM01631 -2	GAPDH	0.634859552	down
DM01631 - 3	GAPDH	0.661819346	down
BEHP	housekeeper	fold change	array direction
DM00871 -1	GAPDH	1.155635478	down
DM00871 - 2	GAPDH	0.746744391	down
DM00871 - 3	GAPDH	0.783870045	down
PentaBDE	housekeeper	fold change	array direction
DM05899 - 1	GAPDH	0.77897343	down
DM05899 - 2	GAPDH	0.829116092	down
DM05899 - 3	GAPDH	0.71185042	down
BEHP	housekeeper	fold change	array direction
DM00174 - 1	GAPDH	1.088125872	down
DM00174 - 2	GAPDH	0.612102623	down
DM00174 - 3	GAPDH	0.289542183	down
FM550 0.0486 µg/L	housekeeper	fold change	array direction
DM00800 - 1	actin	0.380502053	down
DM00800 - 2	actin	0.045625246	down
DM00800 - 3	actin	1.691032326	down
FM550 0.0486 ng/L	housekeeper	fold change	array direction
DM09109 - 1	actin	108.2260689	up
DM09109 - 2	actin	61.30378471	up
DM09109 - 3	actin	61.30378471	up

Table S8. qPCR verification of microarray results. 25 out of 29 experiments agreed with microarray data; samples highlighted in red did not. DMX represents Gene ID from microarray; more information about each gene is in Table S4. The cycle threshold (CT) of the control probe was subtracted from that of the probe of interest, and the difference was then transformed ($2^{\wedge\text{-(change CT)}}$). The control values were averaged, and each value from exposed samples was divided by the control values to get the fold change in transcription. Array direction was determined from microarray data.

Biological Pathways Affected by Exposure to Different Firemaster550 Concentrations			
KEGG Biological Pathway	0.0486 mg/L	0.0486 µg/L	0.0486 ng/L
Ribosome	0.06		
Peroxisome		0.03	
Fatty acid metabolism		0.09	
Glutathione metabolism		0.10	
Valine, leucine and isoleucine degradation		0.07	
Amino sugar and nucleotide sugar metabolism			0.10
Glycosphingolipid biosynthesis - globo series			0.02

Table S9. KEGG pathways analysis of gene transcription data from *Daphnia magna* found results for three of the five FM550 concentrations tested (0.0486 mg/L, 0.0486 µg/L, or 0.0486 ng/L). P-values ≤ 0.1 are considered significant.

GO Term	Name	FDR	single test p-Value	# in test group	# in ref. group	# non annot test	# non annot ref. group	Over/ Under
GO:0015671	oxygen transport	2.00E-02	1.30E-05	4	1	119	2907	over
GO:0015669	gas transport	2.00E-02	1.30E-05	4	1	119	2907	over
GO:0019825	oxygen binding	2.00E-02	1.30E-05	4	1	119	2907	over
GO:0005344	oxygen transporter activity	2.00E-02	1.30E-05	4	1	119	2907	over
GO:0005833	hemoglobin complex	4.70E-02	3.60E-05	4	2	119	2906	over

Table S10. Gene ontology enrichment analysis with Blast2Go on largest gene cluster from HOPACH output of FM550 data found over-representation of functions associated with oxygen binding and transport. Ref.group: Reference group.

FIGURES

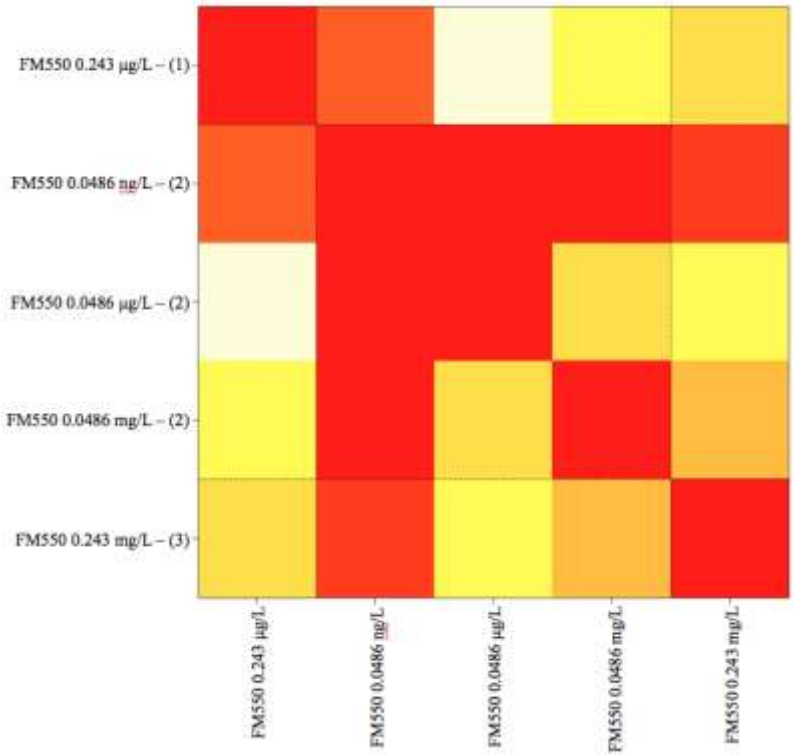

Figure S1. Chemical clustering of FM550 dose-response gene transcription data with HOPACH. Colors show similarity of chemical concentration gene transcription profiles – red is most similar, followed by dark orange and light orange. Yellow and white are least similar.

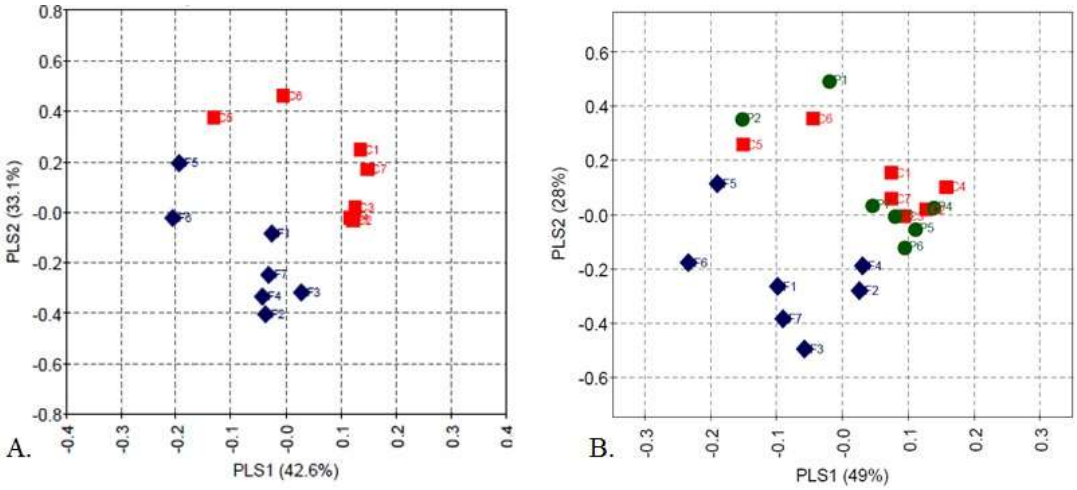

Figure S2. A. PLS-DA plots of metabolic data from FM550-exposed (blue diamond) and control (red square) daphnids. B. FM550, control and pentaBDE-exposed (green circle) daphnids. Each dot represents one biological replicate of 40 animals exposed to 1/10 LC_{50} FM550 or pentaBDE or solvent control.

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