

Evaluating the bioactivity of the ubiquitous tire preservative 6PPD and its quinone transformation product

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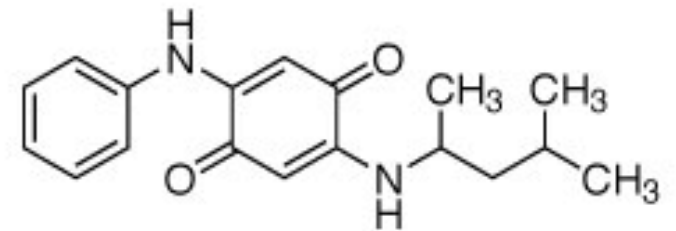
² US EPA, Biomolecular and Computational Toxicology Division

³ US EPA, Region 10, Laboratory and Applied Science Division

* The contents of this presentation neither constitute, nor necessarily reflect US EPA policy.

Following publication of Tian et al 2021 R10 contacted ORD to help generate additional data on 6PPD, 6PPD-Q

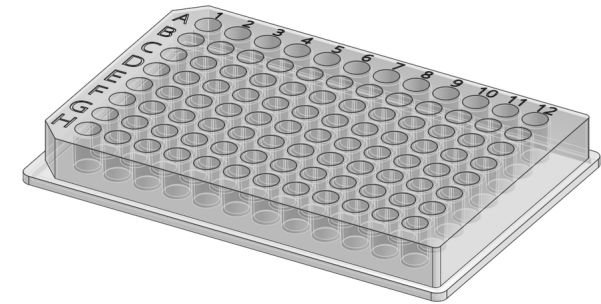
- Toxicity to coho salmon had been characterized (1 hour LC50 \approx 95 ng/L)
- Mode of action was unknown
- Understanding of mode of action is important to defining what other species would likely be susceptible.
- 6PPD-quinone and associated labeled analytical standards were not commercially available for several months
- Around May 2021 Region 10 laboratory was able to obtain 10 mg 6PPD-Q for testing
 - Not enough mass for large scale aquatic exposure studies



Regional Research Partnership Program (R2P2)

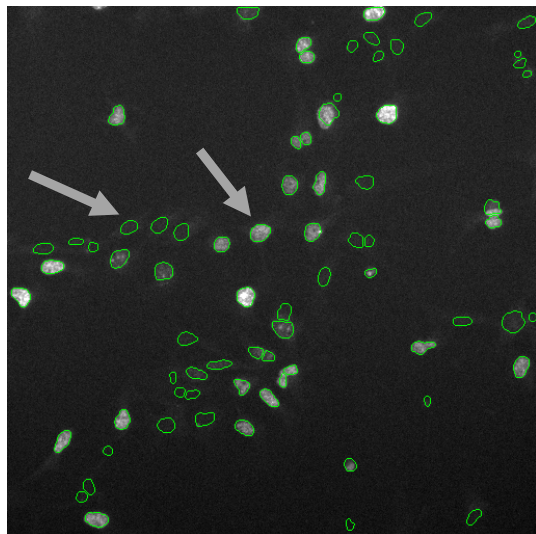
- Objective: Use CCTE's existing battery of alternative assays to generate additional hazard information.
- Hypothesized MoA: Neurotoxicity, mitochondrial uncoupling*
- Selected assays
 - Neurotox/DNT screen (Shafer)
 - Zebrafish embryo toxicity (Padilla)
 - Ecological high throughput transcriptomics (Villeneuve)

*Academic lab planning to run Agilent Seahorse assay

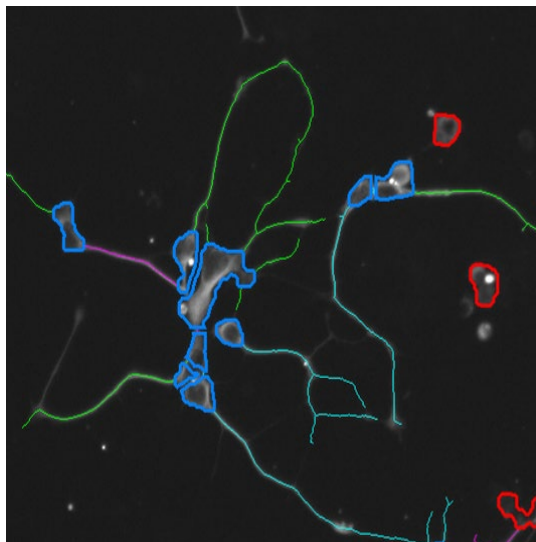


loss of equilibrium, gaping, pectoral fin splaying, death within 1-4 hours

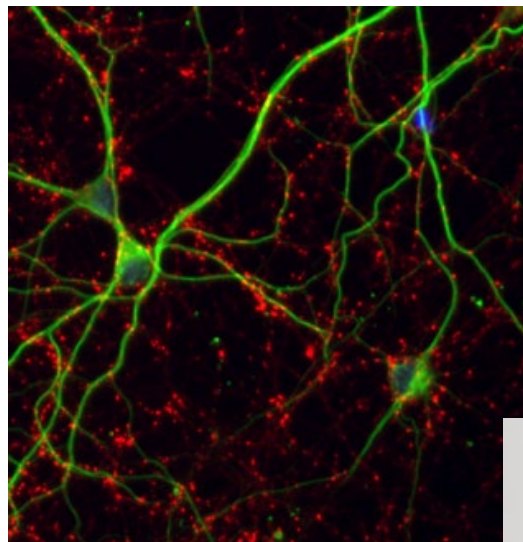
Proliferation



Neurite Outgrowth



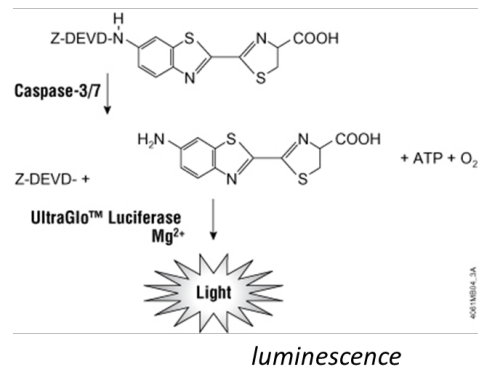
Synaptogenesis



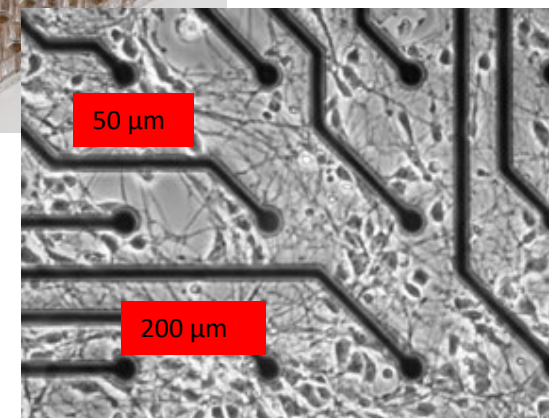
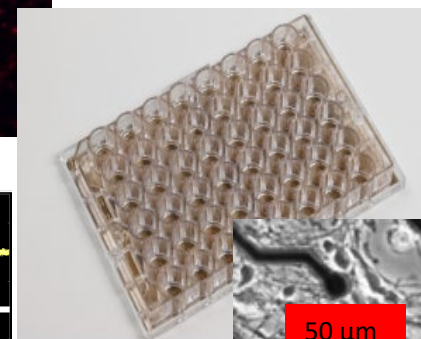
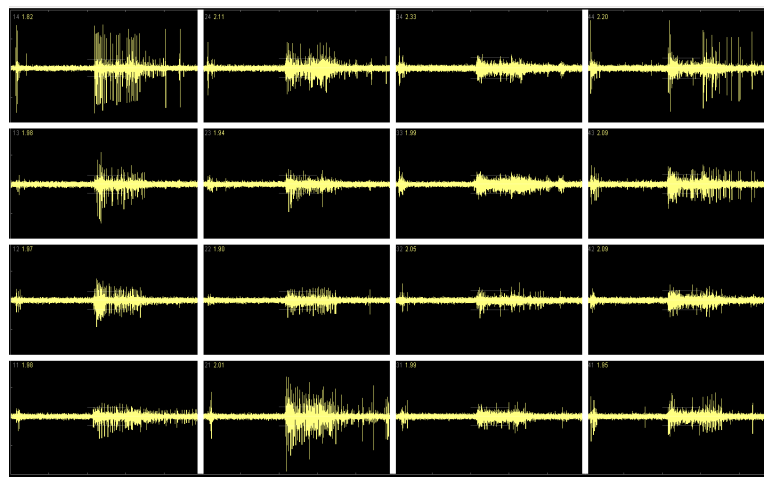
High content imaging

Micro-electrode arrays

Apoptosis

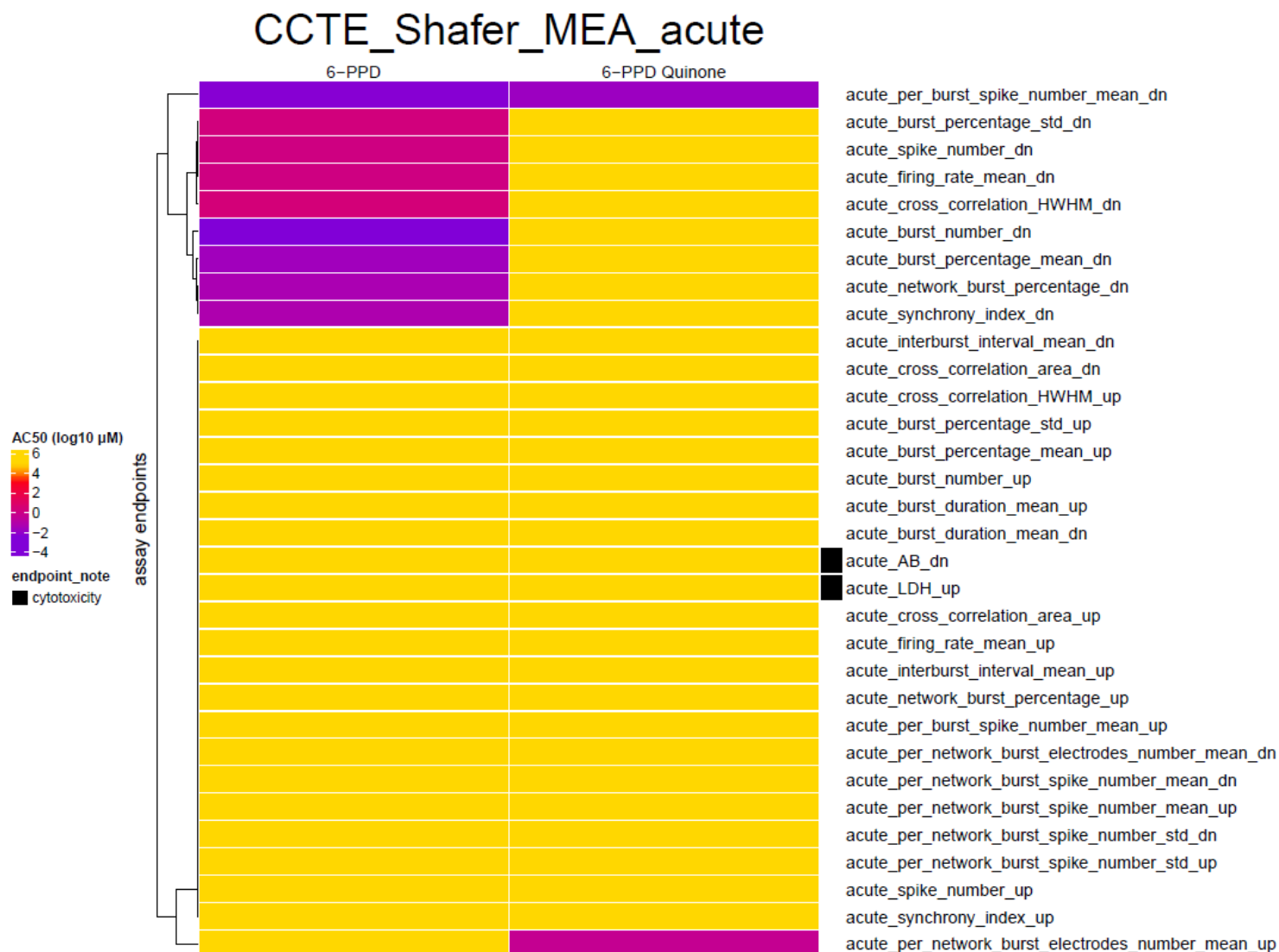


Network Function and Formation





Acute Neurotoxicity (microelectrode array acute assay)



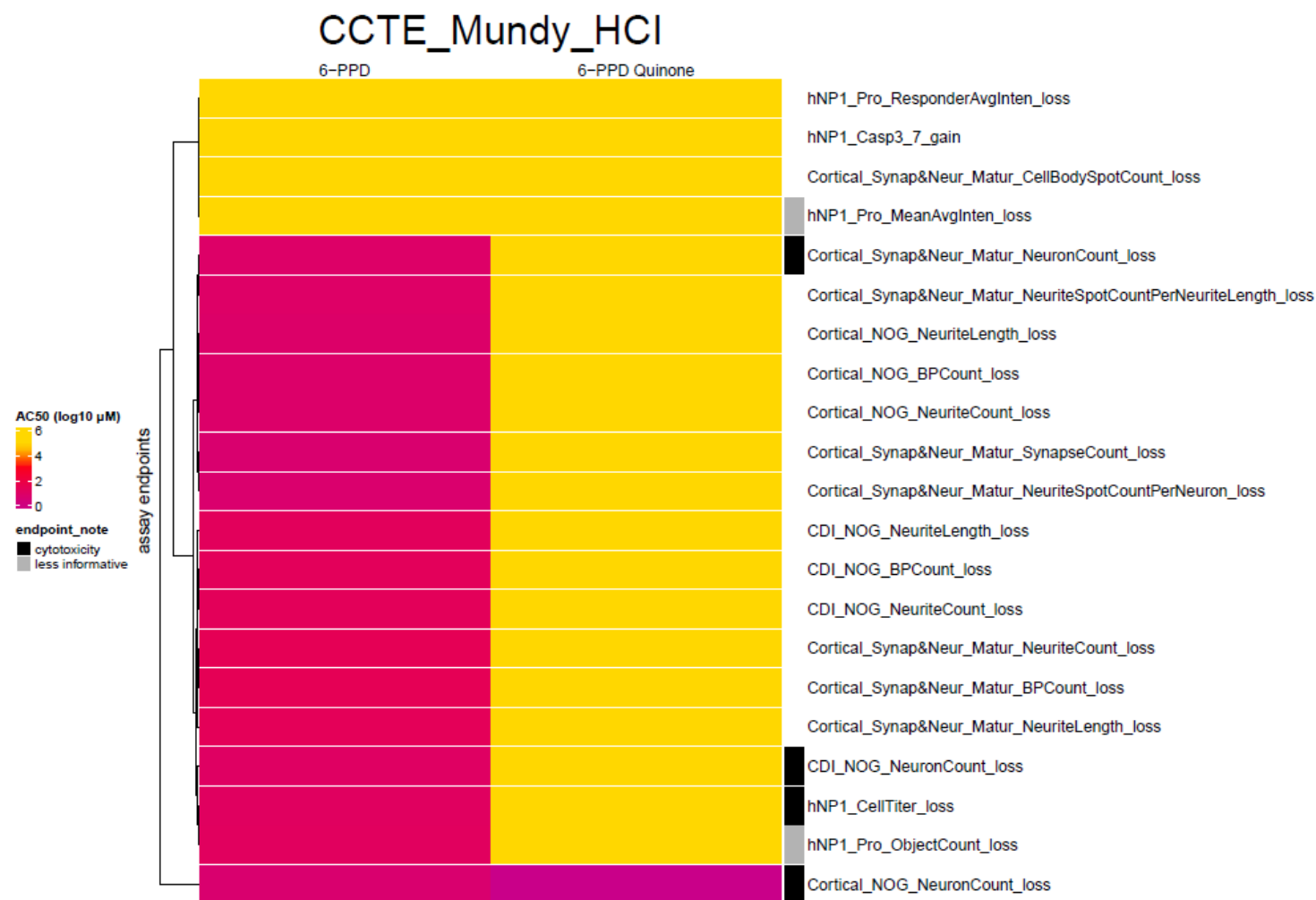
6PPD does elicit some responses associated with neurotoxicity – not highly potent

6PPD-Q – less active, and less potent as a neurotoxicant

Unlikely to be the driver of coho toxicity

This presentation does not reflect EPA Policy. Data are preliminary. Do not cite or quote.

Effects in High Content Imaging Assays



6PPD does elicit some DNT-linked responses, although modest potency

6PPD-Q – appears less active, and less potent as a developmental neurotoxicant

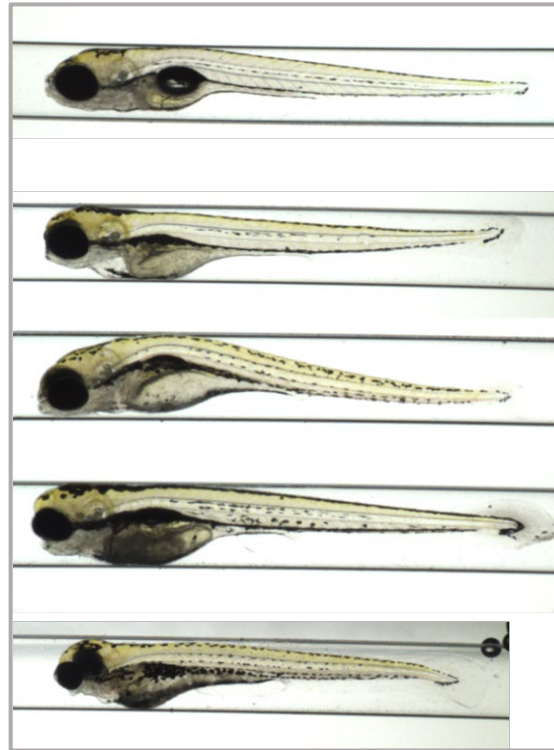
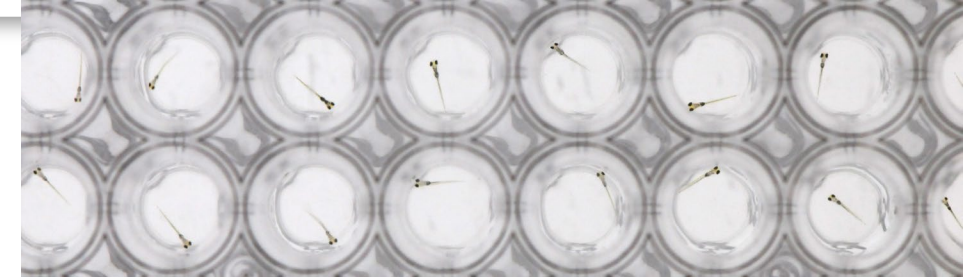
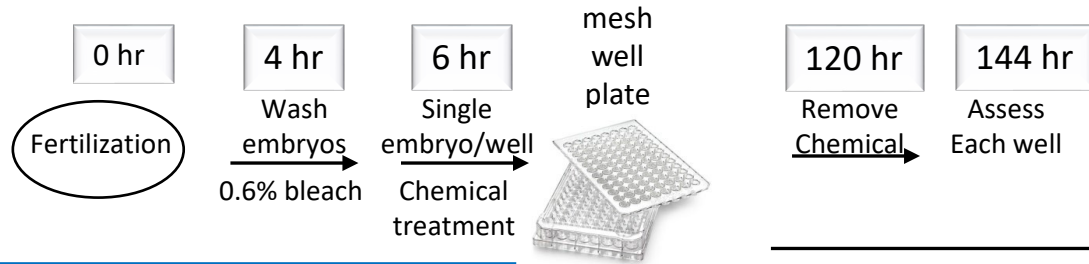
Zebrafish Embryo-larval Toxicity

- At the time of R2P2 initiation, only available toxicity data for 6PPD-Q was for juvenile and adult life stages of coho salmon
- Sensitivity of other species and embryo-larval life stages were unknown

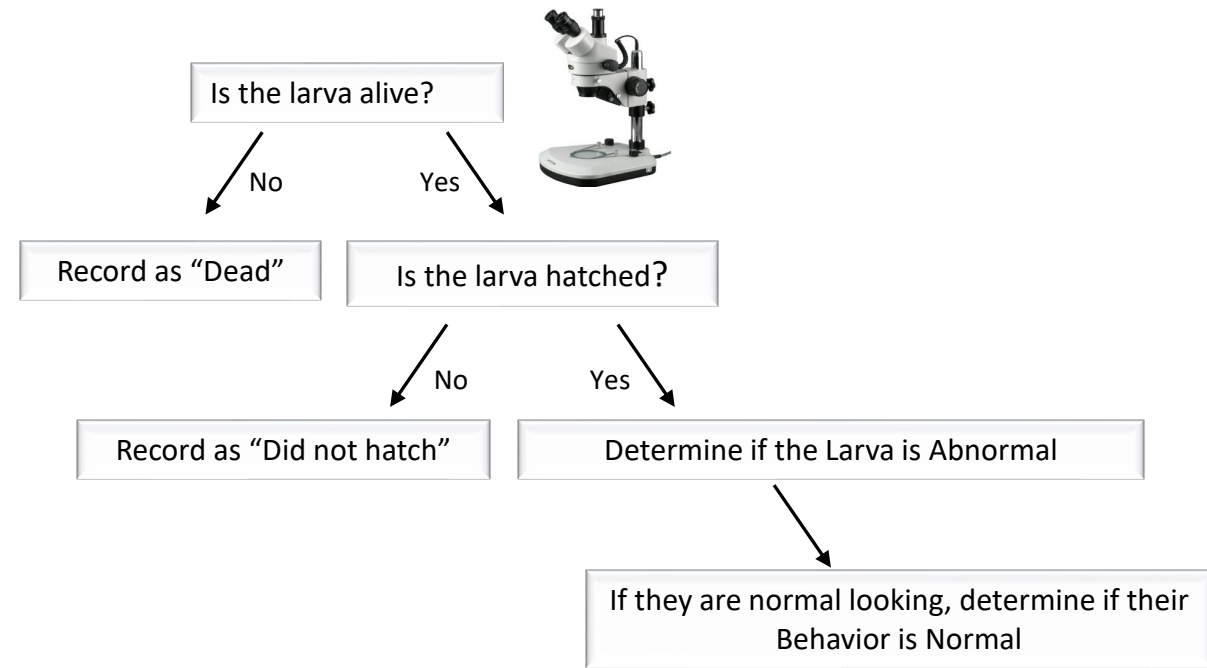




Zebrafish Developmental Assay



Normal

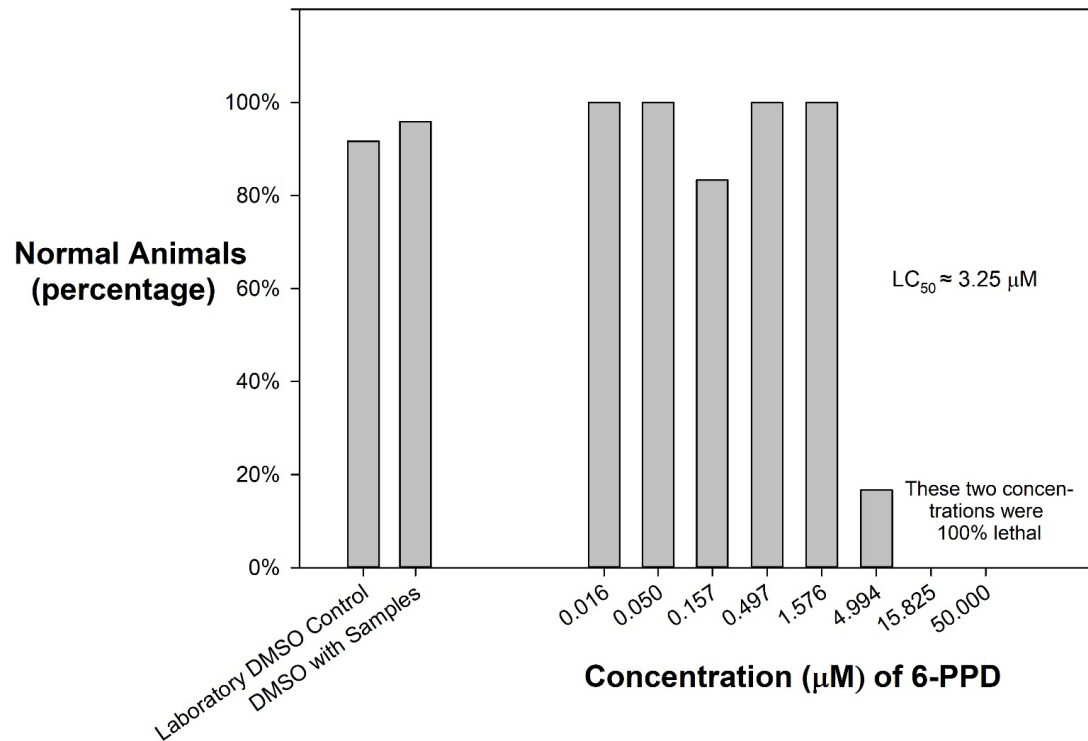




6PPD, but not the Quinone Was Toxic to the Developing Zebrafish

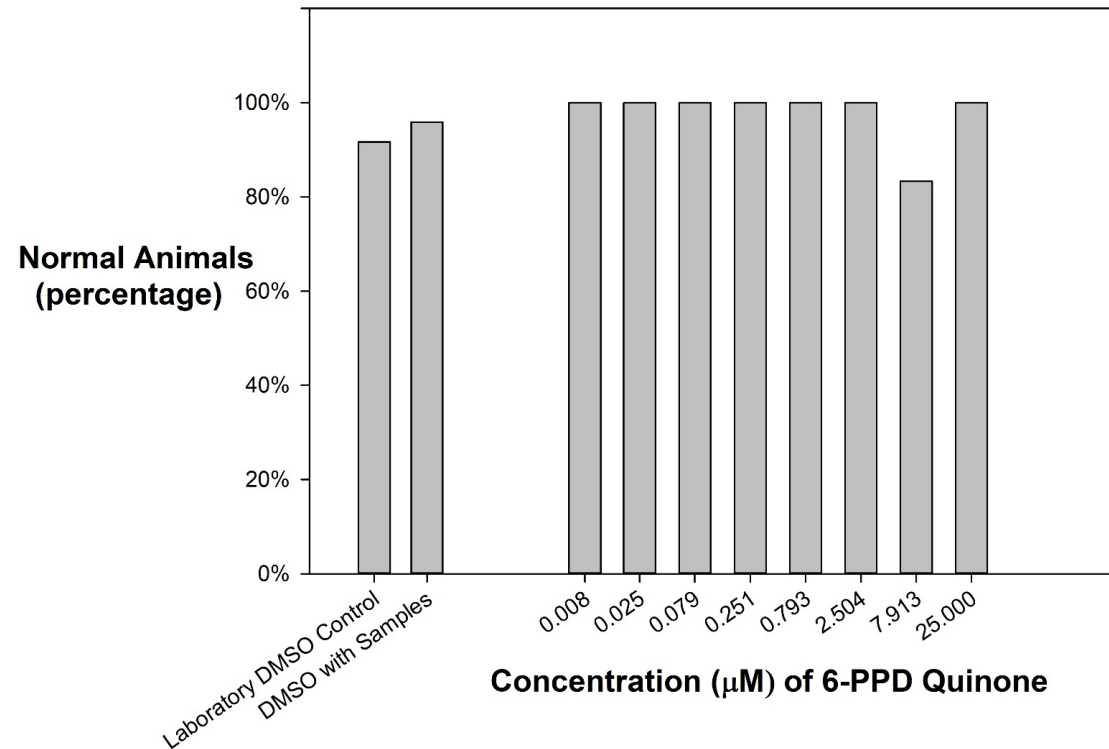
LC₅₀ = 3.25 μ M (\approx 872 μ g/L)
 \approx 3.5 x less sensitive than coho salmon
(LC₅₀ 6PPD \approx 250 μ g/L)

6 PPD Developmental Toxicity



No toxicity up to 25 μ M ($>$ 7 mg/L)
At least 70,000x less sensitive than
coho salmon

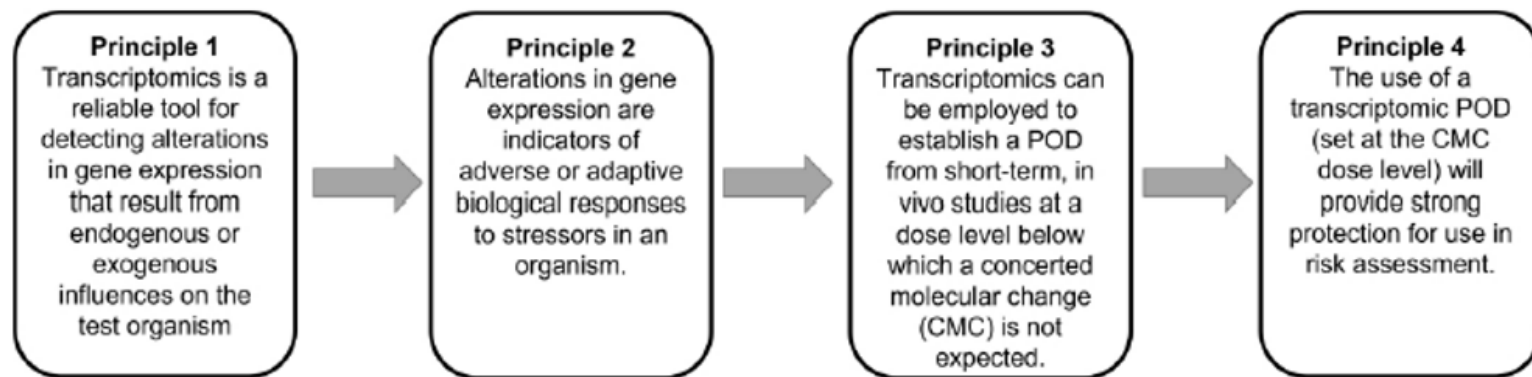
6 PPD-Quinone Developmental Toxicity



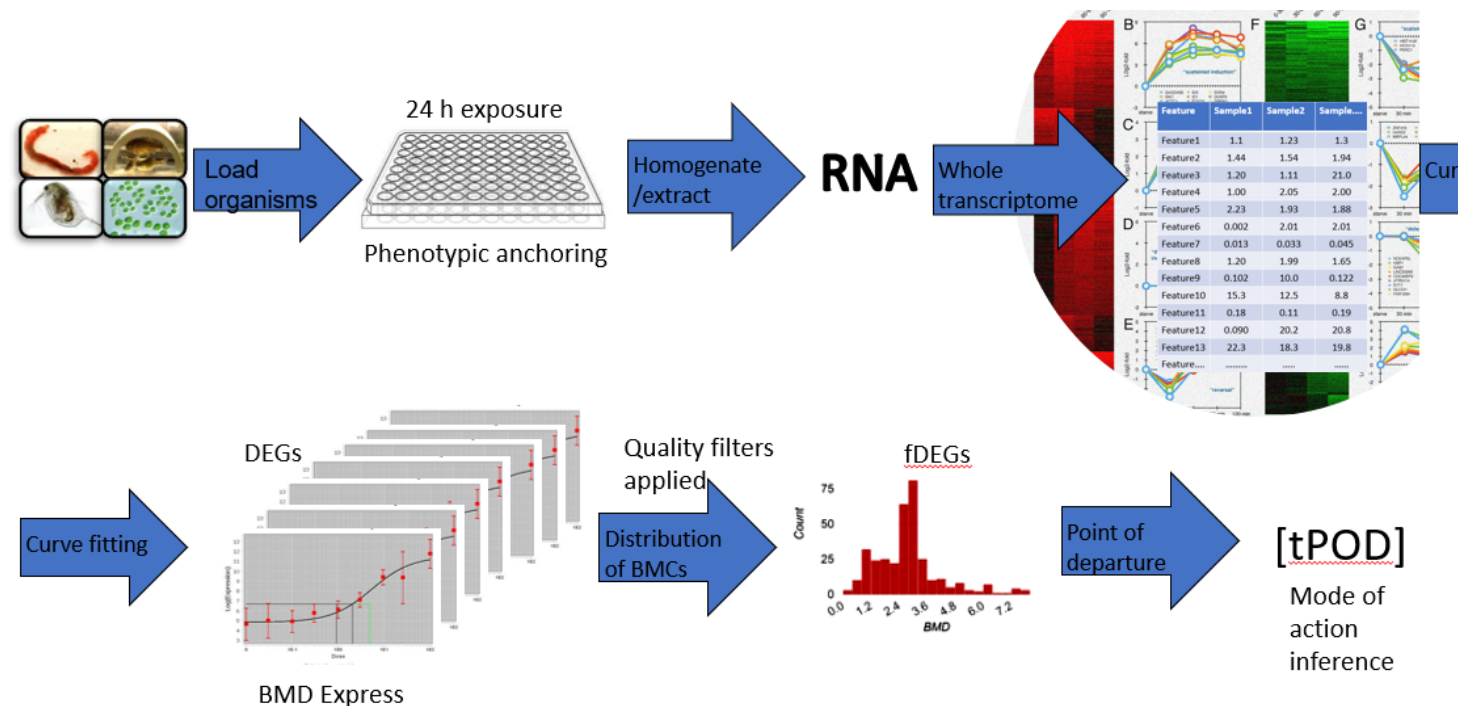


Ecological High Throughput Transcriptomics

- Larval fathead minnows
- 24 h exposure
- Whole transcriptome sequencing



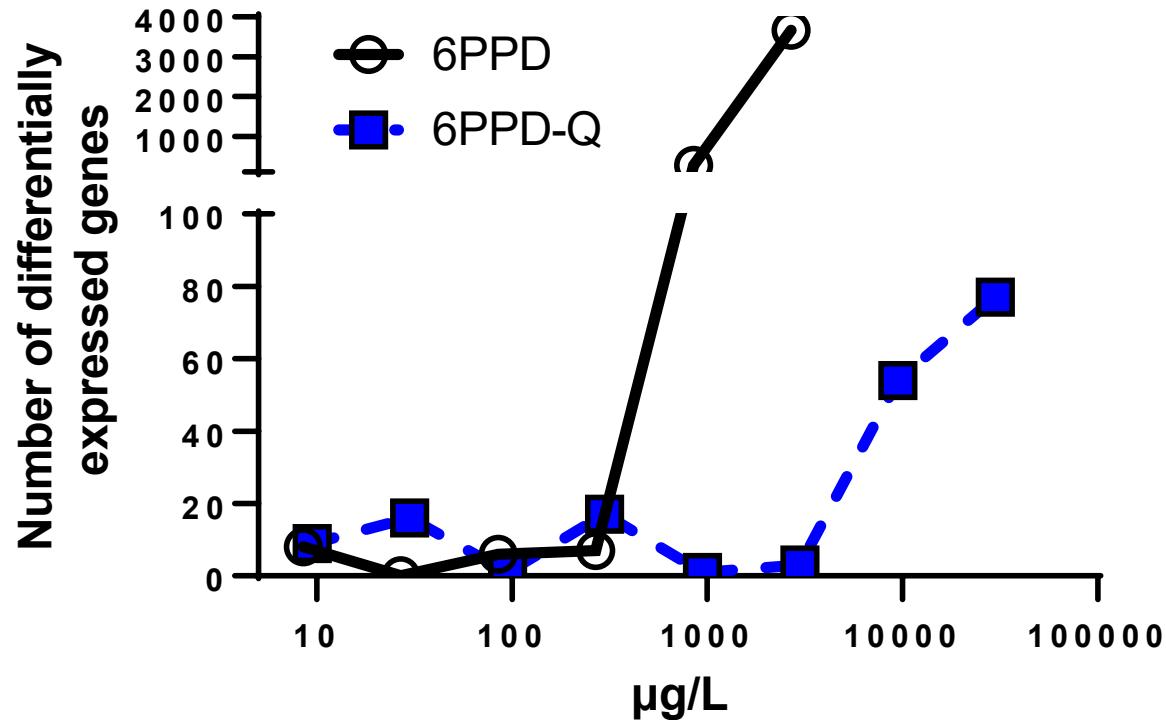
Johnson KJ, Auerbach SS, Stevens T, Barton-Maclaren TS, Costa E, Currie RA, Dalmas Wilk D, Haq S, Rager JE, Reardon AJF, Wehmas L, Williams A, O'Brien J, Yauk C, LaRocca JL, Pettit S. A Transformative Vision for an Omics-Based Regulatory Chemical Testing Paradigm. *Toxicol Sci.* 2022 Nov 23;190(2):127-132. doi: 10.1093/toxsci/kfac097.





Larval fathead minnows, quite insensitive to 6PPD-Q

6PPD more potent than 6PPD-Q in larval fathead minnows



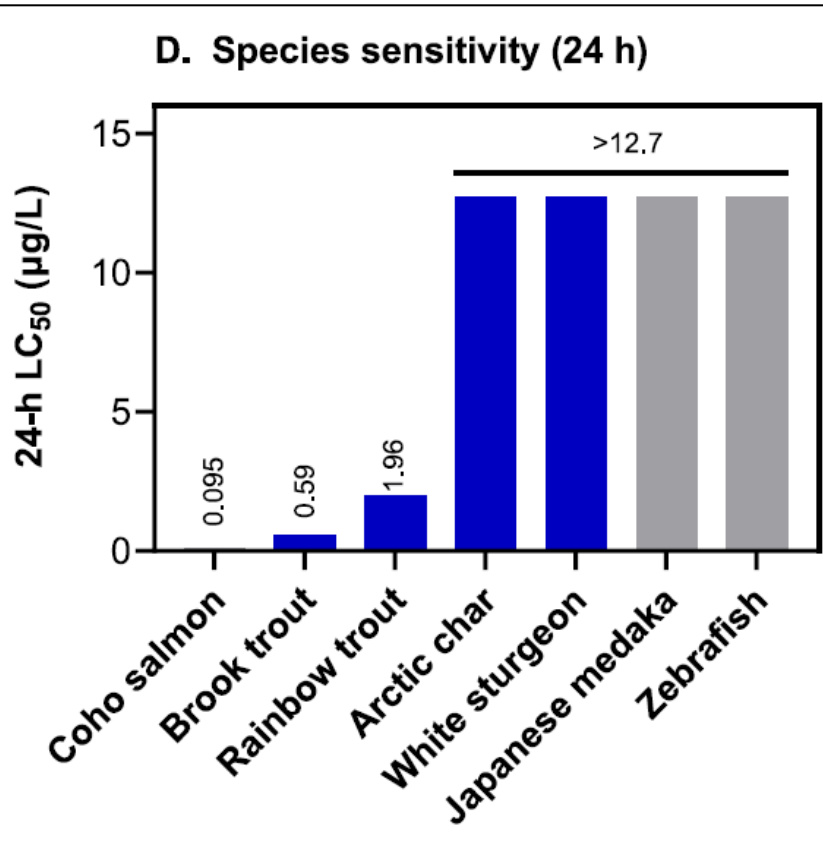
Substance	LC50 (24 h acute; µg/L)	Median BMD (µg/L)	tPOD (µg/L)
6PPD	≈8600	804	348
6PPD-Q*	>29800	19370	2324

*Tolerated a concentration over 20,000 times greater than that which causes toxicity to coho salmon.

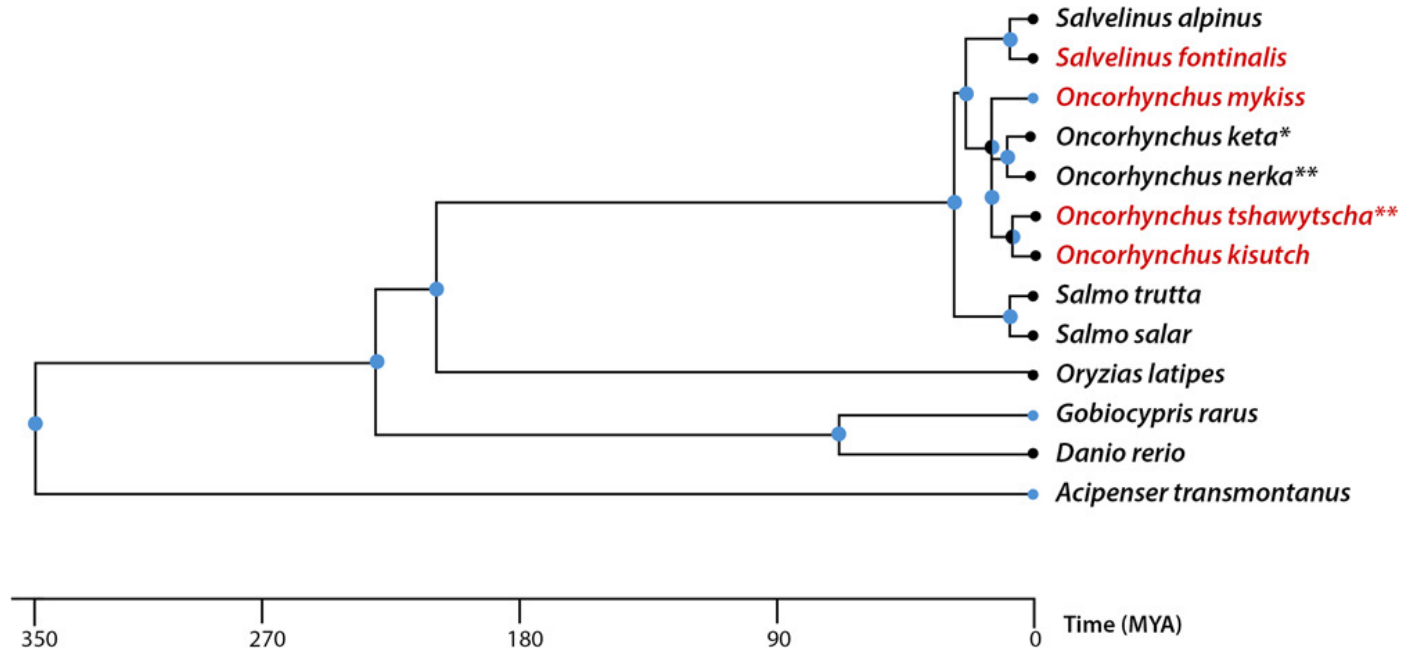
Transcriptomic profile provided no insight into toxic mode of action



Our results are consistent with contemporary studies showing large differences in species sensitivity, with only some salmonids and trout being sensitive, but life stage may affect sensitivity



Brinkmann, M., D. Montgomery, S. Selinger, J. G. P. Miller, E. Stock, A. J. Alcaraz, J. K. Challis, L. Weber, D. Janz, M. Hecker, and S. Wiseman. 2022. Acute Toxicity of the Tire Rubber-Derived Chemical 6PPD-quinone to Four Fishes of Commercial, Cultural, and Ecological Importance. Environmental Science & Technology Letters.

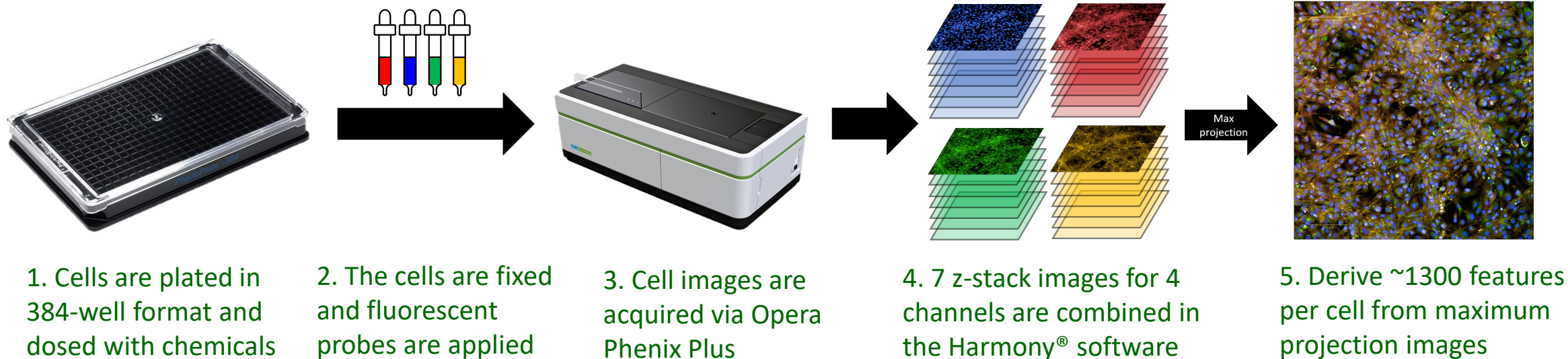


Preliminary phylogenetic tree of species tested and found sensitive or insensitive to 6PPD-Q toxicity

Foldvik A, Kryuchkov F, Sandodden R, Uhlig S. Acute Toxicity Testing of the Tire Rubber-Derived Chemical 6PPD-quinone on Atlantic Salmon (*Salmo salar*) and Brown Trout (*Salmo trutta*). Environ Toxicol Chem. 2022 Dec;41(12):3041-3045. doi: 10.1002/etc.5487.

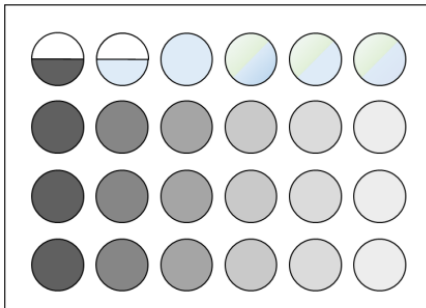
Add On: High Throughput Phenotypic Profiling (HTPP)

- First tier hazard evaluation
- Based on the Cell Painting method (Bray et al. 2016, doi.org/10.1038/nprot.2016.105)
- Fluorescent probes label cellular structures and organelles
- Used to screen chemicals in concentration/response format

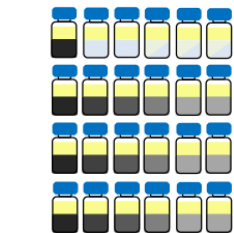


- Rainbow Trout gill cell line
- ATCC recommended for *in vitro* toxicology
- OECD TG 249 to predict acute toxicity in a plate reader assay


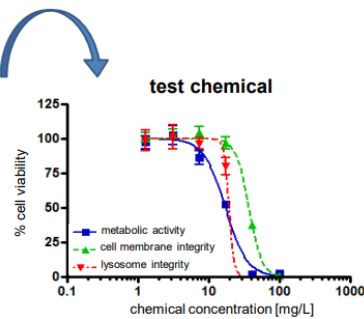
day 3
sampling and measurement
of cell viability



sampling at end of
exposure
500 µL sample per
well and vial



measurement of cell viability




Section 2
Effects on Biotic Systems

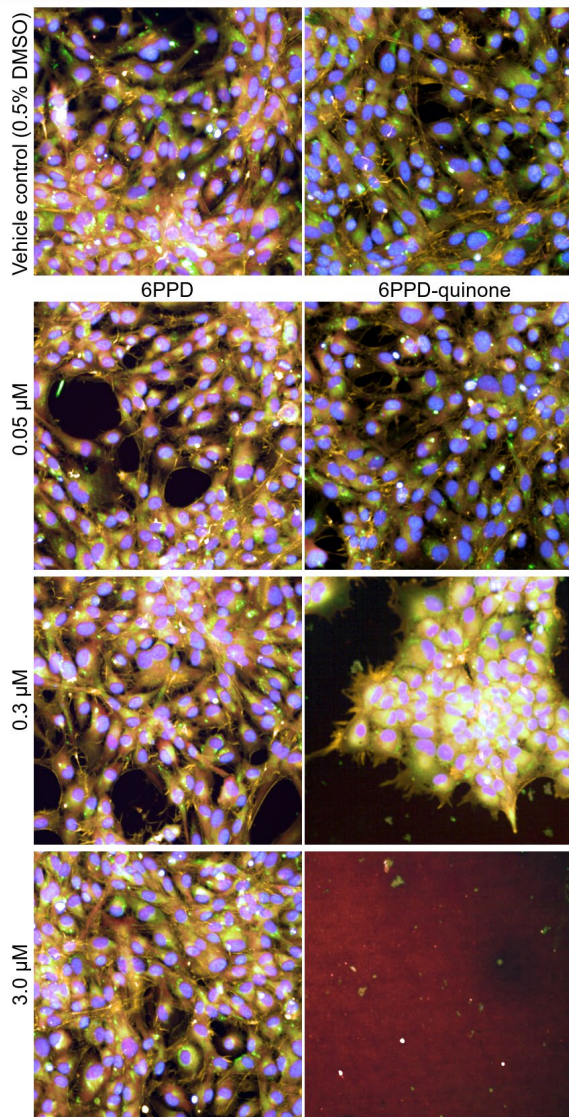
Test Guideline No. 249
Fish Cell Line Acute Toxicity:
The RTgill-W1 cell line assay

14 June 2021

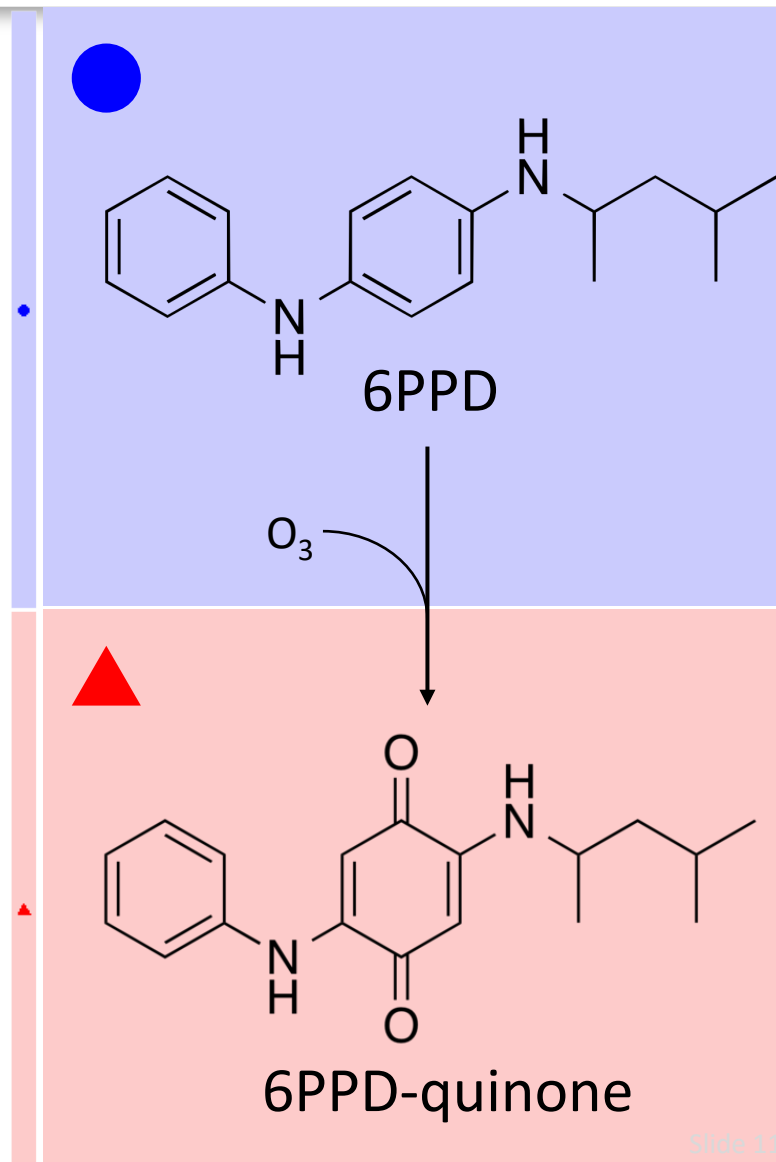
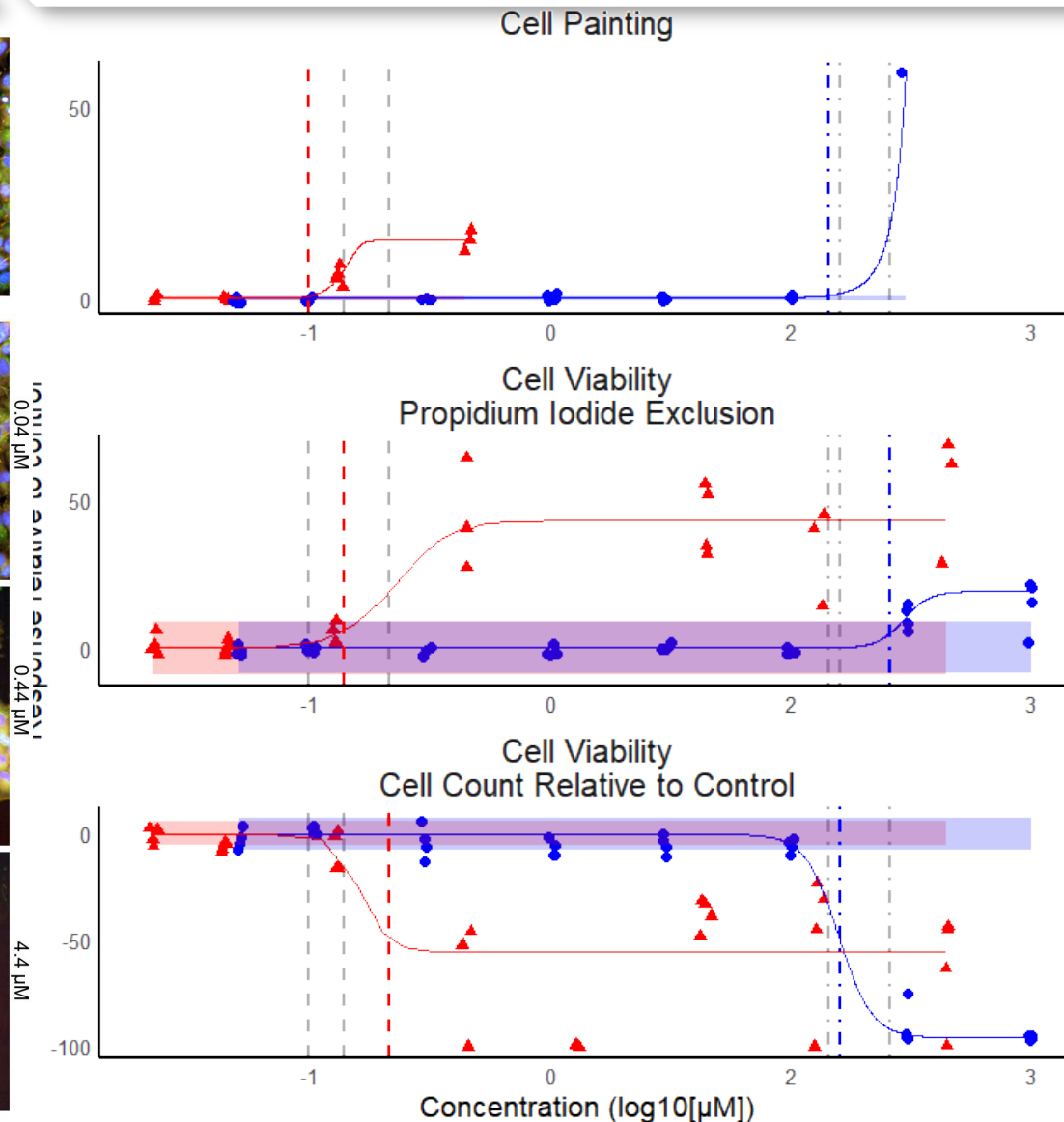
OECD Guidelines for the
Testing of Chemicals



6PPD-quinone, the oxidation product of 6PPD, is more toxic in RTgill-W1 cells



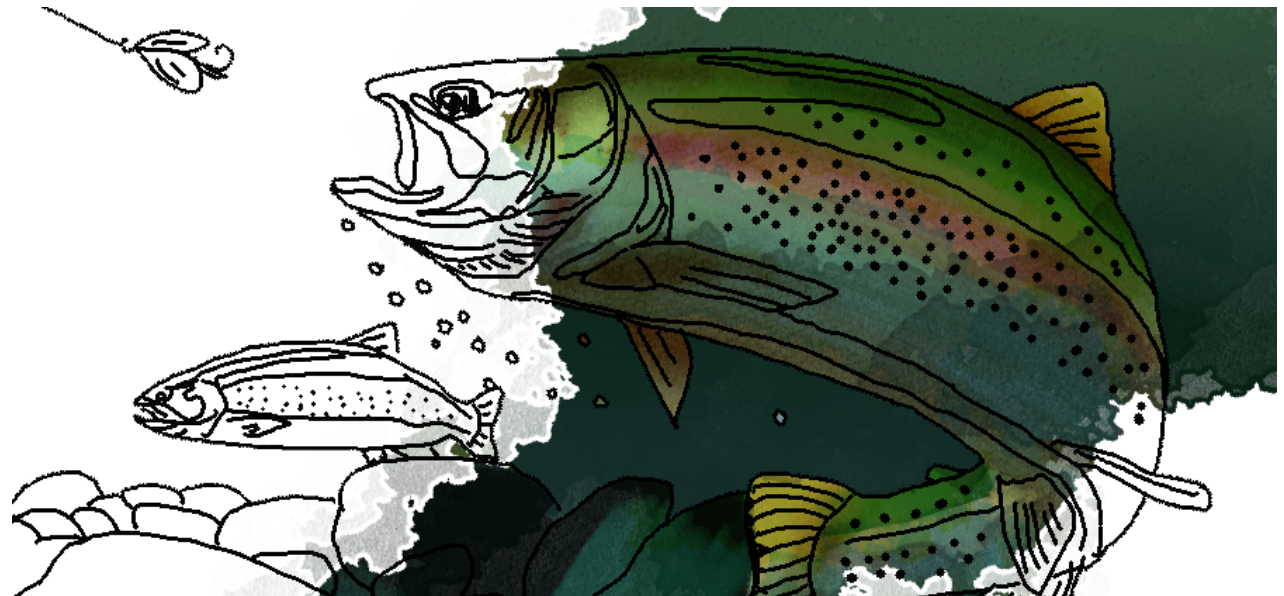
DNA Mitochondria AGP RNA & ER





- ✓ 6PPD-Q was substantially ($\approx 1000 \times$) more potent than 6PPD in this system
- ✓ 6PPD-Q was the second most potent compound out of ≈ 250 screened
 - ✓ Second only to rotenone
- Appears viable as a high throughput screen for potential 6PPD replacements and their transformation products
- Suitable to screen urban run-off samples
- In vitro potency does not directly equate to in vivo effect concentrations, but relative potency can be expressed as 6PPD-Q equivalents
- Proposed as a 2023 Region 10 Regional Applied Research Project

RTgill-W1 Cell Painting Assay





Acknowledgements

NT/DNT Assays:

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- Jackson Keever (ORAU)
- Amy Carpenter (ORISE)

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- Catherine Gockel (WD)
- Barry Pepich (Lab)

Zebrafish Assays:

- Kimberly Jarema
- Bridgett Hill (ORISE)
- Morgan Lowery

RTgill-W1 HTPP:

- Felix Harris
- Johanna Nyffeler

Eco-HTTr Assays:

- Kendra Bush (ORISE)
- Monique Hazemi (ORISE)
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- Michelle Le (ORISE)
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- Emma Stacy