Toxicological Assessment of PFAS using Seahorse Extracellular Flux Analysis

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1. Advancing Non-Animal testing strategies for PFAS

The European Commission is committed to reducing animal testing by supporting the development and regulatory uptake of New Approach Methodologies (NAMs) - mechanism-based, *in vitro* and *in silico* tools that align with the 3Rs (Replacement, Reduction, Refinement). This shift is especially important for complex chemical groups like per- and polyfluoroalkyl substances (PFAS)- a large family of persistent, bioaccumulative, and potentially toxic compounds. PFAS are widely used in products like textiles, cookware, and firefighting foams, and are now recognised globally as "forever chemicals" due to their environmental persistence and resistance to degradation.

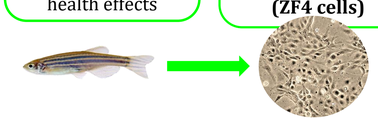
Legacy PFAS such as PFOA and PFOS are being phased out, but are often replaced by short-chain or alternative PFAS, whose toxicological profiles remain poorly understood. Traditional animal-based toxicity testing is not scalable for the 12,000+ PFAS in use- hence the urgent need for human-relevant, high-throughput testing strategies.
This study aims to evaluate PFAS-induced bioenergetic disruption using: (i) Seahorse extracellular flux analysis to assess mitochondrial and glycolytic function; (ii) *In vitro* cytotoxicity assays for comparative analysis (CCK-8 Assay); and (iii) Integration with the Adverse Outcome Pathway (AOP) framework to support regulatory decision-making.

2. Materials and method

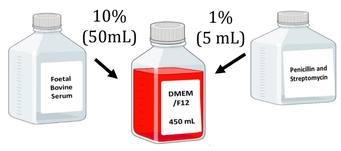
Culturing of ZF4

Zebrafish are a relevant model for studying aquatic toxicity and human health effects

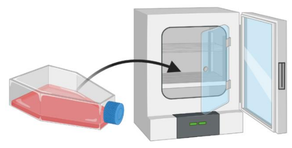
Assessment of PFAS using 1-day-old **Zebrafish embryo fibroblast cells (ZF4 cells)**



1 Cell culture media
ZF4 cells cultivated in classical DMEM/F12 media with Foetal Bovine Serum and antibiotics.



2 Incubation
In a humidified atmosphere of 5% CO_2 at 28 °C.

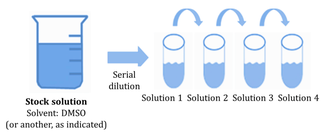


3 Subculture (80 % confluence)
Cells were washed with PBS, detached with trypsin (5min, 28°C), and counted before dilution in fresh medium and seeding.

Screening of 19 PFAS

**List of PFAS analysed shown in Figure 6*

1 Preparation of the stock solution



2 Transfer cells and PFAS exposure

- 40,000 and 20,000 cells/well were seeded in Seahorse XF cell culture plates (80 µL/well) and 96-well plates (100 µL/well) for Seahorse analysis and CCK-8 assay, respectively.
- Cells were exposed to different concentrations of PFAS (10–1000 µM) for 24 or 48 hours at 28 °C and 5% CO_2.
- Three replicates per condition were prepared, along with a positive control (10% DMSO), a negative control, and a blank control. At least two independent experiments were conducted.

Seahorse Analyser

XF metabolic profiling was performed to evaluate mitochondrial respiration and glycolytic function following PFAS exposure. Mitochondrial toxicity was identified and quantified using the mitochondrial toxicity index.

CCK8 Assay

Absorbance was measured at 460 and 800 nm using a microplate reader to assess cellular metabolic activity at each exposure time point.

3. Exploring PFAS toxicity

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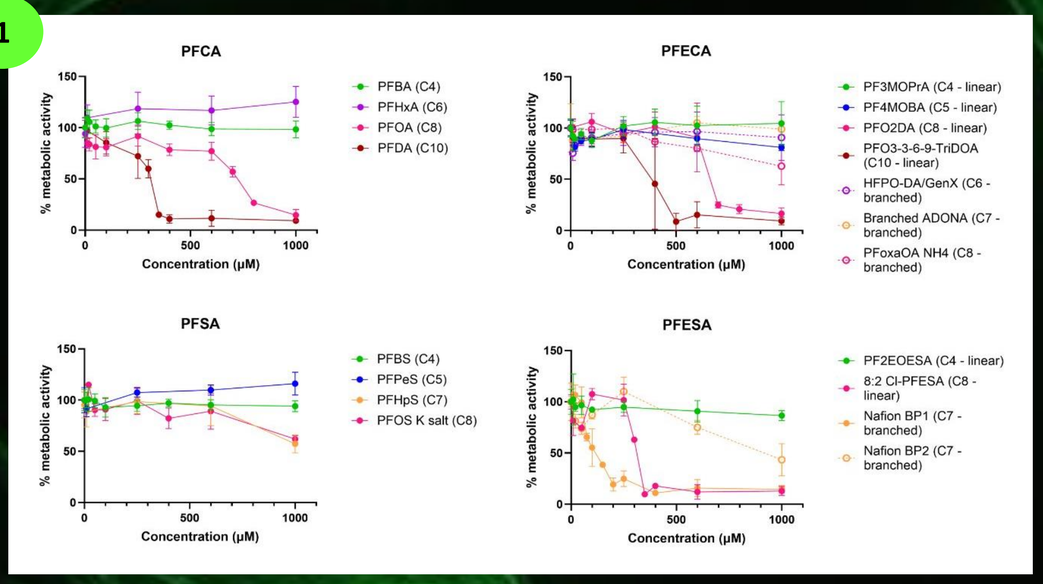

Figure 1. Metabolic Activity (CCK8 Assay) of ZF4 cells exposed for 24 hours to increasing concentrations of PFAS compounds, grouped by chemical class.

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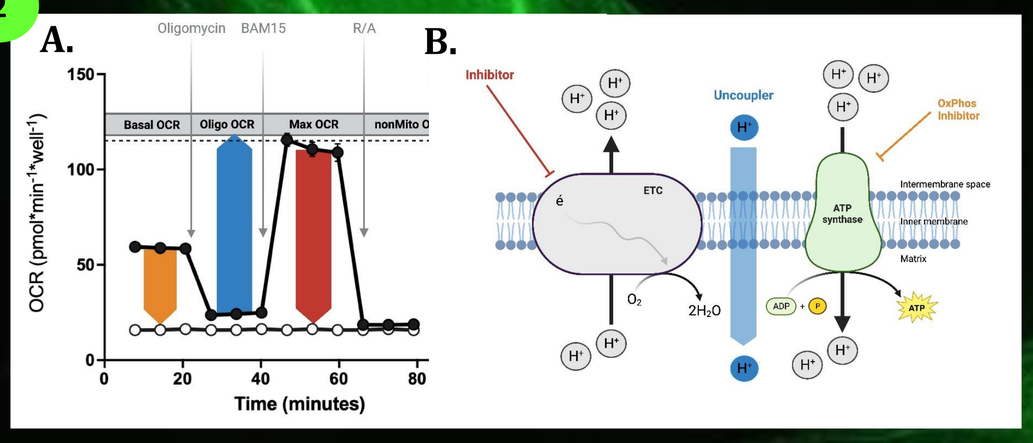

Figure 2. Sites of mitochondrial toxicity identified by an XF MitoTOX assay using XFe96 Extracellular Flux (XF) Analysis. (A) Assay kinetic graph illustrating control groups (vehicle and Rot/AA) with injections. The effect of inhibitors, uncouplers, and OxPhos inhibitors are detected by measuring changes in maximal Oxygen Consumption Rate (OCR,) Oligo OCR, and Basal OCR, respectively. **(B)** Compounds that exert effects on transport, Tricarboxylic Acid cycle, Fatty Acid Oxidation, and/or Electron Transport Chain, or other upstream processes that result in decreased basal and maximal OCR are categorized as inhibitors. Compounds that act as protonophores that uncouple the Electron Transport Chain from the OxPhos machinery resulting in increases in basal and oligo OCR are categorized as uncouplers. Compounds that specifically suppress the OxPhos machinery (i.e., ATP synthase, adenine nucleotide transporter, inorganic phosphate transporter) are categorized as OxPhos inhibitors and decrease basal OCR without significant effects on max OCR.

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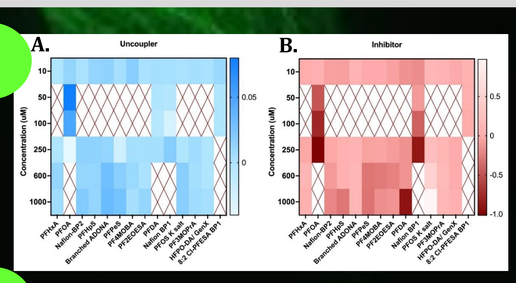

Figure 3. Heat maps of mitochondrial toxicity identified by the XF MitoTOX assay. ZF4 cells were treated with 14 different PFAS at multiple concentrations for 24h. Mitochondrial toxicity was examined and identified using the MitoTOX assay with an XFe96 XF analyser. (A) Effect of different PFAS on mitochondrial uncoupling. (B) Inhibitory effect of different PFAS compounds on maximal respiratory capacity. Data are means from three independent experiments each containing four technical replicates per treatment.

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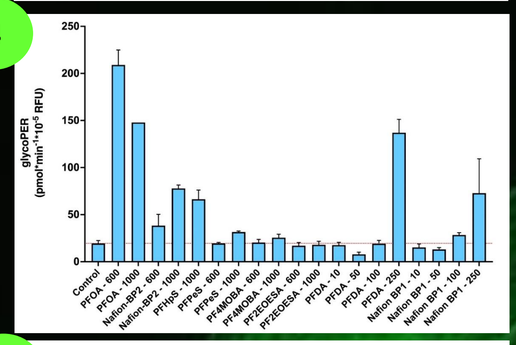

Figure 4. Basal glycolytic activity from ZF4 cells treated with mitochondrial toxic PFAS congeners. ZF4 cells were treated with PFAS that were positive for mitochondrial toxicity. Glycolytic activity (glycoPER) was examined by real-time metabolic profiling using an XFe96 analyser. Data are means from three independent experiments each containing four technical replicates per treatment.

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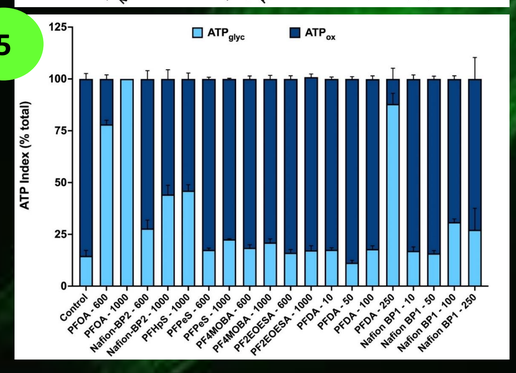

Figure 5. ATP supply fluxes from ZF4 cells treated with mitochondrial toxic PFAS congeners. ZF4 cells were treated with PFAS that were positive for mitochondrial toxicity. Mitochondrial and glycolytic ATP supply flux (ATPox and ATPglyc, respectively) was examined by real-time metabolic profiling using an XFe96 analyser. Data are means from three independent experiments each containing four technical replicates per treatment.

6

		Chain length	C4	C5	C6	C7	C8	C10
PFCA COOH		Name	PFBA		PFHxA		PFOA	PFDA
		CCK8	>1000 µM		>1000 µM		ca 750 µM	ca 300 µM
		Seahorse			no effect		INHIB (600µM)	INHIB (250µM)
		Cytotox (Cyquant, Trypan blue)			no effect		600µM	ca 250 µM
PFECA O-COOH	linear	Name	PF3MOPrA	PF4MOBA			PFO2DA	PFO3-3-6-9-TriDoA
		CCK8	>1000 µM	>1000µM			ca 650 µM	ca 400 µM
		Seahorse	no effect	likely INHIB (600µM)				
		Cytotox (Cyquant, Trypan blue)	no effect	no effect				
	branched	Name			HDPO-DA/GenX	Branched Adona	PFoxaOA NH4	
		CCK8			>1000µM	>1000µM	>1000µM	
		Seahorse			no effect	no effect		
		Cytotox (Cyquant, Trypan blue)			no effect	no effect		
PFSA SO3		Name	PFBS	PFPeS		PFHpS	PFOS K salt	
		CCK8	>1000 µM	>1000 µM		ca 1000 µM	ca 1000 µM	
		Seahorse		likely INHIB (600µM)		likely INHIB (1000µM)	no effect	
		Cytotox (Cyquant, Trypan blue)		no effect		ca 900 µM	750 µM	
PFESA O-SO3		Name	PF2EOESA			Nafion-BP2		
		CCK8	> 1000 µM			ca 1000 µM		
		Seahorse	likely INHIB (600µM)			likely INHIB (600µM)		
		Cytotox (Cyquant, Trypan blue)	no effect			ca 800µM		
		Name				Nafion-BP1	8:2Cl-PFESA	
		CCK8				100µM	ca 300 µM	
		Seahorse				INHIB (100µM)	no effect	
		Cytotox (Cyquant, Trypan blue)				ca 150 µM	ca 200µM	

Figure 6. Summary of PFAS Toxicity by chain length and functional group. Three assays were used to assess PFAS toxicity in ZF4 cells: CCK8 for metabolic activity, Seahorse for mitochondrial respiration and glycolytic function, and cytotoxicity staining (CyQUANT and/or Trypan Blue). The colour coding in the table summarises toxicity levels: red indicates strong effects, yellow moderate inhibition, and green no observed impact. Overall, short-chain PFAS such as PFBA, PFBS, and PF2EOESA, showed minimal or no toxic effects across all assays. In contrast, mid- and long-chain PFAS, including Nafion BP1, PFOA, PFDA, and 8:2Cl-PFESA demonstrated greater cytotoxicity and metabolic disruption of ZF4 cells, with effects observed at concentrations as low as 250–600 µM.
These findings confirmed, in ZF4 cell line, the known trend that perfluoroalkyl carboxylic acid toxicity increases with carbon chain length. The three most toxic compounds tested - Nafion BP1, PFDA and PFOA - consistently caused significant impairment of cellular metabolism.

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