

Supplementary Material: Is Mixtures Additivity Supported by Empirical Data? A Case Study of Developmental Toxicity of PFOS and 6:2FTS in Wildtype Zebrafish Embryos

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Table S1. Relative potency of PFOS and 6:2 FTS to aquatic organisms based on matched test species, effect endpoints, and points of departure (POD).

Test Species	Study Duration (days)	Effect Endpoint	POD	Concentration (µg/L) ¹		Relative Potency ²	Ref.
				PFOS	6:2 FTS		
Eastern Tiger Salamander <i>Ambystoma tigrinum</i>	31	body condition	LOAEL ³	>1000	<10	<1:0.01	[35,36]
Eastern Tiger Salamander <i>Ambystoma tigrinum</i>	31	snout vent length	LOAEL	100	<10	<1:0.1	[35,36]
Water Flea <i>Daphnia magna</i>	2	immobility/ lethality	EC ₅₀	672,000	>109,000	>1:0.2	[37,38]
Northern Leopard Frog <i>Lithobates pipiens</i>	40	developmental de- lay	LOAEL	200	>1000	>1:5	[35,36]
Rainbow Trout <i>Oncorhynchus mykiss</i>	4	survival	LC ₅₀	22,000	>107,000	>1:5	[37,38,39]
Zebrafish, <i>Danio rerio</i>	7	survival	LC ₅₀	1000	8000	1:8	[40]
Moor Frog, <i>Rana arvalis</i>	7	survival	LC ₅₀	1400	10,500	1:8	[40]
American Toad <i>Anaxyrus americanus</i>	30	snout vent length & body mass	NOAEL	100	>1000	>1:10	[35,36]
Northern Leopard Frog <i>Lithobates pipiens</i>	40	developmental de- lay	NOAEL	10	1000	1:100	[35,36]

EC₅₀ = effective concentration that gives half-maximal response; LOAEL = lowest observed adverse effect level; POD = point of departure; LC₅₀ = lethal concentration that causes 50% mortality; NOAEL = no observed adverse effect level; ¹ Values with ">" are unbounded for the study. Unbounded NOAELs are the highest treatment group tested. Unbounded LOAELs are the highest treatment group tested; ² Relative potency based on the ratio of PODs (i.e., 6:2 FTS / PFOS). Results with ">" reflect an unbounded NOAEL or LC₅₀ for 6:2 FTS (numerator) or unbounded LOAEL for PFOS (denominator). Results with "<" reflect an unbounded NOAEL for PFOS (denominator) or unbounded LOAEL for 6:2 FTS (numerator); ³ PFOS toxicity study for *A. tigrinum* supports an unbounded NOAEL for PFOS, meaning the NOAEL is > 1,000 µg/L, and the LOAEL would occur at an even higher concentration.

Table S2. Nominal and measured concentrations of PFOS and 6:2 FTS in stock solutions used for three experiments: 1) PFOS; 2) 6:2 FTS; and 3) binary mixtures of PFOS + 6:2 FTS at a target 1:10 concentration ratio. Values are presented for each experiment/chemical group in units of µg/L (top) and µM (bottom). Molecular weights (g/mol or µg/µM) are 500.13 for PFOS (CASRN 1763-23-1) and 428.17 for 6:2 FTS (CASRN 27619-97-2).

Experiment & Chemical		Control		T1		T2		T3		T4		T5	
		N	M	N	M	N	M	N	M	N	M	N	M
Concentration (µg/L)													
1- PFOS	PFOS	0	0	0.10	0.76	2.0	3.2	60	49.6	600	2070	1980	7480
	6:2 FTS	0	0	0	0	0	0	0	0	0	0	0	0
2- 6:2 FTS	PFOS	0	0	0	0.028	0	0	0	0	0	0	0	0
	6:2 FTS	0	1.9	1.0	1.2	20.0	23.3	600	731	6000	6,330	19,800	15,500
3- PFOS + 6:2 FTS	PFOS	0	0	0.10	0.075	2.0	0.63	60	29.0	600	241	1980	1570
	6:2 FTS	0	0	1.0	1.3	20.0	21.0	600	683	6000	5,830	19,800	15,300
	Ratio ¹	0	0	1:10	1:17	1:10	1:33	1:10	1:24	1:10	1:24	1:10	1:10
Concentration (µM)													
1- PFOS	PFOS	0	0	0.0002	0.0015	0.0040	0.0064	0.120	0.099	1.2	4.1	4.0	15.0
	6:2 FTS	0	0	0	0	0	0	0	0	0	0	0	0
2- 6:2 FTS	PFOS	0	0	0	0.00006	0	0	0	0	0	0	0	0
	6:2 FTS	0	0.0045	0.0023	0.00285	0.0467	0.544	1.4	1.7	14.0	14.8	46.2	36.3
3- PFOS + 6:2 FTS	PFOS	0	0	0.0002	0.00015	0.004	0.00126	0.12	0.058	1.2	0.48	4.0	3.1
	6:2 FTS	0	0	0.0023	0.00304	0.0467	0.049	1.4	1.6	14.0	13.6	46.2	35.6

N = nominal concentration; M = measured concentration.

1. Ratio = 6:2 FTS / PFOS, equivalently expressed as the proportion PFOS: 6:2 FTS. The nominal concentration ratio for each treatment group of the binary experiment was 1 part PFOS per 10 parts 6:2 FTS (1:10). Measured concentration ratios ranged from 1:10 to 1:33.

Table S3. Mortality rate and hatch rate by experiment, treatment, replicate, and days post fertilization.

Experiment	Treatment	Replicate	Count ¹		1 dpf		2 dpf		3 dpf		4 dpf		5 dpf	
			Start	End	MR	HR	MR	HR	MR	HR	MR	HR	MR	HR
1- PFOS	0	A	20	15	25%	0%	25%	0%	25%	75%	25%	75%	25%	75%
	0	B	20	13	35%	0%	35%	0%	35%	65%	35%	65%	35%	65%
	0	C	20	15	30%	0%	30%	5%	25%	75%	25%	75%	25%	75%
	1	A	20	14	30%	0%	30%	0%	30%	70%	30%	70%	30%	70%
	1	B	20	15	25%	0%	25%	0%	25%	75%	25%	75%	25%	75%
	1	C	20	13	35%	0%	35%	0%	35%	65%	35%	65%	35%	65%
	2	A	20	15	25%	0%	25%	0%	25%	75%	25%	75%	25%	75%
	2	B	20	12	35%	0%	35%	0%	40%	60%	40%	60%	40%	60%
	2	C	19	11	32%	0%	32%	5%	42%	63%	42%	63%	42%	63%
	3	A	20	15	35%	0%	35%	5%	25%	75%	25%	75%	25%	75%
	3	B	20	13	35%	0%	35%	0%	35%	65%	35%	65%	35%	65%
	3	C	20	11	45%	0%	45%	0%	45%	55%	45%	55%	45%	55%
	4	A	20	13	35%	0%	35%	0%	35%	65%	35%	65%	35%	65%
	4	B	20	18	10%	0%	10%	0%	10%	90%	10%	90%	10%	90%
	4	C	20	17	15%	0%	15%	25%	15%	85%	15%	85%	15%	85%
	5	A	20	17	15%	0%	15%	0%	15%	85%	15%	85%	15%	85%
	5	B	20	12	40%	0%	40%	0%	40%	60%	40%	60%	40%	60%
	5	C	20	13	35%	0%	35%	0%	35%	65%	35%	65%	35%	65%
2- 6:2 FTS	0	A	20	7	60%	0%	60%	0%	65%	40%	65%	40%	65%	40%
	0	B	20	8	55%	0%	55%	0%	60%	40%	60%	40%	60%	40%
	0	C	20	9	50%	0%	55%	0%	55%	45%	55%	45%	55%	45%
	1	A	20	7	60%	0%	65%	5%	65%	35%	65%	35%	65%	35%
	1	B	20	4	55%	0%	80%	5%	80%	20%	80%	20%	80%	20%
	1	C	20	13	50%	0%	35%	25%	35%	65%	35%	65%	35%	65%
	2	A	21	6	62%	0%	71%	0%	71%	29%	71%	29%	71%	29%
	2	B	20	6	60%	0%	65%	5%	70%	30%	70%	30%	70%	30%
	2	C	20	9	50%	0%	55%	40%	55%	45%	55%	45%	55%	45%
	3	A	20	6	60%	0%	70%	0%	70%	30%	70%	30%	70%	30%
	3	B	20	7	65%	0%	65%	5%	65%	35%	65%	35%	65%	35%
	3	C	20	5	75%	0%	75%	5%	75%	25%	75%	25%	75%	25%
	4	A	20	6	65%	0%	70%	5%	70%	30%	70%	30%	70%	30%
	4	B	20	7	60%	0%	65%	0%	65%	35%	65%	35%	65%	35%
	4	C	20	8	55%	0%	60%	5%	60%	40%	60%	40%	60%	40%
	5	A	20	7	65%	0%	65%	0%	65%	35%	65%	35%	65%	35%
	5	B	20	5	70%	0%	75%	0%	75%	25%	75%	25%	75%	25%
	5	C	20	6	65%	0%	65%	0%	70%	30%	70%	30%	70%	30%
3- PFOS + 6:2 FTS	0	A	20	10	50%	0%	50%	5%	50%	50%	50%	50%	50%	50%
	0	B	20	14	20%	0%	20%	10%	25%	75%	30%	75%	25%	75%
	0	C	20	15	25%	0%	25%	5%	25%	75%	25%	75%	25%	75%
	1	A	20	13	30%	0%	30%	5%	30%	70%	35%	70%	35%	70%
	1	B	20	14	25%	0%	25%	5%	30%	70%	30%	70%	30%	70%
	1	C	20	10	40%	0%	40%	50%	40%	60%	50%	60%	50%	60%
	2	A	20	12	35%	0%	40%	5%	40%	60%	40%	60%	40%	60%
	2	B	20	16	15%	0%	15%	20%	15%	85%	20%	85%	20%	85%
	2	C	20	14	30%	0%	30%	35%	30%	70%	30%	70%	30%	70%
	3	A	20	10	35%	0%	40%	0%	50%	60%	50%	60%	50%	60%
	3	B	21	10	52%	0%	52%	14%	52%	48%	52%	48%	52%	48%

Experiment	Treatment	Replicate	Count ¹		1 dpf		2 dpf		3 dpf		4 dpf		5 dpf	
			Start	End	MR	HR	MR	HR	MR	HR	MR	HR	MR	HR
	3	C	21	13	38%	0%	38%	10%	38%	62%	38%	62%	38%	62%
	4	A	19	12	37%	0%	37%	5%	37%	63%	37%	63%	37%	63%
	4	B	20	15	25%	0%	25%	0%	25%	75%	25%	75%	25%	75%
	4	C	20	15	25%	0%	25%	5%	25%	75%	25%	75%	25%	75%
	5	A	19	12	37%	0%	37%	11%	37%	63%	37%	63%	37%	63%
	5	B	20	13	25%	0%	30%	10%	30%	70%	35%	70%	30%	70%
	5	C	20	11	45%	0%	45%	15%	45%	55%	45%	55%	45%	55%

dpf = days post fertilization; HR = hatch rate; MR = mortality rate.

1. Counts began after the eggs were dispensed into the Petri dishes.

Table S4. Morphological measurements by experiment, treatment, and replicate.

Experiment	Treatment	Replicate	Body Length (mm)	Swim Bladder Area (mm ²)	Yolk Sac Area (mm ²)
1- PFOS	0	A	3.99	0.0575	0.087
	0	B	3.83	0.0704	0.0943
	0	C	3.82	0.0638	0.0894
	1	A	3.75	0.0497	0.0385
	1	B	3.81	0.0385	0.0341
	1	C	3.85	0.0421	0.0459
	2	A	3.84	0.0348	0.0469
	2	B	3.84	0.041	0.0402
	2	C	4.04	0.0388	0.0425
	3	A	3.7	0.033	0.0597
	3	B	3.69	0.0356	0.0558
	3	C	3.72	0.0382	0.0496
	4	A	3.63	0.0371	0.0669
	4	B	3.53	0.0333	0.0628
	4	C	3.58	0.0281	0.0583
2- 6:2 FTS	5	A	3.75	0.0485	0.0869
	5	B	3.71	0.0506	0.0813
	5	C	3.65	0.0424	0.0742
	0	A	3.9	0.0617	0.0601
	0	B	3.73	0.0571	0.0649
	0	C	3.69	0.0597	0.0523
	1	A	3.84	0.053	0.0562
	1	B	3.7	0.0583	0.0615
	1	C	3.65	0.0516	0.0529
	2	A	3.73	0.0473	0.0629
	2	B	3.68	0.0568	0.0627
	2	C	3.8	0.0544	0.0737
	3	A	3.62	0.0572	0.0565
	3	B	3.81	0.0549	0.0587
	3	C	3.59	0.0483	0.0652
	4	A	3.61	0.0531	0.0612
	4	B	3.99	0.0434	0.0577
	4	C	3.83	0.0558	0.0639
	5	A	3.59	0.0427	0.0569
	5	B	3.71	0.041	0.0561
	5	C	3.76	0.0559	0.0562

Experiment	Treatment	Replicate	Body Length (mm)	Swim Bladder Area (mm ²)	Yolk Sac Area (mm ²)
3- PFOS + 6:2 FTS	0	A	3.77	0.0531	0.0655
	0	B	3.64	0.0524	0.0634
	0	C	3.64	0.0534	0.0526
	1	A	3.6	0.0493	0.0784
	1	B	3.79	0.0527	0.0507
	1	C	3.63	0.0521	0.0523
	2	A	3.77	0.0473	0.0638
	2	B	3.66	0.0491	0.0541
	2	C	3.74	0.0454	0.057
	3	A	3.82	0.055	0.0614
	3	B	3.75	0.0489	0.0616
	3	C	3.53	0.0463	0.0601
	4	A	3.67	0.0564	0.0799
	4	B	3.69	0.054	0.0779
	4	C	3.56	0.0703	0.0614
	5	A	3.24	0.0319	0.072
	5	B	3.45	0.026	0.0797
	5	C	3.32	0.0204	0.064

Table S5. Results of pairwise comparisons for effects on mean SBA using R package *emmeans*.

Experiment	Treatment (µg/L) ¹	Emmean [LCL, UCL] ² sq mm	Contrast with T0		df	T Ratio	p-value ⁵
			sq mm ³	% change ⁴			
1-PFOS	T0 (0.00)	0.0639 [0.0581, 0.0697]	N/A	N/A	N/A	N/A	N/A
	T1 (0.76)	0.0434 [0.0376, 0.0493]	0.02047	32%	12	5.396	0.002
	T2 (3.2)	0.0382 [0.0324, 0.0440]	0.02570	40%	12	6.775	< 0.001
	T3 (49.6)	0.0356 [0.0298, 0.0414]	0.02830	44%	12	7.261	< 0.001
	T4 (2,070)	0.0328 [0.0270, 0.0387]	0.03107	49%	12	8.190	< 0.001
	T5 (7,480)	0.0472 [0.0413, 0.0530]	0.01673	26%	12	4.411	0.009
2-6:2 FTS	T0 (1.9)	0.0595 [0.0528, 0.0662]	N/A	N/A	N/A	N/A	N/A
	T1 (1.2)	0.0543 [0.0476, 0.0610]	0.00520	9%	12	1.187	0.83
	T2 (23.3)	0.0528 [0.0461, 0.0596]	0.00667	11%	12	1.522	0.66
	T3 (731)	0.0535 [0.0467, 0.0602]	0.00603	10%	12	1.378	0.74
	T4 (6,330)	0.0508 [0.0440, 0.0575]	0.00873	15%	12	1.994	0.40
	T5 (15,500)	0.0465 [0.0398, 0.0533]	0.01297	22%	12	2.961	0.10
3-PFOS + 6:2 FTS	T0 (0 + 0)	0.0530 [0.0469, 0.0590]	N/A	N/A	12	N/A	N/A
	T1 (0.075 + 1.3)	0.0514 [0.0453, 0.0574]	0.00160	3%	12	0.409	0.99
	T2 (0.63 + 21.0)	0.0473 [0.0412, 0.0533]	0.00570	11%	12	1.459	0.69
	T3 (29.0 + 683)	0.0501 [0.0440, 0.0561]	0.00290	5%	12	0.7452	0.97
	T4 (241 + 5,830)	0.0602 [0.0542, 0.0663]	−0.00727	−14%	12	−1.860	0.47
	T5 (1,570 + 15,300)	0.0261 [0.0201, 0.0321]	0.02687	51%	12	6.875	< 0.001

N/A = not applicable; pairwise contrasts are T1 through T5 vs T0; SBA = swim bladder area; sq mm = square millimeters;

¹ T0 = control group. Concentrations (in parentheses) of treatments increase from T1 to T5; ² Confidence level = 0.95. Pooled standard error for the mean is 0.00276 mm²; ³ Contrast with T0 is the difference in means: [T0 – Ti]. Pooled standard error for the mean of contrasts is 0.00391 mm²; ⁴ Percent change is 100% × [T0–Ti]/T0; ⁵ Statistically significant contrasts (*p* < 0.05) are highlighted in bold.

Table S6. Rank order of points of departure (POD) for zebrafish toxicity studies with PFOS including results from this study (bold font).

Rank	Study	Endpoint	Abbreviation	PFOS (µg/L)	POD ¹
1.5	This study	swim bladder area	SBA	0.76	LOAEL*
1.5	This study	yolk sac area	YSA	0.76	LOAEL*
3	This study	body length	BL	50	NOAEL
4	Huang et al., 2010 [41]	cell death, swimming behavior, malformations	Development	1120	EC ₅₀
5	This study	swim bladder area	SBA	1739	BMDL
6	Vogs et al., 2019 [42]	uninflated swim bladder and curved spine	SBA	1900	EC ₅₀
7.5	Jantzen et al., 2016 [43]	body length	BL	2000	LOAEL
7.5	Jantzen et al., 2016 [43]	yolk sac area	YSA	2000	LOAEL
9	This study	body length	BL	2066	LOAEL
10	Huang et al., 2010 [41]	mortality rate	MR	2200	LC ₅₀
11	Hagenaars et al., 2014 [44]	inflated swim bladder	SBA	2290	EC ₅₀
12	Martinez et al., 2019 [45]	swim bladder inflation	SBA	2500	LOAEL
13.5	Martinez et al., 2019 [45]	body length	BL	5000	LOAEL
13.5	Martinez et al., 2019 [45]	yolk sac area	YSA	5000	LOAEL
15	Hagenaars et al., 2014 [44]	developmental lethality	MR	6250	LC ₅₀
16	Sant et al., 2017 [46]	exocrine pancreas morphology	Development	8000	LOAEL
17	Kherzi et al., 2017 [47]	uninflated swim bladder, less developed gut, curved spine	SBA	8002	LOAEL
18	Martinez et al., 2019 [45]	mortality rate	MR	9100	LC ₅₀
19.5	Annunziato et al., 2020 [48]	body length	BL	17,640	LOAEL
19.5	Annunziato et al., 2020 [48]	yolk sac area	YSA	17,640	LOAEL
21	Annunziato et al., 2020 [48]	developmental lethality	MR	31,000	LC ₅₀
22	Ding et al., 2013 [49]	mortality	MR	50,510	LC ₅₀
23.5	Ding et al., 2013 [49]	mortality rate	MR	54,400	LC ₅₀
23.5	Ding et al., 2013 [49]	mortality rate	MR	54,900	LC ₅₀

EC₅₀ = effective concentration that gives half-maximal response; LOAEL= lowest observed adverse effect level; POD = point of departure; LC₅₀ = lethal concentration that causes 50% mortality; NOAEL= no observed adverse effect level; ¹ This study yields unbounded LOAELs (shown as LOAEL*) for SBA and YSA.

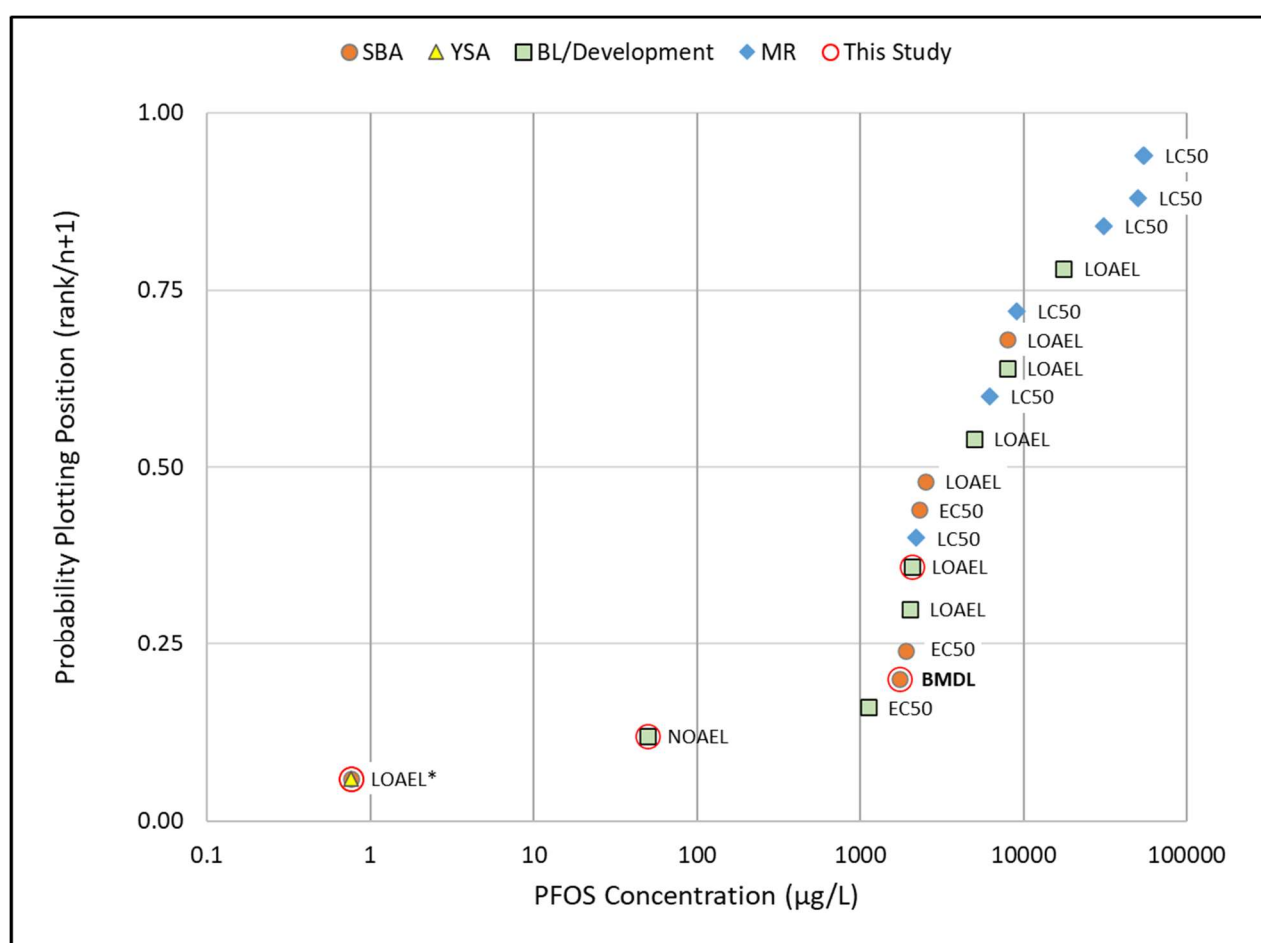


Figure S1. Cumulative probability distribution of $n=24$ points of departure (POD) from zebrafish toxicity studies with PFOS (see Table S6) shown on a log base 10 scale. SBA = swim bladder area, YSA = yolk sac area, BL = body length, MR = mortality rate. Selected PODs from this study are highlighted with a red circle. The benchmark dose lower bound (BMDL) for this study, approximately 1,700 $\mu\text{g/L}$, corresponds to the 20th percentile. The BMDL is the 95% lower confidence limit for the benchmark response at one control group standard deviation, and is most analogous to a NOAEL POD. LOAEL* is an unbounded LOAEL for SBA and YSA from this study.

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