Subchronic inhalation exposure study of an airborne polychlorinated biphenyl (PCB) mixture resembling the Chicago ambient air congener profile

Xin Hu[1]; Andrea Adamcakova-Dodd[2]; Hans-Joachim Lehmler[1,2]; Dingfei Hu[3]; Keri Hornbuckle[1,2,3]; Peter S Thorne[,1,2,3]*

Interdisciplinary Graduate Program in Human Toxicology[1], Department of Occupational and Environmental Health[2] and Department of Civil and Environmental Engineering[3], University of Iowa, Iowa City, Iowa 52242

*Corresponding Author

Peter S. Thorne, PhD

University of Iowa

Dept. Occupational & Environmental Health

105 River St., S341A CPHB

Iowa City, IA 52242 USA

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Chemicals. All chemicals were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise stated. IUPAC identities, numbered PCB 1 (monochlorobiphenyl) through PCB 209 (decachlorobiphenyl) were used for congener identification (1). Florisil (60-100 mesh), sodium sulfite, sulfuric acid (concentrated) and pesticide grade solvents were purchased from Fisher Sci. (Pittsburgh, PA). Tetrabutylammonium hydrogen sulfate was purchased from JT Baker (Phillipsburg, NJ). Diatomaceous earth was obtained from Dionex (Sunnyvale, CA). 3,5-dichlorobiphenyl (PCB 14) and 2,2',3,4,4',5,6,6'-octachlorobiphenyl (PCB 204) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Deuterium labeled 2,3,5,6-tetrachlorobiphenyl (d-PCB 65) was purchased from CDN isotopes (Quebec, Canada). Ongoing precision and recovery (OPR) standard was purchased as a ready mixture (WHO / NIST/ NOAA congener list) from AccuStandard (New Haven, CT). PCB 3 was synthesized in the laboratory (with a purity of >99%, based on the relative peak) and added to the AccuStandard mixture to represent lower chlorinated PCBs. After dilution it contained 410 ng/mL of PCB 3 and 380 ng/mL each of the following in isooctane: PCBs 8, 18, 28, 44, 52, 66, 77, 81, 101, 105, 114, 118, 123, 126, 128, 138, 153, 156, 157, 167, 169, 170, 180, 187, 189, 195, 206, and 209. For the OPR spike, 250 µL was added for each set of samples (*1*).

Toxicity Assessment (Cytochrome P450 assay and Glutathione assay). Cytochrome P-450 1A1, 1A2, 2B1 and 2B2 activities were estimated by the O-dealkylation of the ethyl-, methoyl-, pentyl- and benzyl- ethers of phenoxazone (EROD, MROD, PROD and BROD) respectively, as described previously (*2, 3*). The organ tissue, liver or lung in our case, was homogenized in sucrose solution and centrifuged at 10,000 g for 20 min. The supernatant was again centrifuged for another 1 hr at 100,000 g. The resultant supernatant contained the cytosolic fraction and was aliquoted. The microsome pellets were resuspended in ice-cold sucrose/EDTA solution and protein concentration was measured for each aliquot using Lowry protein assay. Ethoxy-, methoxy-, pentoxy- and benzyloxyresorufin were used as substrate for dealkylation. The reaction rate was measured by detecting the increase of the fluorescent metabolite, resofurin, using a Perkin-Elmer LS 55 spectrofluorometer at excitation wavelength of 550 nm and emission wavelength of 585 nm.

The determination of glutathione levels was carried out according to the GSH-5,5'-dithio-bis-(2-nitro-benzoic acid) (DTNB) recycling assay (*4*). A portion of liver and lung were perfused with saline and then immediately homogenized in 5% 5-sulfosalicylic acid (5-SSA) (w/v) after euthanization. The supernatant from the homogenate were diluted and aliquoted. The reaction was started by adding DTNB into mixture of samples, NADPH and glutathione reductase (GR) to measure total glutathione amount. The reaction was monitored at 412 nm in a Beckman DU-670 spectrophotometer for 5 min and the concentrations of glutathione was determined by comparing the rate of absorbance change to a standard curve obtained with authentic GSH or GSSG. Glutathione disulfide (GSSG) was titrated by the same method after quenching GSH with 2-vinylpyridine.

PCB extraction procedure. The extraction of PCBs from serum and tissue samples was performed by pressurized liquid extraction (PLE) as described elsewhere (*5*, *6*). Briefly, each set of tissue samples was accompanied by a method blank, a tissue blank and an ongoing precision and recovery (OPR) spike sample. Each sample was spiked with a surrogate standard including PCB 14 (100 ng) and deuterium-labeled PCB 65 (d-PCB 65, 100 ng) in hexane. After pre-extraction of PLE cells containing Florisil and diatomaceous earth, about 1 g of liver, lung, brain, 0.4 g adipose tissue samples or serum were thoroughly homogenized into diatomaceous earth and divided to two halves for PCB analysis (Fraction A) and for lipid extraction (Fraction B). Fraction A was placed on top of Florisil in PLE cells and spiked with surrogate standards. Loaded cells were then extracted with hexane-acetone (1:1 v/v) at 120 °C, 10342 kPa (1500 psi) in one static cycle of 5 min using an ASE200 system (Dionex, Sunnyvale, CA). After concentrating the extract to 0.75 mL (TurboVap II, Caliper Life Sciences Inc., Hopkinton, MA), sulfur impurities were removed by mixing the extract with 2-propanol and tetrabutylammonium sulfite, and adding nanopure water afterwards. The organic layer was then mixed with concentrated sulfuric acid and transferred to vials after standing overnight. Fraction B was extracted using chloroform-methoanol (2:1 v/v) under the same condition using ASE200. The total lipid content was determined gravimetrically after evaporation of solvents to dryness. Total cholesterol and triglycerides in serum samples were determined using a commercial test kit (Trig/GB and Chol tests for Roche/Hitachi 917 system; Roche Diagnostics, Indianapolis, IN). Blood lipids were calculated using the formula where phospholipid and free cholesterol contents were predicted from total cholesterol (*7*):

$$Total\ lipids = 2.27 \times Total\ cholesterol + Total\ triglycerides + 62.3\ mg/dL$$

Each XAD cartridge was loaded with 10 g of pre-extracted XAD-2 resin packed with filters and cleaned glass wool. After collection, all samples were placed in sealed zip-lock bags, stored at 4°C until analysis and were later extracted using the same protocol as above.

GC/MS/MS Quantification. The XAD extracts were spiked with 100 ng of PCB 204 as internal standard prior to analysis, while the tissue extract was concentrated to 100 µl for detection of low PCB levels and then spiked with 20ng of PCB 204. PCB congeners were analyzed using GC/MS/MS modified from the EPA method 1668A (*1*) as described previously (*8*, *9*). The quantification of PCB congeners used an Agilent 6890N GC with an Agilent 7683 series autosampler coupled to a Waters Micromass Quattro micro GC MS (Milford, MA), operating under electron impact positive mode at 70 eV and multiple reaction monitoring mode with a trap current of 200 µA. This method separated the 209 congeners into about 170 peaks.

Quality assurance / quality control measures. The quality of the analytical method was assessed by the method blank samples, and the recovery of ongoing precision and recovery standard. Every sample was spiked with surrogates and each PCB mass was corrected for recovery. The method detection limit (MDL) was calculated from blank samples analyzed in parallel to all tissue and blood samples according to EPA formula:

$$MDL = t_{n-1} \times SD + \bar{x}$$

where $\bar{x}$ = the mean of replicates of blank measures, t_{n-1} is Student's t-value for (n-1) degrees of freedom at 99% confidence level, and SD is standard deviation of the replicates. In parallel to XAD samples, one method blank of XAD and four sham samples that collected laboratory air were analyzed. ∑PCBs of 8 ng and 333±114 ng were detected respectively, in contrast to the high levels detected in the CAM vapor (969400±25410 ng). Standard Reference Material 1944, New York, New Jersey Waterway sediment (SRM 1944, National Institutes of Standards and Testing) was also analyzed and recently reported to assess the GC/MS/MS quantification quality (*9*), with an acceptable quantification results with respect to the certified values (mean difference between the measured and certified values was 15 ± 15%).

Table S1.1 Method detection limits (MDL) for all PCB congeners determined from blank samples analyzed in parallel with tissue and blood samples. All congeners had MDLs less than 0.02 ng except those that are listed. Values are expressed in ng[a].

PCB	MDL	PCB	MDL	PCB	MDL	PCB	MDL
1	--	52	2.65	107/124	--	162	--
2	--	54	--	108/123	0.55	165	--
3	0.17	55	--	110/115	1.38	167	--
4	--	56	--	111	0.06	169	--
5	--	57	--	112	0.29	170	--
6	--	58	--	114	--	171/173	--
7	--	59	--	117	--	172	--
8	--	60	--	118	3.58	174	--
9	--	61/70/74/76	--	120	--	175	--
10	--	62/75	--	121	1.61	176	--
11	--	63	--	122	2.53	177	--
12/13	--	64	--	126	--	178	0.03
15	--	66	--	127	--	179	--
16	--	67	--	129/138/163	--	180/193	--
17	--	68	--	130	0.06	181	--
18/30	--	72	--	131	--	182	--
19	--	77	--	132	--	183	--
20/28	--	78	0.03	133	--	184	--
21/33	--	79	--	134/143	--	185	--
22	--	80	--	135/151	--	186	--
23	--	81	0.30	136	4.79	187	--
24	--	82	0.49	137/164	0.23	188	--
25	--	83/99	0.64	139/140	--	189	--
26/29	--	84	0.13	141	--	190	--
27	--	85/116	3.91	142	0.12	191	--
31	--	86/87/97/109/119/125	0.29	144	0.20	192	--
32	--	88	--	145	0.58	194	--
34	0.02	89	--	146	0.12	195	--
35	--	90/101/113	2.73	147/149	2.76	196	--
36	--	91	--	148	--	197	--
37	--	92	--	150	--	198/199	--
38	--	93/100	--	152	1.53	200	--
39	--	94	0.80	153/168	--	201	--
40/41/42/71	--	95	5.52	154	--	202	--
43/73	--	96	--	155	--	203	--
45/51	--	98/102	--	156/157	--	205	--
46	--	103	--	158	--	206	--
48	--	104	--	159	0.76	207	0.13
49/69	--	105	--	160	--	208	--
50/53	--	106	--	161	--	209	0.34

[a]All samples were injected from a final volume of 100 µL.

Table S1.2 LOQ/LOD for the congeners that have TEF values. LOD is calculated from the method detection limit (MDL) and LOQ is from the minimal level of detection: ML = x + 10 SD; where x = the mean of replicates of blank measures analyzed in parallel to all tissue and blood samples and SD is standard deviation of the replicates.

PCB	TEF	LOD	LOQ/LOD
77	0.0001	<0.02	--
81	0.0003	0.3	2.07
126	0.1	<0.02	--
169	0.03	<0.02	--
105	0.00003	<0.02	--
114	0.00003	<0.02	--
118	0.00003	3.58	2.07
123	0.00003	<0.02	--
156	0.00003	<0.02	--
157	0.00003	<0.02	--
167	0.00003	<0.02	--
189	0.00003	<0.02	--

-- indicates that both the LOD and LOQ are less than 0.02 ng in a final volume of 100 µL.

Table S2. Recovery rates of spiked surrogate standards during the PCB extraction process from XAD samples of the generated CAM vapor and from rat liver, lung, blood, adipose tissue and brain after subchronic exposure to the vapor. Values are expressed as mean ± standard deviation. Surrogates are injected into every sample and each PCB mass is corrected for recovery.

	CAM vapor (n=15)	Tissue (n=13)				
		Lung	Blood	Liver	Brain	Adipose tissue
PCB 14	65±8%	31±11%	49±16%	64±12%	84±14%	40±10%
d-PCB 65	100±14%	42±14%	65±22%	74±10%	61±15%	63±28%

Table S3. Recovery rates of spiked ongoing precision and recovery standards during the PCB extraction process from XAD samples of the generated CAM vapor and from rat blood and tissue after subchronic exposure to the vapor. Values are corrected for surrogate recovery, expressed as mean recovery rate and standard deviation (SD).

Congener	CAM vapor	Tissue (n=5)	
		Average	SD
3	105.63%	82.44%	29.49%
8	118.68%	95.51%	20.23%
18	111.97%	95.09%	20.71%
28	111.93%	96.90%	21.71%
44	102.22%	95.53%	19.18%
52	112.19%	95.14%	19.79%
66	109.06%	98.34%	17.70%
77	115.14%	101.85%	17.17%
81	116.42%	98.92%	16.36%
101	118.01%	97.21%	15.46%
105	135.37%	105.67%	13.44%
114	115.65%	102.14%	15.01%
118	133.67%	103.22%	14.19%
123	123.27%	109.52%	10.71%
126	125.09%	105.90%	16.99%
128	131.14%	102.66%	12.85%
138	134.85%	110.33%	15.00%
153	126.76%	107.76%	15.35%
156+157	132.97%	109.92%	16.48%
167	127.08%	110.36%	17.10%
169	131.09%	110.32%	15.69%
170	132.60%	114.31%	20.92%
180	129.81%	113.05%	22.66%
187	129.51%	112.09%	20.27%
189	134.91%	113.68%	22.46%
195	126.08%	111.91%	24.18%
206	134.00%	109.25%	19.96%
209	134.42%	106.40%	18.88%

Table S4. Concentrations of prevailing PCB congeners that accounted for more than 1% in at least one rat tissue after subchronic exposure to the CAM vapor and the chemical structures of these congeners. Values are expressed as ng/g lipid weight. Data shown are the mean of n=6 for lung, blood, liver and brain and n=4 for adipose tissue. Values in bold indicates that the congener accounted for over 5% in the corresponding tissue.

Number of Chlorines	Congener	Lung	Blood	Liver	Brain	Adipose	Chemical Structure
Di	8	28.67	62.98	41.76	**14.55**	47.76	2,4'
	15	**79.47**	68.22	21.23	0.00	7.33	4,4'
Tri	18*/30	8.58	53.35	16.16	1.62	11.30	2,2',5
	20/28*	**619.47**	**555.24**	**277.75**	**115.45**	**881.39**	2,4,4'
	26*/29	19.91	37.88	6.63	0.00	0.00	2,3',5
	31	19.53	31.35	16.99	0.00	12.05	2,4',5
Tetra	49*/69	7.97	56.89	0.00	0.00	9.38	2,2',4,5'
	52	18.42	74.01	32.17	0.40	25.18	2,2',5,5'
	55	0.00	140.52	12.36	0.00	0.00	2,3,3',4
	60	45.17	37.94	28.72	0.66	65.43	2,3,4,4'
	61/70*/74*/76	**88.30**	196.43	**62.70**	3.21	**211.77**	2,3',4',5; 2,4,4',5
	66	**116.30**	**215.35**	**84.79**	**14.88**	**430.33**	2,3',4,4'
Penta	83/99*	**82.77**	113.58	**89.95**	0.00	130.14	2,2',4,4',5
	85*/116	1.61	4.99	14.30	4.40	104.55	2,2',3,4,4'
	86/87*/97*/109/119/125	0.00	0.00	14.62	1.62	10.97	2,2',3,4,5'; 2,2',3,4',5'
	90/101*/113	7.66	**166.14**	28.88	4.34	93.73	2,2',4,5,5'
	95	0.00	105.37	20.31	0.80	24.83	2,2',3,5',6
	105	**76.13**	**165.12**	59.36	9.72	**336.92**	2,3,3',4,4'
	110*/115	0.23	50.96	30.43	2.12	36.63	2,3,3',4',6
	112	23.95	35.72	0.00	0.00	0.00	2,3,3',5,6
	118	**98.69**	**426.64**	**130.52**	**28.92**	**640.72**	2,3',4,4',5
	126	0.00	0.00	16.22	0.64	33.31	3,3',4,4',5
Hexa	129/138*/163	73.22	**172.13**	61.04	0.00	**196.99**	2,2',3,4,4',5'
	147/149*	17.47	52.25	16.16	0.18	16.96	2,2',3,4',5',6
	153*/168	42.75	22.26	31.58	4.24	179.29	2,2',4,4',5,5'
	156*/157	0.00	89.74	6.21	0.00	18.72	2,3,3',4,4',5

*Major congeners of the coeluting congener set in Aroclor 1242 and 1254.

Table S5. Total cell counts and differential cell counts, levels of total protein and LDH activity BAL fluid from rats subchronically exposed to the CAM vapor. Values are expressed as mean ± SE. None of these outcomes measures was significantly different between sham and CAM-exposed rats.

Exposure Group	Total Cells (10^3 per mL BAL)	Differential Cells (10^3 per mL BAL)			Total protein (μg/mL)	LDH activity (U/L)
		Macrophages	Neutrophils	Lymphocytes		
PCB	31.74 ± 3.36	31.64 ± 3.24	0.30 ± 0.10	0.27 ± 0.09	86.9 ± 5.4	31 ± 2
Sham	32.07 ± 2.46	31.58 ± 2.40	0.23 ± 0.09	0.26 ± 0.12	105.2 ± 7.2	34 ± 2

Table S6. Concentration [pg/mL (mean ± SE)] of cytokines in BAL fluid from rats subchronically exposed to the CAM vapor. Values are expressed as mean ± SE. LLOD = lower limit of detection. None of these outcomes measures was significantly different between sham and CAM-exposed rats.

	GM-CSF	IFN-γ	IL-1α	IL-1β	IL-2	IL-4	IL-6	IL-10	IL-12	TNF-α
PCB	1.61± 0.00	1.30 ± 0.16	15.03 ± 2.45	11.30 ± 2.15	150.63 ± 6.10	9.95 ± 1.19	5.18 ± 0.92	90.38 ± 14.55	3.02 ± 0.55	13.21 ± 5.77
Sham	11.34 ±7.91	7.71 ± 4.71	83.20 ± 46.91	42.08 ± 23.04	242.11 ± 85.16	39.02 ± 20.36	20.22 ± 11.03	81.63 ± 5.80	28.04 ± 16.03	19.21 ± 7.02
LLOD	1.61	1.38	9.93	5.36	36.10	11.31	2.72	75.83	2.90	0.35

Table S7. Blood parameters measured from rats subchronically exposed to the CAM vapor. Hematocrit (HCT) was significantly elevated in PCB exposed rats ($p < 0.0000$). No other difference was found.

		WBC	RBC	HGB	HCT	NEUT	LYMPH	MONO	EO	BASO	NEUT	LYMPH	MONO	EO	BASO
		$10^3/\mu L$	$10^6/\mu L$	g/dL	%	$10^3/\mu L$					%				
PCB	Mean	2.27	8.82	16.58	62.03	0.49	1.87	0.05	0.05	0.00	19.93	76.14	1.82	2.01	0.11
	SE	0.16	0.10	0.19	0.53	0.07	0.16	0.02	0.01	0.00	2.57	2.60	0.53	0.23	0.06
Sham	Mean	2.84	8.44	15.89	49.23	0.36	2.35	0.08	0.05	0.00	14.05	81.52	2.45	1.98	0.00
	SE	0.39	0.14	0.29	0.80	0.03	0.37	0.02	0.00	0.00	1.63	1.60	0.47	0.21	0.00

Abbreviation:
WBC – white blood cell
RBC – red blood cell
HGB – hemoglobin
HCT – hematocrit
NEUT – neurtrophils
LYMPH – lymphocyte
MONO – monocyte
EO – eosinophil
BASO - Basophil

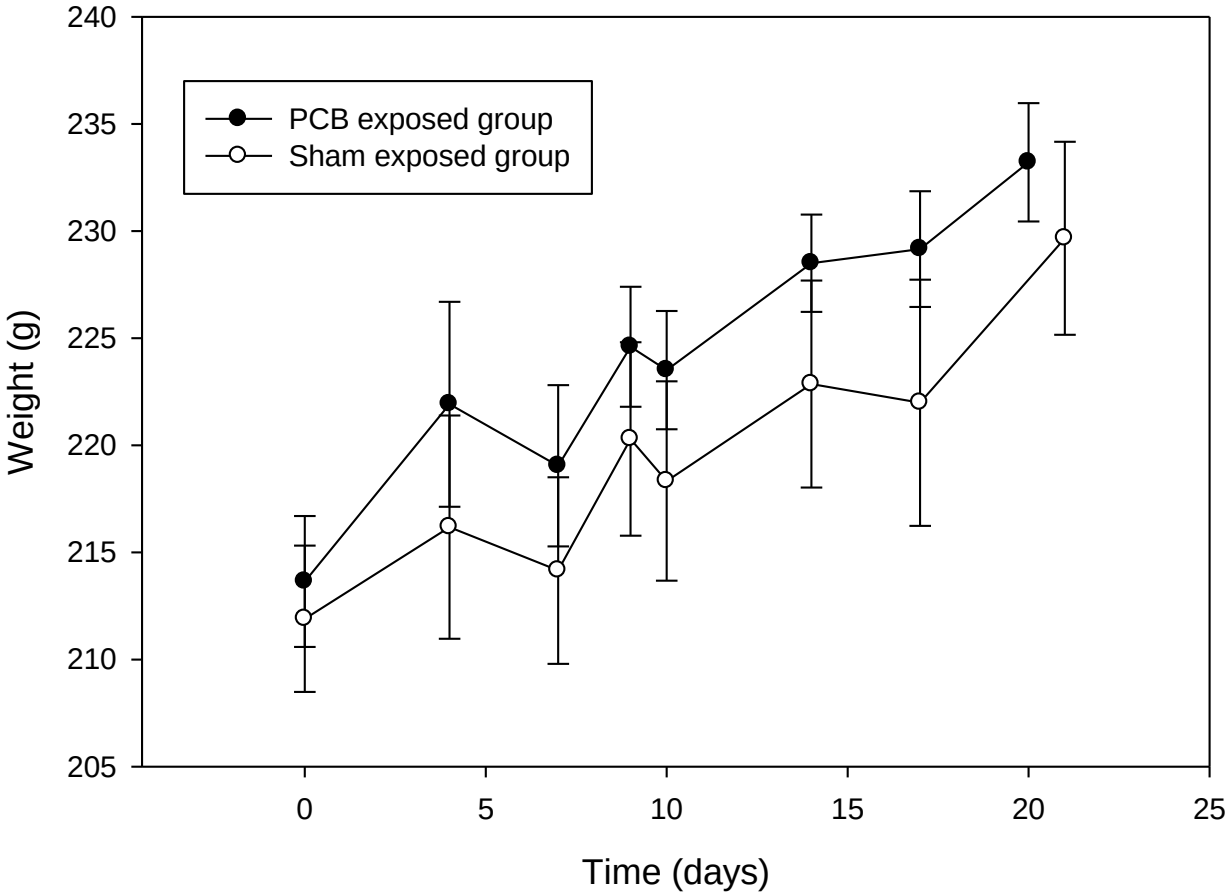

Figure S1. Body weight gain in rats during 20 d subchronic inhalation exposure to the CAM vapor. No significant effects of PCB exposure were observed.

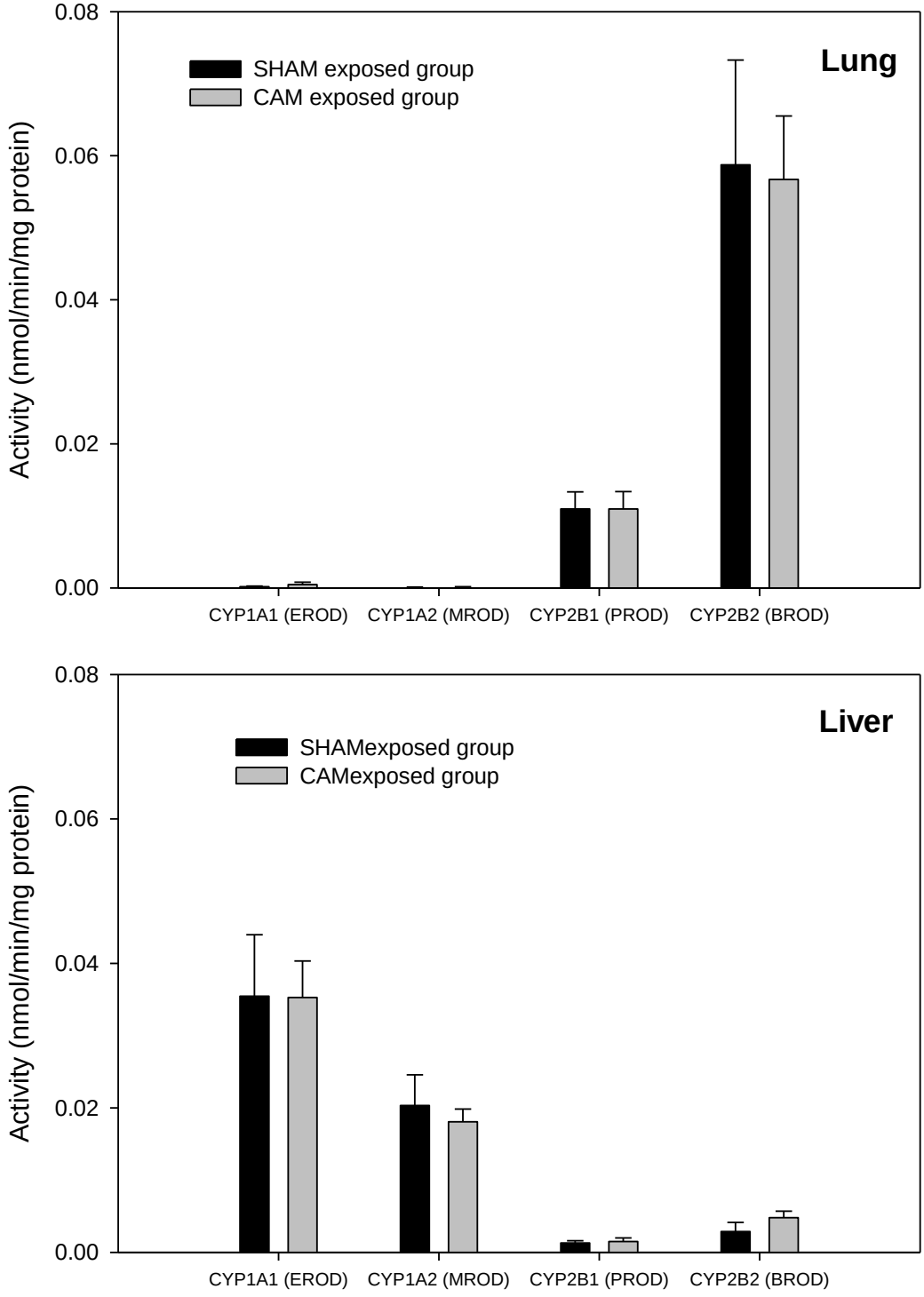

Figure S2. Effect of PCB exposure on CYP1A and CYP2B activities in rat lung and liver after subchronic exposure to the CAM vapor. No significant differences between the exposed group and the sham group were found.

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