Canada

Chemical toxicity assessment using high-throughput transcriptomics and next generation sequencing:
A case study of per- and polyfluoroalkyl substances

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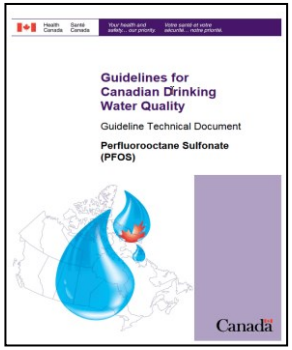
Per- and Polyfluoroalkyl Substances (PFAS)

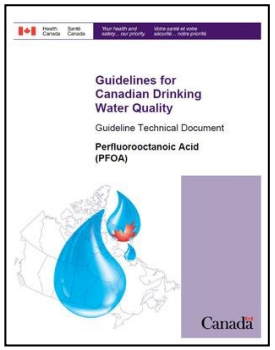
- Per- and poly-fluoroalkylated substances (PFAS) are a class of chemicals that are ubiquitously found in the environment due to their wide use, persistence and high mobility.
- PFAS can contaminate drinking water and soil in proximity to locations where fire-fighting foams are used including fire fighting training areas at airports and military bases.
- There is a growing body of knowledge on PFOS and PFOA toxicity; however, little is known about the many other PFAS




Exposure and Regulation in Canada

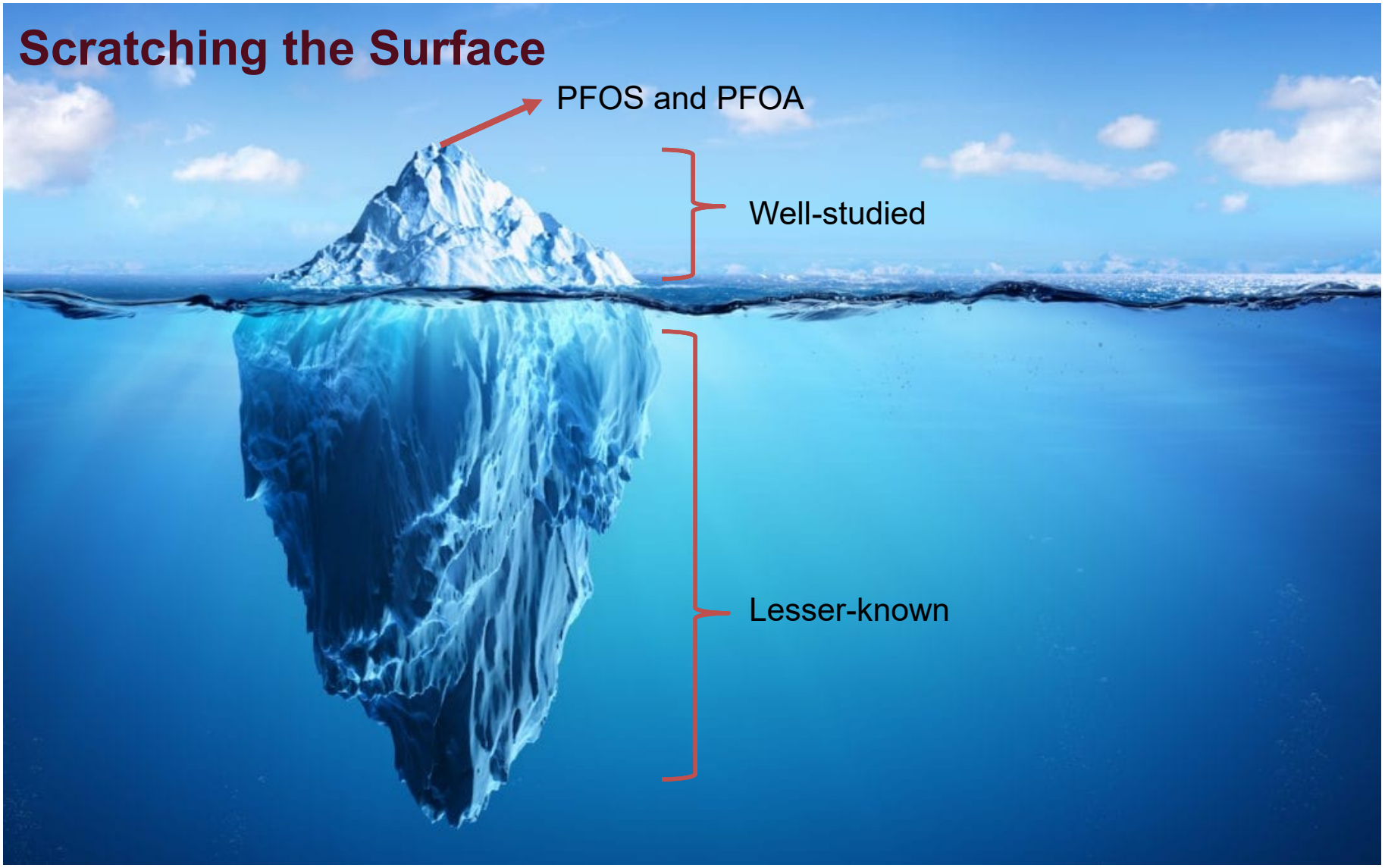
- Chemical persistence and broad use have led to wide-spread human exposure
- **Drinking water screening values** have been developed for PFOS, PFOA and 9 additional PFAS at the request of several provinces
- In 2018, **drinking water guidelines** were published for PFOS and PFOA, and screening values were updated
- **Soil Quality Guidelines** for PFOS and PFOA are currently being developed. **Soil screening values** are currently available for 9 PFAS compounds (including PFOS and PFOA).





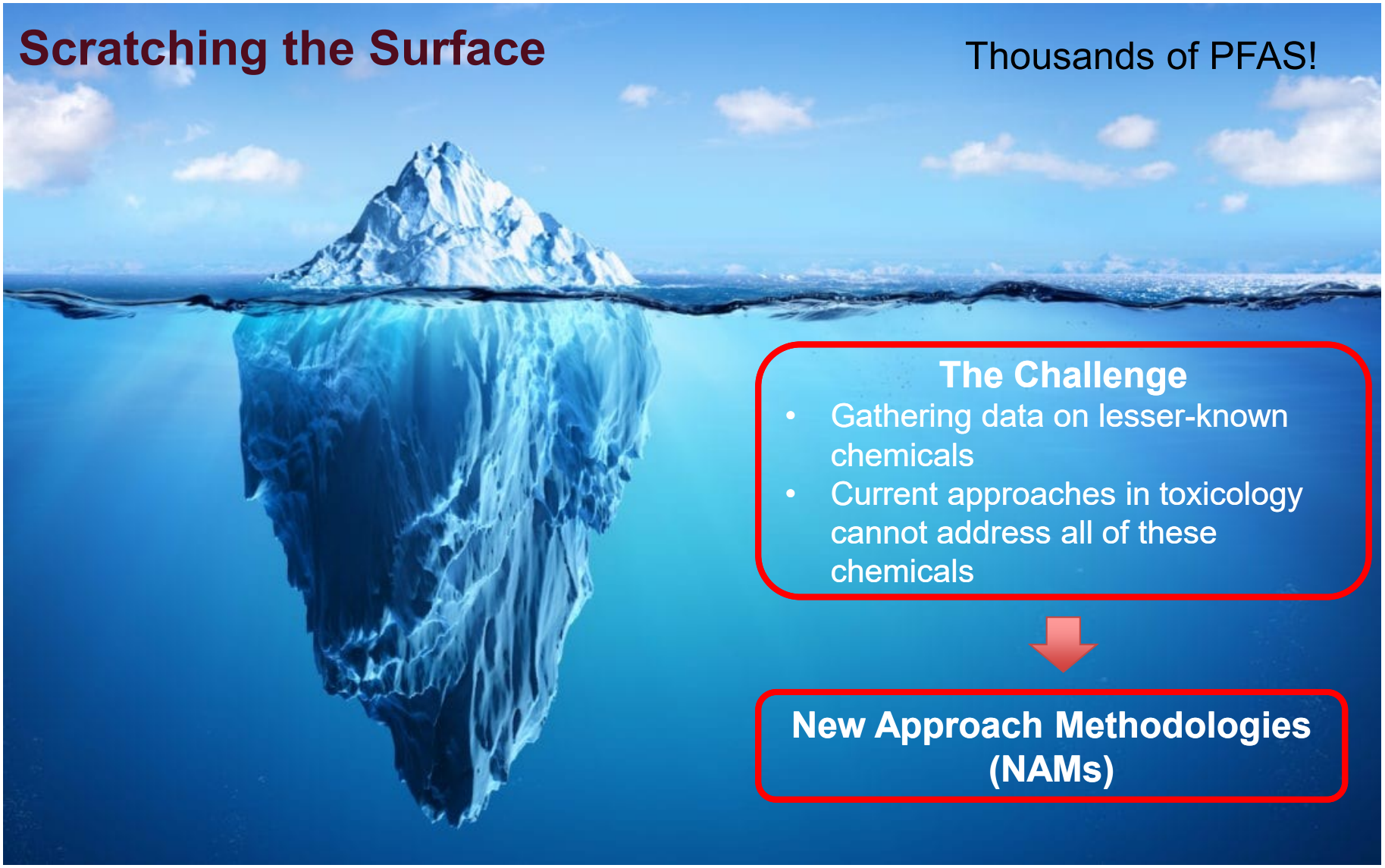
2018 https://www.canada.ca/en/health-canada/services/environmental-workplace-health/reports-publications/water-quality.html#tech_doc

Scratching the Surface



Scratching the Surface

Thousands of PFAS!



The Challenge

- Gathering data on lesser-known chemicals
- Current approaches in toxicology cannot address all of these chemicals

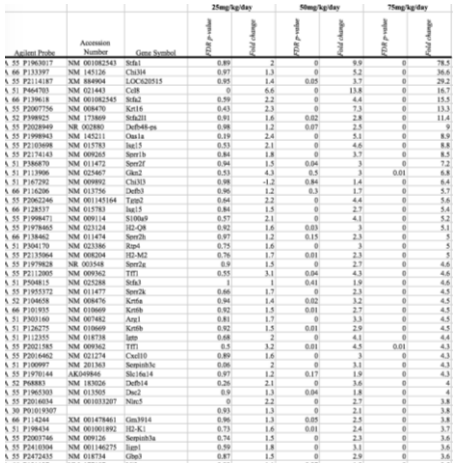
New Approach Methodologies (NAMs)

New Approach Methodologies

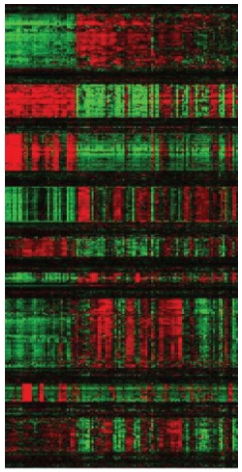
- The term new approach methodologies (NAMs) has been adopted as a broadly descriptive reference to any non-animal technology, methodology, approach, or combination thereof that can be used to provide information on chemical hazard and risk assessment.” (NIEHS roadmap, 2017)
- US EPA directive to reduce (by 2025)
  and subsequently eliminate (by 2035)
  the use of animal models in toxicological assessment

Long-term vision of transcriptomics in regulatory decision making

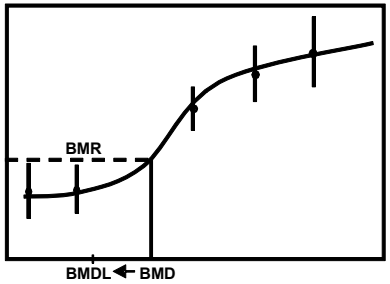
Large gene lists



Extract predictive signatures and pathways



Dose-response modeling



Case studies

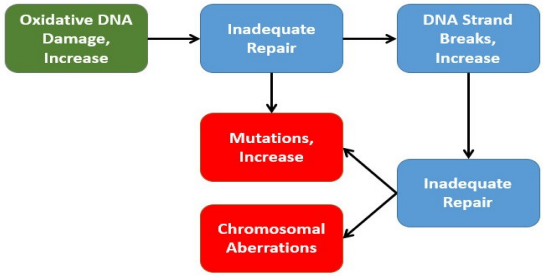
Risk assessment

At what dose do effects occur?

Human exposure levels?

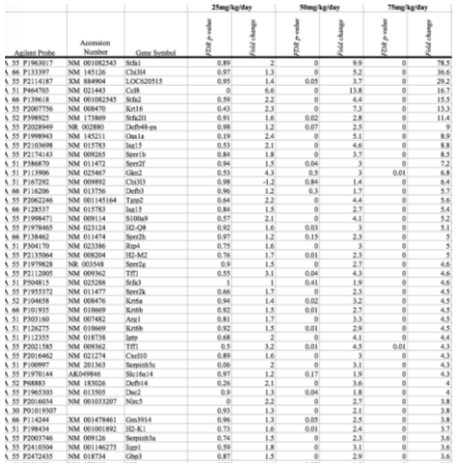
Hazard identification
Mode of action analysis

Align to AOPs



Short-term

Large gene lists



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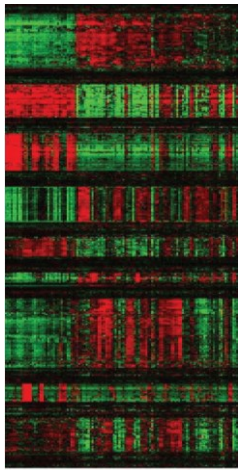
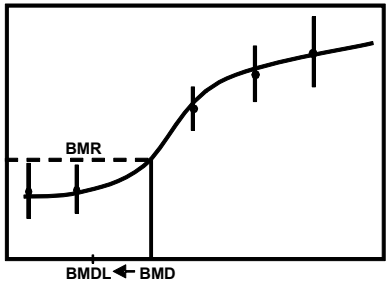


Dose-response modeling



At what dose do effects occur?

- Identify similarities in gene expression (mode of action across chemicals)
- Compare chemical potency (Dose-response)
- Compare human exposure levels for risk evaluation (where applicable)

Objectives and Approach

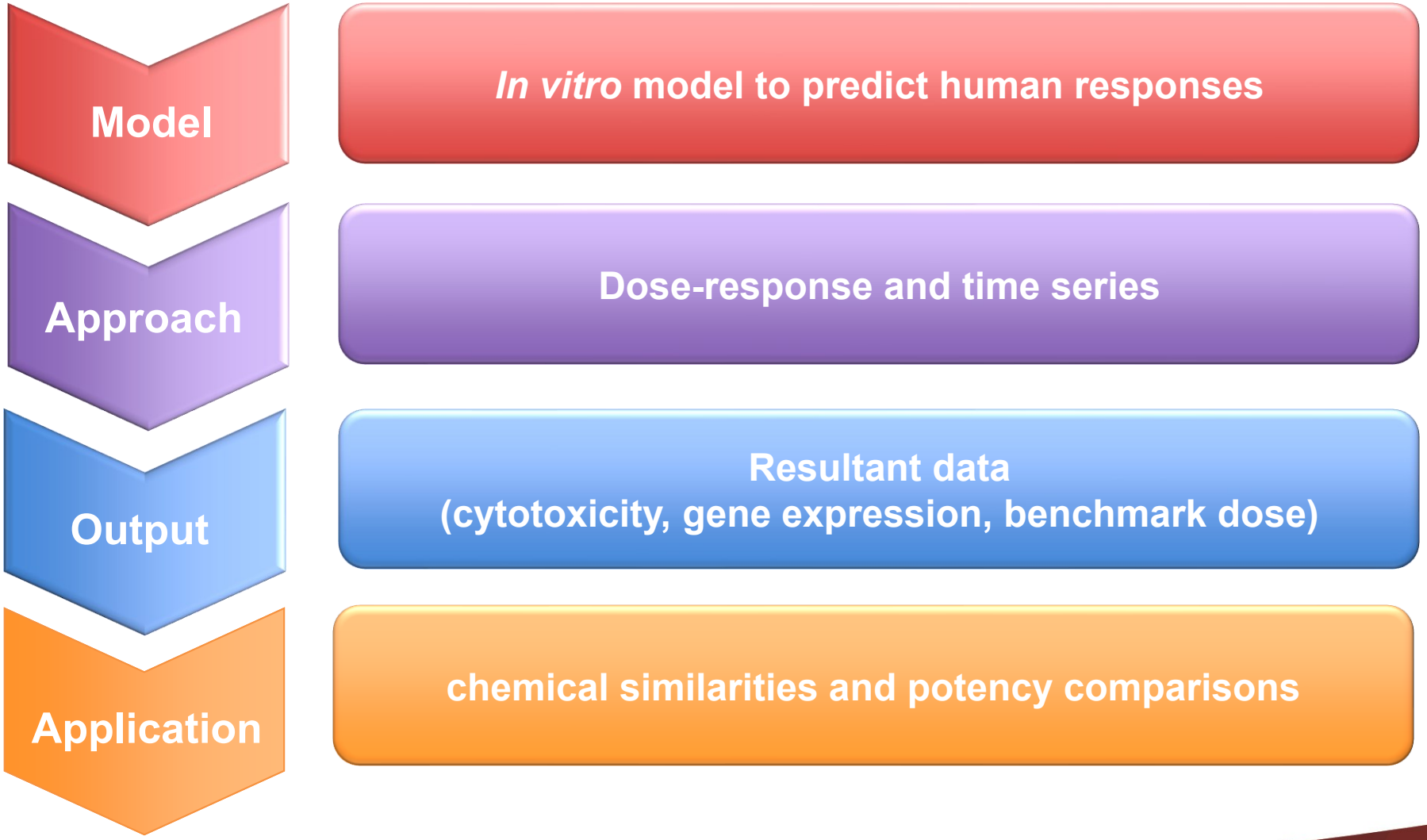
- **Overarching:** Use gene expression profiling to acquire information on PFAS to for application toward human health risk assessment
  - Conduct a high-throughput transcriptomic dose-response and time series analysis of primary human liver spheroids exposed to PFAS

- Experiment 1 - Microscopy
  - Microscopic characterization of biochemical responses of spheroids to PFAS (staining for markers of toxicity)

- Experiment 2 - Time-series, dose-response analysis of prototype PFAS
  - Cytotoxicity assessment and Tempo-Seq analysis
  - Development of bioinformatics pipeline

- Experiment 3 - Prioritize PFAS and mixtures; time- and dose-response
  - Establish potency ranking within the class of PFAS

Overview


Model
In vitro model to predict human responses
Approach
Dose-response and time series
Output
Resultant data
(cytotoxicity, gene expression, benchmark dose)
Application
chemical similarities and potency comparisons

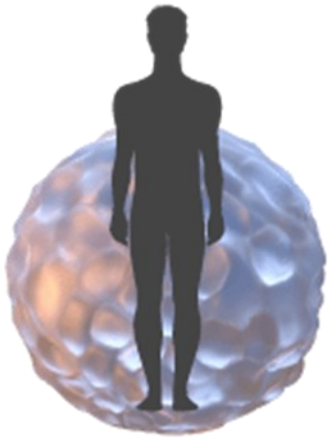
Confirming the model

Model

***In vitro* model to predict human responses**

Primary human cell spheroids:

- Spheroid hepatocytes and Kupffer cells
- Pooled samples from 10 donors
- Toxicological model representation of liver tissue



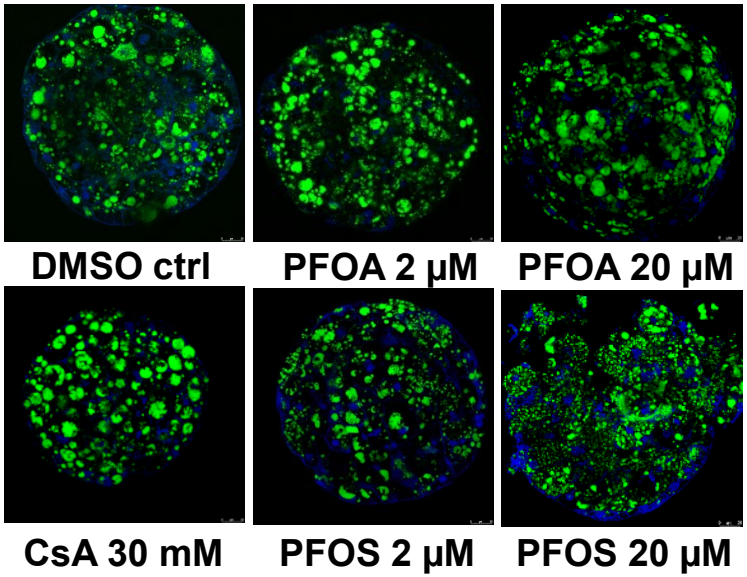
Microscopy images of primary human liver spheroids

Model

***In vitro* model to predict human responses**

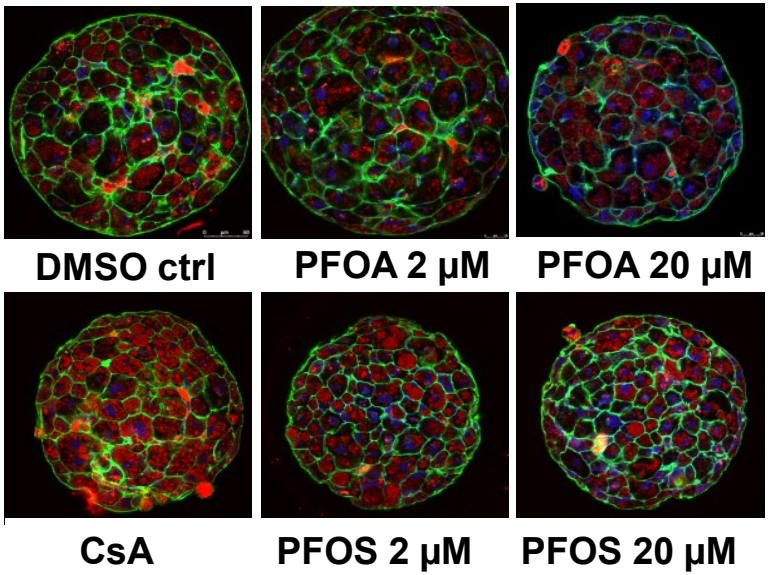
Lipid accumulation

Blue=nuclei, Green=lipids



Xenobiotic metabolism marker

Red=CYP3A4, Blue=Nuclei, Green=actin



(100x magnification) • Cyclosporin A (CsA) – known inducer of steatosis

Objectives and Approach

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Experimental design

Approach

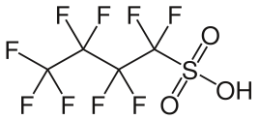
Overview of experimental design

Exposure

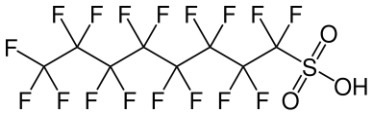
- 4 PFAS (replicates = 4)
- Dose range (0 to 100 µM)
- Time series (1, 4, 10 and 14 days)

Assessment

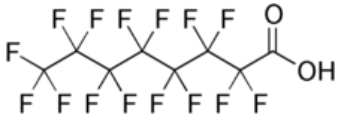
1. Cytotoxicity
2. Genomic responses
3. Predicting mode of action, and potency



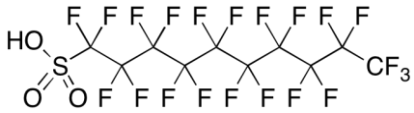
PFBS



PFOS



PFOA



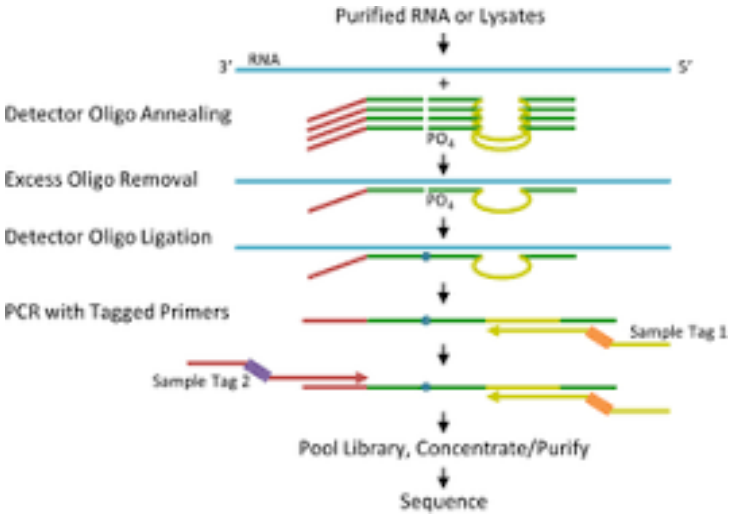
PFDS

High-throughput transcriptomics

Approach

Data generation and handling

- TempO-seq platform (Biospyder) library generation for sequencing from lysates
  - **S1500** gene panel (3000 genes)
- DNA sequencing to quantify the abundance of sister probes that target RNA molecules of interest
- Production of gene expression data



Bioinformatics pipeline development

Approach

Data generation and handling

Raw Data → Gene expression profiles
(Control QA/QC metrics)

Remove outliers

Data visualization (SAV)[2]
Illumina sequence analysis

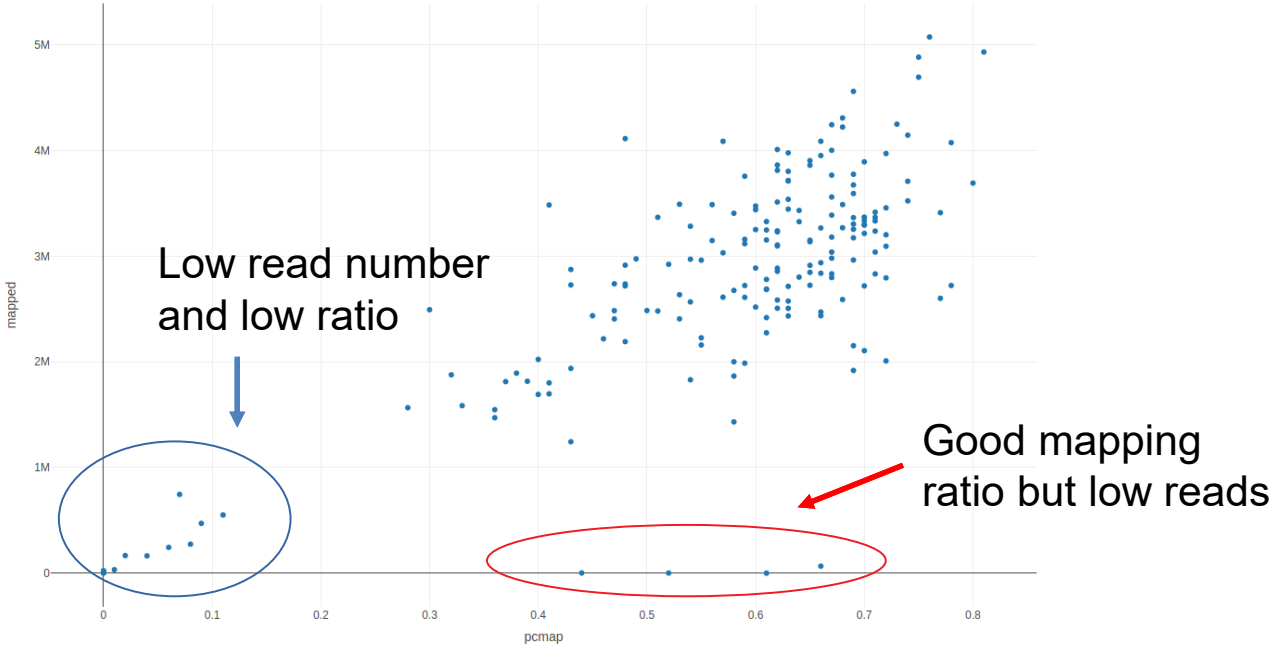
Reads extracted from the bcl files from the sequencer (with bcl2fastq v. 2.20.0.42)
→ Fastq files are processed with the “pete.star.script_v3.0” (supplied by Biospyder)
→ Script uses star v.2.5 to align the reads and the qCount function from QuasR

Examine sample coverage and mapping

Approach

Data generation and handling

Total mapped reads versus the percentage of mapped reads for each sample

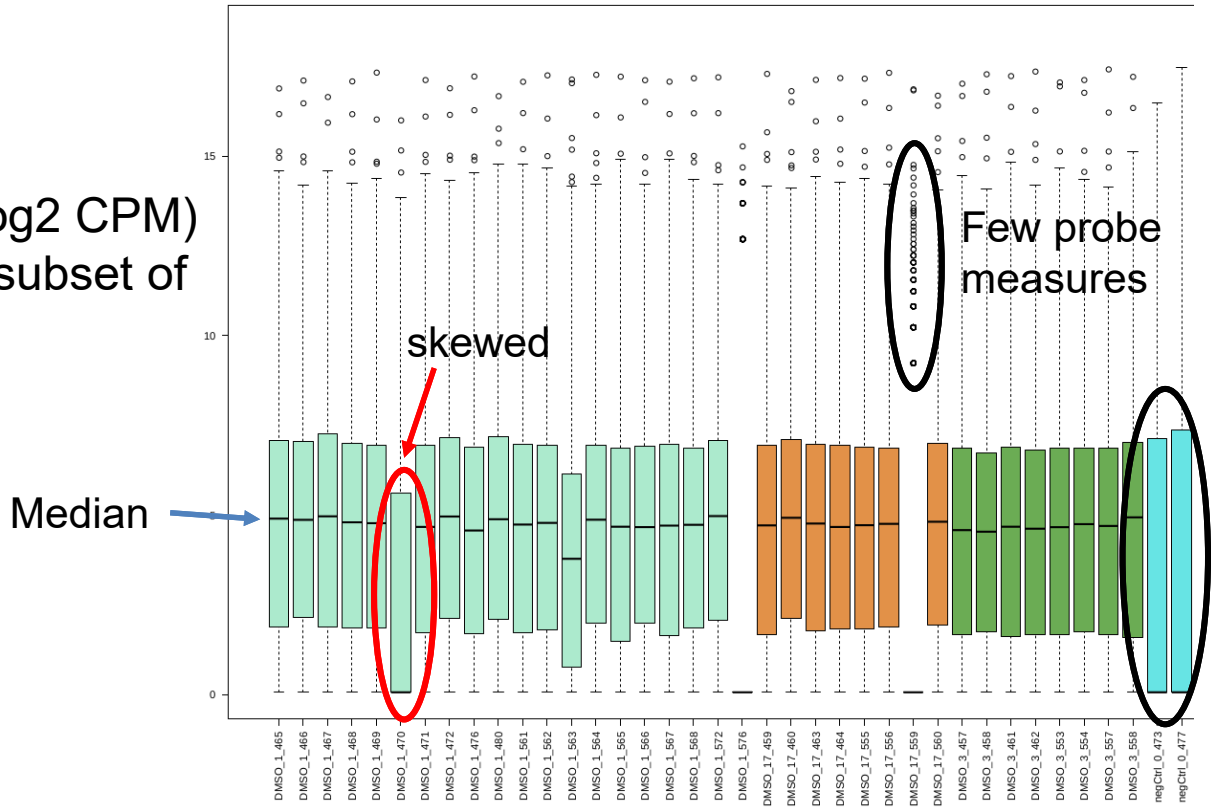


Probe distribution among each sample

Approach

Data generation and handling

Probe measure (log2 CPM) distributions for a subset of samples.

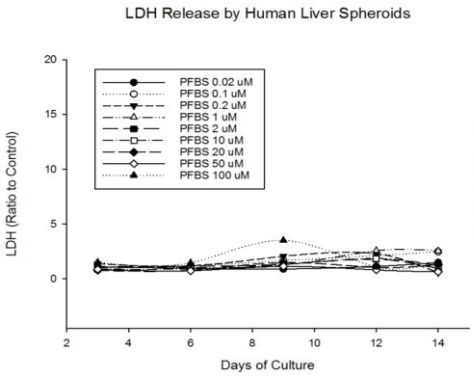


Specific PFAS induce cytotoxicity at higher exposures

Output

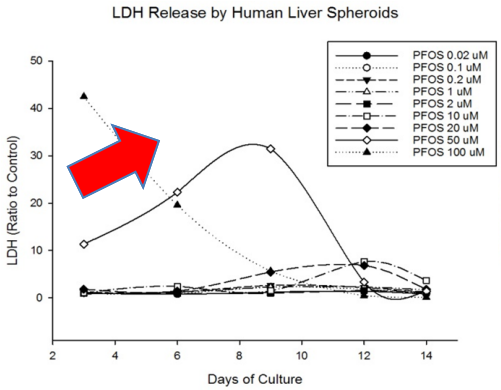
Cytotoxicity over 14-Day exposure (Lactate dehydrogenase assay - LDH)

PFBS



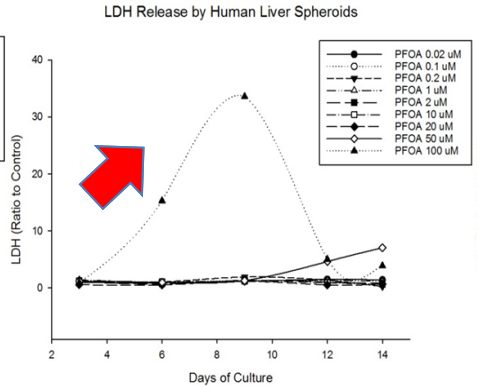
- No observed cytotoxicity

PFOS



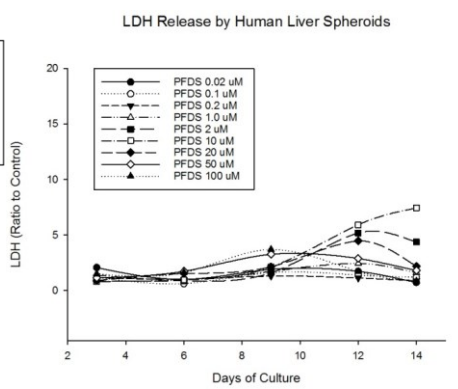
- 100 µM cytotoxic (Day 8 – cell death)
- 50 µM steadily ↑ cell death

PFOA



- 100µM ↑ Cytotoxicity until Day 8
- 50µM ↑ Cytotoxicity after Day 8

PFDS



- ↑ Cytotoxicity 50 & 100 µM (Day 8/10)
- ↑ Cytotoxicity at 2, 10, and 20 µM (Day 10/12)

Increasing number of expressed genes with exposure

Output

Differentially Expressed Genes (DEGs)

Gene expression

Exposure vs. Control (DMSO)

- 1.5-fold change
- p-value < 0.05 (FDR adjusted)

Expected patterns

- ↑ DEGS from low to high dose
- Potency:
- PFOS >> PFBS

X → removed samples (cytotoxicity)

1-Day

µM	0.02	0.1	0.2	1	2	10	20	50	100
PFOS	1	85	3	6	51	167	277	X	X
PFOA	0	8	36	8	19	79	69	227	465
PFDS	0	1	0	6	22	59	81	177	186
PFBS	0	1	0	5	49	5	0	44	73

4-Day

µM	0.02	0.1	0.2	1	2	10	20	50	100
PFOS	0	3	7	20	35	246	285	X	X
PFOA	1	0	17	25	12	30	68	186	822
PFDS	0	3	0	2	4	268	211	220	274
PFBS	7	2	0	15	0	3	0	23	84

10-Day

µM	0.02	0.1	0.2	1	2	10	20	50	100
PFOS	2	7	4	6	60	163	466	X	X
PFOA	14	7	2	4	10	82	101	593	X
PFDS	0	30	0	43	40	134	175	232	231
PFBS	2	1	0	1	2	0	7	54	76

14-Day

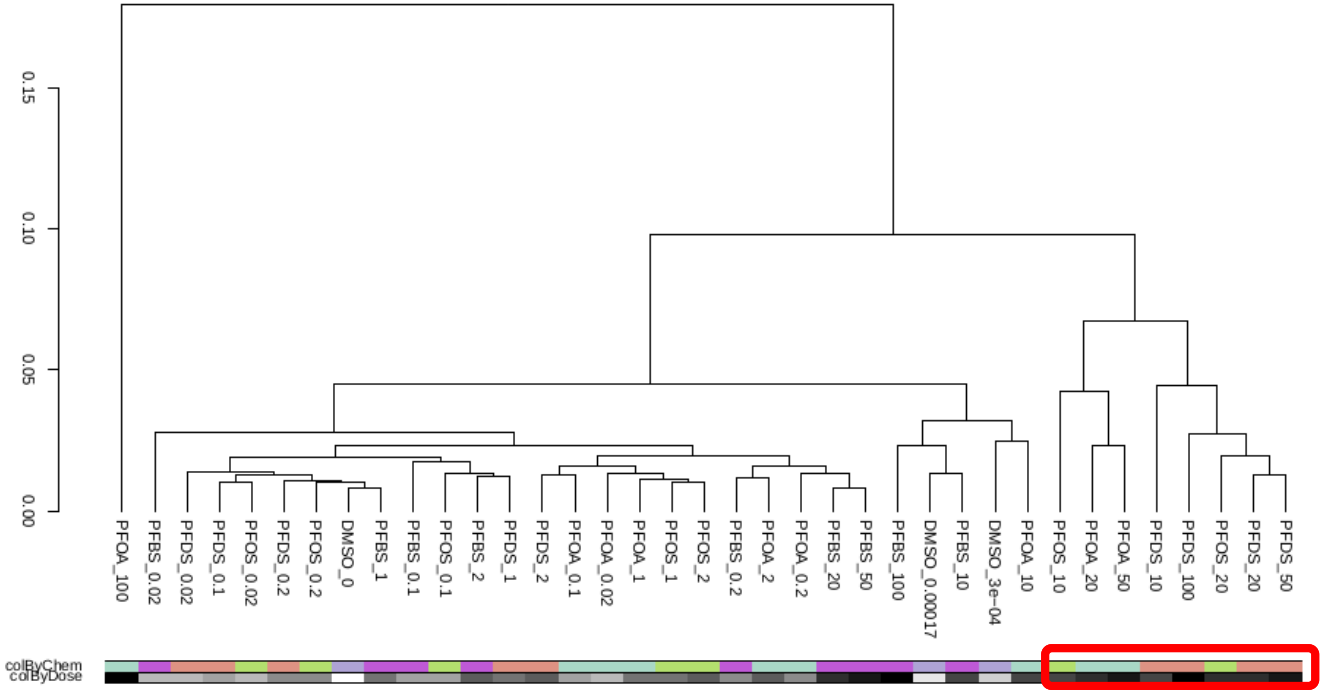
µM	0.02	0.1	0.2	1	2	10	20	50	100
PFOS	0	0	8	5	8	246	373	X	X
PFOA	2	1	3	2	9	66	87	X	X
PFDS	1	2	1	3	96	171	173	187	378
PFBS	0	0	0	0	0	4	1	2	71

Establishing correlation between expression profiles

Output

Differentially Expressed Genes (DEGs)

Hierarchal Cluster Analysis
(consolidated replicates by concentration at 1-Day exposure)



Clustering patterns indicate similarities between sample gene expression profiles

"hclust () function"

Clustering of high concentration samples

Highest source of variation with dose

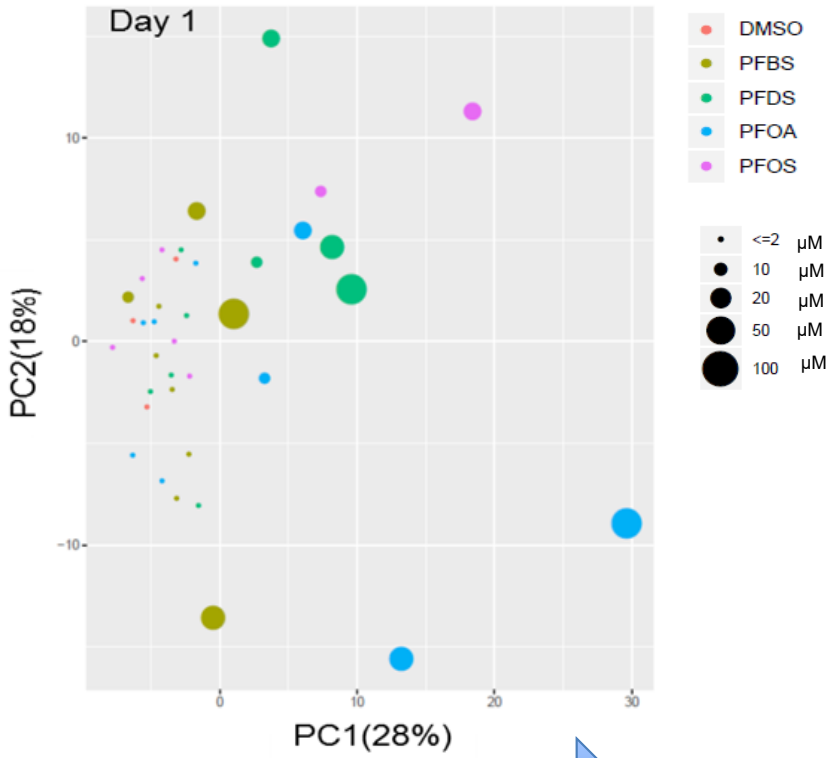
Output

Differentially Expressed Genes (DEGs)

Method to reveal correlations with experimental conditions

- Group
- Dose

Principal component analysis (PCA)



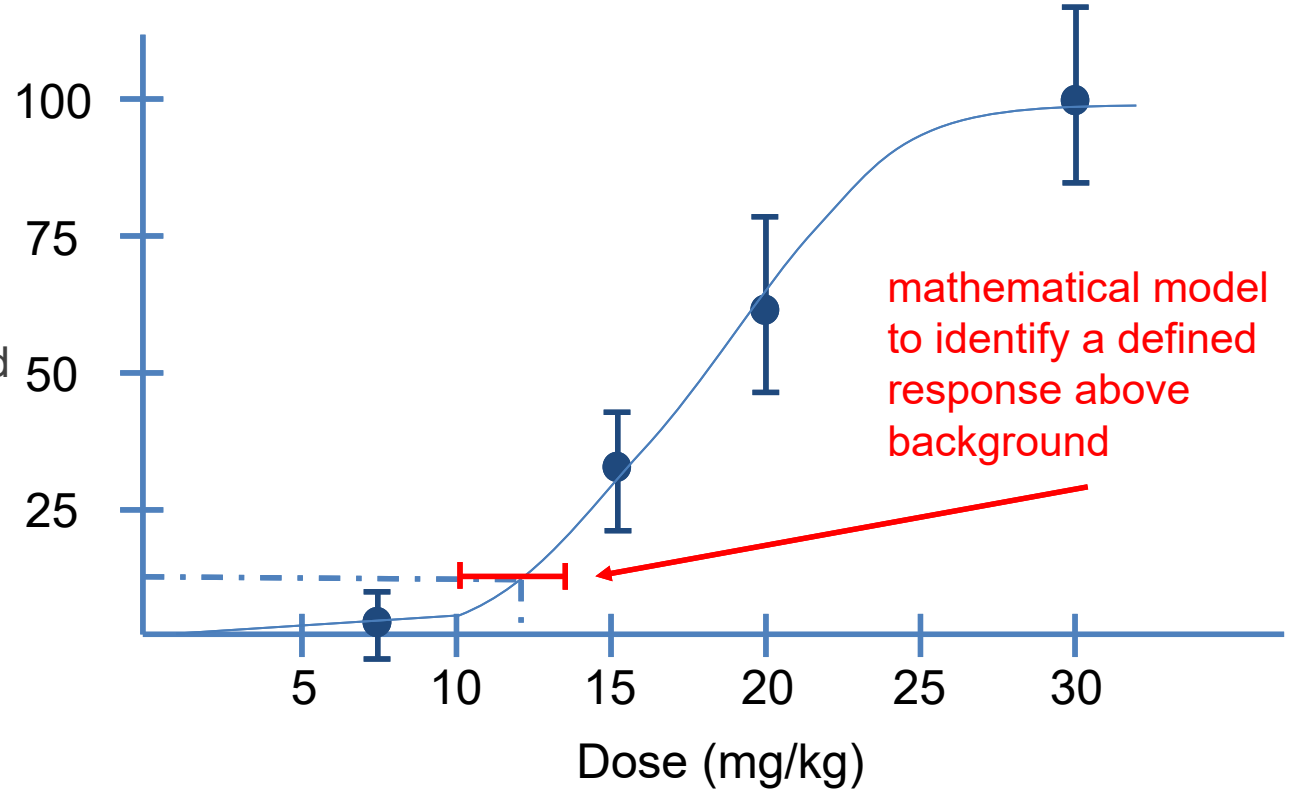
Correlation with dose

What is a benchmark dose?

Output

Benchmark dose (BMD)

1. 3000 genes (S1500 panel)
2. Subset of genes exhibiting a change in response (William Trend Test)
3. Filtered genes are matched to best-fit models
   - BMD based on BMR



Here, we use 1 standard deviation

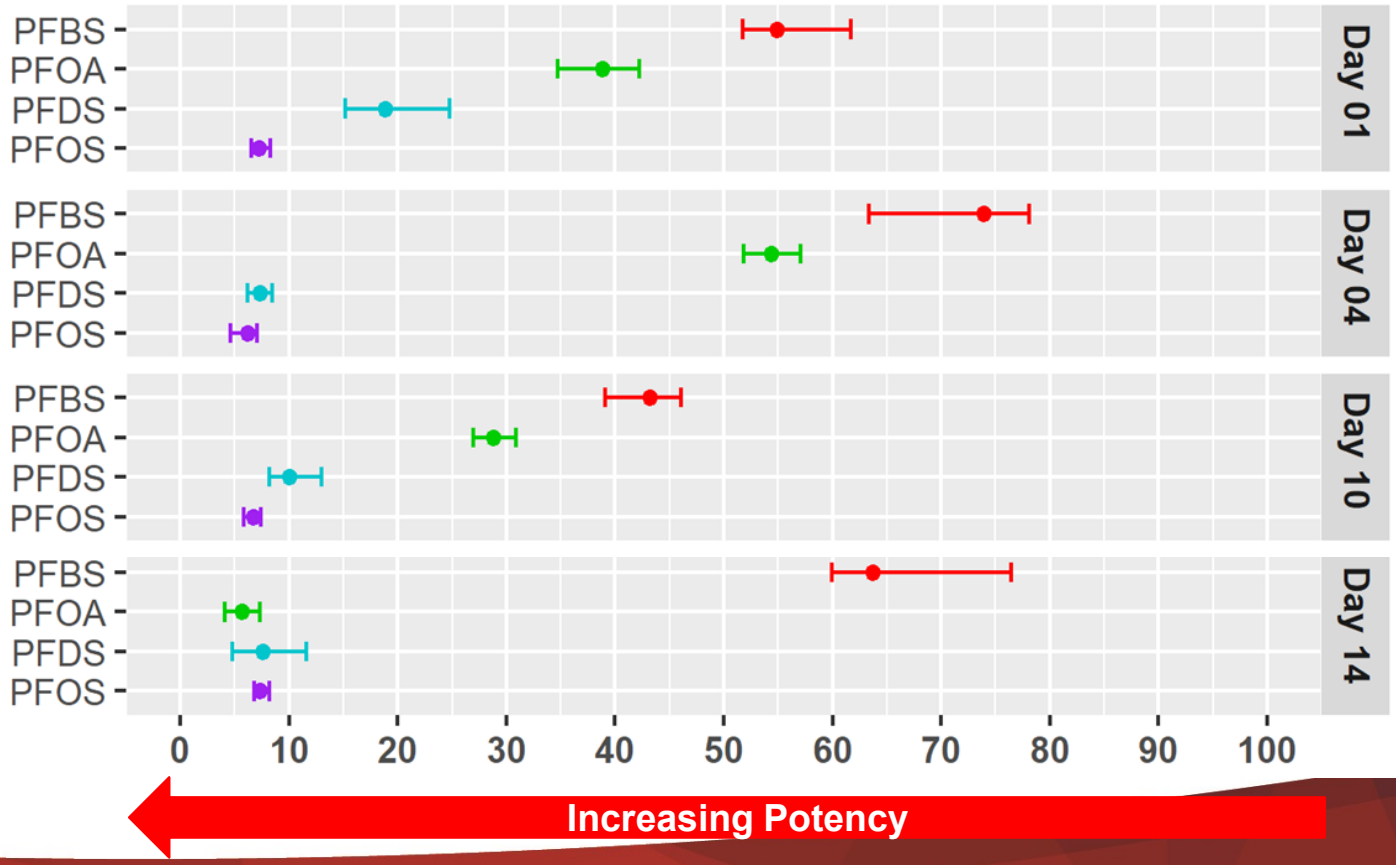
Increasing potency of PFAS with exposure time

Output

Benchmark dose (BMD)

- PFBS – Least potent
- PFOS – Most Potent
- PFOA – Increase potency with time
- PFDS – Equipotent to PFOS by Day 14

Median gene BMDs (95 % confidence interval)



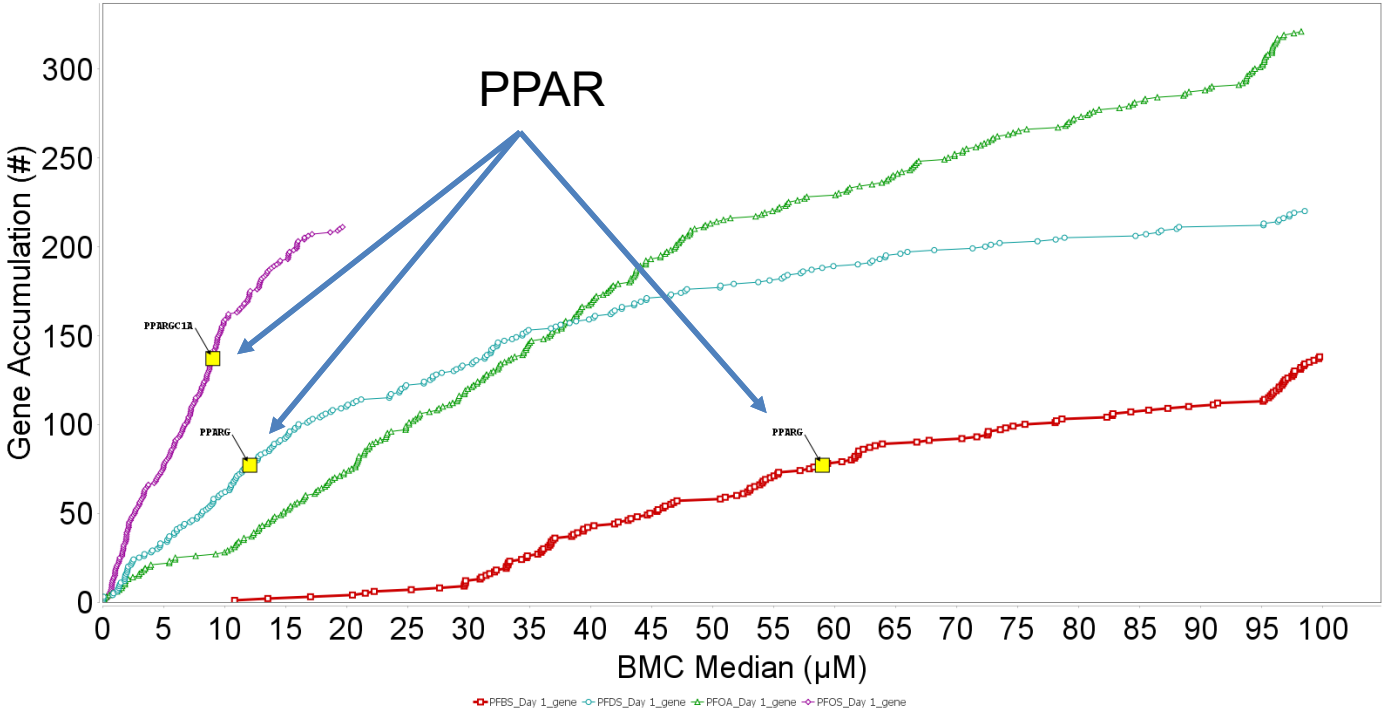
Accumulation plots an indication of transcriptional activity

Output

Benchmark dose (BMD)

- PFBS
- PFOS
- PFOA
- PFDS

BMD accumulation plots – gene level

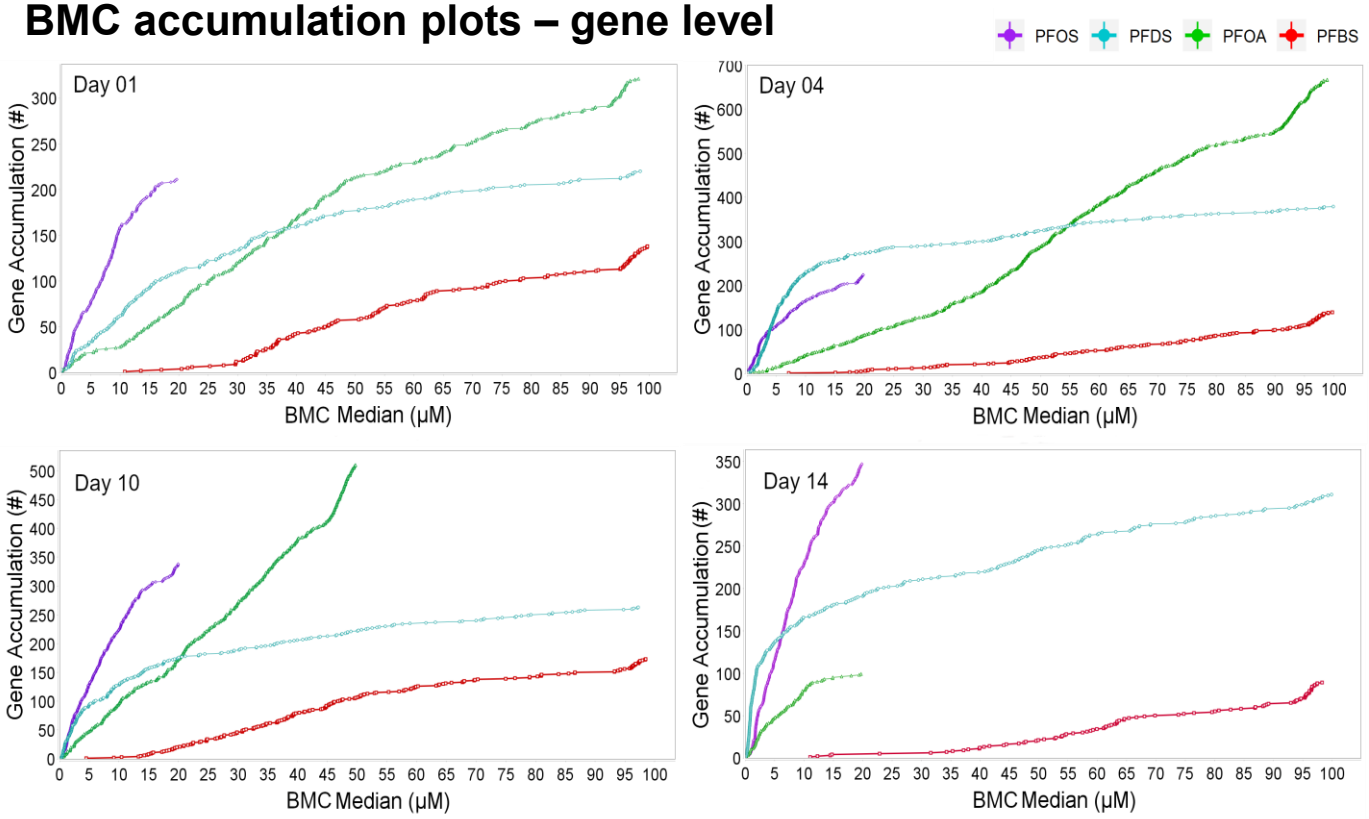


Initiation of transcription activity at low exposures

Output

Benchmark dose (BMD)

BMC accumulation plots – gene level



- Lowest effects occur at similar concentrations for PFOS, PFOA, PFDS (similar potencies)
- Transcriptional activity initiated: 1 – 15 µM

Relevance of exposure in humans

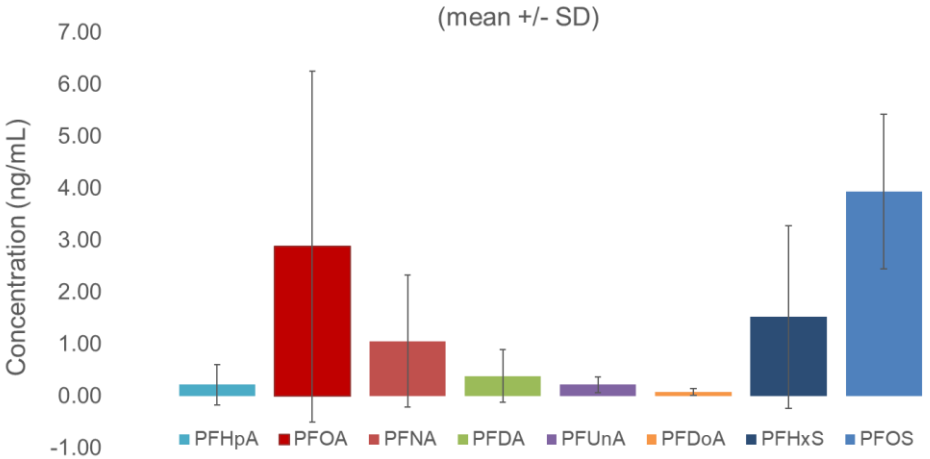
Application

Toxicological relevance

- **Risk**: The *likelihood* that harm from a specific hazard will occur

(https://toxedfoundation.org/hazard-vs-risk/)

RISK = HAZARD x EXPOSURE



Levels of PFAS in human blood (ng/mL)
(Reardon *et al*. 2019)

Determining the level of risk

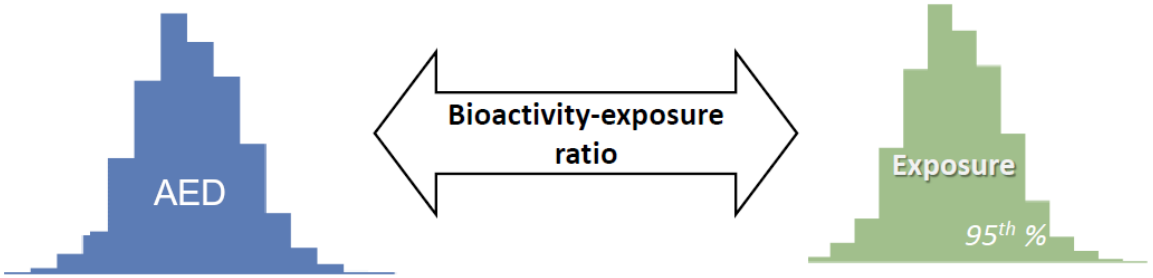
Application

Bioactivity Exposure Ratio (BER)

Transcriptional BMD value (μM)

Conversion factor*

Administered equivalent dose (mg/kg-BW/day)



Upper limit of the daily population exposure

(https://comptox.epa.gov/dashboard)

*The reverse dosimetry approach (Wetmore *et al.*, 2015) for PFOA and PFOS (as described in Wambaugh et al. 2013) was used to determine a conversion factor to calculate the administered equivalent dose from the benchmark concentration estimate of *in vitro* models.

PFOS and PFOA pose risk at current exposure levels

Application

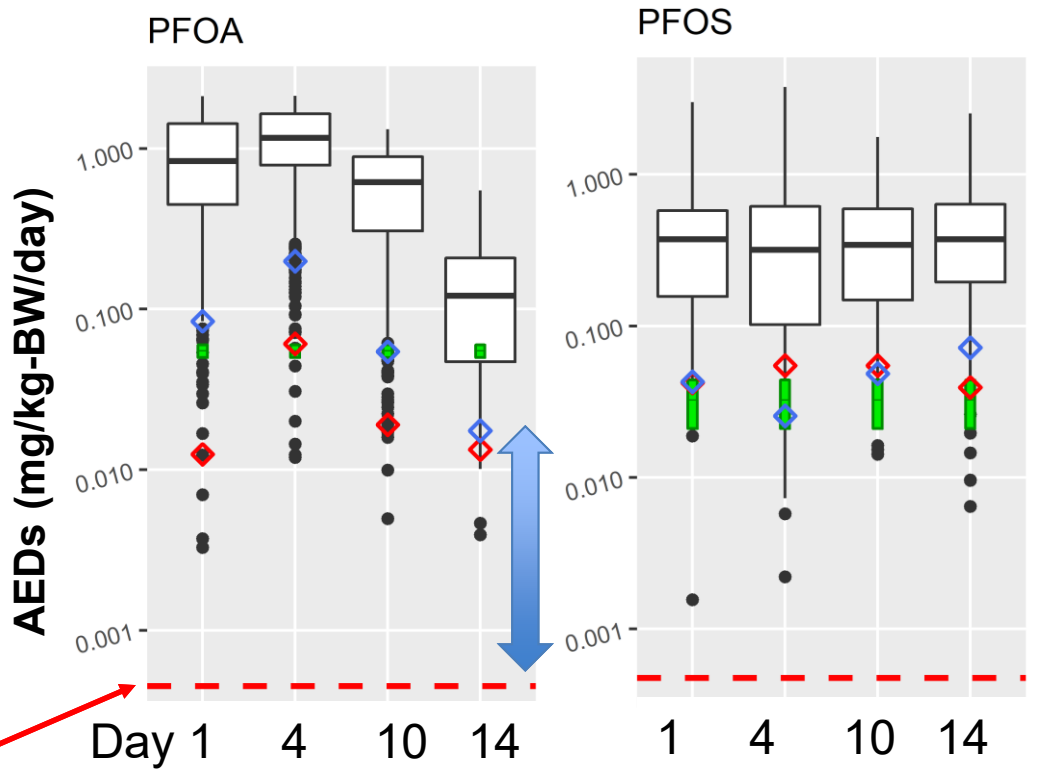
Bioactivity Exposure Ratio (BER)

BER derived by 2 ways:

- **5th percentile gene**
- **Lowest pathway**

- Both approaches were consistent with the apical endpoint BER
  - **Animal PoD**

- BERs **<100**, indicating a narrow margin for highly exposed humans levels



(https://comptox.epa.gov/dashboard).

Summary and conclusions

- This study developed a transcriptomic pipeline to distinguish similarities & differences between model PFAS

- The transcriptional BER was highly consistent with the apical endpoint BER in the HC drinking water guidelines → **confidence to this NAM**
  - PFAS exposure →transcriptional changes →BMD modelling for potency
    - PFAS became more potent over time
    - Longer-chain PFAS had similar patterns of potency
      - short-chain PFBS did not follow this pattern

  - Demonstrates the efficiency of high-throughput transcriptomics to provide valuable data to facilitate risk assessment

Rowan-Carroll *et al*. (preprint). High-throughput transcriptomic analysis of human primary hepatocyte spheroids exposed to per- and polyfluoroalkyl substances (PFAS) as a platform for relative potency characterization

Objectives and Approach

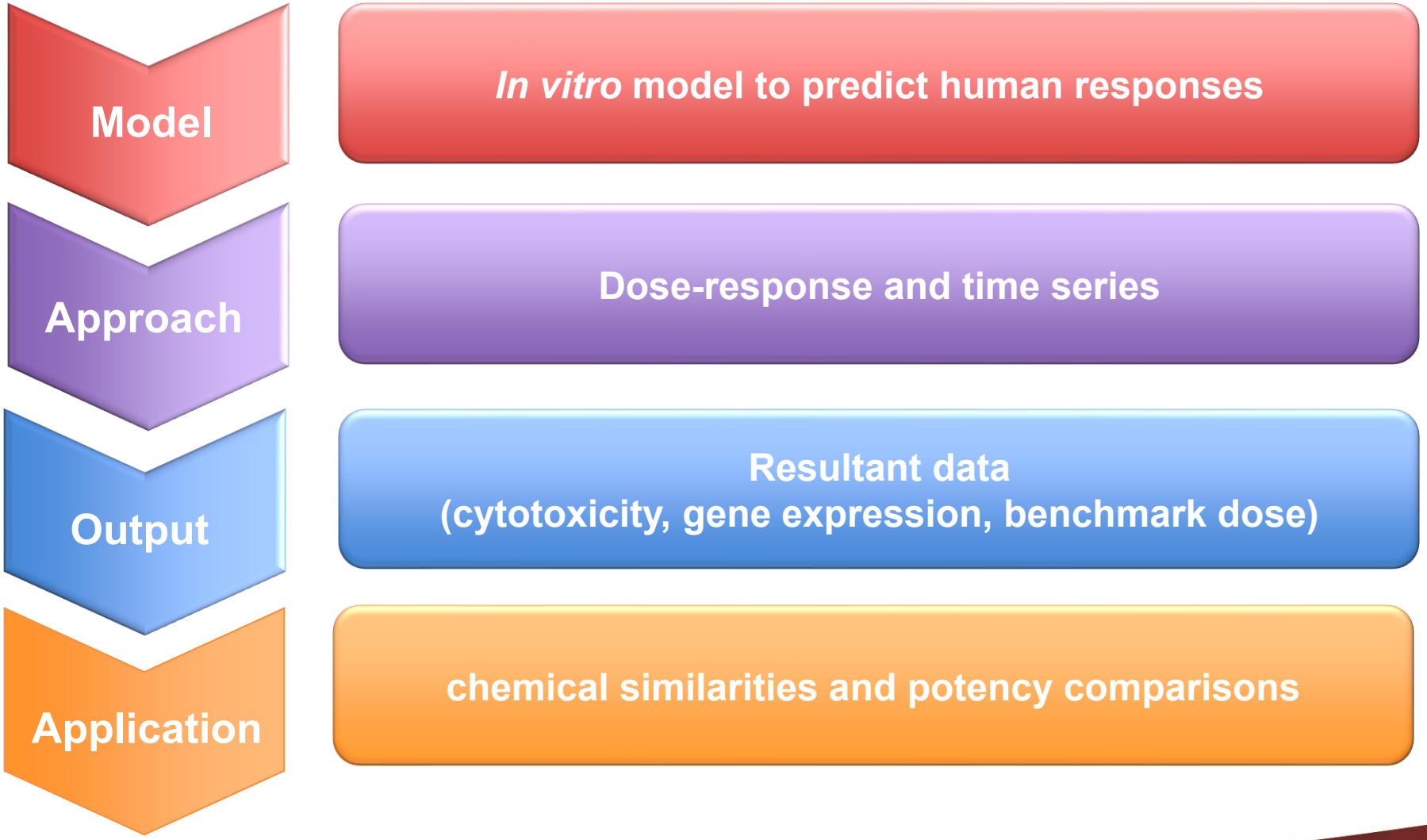
- **Overarching:** Use gene expression profiling to acquire information on PFAS to facilitate read-across for human health risk assessment
  - Conduct a high-throughput transcriptomic dose-response and time series analysis of primary human liver spheroids exposed to PFAS

- Experiment 1 - Microscopy
  - Microscopic characterization of biochemical responses of spheroids to PFAS (staining for markers of toxicity)

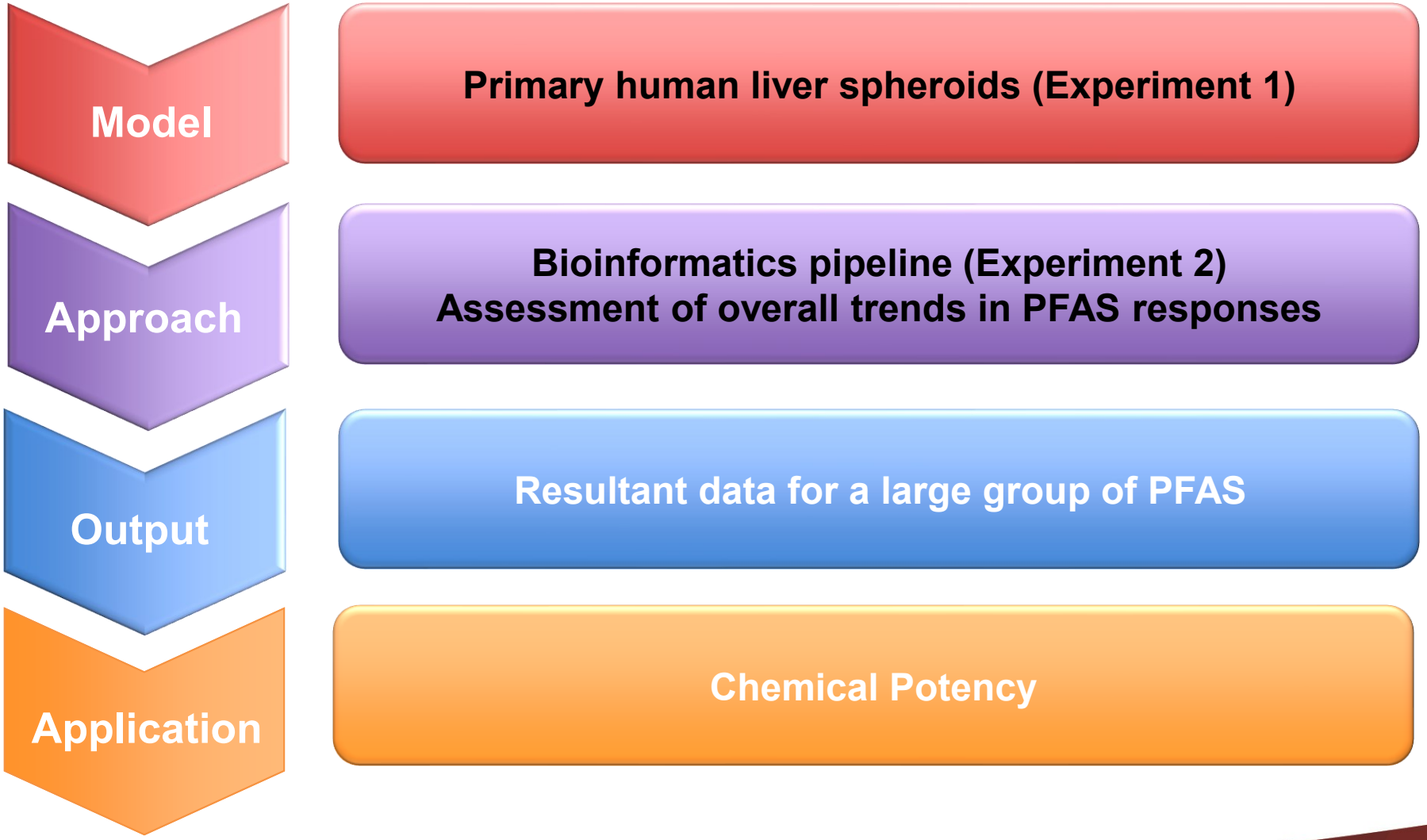
- Experiment 2 - Time-series, dose-response analysis of prototype PFAS
  - Cytotoxicity assessment and Tempo-Seq analysis
  - Development of bioinformatics pipeline

- Experiment 3 – Prioritizing PFAS as a class; time- and dose-response
  - Establish potency ranking within the class of PFAS

Overview


Model
In vitro model to predict human responses
Approach
Dose-response and time series
Output
Resultant data
(cytotoxicity, gene expression, benchmark dose)
Application
chemical similarities and potency comparisons

Overview


Model
Primary human liver spheroids (Experiment 1)
Approach
Bioinformatics pipeline (Experiment 2)
Assessment of overall trends in PFAS responses
Output
Resultant data for a large group of PFAS
Application
Chemical Potency

Scaling up to a larger number of PFAS

Approach

Overview of experimental design

Exposure

- 23 PFAS
- Dose range (0 to 100 µM)
- Time series (1, and 10)

Data handling

- Use bioinformatics pipeline developed as part of experiment 2

Assessment

1. Cytotoxicity (same approach as from experiment 2)
2. Genomic responses
3. Potency rankings

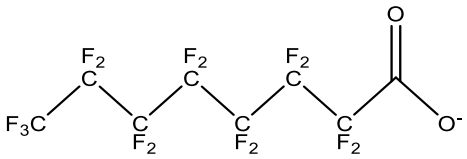
Scaling up to a larger number of PFAS

Approach

Overview of experimental design

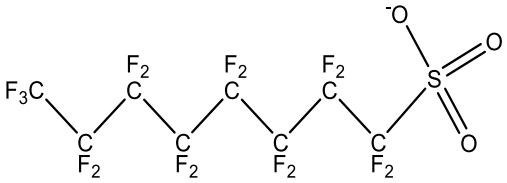
Categorizing PFAS

Perlfuoroalkyl carboxylates (PFCAs)



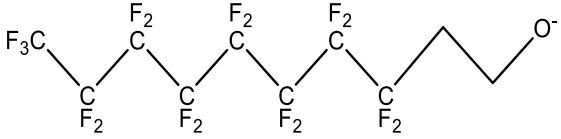
Perfluorooctanoate (PFOA)

Perlfuoroalkyl suflonates (PFSAs)



Perfluorooctane sulfonate (PFOS)

PFAS precursors



8:2 Fluorotelomer alcohol (8:2 FTOH)

Longer-chain PFAS increase in DEGs with exposure

Output

Differentially expressed genes (DEGs)

- Shorter-chain PFAS do not show trend in DEGs
- Longer-chain PFAS increased DEGs with exposure conc.

	# of carbons		Concentration (μM)					
			0.2	2	10	20	50	100
PFCAs	4	PFBA	5	42	1	10	15	58
		PFPeA	-	1	2	2	-	-
		PFHxA	119	43	22	60	101	24
		PFHpA	-	-	1	4	14	51
		PFOA	-	11	72	71	229	491
		PFNA	3	-	37	167	236	785
		PFDA	-	4	1	70	364	-
		PFUnA[A]	40	20	119	227	826	-
	14	PFTeA[B]	46	9	128	55	69	43
PFSAs	4	PFBS	-	34	7	-	50	72
		PFHxS	47	-	-	12	11	16
		PFHpS	-	1	14	26	61	225
		PFOS	1	50	171	295	-	-
	10	PFDS	-	17	49	75	177	190

Concentration of [A] PFUnA (0.13, 1.3, 6.5, 13, 34, 66 μM) [B] PFTeDA (0.06, 0.67, 3.35, 6.7, 17, 33 μM)

Most PFAS precursors have no discernable trend

Output

Differentially expressed genes (DEGs)

- Exception of PFOSA that increased in DEGs
- Non-monotonic dose response
  - DEG spike

Precursors	Concentration (μM)					
	0.2	**2**	**10**	**20**	**50**	**100**
Acid 5:3	-	3	-	9	8	23
MonoPAP 6:2	-	-	7	190	6	7
MonoPAP 8:2	1	-	-	-	1	3
FtOH 6:2	12	55	43	21	67	32
FtOH 8:2	-	7	-	9	-	-
FTS 4:2	-	10	19	10	5	1
FtS 6:2	3	5	15	6	69	7
FtS 8:2	4	29	56	127	212	237
PFOSA	10	30	18	141	799	-

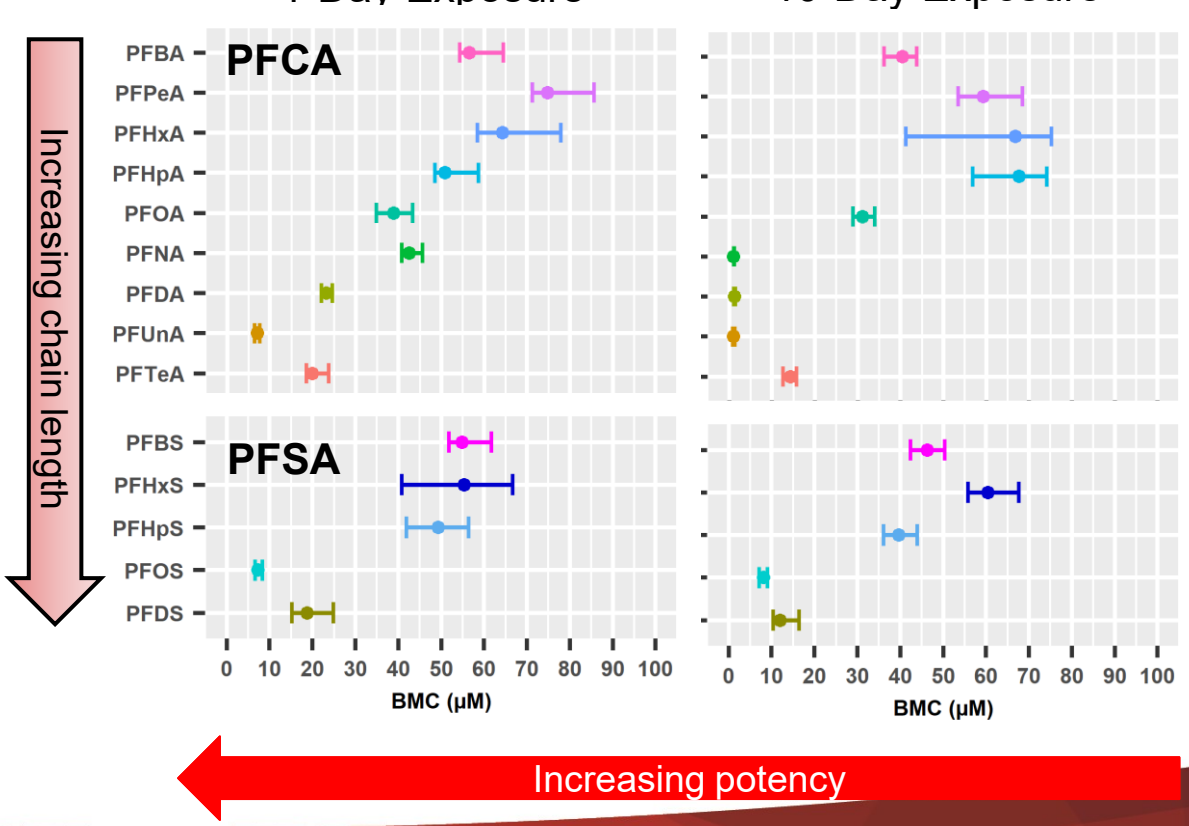
Increase potency with carbon chain length

Output

Benchmark Dose

- Increasing potency with chain-length within both subgroups
- Increased cytotoxicity of longer-chain length PFCAs
  - PFNA
  - PFDA
  - PFUnA

Median gene BMDs (95 % confidence interval)

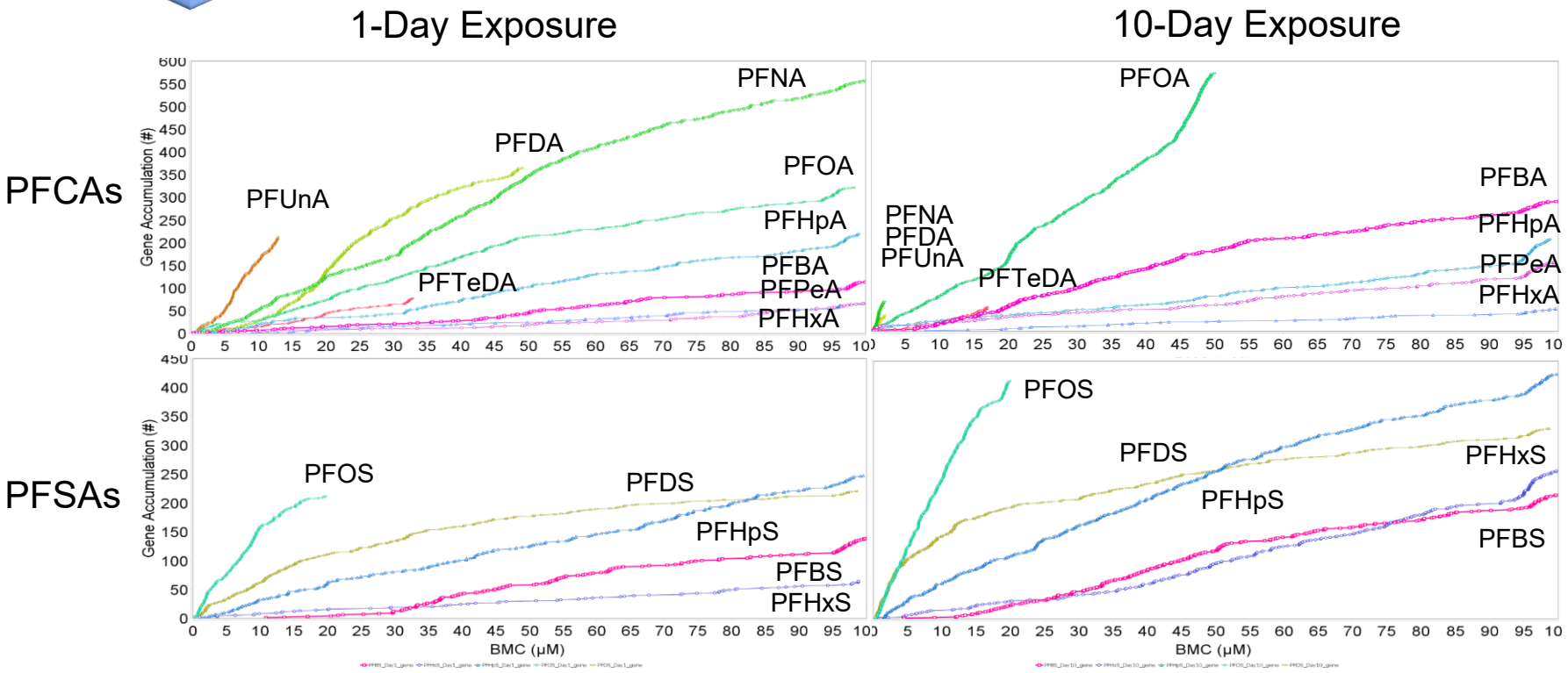


Gene accumulation plots get messy

Output

Benchmark Dose

BMD accumulation plots – gene level

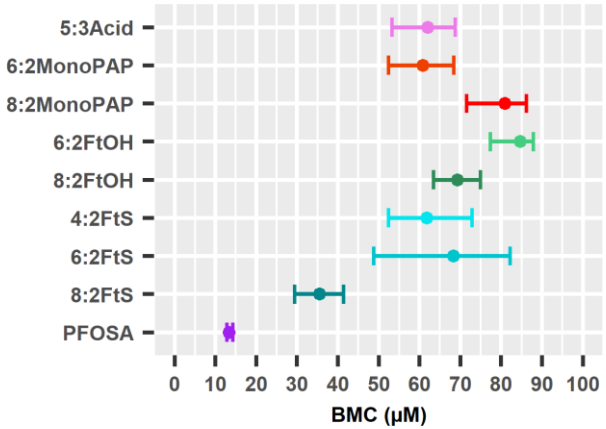


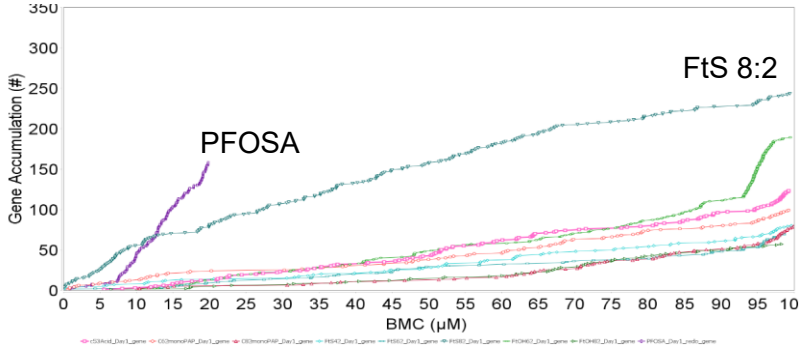
PFAS precursors have no discernable trend

Output

Benchmark Dose

1-Day Exposure





- PFOSA (a PFOS precursor) was found to exhibit the highest potency and transcriptional activity of this subgroup

Experiment 3 - Summary

- Relationships between chain length & extent of transcriptional alterations, and potency that emerged that can be used to inform read-across

- PFAS cause cytotoxicity in human liver cell spheroids & transcriptional changes at similar concentrations to PFOS and PFOA, suggesting these chemicals are harmful to human liver

- This case study is building confidence in the application of transcriptomic BMD modelling for:
    - Potency comparisons
    - Chemical prioritization, scoping/screening assessments

Reardon *et al*. (preprint). High-throughput transcriptomics and benchmark concentration modeling for potency ranking of per- and polyfluoroalkyl substances (PFAS) in exposed human liver cell spheroids

Thank You!

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