Developmental Toxicity of ToxCast™ Phase I and II Chemicals to Worms and Fish

EPA CompTox CoP
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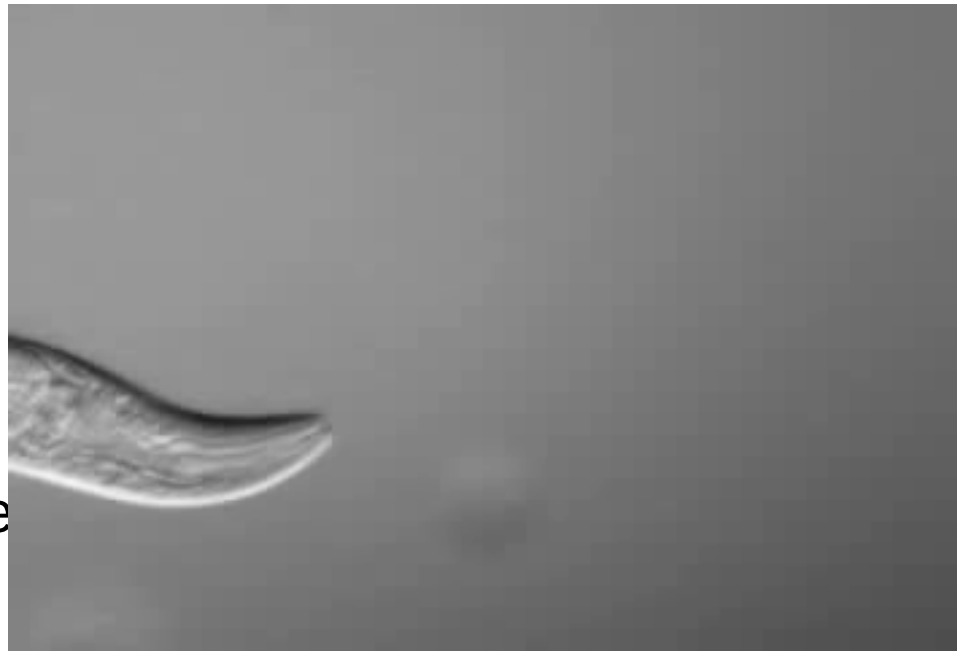
Outline

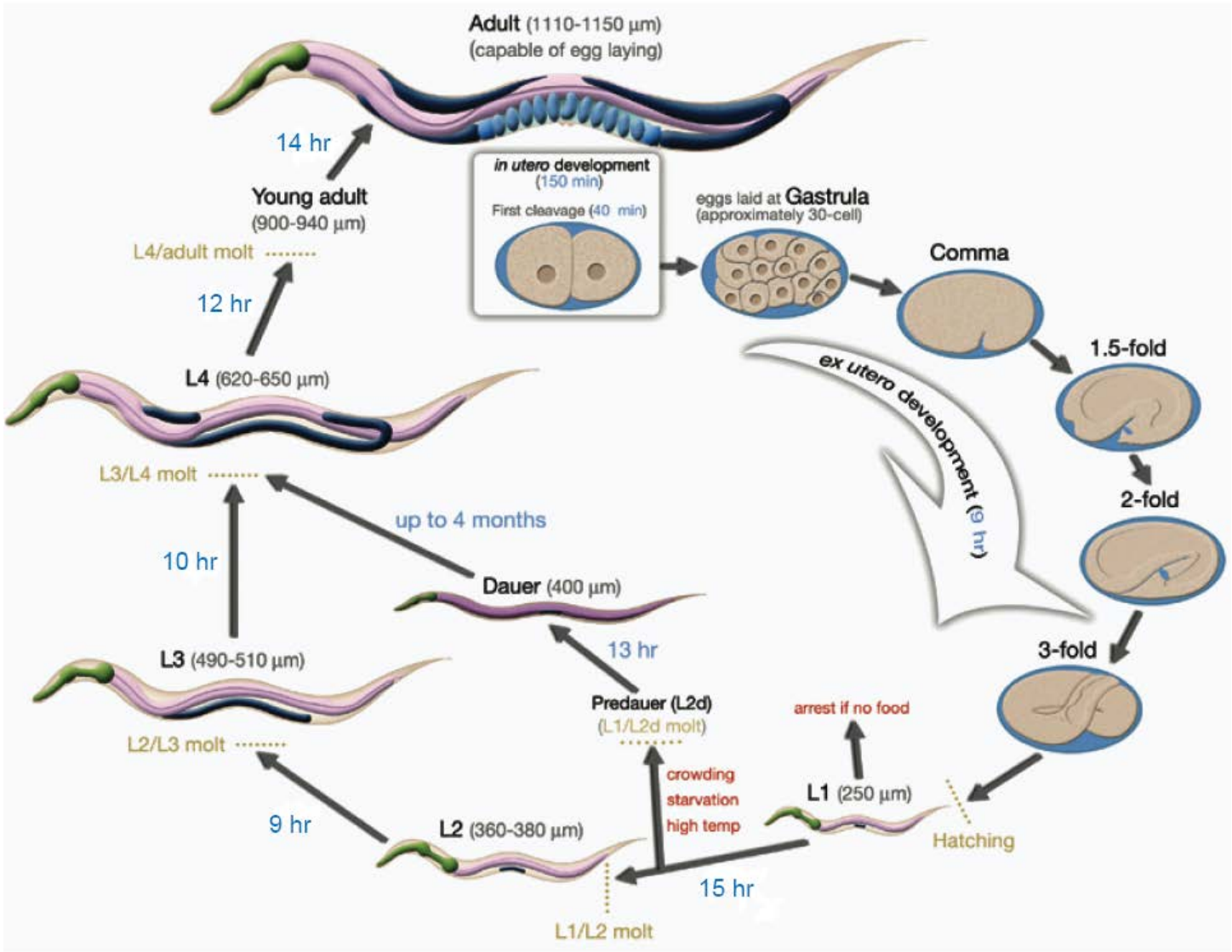
- Introduction to *C. elegans*
  - WormTox Larval Growth and Development Assay
- ToxCast Phase I&II Chemical Library
  - Effects on *C. elegans* larval development
  - Comparisons to two zebrafish embryonic development assays
  - Comparisons to rabbit and rat development
    - Rat_Dev and Rabbit_Dev composite indices in ToxRefDB
- Data Analysis Methods
  - LECs
    - t-test and Effect Size Threshold
  - Concentration Response
    - Hill model fit AC50s and Isotonic Regression

Characteristics of *C. elegans*

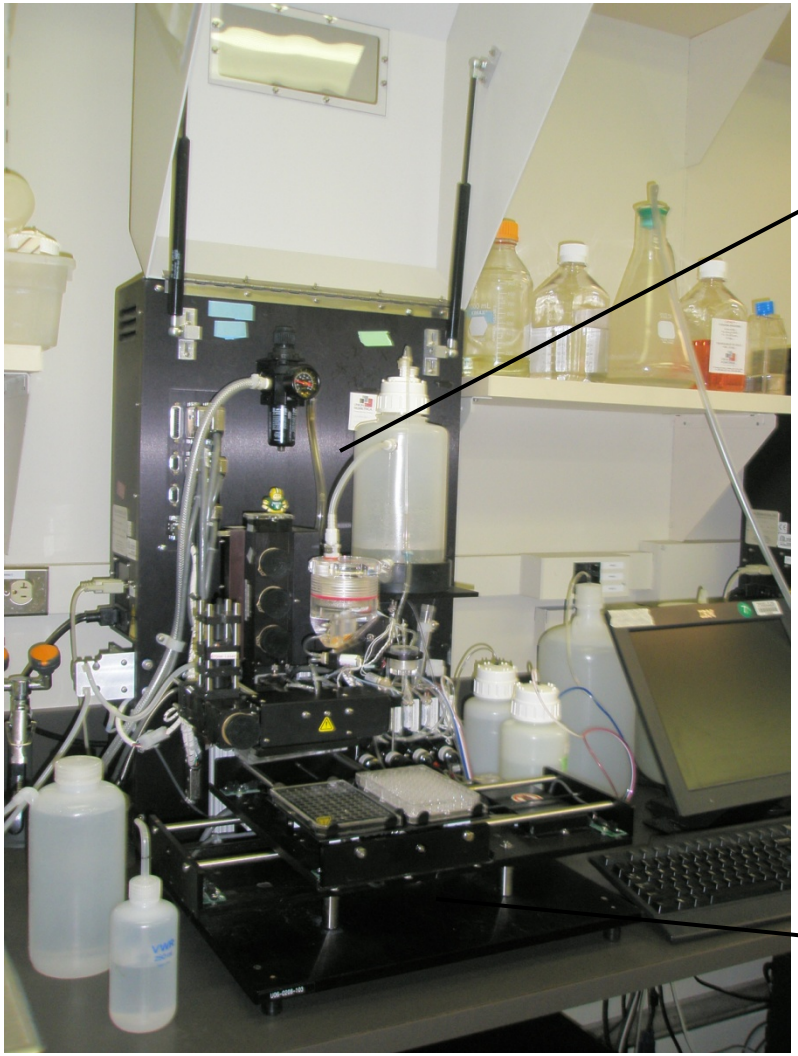
- Non-parasitic nematode
- ~ 1 mm in length
- Transparent
- 10 day life span
- Highly differentiated digestive, reproductive, muscular, and nervous systems



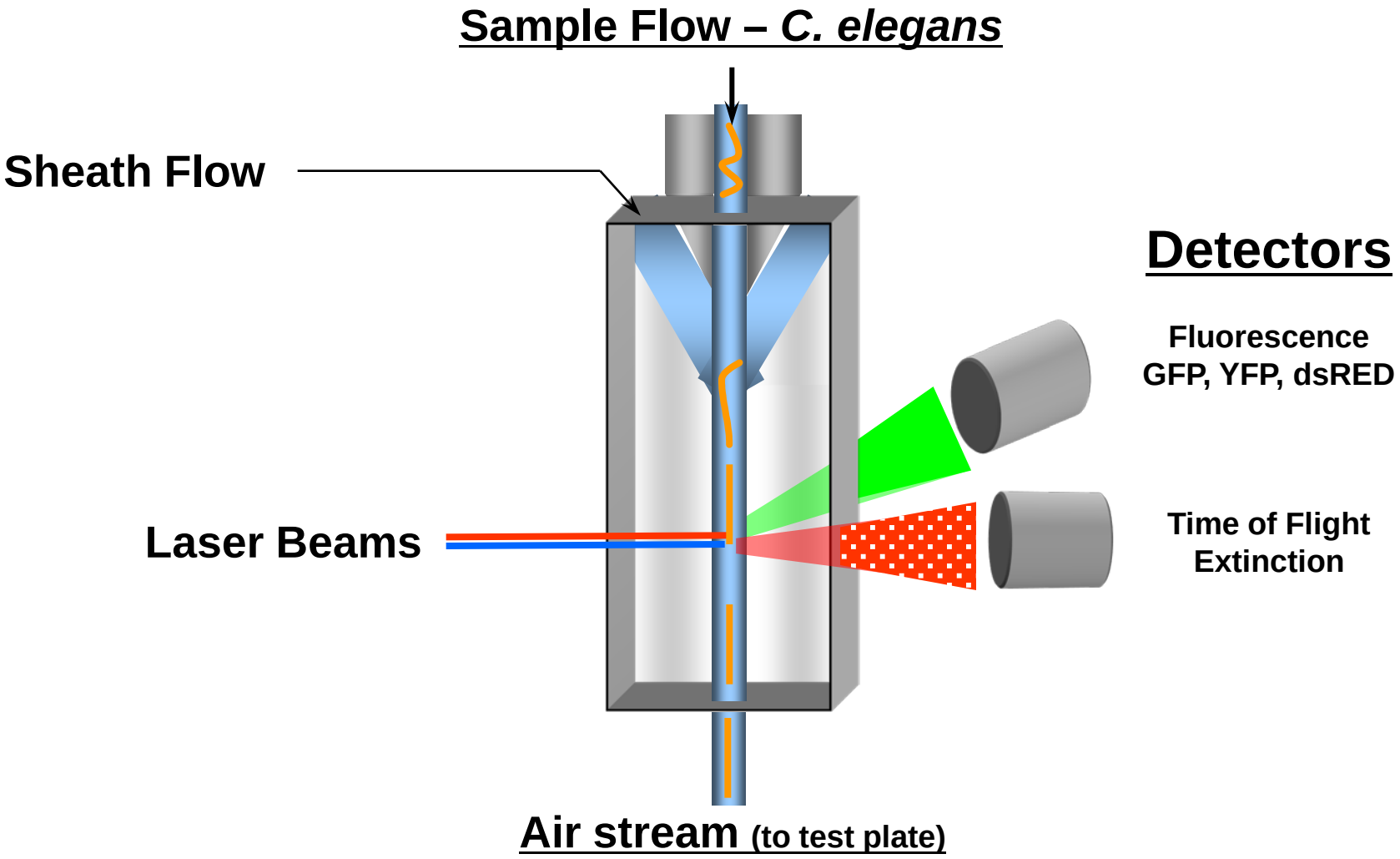
Development of *C. elegans*



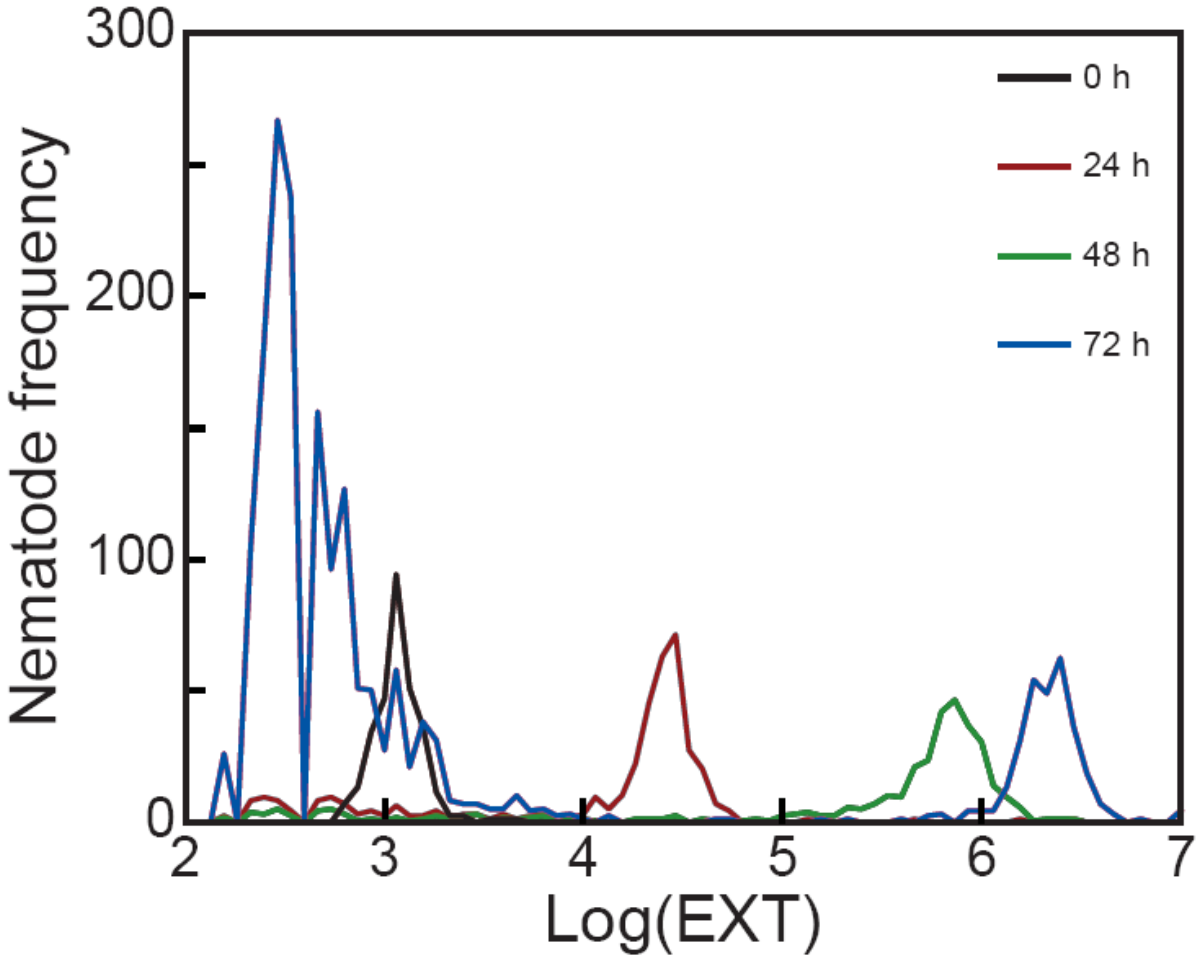
COPAS Biosort



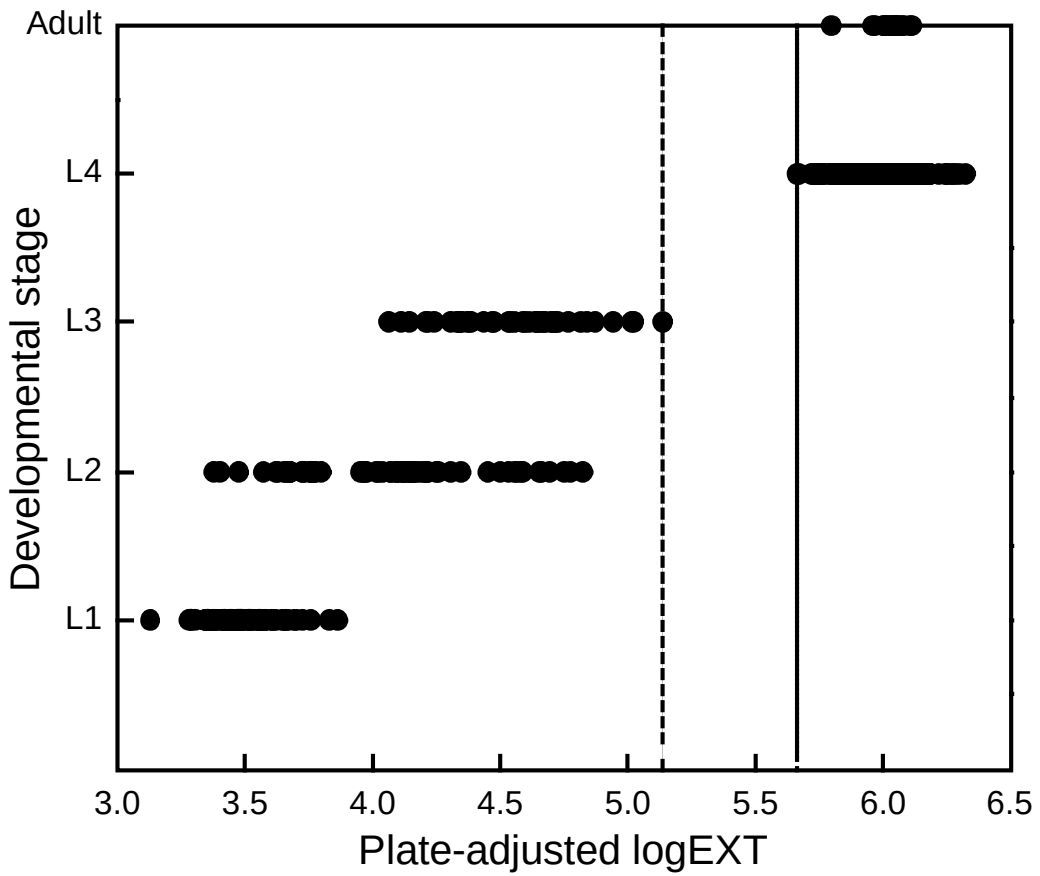
COPAS Biosort Optics



Growth of untreated *C. elegans*



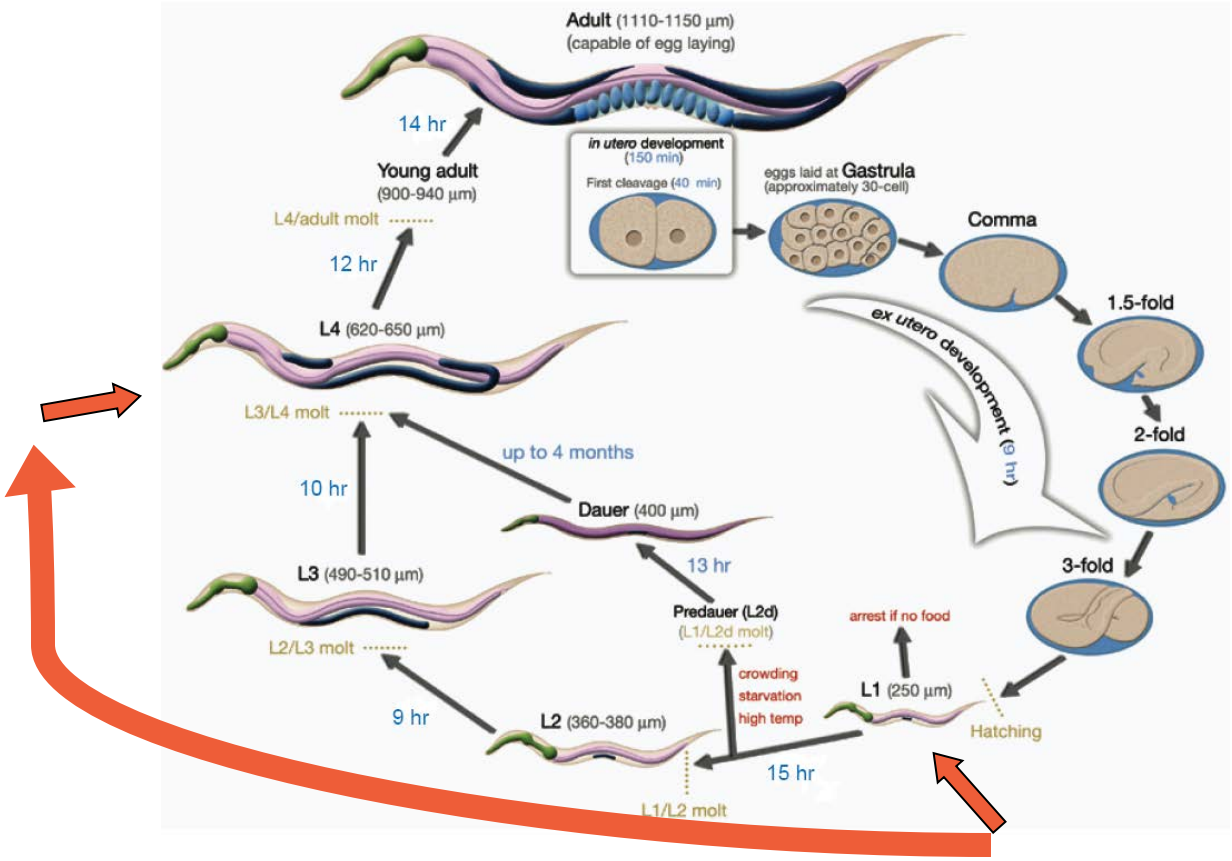
Linking log(EXT) to larval stage



- Effect size threshold provides direct link between the biological response (development) and the measured value (EXT)

C. elegans Growth Protocol

- Load 50 L1 stage nematodes to each well of a 96-well plate using COPAS Biosort
- Incubate at 20˚ C for 48 hours
- Measure size of nematodes (EXT) using Biosort

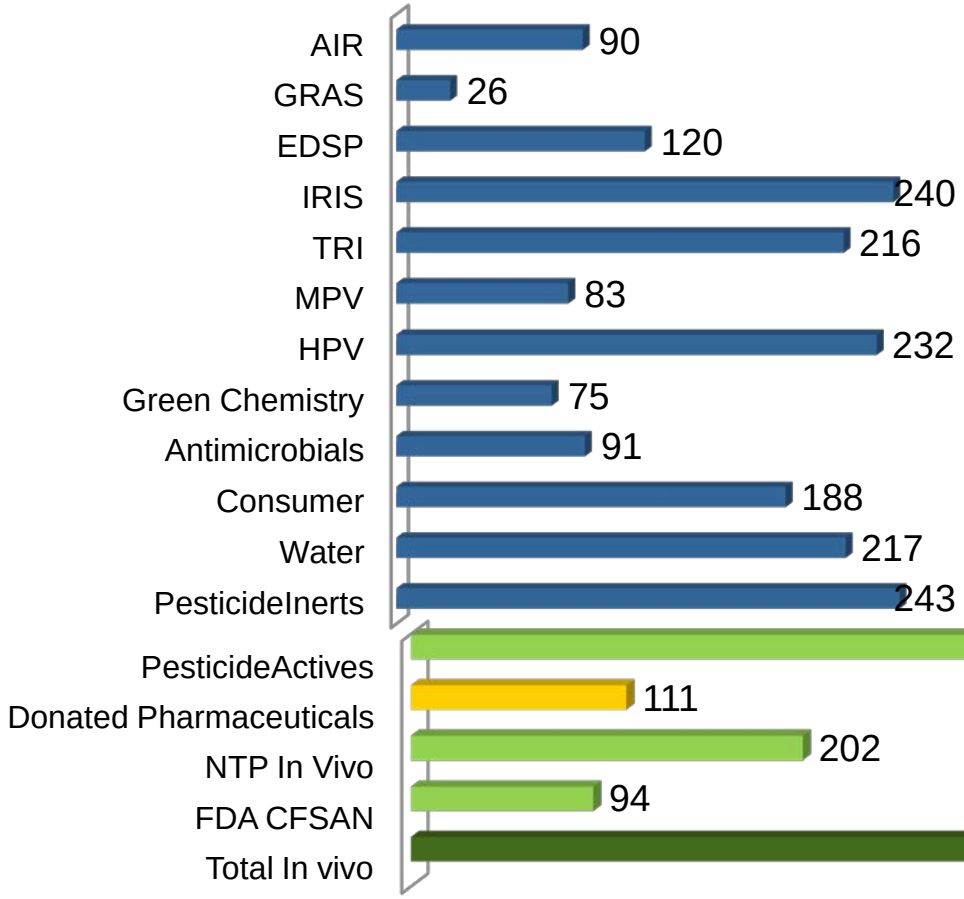


Z-factor

Positive control	Mean Z-factor	Std dev
4% DMSO	0.698	0.175
Parathion	0.779	0.0676
Dichlorvos	0.859	0.0336

- Z-factor provides measure of dynamic range and variability of response
- Excellent Z-factors range from 0.5-1
- Results from *C. elegans* growth assay highly consistent with clear separation between affected and unaffected groups

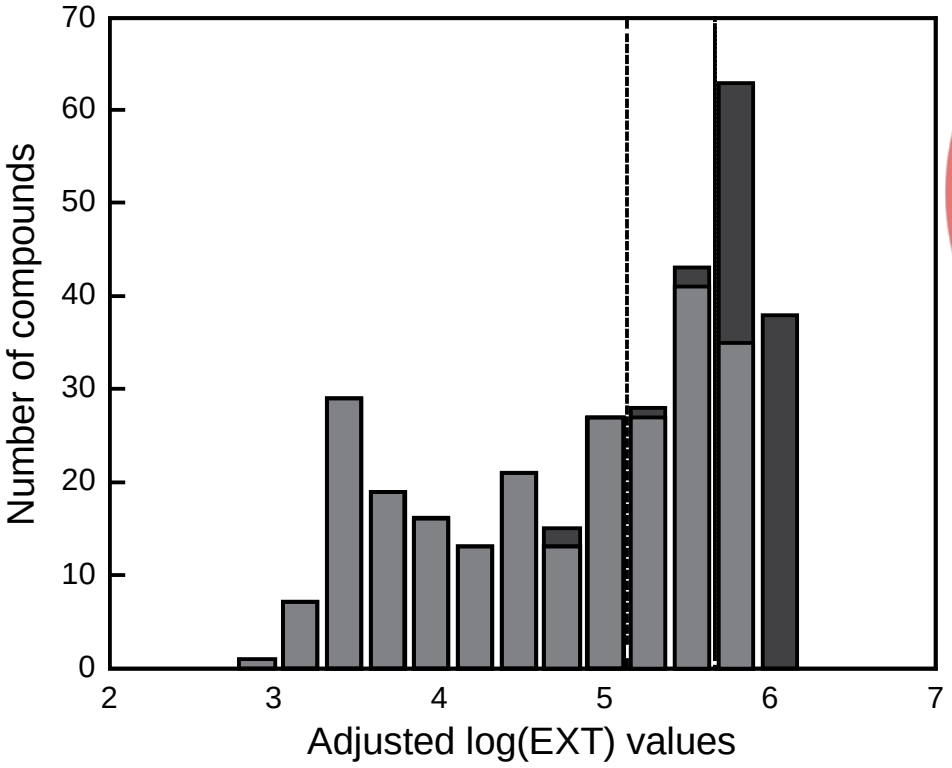
ToxCast PhI&PhII 960

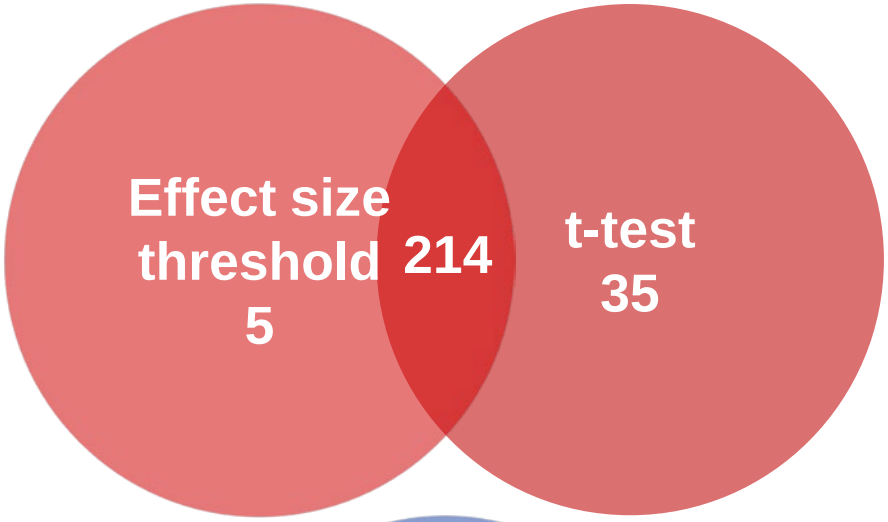


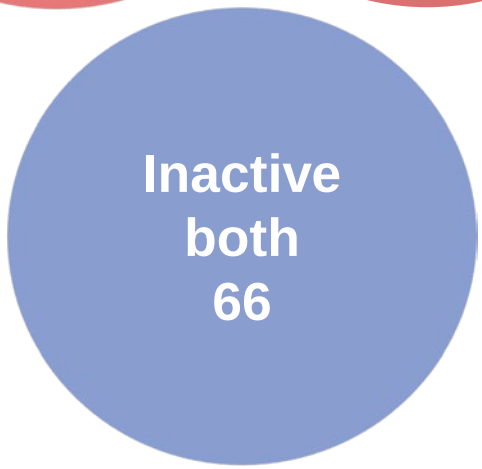
- 960 Total chemicals → 2740 total overlaps across 16 diverse inventories
- Excellent coverage of multiple high-interest inventories
- Broad diversity of chemical-use and types
- Large overlap with data-rich inventories

Slide provided by Ann Richard, U.S. EPA

Comparison between t-test and log(EXT) effect size threshold







*Phase I chemicals identified as active at the highest concentration tested (200 µM) using two analytical methods.

C. elegans LECs: Phase I & II

LEC (µM)	0.5	1	5	10	50	100	200
PHASE I	6.5% (19)	1.7% (5)	3.4% (10)	4.1% (12)	15.7% (46)	8.5% (25)	30.7% (90)
PHASE II	2.4% (16)	1.3% (9)	5.2% (35)	5.2% (35)	12.7% (86)	7.5% (51)	24.3% (164)
TOTAL	35	14	45	47	132	76	254
CUMULATIVE TOTAL	**35**	**49**	**94**	**141**	**273**	**349**	**603**

- 207/293 (71%) of Phase I active up to 200 µM; 86 inactive
- 396/676 (59 %) of Phase II active up to 200 µM; 280 inactive
- 603/969 (62 %) of Phase I & II active up to 200 µM; 366 inactive

Replicates: Phase I

Chemical	Mean size at 200 µM*	LEC	Hazard	AC50
Bensulide	3.71	50	active	16.3
	3.83	50	active	13.7
	3.49	100	active	79.8
Dibutyl phthalate	5.58	200	active	---
	5.26	50	active	21.3
Diclofop-methyl	4.92	200	active	179
	4.46	50	active	179
	4.47	50	active	56
EPTC	6.02	---	inactive	---
	5.70	---	inactive	---
Fenoxaprop-ethyl	5.01	100	active	76.7
	5.36	50	active	46.0
IPBC	3.00	200	active	138.7
	3.34	100	active	74.3

Replicates: Phase II

Chemical	Phase	Mean size	LEC	Hazard
Allethrin	II	5.39	100	active
	II	4.93	50	active
	II	5.22	200	active
Azoxystrobin	I	5.51	200	active
	II	5.60	200	active
	II	5.43	200	active
	II	5.44	50	active
Bisphenol A	I	5.37	200	active
	II	5.57	200	active
	II	5.38	200	active
	II	5.52	200	active

Replicates: Phase II

Chemical	Phase	Mean size	LEC	Hazard
Clorophene	I	3.61	200	active
	II	3.87	10	active
	II	3.83	50	active
	II	3.65	50	active
	II	3.92	0.5	active
	II	3.79	50	active
	II	3.91	50	active
Mancozeb	I	5.75	---	inactive
	II	5.35	200	active
	II	5.37	200	active
	II	5.24	100	active
	II	5.47	0.5	active
	II	5.24	100	active
	II	5.29	200	active

Replicates: Phase II

Chemical	Phase	Mean size	LEC	Hazard
Oryzalin	I	3.97	50	active
	II	4.95	50	active
	II	4.72	10	active
	II	4.57	10	active
PFOS	I	3.66	200	active
	II	3.06	5	active
	II	3.22	0.5	active
	II	3.39	5	active
Triadimenol	I	4.99	200	active
	II	5.63	200	active
	II	5.94	---	inactive
	II	5.79	---	inactive
Triclosan	I	3.98	50	active
	II	3.83	10	active
	II	4.06	50	active
	II	4.15	10	active

Most active in Phase I screen:
Decreased growth at lowest concentration (0.5 µM)

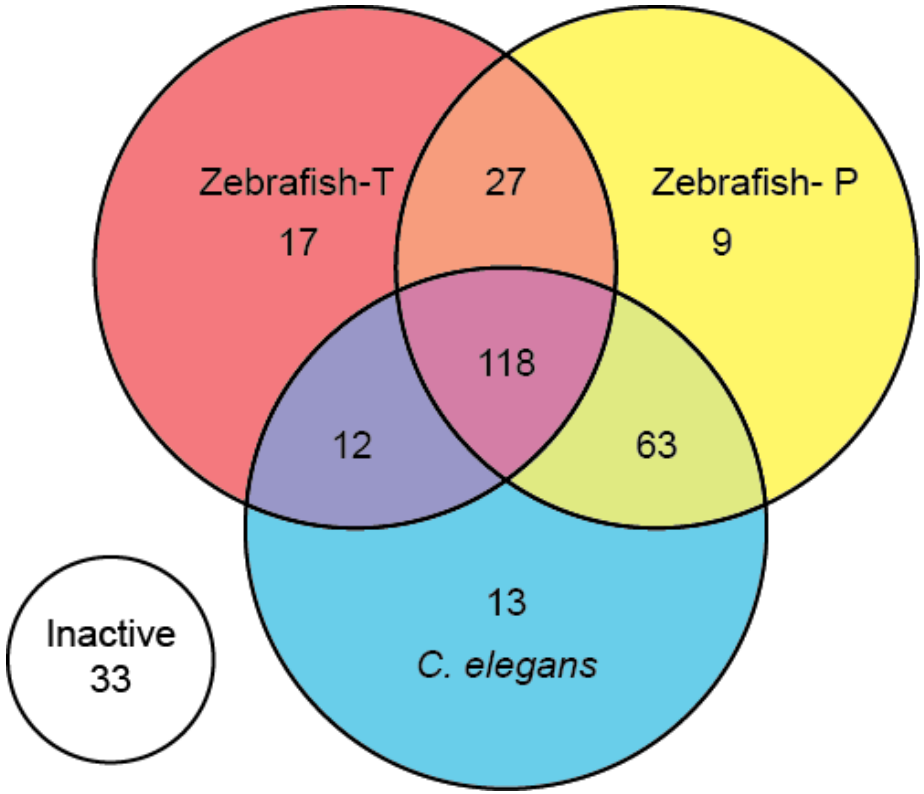
- Milbemectin
- Chlorpyrifos oxon
- Abamectin
- Dicofol
- Tebufenpyrad
- Parathion
- Fentin
- (Z,E)-Fenpyroximate
- Methylene bis(thiocyanate)
- Oxyfluorfen
- Coumaphos
- Methoxychlor
- Quinoxyfen
- Isazofos
- Pyridaben
- Fenamiphos
- Emamectin benzoate
- Pyriproxyfen
- Molinate
- Mesosulfuron-methyl

Most active in Phase II screen:
Decreased growth at lowest concentration (0.5 µM)

- Tributyltin chloride
- Stannane, tributyl…
- Hexamethyl-p-rosaniline
- Benzo[b]fluoranthene
- O-Ethyl O-phenylphos
- Mancozeb
- N-Lauryl-2-pyrrolidone
- Phenylmercuric acetate
- 4-Dodecylbenzenesulfonic acid
- Octrizole
- Heptadecafluorooctanesulfonic acid
- Octhilinone
- 6-chloro-…-indole-1-carboxamide
- 3,4,4'-Trichlorocarbanilide
- Chlorpyrifos
- Darbufelone mesylate
- p,p’-DDD
- Clorophene
- PFOS

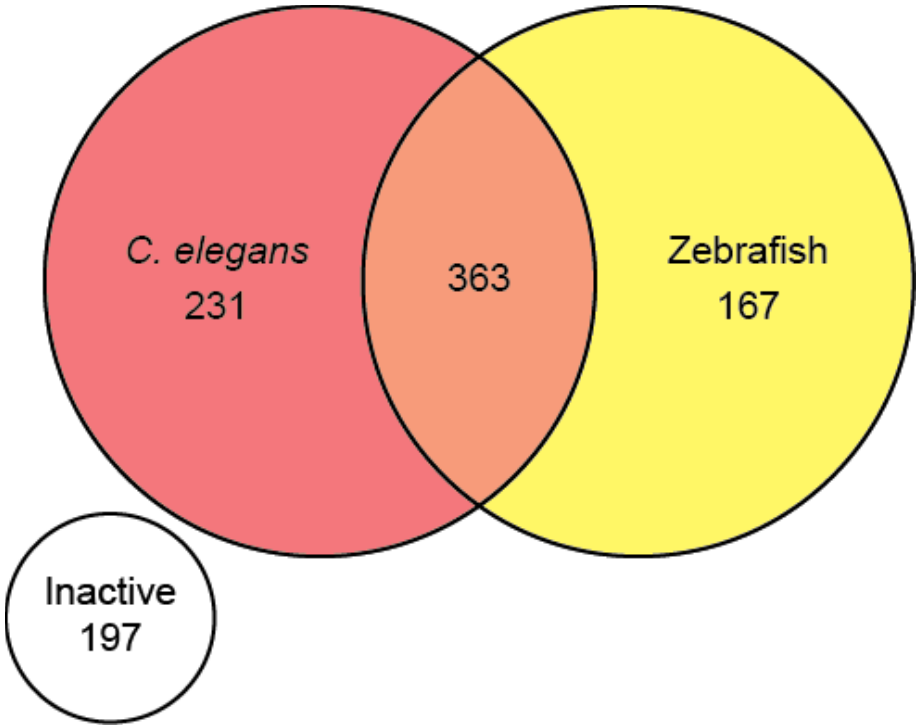
INTERSPECIES COMPARISONS

Comparison of Phase I compound activity in *C. elegans* and zebrafish



- **Concordance**
  - Worms and Tanguay fish = 59%
  - Worms and Padilla fish = 79%
  - Tanguay and Padilla fish = 65%
  - Rat and rabbit = 58%
- 292 unique compounds

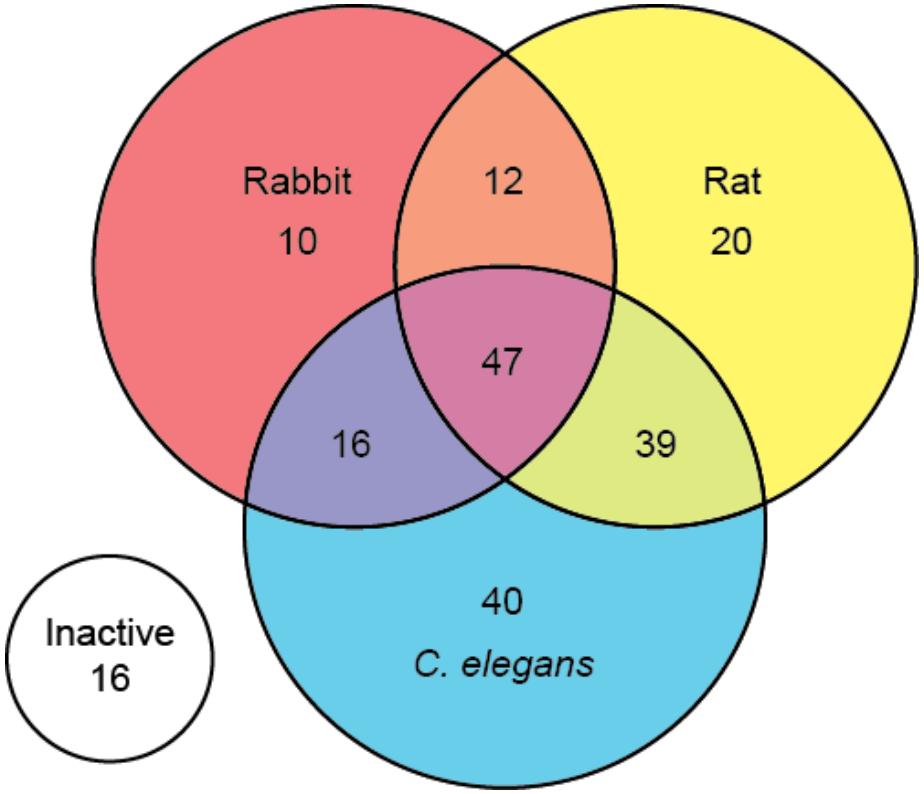
Comparison of Phase I&II compound activity in *C. elegans* and zebrafish (Tanguay)



- **Concordance**
  - 958 unique compounds
  - 58.5%
- **Potency rank**
  - Compare rank of LELs
  - Kendall's tau = 10.3% ($p = 4.022e^{-5}$)
  - Slight but significant correlation

*Compounds identified as active if no LEC up to highest concentration tested: in C. elegans (200 µM) and zebrafish (64 µM).

Prediction of rat and rabbit developmental outcomes*



- **Balanced Accuracy**
  - (Sensitivity+Specificity)/2
  - Worms predicting rabbits = 52.7%
  - Worms predicting rats = 52.3%
- Worms have greatest number of actives = higher sensitivity than specificity

*ToxRef database composite LECs for 27 fetal developmental outcomes in rat and 26 in rabbits including weight reduction, structural abnormalities, and general fetal pathology (Knudsen et al. 2009)

Prediction of rat and rabbit developmental outcomes*

	Predictor species		
Predicted	***C. elegans***	**Zebrafish (P)**	**Zebrafish (T)**
Rabbits	BA = 52.7% Sensitivity= 74.1% Specificity= 31.3%	BA = 44.6% Sensitivity= 68.2% Specificity= 20.9%	BA = 49.6% Sensitivity= 60.0% Specificity= 39.1%
Rats	BA = 52.3% Sensitivity= 72.9% Specificity= 31.7%	BA = 52.2% Sensitivity= 76.3% Specificity= 28.0%	BA = 50.6% Sensitivity= 61.0% Specificity= 40.2%

*ToxRef database composite LECs for 27 developmental outcomes in rats and 26 in rabbits

**200 unique chemicals with data for all species

Activity across chemical classes

Chemical class	*C. elegans*	Zebrafish^{P}	Zebrafish^{T}	Rat	Rabbit
Amides	14/24	18/24	18/24	10 / 21	8 / 22
Anilides	9/14	11/14	12/14	7 / 14	6 / 14
Carbamate	8/15	12/15	10/15	10 / 14	7 / 14
Conazole	18/18	18/18	16/18	16/16	11/16
Organophosphate	28/35	30/35	20/35	8/25	6/25
Phenoxy	8/12	11/12	4/12	6/8	3/11
Pyrethroid	11/12	12/12	8/12	6/12	4/10
Pyridine	7/10	6/10	4/10	3/7	3/6
Urea	5/8	6/8	5/8	5/6	3/5

Chemical class concordance

Chemical Class	*C. elegans* and Zebrafish^{P}	*C. elegans* and Zebrafish^{T}	Zebrafish^{P} and Zebrafish^{T}
Amide	18/24 = 0.75	12/24 = 0.50	18/24 = 0.75
Anilide	12/14 = 0.86	7/14 = 0.50	9/14= 0.64
Carbamate	9/15 = 0.60	11/15 = 0.73	11/15 = 0.73
Conazole	18/18 = 1	16/18 = 0.89	16/18 = 0.89
Organophosphate	29/35 = 0.83	17/35 = 0.48	19/35 = 0.54
Phenoxy	9/12 = 0.75	4/12 = 0.33	5/12 = 0.42
Pyrethroid	11/12 = 0.92	7/12 = 0.58	8/12 = 0.67
Pyridine	9/10 = 0.90	5/10 = 0.50	6/10 = 0.60
Urea	3/8 = 0.38	4/8 = 0.50	3/8 = 0.38

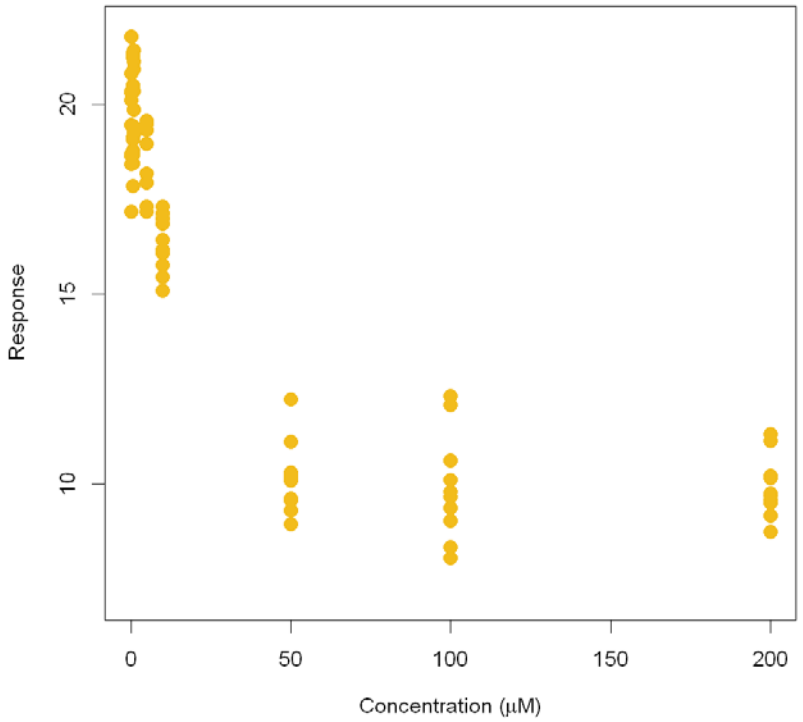
Prediction by chemical class: BAs

Chemical Class	*C. elegans* to Rat	Zebrafish^{P} to Rat	Zebrafish^{T} to Rat	*C. elegans* to Rabbit	Zebrafish^{P} to Rabbit	Zebrafish^{T} to Rabbit
Amide	0.623	0.632	0.536	0.759	0.714	0.420
Anilide	0.571	0.571	0.500	0.813	0.688	0.333
Carbamate	0.500	0.700	0.425	0.643	0.714	0.714
Conazole	---	---	---	0.500	0.500	0.409
Organophosphate	0.434	0.526	0.360	0.496	0.579	0.596
Phenoxy	0.500	0.750	0.750	0.521	0.333	0.313
Pyrethroid	0.417	0.500	0.333	0.375	0.500	0.333
Pyridine	0.625	0.750	0.417	0.333	0.500	1.00
Urea	0.300	0.300	0.9000	0.750	0.167	0.750

CONCENTRATION RESPONSE

Concentration Response

- LECs identify chemicals with significant response at each concentration
- But, C-R data collected for every chemical
- Can determine additional measures of chemical toxicity (efficacy, potency).

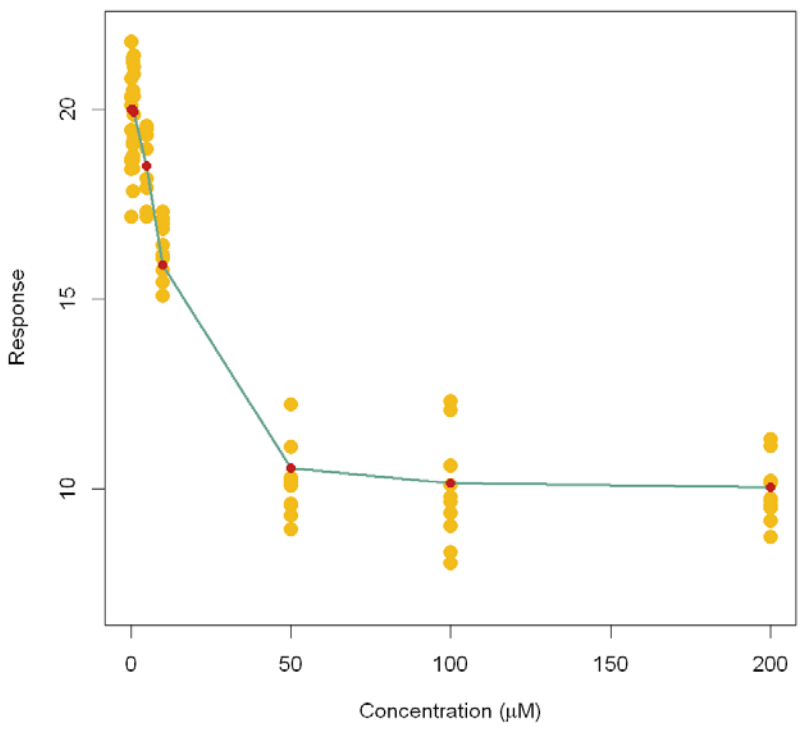


Concentration Response

- Hill equation used to model concentration-response
  - Calculated AC50s for active chemicals but problematic
- With the *C. elegans* growth assay, see increasing risk with increasing exposure
  - I.e., the response is *monotonic*.
- Isotonic regression provides a good balance of complexity and simplicity

Isotonic regression

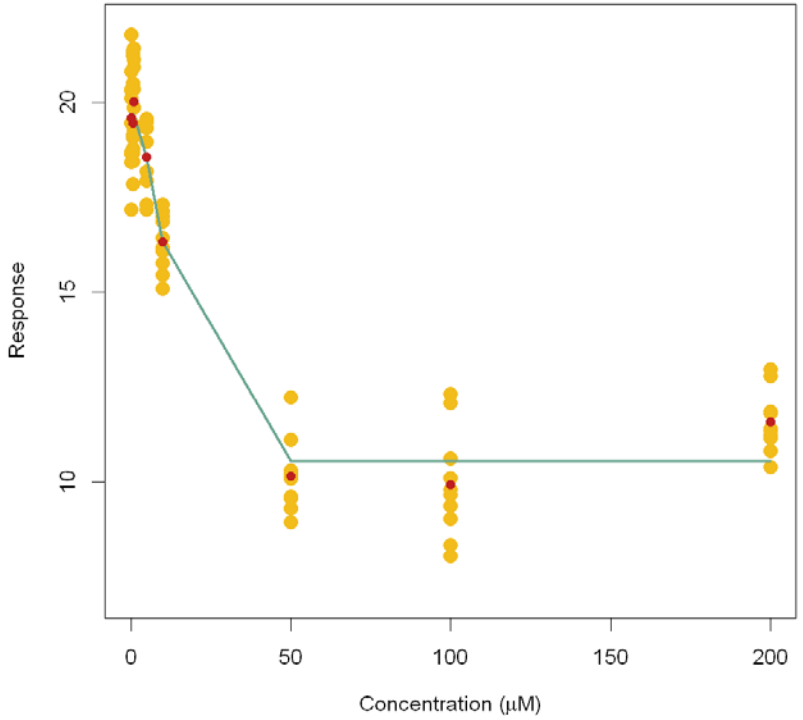
- Fits model using pool adjacent violators algorithm (PAVA[2])
- Linear interpolation between fitted values
- If means are decreasing, the isotonic fit is identical to the data means



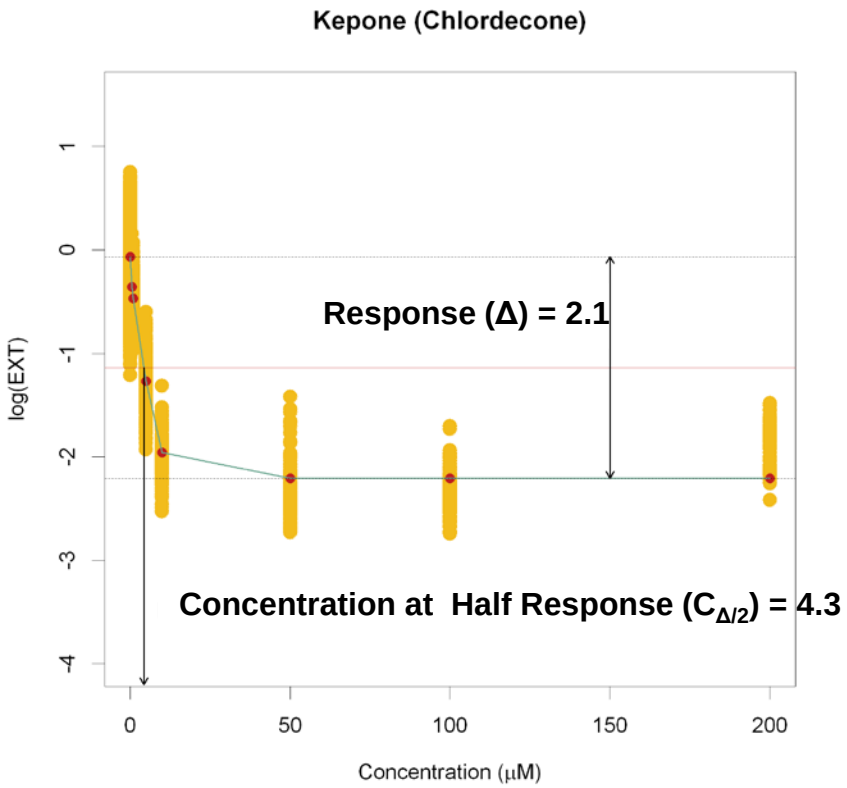
[2]Barlow, R. E., Bartholomew, D., Bremner, J. M. & Brunk, H. D. (1972), *Statistical inference under order restrictions; the theory and application of isotonic regression*, Wiley, New York.

Isotonic regression

- Otherwise, isotonic regression finds a solution that smooths the non-monotonic region

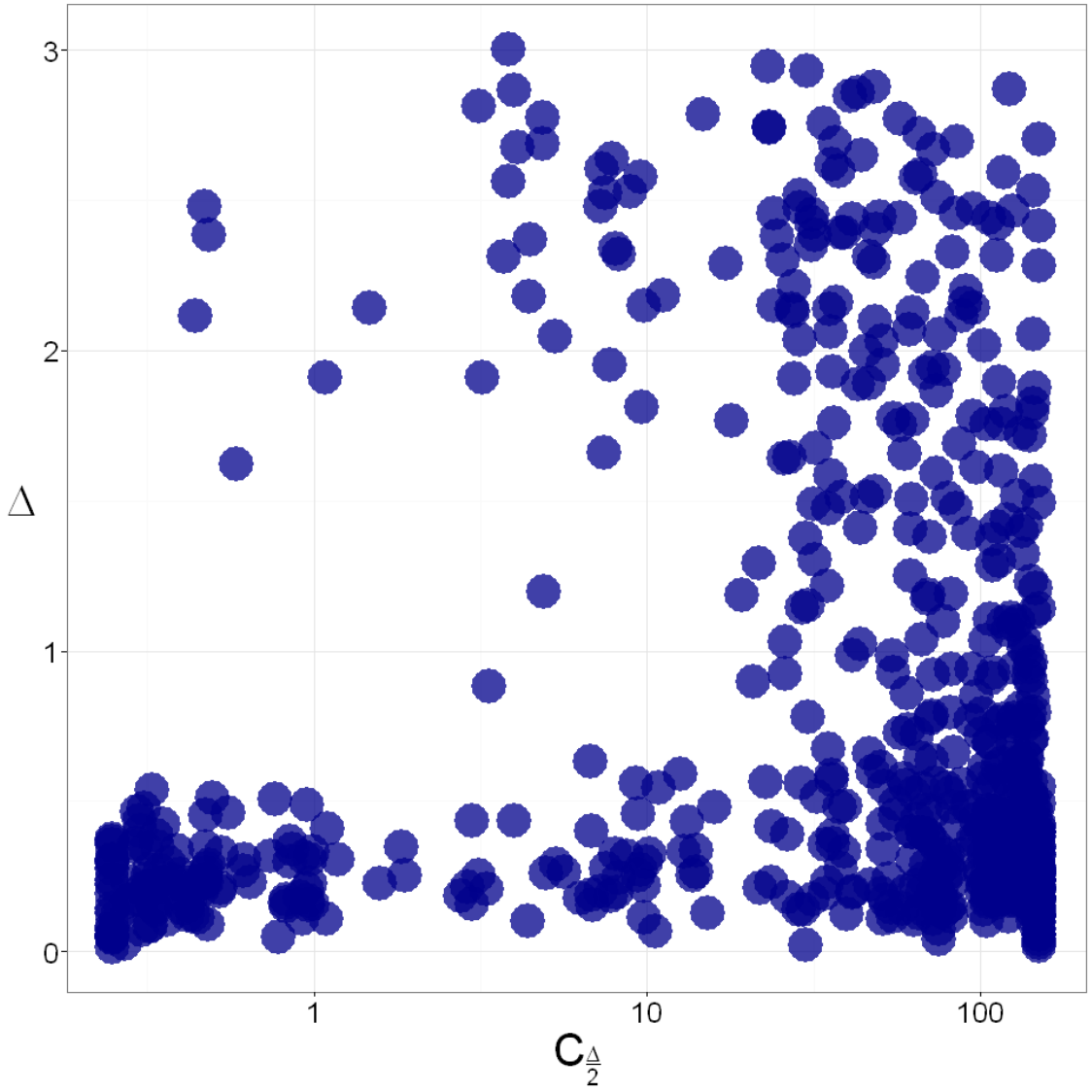


Toxicity parameters

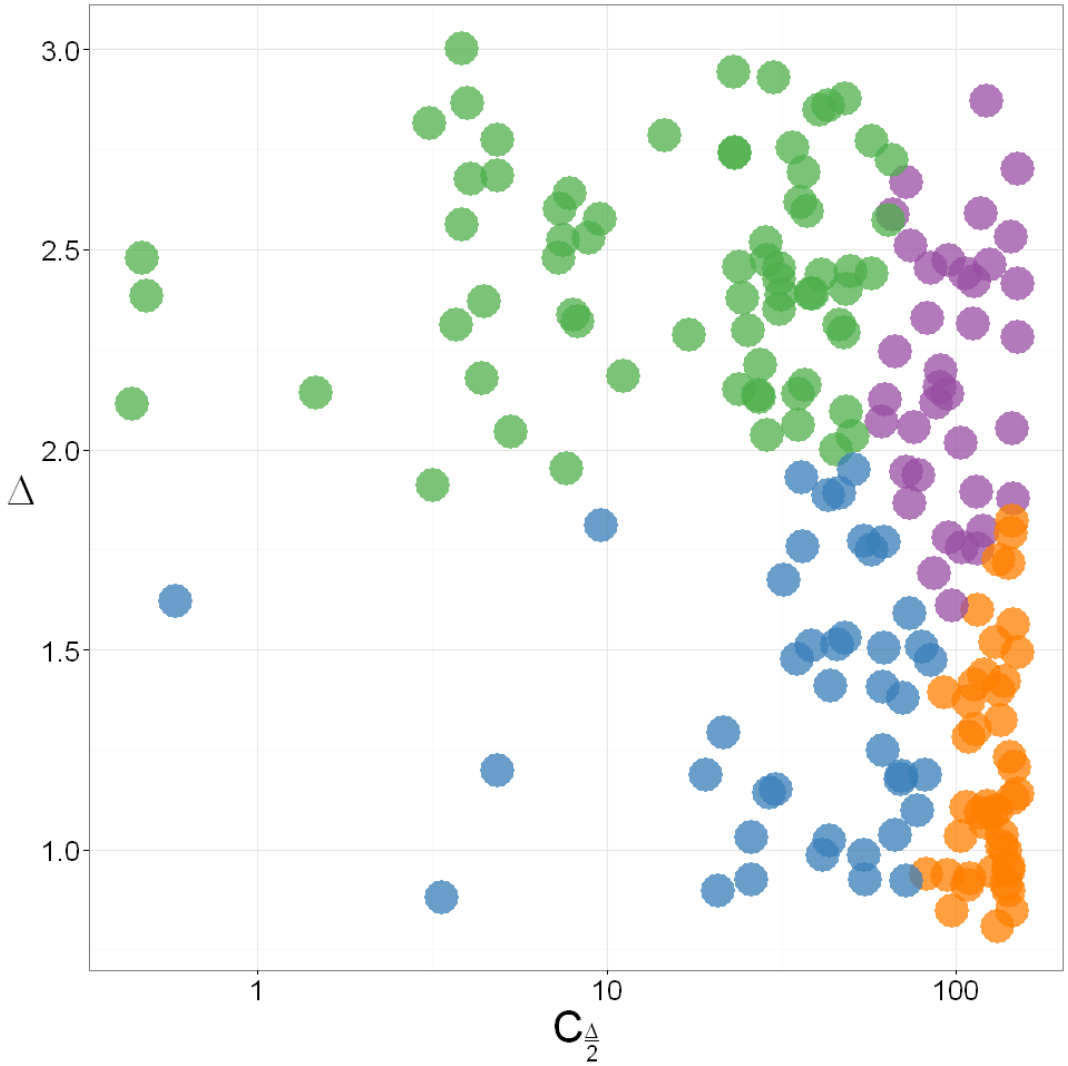


- For each of the Phase II chemicals:
  - Fit isotonic regression model and calculate:
    - change in response between control and highest concentration (Δ)
    - concentration where half of Δ is reached ($C_{\Delta/2}$)

Summary of toxicity parameters

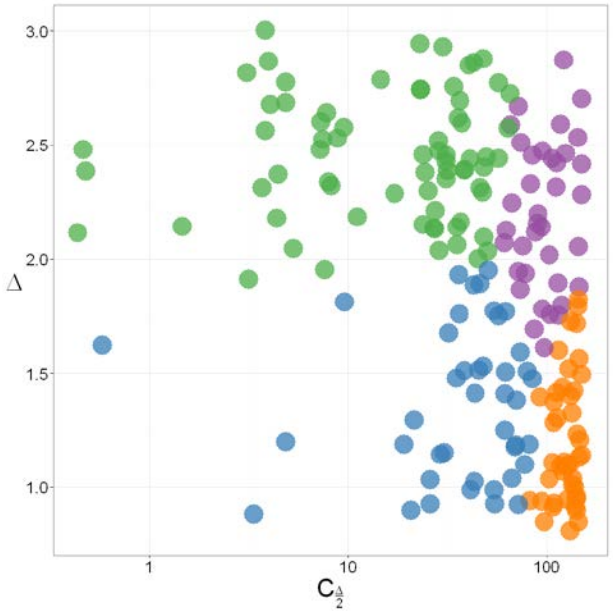


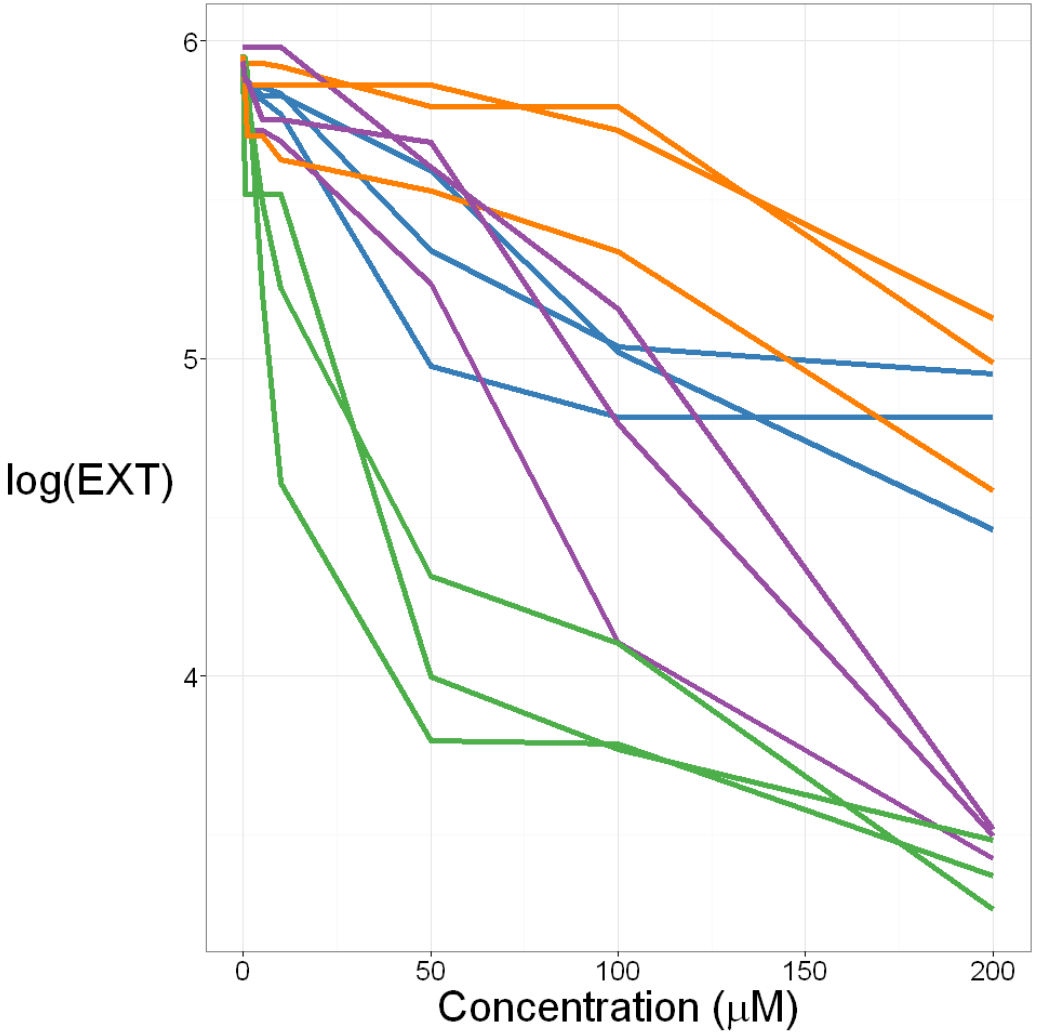
Summary of toxicity parameters



- Active chemicals from Phase II
- Color: cluster assigned by PAM
- Clusters do not represent formal toxicity classes

Summary of toxicity parameters





Randomly selected 3 concentration response curves from each of the 4 clusters.

Summary

- *C. elegans* growth assay
  - consistent and reliable according to Z-factor
  - predicts rat and rabbit development at least as well as zebrafish
- Two approaches to rank compound activity
- Single concentration → LEC
  - t-test: so much power that even small size differences are statistically but not biologically relevant
  - Effect size threshold: adds biological relevance
- Concentration-response
  - AC50
  - Δ and $C_{\Delta/2}$ → Integrate size thresholds to identify chemicals of most concern

Questions/comments?

Boot-strapped t-statistics

- Data have plate effects, column effects, and well effects
- Using data from 24 control plates
  - Adjusted for plate effects by subtracting plate means
  - For each treatment group (column), 12500 samples of 4 well means with corresponding well means from the vehicle control were drawn and t-statistics calculated
  - Formed 8 null distributions for t-statistics that include column effects between designated treatment column and vehicle control column
- For each compound/concentration, t-statistics were calculated using 4 well means with 4 plate control well means and compared to the null distributions
  - If fewer than 0.05/#(compounds) observations from the null distributions were found to the right of the estimated t-statistic, the compound was called significantly different from controls