Assessing effects of mixtures of Per- and Polyfluoroalkyl Substances (PFAS) using transcriptomic points of departure

Gregory Addicks

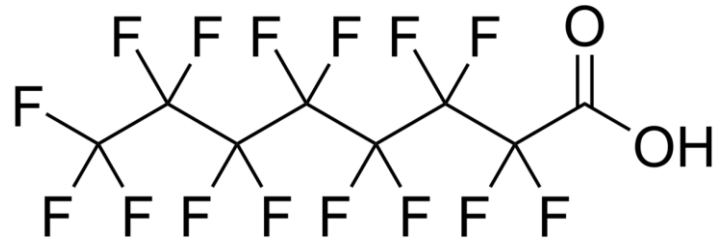
DSRG presentation - March 7th 2023

Overview

- Introduction
  - PFAS chemicals and why mixtures are a concern
- Main Question
  - Do PFAS have synergistic or antagonistic effects or are their effects additive?
- Methodology
  - Chemicals and mixtures – Cells and treatments – Sequencing and QC
  - Data processing and BMC generation with BMD Express
  - Using data to predict mixture potency
- Results
  - Comparison of predicted mixture potency to empirical mixture potency

The Miracle of PFAS

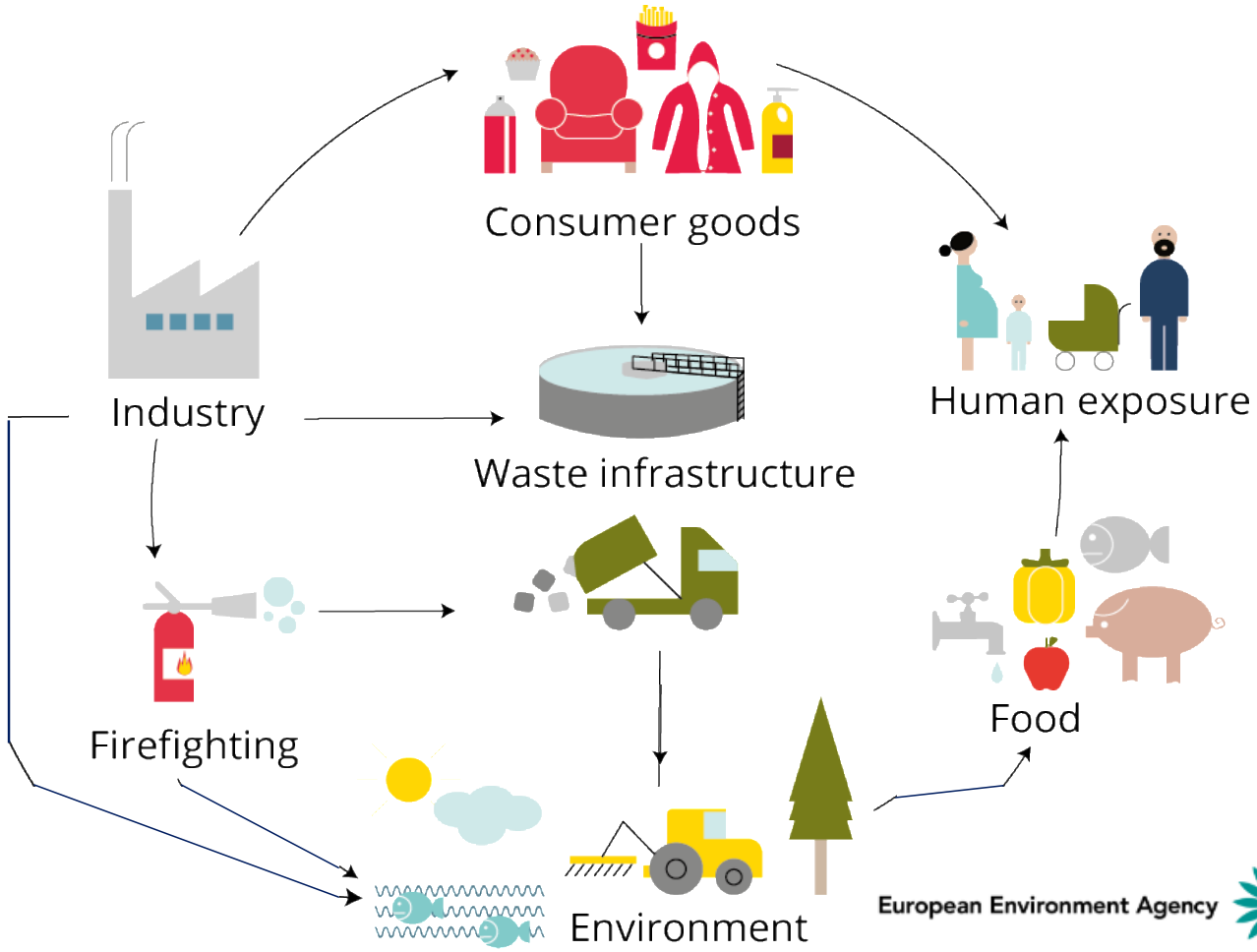
PFAS
PFOA (8C)



PFAS are Per and Polyfluoroakyl Substances

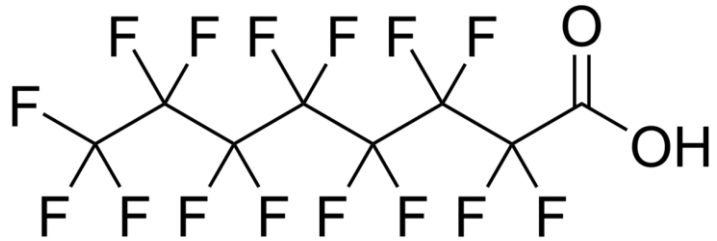
- Hydrophobic
- Lipophobic
- Heat and Fireproof
- Non-stick
- Very low surface tension
  - Helps stuff flow and/or stick

- Breathable waterproof fabrics
- Non-stick cookware
- Waxed paper
- Waterproof makeup
- Stain guard
- Fire fighting foam
- Industrial processes



The Problem with PFAS

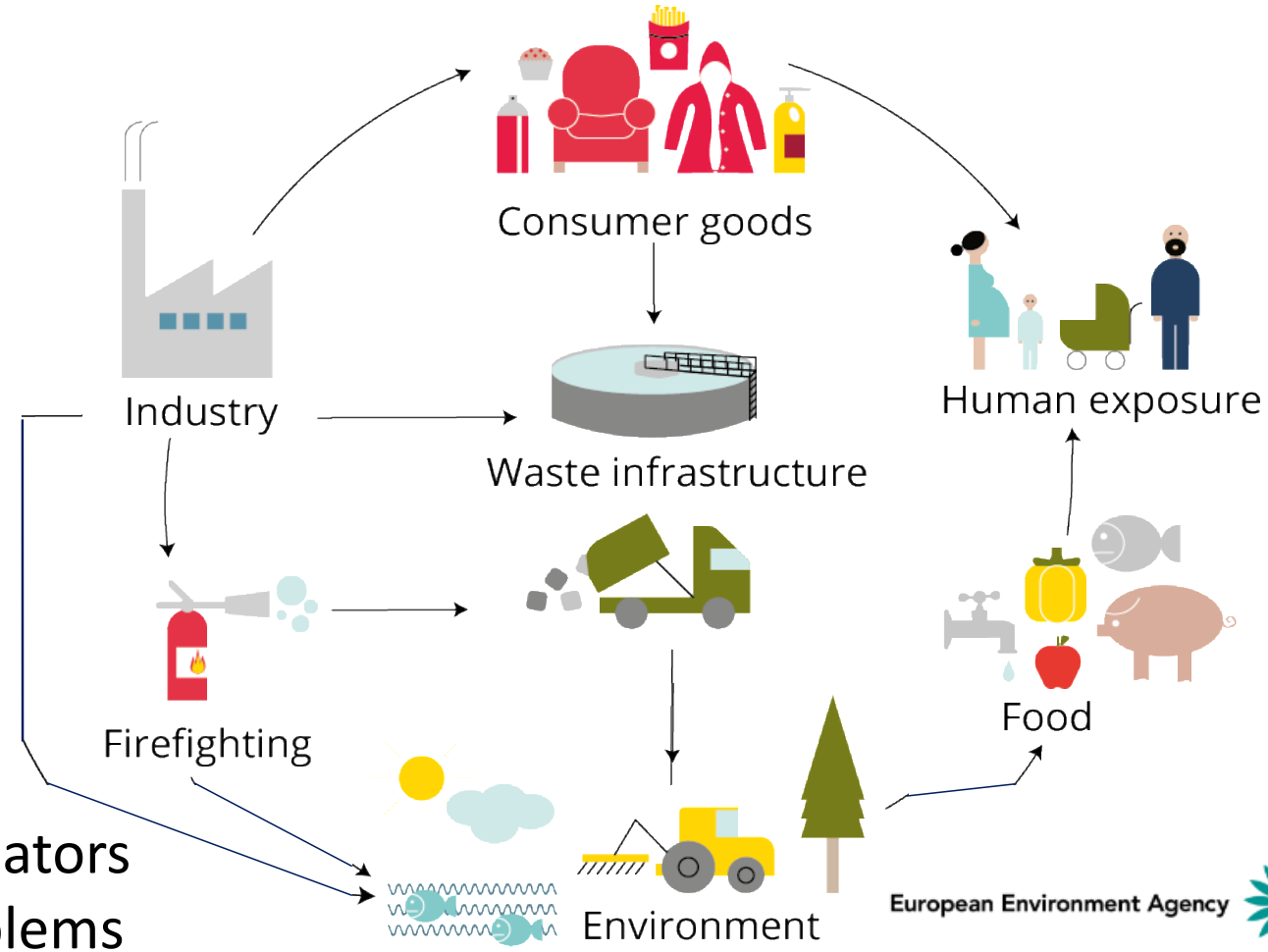
PFAS
PFOA (8C)



PFAS are Per and Polyfluoroakyl Substances

- Hydrophobic
- Lipophobic
- Heat and Fireproof
- Non-stick
- Very low surface tension
  - Helps stuff flow and/or stick

- Do not break down in environment
- Mobile in the environment
- Accumulate in biological organisms
- Resemble metabolic substrates
- Interact with cellular metabolism regulators
- Associated with numerous health problems



The PFAS Problem

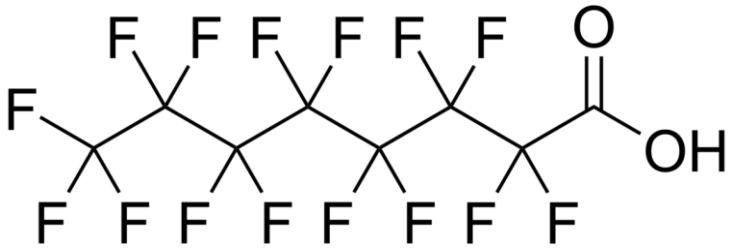
PROTEIN | SCIENCE

Unveiling the binding mode of perfluorooctanoic acid to human serum albumin

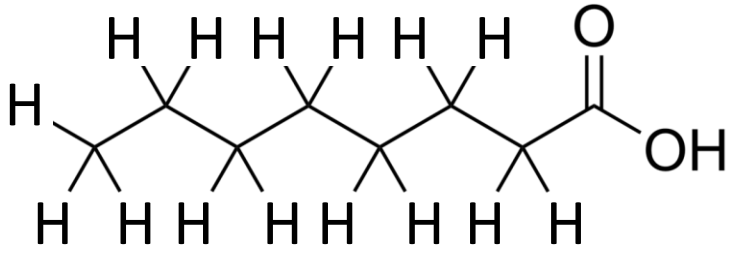
Lorenzo Maso,

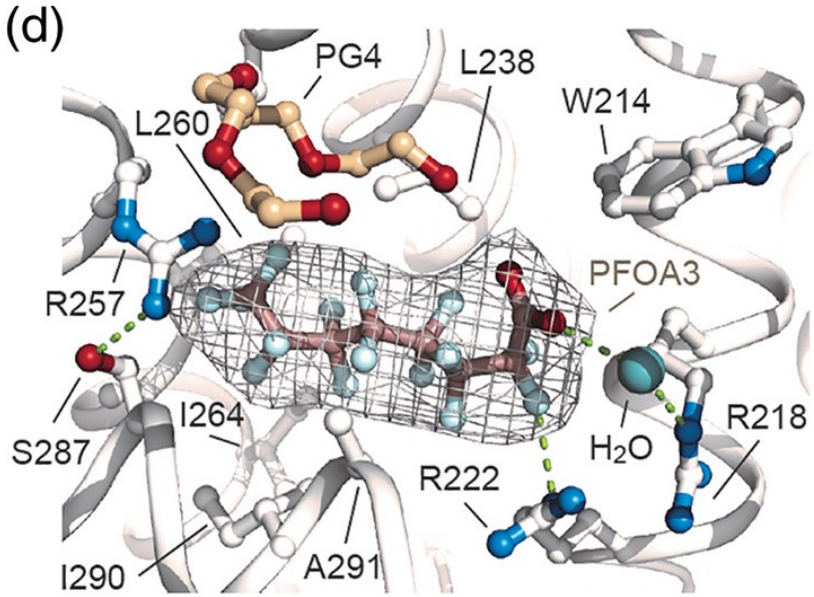
07 February 2021 | https://doi.org/10.1002/pro.4036 |

PFAS
PFOA (8C)



Fatty Acid
Capyrlic Acid
(8C)





- Resemble metabolic substrates
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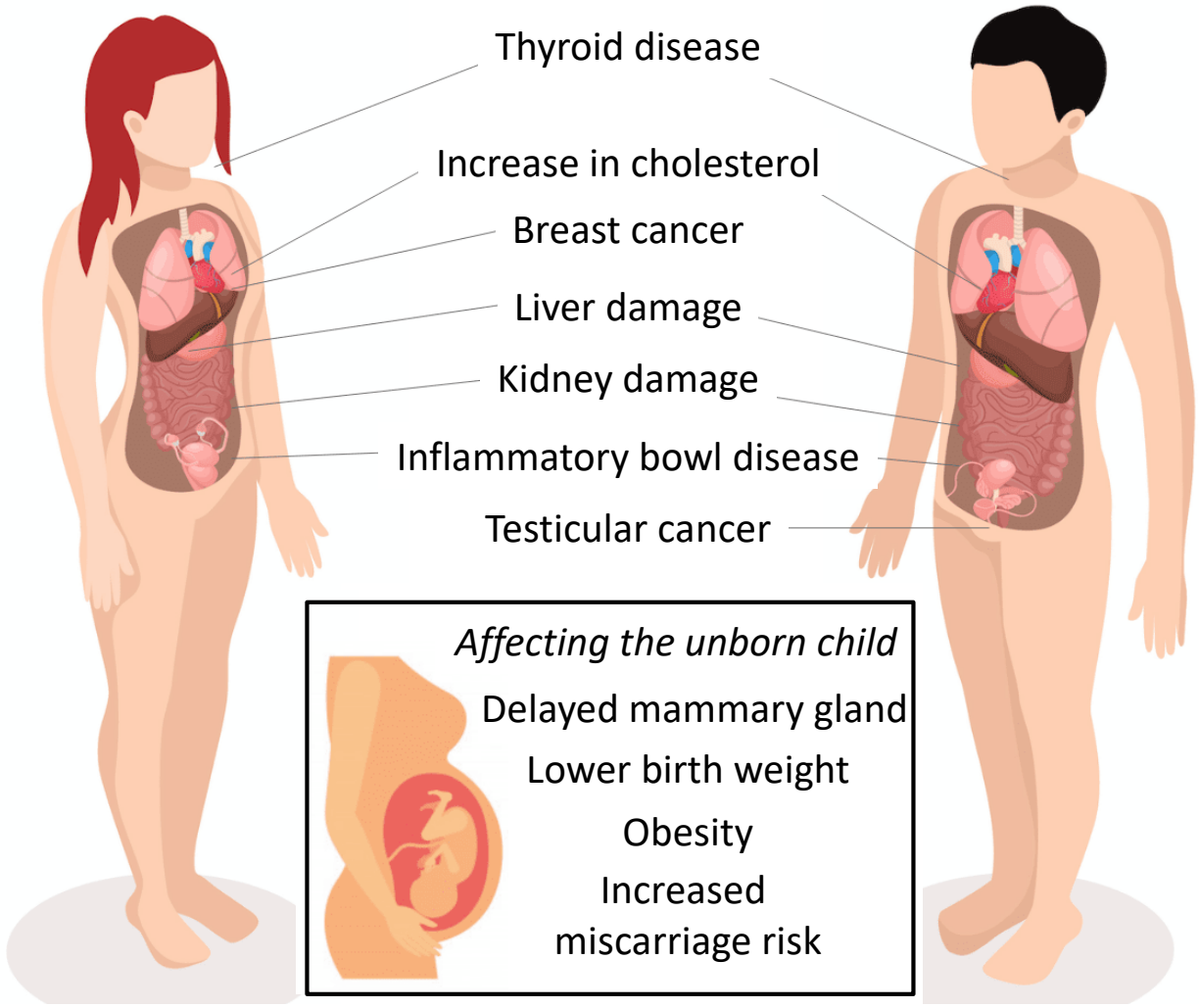


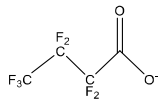
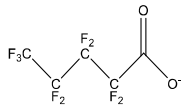
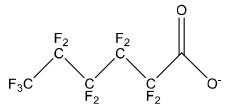
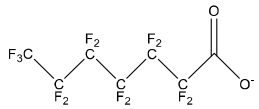
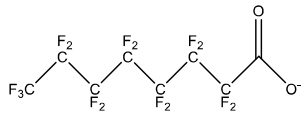
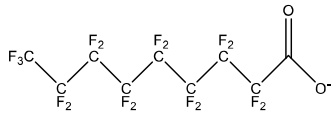
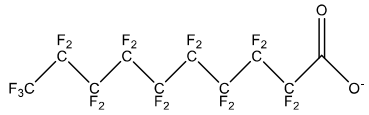
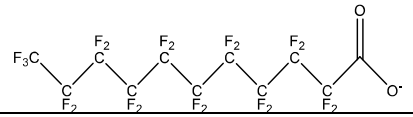
European Environment Agency

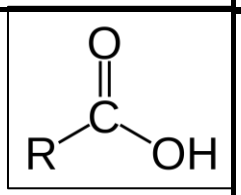
- Associated with numerous health problems

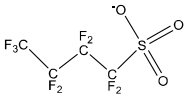
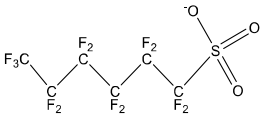
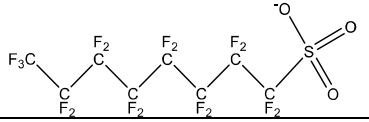
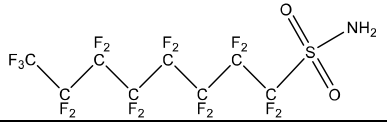
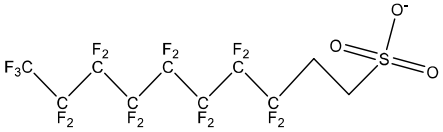
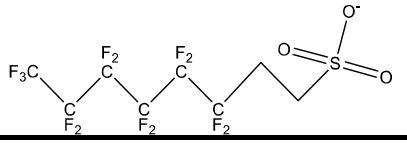
Project Overview - Assess PFAS mixtures

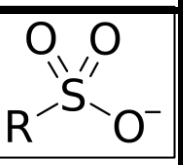
- Assess potency of PFAS mixtures
  - PFAS are in the environment– exposure to multiple PFAS is supported by biological screening surveys worldwide
  - Do PFAS mixtures have additive, synergistic or antagonistic effects?
- Human Liver Spheroids exposed to PFAS and PFAS mixtures
- Transcriptomic analysis of overall change to gene expression
- In-vitro
  - Exposures are most relevant to concentrations at plasma / cellular level
    - Data is not directly usable for regulatory purposes
    - Exposures need in-vitro to in-vivo extrapolation

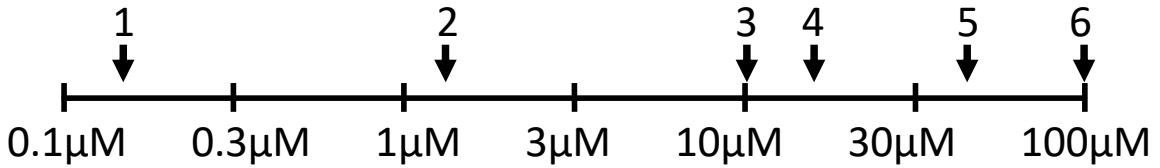
PFAS used in this study

Class	Name (Acronym)	Structure
Perfluoroalkyl carboxylates (PFCAs)	Perfluorobutanoate (PFBA)	
	Perfluoropentanoate (PFPeA)	
	Perfluorohexanoate (PFHxA)	
	Perfluoroheptanoate (PFHpA)	
	Perfluorooctanoate (PFOA)	
	Perfluorononanoate (PFNA)	
	Perfluorodecanoate (PFDA)	
	Perfluoroundecanoate (PFUnA)	



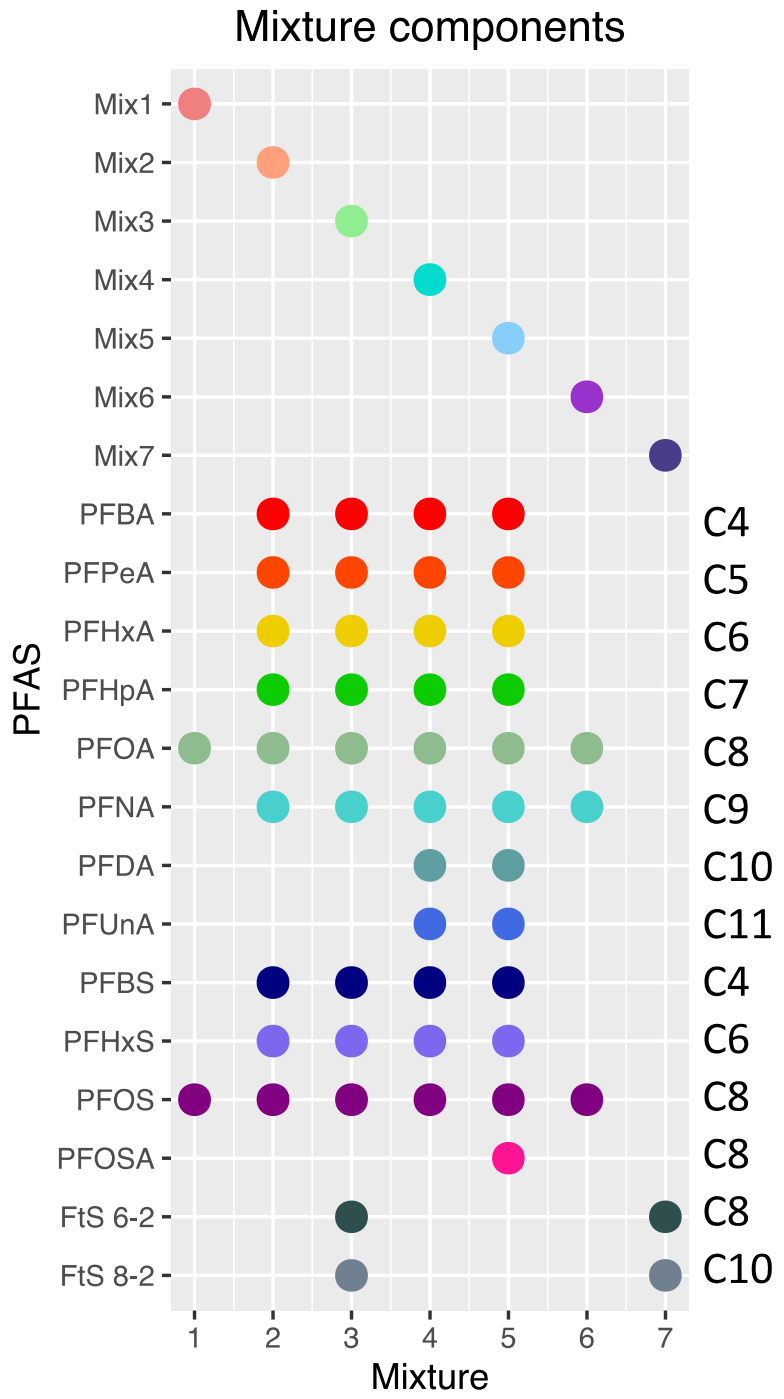
Class	Name (Acronym)	Structure
Perfluoroalkyl sulfonates (PFSAs)	Perfluorobutane sulfonate (PFBS)	
	Perfluorohexane sulfonate (PFHxS)	
	Perfluorooctane sulfonate (PFOS)	
Sulfonamide	Perfluorooctane sulfonamide (PFOSA)	
Sulfonate telomers	8:2 Fluorotelomer sulfonate (8:2 FtS)	
	6:2 Fluorotelomer sulfonate (6:2 FtS)	





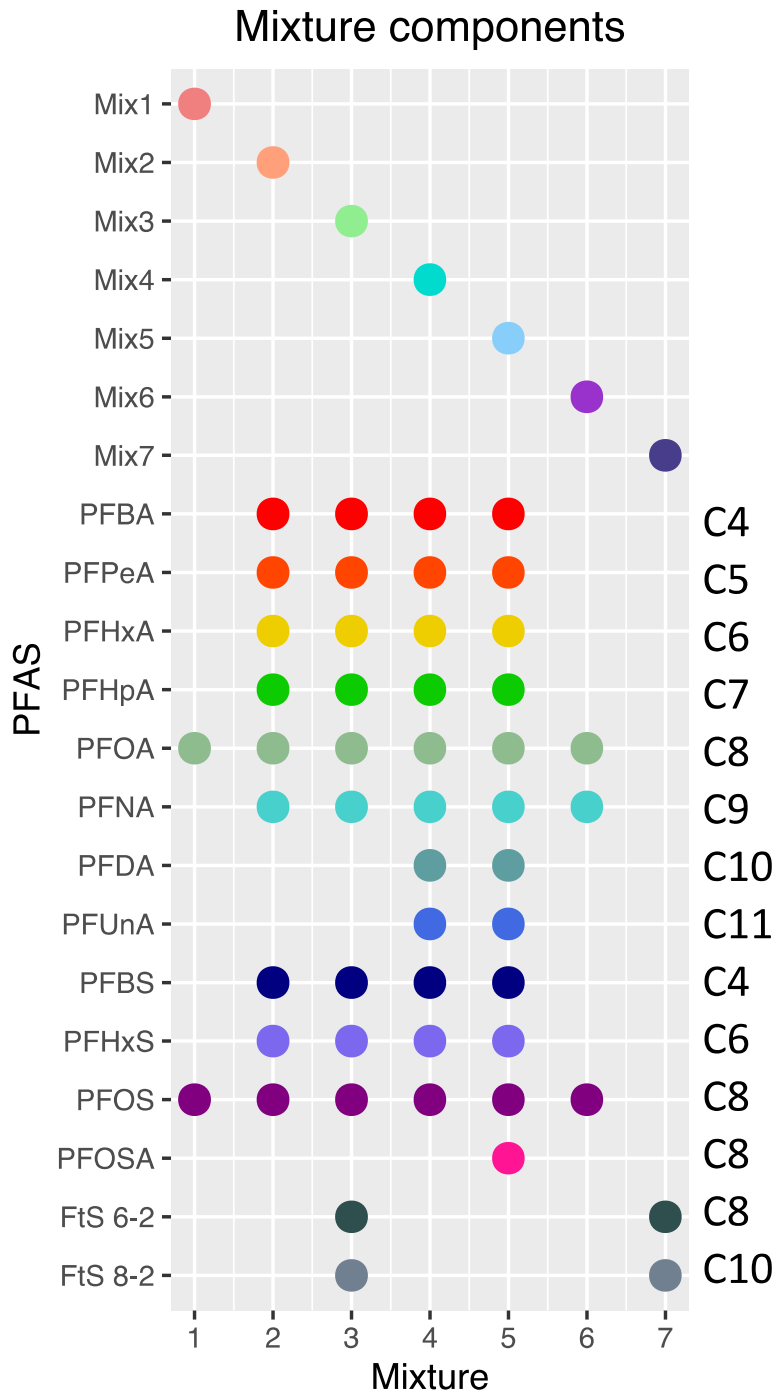
PFAS Mixtures

- 14 PFAS used in study
- 7 mixtures of varying complexity and composition



PFAS Mixtures

- 14 PFAS used in study
- 7 mixtures of varying complexity and composition
- Mixture concentration range similar to PFAS
  - Exposure concentration for mixtures =
    - Combined concentration of all PFAS
    - 2µM Mixture = 1µM POFA + 1µM PFOS



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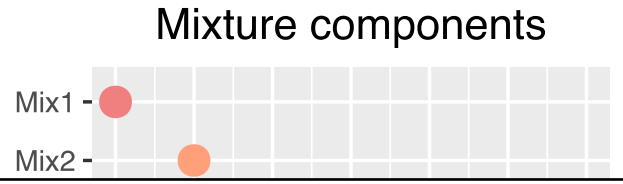
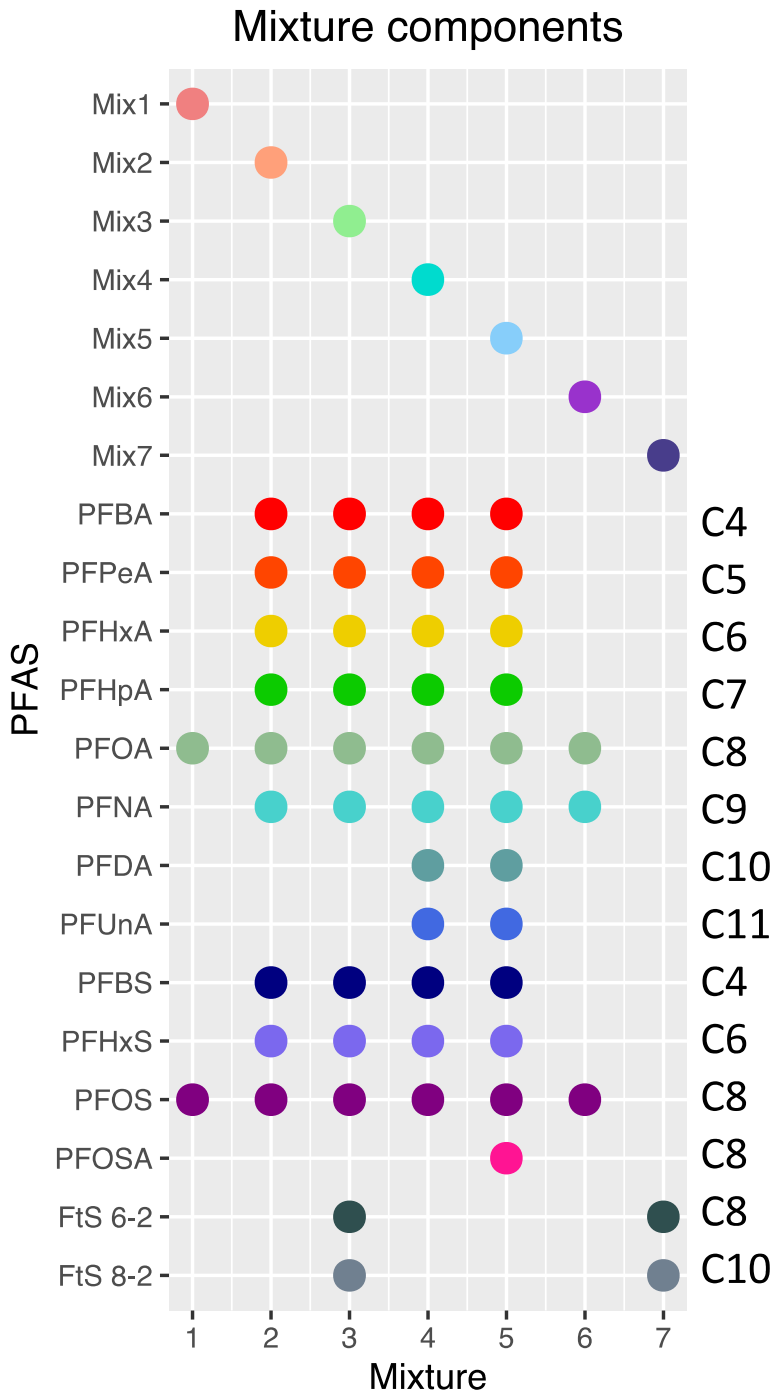


Non Mixtures	----------	Individual PFAS	Concentrations (µM)
Single PFAS		PFBA, PFPeA, PFHxA, PFHpA, PFOA*, PFNA, PFDA, PFUnA, PFBS*, PFOS*, PFHxS, PFOSA, 6:2 FtS, 8:2 FtS † PFUnA	0.2, 2, 10, 20, 50, 100 * also 0.02, 0.1, 1 † 0.13, 1.3, 6.5, 13, 34, 66
Mixture Name (# PFAS in Mix)	Subgroup	Individual PFAS	Concentrations (µM)
1 (2)	PFCAs (1) PFSAs (1)	PFOA PFOS	0.4, 2, 4, 20, 40, 100
2 (9)	PFCAs (6) PFSAs (3)	PFBA + PFPeA + PFHxA + PFHpA + PFOA + PFNA PFBS + PFHxS + PFOS	0.18, 1.8, 9, 18, 45, 100
3 (11)	PFCAs (6) PFSAs (3) Other (2)	PFBA + PFPeA + PFHxA + PFHpA + PFOA + PFNA PFBS + PFHxS + PFOS 6:2 FtS + 8:2 FtS	0.22, 2.2, 11, 22, 55, 100
4 (11)	PFCAs (8) PFSAs (3)	PFBA + PFPeA + PFHxA + PFHpA + PFOA + PFNA + PFDA + PFUnA PFBS + PFHxS + PFOS	0.22, 2.2, 11, 22, 55, 100
5 (12)	PFCAs (8) PFSAs (3) Other (1)	PFBA + PFPeA + PFHxA + PFHpA + PFOA + PFNA + PFDA + PFUnA PFBS + PFHxS + PFOS PFOSA	0.24, 2.4, 12, 24, 60, 100
6 (3)	PFCAs (2) PFSAs (1)	PFOA + PFNA PFOS	0.9, 9, 18, 30, 60, 100
7 (2)	Other (2)	6:2 FtS + 8:2 FtS	0.4, 2, 4, 20, 40

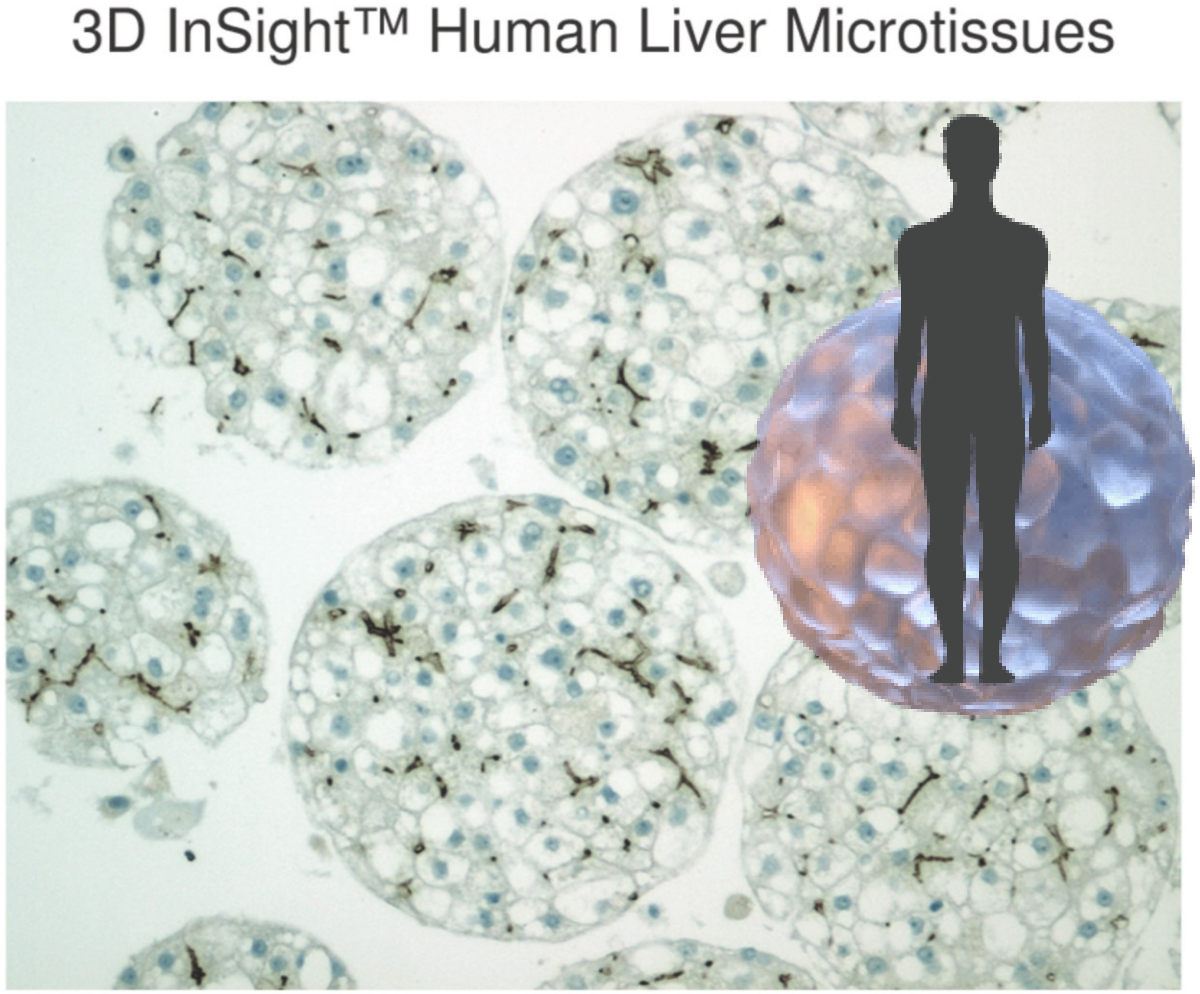
PFAS Mixtures

- 14 PFAS used in study
- 7 mixtures of varying complexity and composition
- Mixture concentration range similar to PFAS
  - Exposure concentration for mixtures =
    - Combined concentration of all PFAS
    - 2µM Mixture = 1µM POFA + 1µM PFOS
  - Mixtures are all equimolar
    - External exposures are variable
    - Each component has opportunity to affect total mixture potency



PFAS exposed Human Liver Spheroids

- Contains cells from 10 donors
  - Donors from both sexes
  - Kupffer cells and hepatocytes
- ~2000 cells per spheroid



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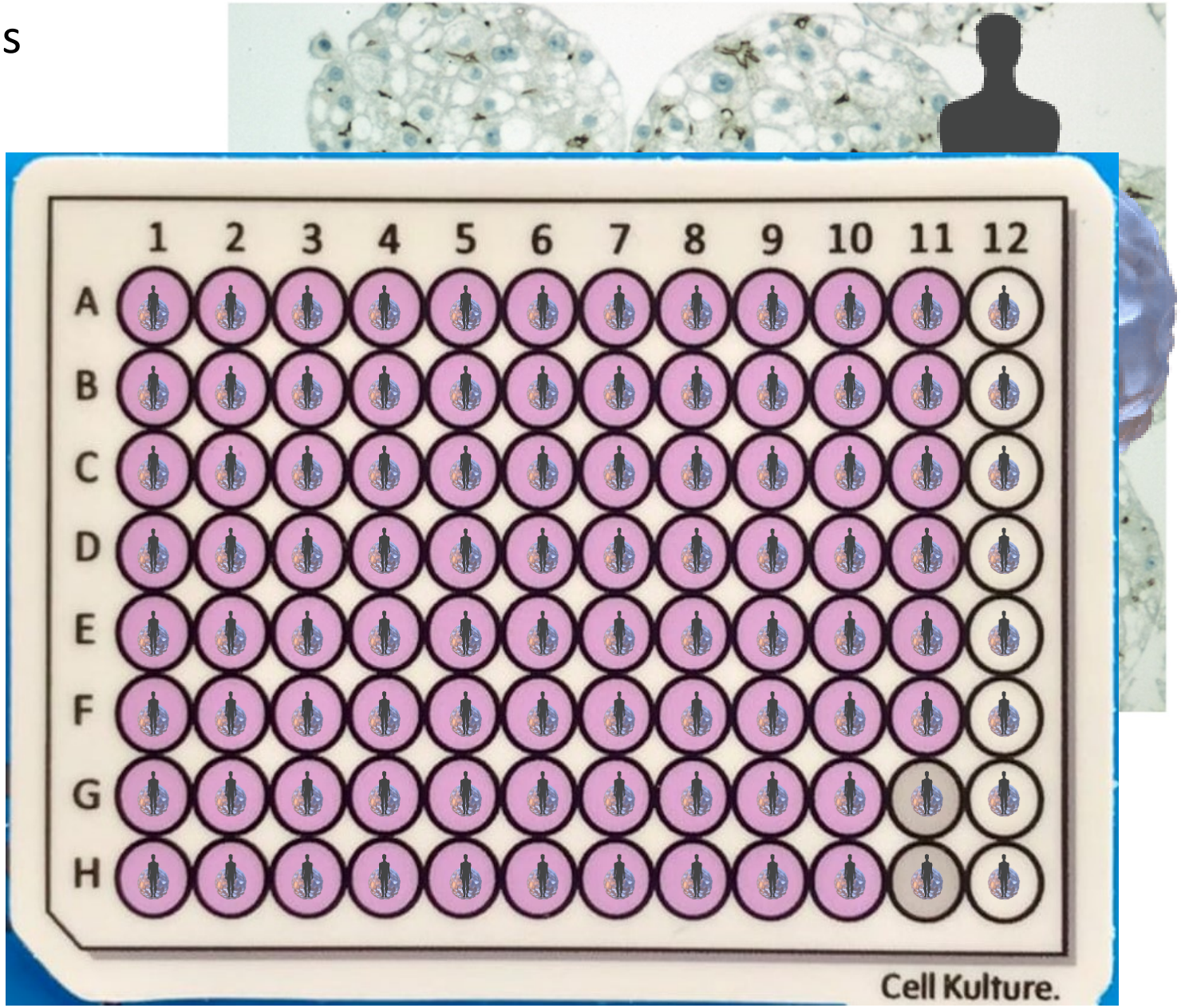
  - One spheroid per well



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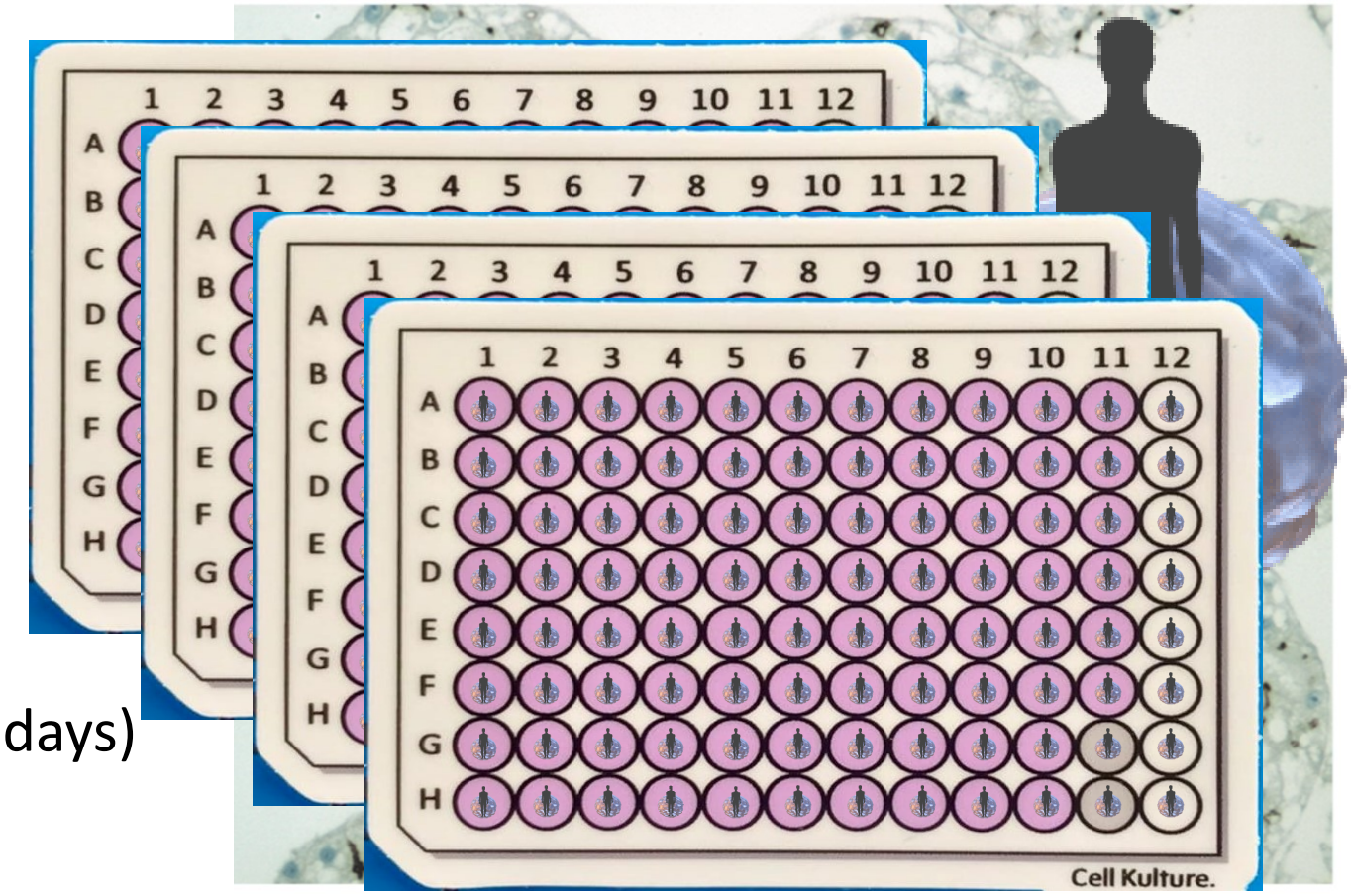
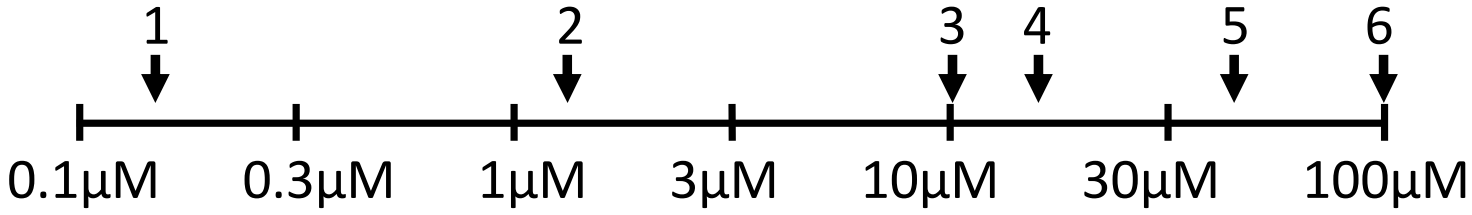
  - One spheroid per well
- 6 – Exposure concentrations
- 4 – Replicate exposures (1 x per plate)
- 2 – Exposure times (24 hours and *10 days)
  - *Media refreshed every 48 hours





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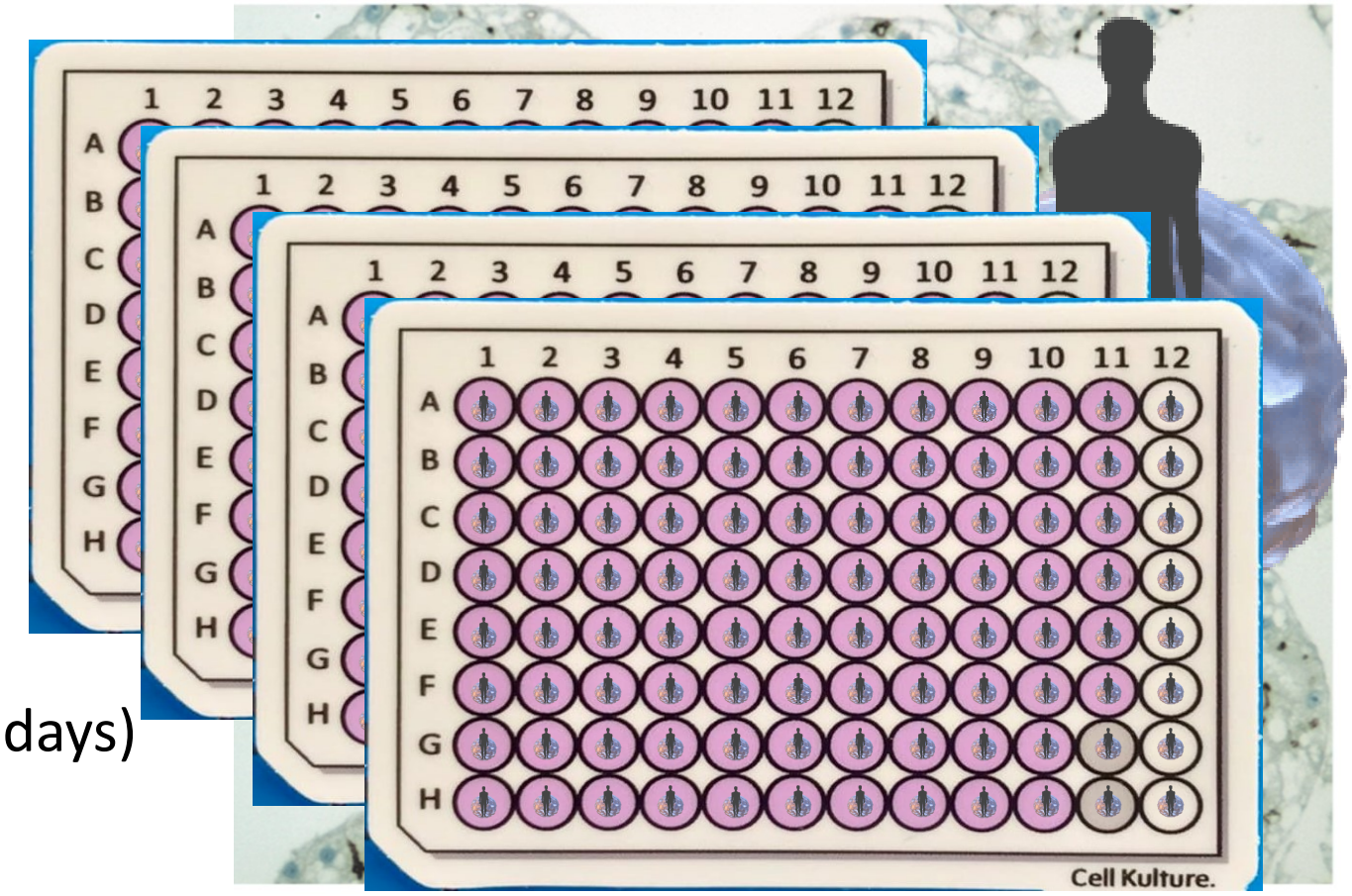
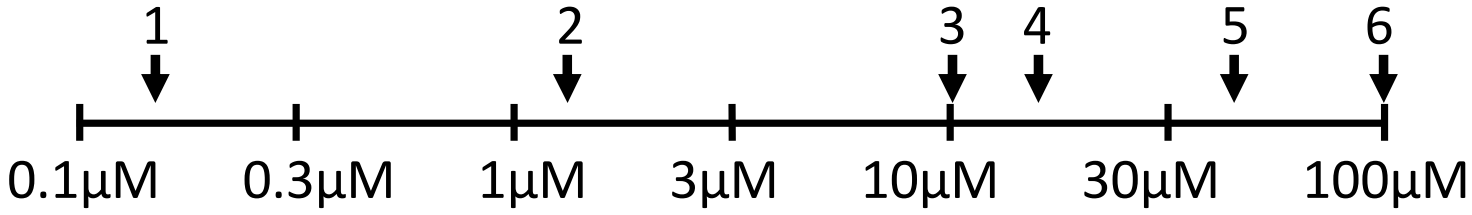
  - One spheroid per well
- 6 – Exposure concentrations
- 4 – Replicate exposures (1 x per plate)
- 2 – Exposure times (24 hours and *10 days)
  - *Media refreshed every 48 hours
- Several controls
  - DMSO
  - Downstream processing
    - Sequencing
    - QC

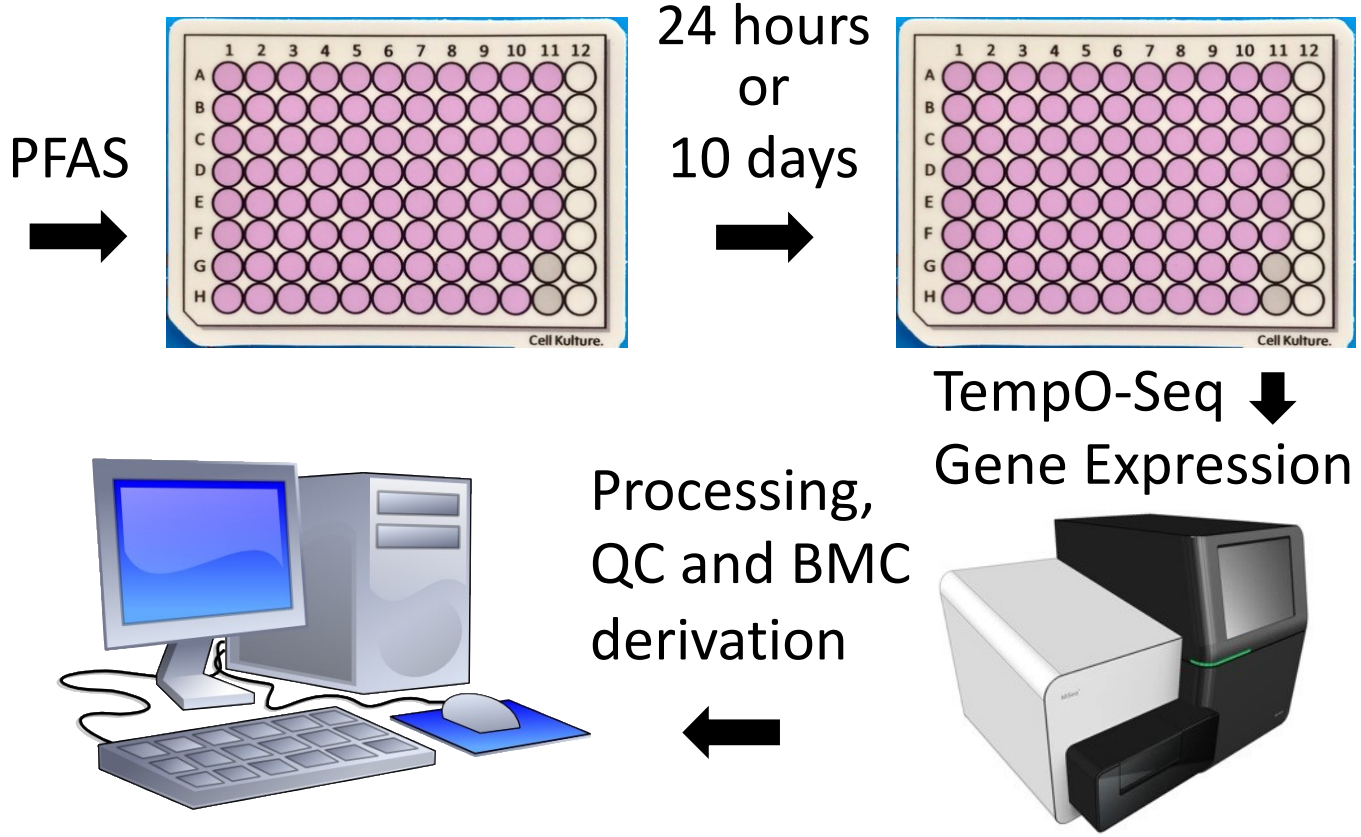




Transcriptomics

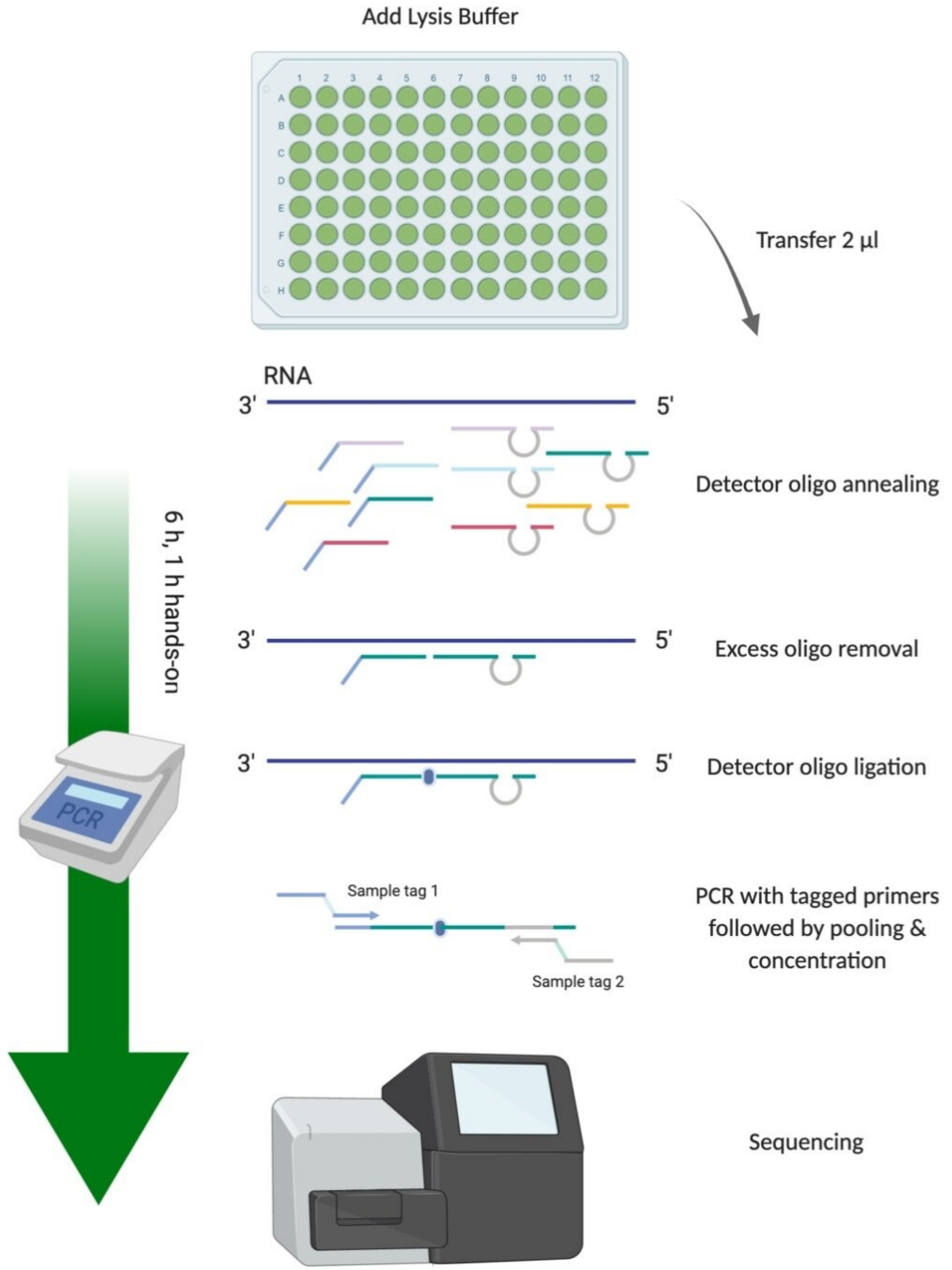
- TempO-Seq
  - Higher throughput
    - (less in house labor)
  - More economical
    - (lower cost for sequences)
- S1500+
  - 2753 genes
  - Captures all pathways

- Quality Control (QC)
  - spheroid loss
  - contamination
  - unexplained excessive variability



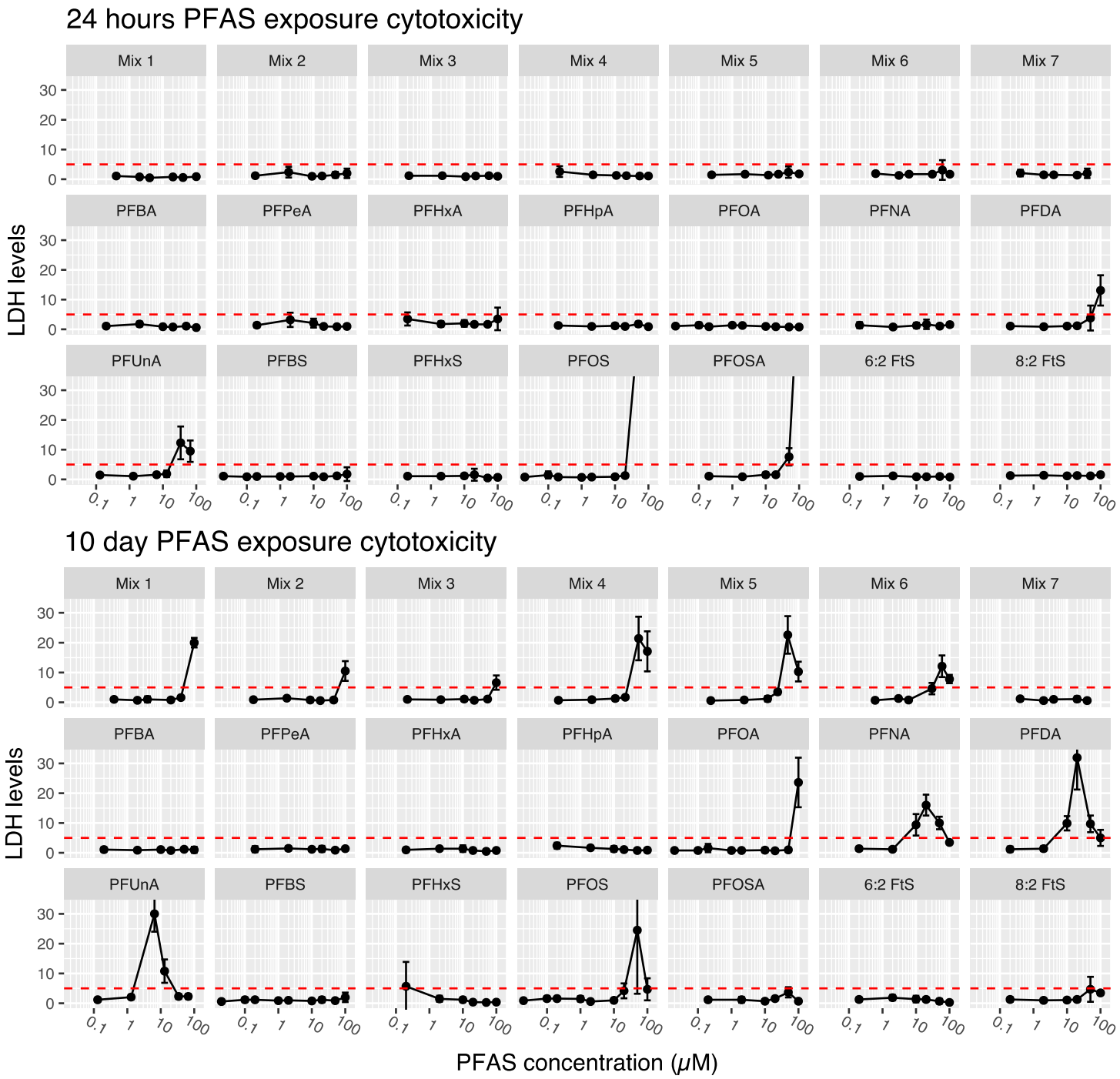
Gene expression measurement

- Transcriptomics
  - Measure cellular compensation response
  - Direct activation of transcription factors
  - Global and specific effects
- TempO-Seq
  - Oligo based gene expression
  - Assesses expression of specific genes
  - Higher number of genes than microarray
  - Sequencing screens out background
  - Higher throughput than RNA seq
- S1500+
  - 2753 genes
  - Representative of diverse biological space
  - More economical that whole transcriptome TempO-Seq



PFAS Cytotoxicity

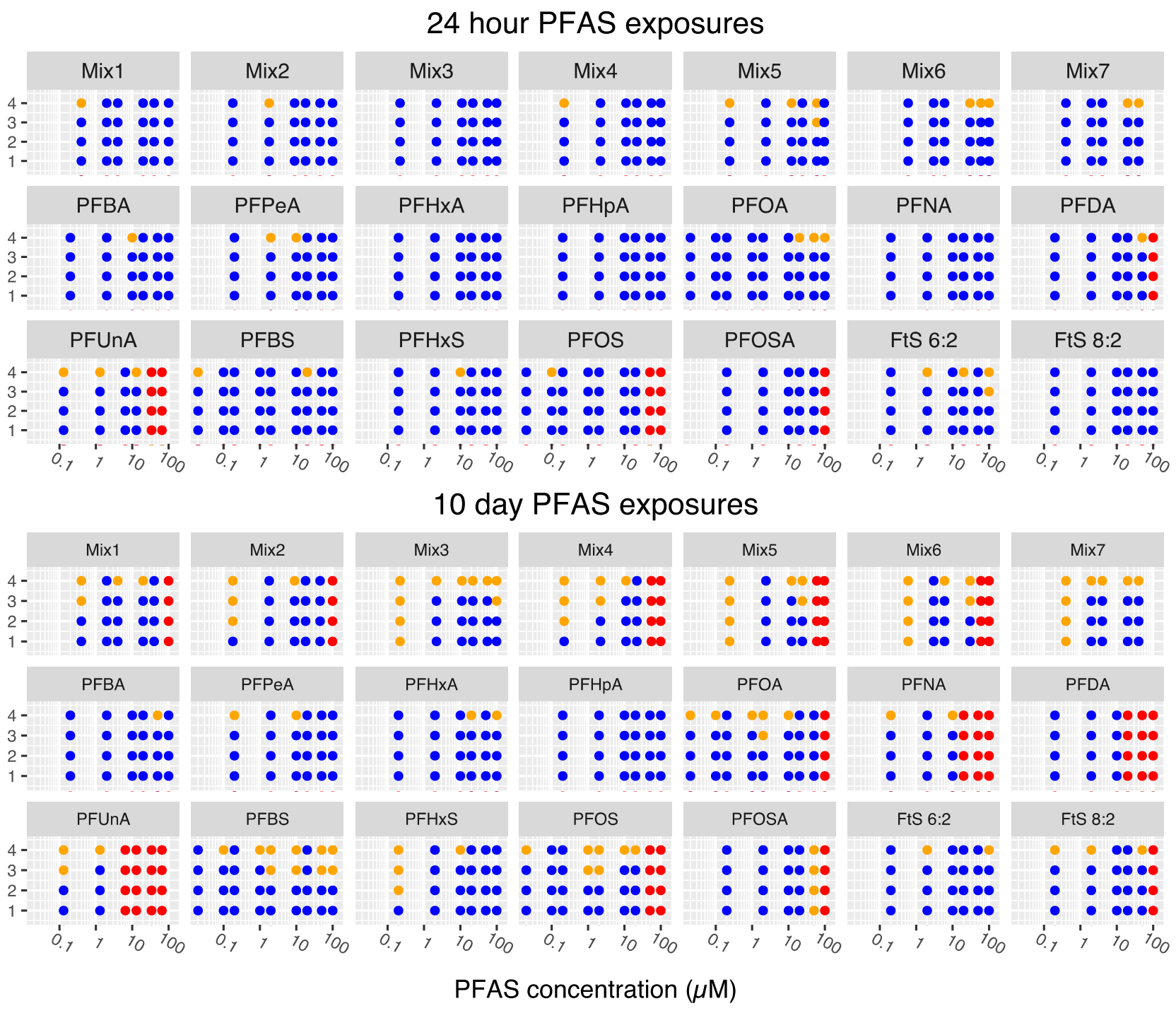
Sequencing data for cytotoxic samples removed from downstream data processing to improve statistical power for data quality control



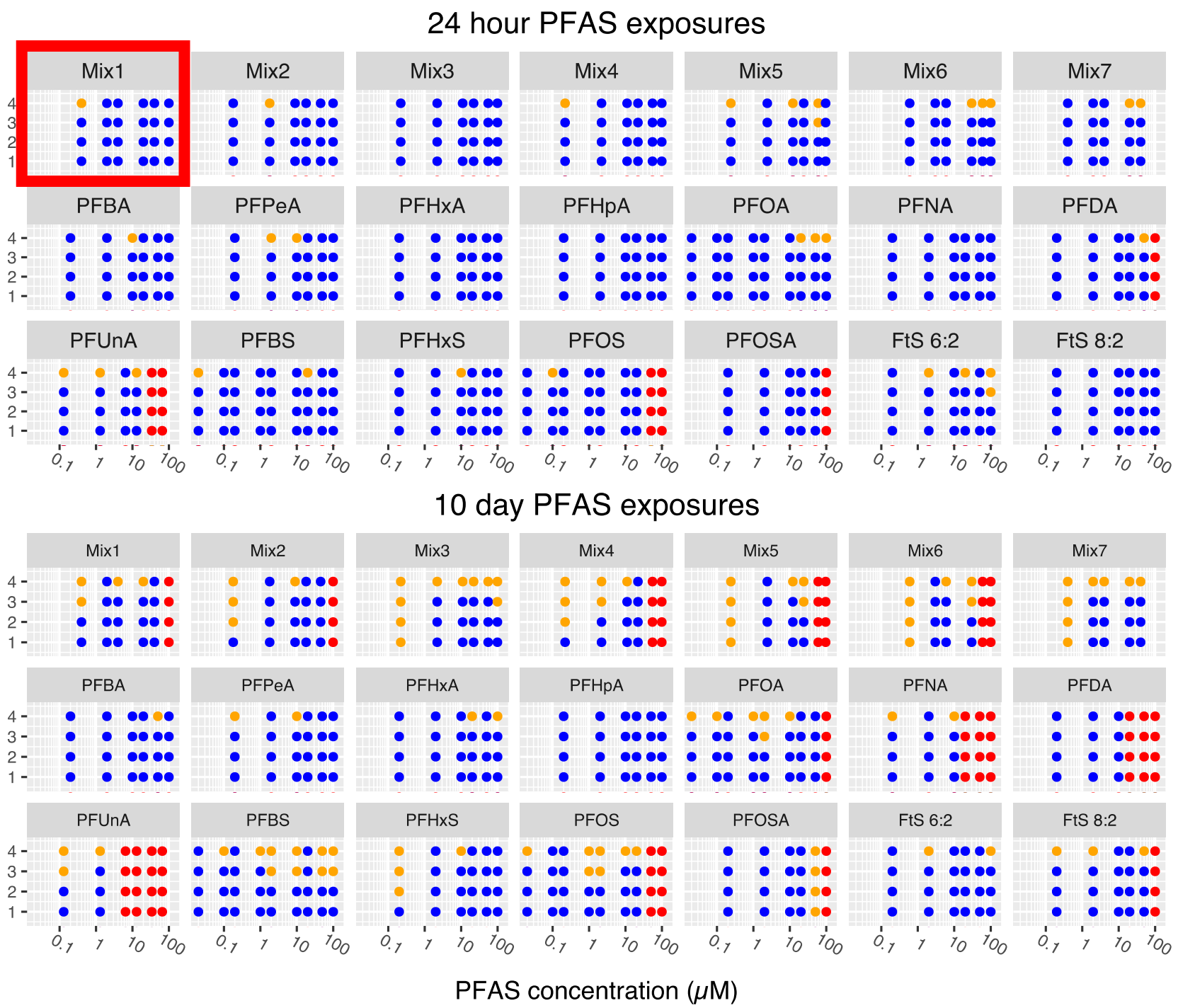
Quality Control (QC)

Sequencing data was screened for several measures to ensure data quality

- Clustering distance
- Number of mapped reads
- Fraction of mapped reads
- Number of active probes
- Number of probes with 80% of signal
- Gini coefficient



Data passing QC for each PFAS or Mixture at 24 hours or 10 days (Exposure Series) is used for BMC determination



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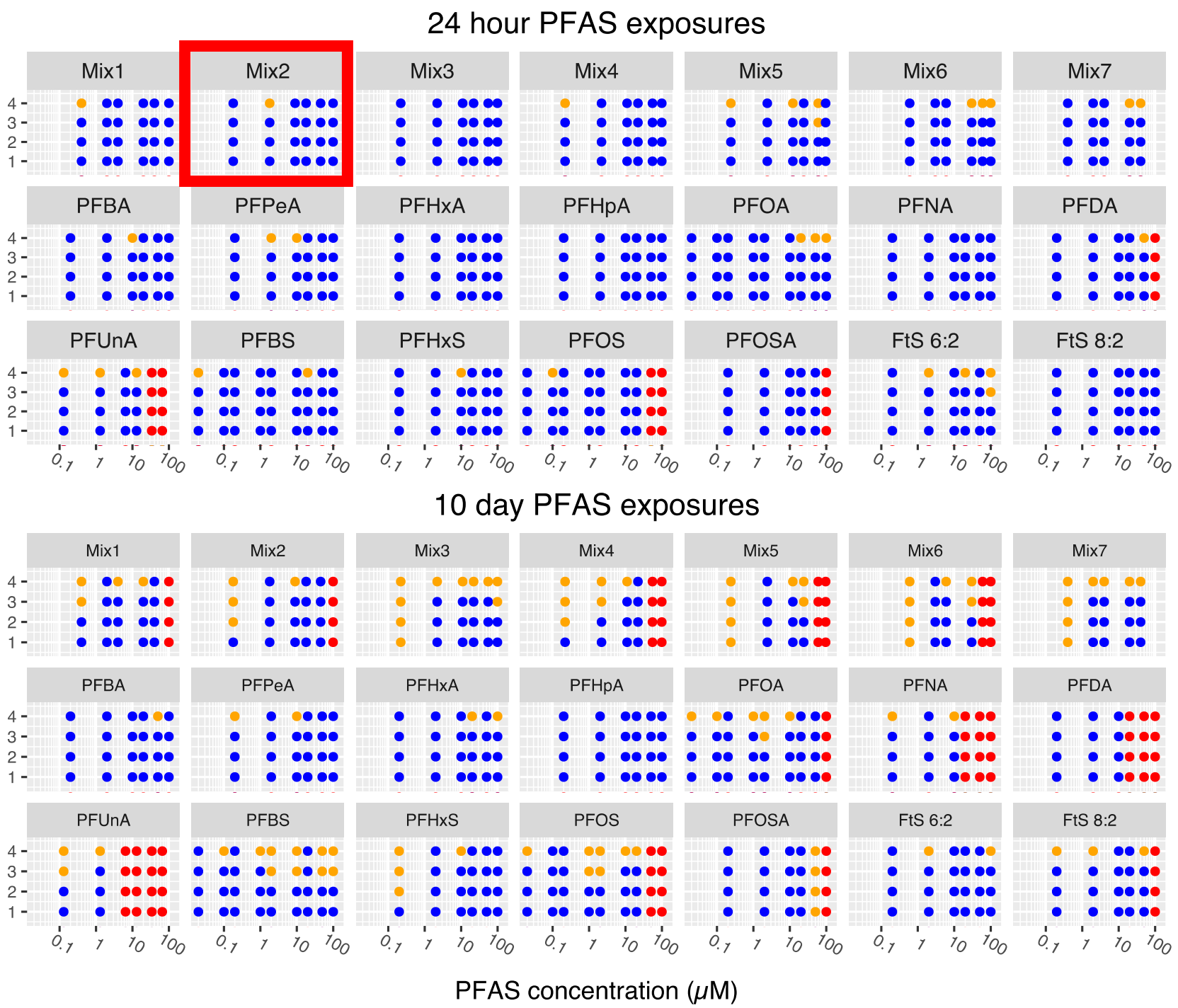

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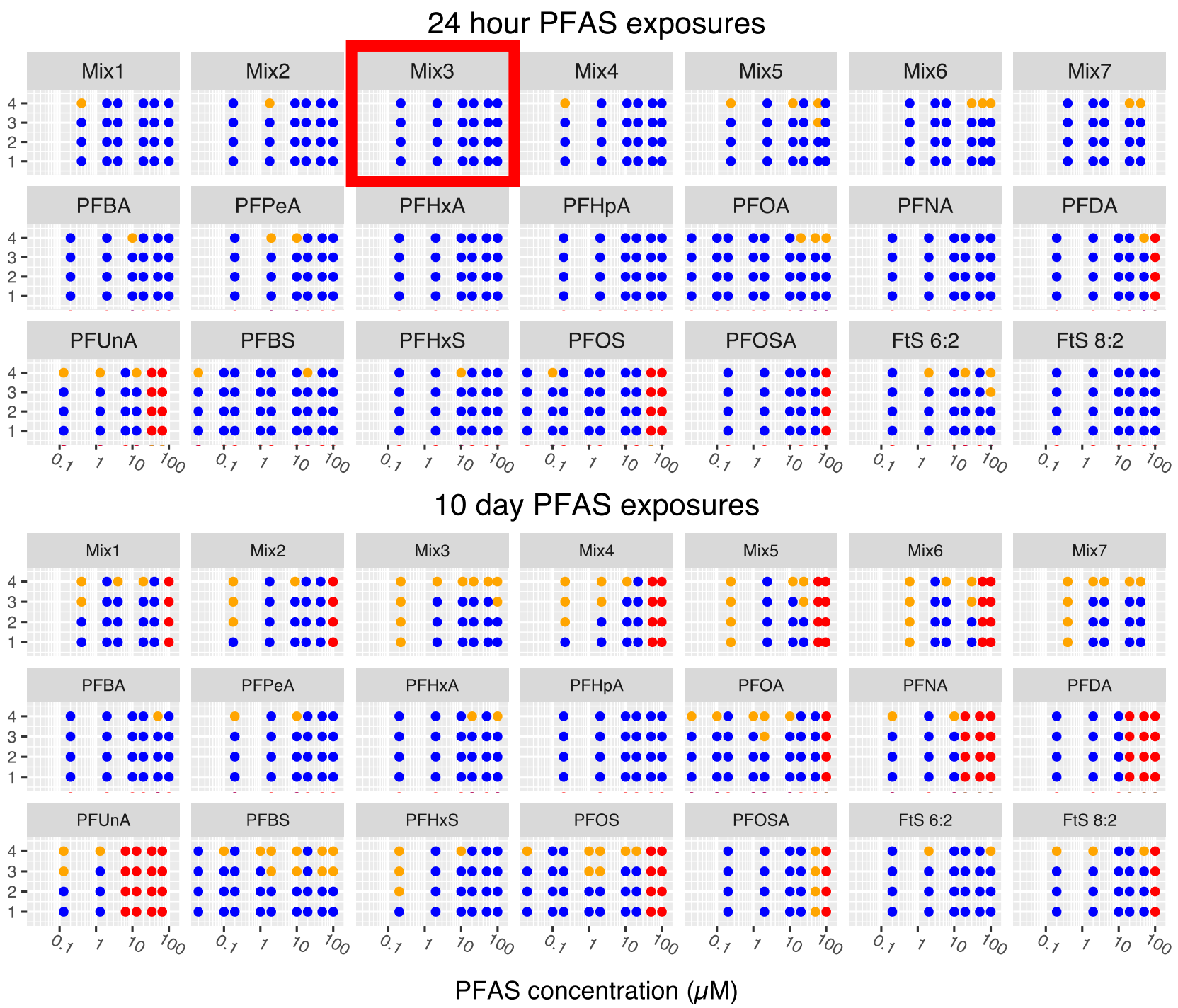
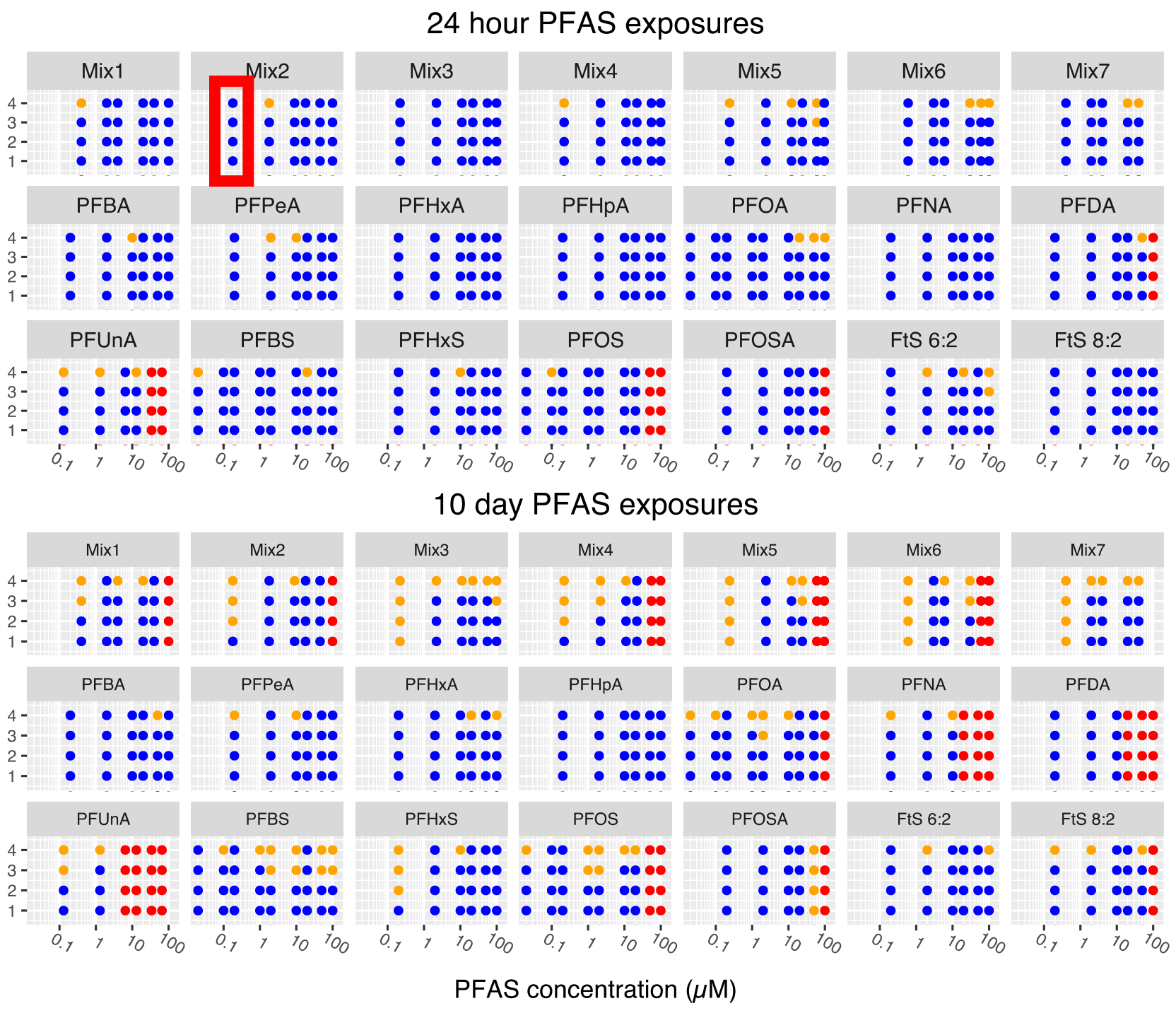


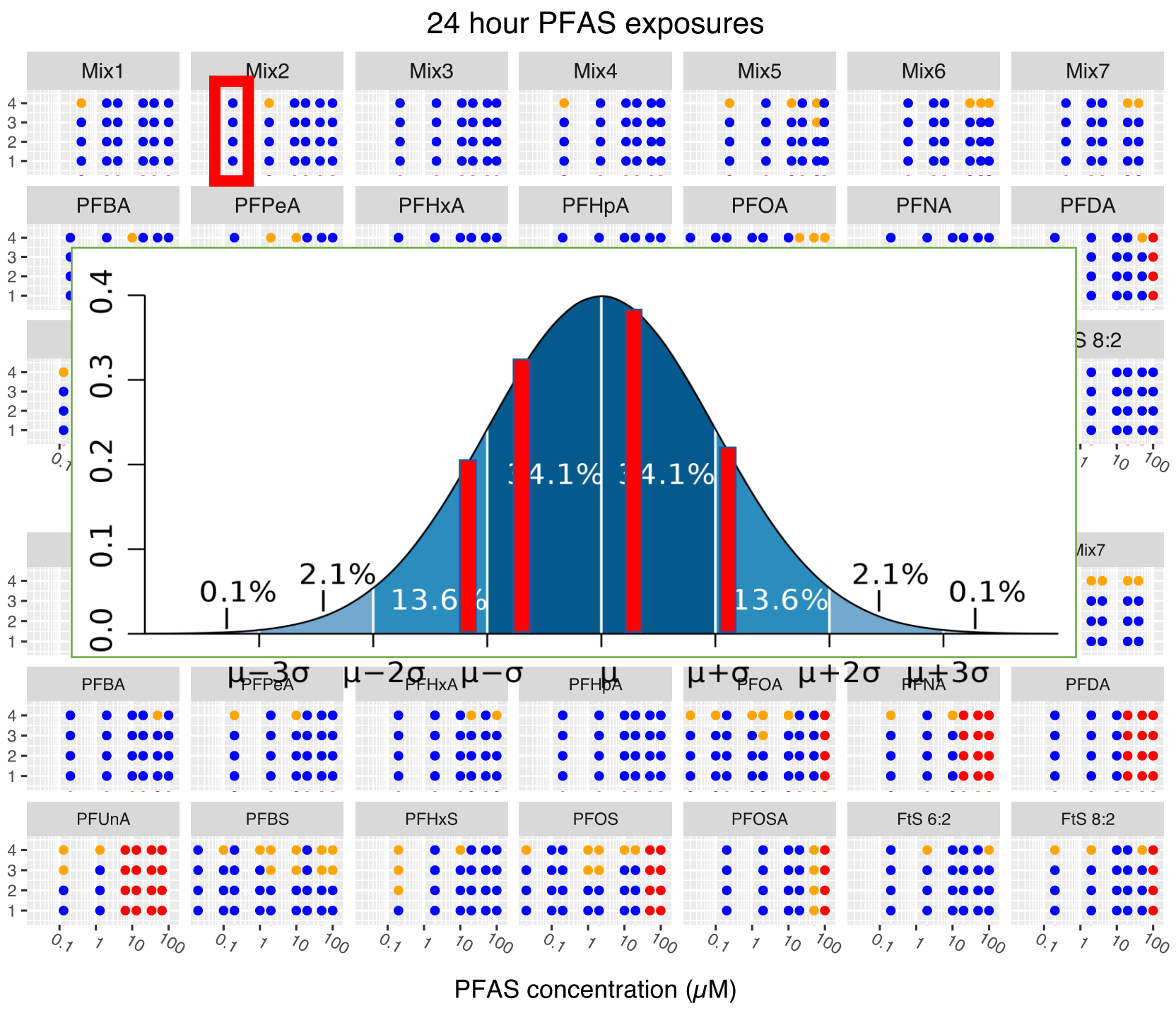
Bootstrapping

- Data for each concentration used for bootstrapping



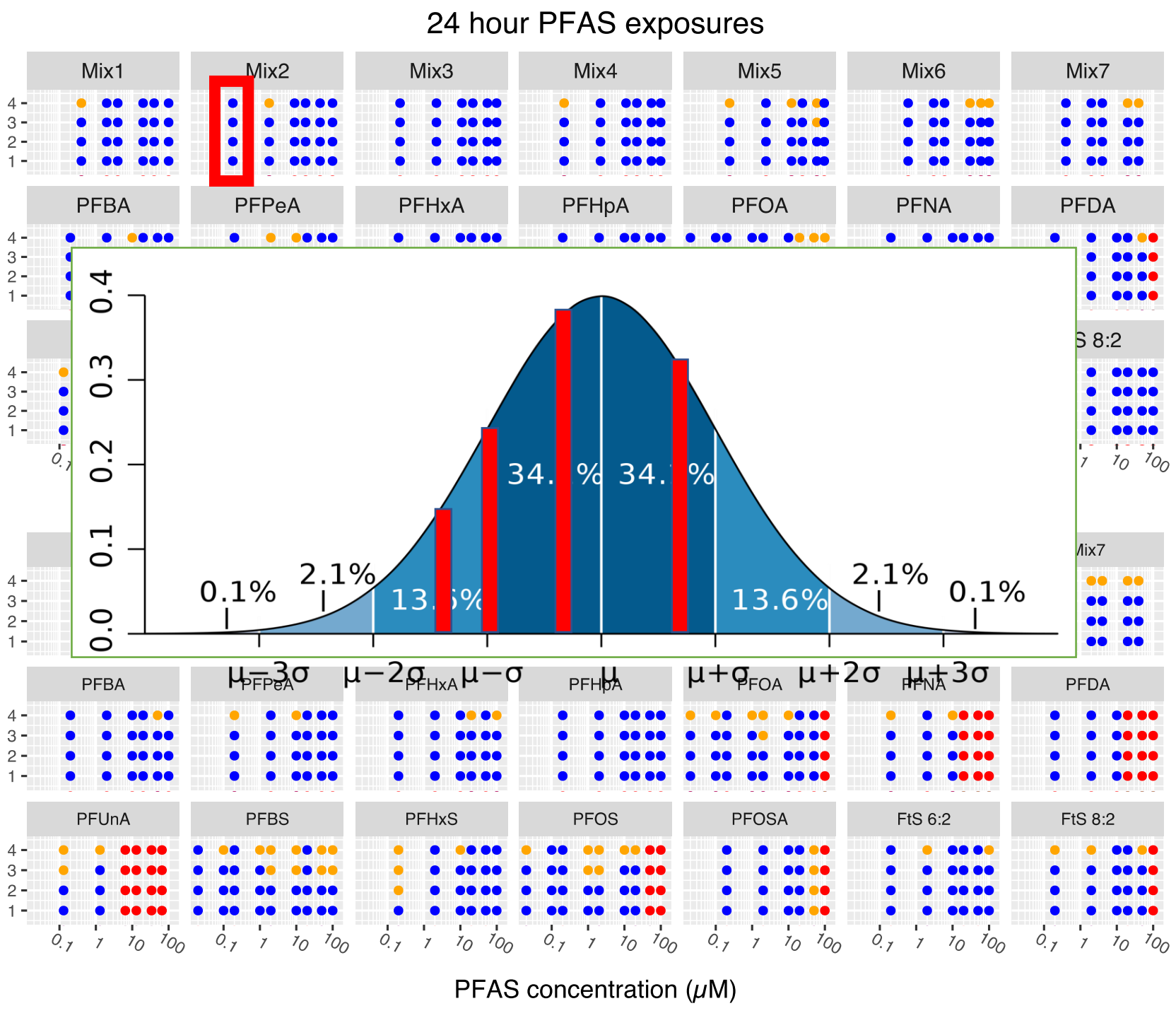
Bootstrapping

- Data for each concentration used for bootstrapping
- Data for each gene, each exposure level and PFAS or mixture used to create a normal distribution



Bootstrapping

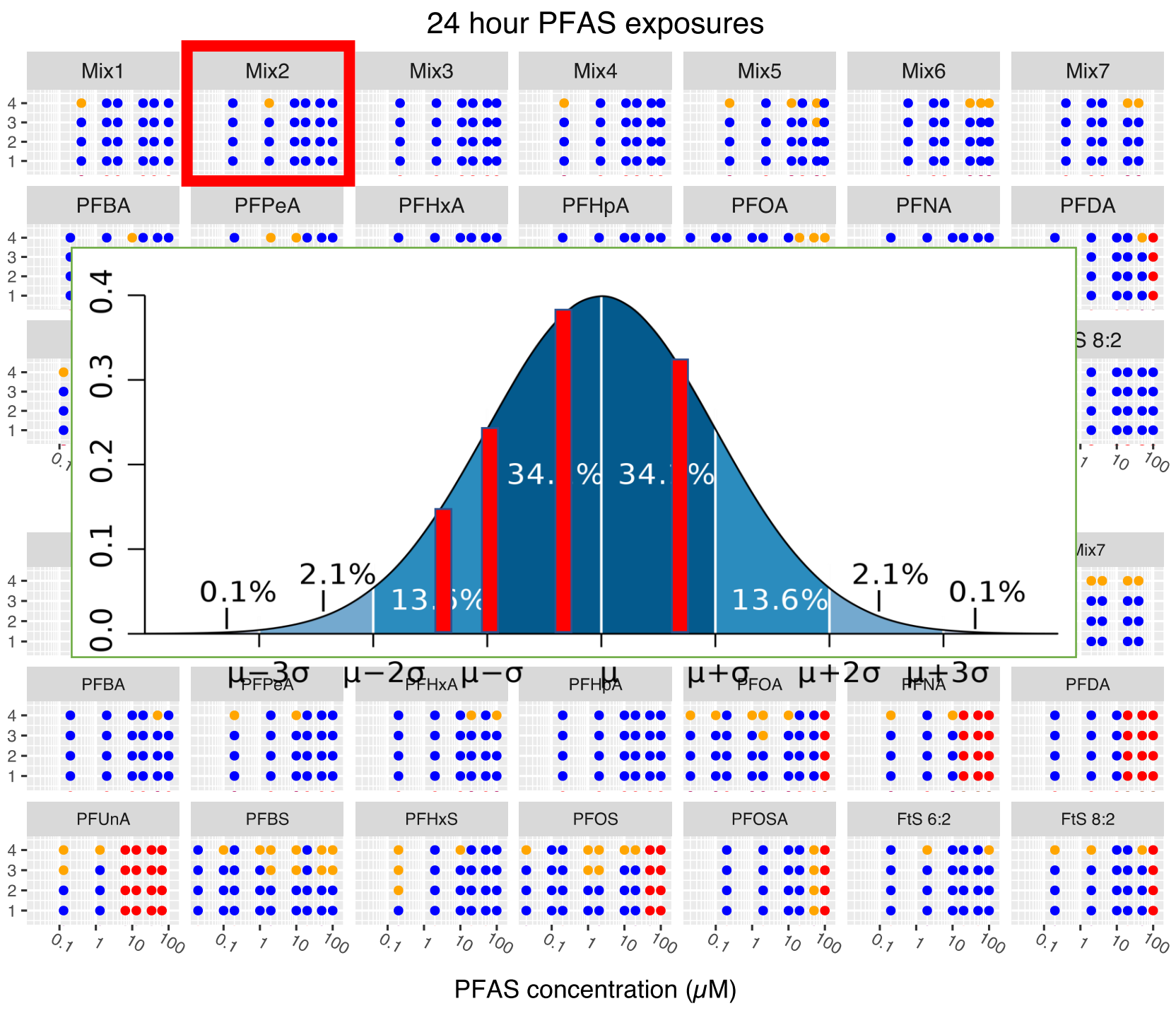
- Data for each concentration used for bootstrapping
- Data for each gene, each exposure level and PFAS or mixture used to create a normal distribution
- Normal distributions are randomly sampled for each gene, exposure level and PFAS or mixture



Bootstrapping

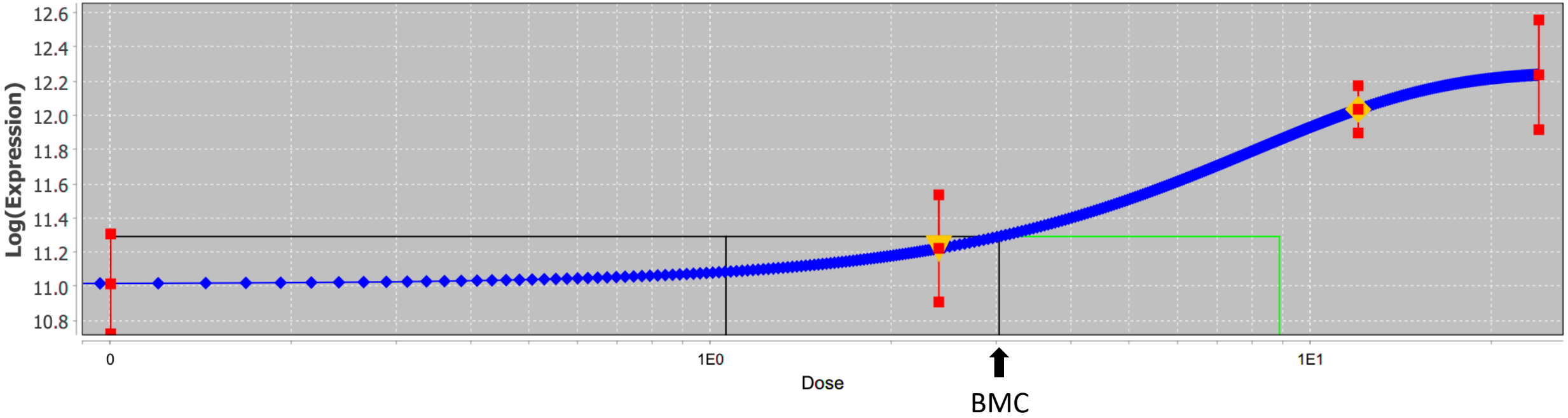
- Data for each concentration used for bootstrapping
- Data for normal distributions are randomly sampled for each gene
- Normal distributions are randomly sampled for each gene, exposure level and PFAS or mixture

- End result is:
- 100 x exposure series simulations (for each gene for each PFAS or mixture and exposure time)



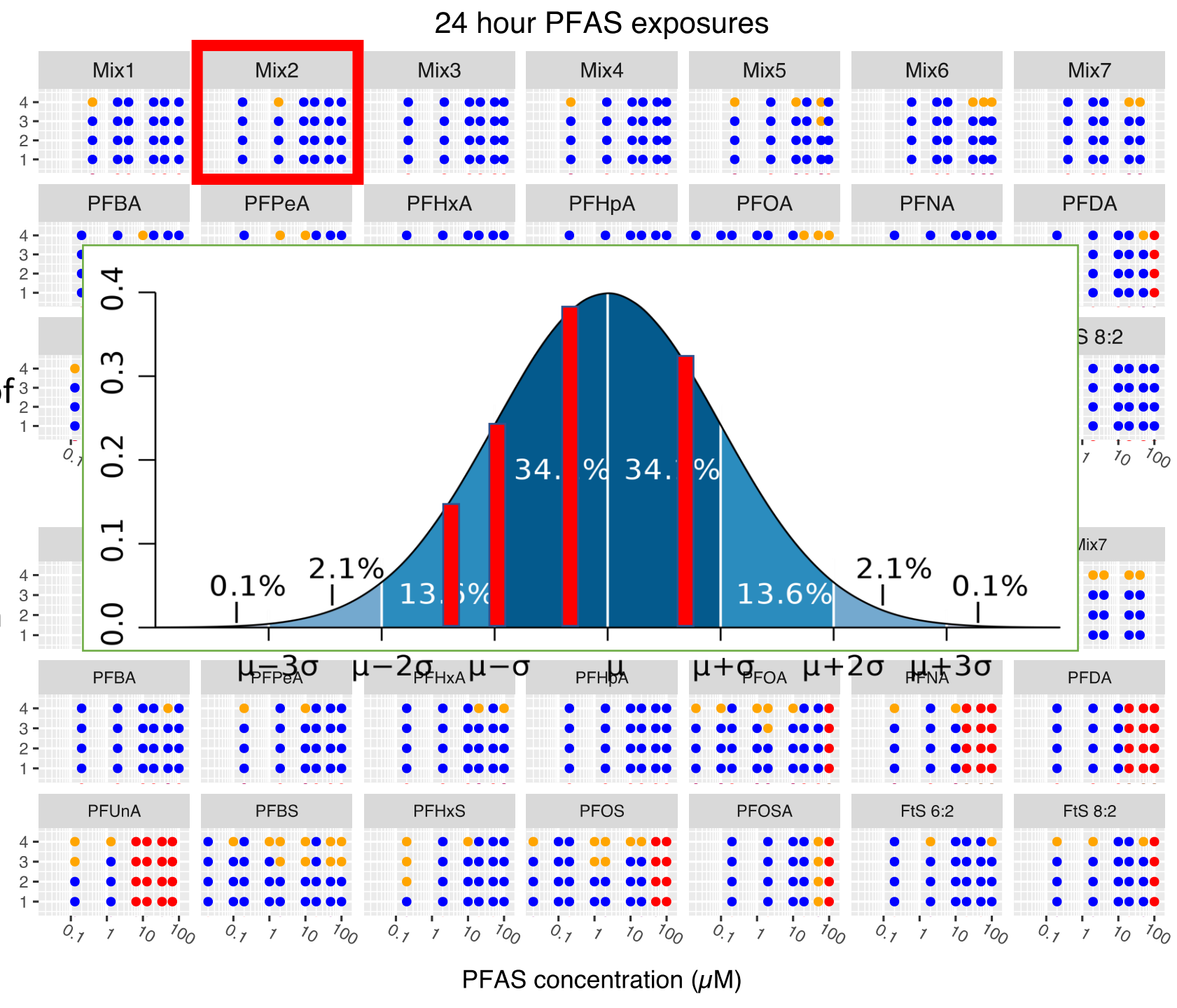
BMC Determination using BMDExpress – Gene Expression

- Gene expression data for each PFAS / Mixture exposure series
  - Filtered for 1.5 fold change in expression
  - Williams trend test p = 0.01
  - Plotted for dose response curve fitting
  - BMC is one standard deviation from the mean of the DMSO control



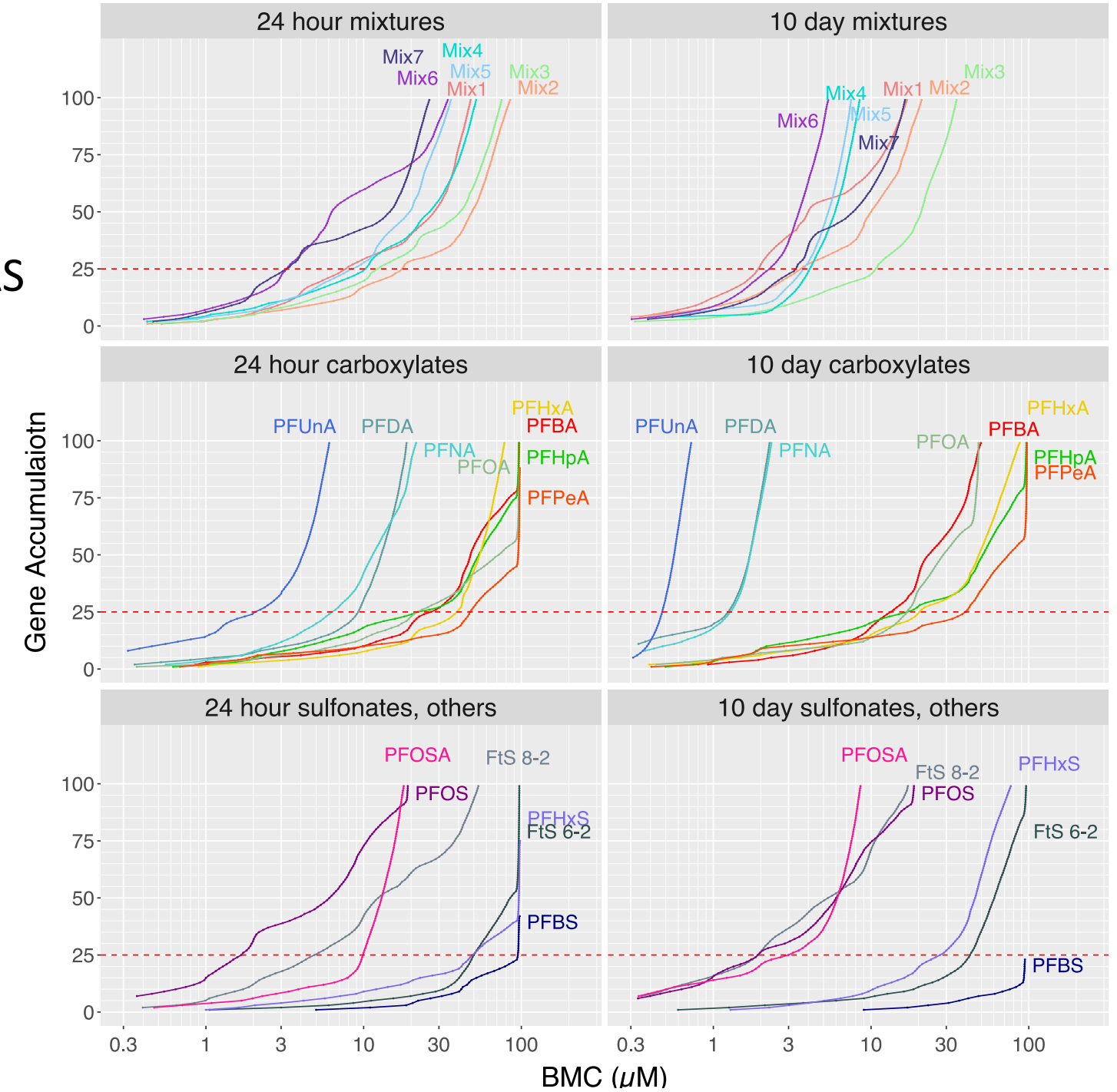
Bootstrapping

- All BMCs for each exposure series are placed in a bootstrap distribution
  - BMCs are sampled the appropriate number of times (depending on total number of BMCs)
  - BMC list is ranked by BMC concentrations
  - Repeated 10,000x resulting in list of potential BMCs for each rank
    - Lowest BMC
    - 2nd lowest BMC…
    - 25th lowest BMC
- For each BMC rank: median 2.5% and 97.5% values used for BMCs and 95% CIs



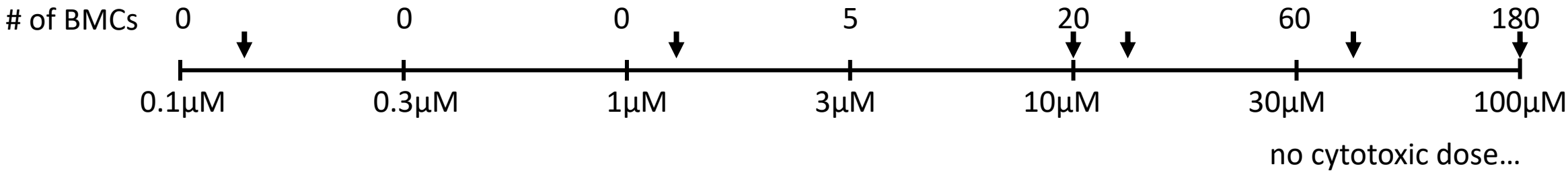
BMC accumulation plots

- BMCs ranked from lowest concentration
  - 10s to 100s of BMCs for each PFAS
  - Total # of BMCs influenced by experimental parameters
    - Exposure concentrations
    - Cytotoxicity
- 25th BMC
  - Concerted molecular response
  - Concentration where gene expression changes broadly
    - Compensation to changes caused by toxicant exposure
    - Direct activation of transcription factors

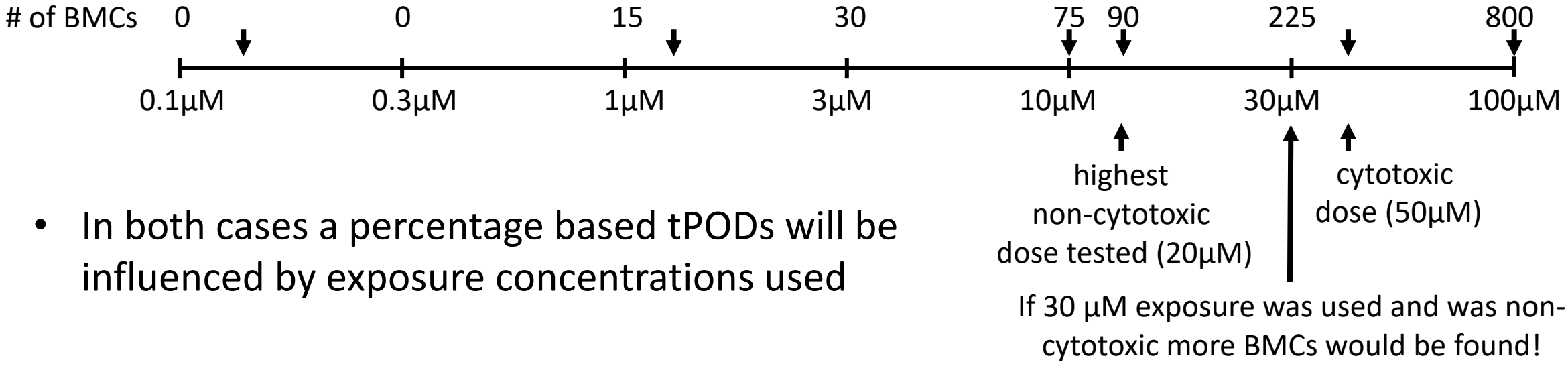


25th BMC – does not require accurate highest BMC

- If highest dose is not cytotoxic more BMCs are possible



- If highest exposure is far from the cytotoxic dose more BMCs are possible

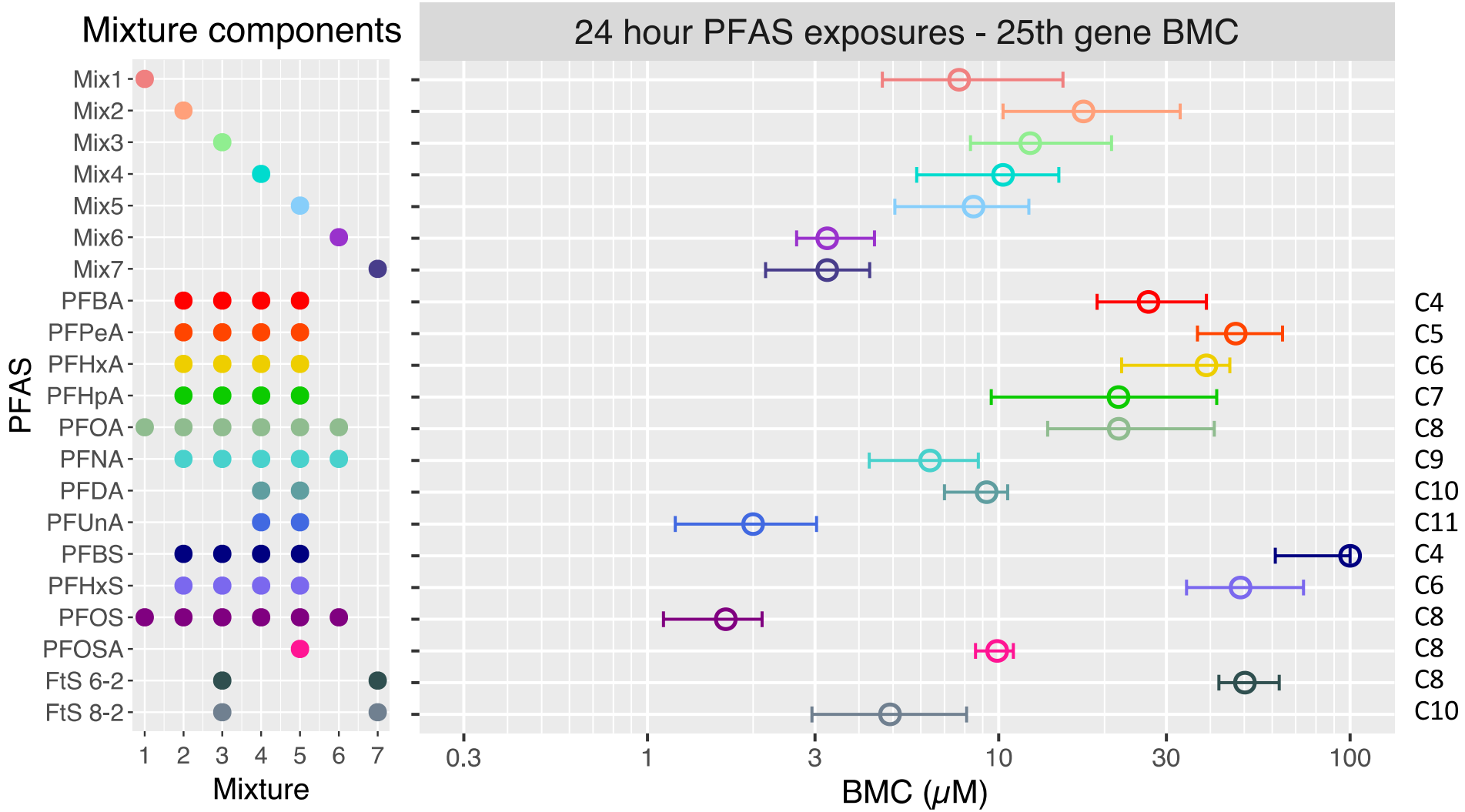


- In both cases a percentage based tPODs will be influenced by exposure concentrations used

25th BMCs for PFAS and mixture exposures

PFAS Potency

- Longer chain PFAS generally more potent
- Different potency depending on type of PFAS
- Mixtures seem to have potency corresponding to their constituents



25th BMCs for PFAS and mixture exposures

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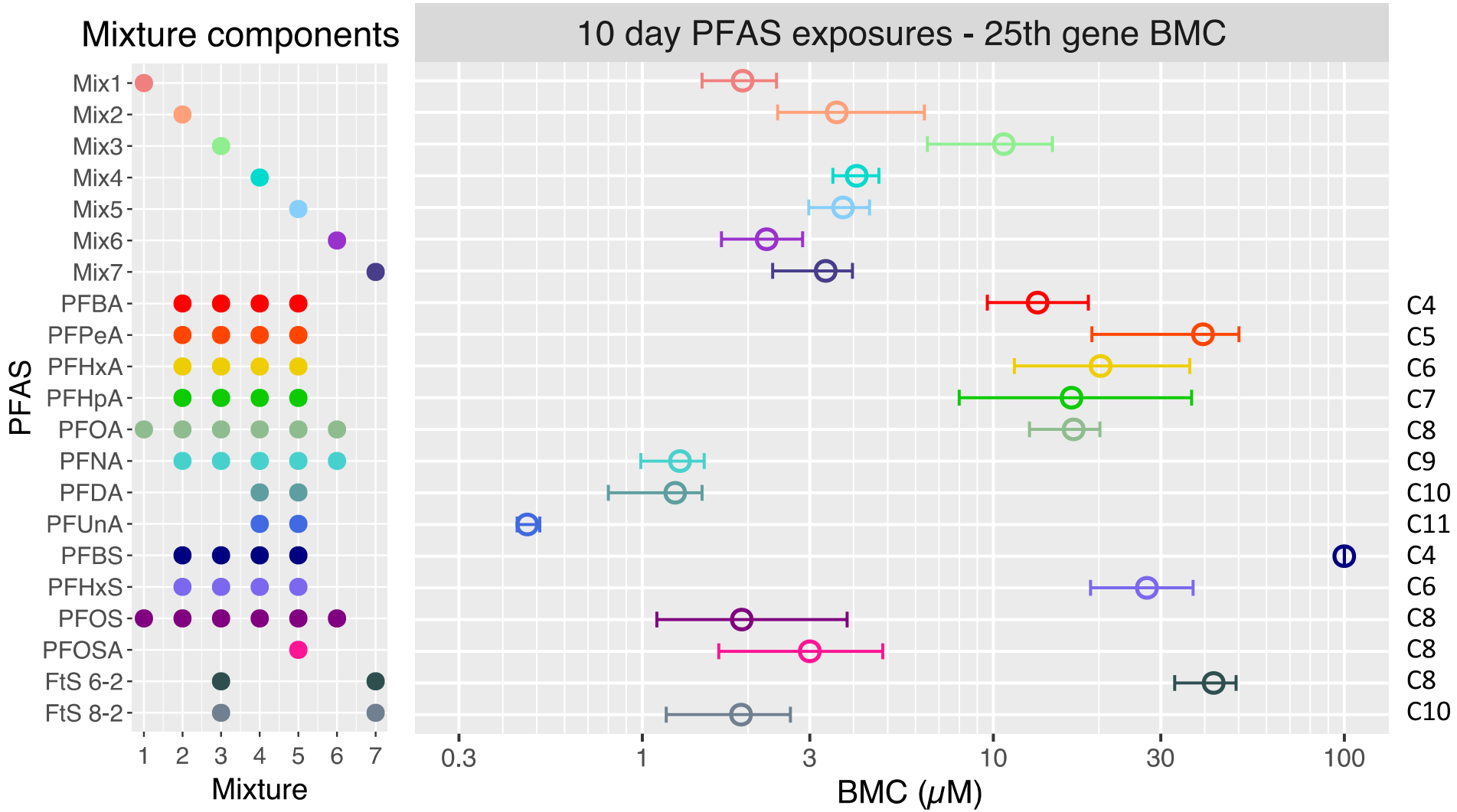
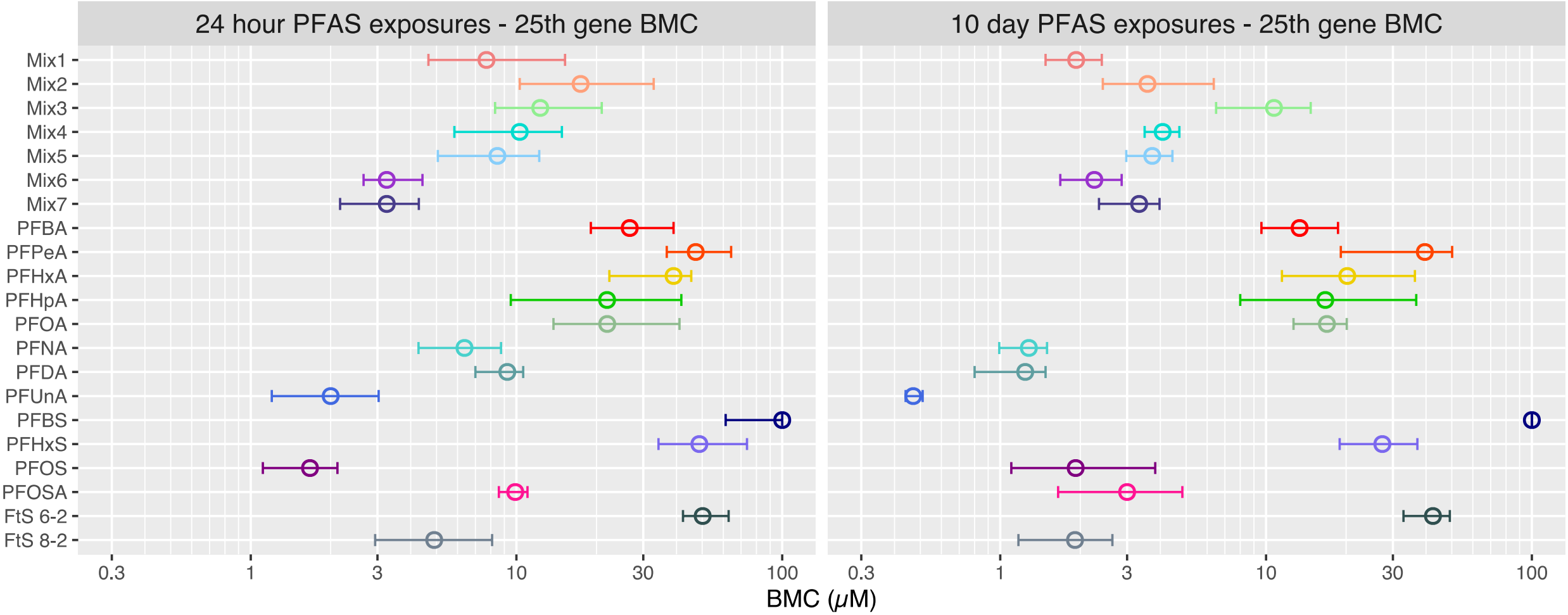
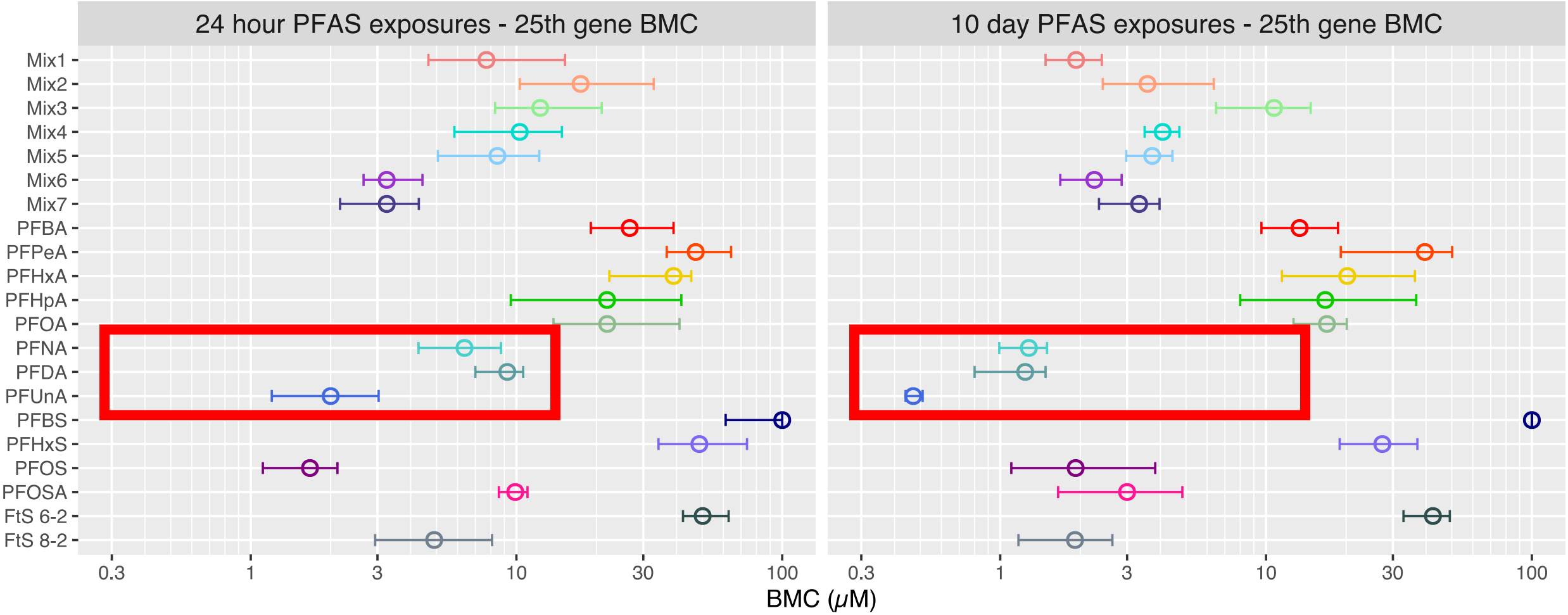


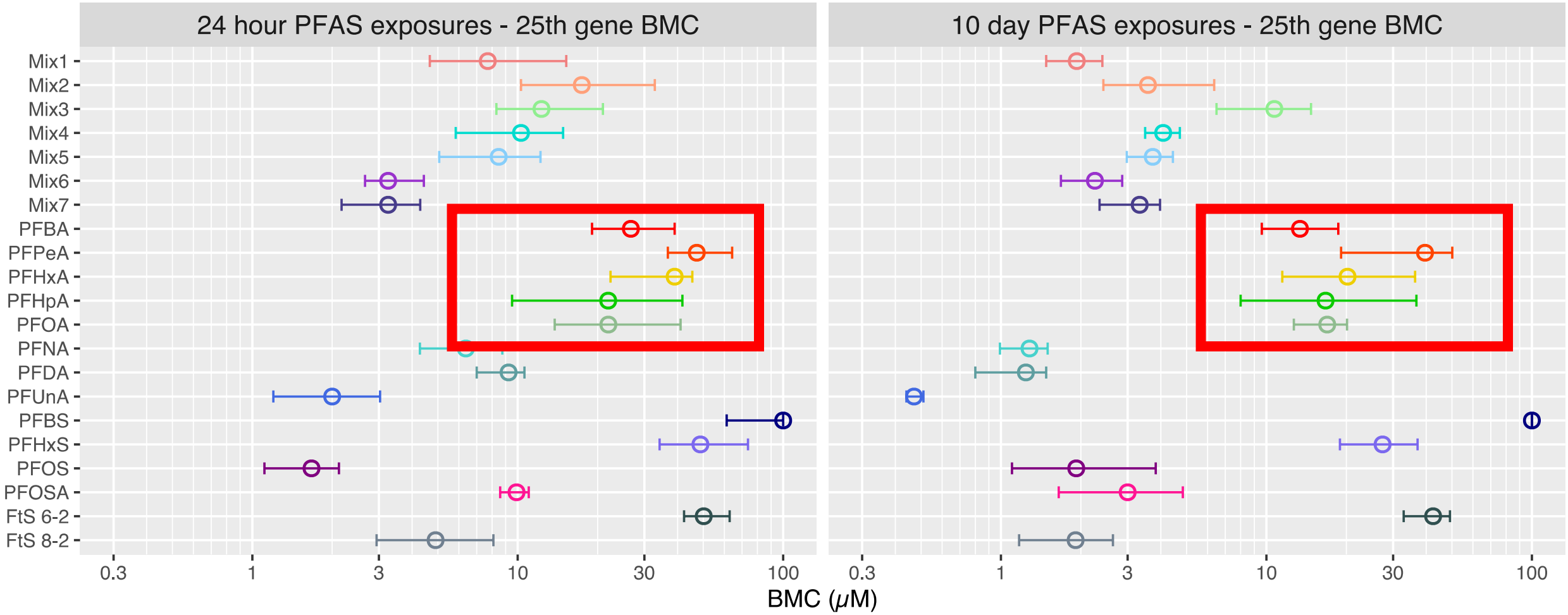
25th BMCs for PFAS and mixture exposures



25th BMCs for PFAS and mixture exposures

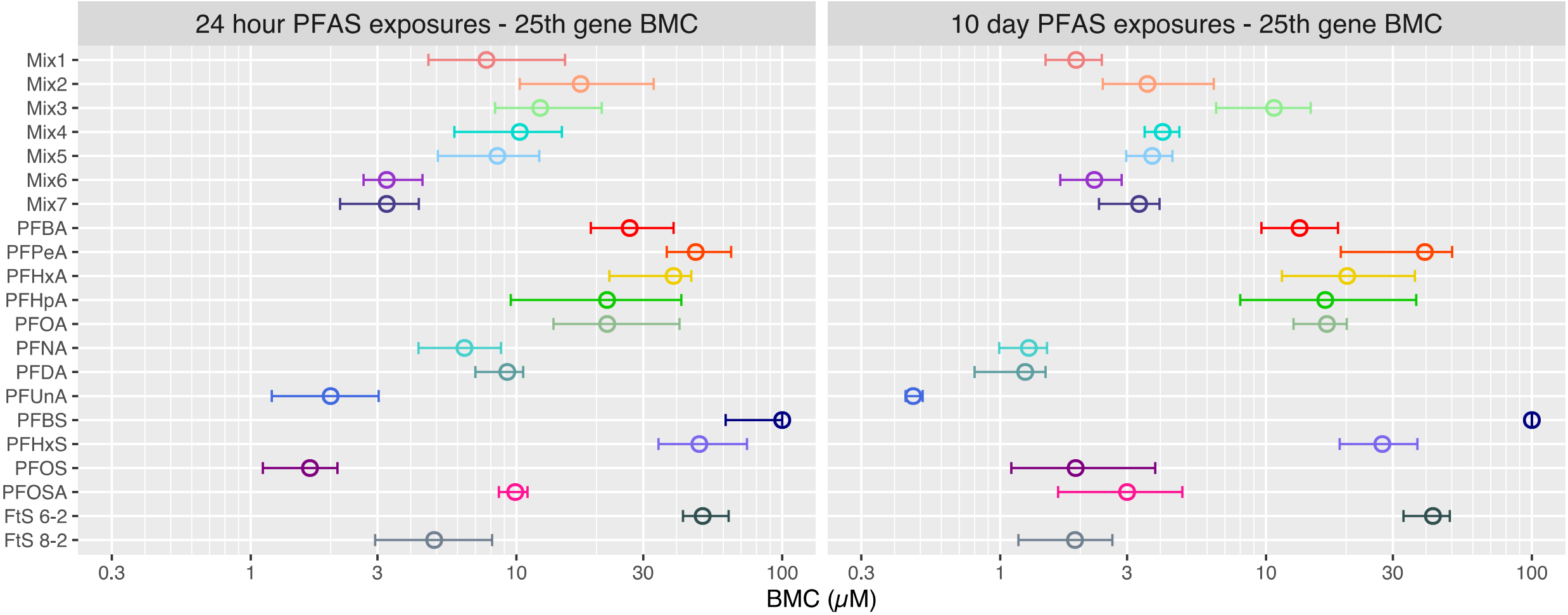


25th BMCs for PFAS and mixture exposures



25th BMCs for PFAS and mixture exposures

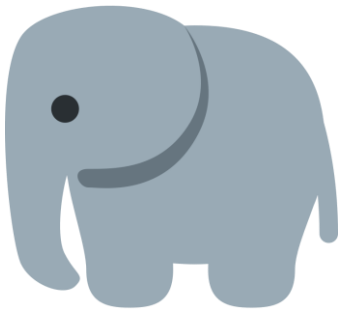
Are mixture potencies comparable to expectation based on single PFAS potencies?



Analysis of Mixtures – Potency and BMC

- Chemicals having different relative potencies have stronger effects at the same dose
- BMCs are the doses of substances that result in the same specified effect
- BMC is reciprocal of Potency

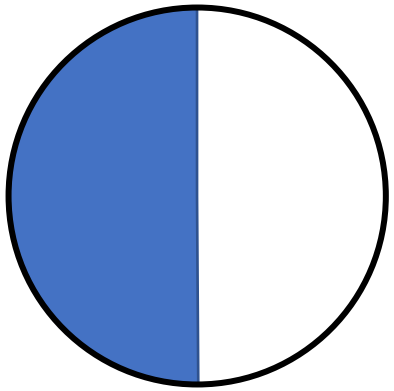
	Relative Potency	BMC
• Morphine	1 x	1
• Hydromorphone	10 x	0.1
• Fentanyl	100 x	0.01
• Sufentanil	1,000 x	0.001
• Carfentanil	10,000 x	0.0001



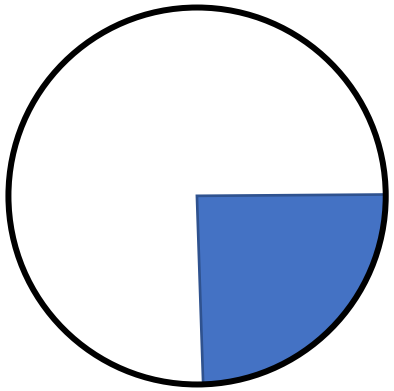
*example only – Dr. Addicks is not a physician or veterinarian

Concentration addition (aka dose addition)

Chemical 1
potency = 1/2 BMC = 2



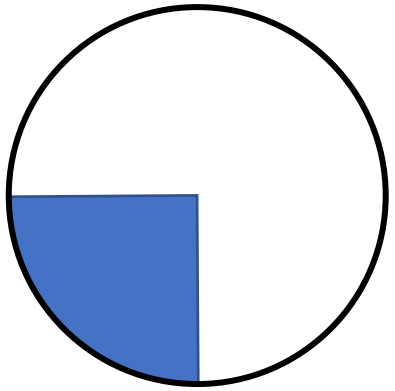
Chemical 2
potency = 1/4 BMC = 4



- Equimolar mixture of chemicals 1 and 2
- Total PFAS concentration in the mixture is the same
- Concentration (and potency) of each PFAS is in mixture is half

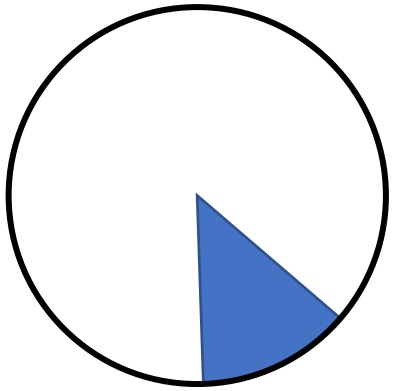
⬇ Mixture of 1 and 2 ⬇

potency = 1/4 BMC = 4



+

potency = 1/8 BMC = 8



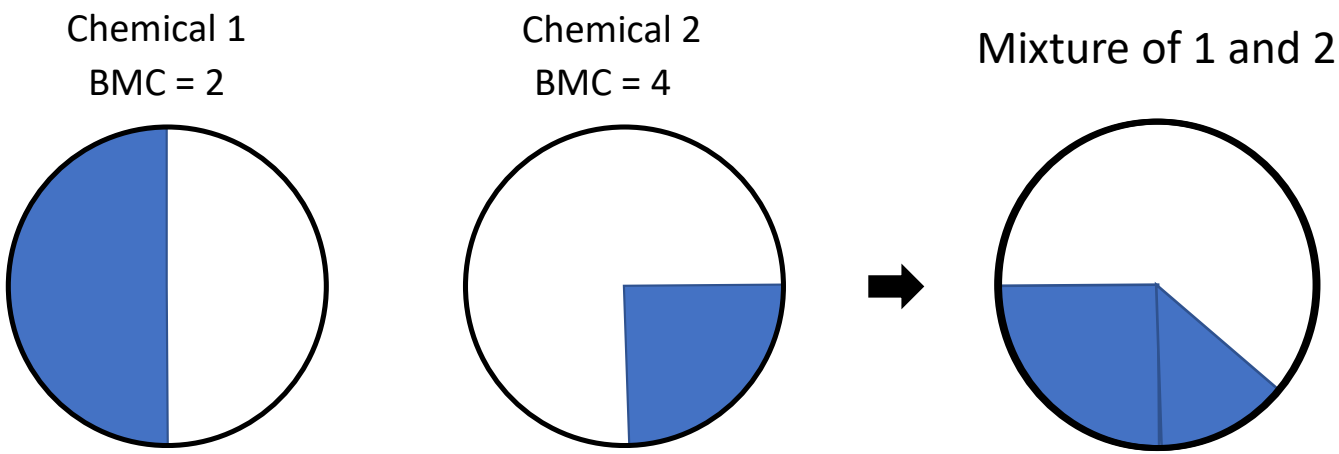
=

potency = 3/8 BMC = 8/3 (2.67)

Predicted mixture BMC calculations - based on dose addition

$$ECx_{Mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1}$$

$$BMC_{mix} = \left(\Sigma \frac{Fraction_i}{BMC_i} \right)^{-1}$$



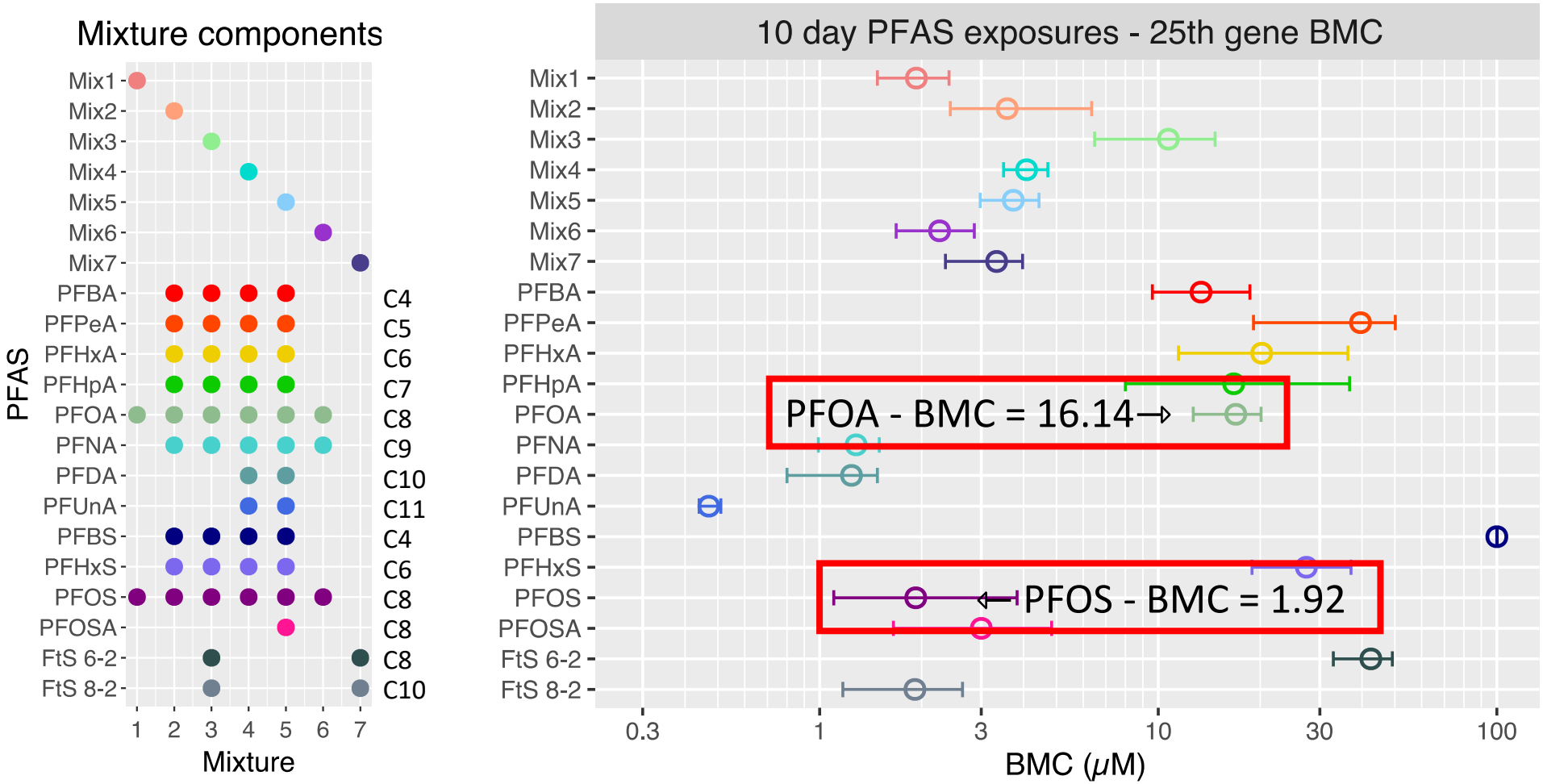
$Fraction_i$ = Fraction of each component in mixture

BMC_i = BMC of each component in mixture

$$BMC_{mix} = \left(\Sigma \frac{0.5}{2} + \frac{0.5}{4} \right)^{-1} = \left(\Sigma \frac{1}{4} + \frac{1}{8} \right)^{-1} = \frac{3}{8}^{-1} = \frac{8}{3} = 2.67$$

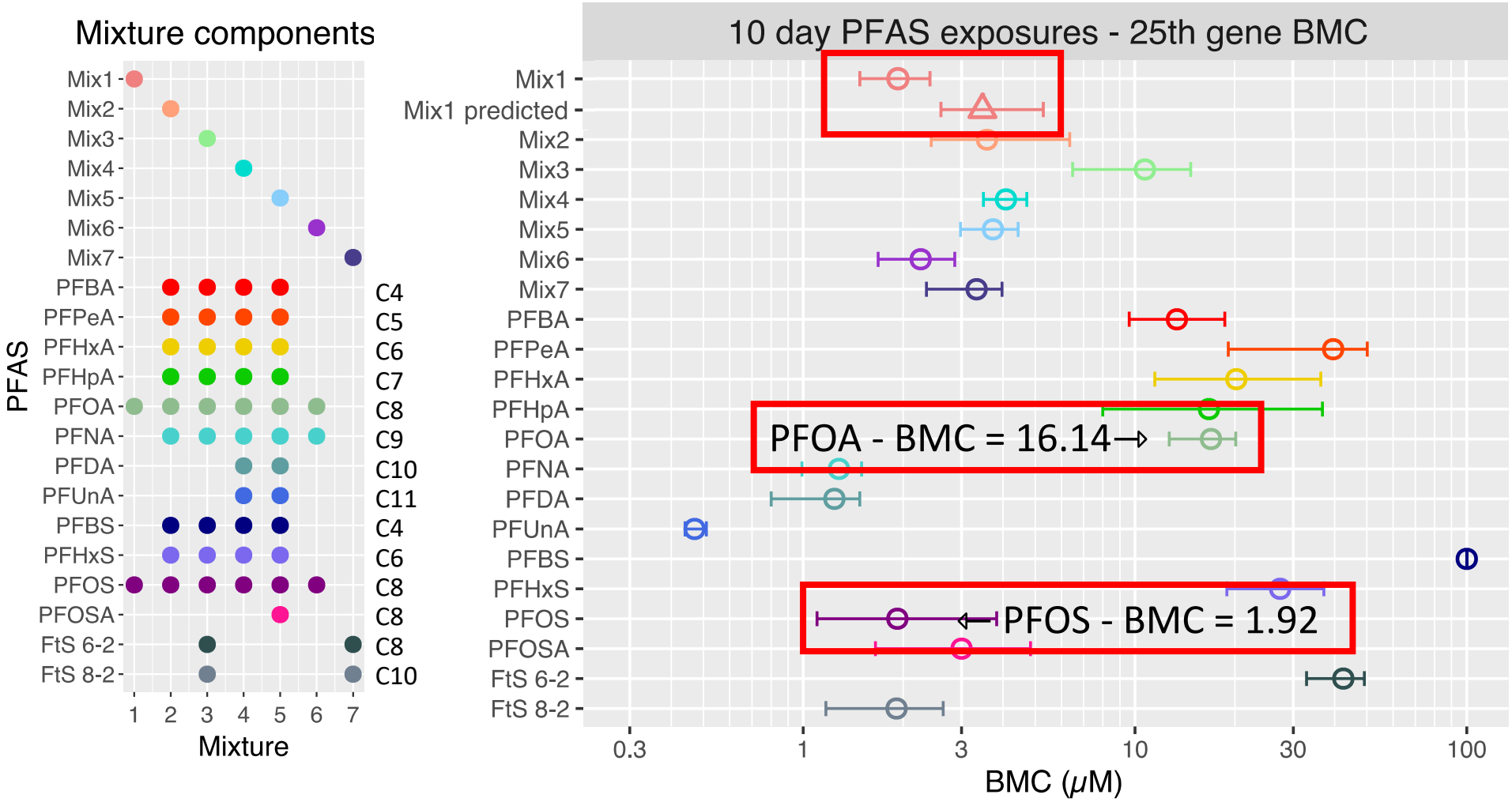
BMC (mixture) of POFA and PFOS =

$$\text{Predicted BMC (mixture)} = \left(\frac{0.5}{16.14} + \frac{0.5}{1.92} \right)^{-1} = (0.0295 + 0.260)^{-1} = (0.290)^{-1} =$$



BMC (mixture) of POFA and PFOS = 3.45

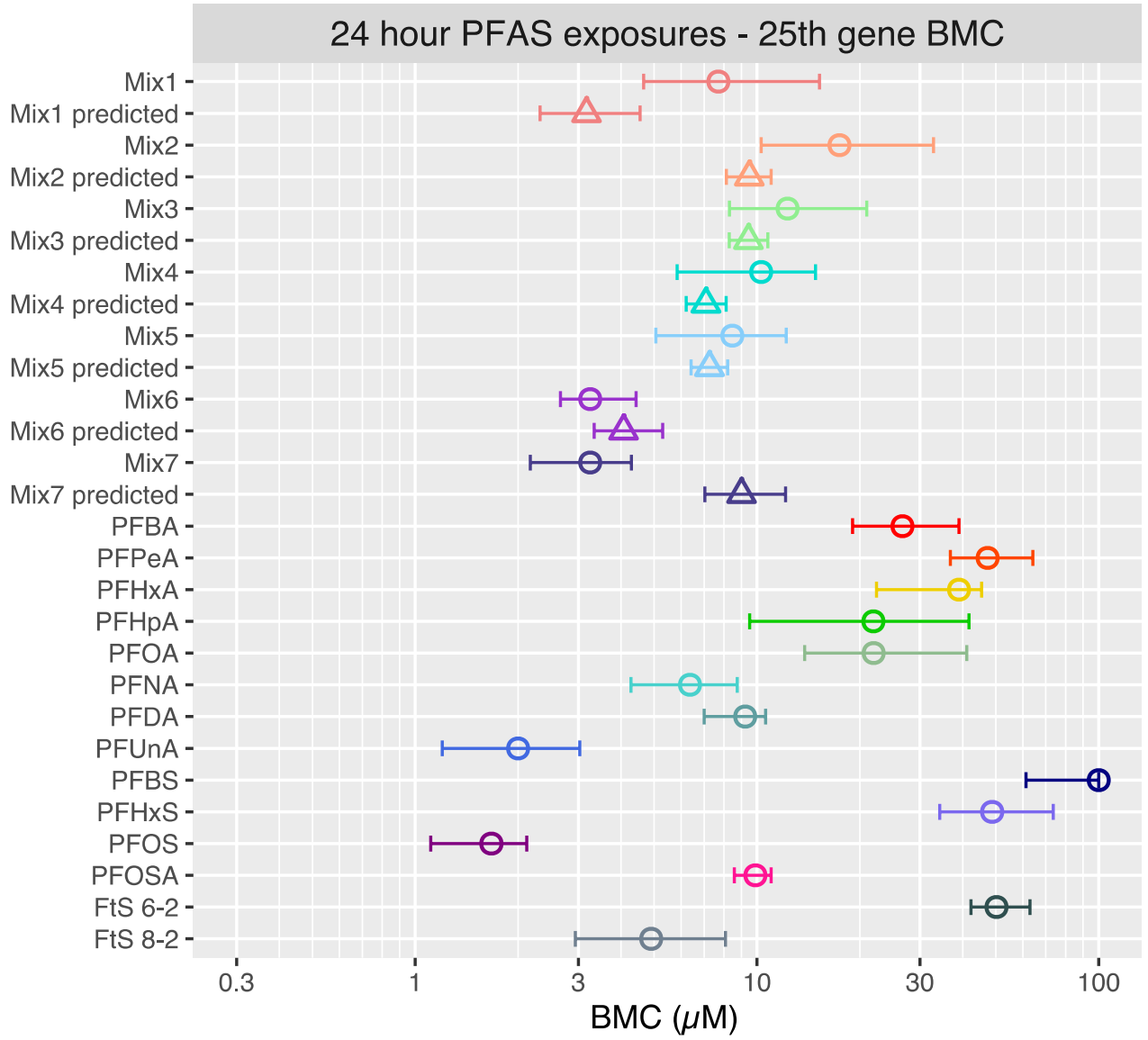
$$\text{Predicted BMC (mixture)} = \left(\frac{0.5}{16.14} + \frac{0.5}{1.92} \right)^{-1} = (0.0295 + 0.260)^{-1} = (0.290)^{-1} = 3.45\ \mu M$$

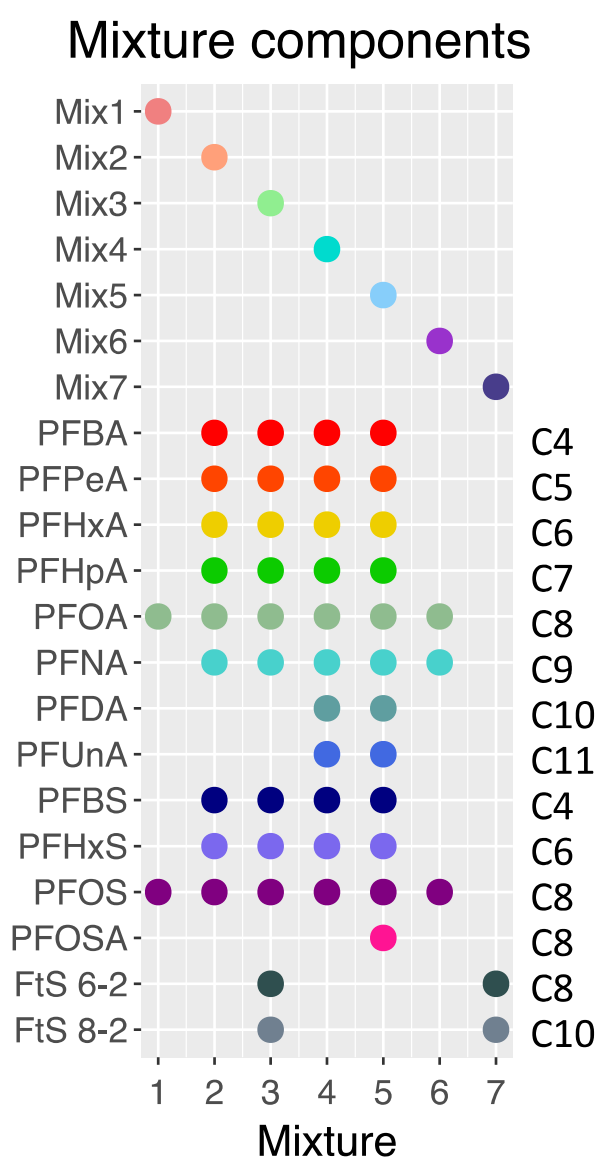


24 hour 25th gene BCMs (+/- BMCL/BMCU)

Concentration Addition predicted BMCs

- Empirical – O
- Predicted – Δ
- No clear deviation from additivity
- Most have overlapping CIs
- Most have less than ± 2 fold differences in BMC
- All have less than ± 2 fold differences in CI endpoints

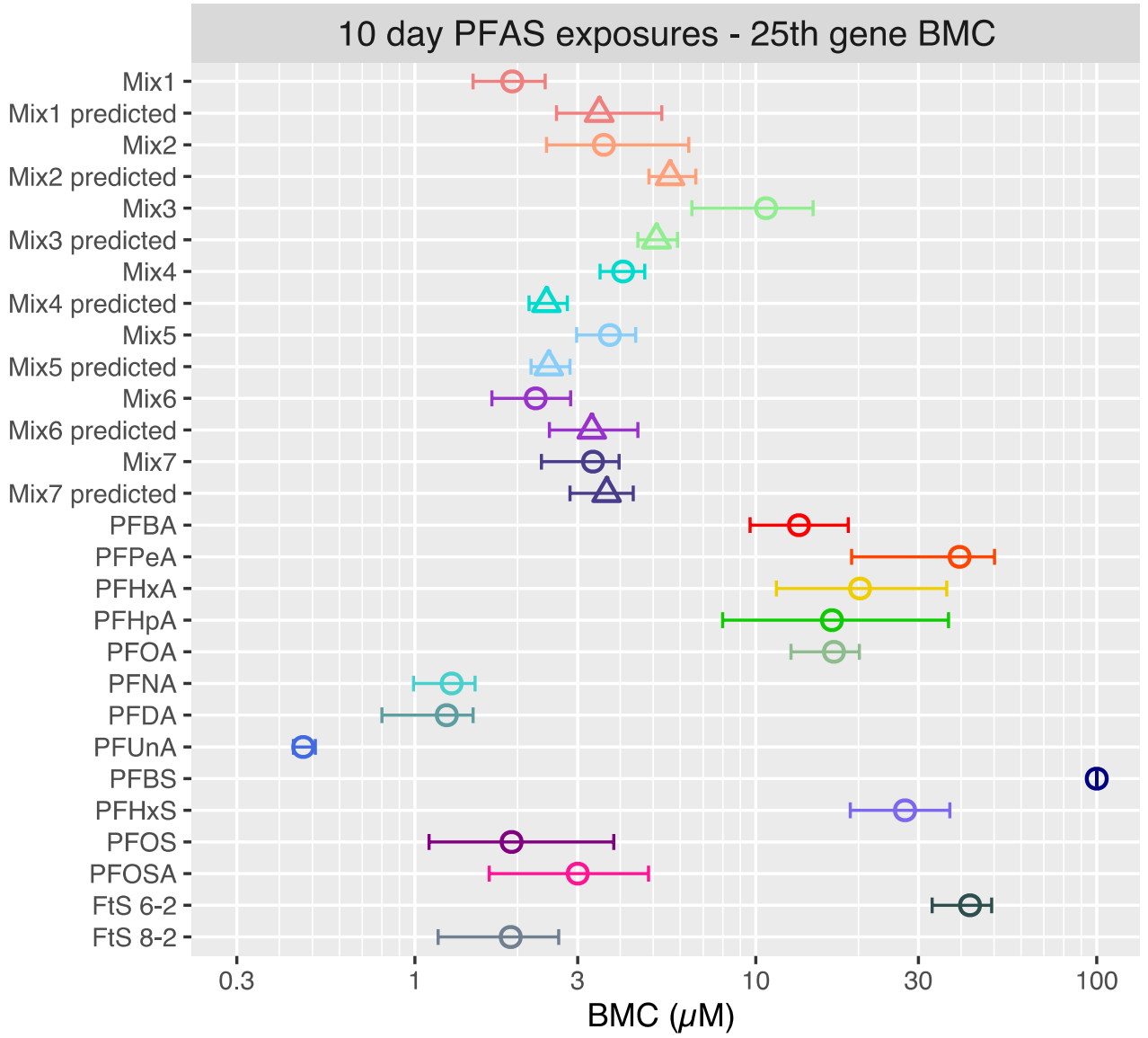


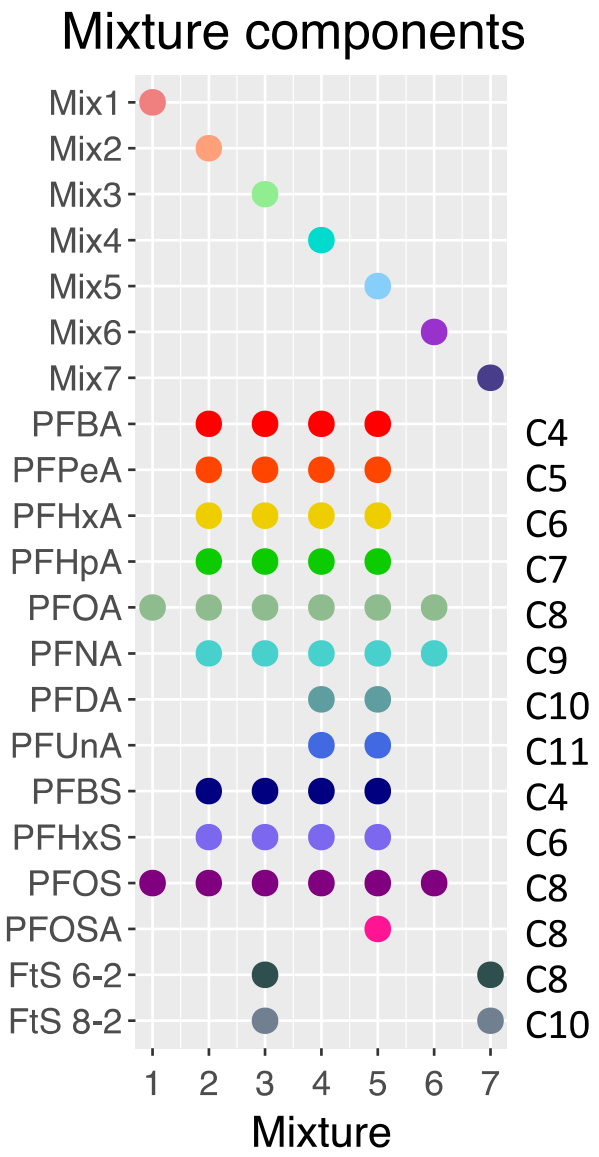


10 day 25th gene BCMs (+/- BMCL/BMCU)

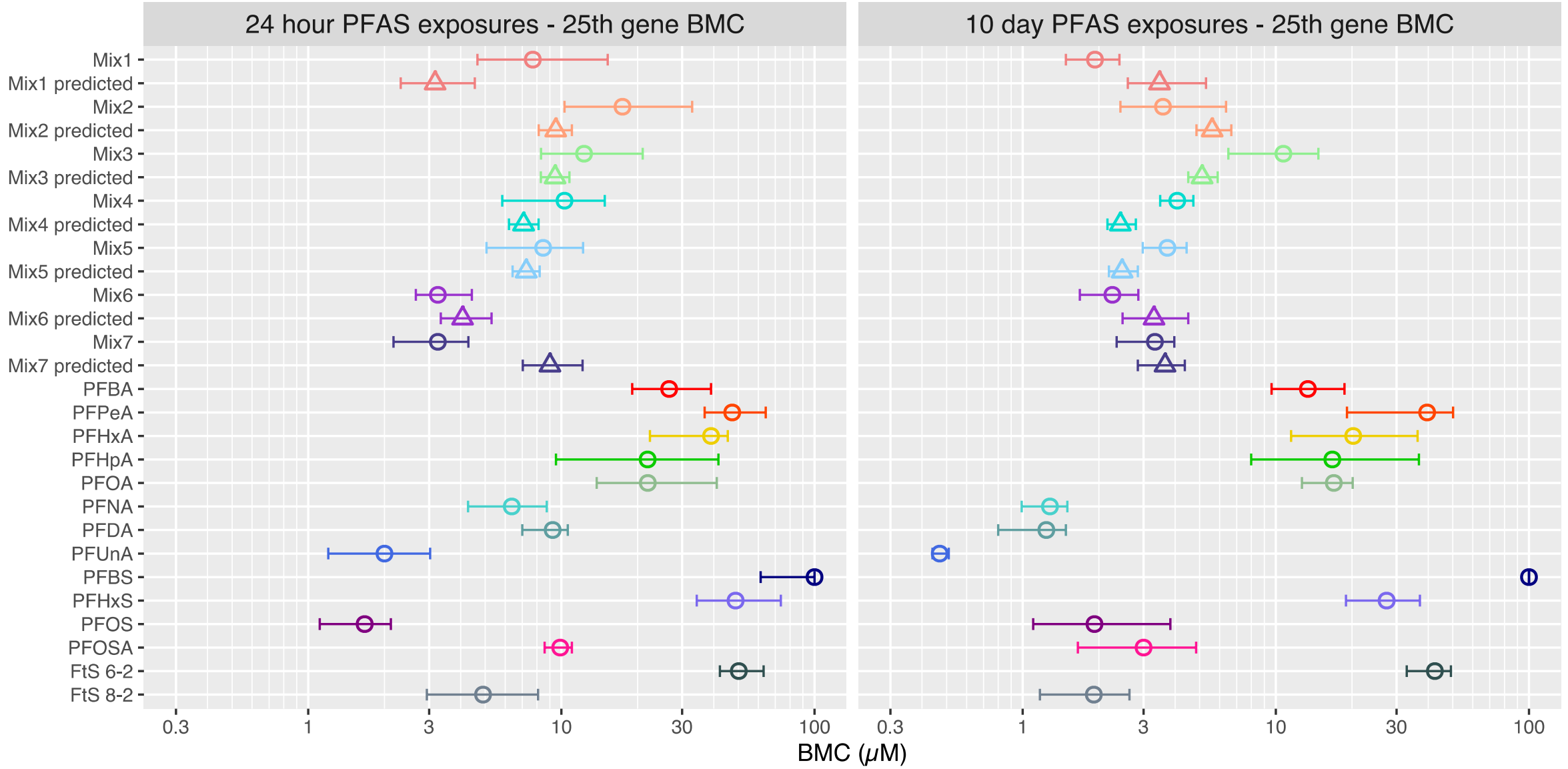
Concentration Addition predicted BMCs

- Empirical – O
- Predicted – Δ
- No clear deviation from additivity
- Most have overlapping CIs
- Most have less than ± 2 fold differences in BMC
- All have less than ± 2 fold differences in CI endpoints

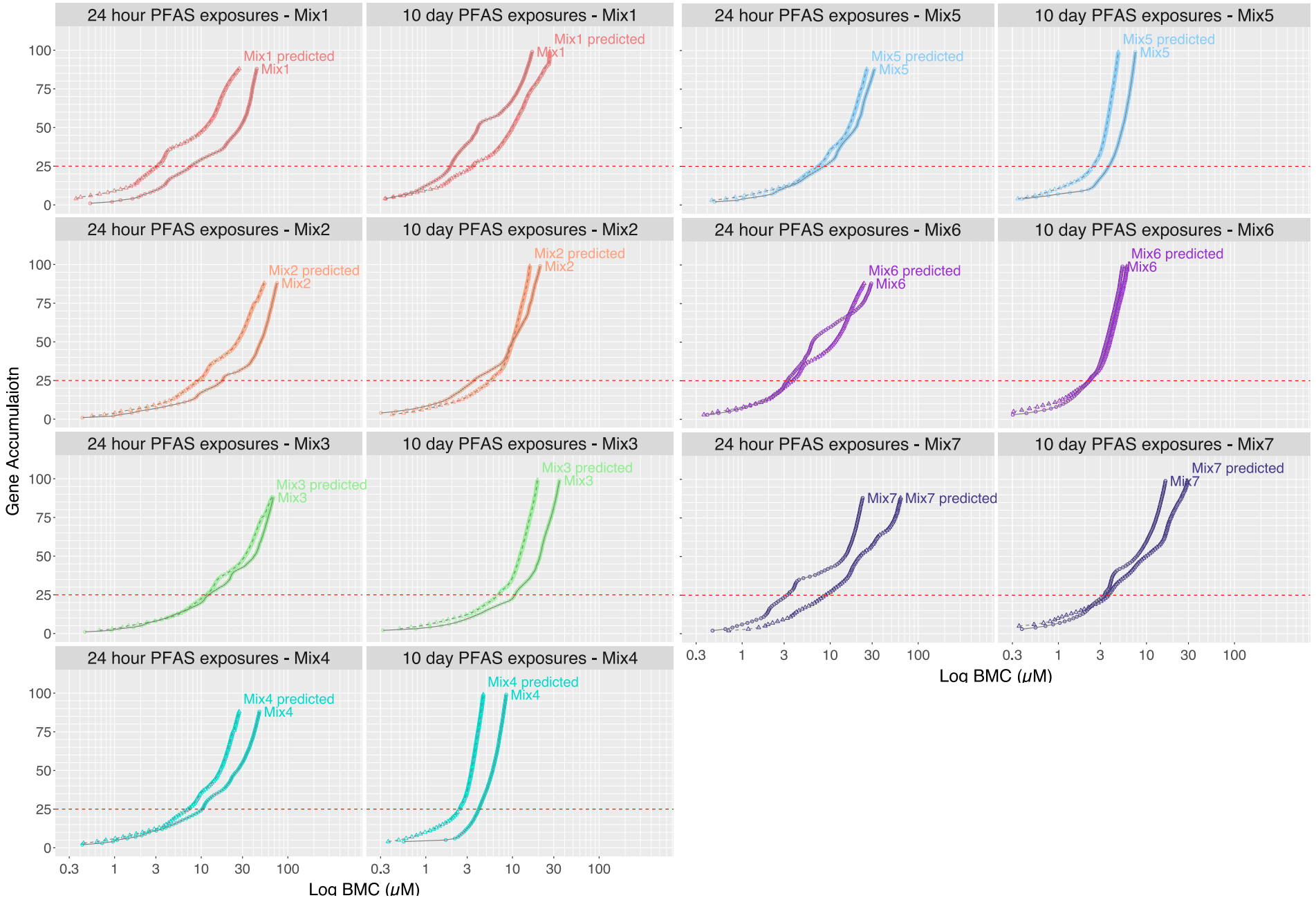




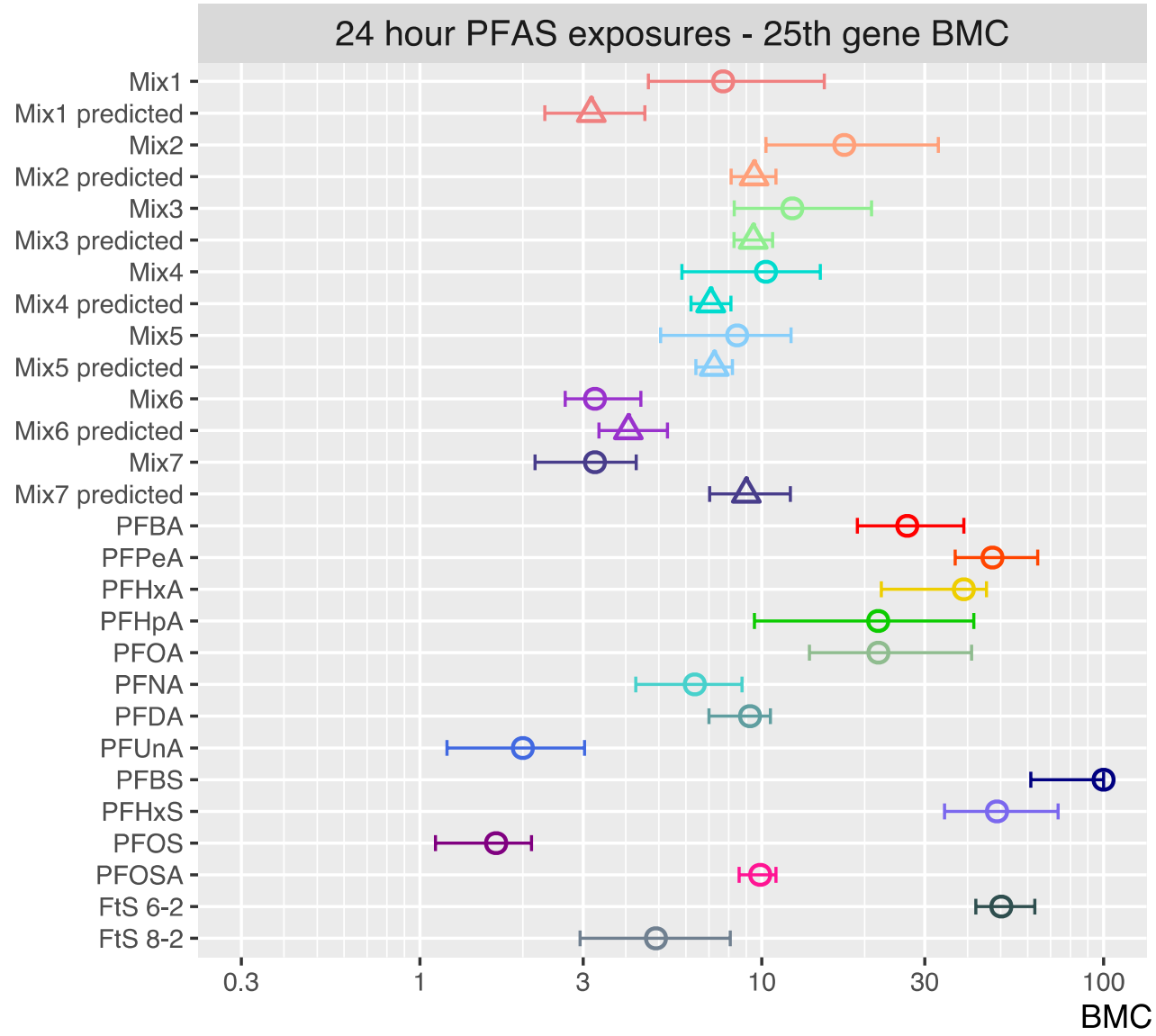
CA predicted PFAS mixture BMCs

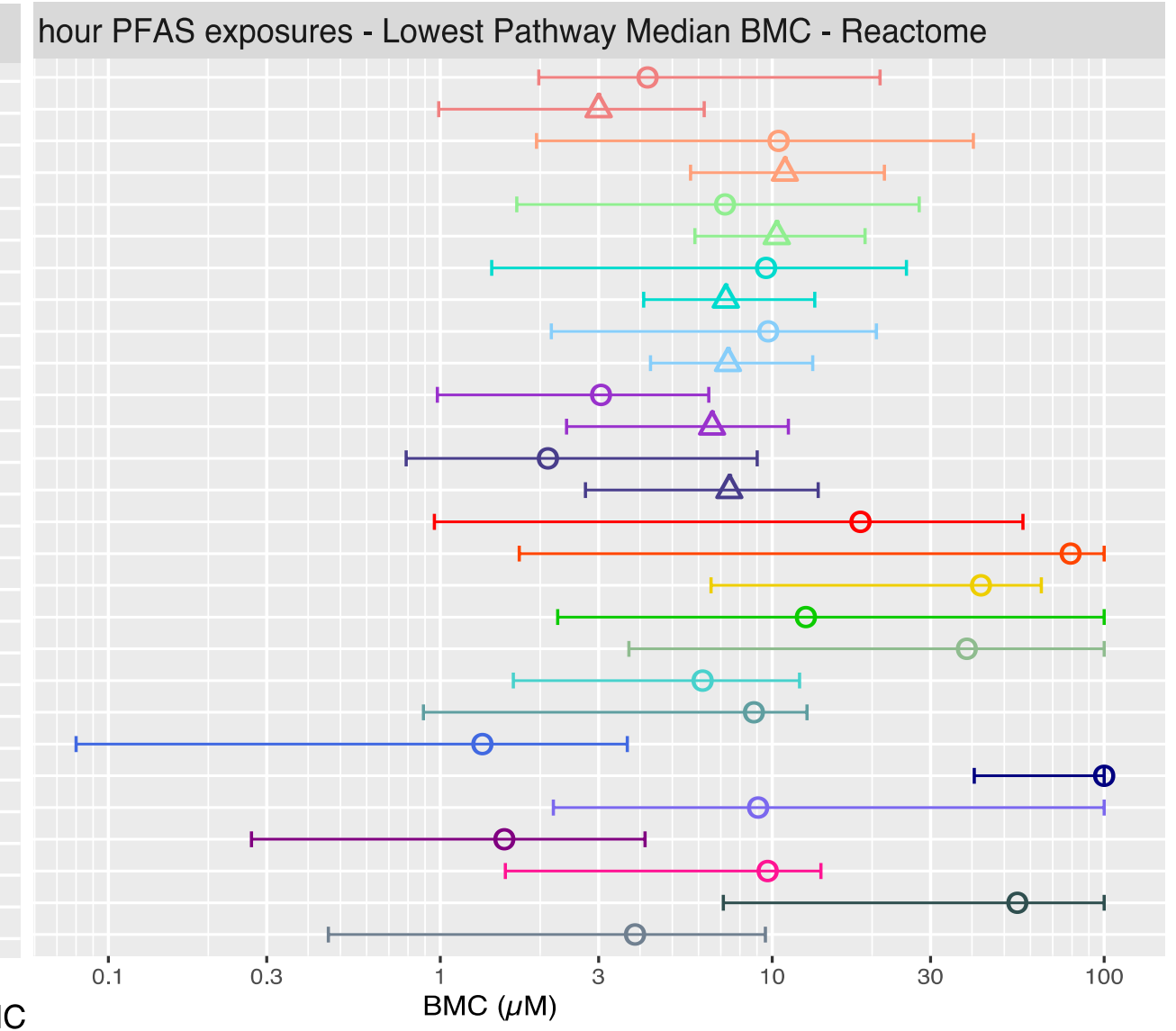


Dose addition calculations done for each BMC independently (RPF calculated for each BMC)



CA predicted PFAS mixture BMCs





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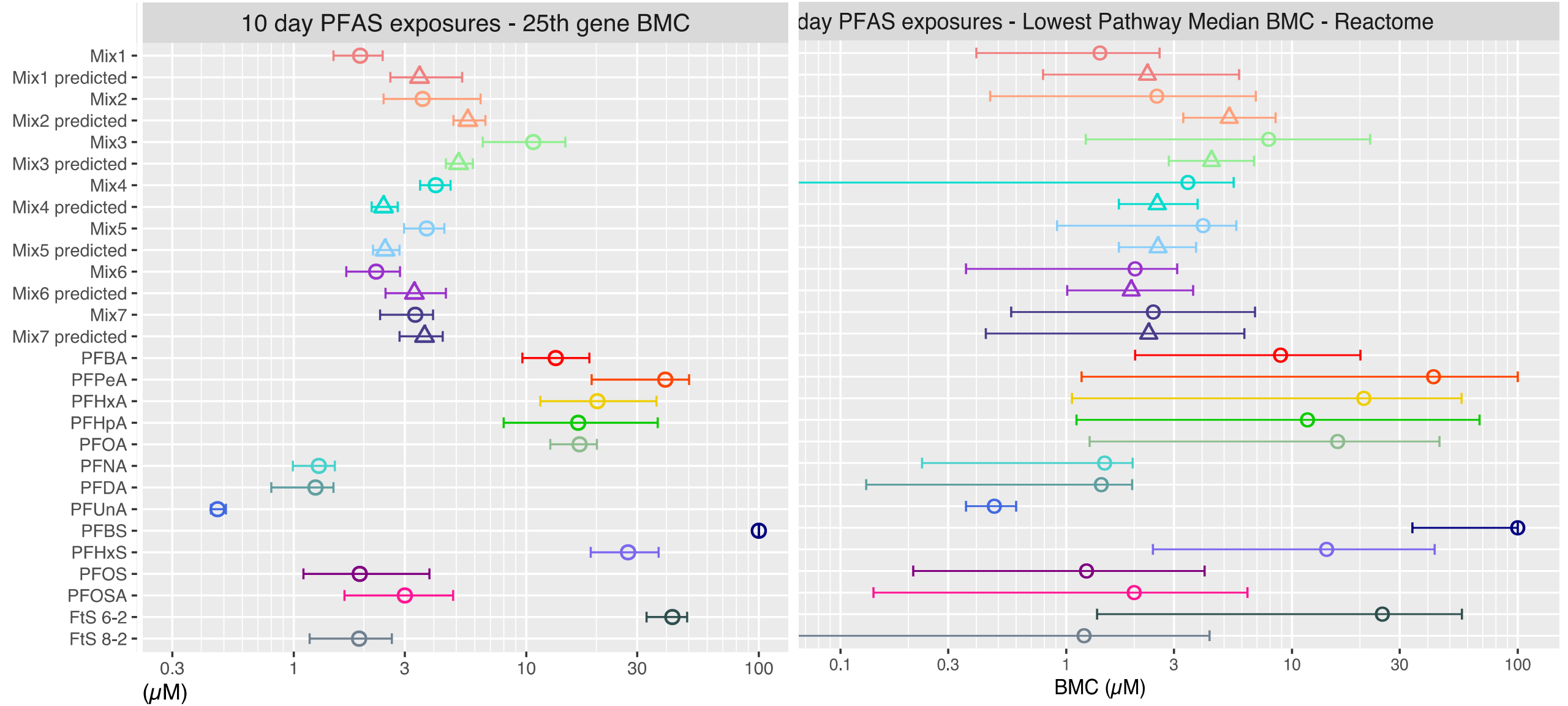

Conclusions

- PFAS mixtures have additive effects in mixtures
  - No evidence of synergistic or antagonistic effects using conservative criteria
  - Debate continues on what qualifies as synergistic or antagonistic
- Findings apply to PFAS concentrations at cellular environment
  - Relative potencies of PFAS differ from some in-vivo data
    - Bioaccumulation or excretion?
  - In-vitro to in-vivo extrapolation data needed to apply this data to human or animal external exposures through food, water, contact etc.
  - Can not exclude PFAS interactions affecting accumulation, persistence or excretion at whole organism level

Acknowledgements

- Ella Atlas and Carole Yauk –
  - designed the project
- Andrea Rowan-Carroll and Karen Leingartner –
  - cell culture, planning, sequencing etc.
- Matthew Meier and Andrew Williams –
  - data processing, computation
- Barbara Wetmore and others at US EPA –
  - PFAS chemicals
- Many others in the background supporting the project