NTP
National Toxicology Program

In Vitro Disposition of Tox21 Chemicals: Initial Results and Next Steps

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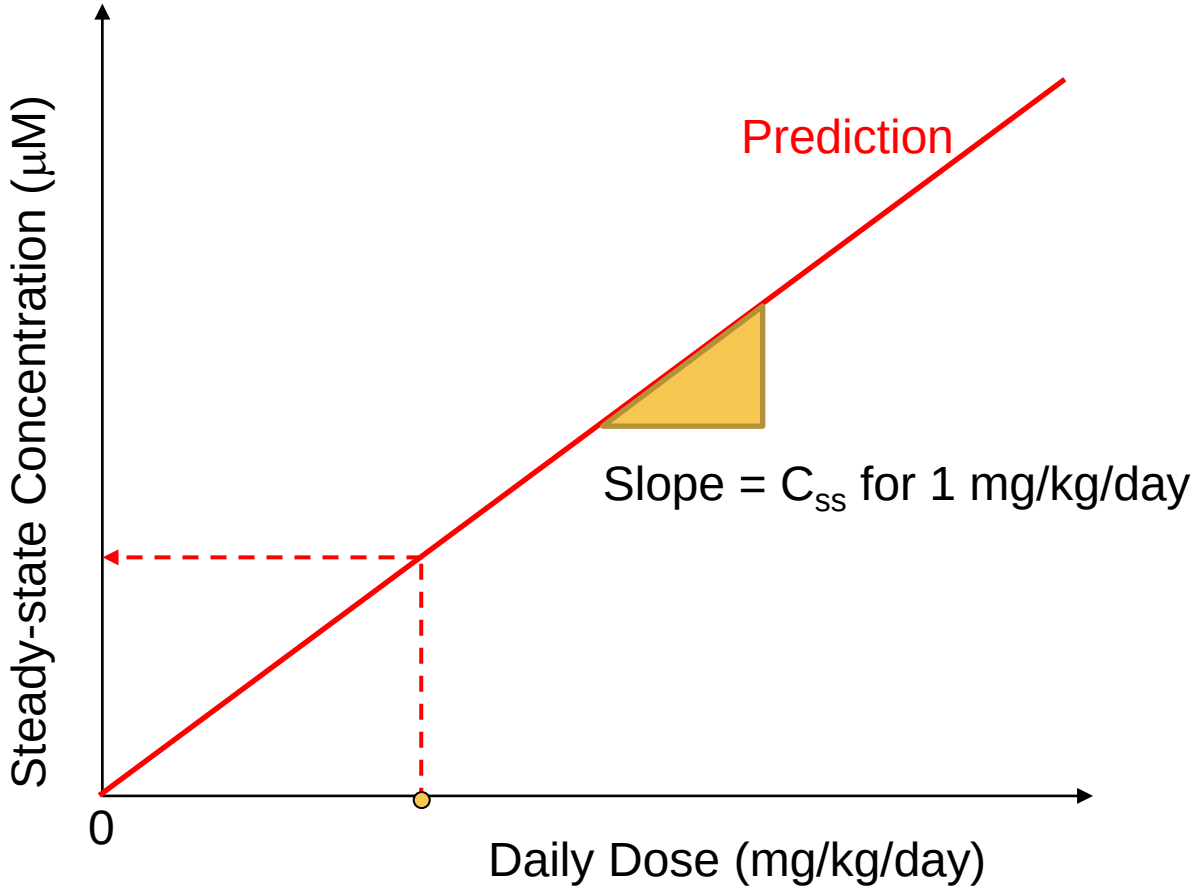
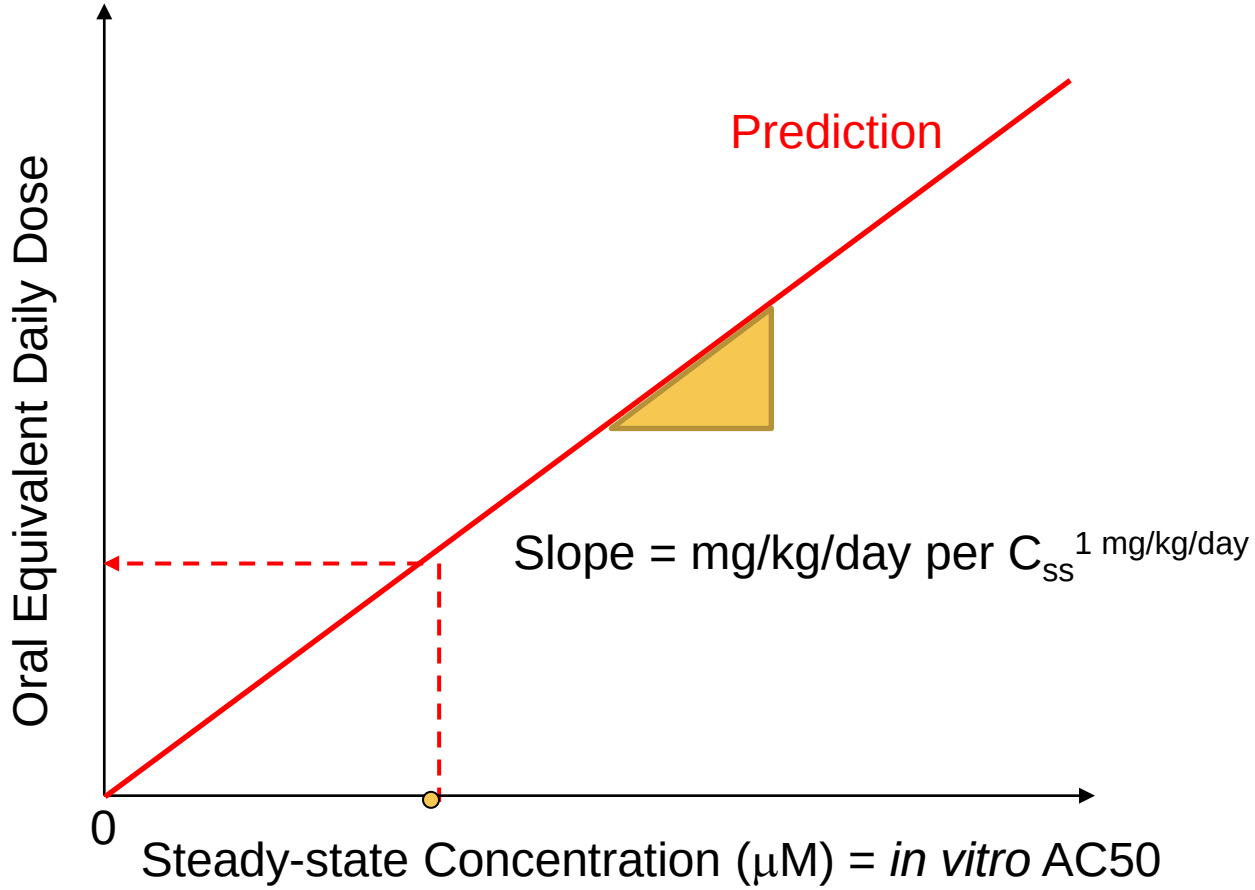
IVIVE predictions currently rely on $C_{nominal}$

- What if the nominal concentration in an assay fails to represent the cellular concentration?
- IVIVE prediction accuracy may be affected.

Steady State IVIVE Assumption

Blood::tissue partitioning ≈ cells::medium partitioning





$$C_{ss} = \frac{\text{oral dose rate}}{(\text{GFR} * \text{F}_{ub}) + \left(\text{Q}_l * \text{F}_{ub} * \frac{Cl_{int}}{\text{Q}_l + \text{F}_{ub} * Cl_{int}}\right)}$$

Wetmore *et al*. (2012)

- Swap the axes (this is the "reverse" part of reverse dosimetry)
- Can divide bioactive concentration by C_{ss} for for a 1 mg/kg/day dose to get oral equivalent dose

The question is…

To date, *in vitro* partitioning has been empirically evaluated for very few chemicals and very few model systems; thus, it is unknown for how many chemicals and to what degree differential chemical partitioning affects the accuracy of IVIVE predictions made across the Tox21 chemical library.

EPA New Approach Methods Work Plan

- Understanding the *in vitro* distribution of chemicals is essential to the future utility of NAMs such as *in vitro* assays in a regulatory context
- This work fits into the **EPA NAMs workplan** under **Objective 3** by helping to “Establish Scientific Confidence in NAMs and Demonstrate Application to Regulatory Decisions”

What factors predominately influence *in vitro* partitioning?

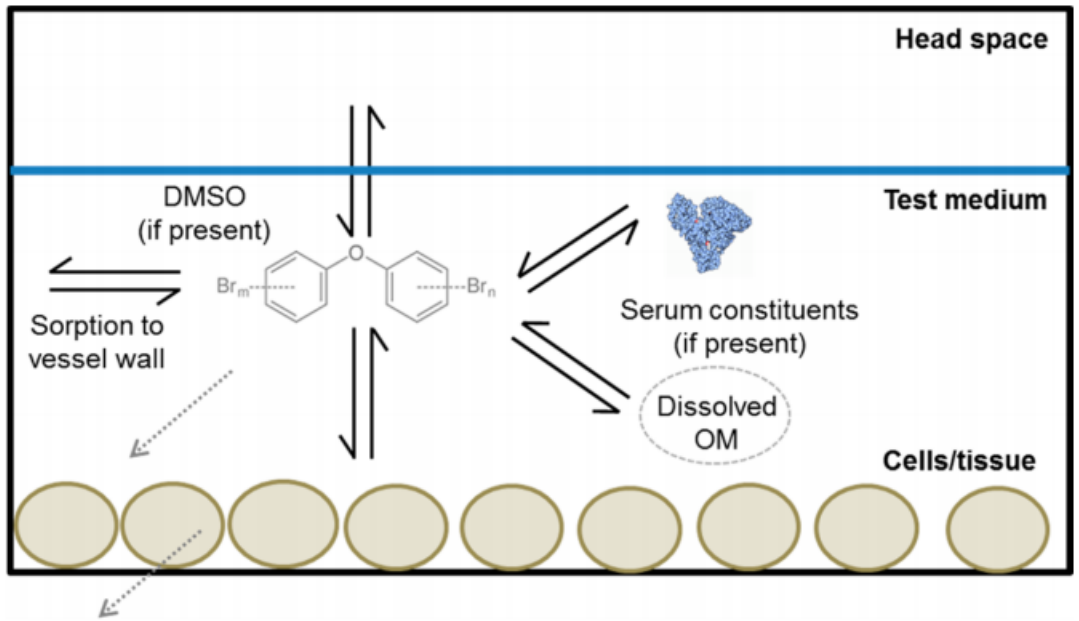
- Armitage et al. (2014) suggest that *in vitro* partitioning relates strongly to **LogK$_{ow}$** and concentration of **serum** in the medium
- Sorption to plastic played a smaller role in determining the cellular concentration

Mass-balance model

$$C_W = \frac{M_T}{K_{AW}V_A + V_W + K_{SaW}V_{Sa} + K_{SlW}V_{Sl} + K_{DW}V_D + K_{CW}V_C} \quad (1)$$

Diagram of *in vitro* compartments

Environmental Science & Technology Article



Armitage et al. 2014 PMID 25014875

Figure 1. Conceptual representation of an in vitro test system. DMSO: dimethyl sulfoxide, an example of a cosolvent. OM: organic matter.

A hypothesis for our work

The physicochemical properties of a given chemical can be used to predict the difference between ‘nominal’ concentration of a chemical in the medium and ‘true’ medium and cellular concentrations.

Unknown unknowns?

In vitro chemical partitioning between media and cells (in metabolically-incompetent cells) is dependent on:

- amount of serum in the media;
- the relative binding of the chemical to serum binding proteins;
- $LogK_{ow}$ of the chemical;
- chemical binding to plastic.

Chemical Selection

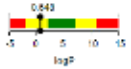
- Approximately 200 chemicals
- 92.5% ToxCast chemicals.
- 44.5% low fraction unbound, 27% moderate, 28.5% high.
- 50% neutral, 30% anionic, and 17% cationic at pH 7.4.
- 60% of the compounds were inactive in Attagene ER, 4.5% were potent at < 0.1 μM, 17.5% were potent at less than 10 μM.
- 20.5% have an existing NTP method.
- 3.5% have radiolabeled compound available somewhere at EPA.
- For these chemicals, the Armitage et al. (2014) model predicts that the cellular concentration will be 100-fold lower than media concentration for 10.5%, will be 3.2-fold lower than media concentration for 14.5%, within 3.2-fold of media concentration for 18%, greater than 3.2-fold the media concentration for 36%, and greater than 100-fold the media concentration for 18%.

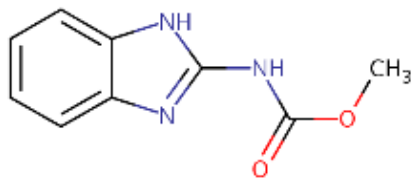
Goals of Pilot

- Sample generation and sample handling workflow
- Are we getting the information that we want?
- Efficient data collection and analysis

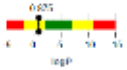
10 Chemical Pilot

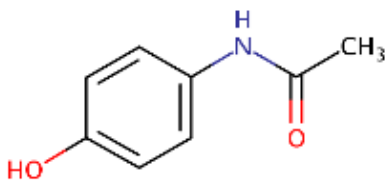
Carbendazim
10605-21-7 | DTXSID4024729



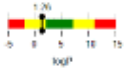


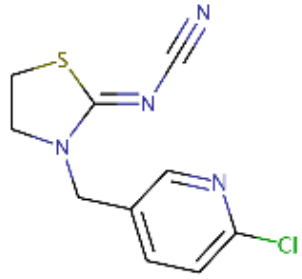
Acetaminophen
103-90-2 | DTXSID2020006



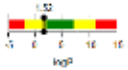


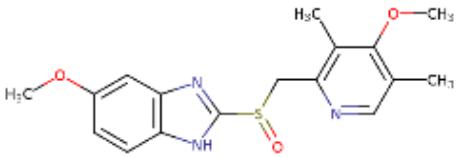
Thiacloprid
111988-49-9 | DTXSID7034961



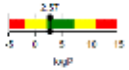


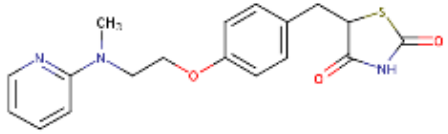
Omeprazole
73590-58-6 | DTXSID6021080



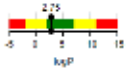


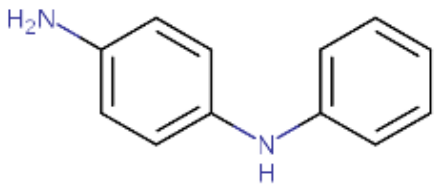
Rosiglitazone
122320-73-4 | DTXSID7037131





N-Phenyl-1,4-benzenediamine
101-54-2 | DTXSID7025895





Atrazine
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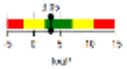

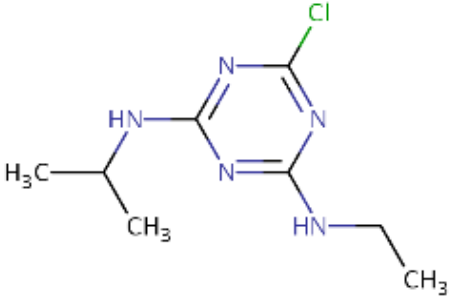

Rifampicin
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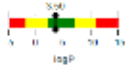

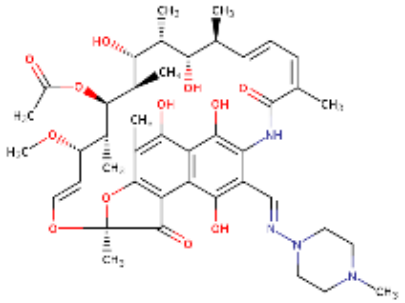

Flusilazole
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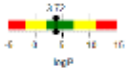
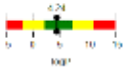
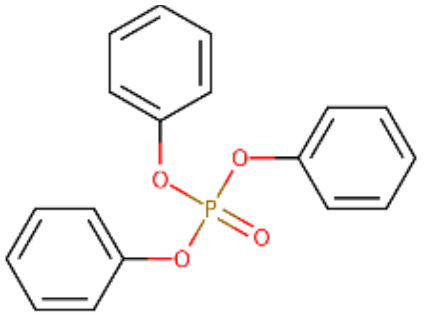


Triphenyl phosphate
115-86-6 | DTXSID1021952





Pilot 1.0 Study Design

Table 1. Sample Calculations		
Design Parameter:	**Multiplier**	**Comments**
Cell Type(s)	1	MCF7
Number of Plates	9	See Plate Matrix
Technical Replicates	4	See Plate Map
Chemicals	10	See Chemical List
Concentrations	1	10 µM
Time Points	3	1, 6, 24 hours
Media Types	2	Either 1% and 10% FBS

Cell Plating

BioTek MultiFlo FX Peristaltic Dispenser

Chemical Dispensing

LabCyte Echo 550 Acoustic Dispenser

Media Transfer

Integra ViaFlo 384 Guided Pipetting System

Acetonitrile Addition

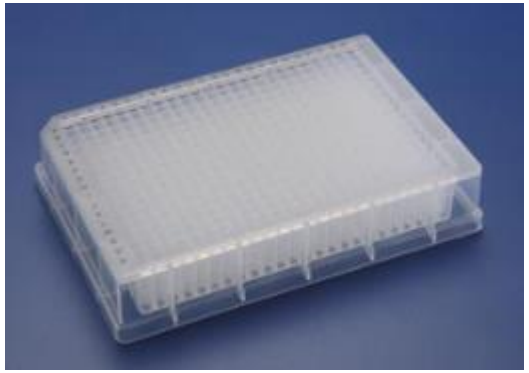
Gyger Certus Flex Solenoid MIcrodispenser

Table 2. Plate Matrix				
Test Plate	**Test Plate Barcode**	**Plating Condition**	**Exposure Duration (hr)**	**Measured Compartment**
A	TC00284721	Medium - cells	1	Medium
		Medium - cells	1	Plastic
B	TC00284722	Medium + cells	1	Medium
		Medium + cells	1	Plastic + Cells
C	TC00284723	Medium + cells	1	Whole Well Crash
D	TC00284724	Medium - cells	6	Medium
		Medium - cells	6	Plastic
E	TC00284725	Medium + cells	6	Medium
		Medium + cells	6	Plastic + Cells
F	TC00284726	Medium + cells	6	Whole Well Crash
G	TC00284727	Medium - cells	24	Medium
		Medium - cells	24	Plastic
H	TC00284728	Medium + cells	24	Medium
		Medium + cells	24	Plastic + Cells
I	TC00284729	Medium + cells	24	Whole Well Crash

Test Plate

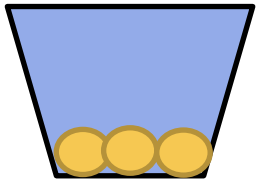
Corning 3985BC Polystyrene

Receiving Plate



PlateOne 384 Deep Well Polystyrene

Sample Handling to Measure Different Compartments

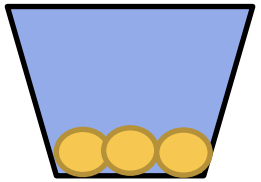


Cells + Media + Chemical

Add ACN

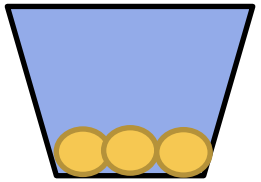
Whole Well Crash

Sample Handling to Measure Different Compartments

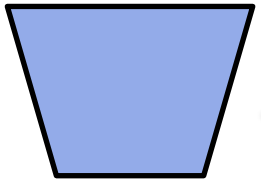


Add ACN → Whole Well Crash

Cells + Media + Chemical

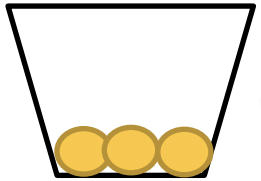


Remove Media →



Media

+



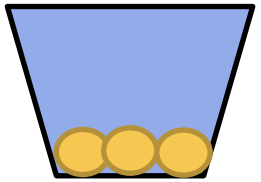
Plastic + Cells

Add ACN → Amount in Media

Add ACN → Amount in Plastic + Cells

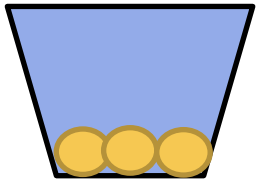
Cells + Media + Chemical

Sample Handling to Measure Different Compartments



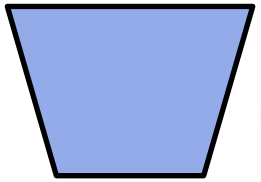
Cells + Media + Chemical

Add ACN → Whole Well Crash



Cells + Media + Chemical

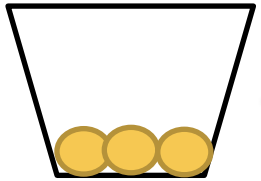
Remove Media →



Media

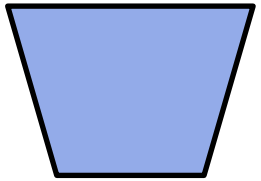
Add ACN → Amount in Media

+



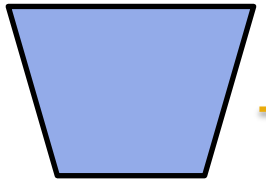
Plastic + Cells

Add ACN → Amount in Plastic + Cells



Media + Chemical

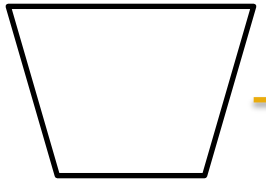
Remove Media →



Media

Add ACN → Amount in Media

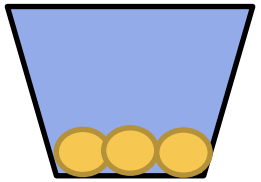
+



Plastic

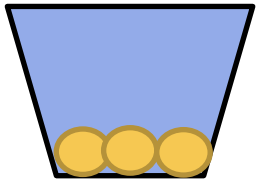
Add ACN → Amount in Plastic

Sample Handling to Measure Different Compartments



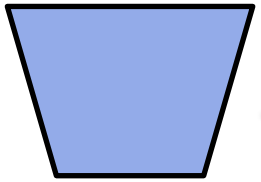
Cells + Media + Chemical

Add ACN → Whole Well Crash



Cells + Media + Chemical

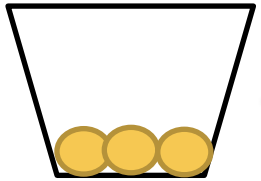
Remove Media →



Media

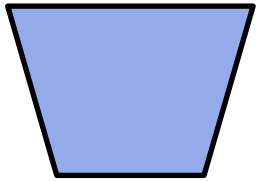
Add ACN → Amount in Media

+



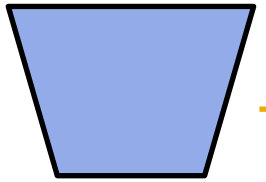
Plastic + Cells

Add ACN → Amount in Plastic + Cells



Media + Chemical

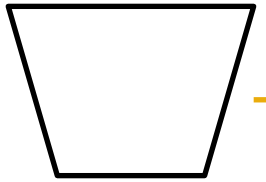
Remove Media →



Media

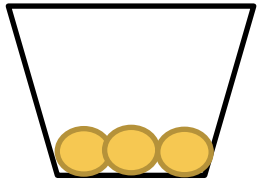
Add ACN → Amount in Media

+



Plastic

Add ACN → Amount in Plastic



Plastic + Cells

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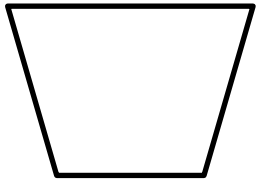


Plastic

↓

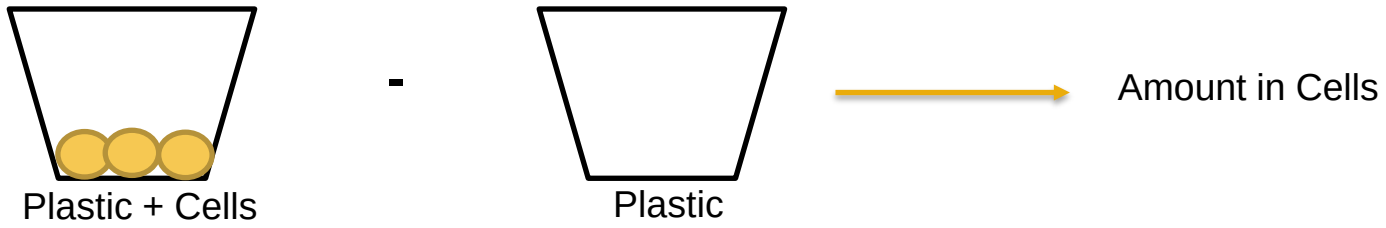
Amount in Cells

Analytical Methods

- LC (Thermo Vanquish)
  - LC method is a 7 minute run on a C18 column
  - Mobile Phase A: Water with 0.1% formic acid
  - Mobile Phase B: Acetonitrile with 0.1% formic acid
  - 10% B to 100% B over 5 minutes
  - 100% B for 1 minute
  - 100% B to 10% B for 1 minute
- MS (Thermo Q Exactive Plus)
  - Targeted Single Ion Monitoring Mode

Calculating Concentrations and Amounts

- Calculating Concentrations in Media and Whole Well Samples
  - Final Conc. = Raw Conc. x Post-Incubation Dilution Factor x Analytical Dilution Factor
- Calculating Amounts
  - Final Amount = Raw Conc. x Post-Incubation Dilution Factor x Analytical Dilution Factor x Volume in Well
- Calculating Concentrations in Cells
  - Final Conc. = (Amount in Plastic + Cells – Amount in Plastic) / (Molecular Weight * Volume of Cells)
  - Volume of Cells = 10,000 cells * 2.0 pL/cell = 20 nL



Individual *vs.* Cassette Analysis

- All samples are incubated individually and then analytically measured both as individual samples (1 chemical) and as cassette samples (5 chemicals) – Goal is to decrease LCMS analysis time

Single Chemical Incubations

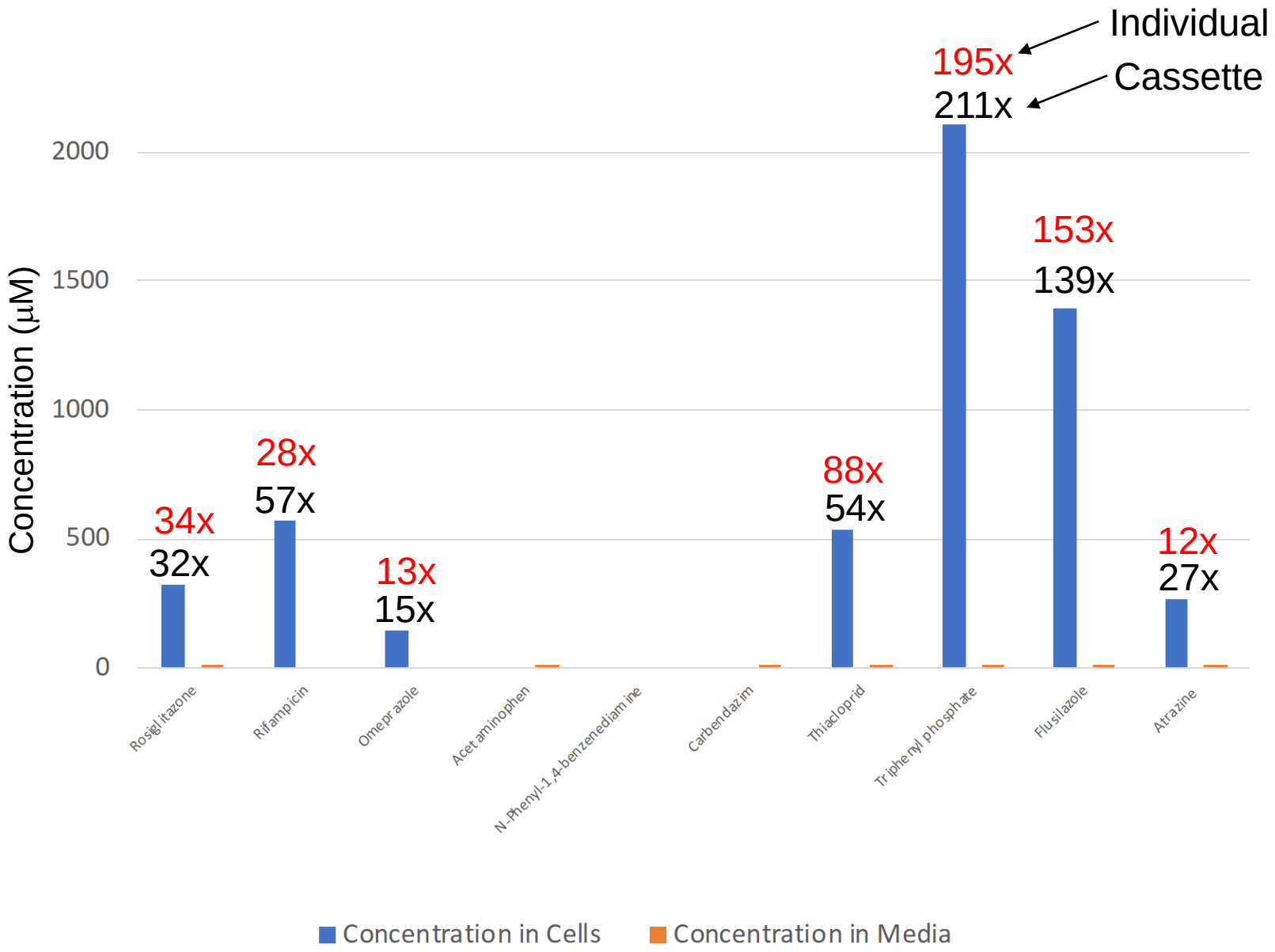
Individual Sample Analysis

All samples are analyzed individually be LCMS

Cassette Sample Analysis

Samples from 5 different chemicals are analyzed via LCMS in a single injection

Concentration in Cells *vs.* Media at 24 hours – Cassette Analysis



Similar results between individual and cassette analysis

Comparing “Plastic + Cells” vs. “Plastic”

Compound	384-well Difference (ng)
Rosiglitazone	2.3
Rifampicin	9.4
Omeprazole	1.0
Acetaminophen	------
N-Phenyl-1,4-benzenediamine	-0.5
Carbendazim	------
Thiacloprid	2.7
Triphenyl phosphate	13.8
Flusilazole	8.8
Atrazine	1.4

Small differences between “Plastic + Cells” and “Plastic” fractions to determine amount of chemical in cells versus bound to plastic creates a challenge from an analytical measurement perspective

Pilot 1.0 Review

- Cassette analysis for analytical measurements produced similar results to individual analysis
  - major reduction in run time
  - 282 days *vs* 56 days
- A challenge is the small differences in the amount of chemical observed in the cells versus the amount bound to plastic

Pilot 2.0

- Move from 384 well format to 96 well format
- Greater number of cells in each well per surface area
- Added wash step to remove residual chemical not actually in cells or bound to plastic
- Single media composition with 10% FBS (no longer looking at 1% FBS media)

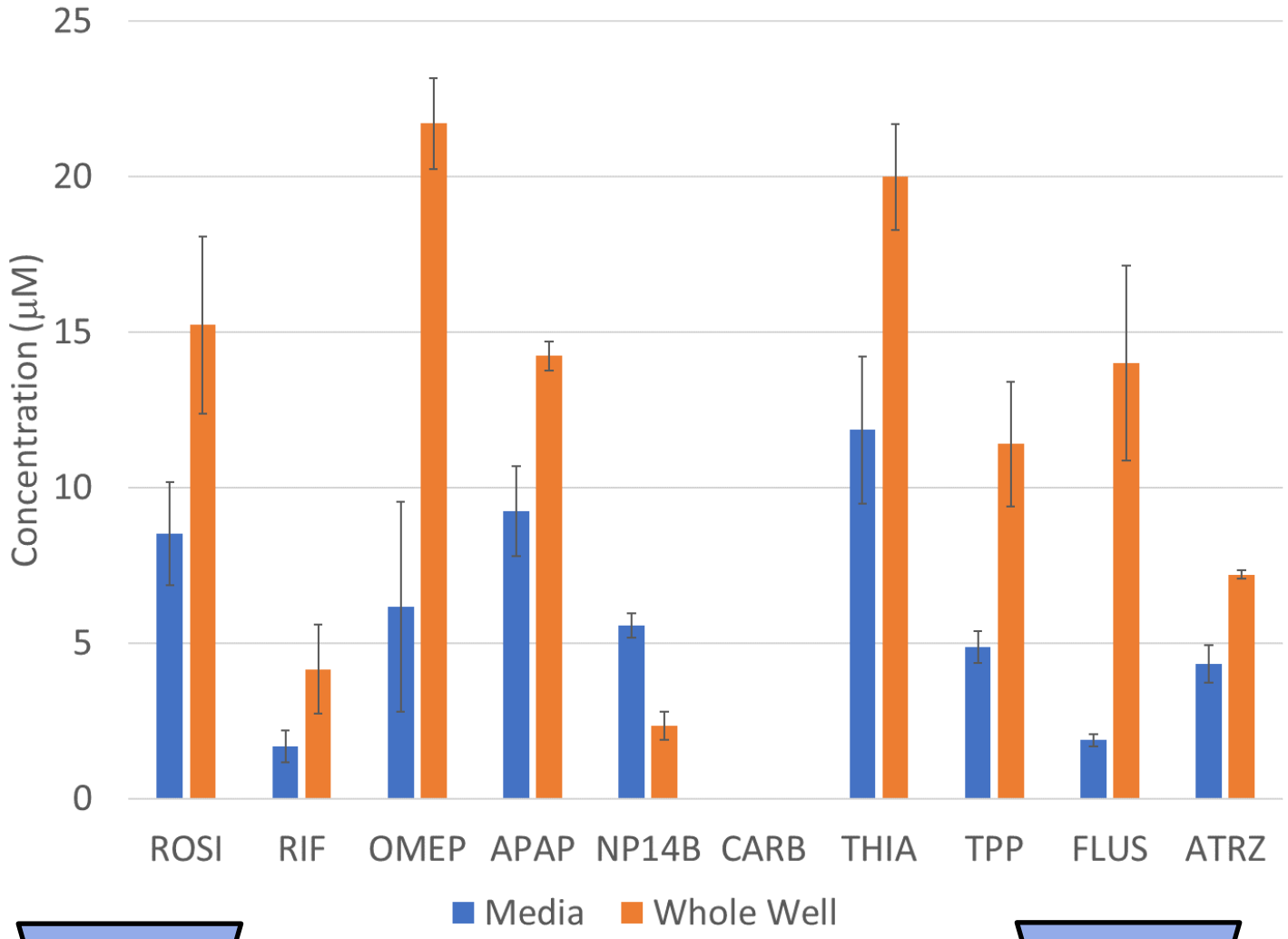
Pilot 2.0 and 2.1

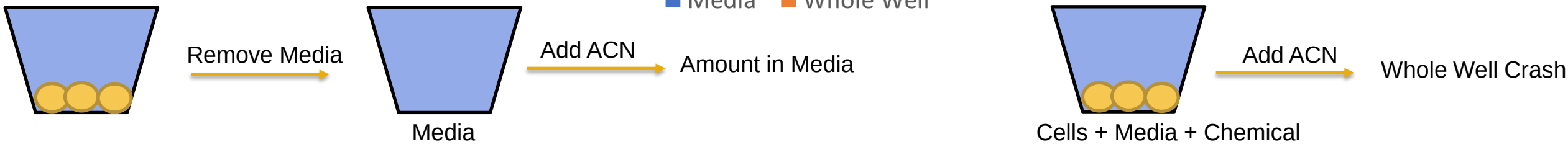
- LCMS drift issues with first pilot 2.0 analytical measurements
- Reanalyzed samples from Pilot 2.0 incubations
- Used a data normalization method to account for LCMS instrument changes over runtime
- Inject a standard every 10 injections that is used to normalize signal intensity across a run

Calculating Concentrations and Amounts for 96-well Format

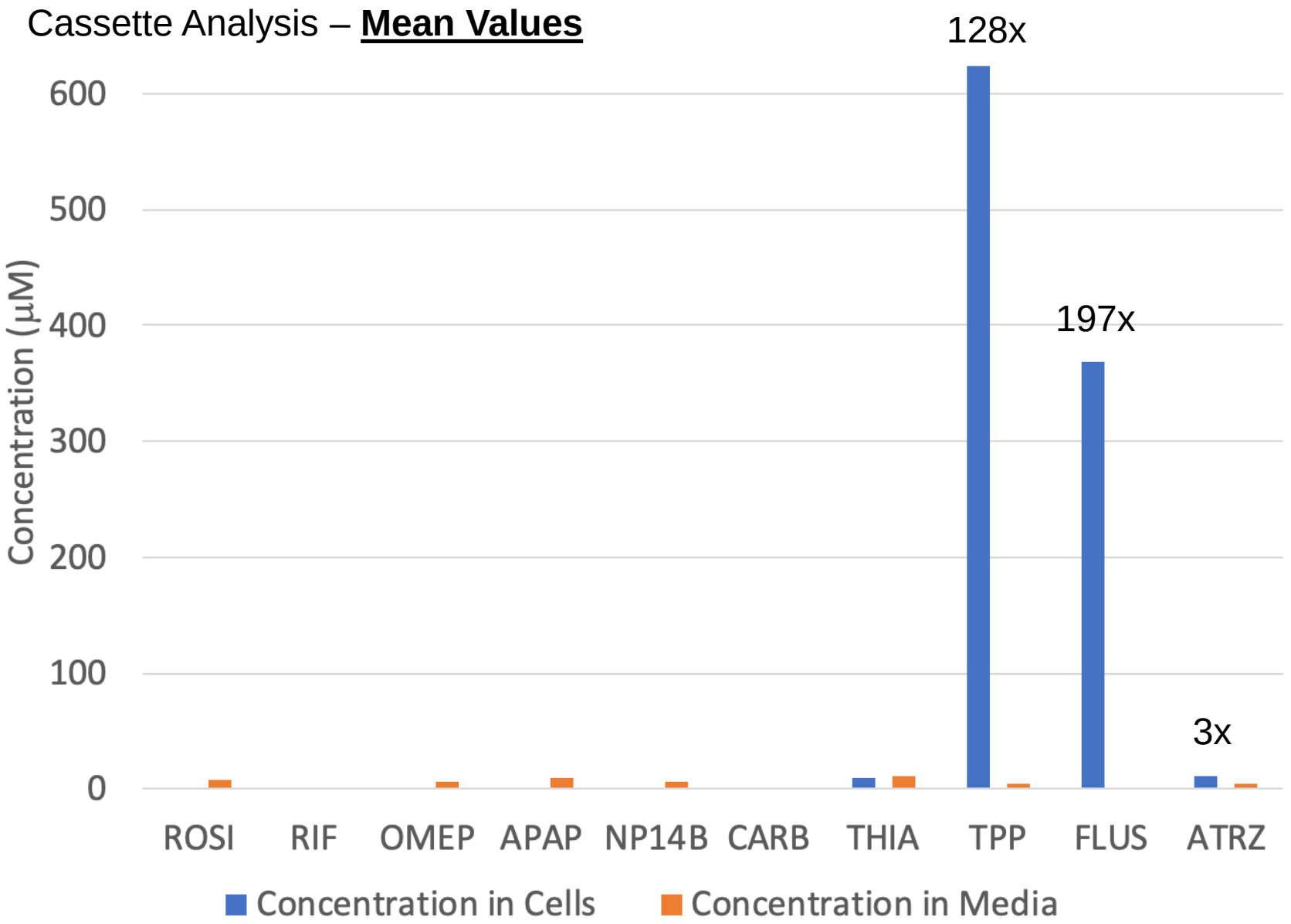
- Calculating Concentrations in Media and Whole Well Samples
  - Final Conc. = Raw Conc. x Post-Incubation Dilution Factor x Analytical Dilution Factor
- Calculating Amounts
  - Final Amount = Raw Conc. x Post-Incubation Dilution Factor x Analytical Dilution Factor x Volume in Well
- Calculating Concentrations in Cells
  - Final Conc. = (Amount in Plastic + Cells – Amount in Plastic) / (Molecular Weight * Volume of Cells)
  - Volume of Cells = 5.11×10^4 cells * 2.0 pL/cell = 102.2 nL

Media *vs.* Whole Well Concentration at 24 Hours





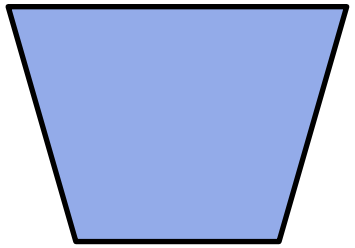
Concentration in Cells *vs.* Media at 24 Hours



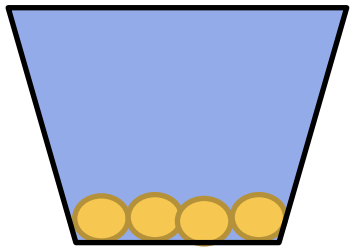
Plastic Binding – 96 Well Plate Format

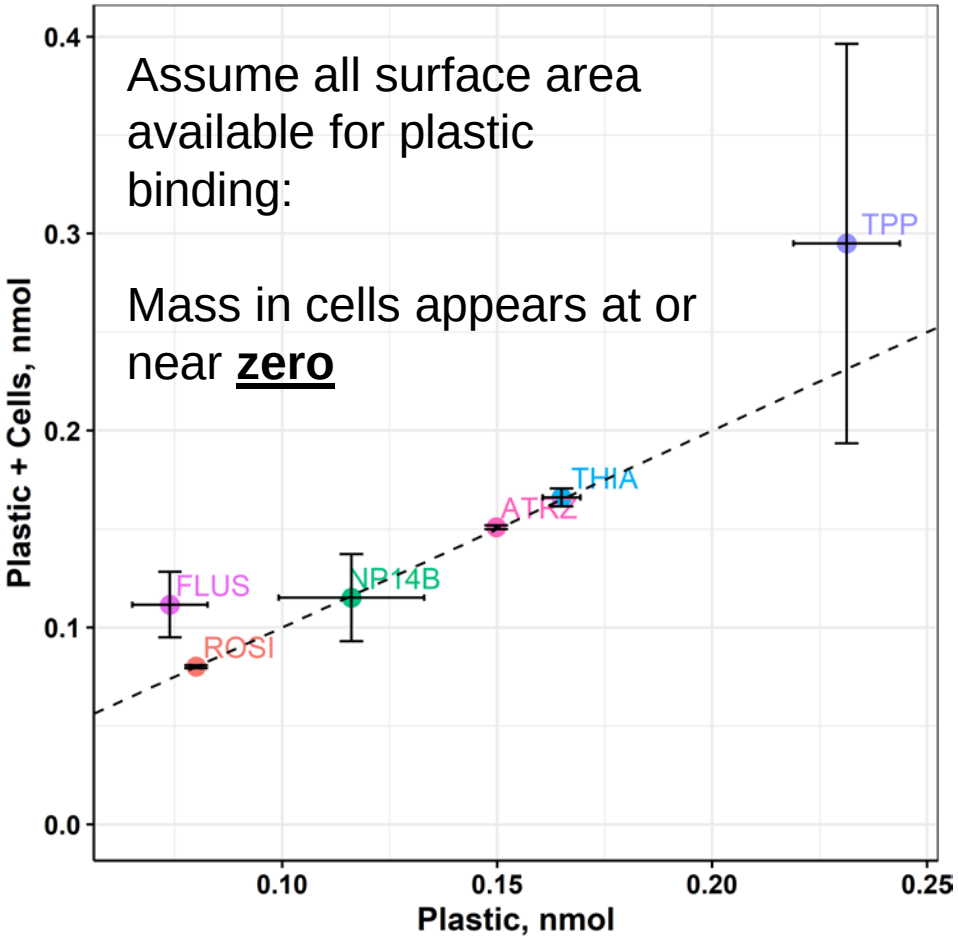
24 hr Cassette

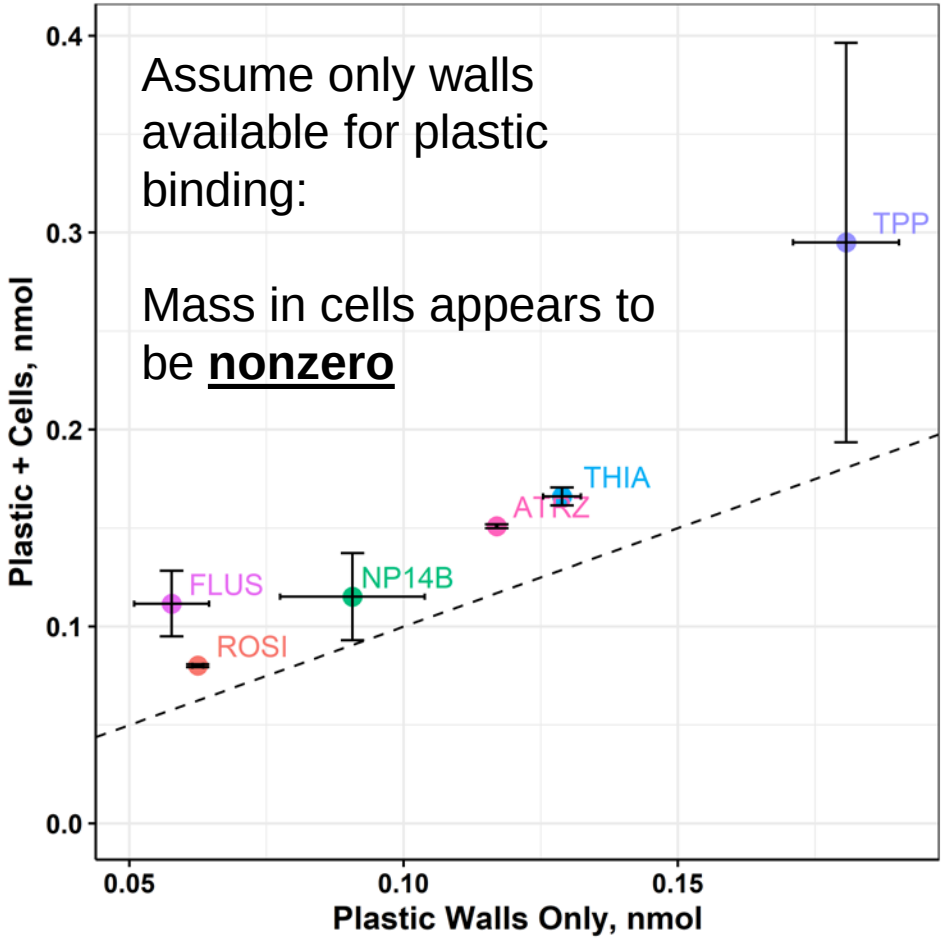
Plastic:



Plastic + Cells:







Is surface area of bottom available for binding of chemical to plastic?

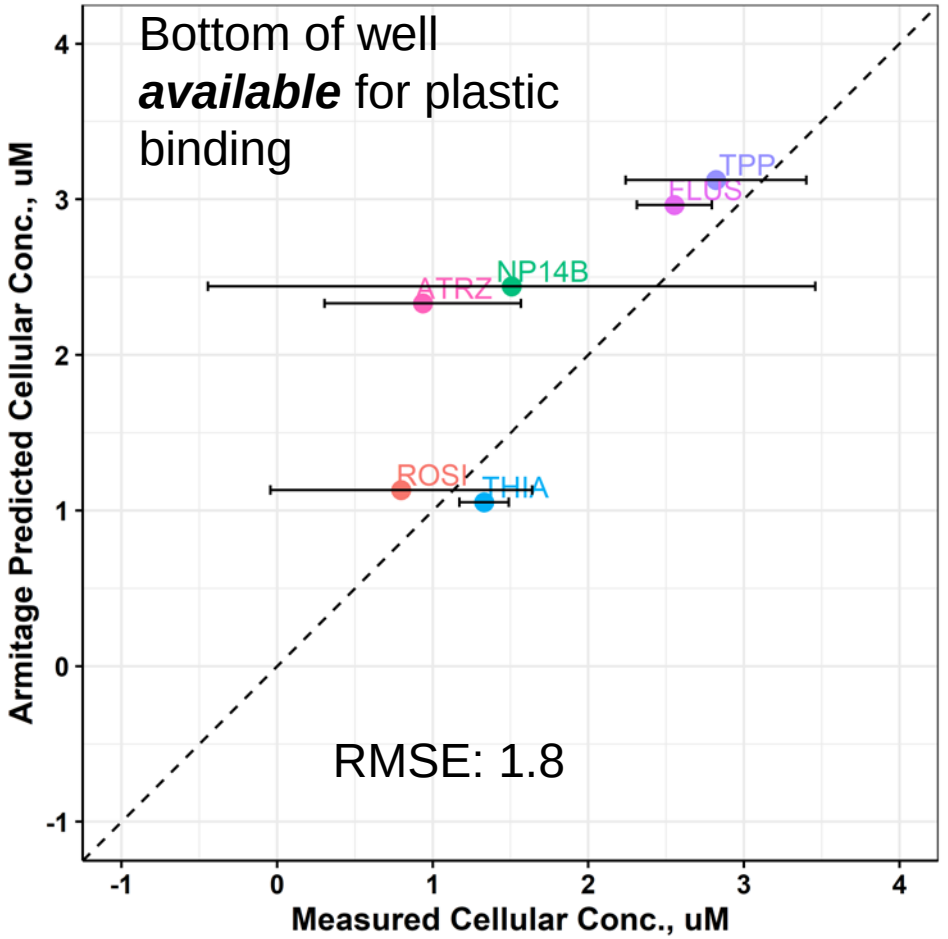
SA total: 137 mm^2

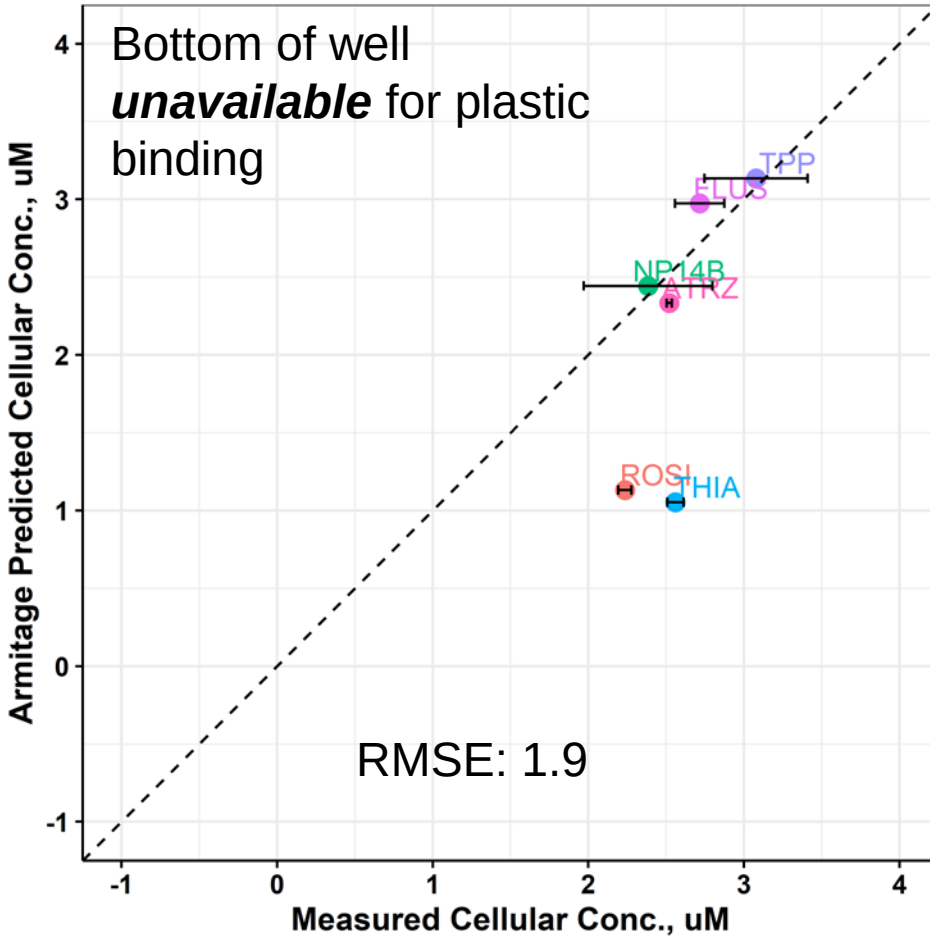
SA walls: 107 mm^2

Armitage Model Predictions

24 hr Cassette – 96 well plate format comparison

In vitro disposition model (Armitage et al. 2014) reasonable prediction of experimental results whether well bottom is included or not





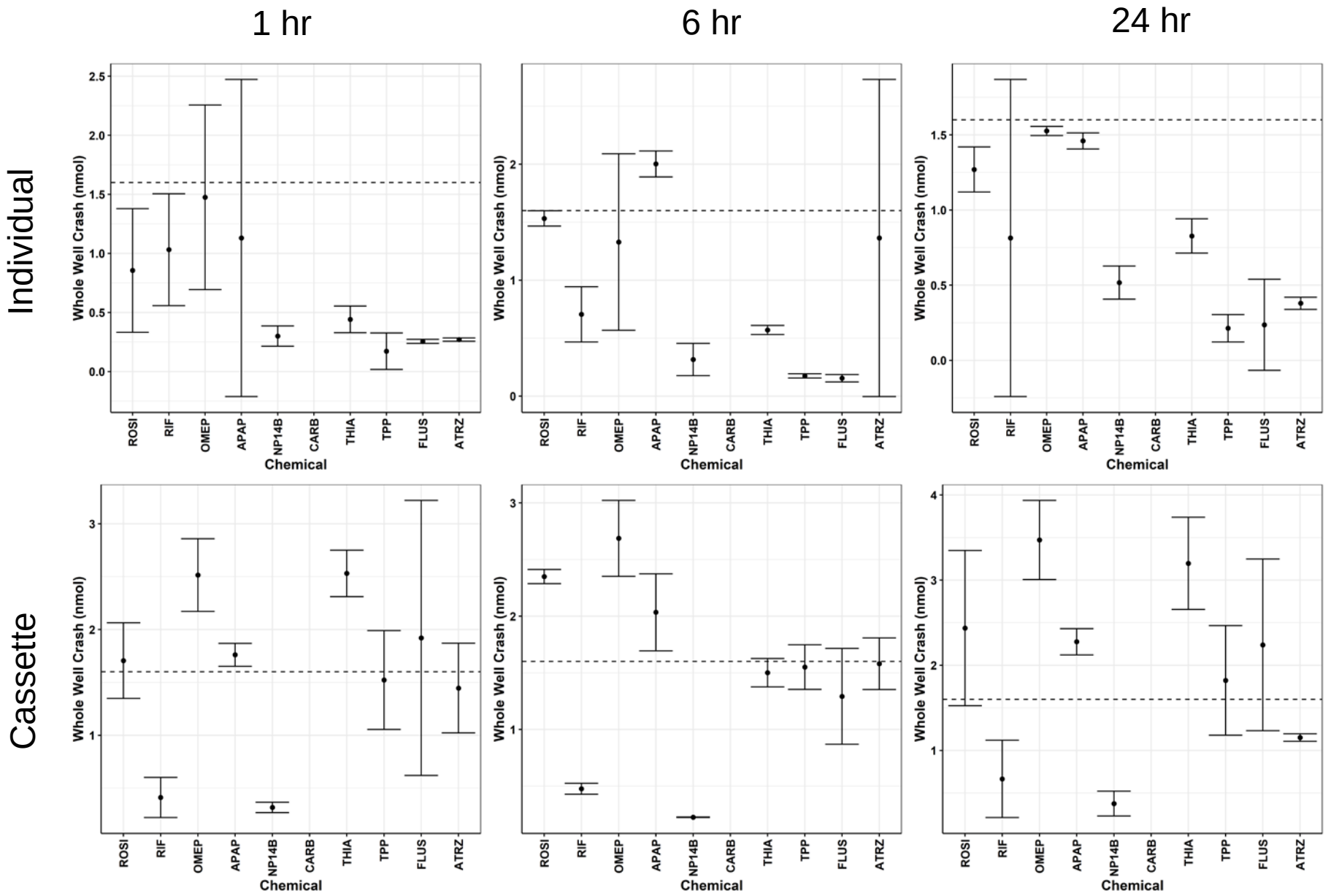
Mass Balances – 96 Well Plate Format

Whole Well Crash – measured total mass balance in experiment with cells

1.6 nmol
theoretical maximum

Error bars ± 2 SD
(4 possible repeated measures – measurements from separate wells)

Mass balances are poor. Cassette method shows generation of mass.



Observations

- Initial results suggest that nominal concentration ≠ cellular concentration
- Most cell concentrations are near zero for 96 well format *unless* we assume the bottom surface area is unavailable to plastic binding
- Armitage model ($LogK_{ow}$ based) reasonable estimate of experimental cell concentration measurement
- Mass balances aren’t great

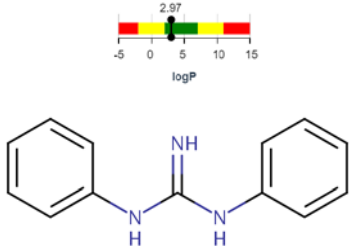
Pilot 3.0

- 20 chemicals
  - 10 chemicals used in previous pilots
  - 10 new chemicals to further cover chemical space
- 3 concentrations – 5, 10, and 20 μM

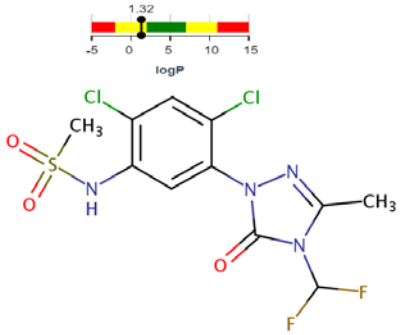
1,3-Diphenylguanidine
Sulfentrazone
Flutamide
Gemfibrozil
Pirimiphos-methyl
Genistein
Oxytetracycline dihydrate
Fluroxypyr
Dinoseb
Butylparaben

Additional 10 Chemicals

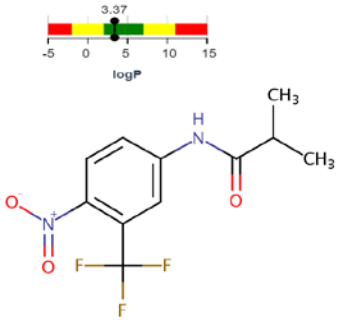
1,3-Diphenylguanidine
102-06-7 | DTXSID3025178



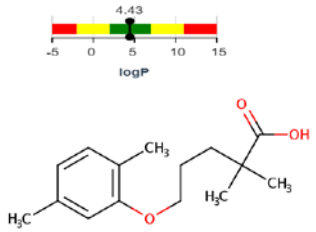
Sulfentrazone
122836-35-5 | DTXSID6032645



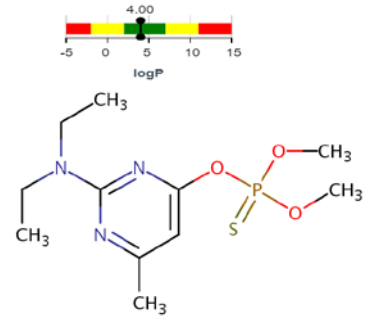
Flutamide
13311-84-7 | DTXSID7032004



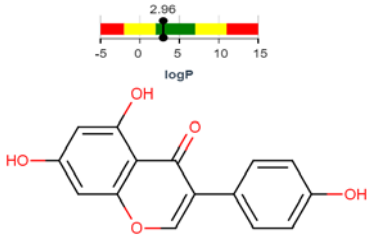
Gemfibrozil
25812-30-0 | DTXSID0020652



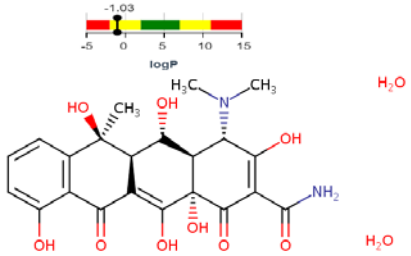
Pirimiphos-methyl
29232-93-7 | DTXSID0024266



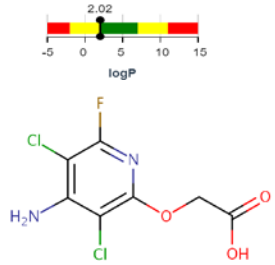
Genistein
446-72-0 | DTXSID5022308



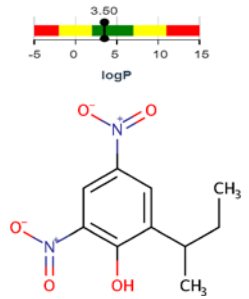
Oxytetracycline dihydrate
6153-64-6 | DTXSID4023412



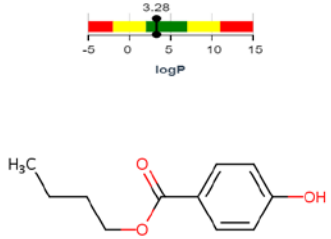
Fluroxypyr
69377-81-7 | DTXSID2034627



Dinoseb
88-85-7 | DTXSID3020207



Butylparaben
94-26-8 | DTXSID3020209



Pilot 3.0 Status

- Analytical methods completed for all 20 compounds
- Incubation completed in February
- LCMS analysis end of 2020/beginning of 2021

Acknowledgements

- NTP
  - Steve Ferguson
  - Nisha Sipes
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