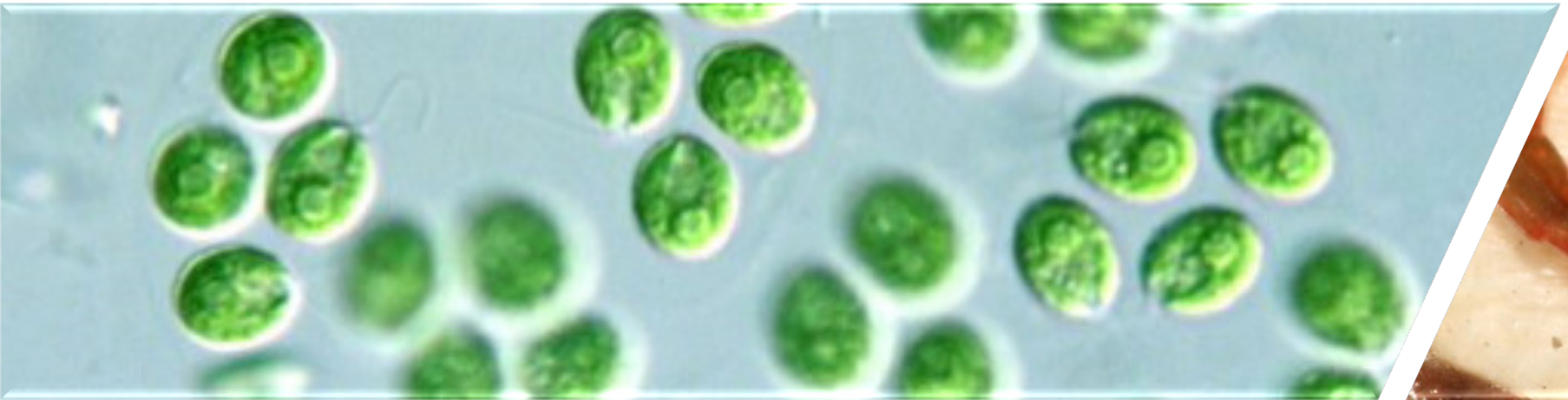
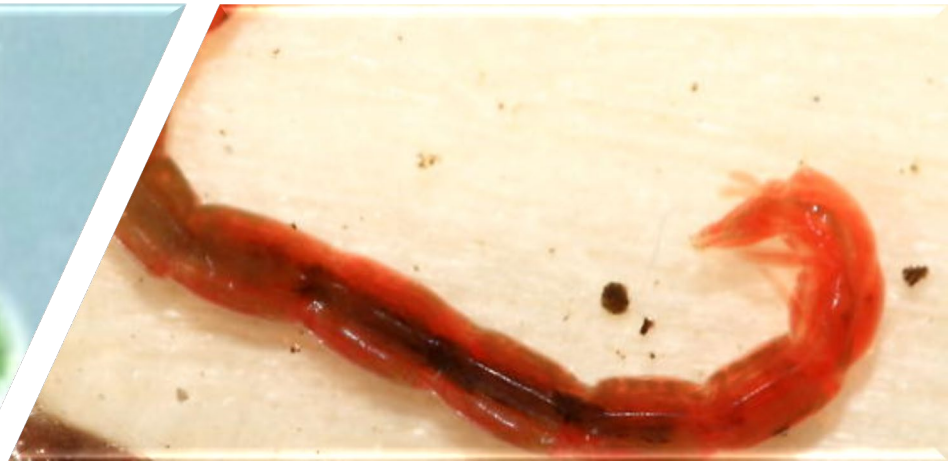
Transcriptomics-based points of departure for ecotoxicology – an update





APCRA Case study: Transcriptomics-based PODs for Ecotoxicology

Hypothesis: 24 h whole body transcriptomics can provide a protective, but not overly protective, point of departure for ecological toxicity.

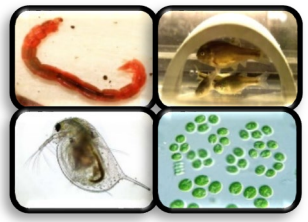
Approach:

1. Generate transcriptomic PODs for ≈ 20 chemicals
   - Initial focus on fathead minnow

2. Compare tPODs with available acute and chronic toxic toxicity data

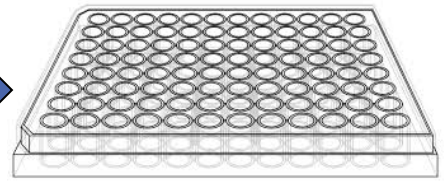
3. Compare tPODs with in vitro-derived PODs

Method Overview



Load organisms

24 h exposure

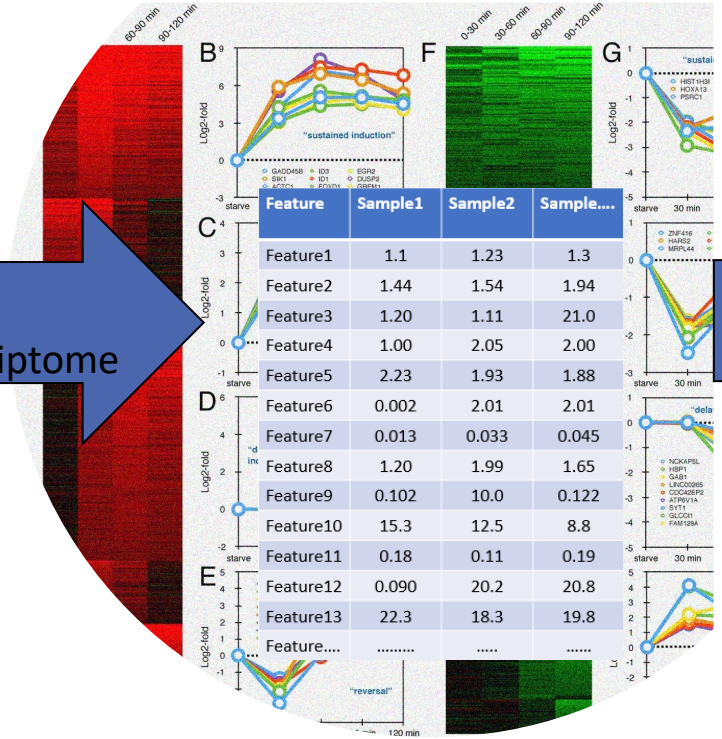


Phenotypic anchoring

Homogenate /extract

RNA

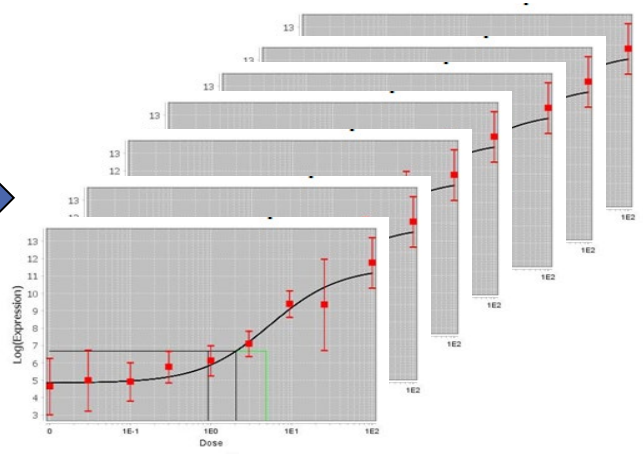
Whole transcriptome



Feature	Sample1	Sample2	Sample....
Feature1	1.1	1.23	1.3
Feature2	1.44	1.54	1.94
Feature3	1.20	1.11	21.0
Feature4	1.00	2.05	2.00
Feature5	2.23	1.93	1.88
Feature6	0.002	2.01	2.01
Feature7	0.013	0.033	0.045
Feature8	1.20	1.99	1.65
Feature9	0.102	10.0	0.122
Feature10	15.3	12.5	8.8
Feature11	0.18	0.11	0.19
Feature12	0.090	20.2	20.8
Feature13	22.3	18.3	19.8
Feature....	.....	.....	.....

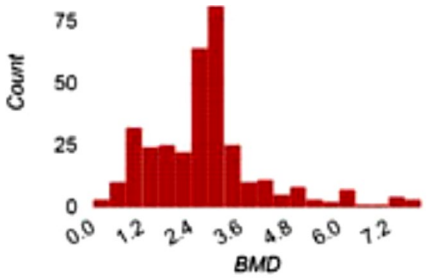
Curv

ve fitting



BMD Express

Distribution of BMDs



Point of departure

[tPOD]

Case study Progress

- Exposures have been completed for 22 chemicals.
  - 7 mode of action groupings
  - 3-4 chemicals per MoA group
- Sequencing complete and tPOD generated for 10 chemicals to date
  - 3 metals
  - 3 SSRIs
  - 4 neonicotinoid / related
- Contracts and funds in place to generate data for next 12.

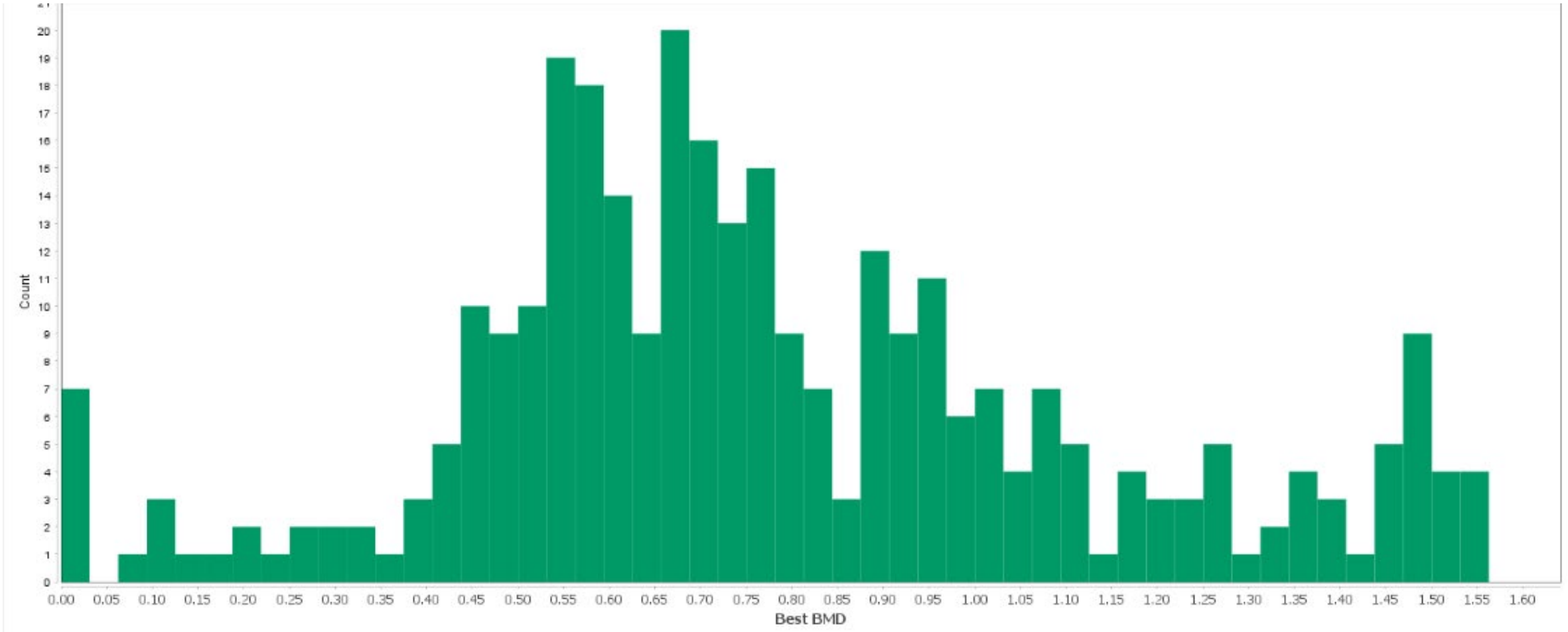
	Test Chemical	Assay Completed	Library Prep	tPOD
1	$CuSO_4$	X	X	X
2	$ZnSO_4$	X	X	X
3	$NiSO_4$	X	X	X
4	Clothinidin	X	X	X
5	Flupyradifurone	X	X	X
6	Imidacloprid	X	X	X
7	Thiacloprid	X	X	X
8	Sertraline	X	X	X
9	Fluoxetine	X	X	X
10	Paroxetine	X	X	X
11	Dibutyl phthalate	X		
12	DEHP	X		
13	Benzyl butyl pthalate	X		
14	Parathion	X	X	
15	Fenthion	X	X	
16	Methidathion	X	X	
17	Bisphenol A	X	X	
18	4-nonyl phenol	X		
19	Estrone	X	X	
20	Methoxyfenozide	X	X	
21	Tebufenozide	X		
22	Halofenozide	X		

Pre-covid

Covid

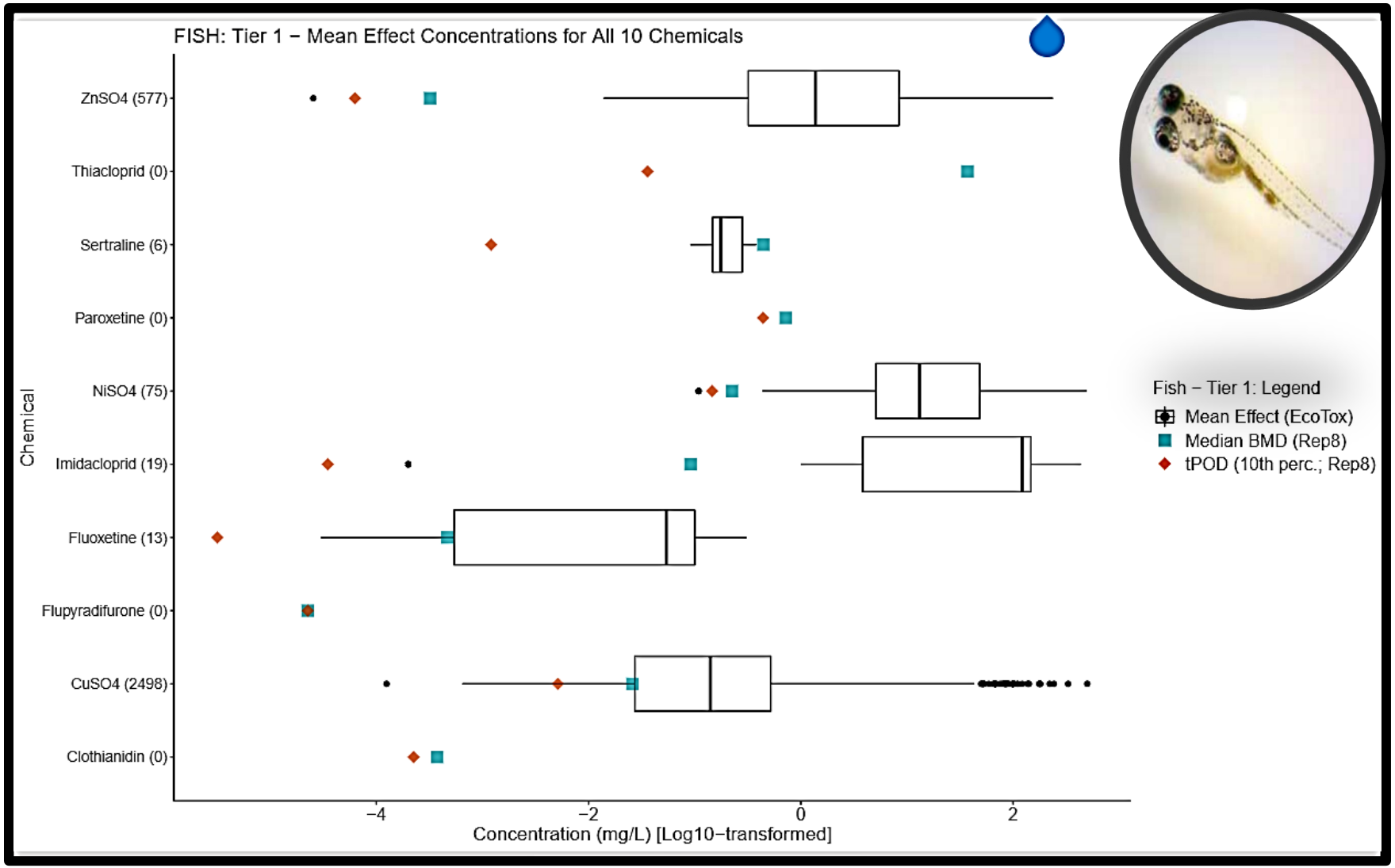
Nominal concentrations only

Analytical exposure verification



Preliminary Results: First 10 chemicals

Comparison with In vivo, **Adverse Effect** Concentrations (Fish)

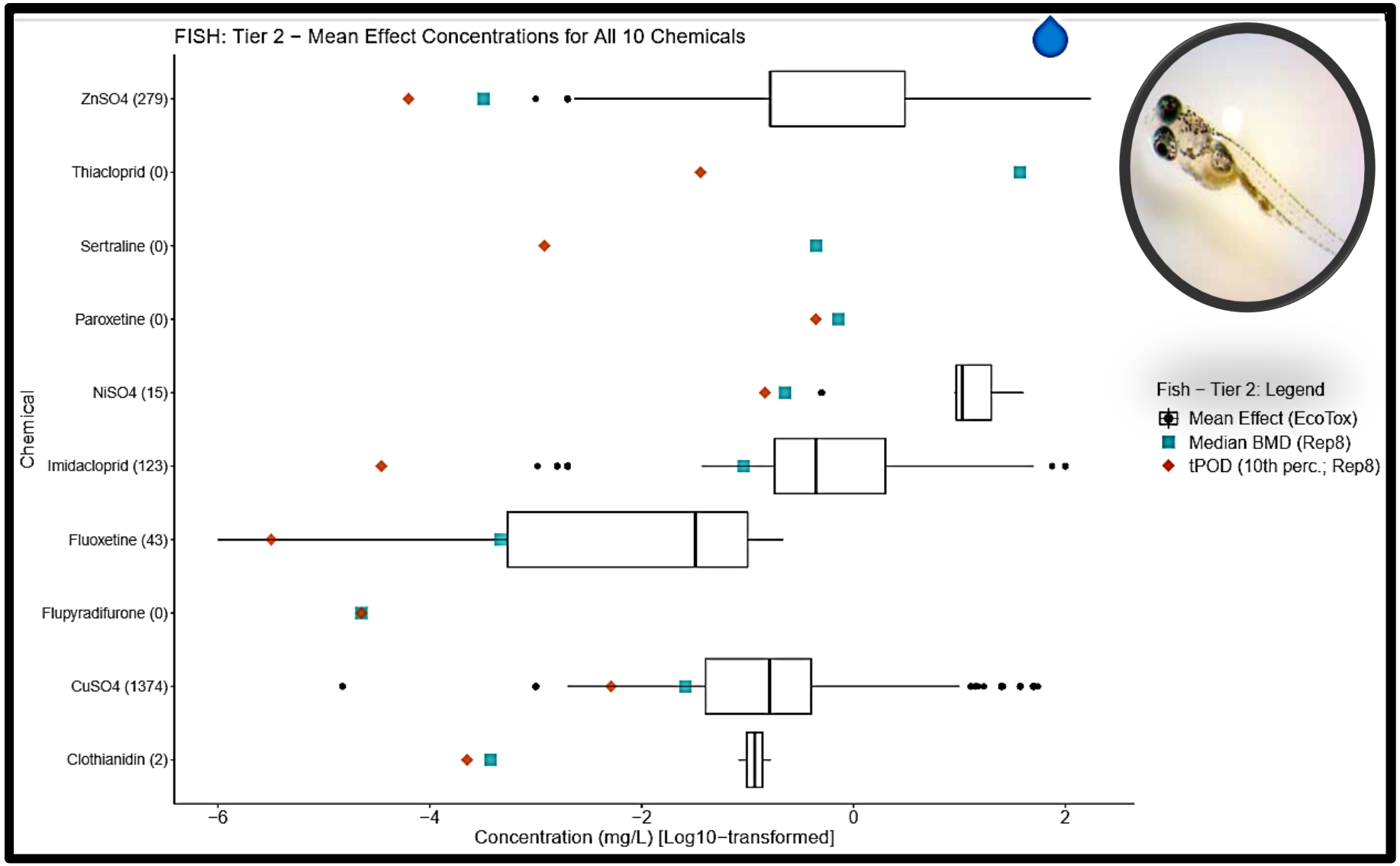


tPODs (10th centile BMD) were generally more sensitive than apical adverse effect concentrations.

tPOD based on median BMD were less protective

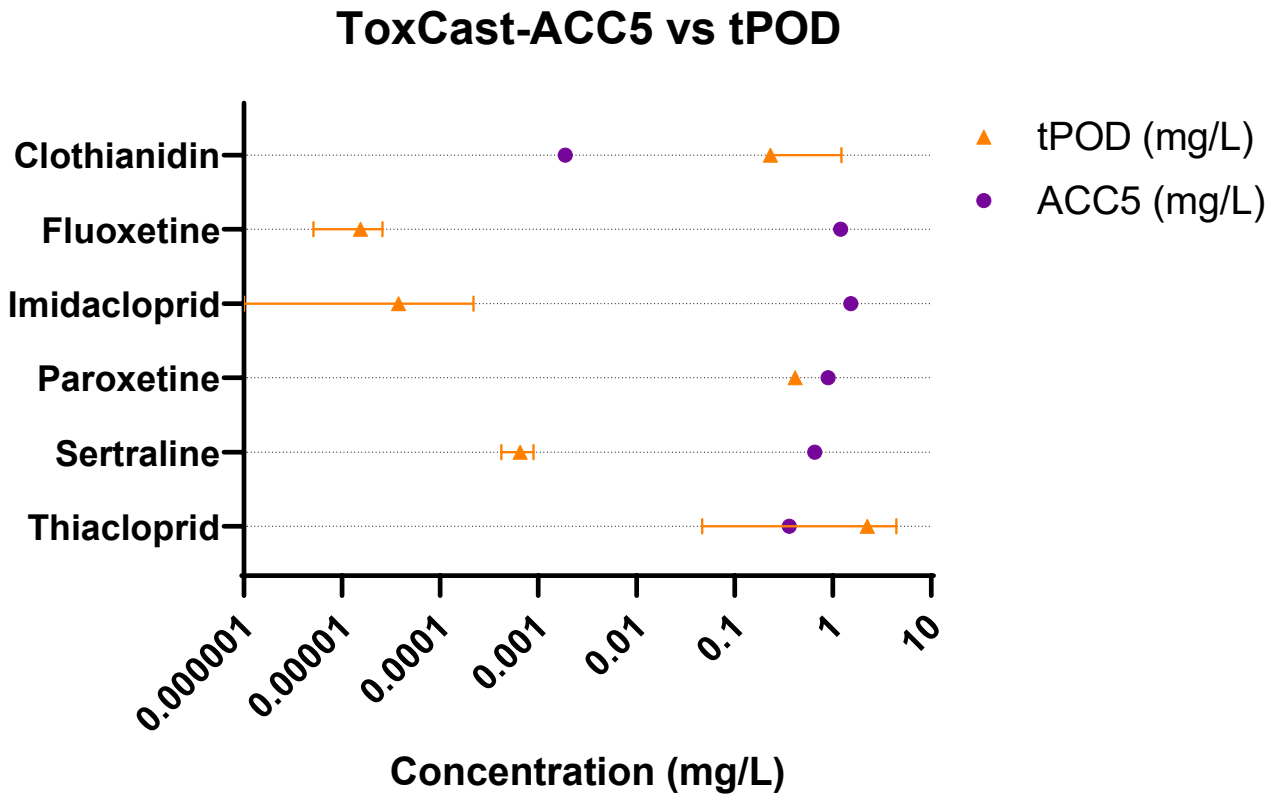
Up to 4 orders of magnitude more protective

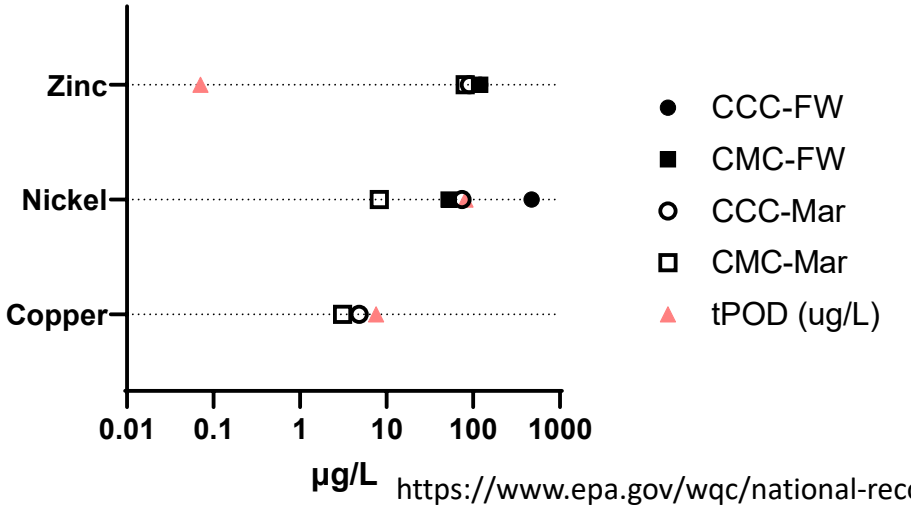
Comparison with In vivo, **Biological Effect** Concentrations (Fish)



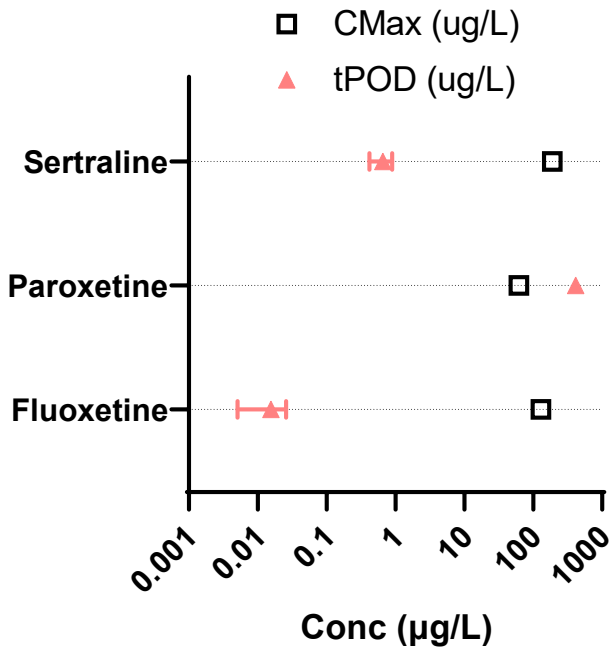
tPODs still generally more sensitive in vivo biological effect concentrations.

Up to 2 order of magnitude lower to overlapping lower quartile





https://www.epa.gov/wqc/national-recommended-water-quality-criteria-aquatic-life-criteria-table



Optimization and Assay Acceptance

Pilot experimental design

11 concentrations + control (½ log spacing)

Concentration range based on in vivo LC50 as upper bound

8 biological replicates (1 fish/well)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

- 700 µl per well
- 24 h static exposure

- 1-2 day post-hatch juveniles
  - Organogenesis mostly complete
  - Prior to indep. Feeding
  - Sufficient RNA

96 samples
Whole genome RNAseq

Approx. $8500 per chemical

Phenotypic observations: Survival, swimming behavior, deformities/abnormalities

Optimization

How much can we reduce gene set size, but still determine tPOD?

How much can we reduce samples size per treatment, maintain power?

Can we estimate biological uncertainty in tPOD estimate?

Performance criteria for an acceptable assay?

In silico Subsampling

Sample ID

Dose

Transcript ID

	A	B	C	D	E	F	G	H
1		Cu_P1_A1	Cu_P1_A10	Cu_P1_A2	Cu_P1_A3	Cu_P1_A4	Cu_P1_A5	Cu_P1_A6
2	Dose	0	0.2	0.00002	0.00006	0.0002	0.0006	0.002
3	FMt000003	6.396698281	7.165080955	6.139417185	5.625463693	5.936491943	5.984797836	6.526667329
4	FMt000004	2.727597133	2.819530386	2.476866196	2.260722943	2.072535549	2.408035192	2.829599109
5	FMt000005	2.666574282	2.757823089	2.957500498	3.294229757	3.698358555	3.209724111	2.681578019
6	FMt000006	4.675333003	4.768559009	4.858302489	5.139450164	4.551132645	4.819989912	4.829061001
7	FMt000008	5.816765605	5.550398239	5.373006159	5.467031575	5.756057101	5.692205772	5.572099115
8	FMt000009	1.921778265	1.337509659	1.842399017	1.364723466	1.820179855	2.227968209	1.720804988
9	FMt000010	0.400578876	1.02468777	0.580341068	1.428654949	1.048759955	1.133331622	0.796197253
10	FMt000011	1.036715489	1.337509659	1.179840942	1.762168511	2.033423943	1.907113212	1.682372652
11	FMt000013	4.339705191	4.231422779	4.457041355	4.158758634	4.390170142	4.287434091	4.354221981
12	FMt000014	0.753428738	1.497088068	1.179840942	1.227677204	0.694880669	1.737858491	1.117537912
13	FMt000016	4.685621982	4.677065189	4.791080697	4.988545119	4.631905782	4.902412506	5.063939077
14	FMt000017	1.15990699	1.392687117	1.548825352	2.575114318	1.623516583	2.058579857	2.227306515
15	FMt000019	2.982521854	1.092914161	1.969452091	3.276966231	2.985273802	2.37954184	2.472248844
16	FMt000020	4.326615646	3.729709358	4.659064377	3.978274305	3.280771887	3.922966112	3.700540563
	Mt000022	0.753428738	0.427434191	0.350624657	0.608894253	0.224966545	0.332838013	0.997448031
18	FMt000026	2.536192677	2.859249401	3.166436765	3.031435449	2.856737829	2.90442897	2.963836436
19	FMt000031	1.886534004	2.036875017	2.086216064	2.709867426	2.474112535	2.463387273	2.898278494
20	FMt000032	4.210281411	3.872414951	4.088442997	3.934904526	3.907492756	4.116679066	3.631612409
21	FMt000034	3.641040659	3.975408278	3.930816133	3.425369888	3.928963604	4.116679066	4.285372312
			200831699	4.413005717	4.267645049	4.435879765	4.332726221	4.354221981
			025579454	3.364405188	3.409614619	2.5591869	2.982290068	3.505201762
			220389325	0.470050181	0.081807702	0.59096166	0.072994421	0.796197253

BMD Express2.2

10^{th} percentile(BMD) = tPOD

In silico Subsampling

How much can we reduce gene set size, but still determine tPOD?

LEGEND

Transition to next replicate set

Sampled data

Fluoxetine	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	B01	B02	B03	B04	B06	B07	B08	B09	B10	B11
Dose	0	1.5E-05	0.00005	0.00015	0.0005	0.0015	0.005	0.015	0.05	0.15	0.5	0	1.5E-05	0.00005	0.00015	0.0015	0.005	0.015	0.05	0.15	0.5
FMt000003	5.69	5.66	5.67	5.98	5.63	5.88	5.46	5.56	5.43	5.69	5.49	6.01	6.01	5.69	5.48	5.53	5.54	5.15	5.61	5.48	5.45
FMt000004	1.85	1.93	1.97	2.01	2.10	1.33	2.12	1.83	1.47	1.71	2.03	2.23	2.13	1.58	1.37	2.15	1.81	2.09	1.51	1.05	1.50
FMt000005	2.78	2.22	2.22	2.36	2.91	3.21	1.92	2.48	2.28	3.24	2.83	3.20	2.30	2.62	1.93	2.62	2.34	3.16	2.90	2.16	2.79
FMt000006	4.63	4.45	4.49	4.45	4.16	4.39	4.88	4.21	4.25	4.35	4.52	3.90	4.06	4.20	4.53	4.09	4.24	4.10	4.12	4.01	4.38
FMt000008	5.21	5.40	5.41	5.15	5.49	5.48	5.37	5.43	5.49	5.37	5.28	5.41	5.38	5.40	5.43	5.40	5.44	5.62	5.61	5.38	5.27
FMt000009	1.72	1.49	1.63	1.65	1.96	1.54	1.30	1.69	1.21	1.32	1.07	1.91	1.44	1.67	1.87	1.75	2.10	1.72	1.58	1.35	1.36
FMt000010	1.14	1.23	1.05	0.53	1.59	1.39	0.51	1.22	1.42	1.57	1.41	1.63	1.35	1.44	1.33	1.47	1.39	1.18	1.86	1.64	1.33
FMt000011	1.75	1.67	1.54	1.50	1.89	1.72	2.30	1.95	1.73	1.66	1.56	2.11	2.26	1.94	2.02	1.78	1.91	2.12	1.58	1.78	1.46
FMt000013	4.32	4.63	4.69	5.39	4.74	4.83	5.06	4.80	4.89	4.69	4.46	4.90	4.77	4.97	4.71	5.05	4.99	4.74	4.84	5.01	4.61
FMt000014	0.97	1.04	1.49	1.65	1.02	1.33	1.18	1.07	1.16	1.37	1.51	1.40	0.74	0.84	1.37	0.70	1.29	1.33	0.98	1.00	1.29
FMt000016	5.14	5.38	5.13	5.42	4.99	4.82	5.09	4.63	4.90	5.03	4.94	4.96	5.02	4.98	5.04	4.96	4.86	4.80	5.13	5.11	5.27
FMt000017	2.51	2.57	2.56	2.61	2.65	2.16	2.40	1.73	2.08	2.26	2.31	2.44	2.10	2.49	2.31	1.98	2.18	2.09	2.44	2.39	2.61
FMt000019	1.60	1.28	1.87	2.01	2.26	1.59	3.13	1.73	3.39	1.14	2.03	0.30	0.74	1.67	2.53	2.74	2.69	2.54	2.98	1.48	1.70
FMt000020	3.29	3.58	3.42	3.64	3.32	3.79	3.86	3.30	3.67	3.50	3.76	3.48	3.82	3.68	3.65	3.81	3.43	3.26	3.85	3.34	3.21

Original expression matrix

Full dataset:
- 31,158 transcripts
- 12 doses, 8 reps per dose
- 96 samples total

Transcript(m), m=100 - 30,000 at random intervals

"Transcript(100) example"

Fluoxetine	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	B01	B02	B03	B04
Dose	0	1.5E-05	0.00005	0.00015	0.0005	0.0015	0.005	0.015	0.05	0.15	0.5	0	1.5E-05	0.00005	0.00015
FMt000003	5.69	5.66	5.67	5.98	5.63	5.88	5.46	5.56	5.43	5.69	5.49	6.01	6.01	5.69	5.48
FMt000004	1.85	1.93	1.97	2.01	2.10	1.33	2.12	1.83	1.47	1.71	2.03	2.23	2.13	1.58	1.37
FMt000005	2.78	2.22	2.22	2.36	2.91	3.21	1.92	2.48	2.28	3.24	2.83	3.20	2.30	2.62	1.93
FMt000006	4.63	4.45	4.49	4.45	4.16	4.39	4.88	4.21	4.25	4.35	4.52	3.90	4.06	4.20	4.53
FMt000008	5.21	5.40	5.41	5.15	5.49	5.48	5.37	5.43	5.49	5.37	5.28	5.41	5.38	5.40	5.43
FMt000009	1.72	1.49	1.63	1.65	1.96	1.54	1.30	1.69	1.21	1.32	1.07	1.91	1.44	1.67	1.87
FMt000010	1.14	1.23	1.05	0.53	1.59	1.39	0.51	1.22	1.42	1.57	1.41	1.63	1.35	1.44	1.33
FMt000011	1.75	1.67	1.54	1.50	1.89	1.72	2.30	1.95	1.73	1.66	1.56	2.11	2.26	1.94	2.02
FMt000013	4.32	4.63	4.69	5.39	4.74	4.83	5.06	4.80	4.89	4.69	4.46	4.90	4.77	4.97	4.71
FMt000014	0.97	1.04	1.49	1.65	1.02	1.33	1.18	1.07	1.16	1.37	1.51	1.40	0.74	0.84	1.37
FMt000016	5.14	5.38	5.13	5.42	4.99	4.82	5.09	4.63	4.90	5.03	4.94	4.96	5.02	4.98	5.04
FMt000017	2.51	2.57	2.56	2.61	2.65	2.16	2.40	1.73	2.08	2.26	2.31	2.44	2.10	2.49	2.31
FMt000019	1.60	1.28	1.87	2.01	2.26	1.59	3.13	1.73	3.39	1.14	2.03	0.30	0.74	1.67	2.53
FMt000020	3.29	3.58	3.42	3.64	3.32	3.79	3.86	3.30	3.67	3.50	3.76	3.48	3.82	3.68	3.65

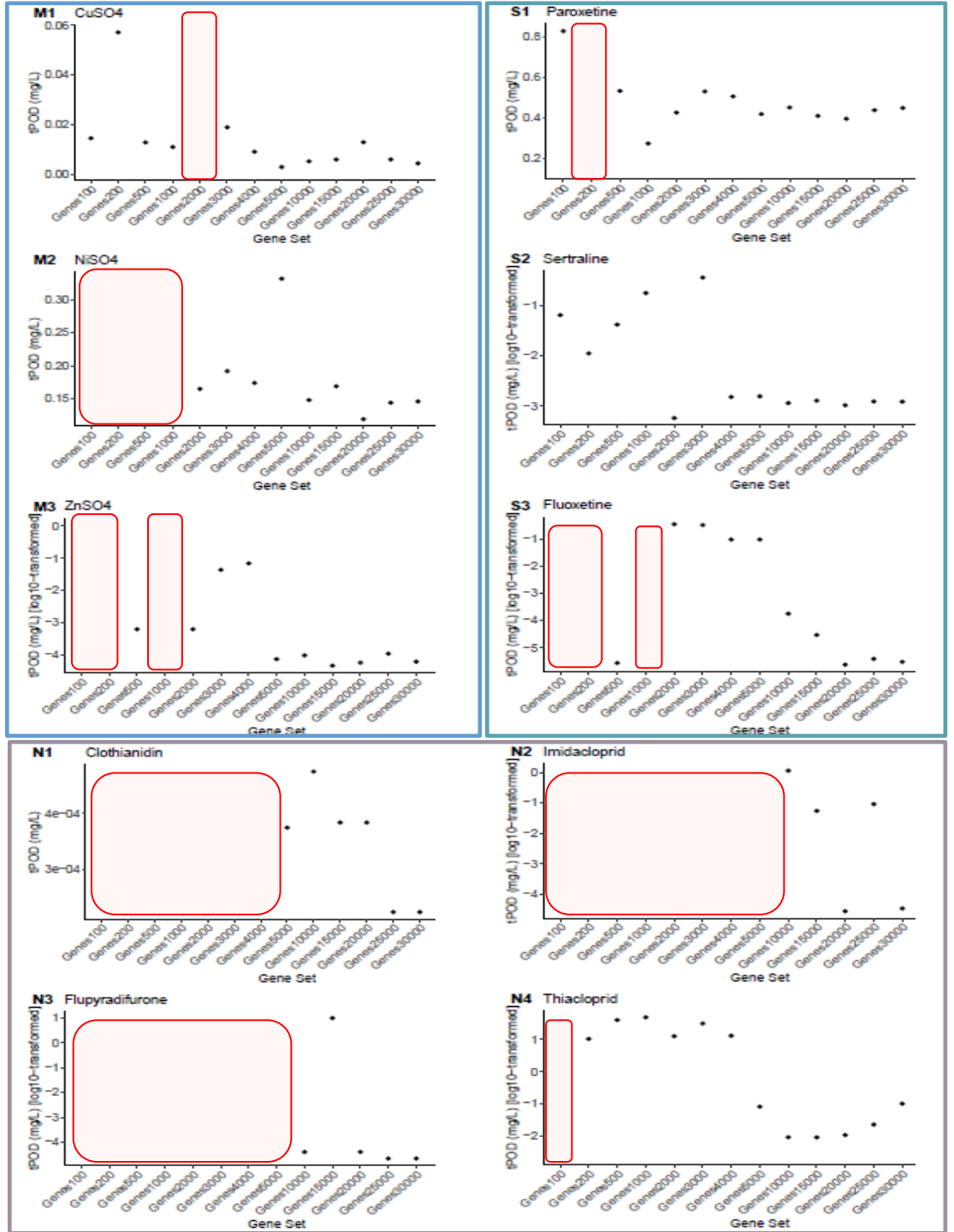
Transcript 100 dataset:
- **100 transcripts**
- 12 doses, 8 reps per dose
- 96 samples total

Variable Transcript Set Sizes: tPOD

Metals | Pharms | Neonics

No tPOD could be estimated

- tPOD could always be estimated when ≥ 10,000 transcripts were analyzed
- ≥ 1,000 was sufficient for 6/10 chemicals



Methods – *In silico* Subsampling

Replicate 3, 12x dataset:
- 31,158 transcripts
- 12 doses, **3 reps per dose**
- 96 samples total
- **12 iterations of each dataset**

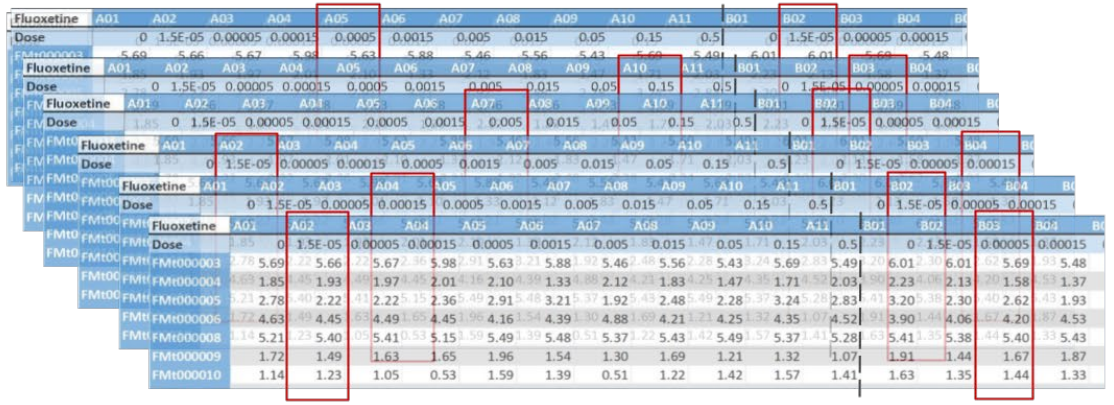
LEGEND

Transition to next replicate set

Sampled data

Replicate(n,12x), n=3 - 7

"Replicate(3,12x) example"



Original expression matrix

Fluoxetine	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	B01	B02	B03	B04	B06	B07	B08	B09	B10	B11
Dose	0	1.5E-05	0.00005	0.00015	0.0005	0.0015	0.005	0.015	0.05	0.15	0.5	0	1.5E-05	0.00005	0.00015	0.0015	0.005	0.015	0.05	0.15	0.5
FMt000003	5.69	5.66	5.67	5.98	5.63	5.88	5.46	5.56	5.43	5.69	5.49	6.01	6.01	5.69	5.48	5.53	5.54	5.15	5.61	5.48	5.45
FMt000004	1.85	1.93	1.97	2.01	2.10	1.33	2.12	1.83	1.47	1.71	2.03	2.23	2.13	1.58	1.37	2.15	1.81	2.09	1.51	1.05	1.50
FMt000005	2.78	2.22	2.22	2.36	2.91	3.21	1.92	2.48	2.28	3.24	2.83	3.20	2.30	2.62	1.93	2.62	2.34	3.16	2.90	2.16	2.79
FMt000006	4.63	4.45	4.49	4.45	4.16	4.39	4.88	4.21	4.25	4.35	4.52	3.90	4.06	4.20	4.53	4.09	4.24	4.10	4.12	4.01	4.38
FMt000008	5.21	5.40	5.41	5.15	5.49	5.48	5.37	5.43	5.49	5.37	5.28	5.41	5.38	5.40	5.43	5.40	5.44	5.62	5.61	5.38	5.27
FMt000009	1.72	1.49	1.63	1.65	1.96	1.54	1.30	1.69	1.21	1.32	1.07	1.91	1.44	1.67	1.87	1.75	2.10	1.72	1.58	1.35	1.36
FMt000010	1.14	1.23	1.05	0.53	1.59	1.39	0.51	1.22	1.42	1.57	1.41	1.63	1.35	1.44	1.33	1.47	1.39	1.18	1.86	1.64	1.33
FMt000011	1.75	1.67	1.54	1.50	1.89	1.72	2.30	1.95	1.73	1.66	1.56	2.11	2.26	1.94	2.02	1.78	1.91	2.12	1.58	1.78	1.46
FMt000013	4.32	4.63	4.69	5.39	4.74	4.83	5.06	4.80	4.89	4.69	4.46	4.90	4.77	4.97	4.71	5.05	4.99	4.74	4.84	5.01	4.61
FMt000014	0.97	1.04	1.49	1.65	1.02	1.33	1.18	1.07	1.16	1.37	1.51	1.40	0.74	0.84	1.37	0.70	1.29	1.33	0.98	1.00	1.29
FMt000016	5.14	5.38	5.13	5.42	4.99	4.82	5.09	4.63	4.90	5.03	4.94	4.96	5.02	4.98	5.04	4.96	4.86	4.80	5.13	5.11	5.27
FMt000017	2.51	2.57	2.56	2.61	2.65	2.16	2.40	1.73	2.08	2.26	2.31	2.44	2.10	2.49	2.31	1.98	2.18	2.09	2.44	2.39	2.61
FMt000019	1.60	1.28	1.87	2.01	2.26	1.59	3.13	1.73	3.39	1.14	2.03	0.30	0.74	1.67	2.53	2.74	2.69	2.54	2.98	1.48	1.70
FMt000020	3.29	3.58	3.42	3.64	3.32	3.79	3.86	3.30	3.67	3.50	3.76	3.48	3.82	3.68	3.65	3.81	3.43	3.26	3.85	3.34	3.21

Full dataset:
- 31,158 transcripts
- 12 doses, 8 reps per dose
- 96 samples total

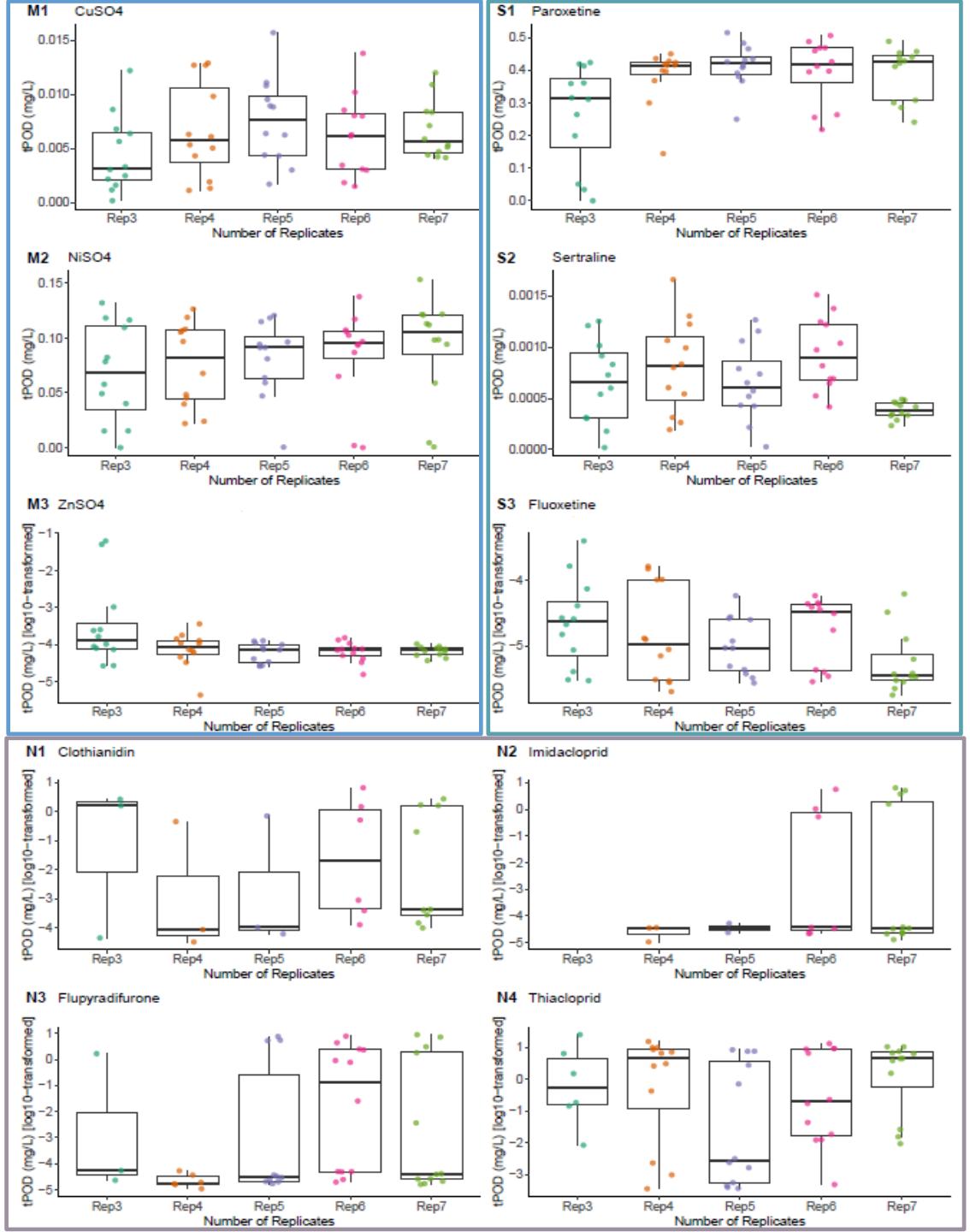
How much can we reduce samples size per treatment, maintain power?

Can we estimate biological uncertainty in tPOD estimate?

Variable Replicate (12x) Sizes: tPOD

Metals | Pharms | Neonics

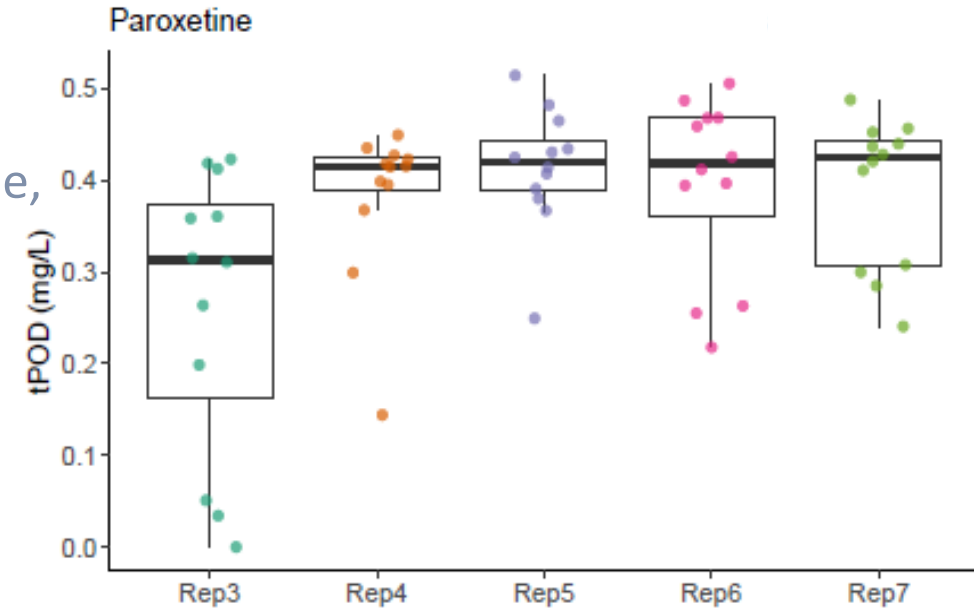
- In silico sub-sampling approach provided a means to estimate the biological variability/uncertainty in the tPOD determination.
- tPODs based on n=4 individuals were, on average, no more variable than those based on >4.
- Uncertainty around tPOD ranged from just 2-3 fold (paroxetine) to up to 6 orders of magnitude (flupyradifone)

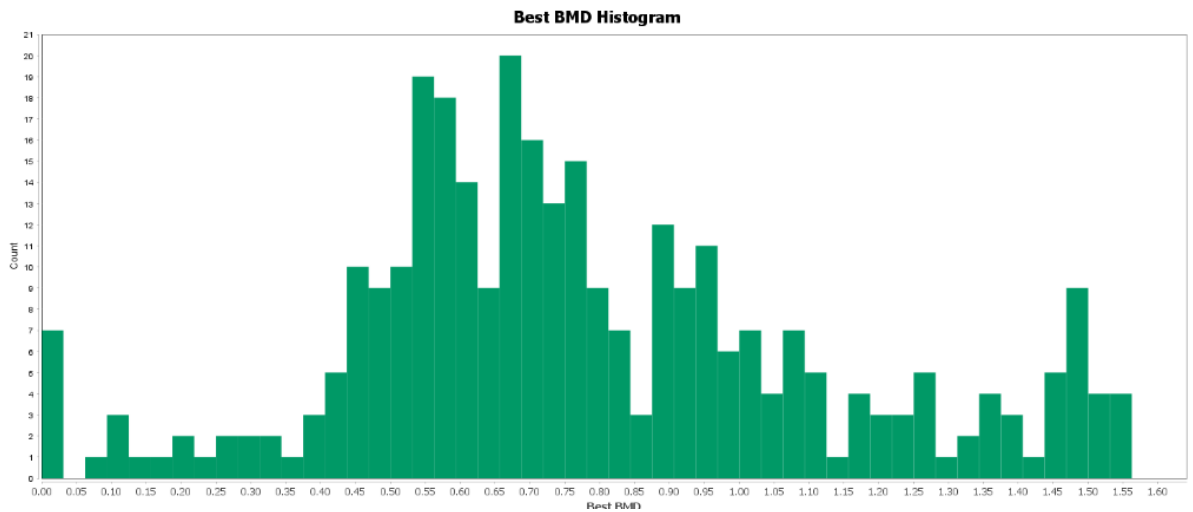


tPOD Variability

Paroxetine (500-600 DEGs)

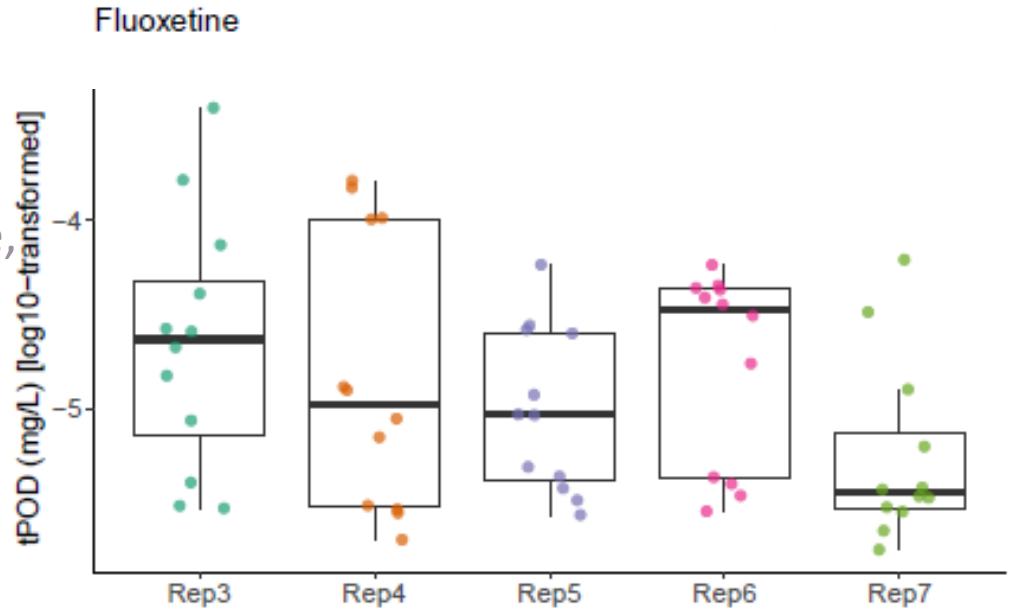
Linear scale, 2-3 fold variability

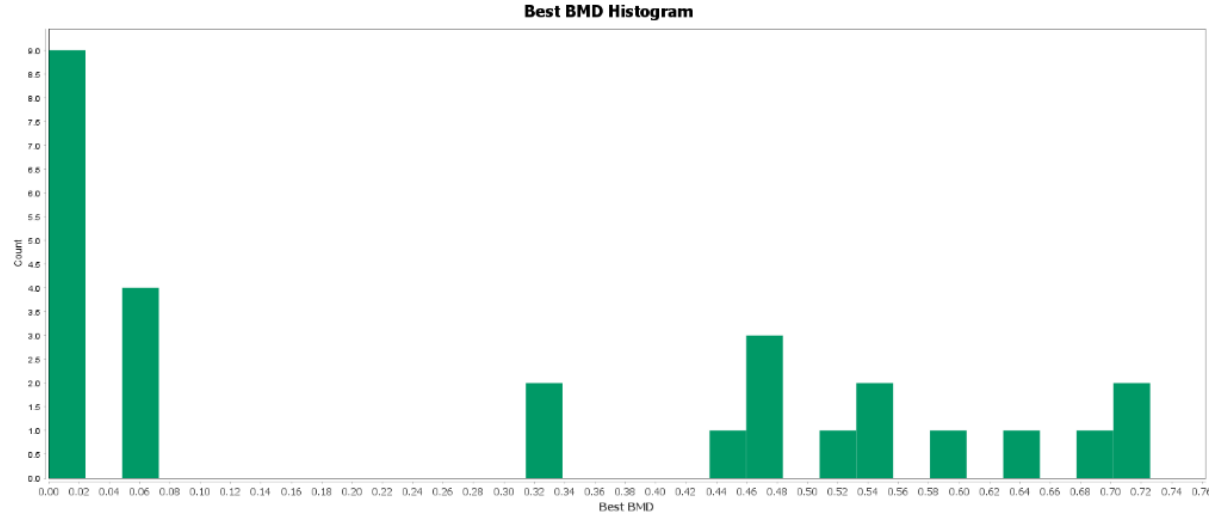




Fluoxetine (25-60 DEGs)

Log 10 scale, 1-1.5 log variability

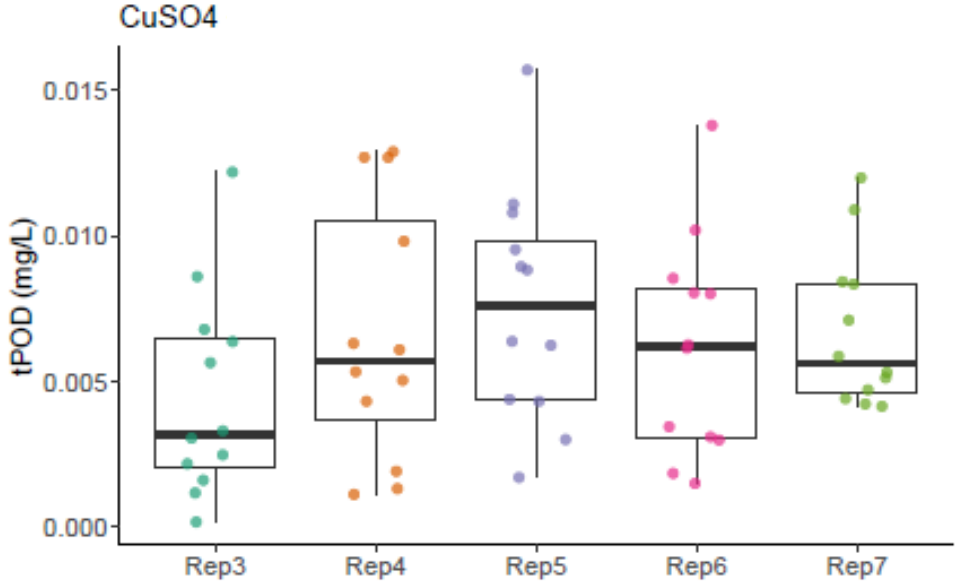




tPOD Variability

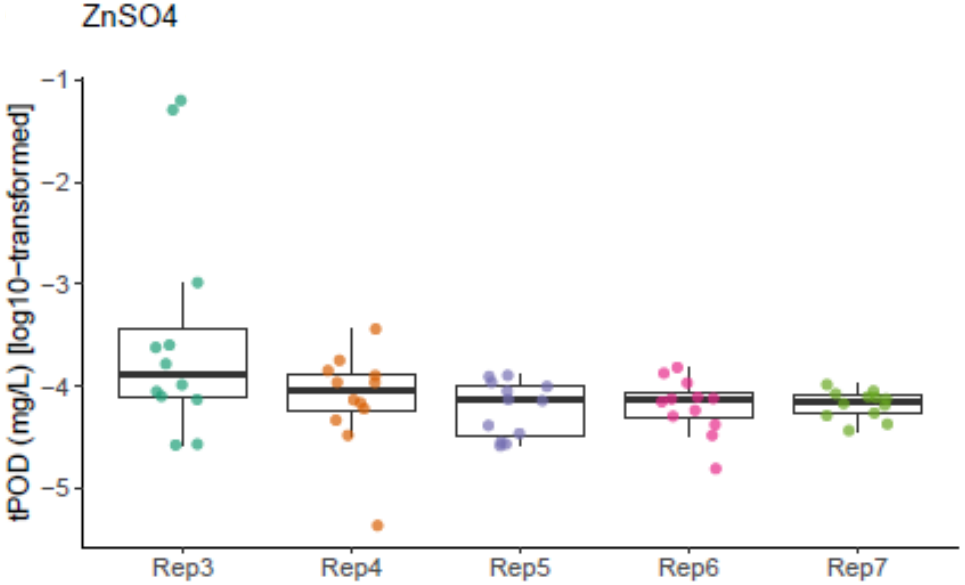
Copper sulfate (100-150 DEGs)

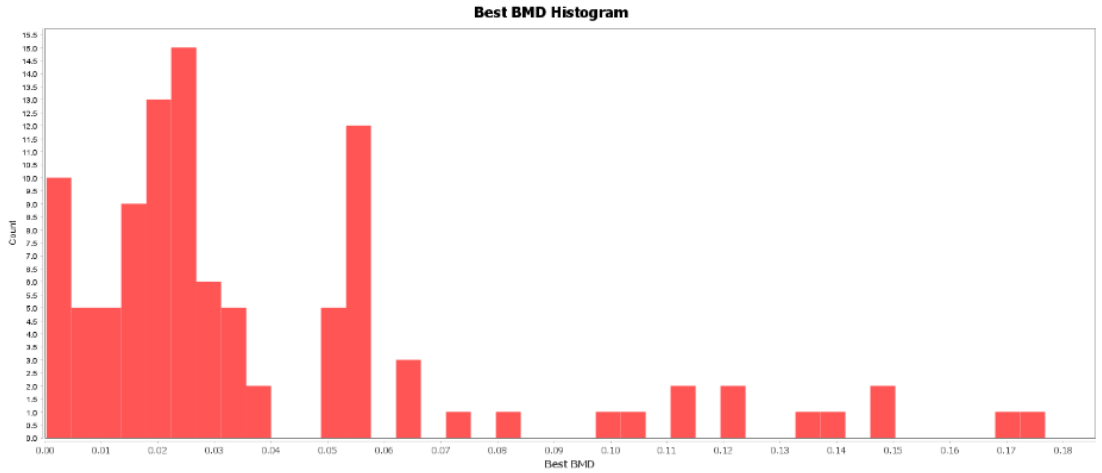
Linear scale, 3-4 fold variability

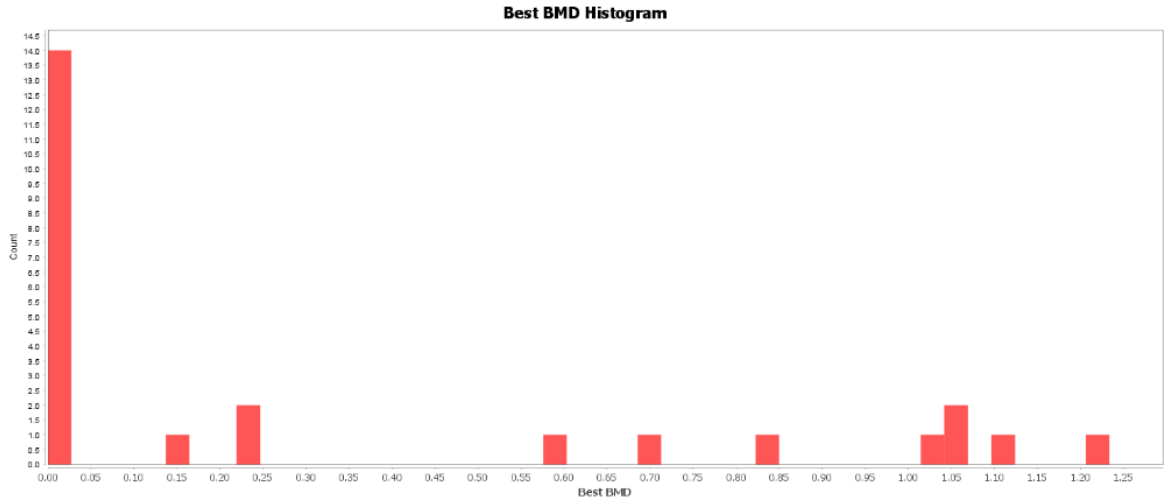


Zinc sulfate (20-60 DEGs)

Log 10 scale, ≈ 1 log ~~variability~~

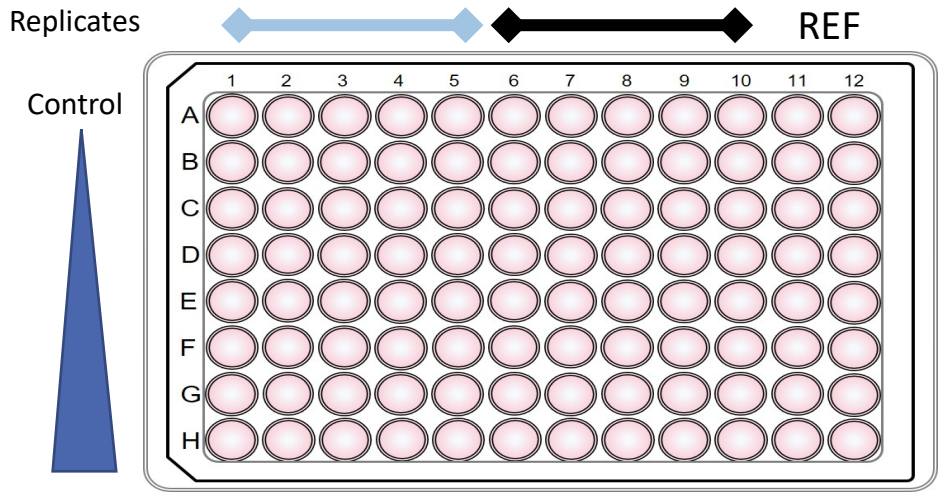






Assay optimization – tentative conclusions

- Minimum gene set size ≈ 10,000
- Minimum biological replication n=4; include n=5 to allow in silico sub-sampling
- Minimum number of DEGs **[TBD]**
- BMD distribution **[TBD]**

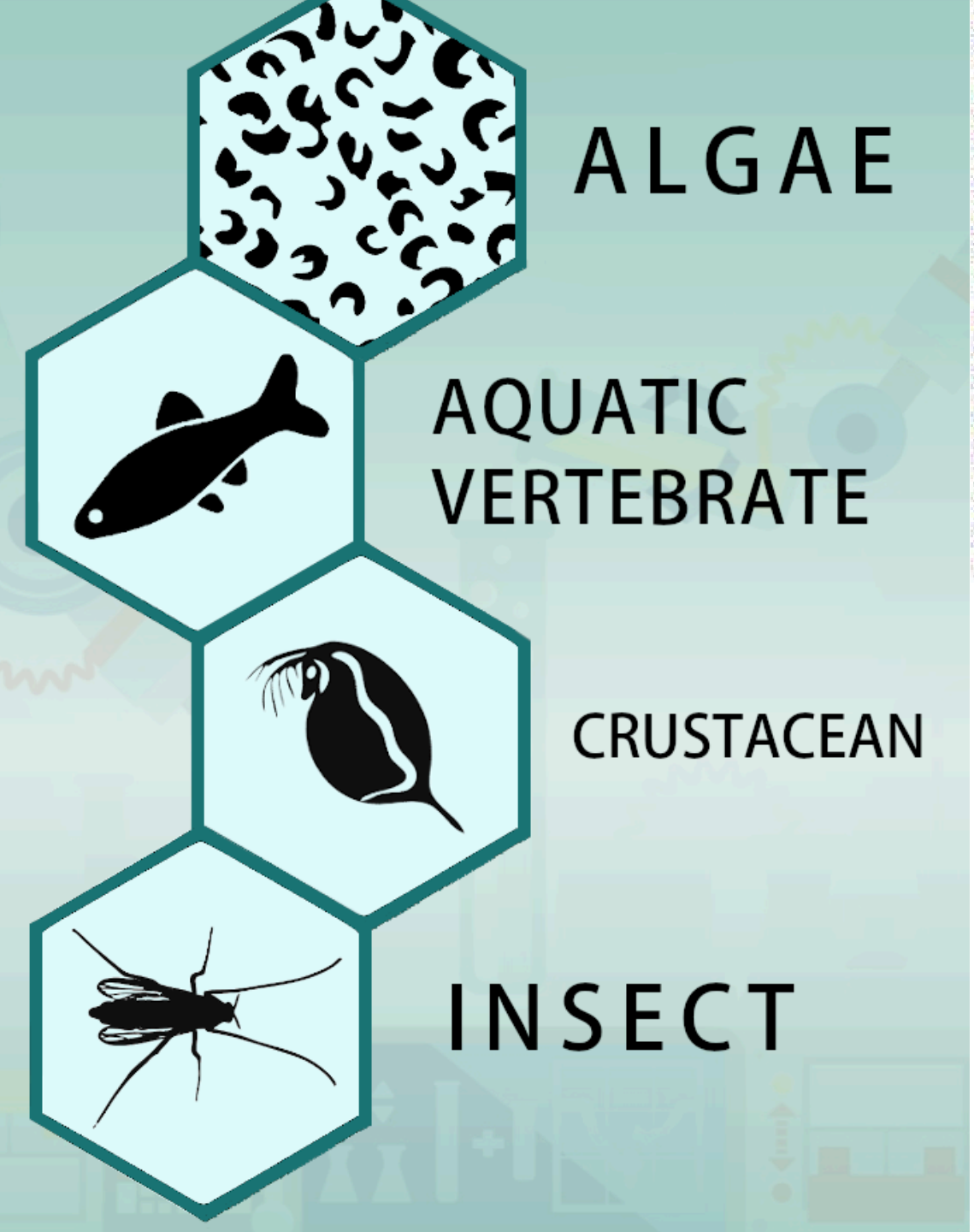


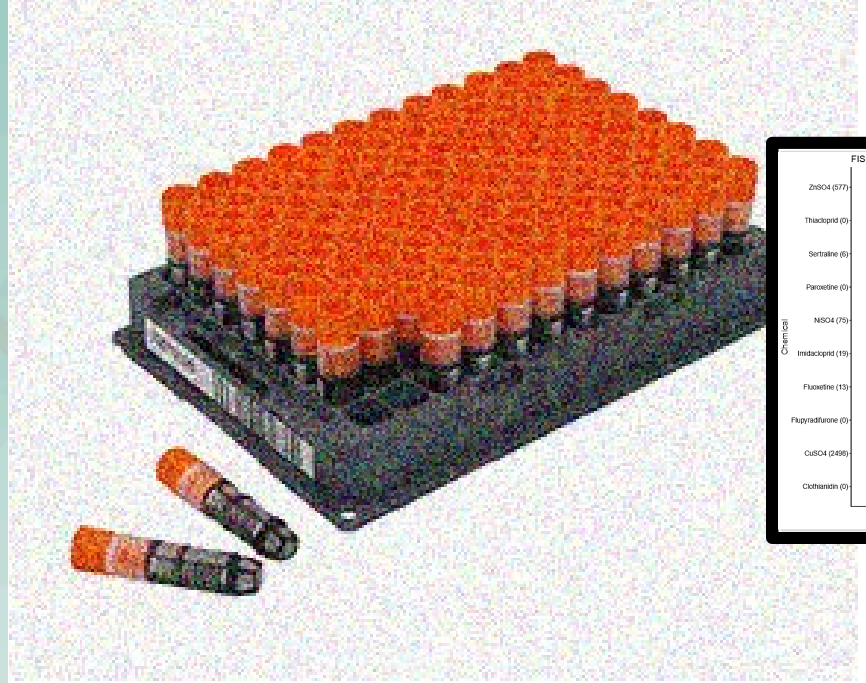
Conceptual illustration – actual layout will be randomized

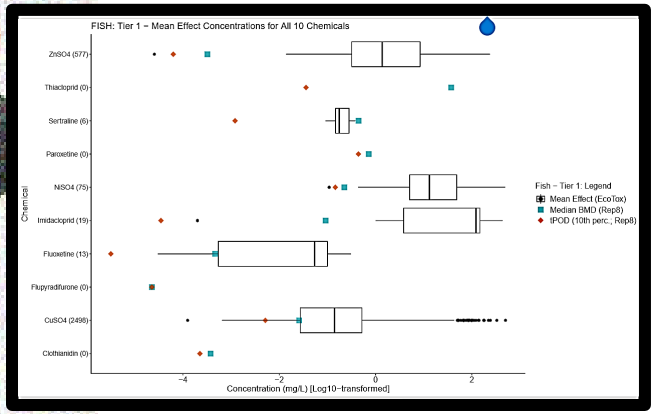
<u>Revised Design</u>:

- n= 5 biological replicates
- n= 8 concentrations
- Reference samples included on each plate

- Currently testing with 3 other species





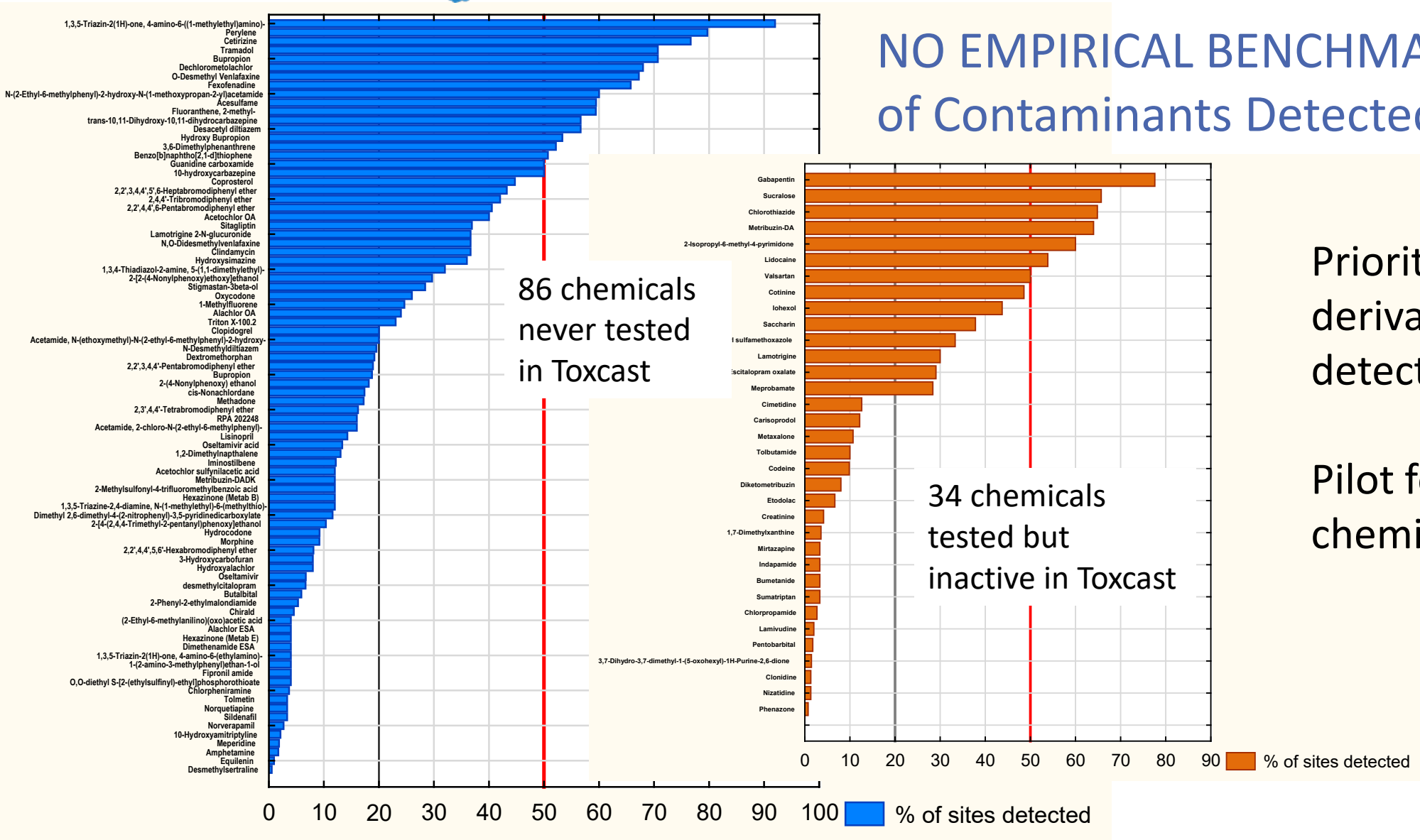


Expand the tPOD to traditional Ecotox benchmark comparisons:

- 50ish chemicals
- Four species
- Testing model prediction of free chemical concentration in plate
- Reduced cost (optimized design; lower cost sequencing)

10 years monitoring emerging contaminants in the Great Lakes

NO EMPIRICAL BENCHMARKS for ≈30% of Contaminants Detected in Water



Prioritize for tPOD derivation based on detection frequencies

Pilot for n≈12 chemicals

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The authors have no conflicts of interest to declare.

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