Development and evaluation of ecological high throughput transcriptomics as a new approach methodology for chronic ecological safety evaluations

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Conventional regulatory approach to chemical safety assessment

Majority are some variation of expose an animal, observe apical effects.

Guideline tests for ecotoxicity alone

>80 for human health

23 Nov 2004 Test No. 202: Daphnia sp. Acute Immobilisation Test OECD

04 Jul 2023 Test No. 218: Sediment-Water Chironomid Toxicity Using Spiked Sediment OECD

21 Jan 2000 Test No. 217: Soil Microorganisms: Carbon Transformation Test OECD

21 Jan 2000 Test No. 216: Soil Microorganisms: Nitrogen Transformation Tes OECD

21 Jan 2000 Test No. 215: Fish, Juvenile Growth Test OECD

21 Sept 1998 Test No. 214: Honeybees, Acut OECD

21 Sept 1998 Test No. 213: Honeybees, Acute OECD

21 Sept 1998 Test No. 212: Fish, Short-term Toxicity Test OECD

04 Apr 1984 Test No. 207: Earthworm, Acute Toxicity Te OECD

04 Apr 1984 Test No. 206: Avian Reproduction Test OECD

04 Apr 1984 Test No. 205: Avian Dietary Toxicity Test OECD

04 Apr 1984 Test No. 204: Fish, Prolonged Toxicity Test: 14-Day Study OECD

26 Jul 2013 Test No. 210: Fish, Early-life Stage Toxicity Test OECD

26 Jul 2013 Test No. 237: Honey Bee (Apis Mellifera) OECD

26 Jul 2013 Test No. 236: Fish Embryo Acute Toxicity OECD

02 Oct 2012 Test No. 229: Fish Short Term Reproducti

: Daphnia magna Reproducti

n Test

Cycle Toxicity Test Using Spike

bition Test (Carbon and Ammonium Oxidatio

OECD

t No. 230: 21-day Fish Assay

225: Sediment-Water Lumbriculus Tox

224: Determination of the Inhibition of the Activity of Anaerobic Bacteria

mary of Considerations in the Report from the OECD Expert Group on Ecotoxicology OECD

17 Aug 2006 Test No. 227: Terrestrial Plant Test: Vegetative Vigour Test OECD

17 Aug 2006 Test No. 208: Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test OECD

11 Jul 2006 Test No. 221: Lemna sp. Growth Inhibition Test OECD

OECD

249: Fish Cell Line Acute Toxicity - The RTgill-W1 cell line assay

250: EASZY assay - Detection of Endocrine Active Substances, acting through estrogen , using transgenic tg(cyp19a1b:GFP) Zebrafish embryos

248: Xenopus Eleutheroembryonic Thyroid Assay (XETA)

203: Fish, Acute Toxicity Test

247: Bumblebee, Acute Oral Toxicity Test OECD

09 Oct 2017 Test No. 246: Bumblebee, Acute Contact Toxicity Test OECD

245: Honey Bee (Apis Mellifera L.), Chronic Oral Toxicity Test (10-Day Feeding)

244: Protozoan Activated Sludge Inhibition Test OECD

29 Jul 2016 Test No. 232: Collembolan Reproduction Test in Soil OECD

29 Jul 2016 Test No. 228: Determination of Developmental Toxicity to Dipteran Dung Flies(Scathophaga stercoraria L. (Scathophagidae), Musca autumnalis De Geer (Muscidae)) OECD

29 Jul 2016 Test No. 226: Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil OECD

29 Jul 2016 Test No. 223: Avian Acute Oral Toxicity Test OECD

29 Jul 2016 Test No. 222: Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei) OECD

29 Jul 2016 Test No. 220: Enchytraeid Reproduction Test OECD

29 Jul 2016 Test No. 243: Lymnaea stagnalis Reproduction Test OECD

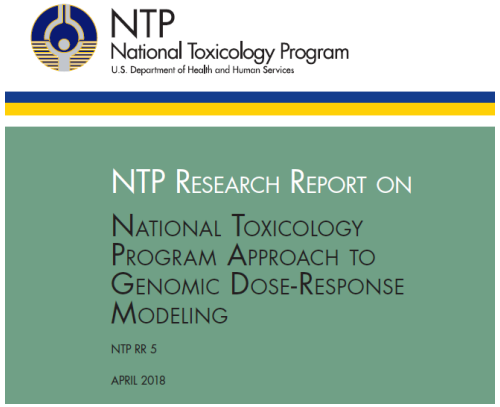
29 Jul 2016 Test No. 242: Potamopyrgus antipodarum Reproduction Test OECD

28 Jul 2015 Test No. 241: The Larval Amphibian Growth and Development Assay (LAGDA) OECD

Transcriptomics Approach

5 day rodent test

Whole transcriptome



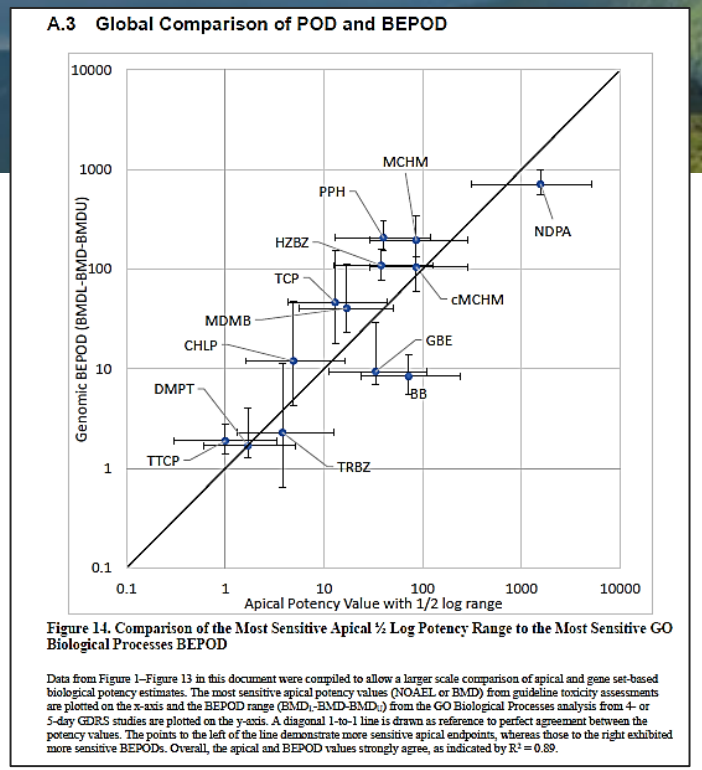
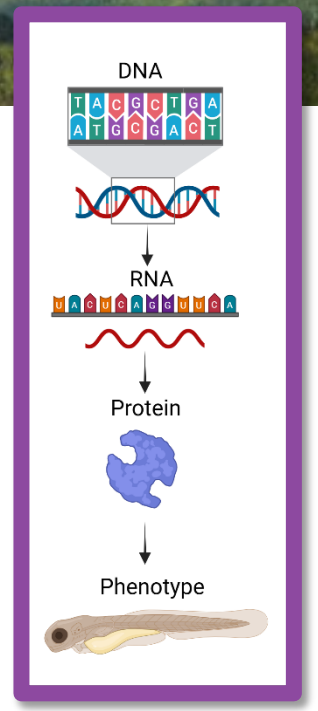
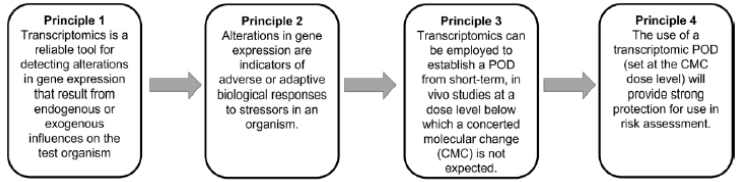


Figure 14. Comparison of the Most Sensitive Apical ½ Log Potency Range to the Most Sensitive GO Biological Processes BEPOD

Data from Figure 1–Figure 13 in this document were compiled to allow a larger scale comparison of apical and gene set-based biological potency estimates. The most sensitive apical potency values (NOAEL or BMD) from guideline toxicity assessments are plotted on the x-axis and the BEPOD range (BMD_L-BMD-BMD_U) from the GO Biological Processes analysis from 4- or 5-day GDRS studies are plotted on the y-axis. A diagonal 1-to-1 line is drawn as reference to perfect agreement between the potency values. The points to the left of the line demonstrate more sensitive apical endpoints, whereas those to the right exhibited more sensitive BEPODs. Overall, the apical and BEPOD values strongly agree, as indicated by $R^2 = 0.89$.



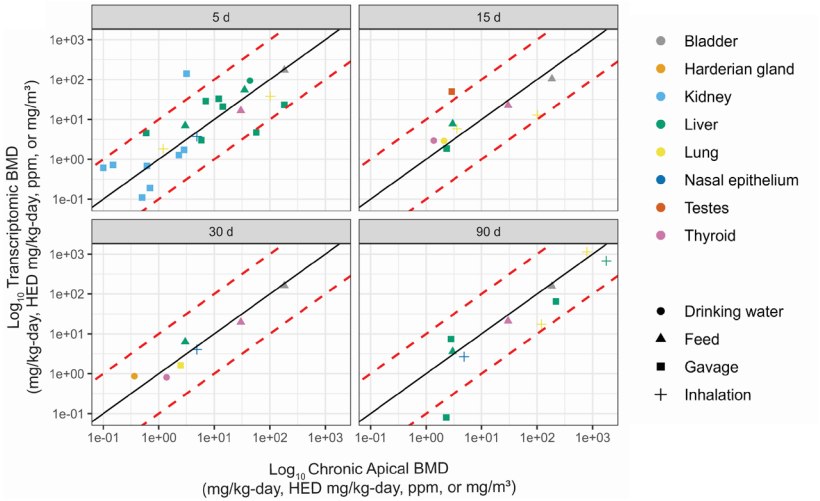


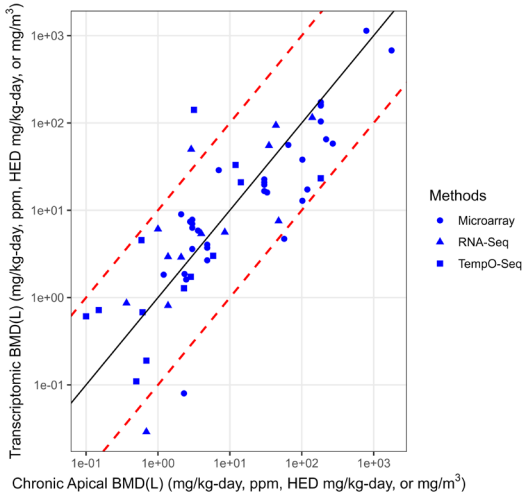
Concept:

- Exposure to stressors alters gene expression
- Δ gene expression not necessarily adverse (e.g., adaptive responses)
- If no concerted change in gene expression, unlikely to be hazardous, even over much longer exposure durations.

Johnson KJ, Auerbach SS, Stevens T, Barton-Maclaren TS, Costa E, Currie RA, Dalmas Wilk D, Haq S, Rager JE, Reardon AJF, Wehmas L, Williams A, O'Brien J, Yauk C, LaRocca JL, Pettit S. A Transformative Vision for an Omics-Based Regulatory Chemical Testing Paradigm. Toxicol Sci. 2022 Sep 27:kfac097. doi: 10.1093/toxsci/kfac097.

Scientific Support Document: Comprehensive literature review supports dose concordance between disruption of gene activity and toxicity





- Literature review identified 140 chemicals in 32 studies
- The transcriptomic BMD was highly correlated with the chronic, apical BMD
- The concordance RMSD (0.561) is similar to the range of inter-study standard deviation estimates for the lowest observable adverse effect levels (LOAELs) for systemic toxicity in repeated dose studies

  (Pham *et al. Comp Toxicol.*, 2020)
- The concordance was robust across exposure durations, exposure routes, species, sex, target tissues, physical chemical properties, toxicokinetic half-lives, and technology platforms

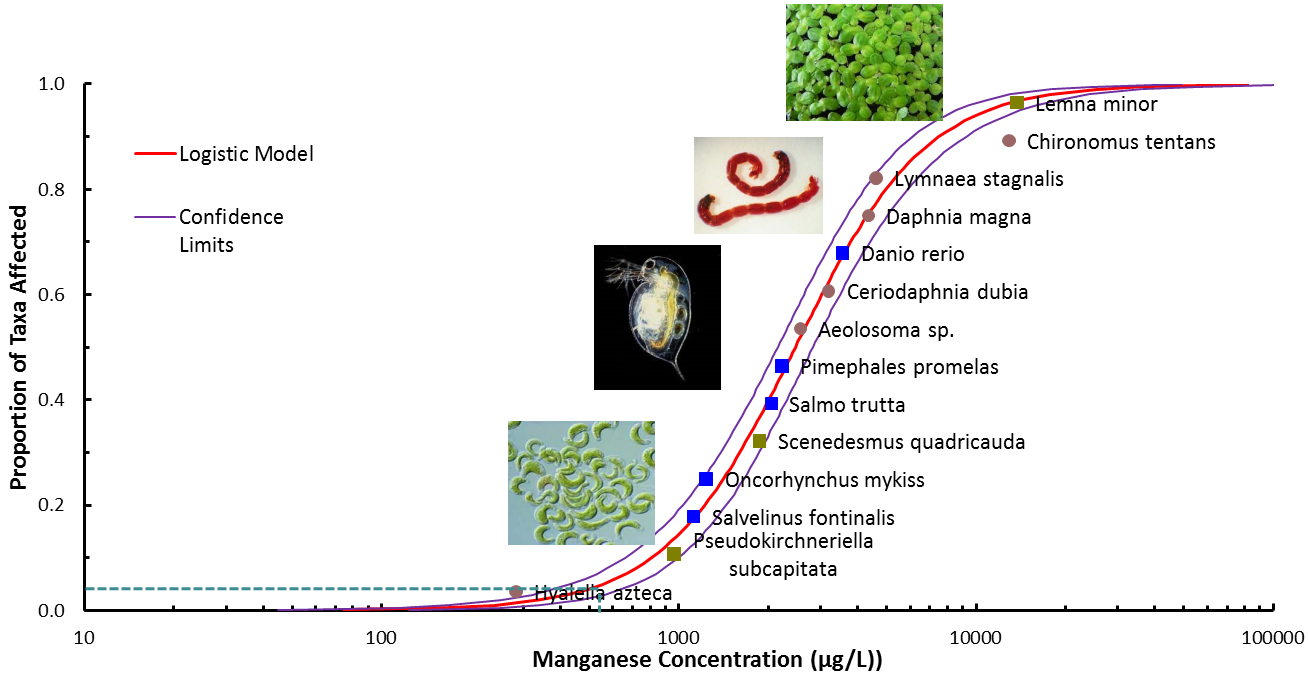
Slide courtesy of Alison Harrill

https://www.epa.gov/bosc/epa-transcriptomic-assessment-products-etap-panel

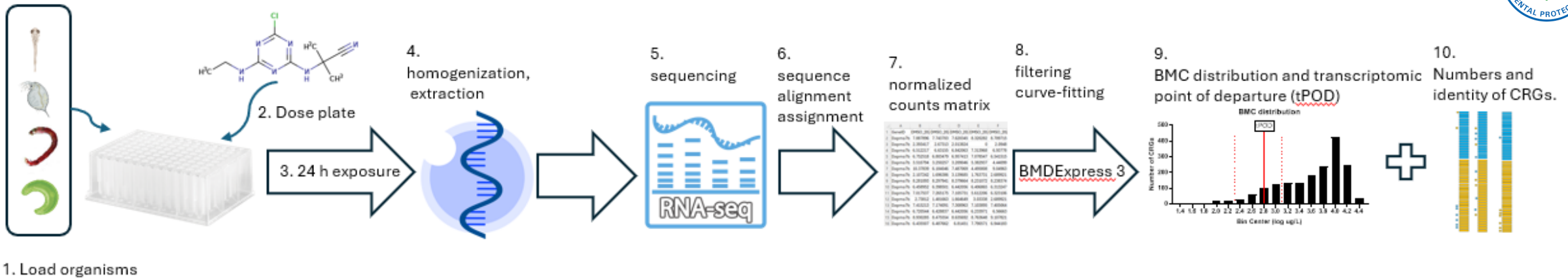
What about Eco?

- Ecological risk assessments face a similar need to estimate exposure concentrations that would not represent appreciable risk of adverse effects following chronic exposure.

- Additional challenge of protecting taxonomically diverse species.



Eco-HTTr: Generalized workflow



- Small organisms exposed in 96 well plate format
  - Genomically, physiologically, trophically diverse
  - Globally harmonized system for classification and labeling of ecological hazard
- 96 well plates
  - Accommodates large array of concentrations x replicates
  - Laboratory automation
  - Standardized format/exposure conditions – repeatability / comparability
  - Chemical masses, extract volumes required are small
- Rapid (24 h), avoids need to feed, avoids need to solution renewals
- Phenotypic endpoints can be assessed

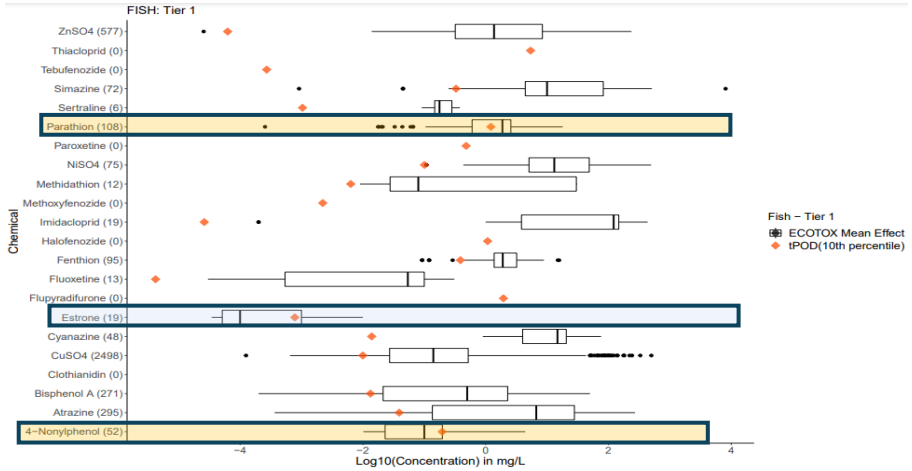
tPOD Utility:

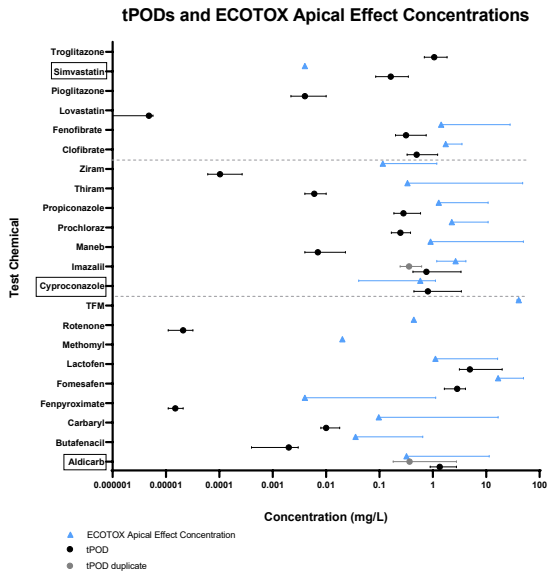
- Identify a concentration below which gene expression is not markedly affected.
- "Safe level" ≈ predicted no effect concentration ($PNEC_{chronic}$)

Gene Expression profile Utility

- Grouping: Insights into relationships between structure and function
- Read-across: Inference based on similarity to better studied chemicals
- Mode of action inference

Comparing tPODs to apical PODs





Protective relative to adverse effect ≈85-95% of time when correct for free chemical in well.

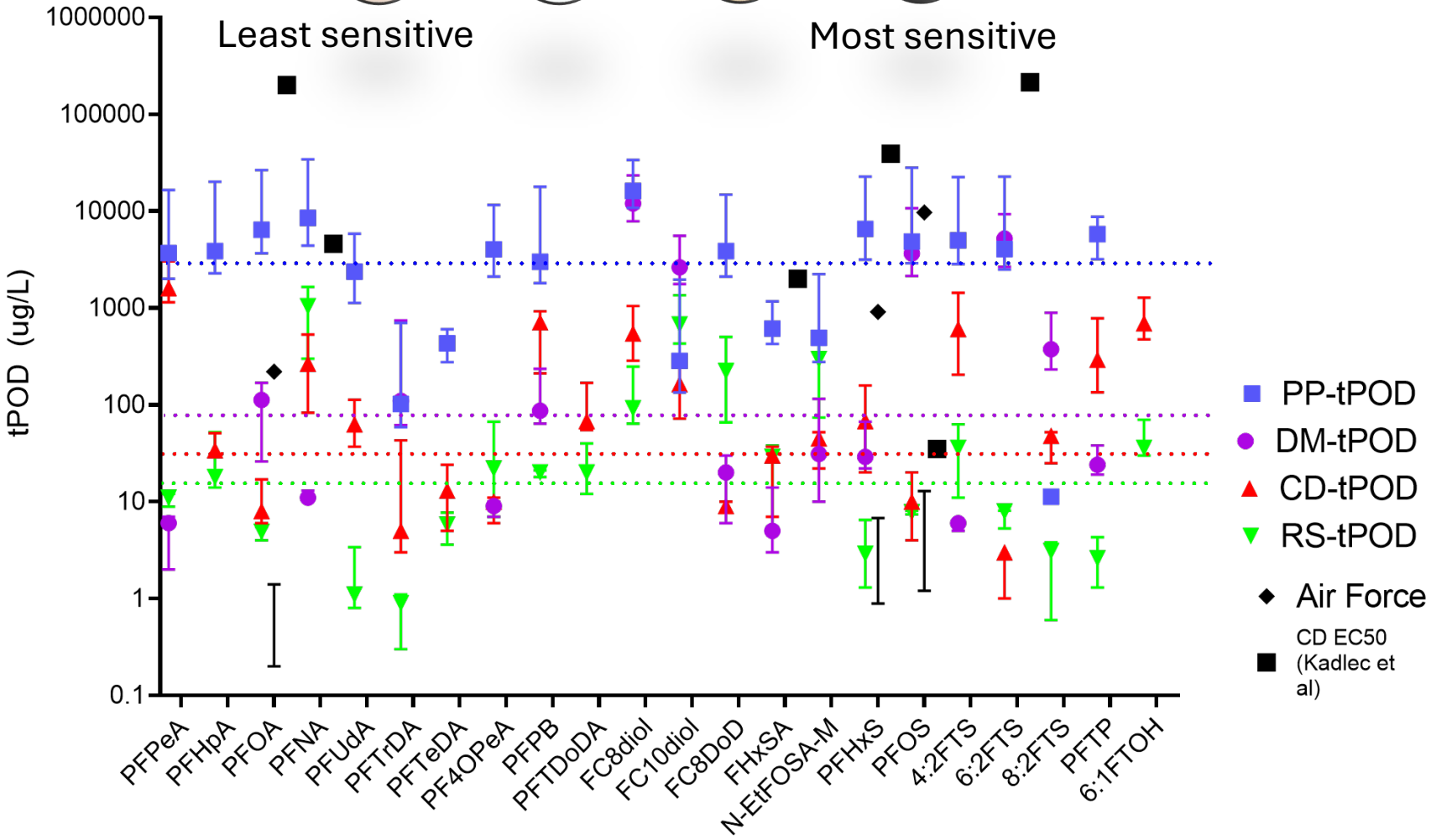
- Survival
- Growth
- Reproduction

tPODs tend to overlap with sub-apical effect concentrations (not overly conservative)

- Gene expression
- Hormone concentrations
- Behavior
- Biomarkers
- Histology ….

Villeneuve, D. L. et al., 2023. doi: 10.1016/j.crtox.2022.100099.
Flynn et al. 2024. doi: 10.1007/s00244-024-01064-y.
Baettig et al. 2026. doi: 10.1016/j.aquatox.2025.107650.

There are taxonomic differences in sensitivity to different chemical classes



Single taxa is not protective of all.

Human cell lines are not protective of ecological taxa.

Most sensitive taxa varies by chemical class.

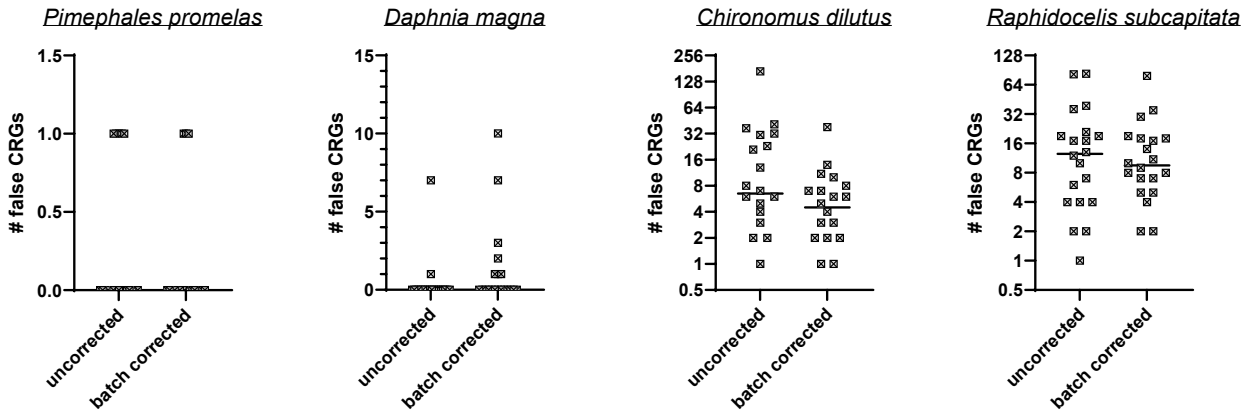
How many taxa = enough representation

Villeneuve et al. 2025a - doi: 10.1002/etc.5825
Flynn et al. 2025a - doi: 10.1093/etojnl/vgaf022

Villeneuve et al. 2025b - doi: 10.1002/etc.5838
Flynn et al. 2026 (in review)

Evaluating Assay Performance – False discovery

Number of concentration-responsive genes (CRGs) detected for controls assigned to “mock treatments”

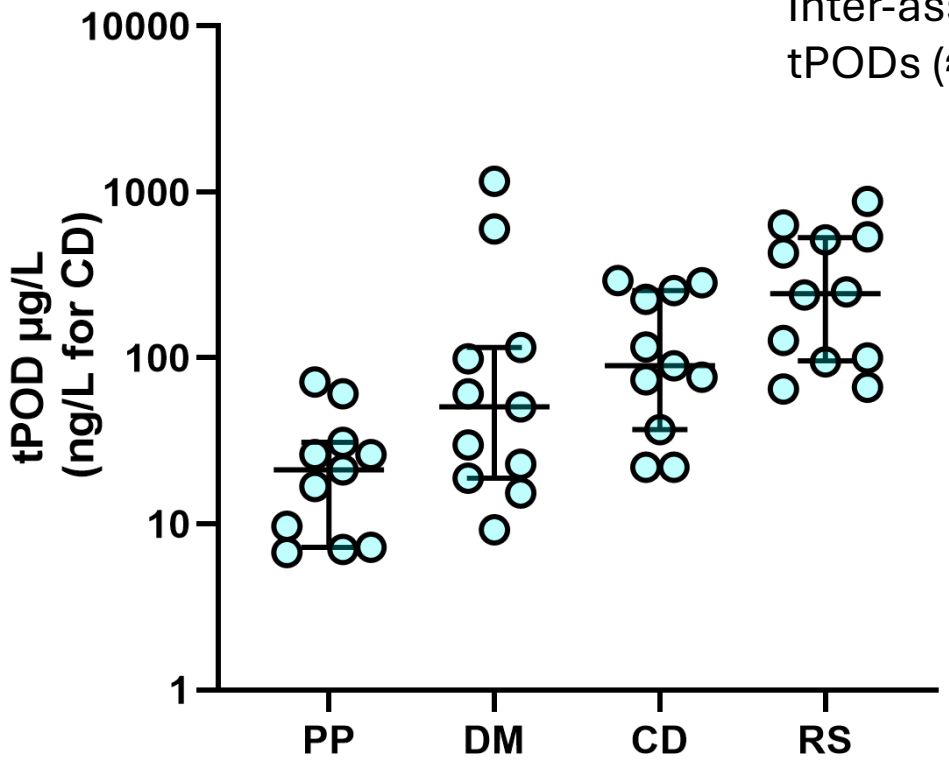


Critical for distinguishing a “positive” from “negative” response in the assays

	Targeted sequencing of 1800-2600 gene features				Whole transcriptome sequencing > 10,000 gene features			
	un-corrected	batch corrected	un-corrected	batch corrected	un-corrected	batch corrected	un-corrected	batch corrected
Taxa	PP	PP	DM	DM	CD	CD	RS	RS
Median	0	0	0	0	7	5	13	10
90th Percentile	1	1	0.9	7	41	14	78	35
Std. Error of Mean	0.1	0.09	0.4	0.6	8	2	5	4
90th P / total features[a]	0.06%	0.06%	0.05%	0.4%	0.3%	0.1%	0.8%	0.3%

<1% of features

Evaluating Assay Performance – tPOD reproducibility



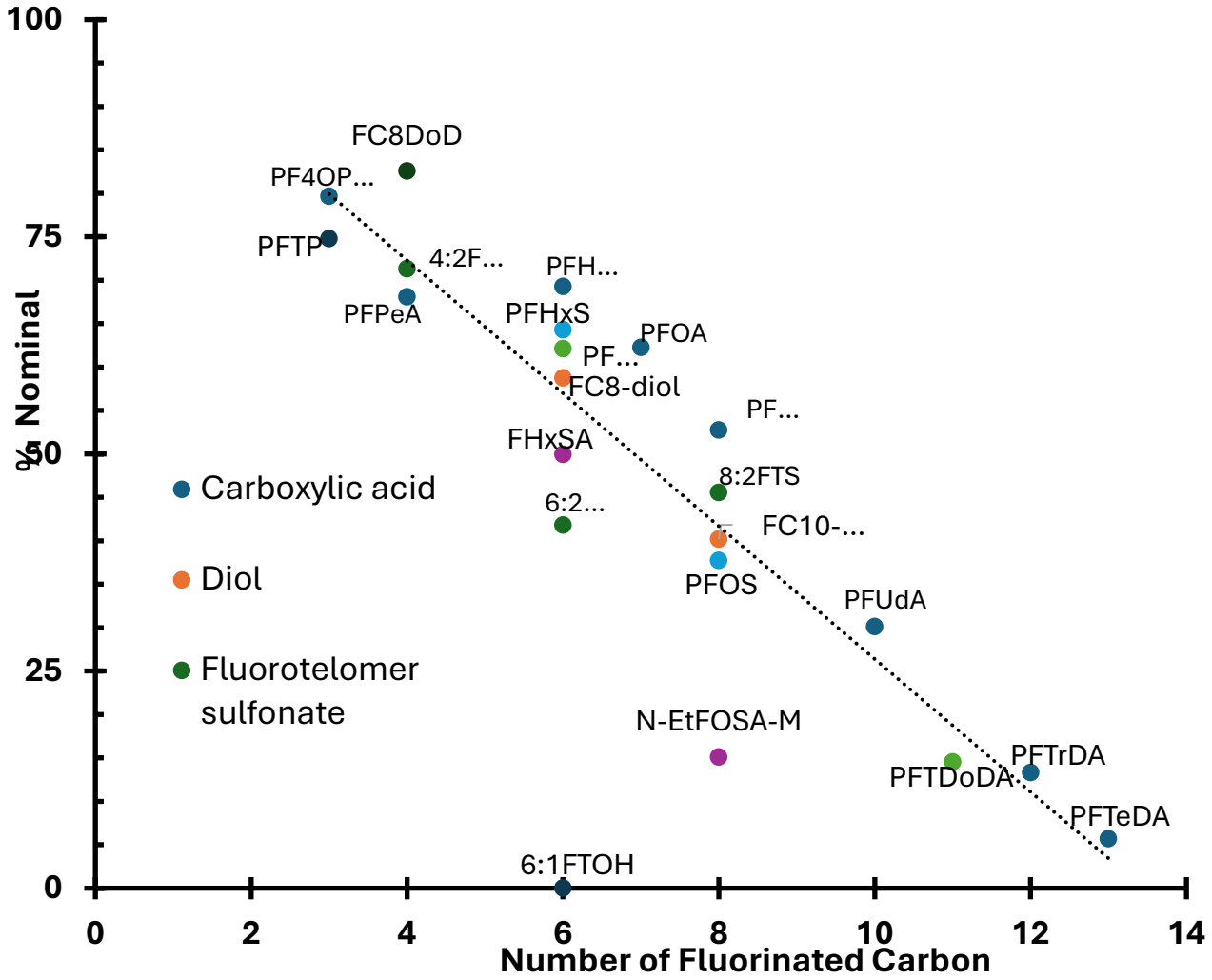
Inter-assay variability of tPODs (≈ factor of 12)

Same chemical tested 11-12 times with the same species (different cohorts)

Define fit-for-purpose for different applications requiring differing levels of precision

Help to define acceptance criteria for the assays

Exposure verification



Villeneuve et al. 2025a - doi: 10.1002/etc.5825

Have observed multiple cases where a lack of exposure verification could lead to erroneous conclusions.

Measured concentration <<< nominal

- Underestimate of potency

Contaminant(s) in stock chemical

- Incorrect attribution of cause

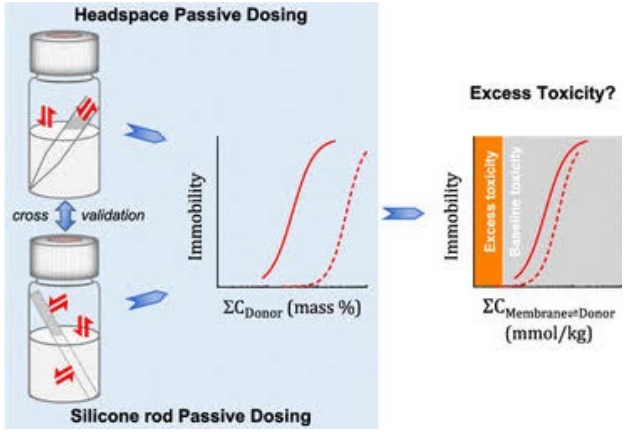
Reactivity (e.g., hydrolysis, unstable in DMSO)

- Unintended mixture testing

On-going R&D:
Adaptations for difficult-to-test (DTT) substances

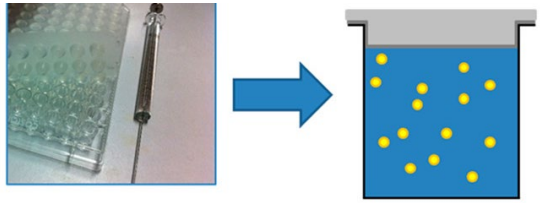
High log K_{ow} (volatile)

High log K_{ow} (non-volatile)



Troc et al. 2021; https://doi.org/10.1021/acs.est.1c00343

Low log K_{ow} (volatile)



Closed System Without Headspace

Stalter et al. 2013; https://doi.org/10.1021/tx400263h

Reactive (non-volatile)

Analytical characterization, potentially including non-targeted analysis.

On-going R&D: Mode of Action Inference

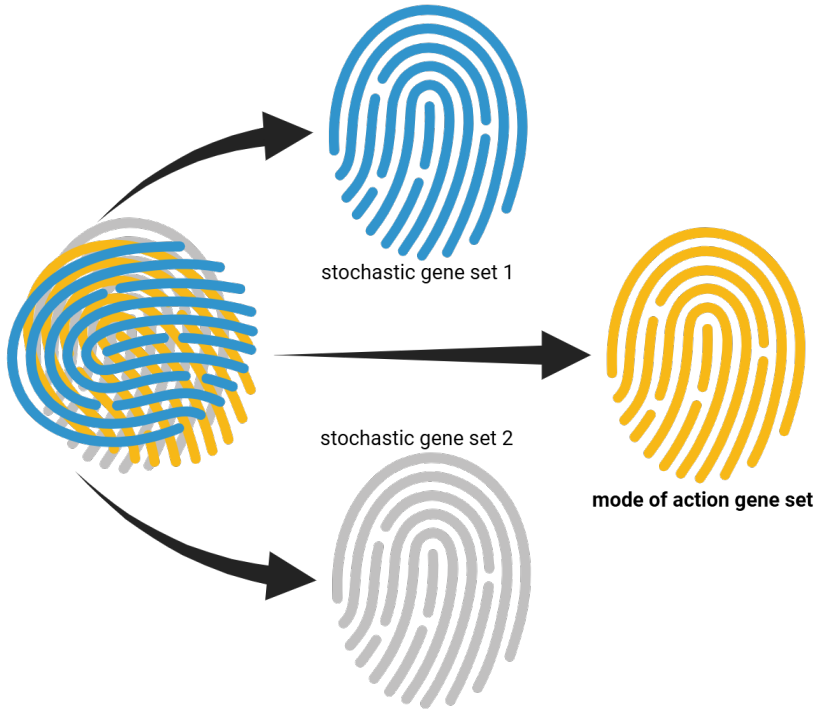
Interest in using gene expression profiles

1. Infer mode(s) of action and MIE's in relevant AOPs
2. Biologically-based read-across to chemicals causing specific effects
3. Attribution of mixture effects to certain chemical classes/groups

Results to date suggest this may challenging:

- Considerable variability/heterogeneity in the identity of CRGs and DEGs in repeated assays (despite fairly stable tPOD) and chemicals with common MoA.

  11-12 repeated assays for the same chemical
  - >65% of CRGs occur in only one trial
  - Nonetheless, a small fraction (e.g., 5-9% of CRGs) detected in all trials



On-going R&D: Considerations for use in specific programmatic contexts

Pesticide risk assessments under FIFRA

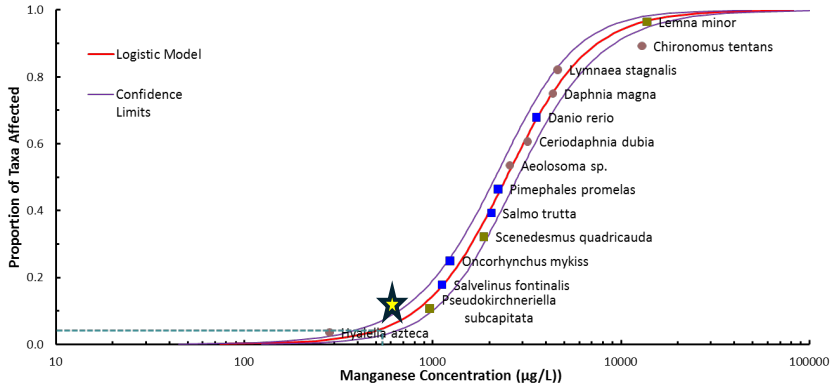
Hypothesis: Minimum tPOD across 4 taxa will yield POD ≤ pesticide benchmarks derived from traditional approaches.

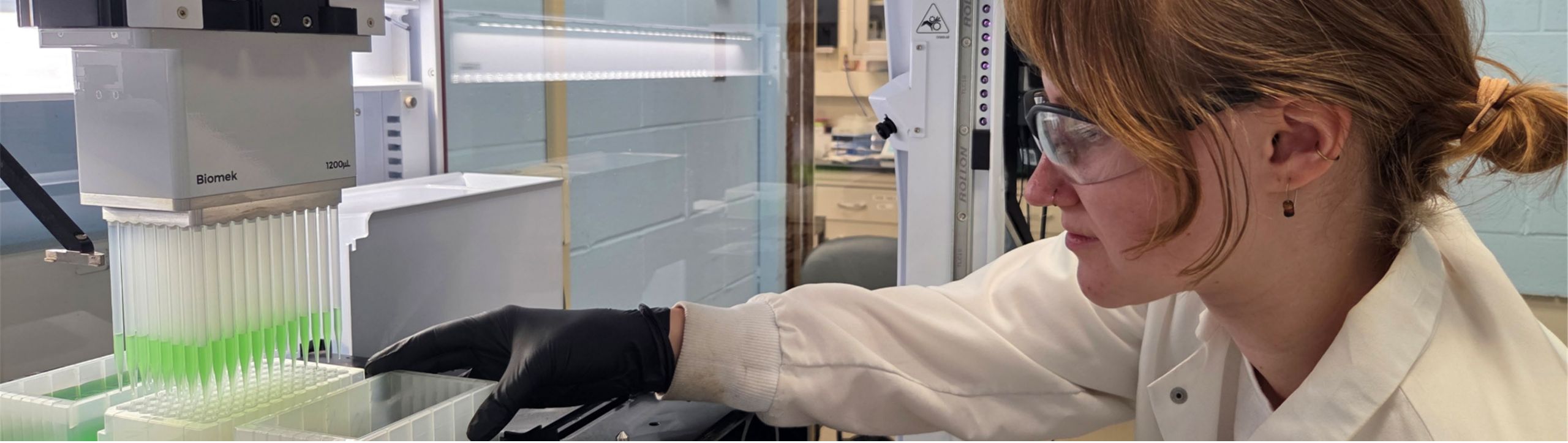
- OPP Aquatic Life Benchmark table
  - Tested 24 ai across four species
  - Includes mix of most potent to f.w. vertebrates, non-vascular plants, f.w. invertebrates
  - Bioassays complete and data in-hand
  - Exposure verification on archived samples in progress

Aquatic Life Criteria/Benchmarks under CWA

Hypothesis: Minimum tPOD across 4 taxa will yield POD ≤ species sensitivity distribution (SSD)-based aquatic life criteria or benchmarks (HC5).

- Provide insight into whether 4 taxa is enough





Conclusions:

- Strong interest in NAMs in both the regulatory and regulated community
- Eco-HTTr efforts to-date suggest tPODs are protective relative to apical adverse outcomes
- Key performance metrics to determine assay acceptance and fit-for-purpose determined
- Exposure verification and optimized strategies for challenging chemical classes are needed
- Mode-of-action inference & grouping will require distinguishing consistent vs stochastic responses
- More tailored analyses of fit-for-purpose and limitations for specific application domains are underway

Acknowledgments

- Kevin Flynn
- Dan Villenueve
- Jenna Cavallin
- Peter Schumann
- Brett Blackwell
- Emma Stacy
- Michelle Le
- John Hoang
- Kendra Bush
- Sara Daley
- Alex Kasperek
- Mackenzie Nash
- Kathy Jensen
- Heather Brown
- Ty Lahren
- Camille Baettig
- Jacob Collins
- Grace Casciano
- Monique Hazemi
- Justin Stewart

Performance x Experimental Design

- Sequenced an entire plate (n=96) controls for each test species.
- Assigned in silico to “mock treatments” to examine effect of different design strategies on FDR.
- Tested n=100 random assignments for each design.

Mean FDR (CRGs) for different rep/trt x concentration combinations

REPLICATES	CONCENTRATIONS 4	5	6	7	8	9	10	11	12
3	35	33	40	43	35	40	37	38	41
4					27				
5					16				
6					10				
7					9				
8	10	8	7	6	5	5	6	**5**	**5**
9					3				
10					2				
11					2				
12					2				

Pool 1

One organism per sample

REPLICATES	CONCENTRATIONS 4	5	6	7	8	9	10	11	12
3	8	7	8	7	**7**	8	7	6	8
4					5				
5					3	**2**			
6					2				
7					1				
8					1				
9					0.4				
10					0.4				
11					0.4				
12					0.4				

Pool 3

Three organisms pooled per sample

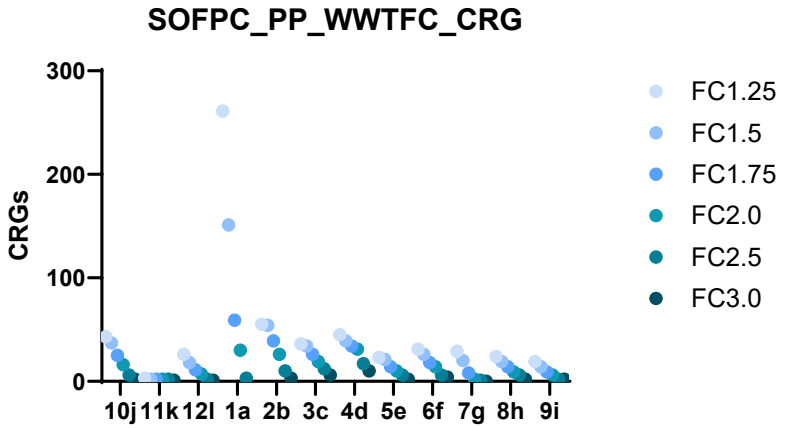
- Pooling (n=3/pool) reduces false discovery of CRGs by a factor of 5.
- Effective experimental design strategy for controlling FDR without inflating sequencing costs.

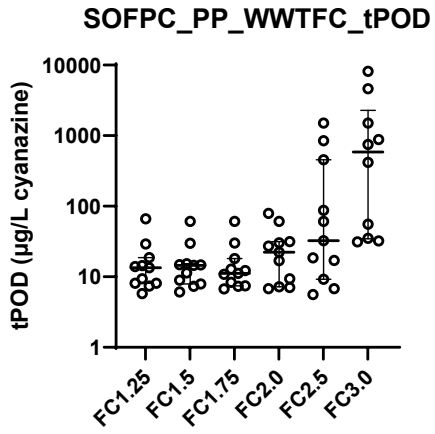
Performance x Analysis settings

- Stringency in defining differentially expressed/concentration responsive genes has to be balanced against inflating tPOD uncertainty due to sparse CRG distribution.

Fold-change cut-off

- Total CRGs and FDR decrease as fold-change cut-off is increased.
- FC≥2.5 leads to inflated tPOD variability due to sparse CRG distribution.





R squared cut off for best fit regression

- Total CRGs and FDR decrease as minimum Rsq is increased.
- Min Rsq 0.75 leads to inflated tPOD variability due to sparse CRG distributions.