Item 12:
Update on US EPA application of the DNT In Vitro Battery

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Disclosure Statement

This work has been funded by the US. Environmental Protection Agency. I have no conflicts to declare.

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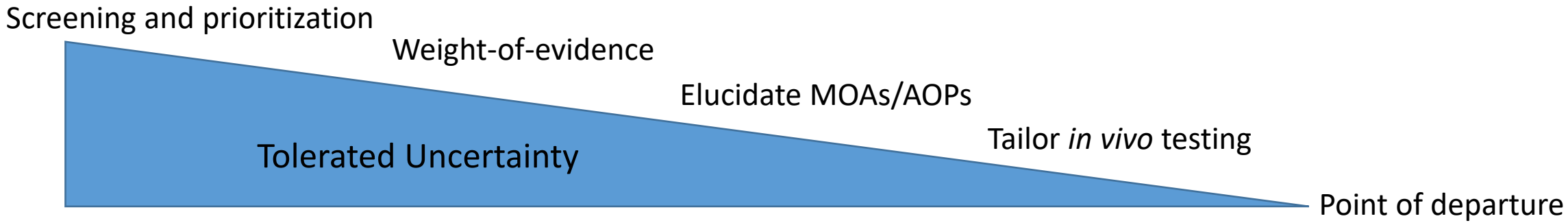
Integrating NAMs into Risk Assessment

- Integration process is a continuum
  - Aid in interpretation of *in vivo* observations
  - Aid in deciding if additional studies are/aren't needed
  - Leverage understanding of underlying biological processes
- Different levels of uncertainty tolerated depending on use context



Examples of the use of DNT NAMs at EPA

I. Screening Level information

- Accelerating the Pace of Chemical Risk Assessment (APCRA), Toxic Substance Control Act (TSCA) chemicals, Perfluoroalkyl Substances (PFAS)

II. Weight of Evidence approach

- **Structure-activity relationships**
  - Apply DNT NAMS to evaluate structurally similar chemicals when one of the chemicals has known effects in a Guideline DNT study
    - Example with compound X and structurally similar analogs
      - DNT Guideline exists for X, should it be required for the analogs?

III. Weight of Evidence approach

- Organophosphates
  - Are PoDs based on AChE inhibition health protective for organophosphates?

Example #1: Screening Level Information for PFAS Compounds

Problem: Perfluoroalkyl substances (PFAS) have recently been identified as environmental contaminants with significant human exposure. Little toxicological information is available for these compounds.

- Structurally diverse
- Except for a few specific congeners, little toxicological information
- Evidence of DNT is ambiguous,
  - epidemiological studies report positive and negative neurodevelopmental effects associated with exposure to PFAS

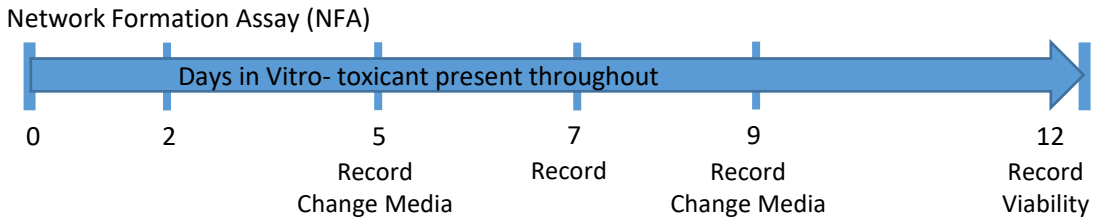
Assembled a PFAS Chemical Library for Research and Methods Development

Test those compounds in NAMs and other assays

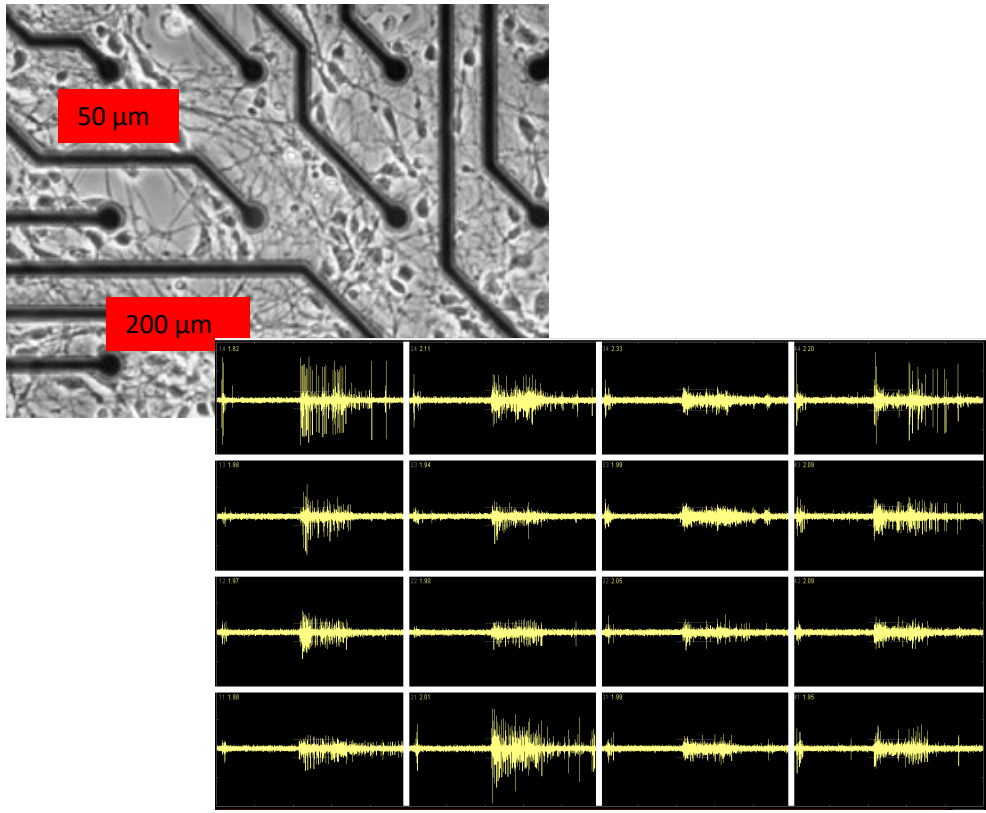
Hepatotoxicity
Developmental toxicity
Mitochondrial toxicity
Developmental neurotoxicity
Endocrine Disruption
General toxicity

PFAS Compounds Tested for Effects on Network Formation*

Test Set of Compounds

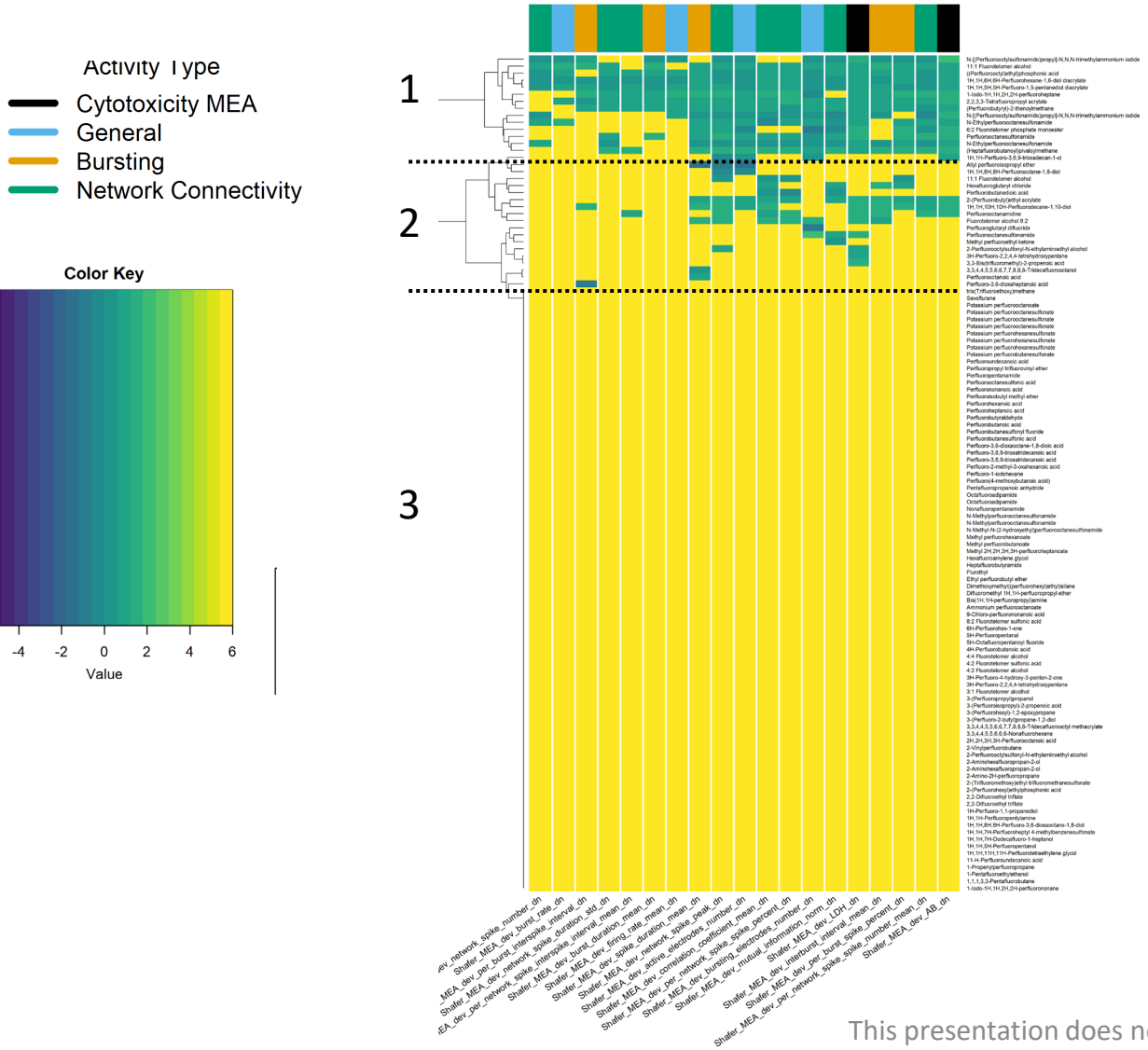


- Original PFAS 150
  - 75 tested in concentration-response (0.03-30 µM)
- Re-procured PFAS
  - 131 Tested single-concentration (30 µM)
  - 42 tested in concentration-response (0.03-30 µM)
- 13 compounds tested as biological replicates



*Other EPA DNT in vitro NAMs data are currently being analyzed

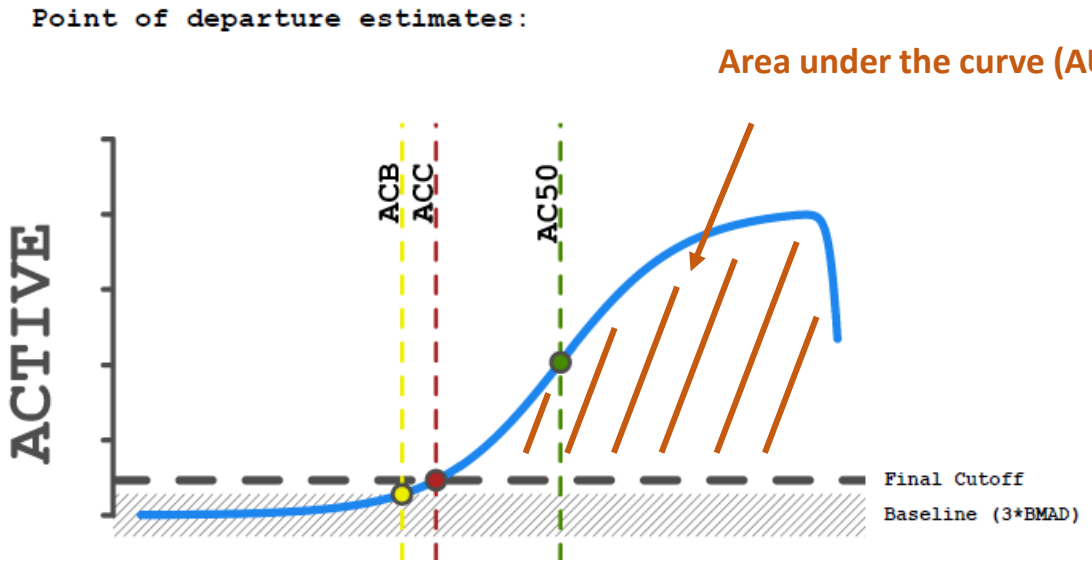
Only a fraction of PFAS compounds disrupt network formation

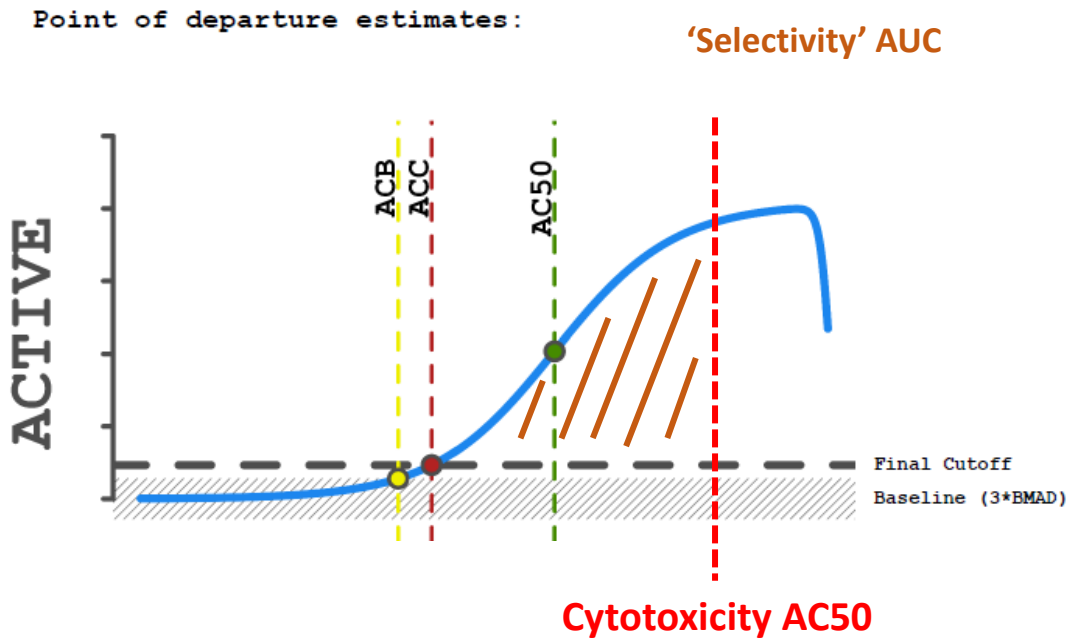


- ~25% of tested compounds were active
- No PFAS compound increased network formation parameters compared to control wells
- Three Groups: 1) “Pan Active” 2) subset of parameters 3) Inactive
- Positive and negative controls gave appropriate responses.
- Replicates gave generally consistent results
- Cytotoxicity was prominent in “Pan Active”

Calculating a 'selectivity' metric

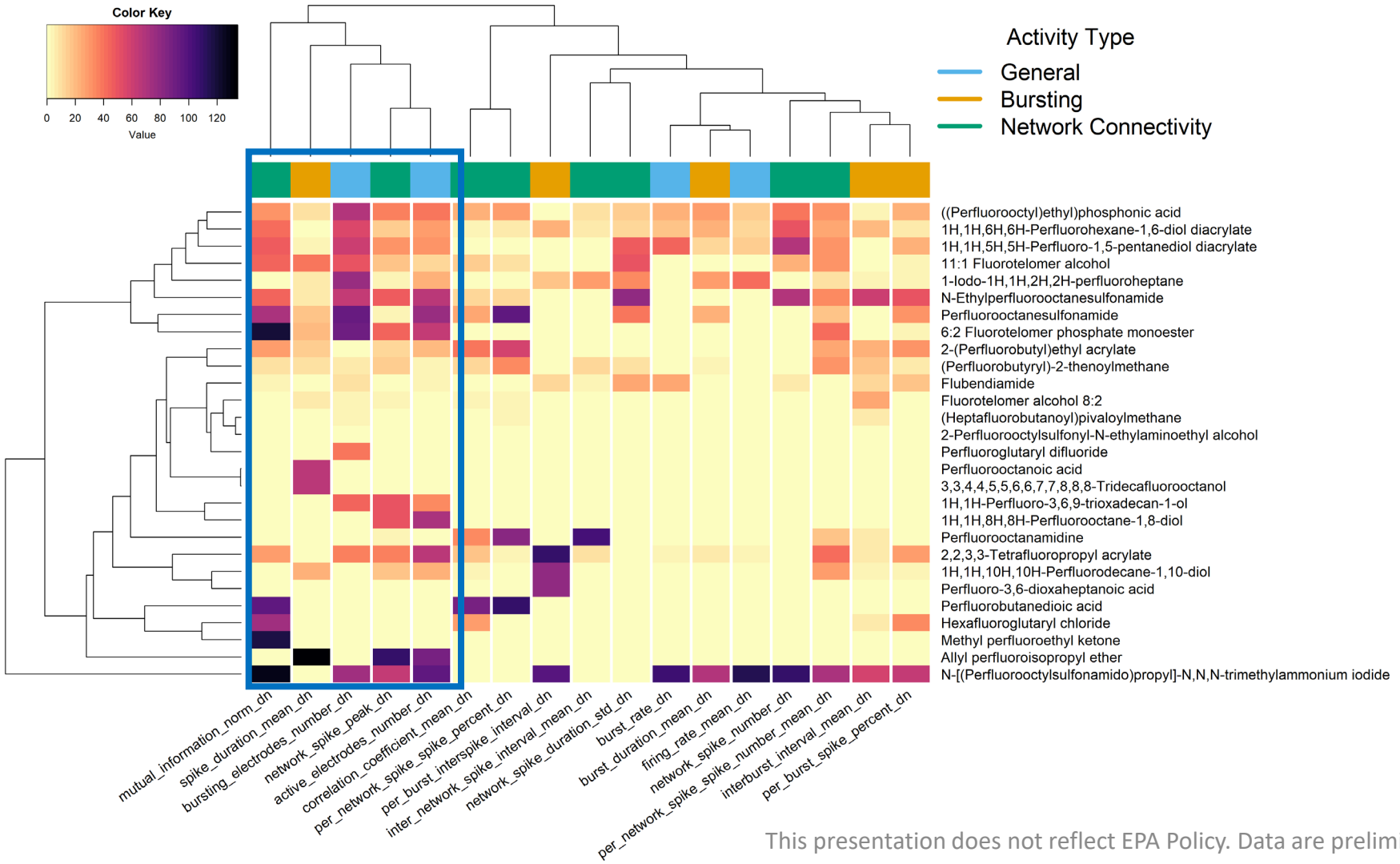
Selectivity: activity at concentrations lower than cytotoxicity.





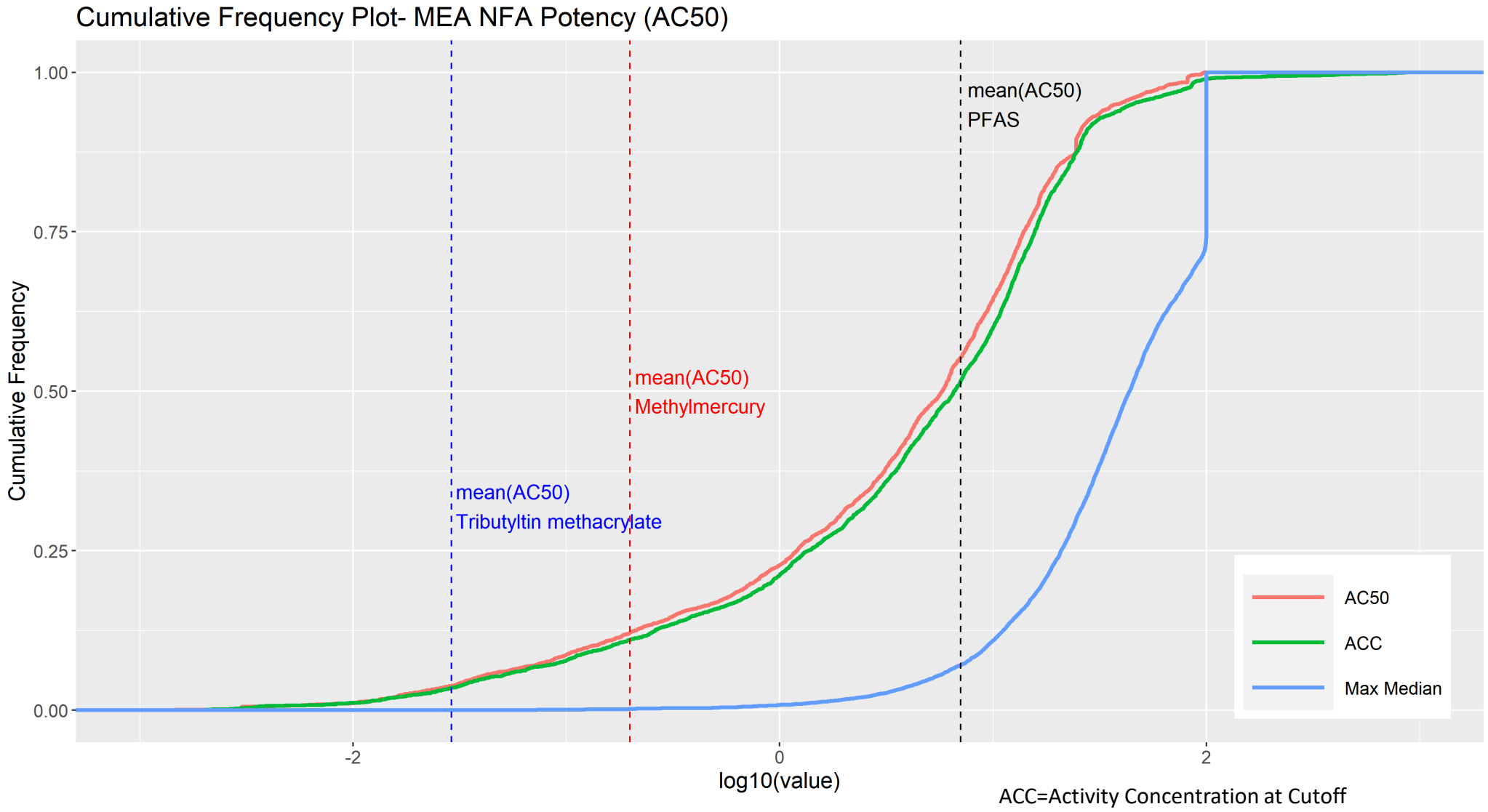
Modified from "The New ToxCast Analysis", Dayne Filer
https://www.epa.gov/sites/production/files/2015-01/documents/the_new_toxcast_analysis_v2.pdf

Selectivity of PFAS Compounds indicates that General activity and Network Connectivity are Altered more than Bursting



Comparison of PFAS AC_{50} to Other Compounds in the NFA

The potency of active PFAS compounds is near the median of potencies for all compounds tested in the NFA



Summary of PFAS effects in the NFA

The NFA identified PFAS compounds that disrupt the formation of neural networks in vitro

- This identifies compounds with a potential *hazard* for DNT
- Can place the potency of these effects into context with other DNT compounds
- Exposures to these compounds have not been considered.

These data* can be used to make inferences for a broader landscape of PFAS:

- Chemical Category and Read-across approaches for additional hazard identification/characterization.
- Bioactive Dose Level (BDL) Approach (*in vitro* to *in vivo* extrapolation to define administered dose equivalent (ADE) values)

*Data are currently being analyzed across all of the different toxicities evaluated.

Example #2: Using WOE and DNT NAMs for Guideline DNT waiver decisions

EPA's Office of Pesticide Programs (OPP)

- Registers pesticides for use in the United States.
- Three new compounds (EX000372, EX000373, EX000374)
  - potentially neurotoxic.

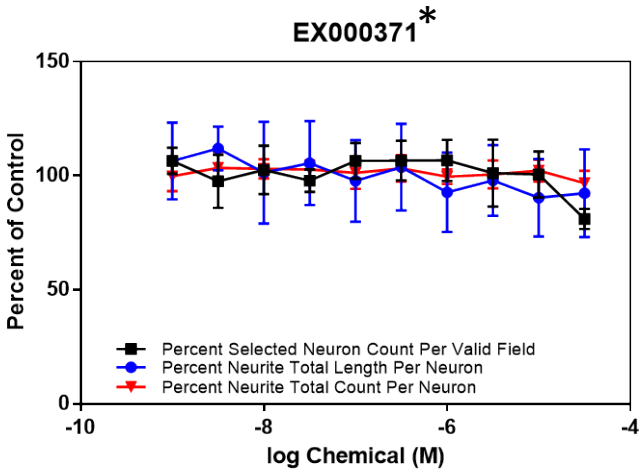
  - A DNT Guideline study existed for "compound X (EX000371)" that was structurally similar to the novel compounds.
    - The DNT Study showed small but statistically significant changes in brain morphology
  - Literature data indicated that EX000371 caused acute neurotoxicity in vivo and altered network activity in vitro following acute exposure.

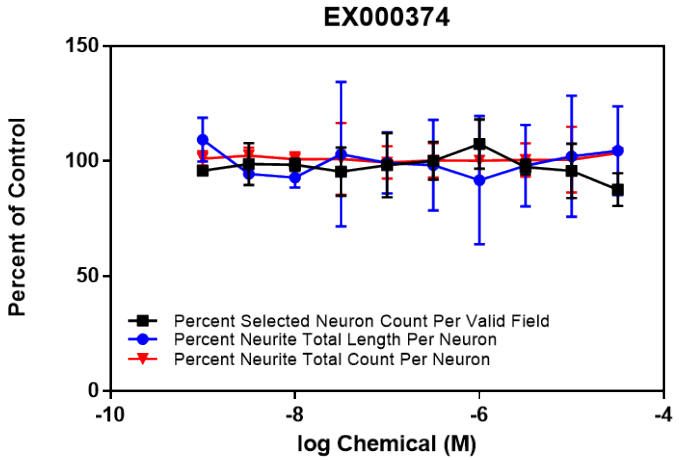
OPP needed to decide whether to request DNT Guideline studies on the new compounds

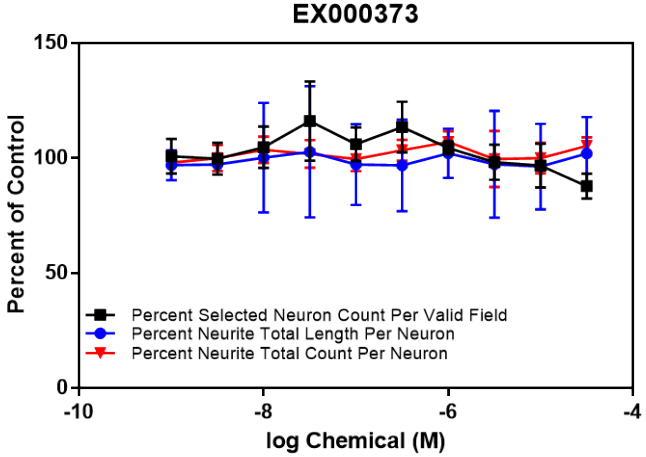
OPP asked EPA's Office of Research and Development to provide data to inform their decision on the novel compounds.

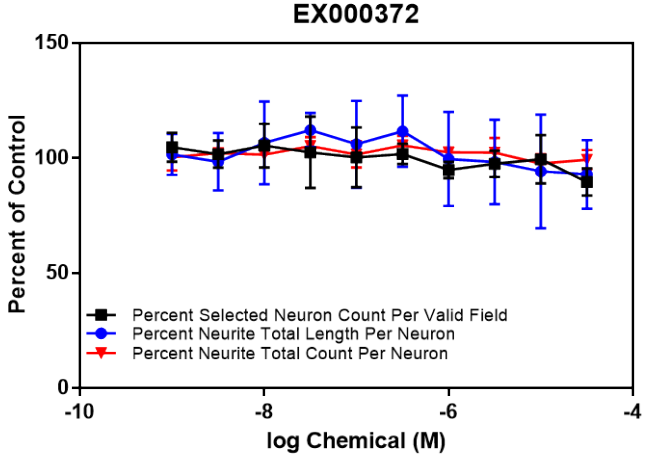
- **Neurite Outgrowth** and **Network Formation** assays were selected based on the of activity of EX000371 in Guideline Study and in vitro, respectively.
- Compounds EX000372, EX000373, EX000374 were tested in these assays, along with appropriate controls

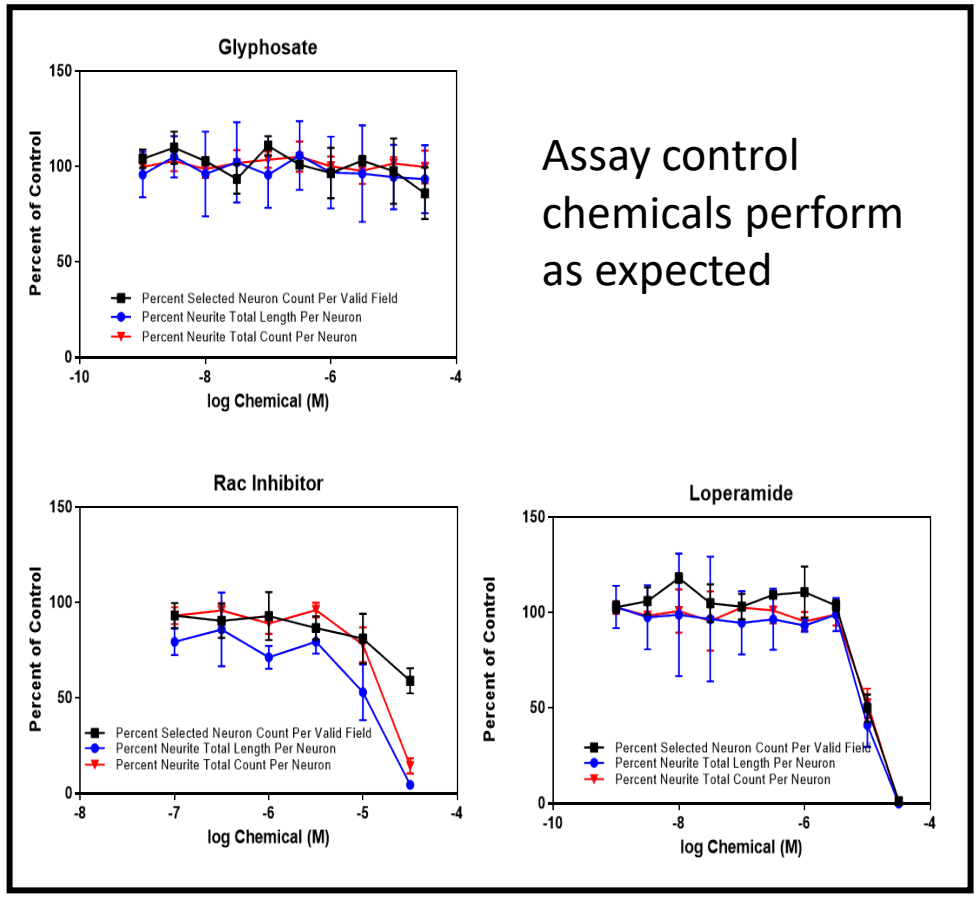
Compound X and analogs lack effects on Neurite Outgrowth









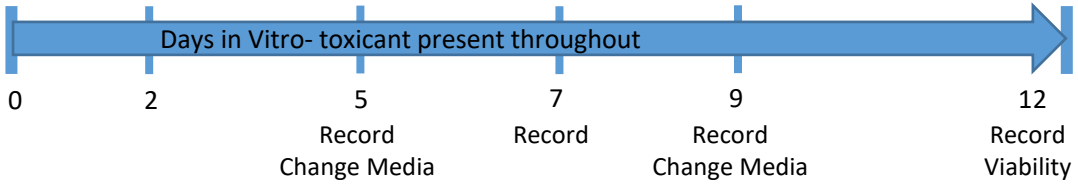


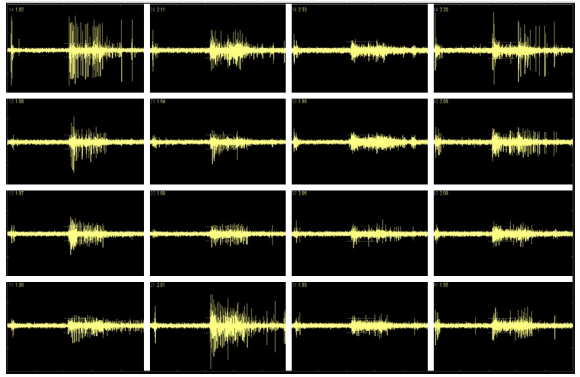
Assay control chemicals perform as expected

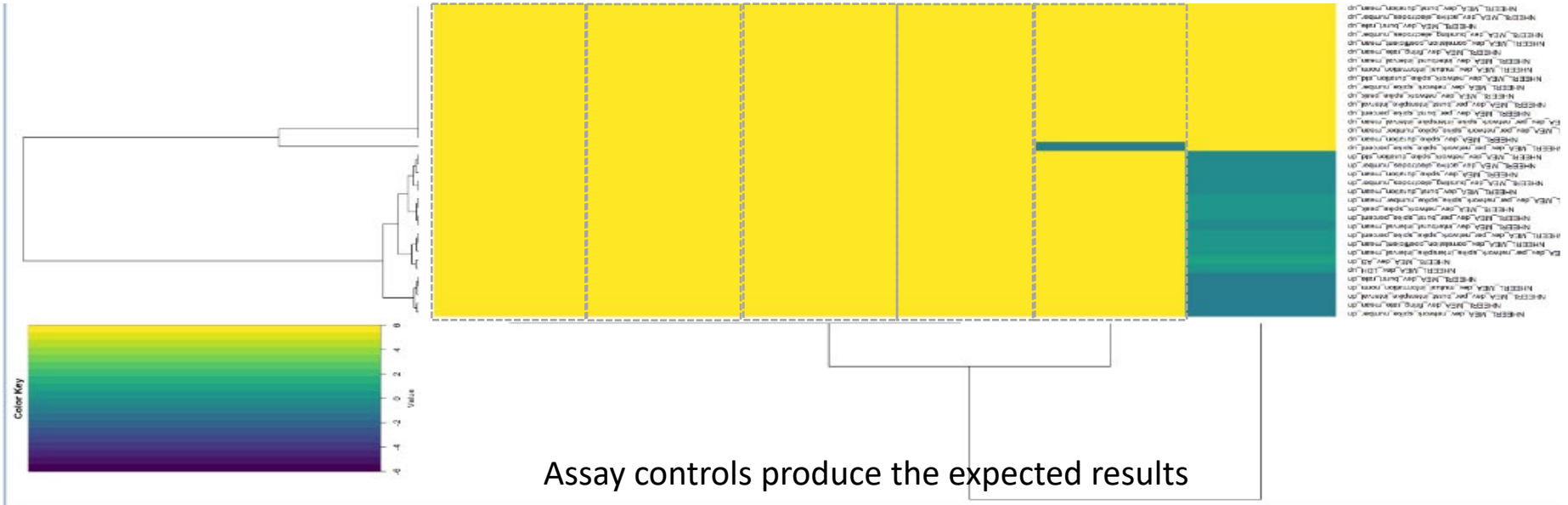
*EX000371 = Compound X

Compound X and analogs lack effects on Network Formation

Network Formation Assay (NFA)

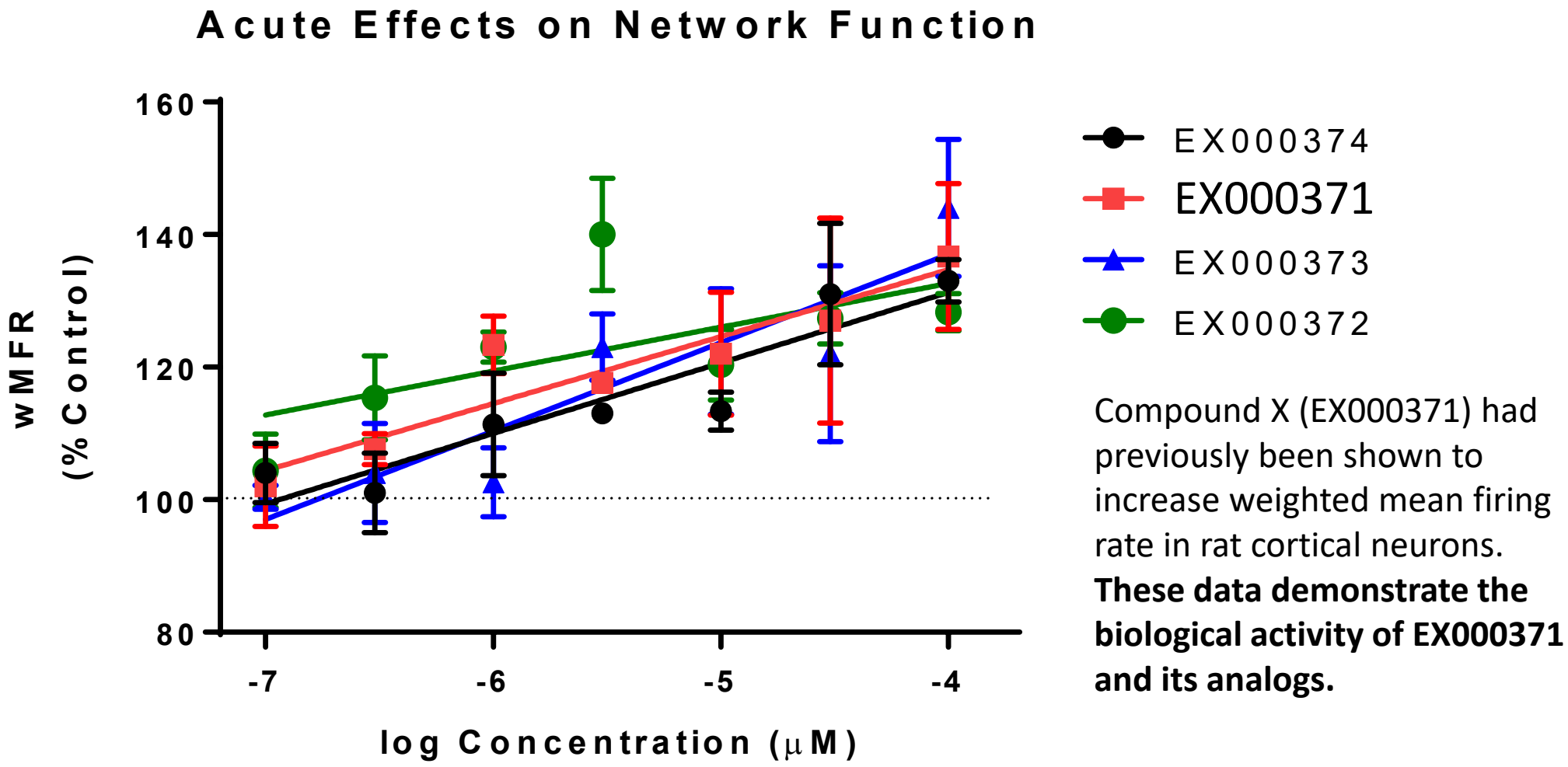






Assay controls produce the expected results

EX000371 and analogs alter Network Function



Compound X (EX000371) had previously been shown to increase weighted mean firing rate in rat cortical neurons. **These data demonstrate the biological activity of EX000371 and its analogs.**

IVIVE indicates appropriate concentration ranges were tested

From Guideline study, NOAEL of EX000371 = **14 mg/kg/day**

Using HTTK and IVIVE

- 1 mg/kg/day = Css values of 0.66 and 2.21 µM in rats and humans, respectively
- 30 µM EX000371 = AED of **45 mg/kg/day** (rats) and 13.5 mg/kg/day (humans)

Summary:

- **At concentrations equivalent to or above the NOAEL from in vivo studies, EX000371 and analogs did not alter Neurite Outgrowth or Network Formation, but did have acute effects on Network Function**
- **No differences were observed between EX000371 and its analogs.**

These data, along with other (e.g. exposure) data, were used to support a decision by OPP to waive the requirement for a DNT Guideline study for compounds EX000372, EX000373 and EX000374. Given the totality of the data, EPA determined that conducting a Guideline DNT for the analogs would not result in a lower point of departure than for EX000371.

Example #3: Using WOE to evaluate the health protective nature of PODs based on AChE inhibition: Organophosphates

Organophosphate (OP) insecticides are currently regulated based on inhibition of acetylcholinesterase (AChE).

Primary Questions:

1. Does the DNT battery indicate sufficient health protection from regulation based on AChE inhibition?

2. Can data from the DNT battery contribute to a Weight of Evidence (WOE) approach for OPs?

Example #3: Organophosphates and DNT

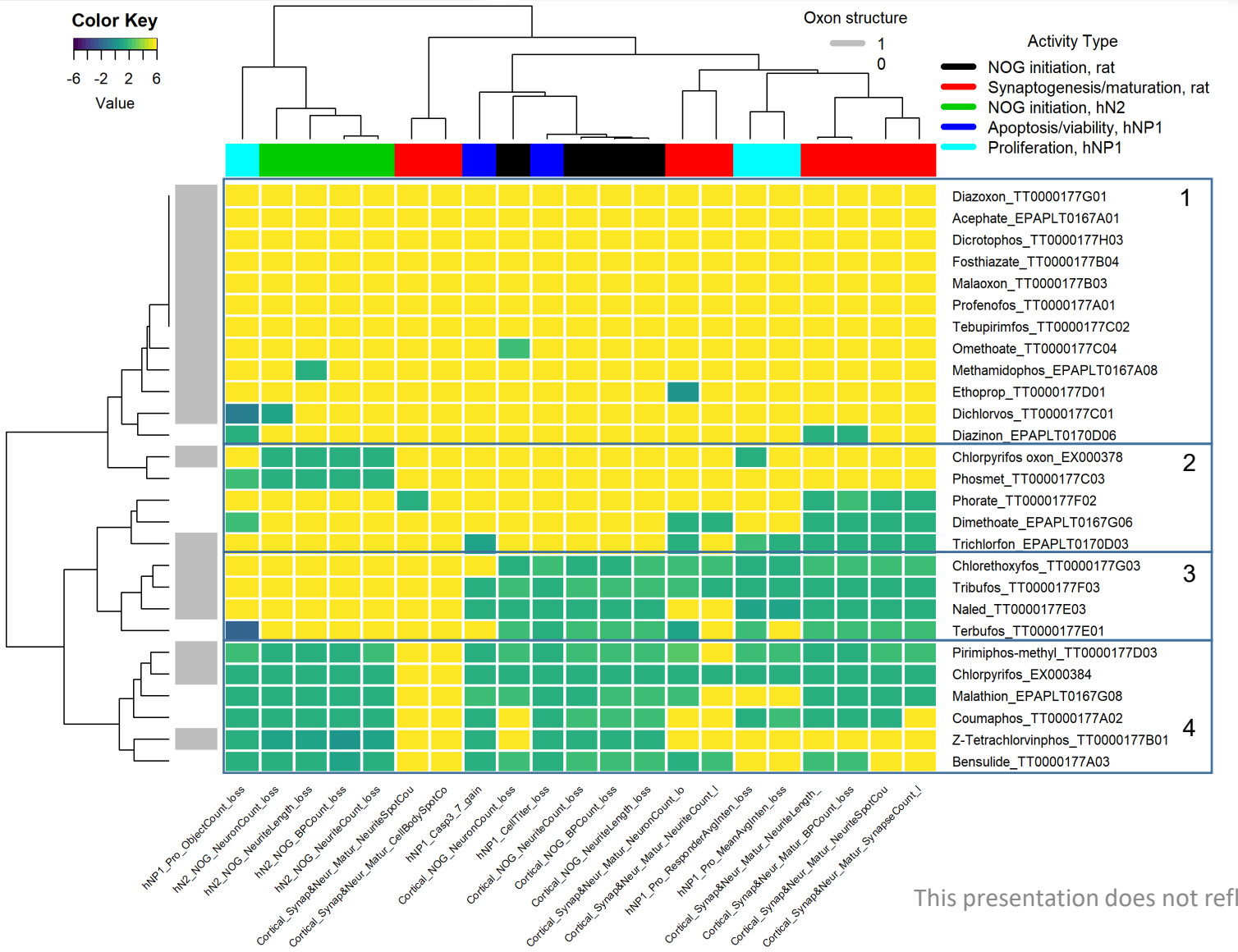
Study Design:

Test 27 Organophosphate insecticides in the EPA DNT assays
8 Parent/oxon pairs
Concentration-response up to 100 µM
Pipeline results through TCPL to generate AC_{50} values
Use HTTK to convert AC_{50} values to AED_{50} values
Compare to BMD/BMDL10 values based on AChE inhibition

Assays:

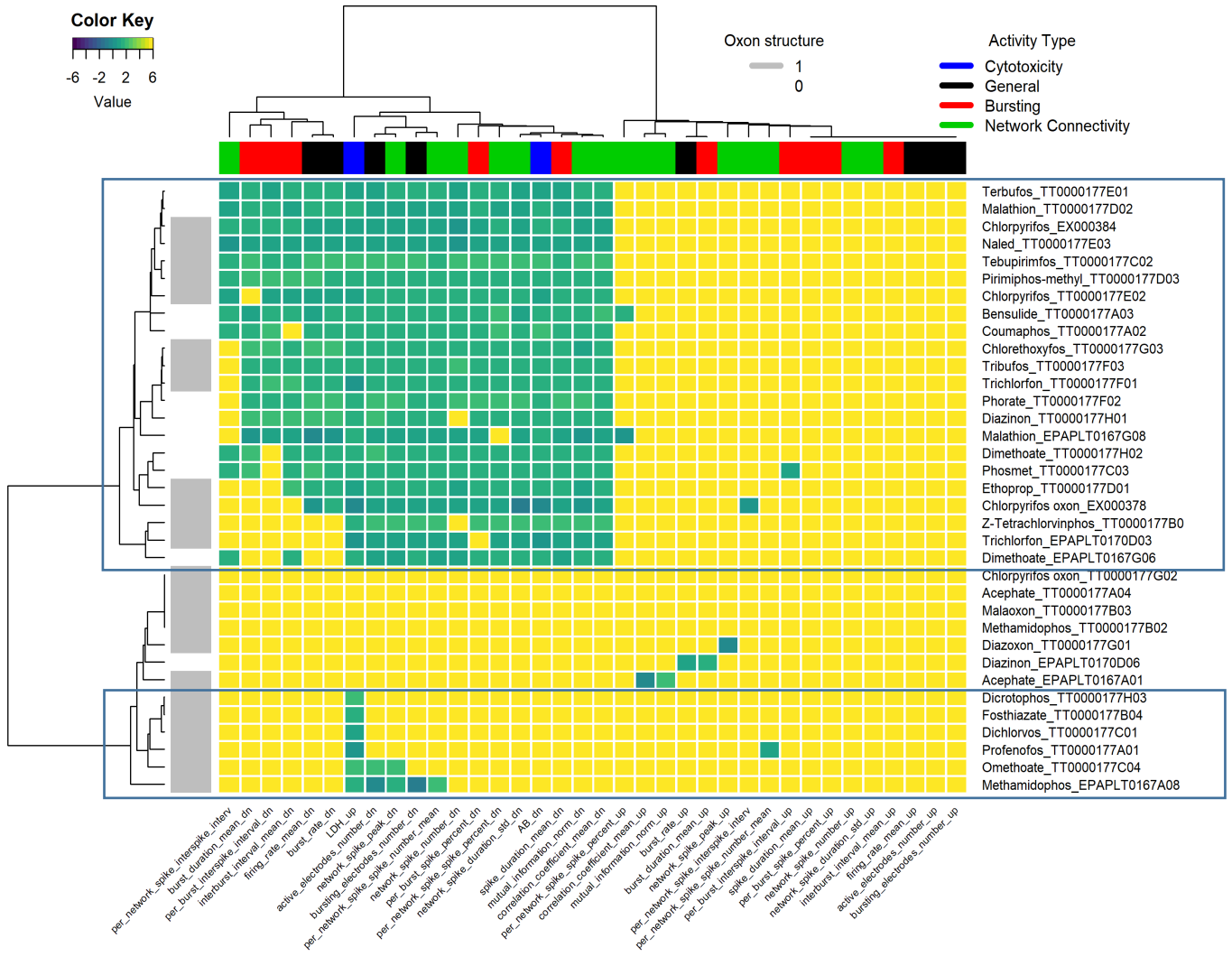
Proliferation	-	human neuroprogenitors (hNP1)	
Apoptosis	-	human neuroprogenitors (hNP1)	High-Content Imaging (HCI) assays
Neurite initiation	-	human neurons (hN2)	
Neurite initiation	-	rat primary neural culture	
Neurite maturation	-	rat primary neural culture	
Synaptogenesis	-	rat primary neural culture	
Network formation (MEA)	-	rat primary neural culture	
Behavior/Anatomy	-	zebrafish (data analysis pending)	

OPs demonstrate differential responses in the HCI assays.



- Cluster 1: negative or with effects in 1-3 endpoints.
- Cluster 2: effects on 5 or more assay endpoints
- Cluster 3: effects on all HCI assay activity types except for NOG initiation in hN2 cells and synaptogenesis in cortical cells
- Cluster 4: widespread effects across activity types

Most OPs decreased MEA NFA activity



- Top active cluster of OPs contains oxon and non-oxon structures.
- These OPs, like the assay performance controls and many other compounds, appear to generally decrease all activity types and most assay endpoints.
- Bottom cluster with minimal actives appears somewhat driven by cytotoxicity in the LDH assay.
- Negative- 0 assay endpoints altered
- Equivocal- 1-3 assay endpoints altered
- Positive- >3 assay endpoints altered

HCI and MEA_NFA assays show consistent results

DTXSID	Chemical	MEA NFA			HCI			
		Neg	Equiv	Pos	1	2	3	4
DTXSID8023846	Acephate	X	X		X			
DTXSID9032329	Bensulide			X				X
DTXSID2032344	Chlorethoxyfos			X			X	
DTXSID4020458	Chlorpyrifos			X,X				X
DTXSID1038666	Chlorpyrifos oxon	X		X		X		
DTXSID2020347	Coumaphos			X				X
DTXSID9020407	Diazinon		X	X		X		
DTXSID5037523	Diazoxon		X		X			
DTXSID5020449	Dichlorvos		X		X			
DTXSID9023914	Dicrotophos		X		X			
DTXSID7020479	Dimethoate			X		X		
DTXSID4032611	Ethoprop			X	X			
DTXSID0034930	Fosthiazate		X		X			
DTXSID9020790	Malaoxon	X			X			
DTXSID4020791	Malathion			X				X
DTXSID6024177	Methamidophos	X	X			X		
DTXSID1024209	Naled			X			X	
DTXSID4037580	Omethoate		X		X			

DTXSID	Chemical	Neg	Equiv	Pos	1	2	3	4
DTXSID4032459	Phorate			X		X		
DTXSID5024261	Phosmet			X		X		
DTXSID0024266	Pirimiphos-methyl			X				X
DTXSID3032464	Profenofos		X		X			
DTXSID1032482	Tebupirimfos			X	X			
DTXSID2022254	Terbufos			X			X	
DTXSID1024174	Tribufos			X			X	
DTXSID0021389	Trichlorfon			X		X		
DTXSID1032648	Z-Tetrachlorvinphos			X				X

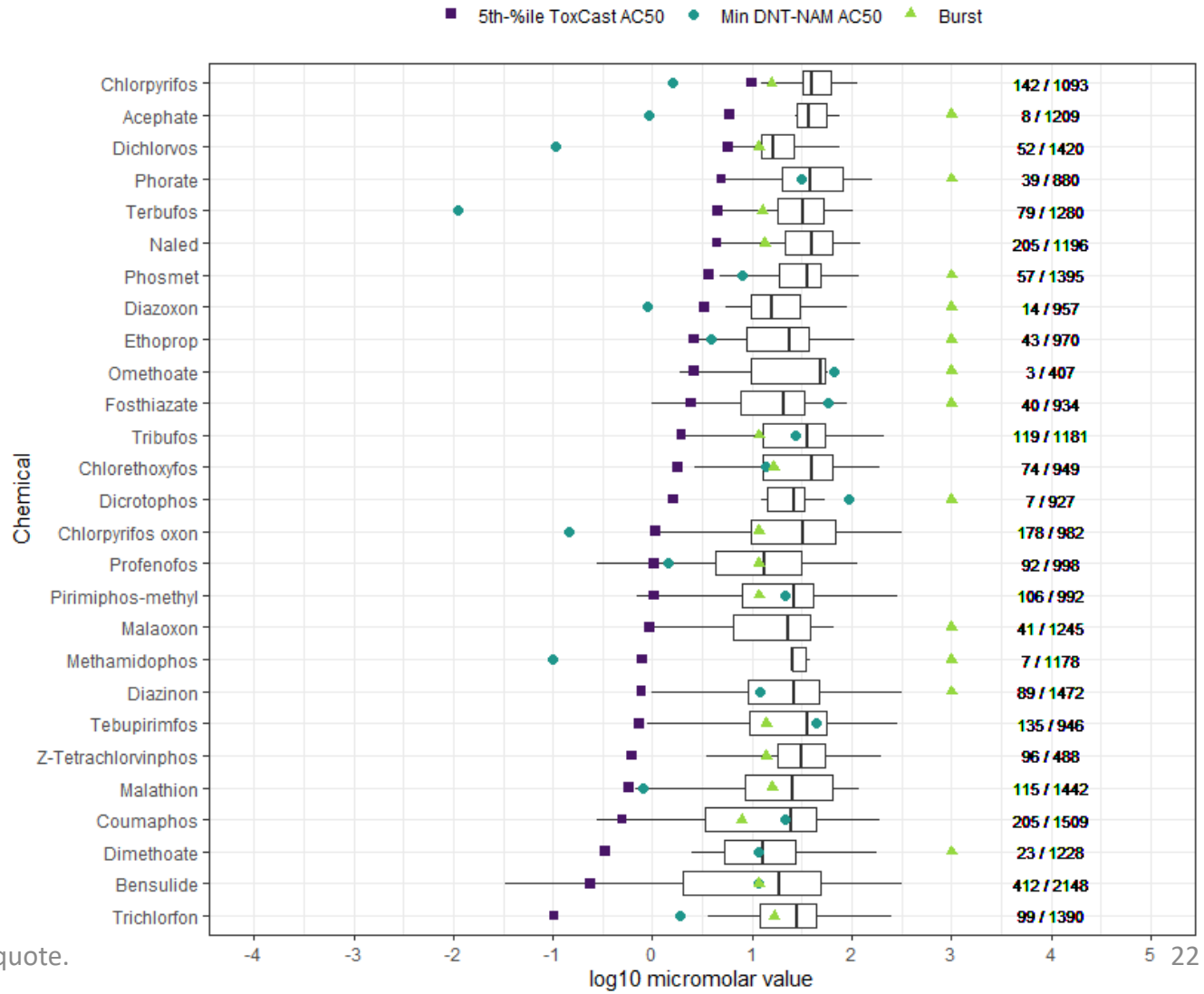
- **If activity is observed in the HCI assays, it is likely that the OP chemical will also be active in the MEA NFA.**

For some OPs, DNT-NAM AC_{50} < bioactivity estimate from the rest of ToxCast.

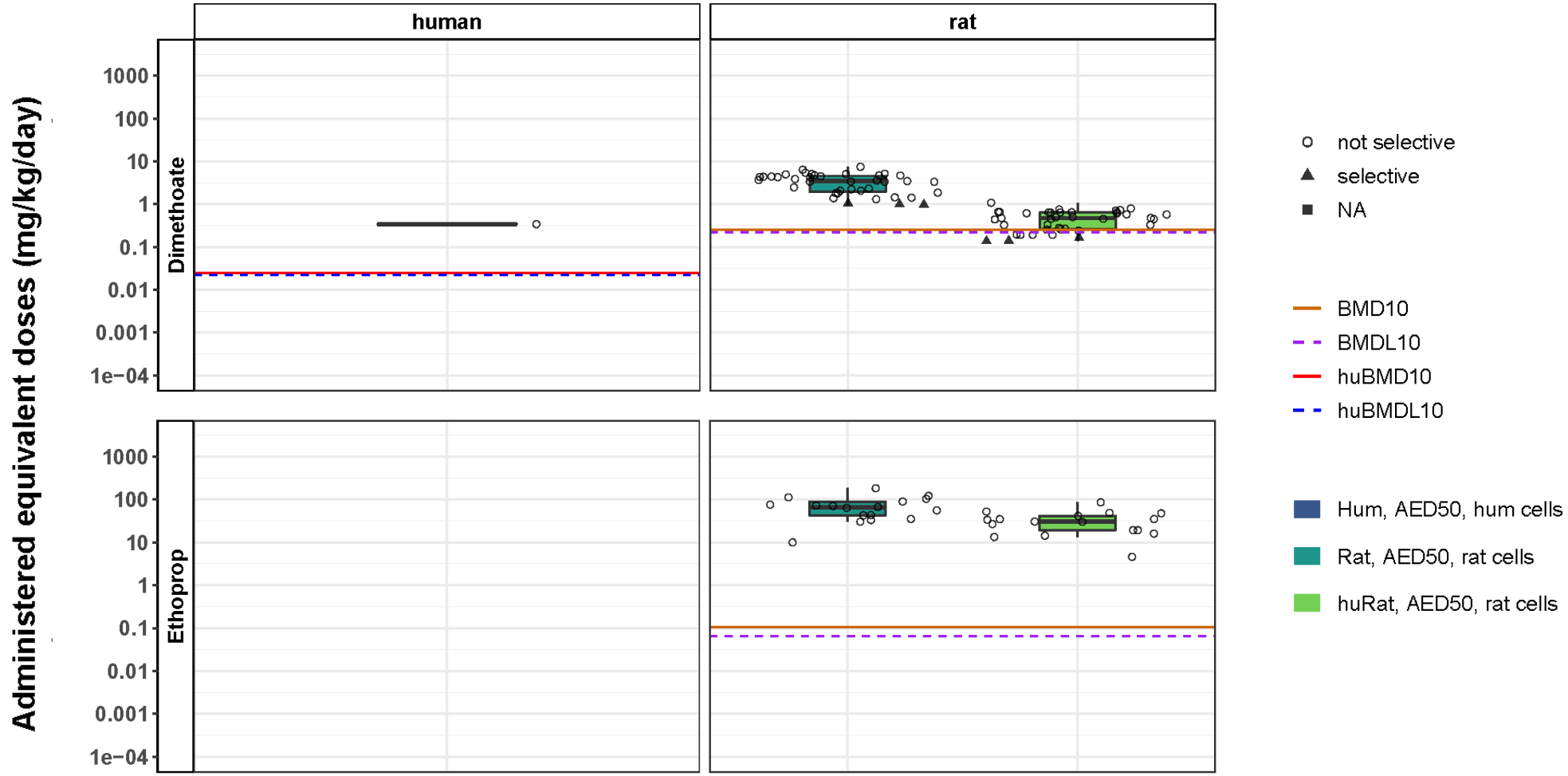
DNT-NAM battery may provide a more potent estimate of bioactivity for substances with minimum DNT-NAM AC50 < 5th percentile of filtered ToxCast AC50 values:

- Chlorpyrifos and chlorpyrifos oxon
- Acephate
- Dichlorvos
- Terbufos
- Diazoxon
- Methamidophos

Suggests that the DNT-NAM battery, in covering some new biology not previously in ToxCast, may yield bioactivity threshold concentrations lower than what is already available for some neuroactive substances in ToxCast.



AED_{50} to $BMD/BMDL_{10}$ comparisons



Summary of the AED_{50} to BMD/BMDL comparison

	Chemicals with AED50 values >>> BMD/BMDL comparator	Chemicals with lowest AED50 within 1 log10 order of magnitude of BMD/BMDL comparator	Chemicals with lowest AED50 approaching BMD/BMDL comparator	Missing in vitro data for comparison
Rat/HuRat	Coumaphos, diazoxon, dicrotophos, ethoprop, fosthiazate, omethoate	acephate, bensulide, chlorpyrifos, chlorpyrifos oxon, diazinon, dimethoate, malathion, methamidophos, and phorate	dimethoate and methamidophos (lower quartile of huRat AED_{50} values dichlorvos (huRat AED_{50}; only one positive rat assay endpoint) overlaps with the BMDL10 value, and it was not based on selective bioactivity in the DNT-NAM battery. malathion (huRat AED_{50} (selective) for also approach the BMD/BMDL10 values.	Malaoxon (negative in all assays)
Human	bensulide, chlorpyrifos, chlorpyrifos oxon, coumaphos, diazinon, dimethoate, malathion, methamidophos, phosmet, pirimiphos-methyl, tribufos, and trichlorfon		dichlorvos, only two AED_{50} values are available for comparison, and these values are centered around the BMD10/10 and BMDL10/10 values. terbufos, only 3 human AED_{50} values are available for comparison, and the lowest one of these values approaches the BMD10/10 value.	**Negative in all assays with human cells:** Acephate, diazoxon, dicrotophos, ethoprop, fosthiazate, omethoate, phorate, profenofos, and tebupirimfos Malaoxon was negative in all assays.

Summary for OP effects in DNT NAMs

- Overall, the BMDs for AChE inhibition are lower than those for DNT NAMS
  - This **decreases uncertainty** that the AChE inhibition values are health protective

- DNT NAM AED_{50} values approached the AChE BMD values for some compounds (dichlorvos, dimethoate, malathion)

- In 2020, a Scientific Advisory Panel reviewed these data and determined that NAMs can be used as part of a weight of evidence approach for decision-making regarding DNT.

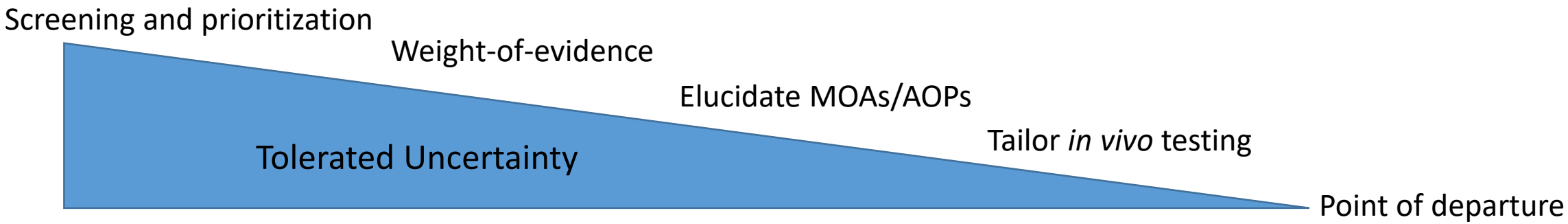
- Future Direction- these OPs will be tested in the other DNT NAMs in the battery in the next year.

Overall conclusion

EPA is currently using DNT NAMS for decisions in the following contexts:

- Screening and Prioritization
- Weight of Evidence

EPA/OPP regularly checks to see if DNT NAMs data are available



Thank you!
Questions?

EPA ORD Colleagues:

- Kathleen Wallace
- Theresa Freudenrich
- Bill Mundy (retired)
- Josh Harrill
- Jasmine Brown
- Katie Paul Friedman
- Melissa Martin
- Kelly Carstens Amy Carpenter (ORISE)
- Seline Choo (ORISE)
- Richard Judson
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EPA Program Office Colleagues

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