Trust Your Gut: Establishing Confidence in Gastrointestinal Models

An Overview of the State of the Science and Contexts of Use

October 11-12, 9:00 a.m.-12:00 noon EDT both days

Characterization of a Human *In Vitro* Intestinal Model for the Hazard Assessment of Engineered Materials

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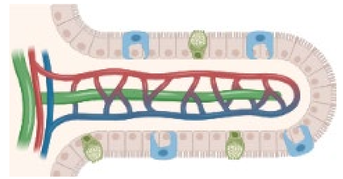
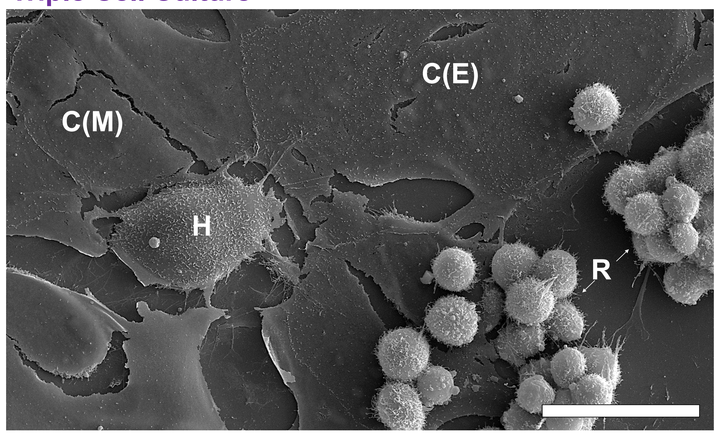
3D Co-culture of the Human Gut Model

Tri-culture assay set-up in Transwell plate

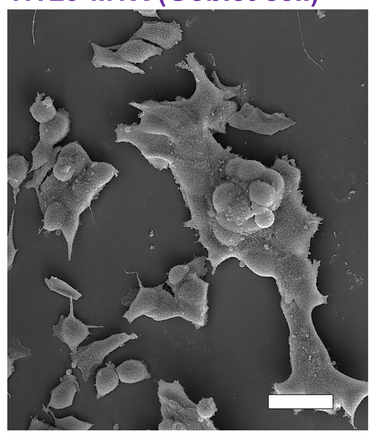
- Intestinal mucous-producing goblet cells (HT29-MTX)
- Colon epithelial cells (undifferentiated Caco-2)
- M-cells (differentiated Caco-2)
- Lymphocytes (Raji B)



Triple Cell Culture

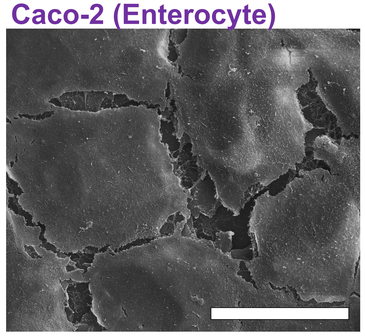


HT29-MTX (Goblet cell)

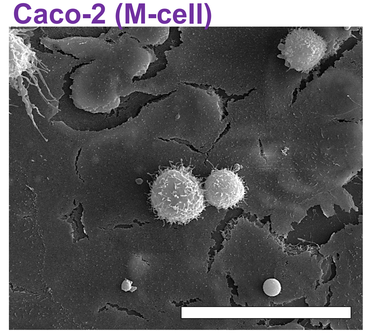


Scanning electron micrograph of the triple cell culture. C(M) is identified as an M-cell, C(E) is identified as a Caco-2 cell, H is identified as an HT29-MTX cell, and R is identified as a Raji B cell

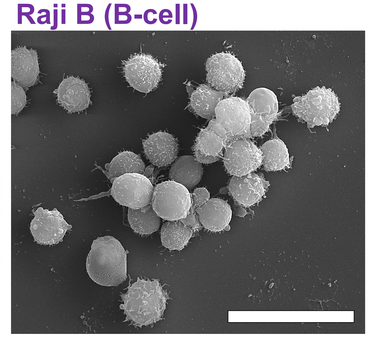
Caco-2 (Enterocyte)



Caco-2 (M-cell)



Raji B (B-cell)



Scale bars represent 30 µm

“Emerging Materials & Environmental Health” Laboratory

Funding & Support

Air Force Research Laboratory (AFRL)

United States Department of Agriculture (USDA)

National Institute for Occupational Safety & Health (NIOSH)

National Institutes of Health (NIH-NIGMS)

C. Gus Glasscock, Jr. Endowed Fund for Excellence in Environmental Sciences

The Department of Environmental Science at BU

The Sayes Group
at Baylor University

Emerging technologies & environmental health

Research & Teaching Program Overview

Human Health Effects

Elucidate molecular initiating events, mechanisms of exposures, and adverse health outcomes

Toxicology & Environmental Health

Predict toxicity by identifying strategies that can anticipate, identify, monitor, and prevent the biological effects of toxic substances

Engineering & Technology

Improve exposure assessment methodologies to detect, characterize, and understand human exposures and associated risks

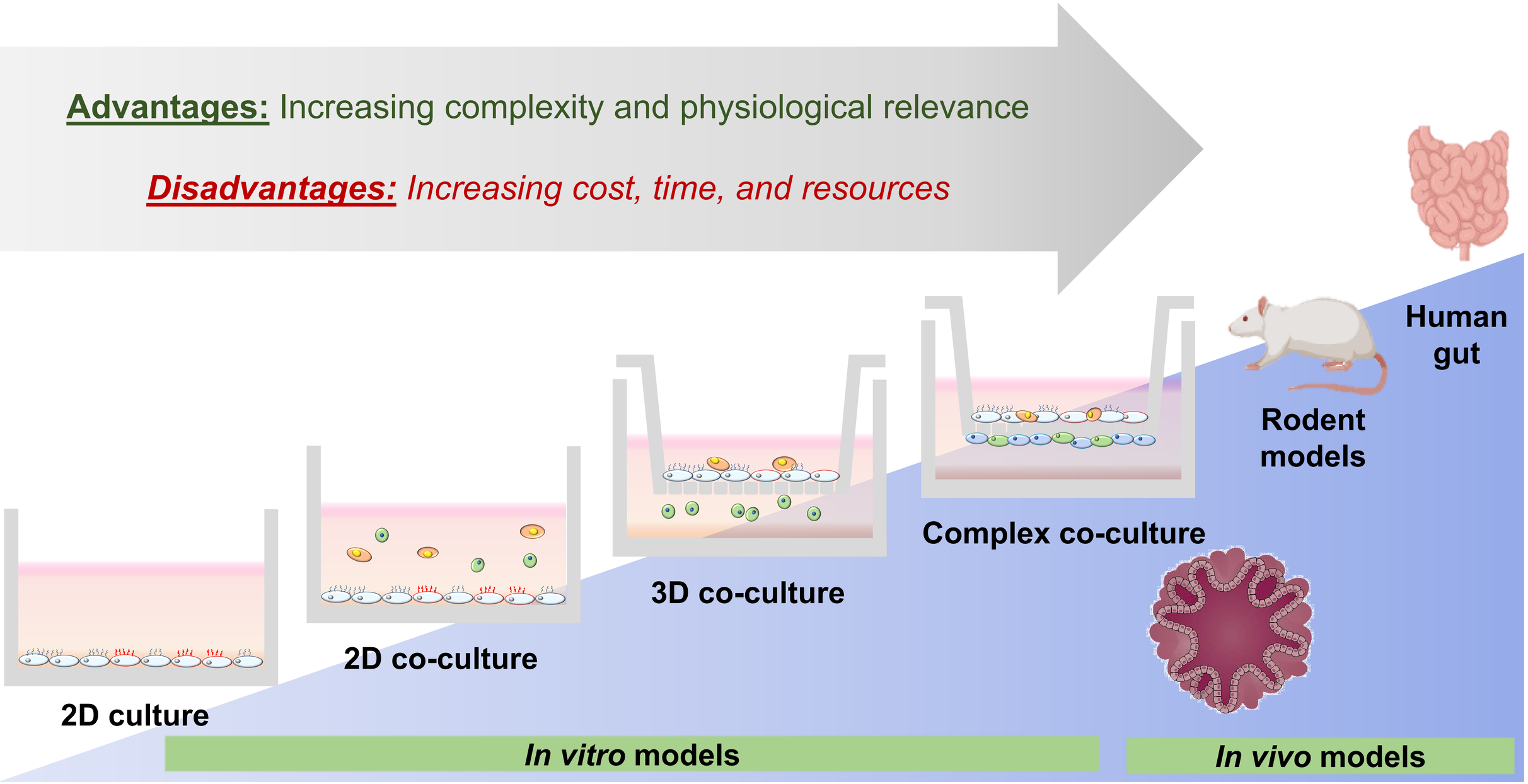
Chemistry & Materials Science

Identify toxicants deemed hazardous and elucidate mechanisms of chemical and biological transformations

Simple-to-Complex Toxicological Models

Advantages: Increasing complexity and physiological relevance

Disadvantages: *Increasing cost, time, and resources*

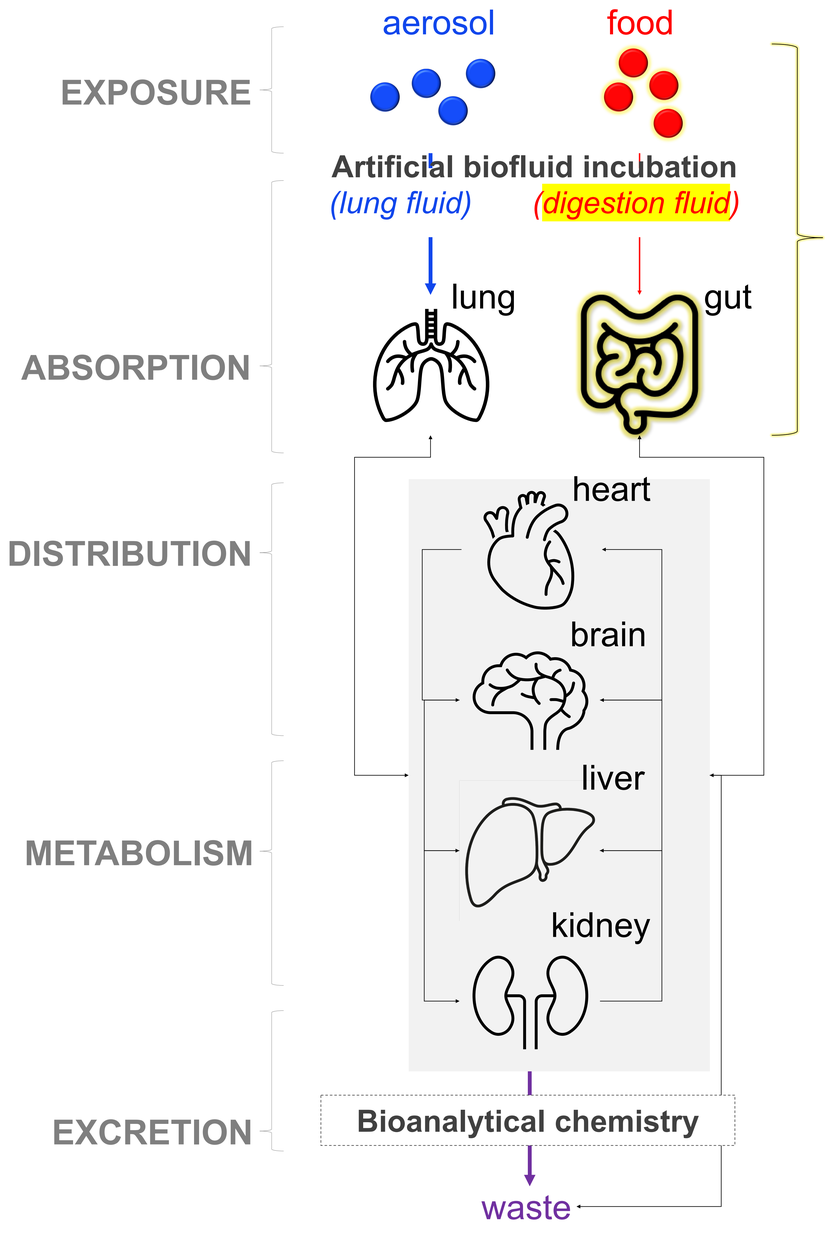


Gut organoid, Vivian S.W. Li, Nature Reviews, 2021.

Using *In Vitro* Models To Mimic Human Gastrointestinal Tract

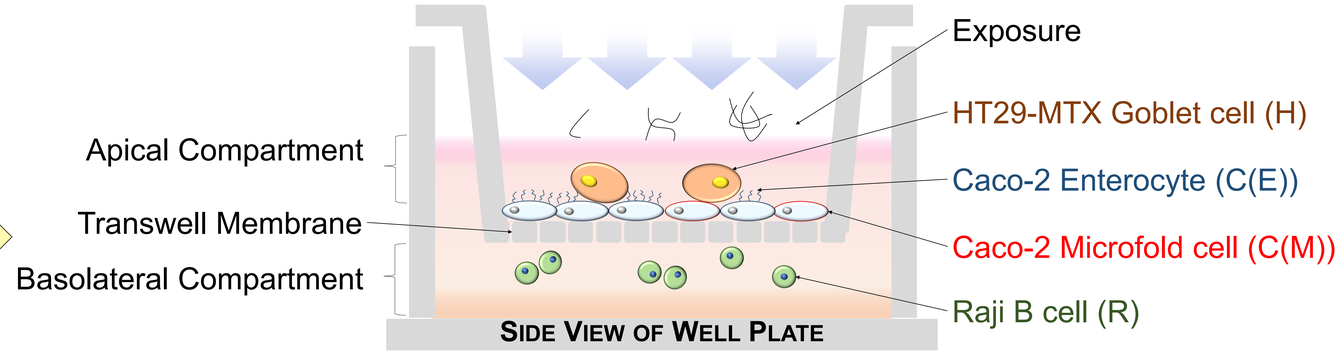
THE SAFETY-BY-DESIGN TOOLBOX OVERARCHING TOXICOLOGY MODEL

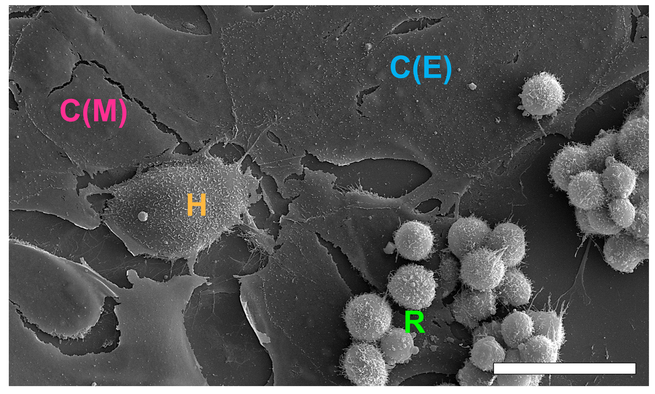
In vitro methodologies inform potential ADME effects



THE SPECIFIC MODEL TO BE UTILIZED IN THIS PROPOSED WORK PLAN

3D gut cell co-culture model identifies and characterizes responses to chemical/particle exposures

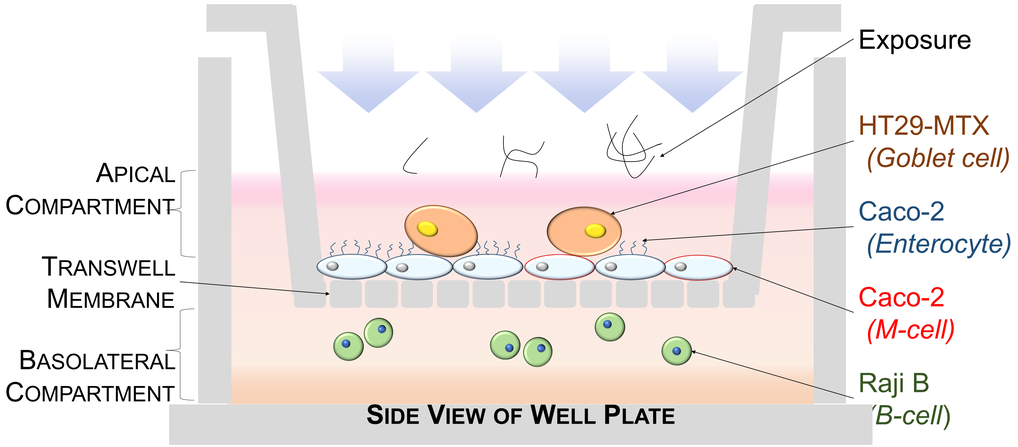




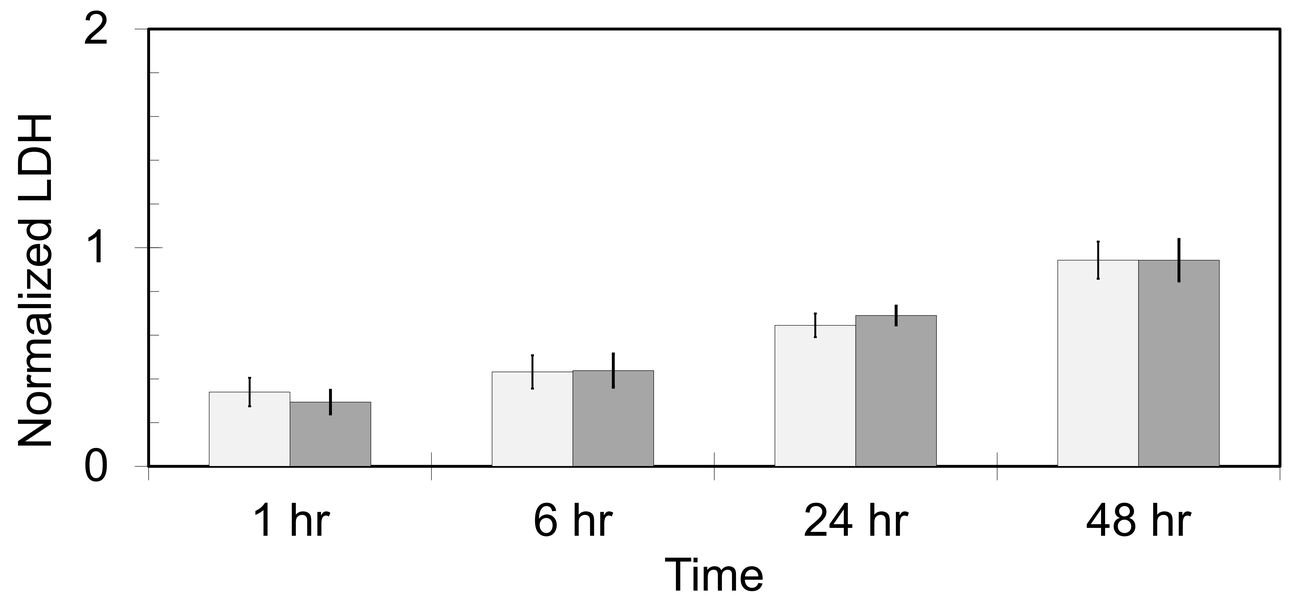
Mode(s)-of-action	*Gut cell co-culture model*	Cell death, inflammation, oxidative stress
Structural endpoints	*Gut cell co-culture model*	Cellular morphology, barrier resistance
Molecular pathways	*Gut cell co-culture model & Zebrafish model*	Gene expression analyses
Developmental endpoints	*Zebrafish model*	Coagulated eggs; somite formation; detached tail; heartbeat

Probe for (Certain) Biological Interactions

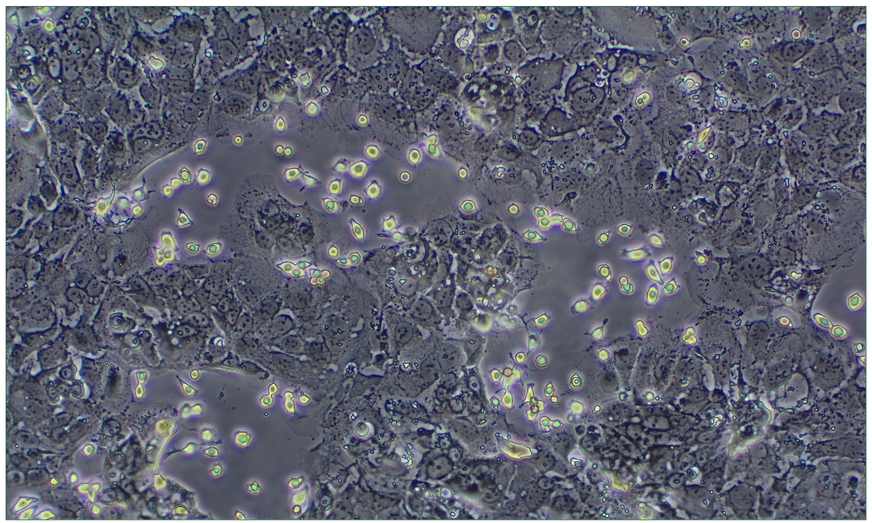
Co-culture model used in these studies



$$\% \, Cytotoxicity = \left[\frac{Cellulose \; expsoure \; LDH \; activity - Spontaneous \; LDH \; activity}{Maximum \; LDH \; activity - Spontaneous \; LDH \; activity}\right] x \; 100$$



Brightfield micrograph showing the normal cellular morphology



We believe that the model is the right tool to probe for:

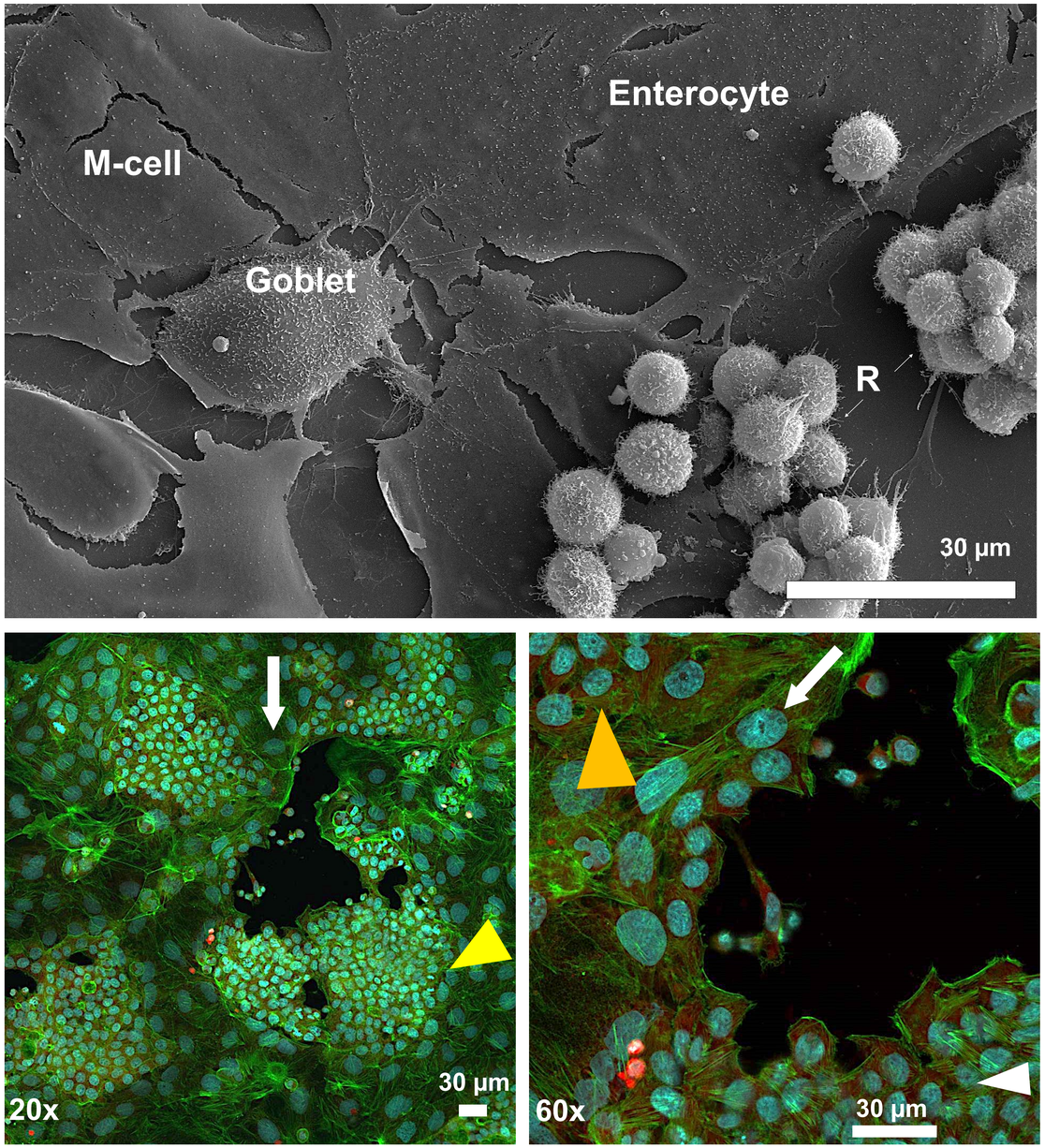
1. Barrier permeability

2. Antigen transport

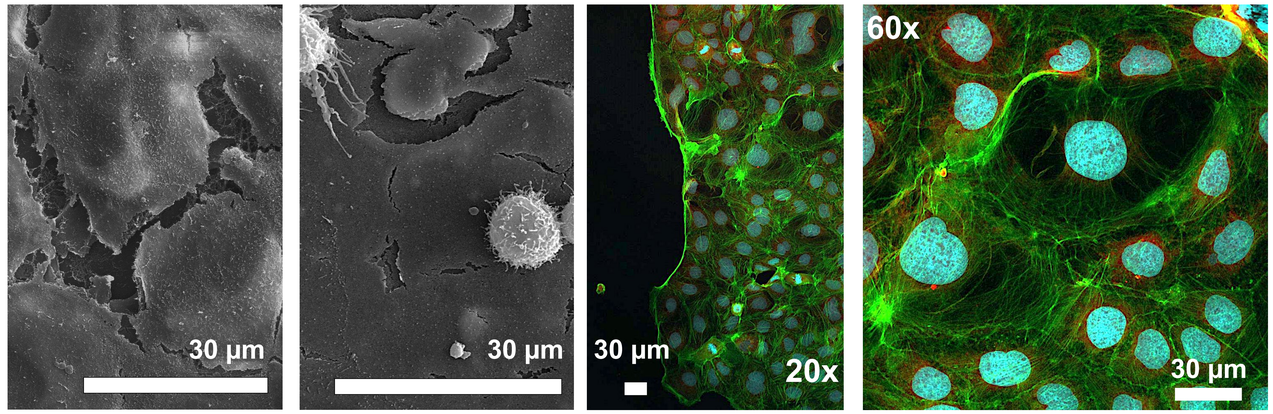
3. Inflammatory response *(inc. oxidative stress)*

Morphological Characterization

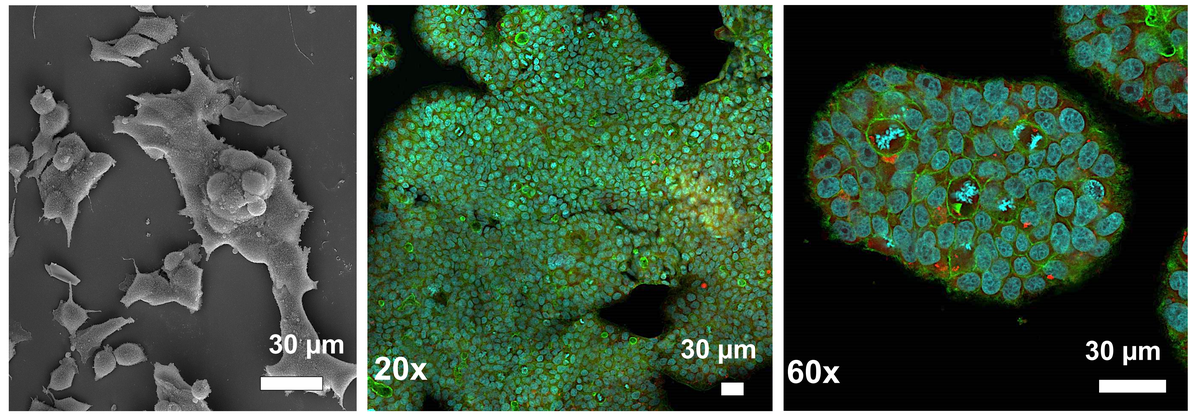
Triple Cell Culture



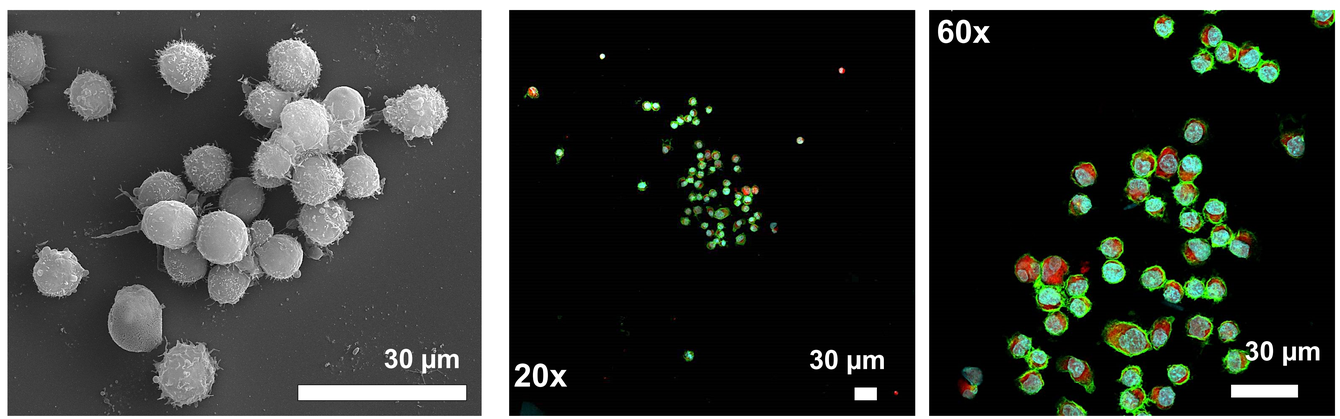
Caco-2 (Enterocyte & Microfold cell)



HT29-MTX (Goblet cell)



Raji B (B-cell)



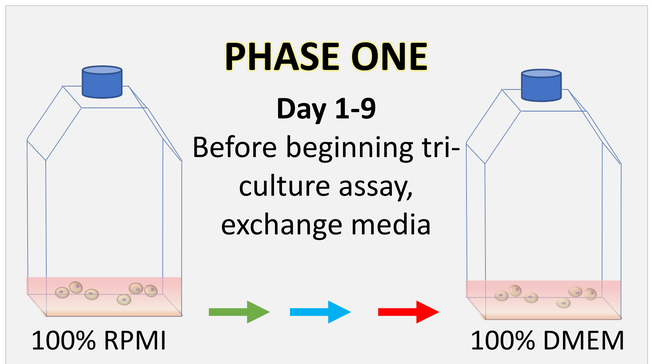
Gibb M, Pradhan SH, …, Sayes CM. (2021). Applied Sciences 27;11(5):2113.

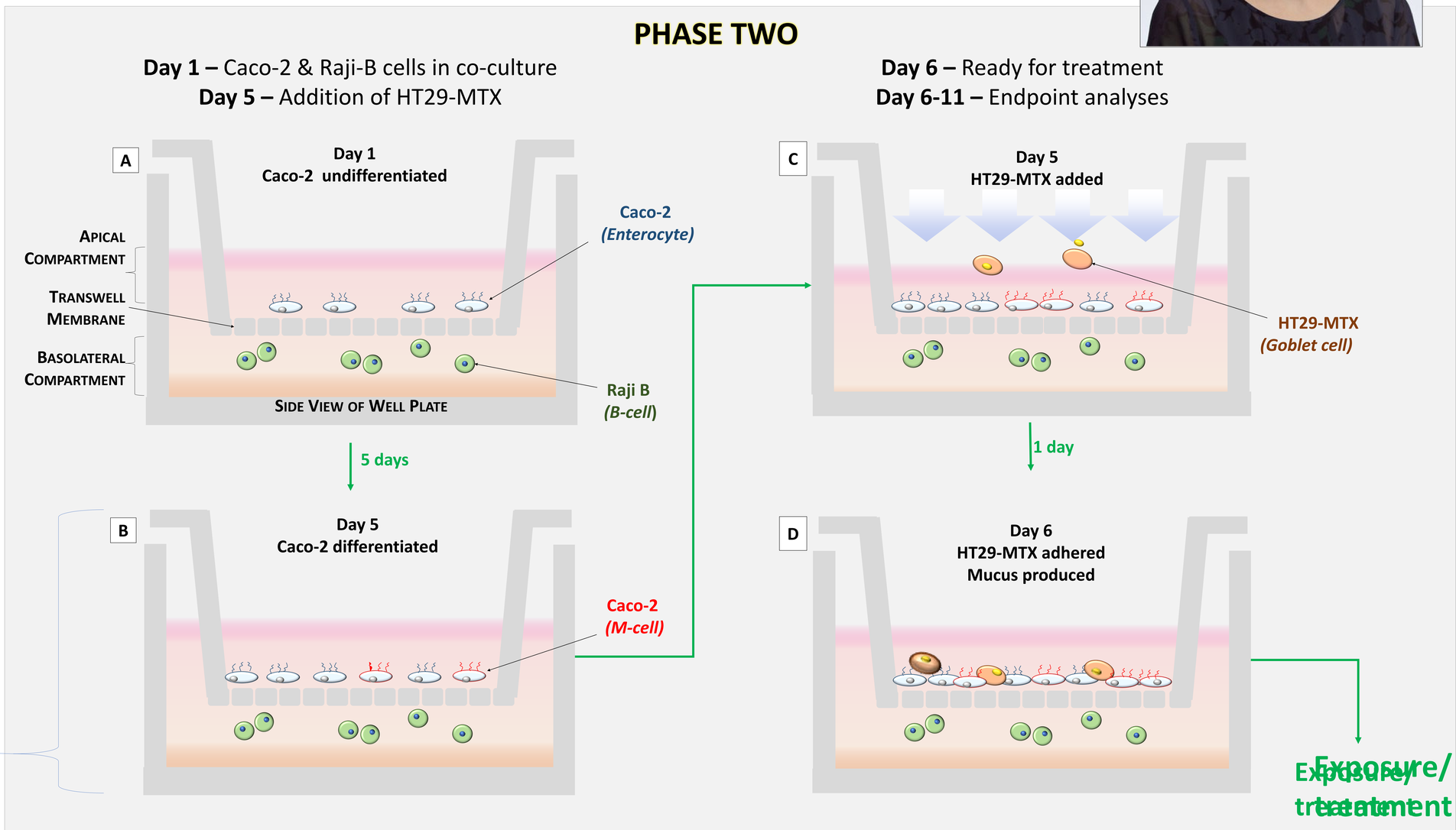
In Vitro Gut Model Set-up

Tri-culture assay progression is divided into 2 phases

Phase one is gradual transition of the Raji B cells into media representative of the entire tri-culture model (apical and basolateral sides).

Phase two includes the differentiation of Caco-2 cells (induced by Raji B cells) into M cells, followed by addition of the mucus producing HT29 cells.





In vitro Ingestion Model Characterization:

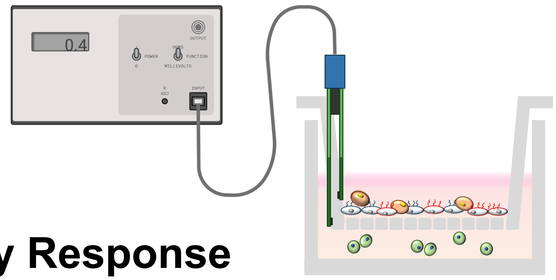
1. Microscopy
2. Cytokine expression
3. Cell surface receptors
4. Transepithelial resistance

Validation of *In Vitro* Gut Model

Validation via 3 methods:

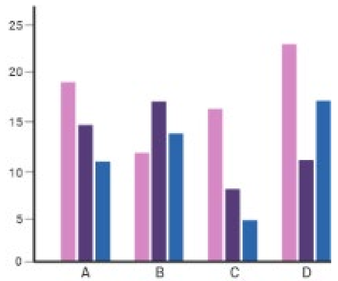
1. Barrier Integrity

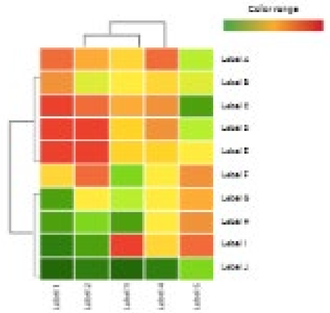
TEER & ZO-1 staining



2. Immune/Inflammatory Response

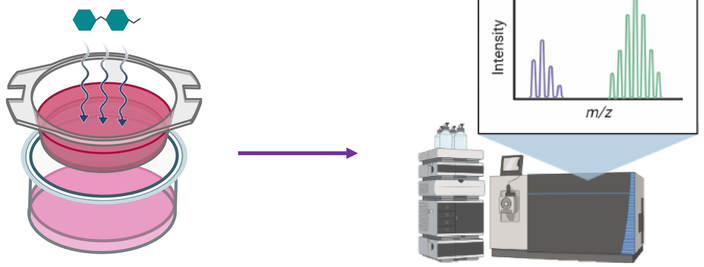
Gene microarray/Luminex

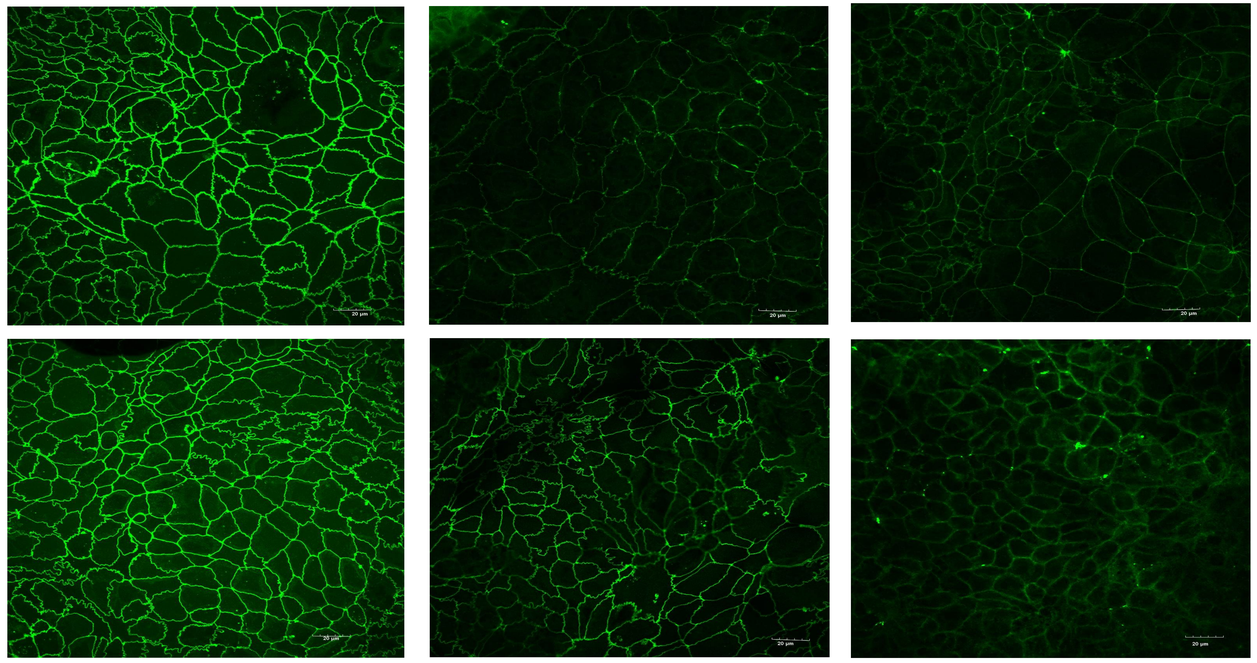




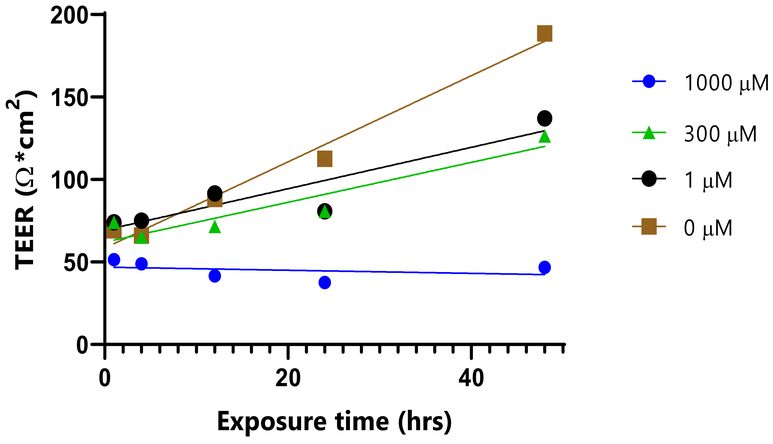
3. Transport

Probe for biomarker in basolateral compartment after translocation from apical compartment of exposed cells (i.e., Dextran dyes)





Above: Tight junction visualization via ZO-1 staining; 48 hrs PFOA treatment



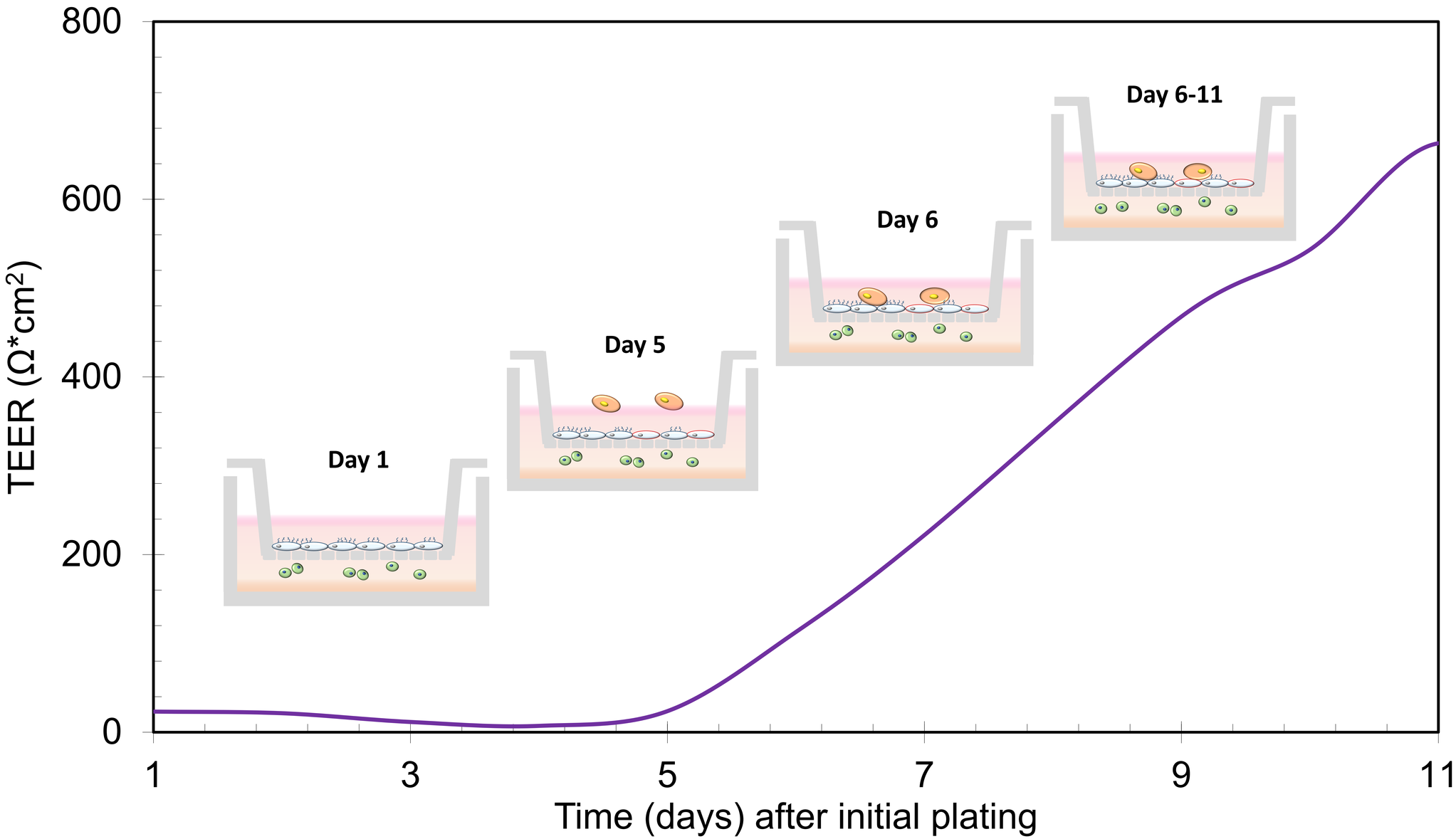
Left: TEER testing of PFOA exposure to tri-culture

Results:

- Epithelial cells form tight junctions
- Tight junctions enable barrier integrity
- If tight junctions do not form, then barrier integrity is lost

Triculture Barrier Permeability

as viewed from the side view of Transwell® insert



Epithelial cells form tight junctions

Tight junctions enable *barrier integrity*

If tight junctions do not form, then *barrier integrity is lost*

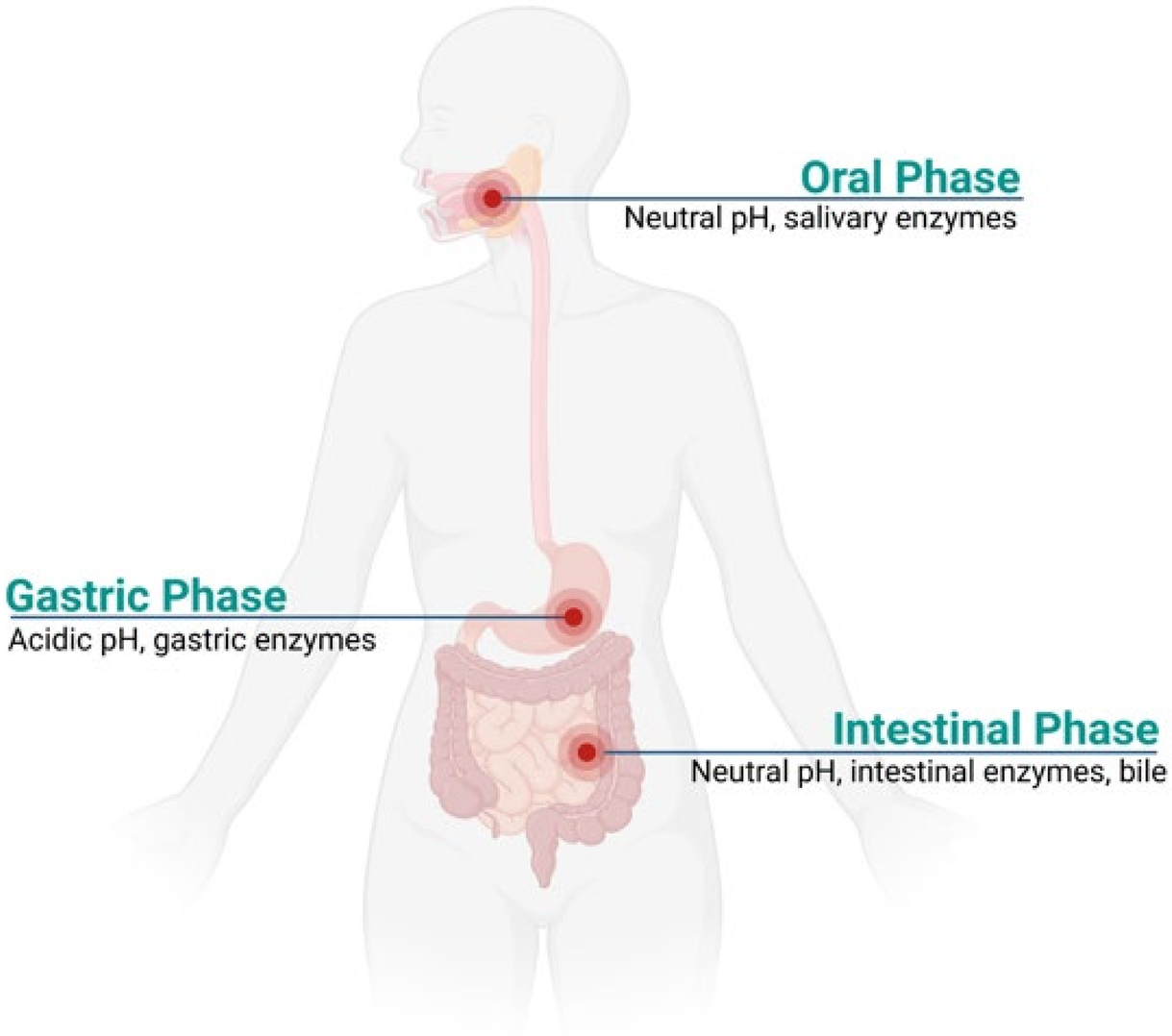
Pradhan SH, Gibb M, Shatkin JA, Sayes CM. Toxicol Res. 2020 9(3):290-301.*

Sevik A., Gibb M., Pradhan S. & Sayes CM (2022). Toxicology In Vitro.*

Sample Preparation Prior To Exposure

Simulated Gastric Fluid Digestion

Simulated Gastric Fluid Digestion



Oral Phase
Mix 1:1 with SSF + Salivary amylase (75 U/ml), 2 min, pH 7

↓

Gastric Phase
Mix 1:1 with SGF + pepsin (2000 U/ml), 2 hrs, pH 3

0.17mM phospholipids (non-standard conditions)

↓

Intestinal Phase
Mix 1:1 with SIF + enzymes, 2 hrs, pH 7

Individual enzymes	**Enzyme extract**
Trypsin (100 U/ml) Chymotrypsin (25 U/ml) Pancreatic lipase (2000 U/ml) Colipase (2:1 molar ratio with lipase) Pancreatic amylase (200 U/ml) Bile (10 mM)	Pancreatin (based on trypsin activity at 100 U/ml) Bile (10 mM)

Minekus M, *et al.* (2014). A standardised static in vitro digestion method suitable for food–an international consensus. *Food Funct.* 5:1113–1124

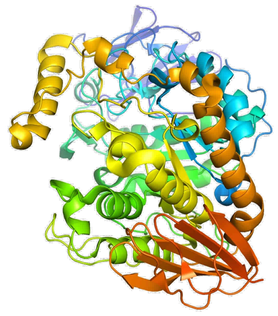
Enzymatic Gastric Digestion

Adapted from
Minekus et al. *Food Funct.*, 2014, 5, 1113

EFSA Approved Method

Salivary Phase

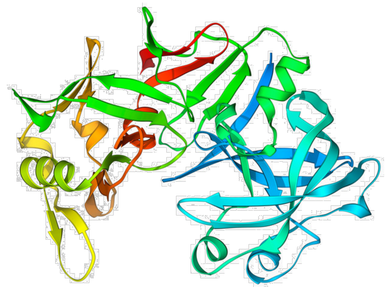
Electrolyte solution
pH = 7
Incubation time: 2 min



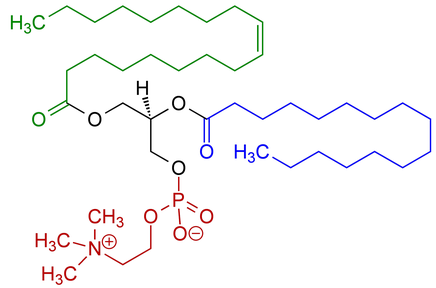
α-Amylase

Gastric Phase

Electrolyte solution
pH = 3
Incubation time: 2 hr



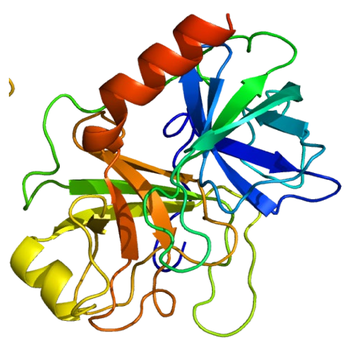
Pepsin



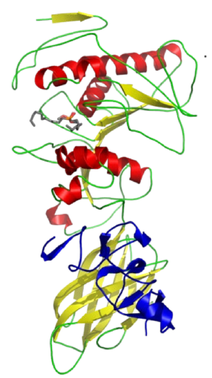
Lecithin

Intestinal Phase

Electrolyte solution
Bile Salts
pH = 7
Incubation time: 2 hr



Trypsin

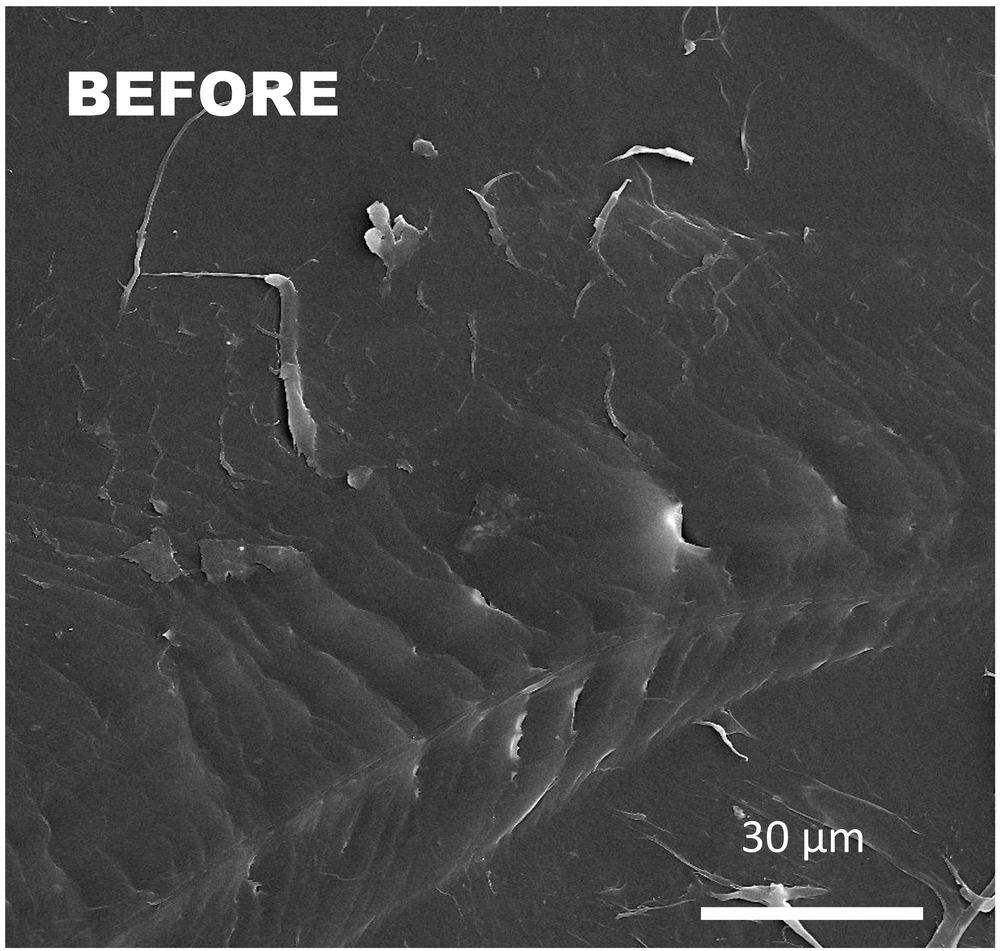


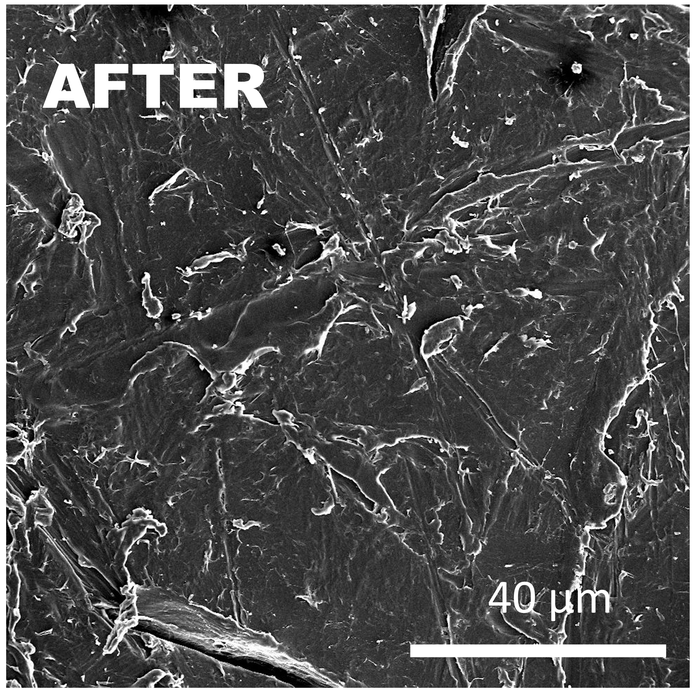
Pancreatic lipase

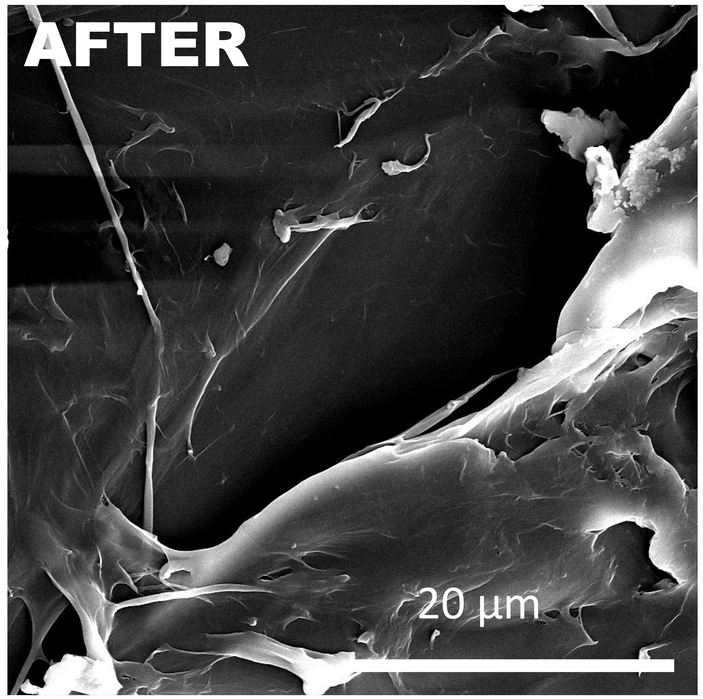
- Chymotrypsin
- Pancreatic amylase
- Pancreatic colipase

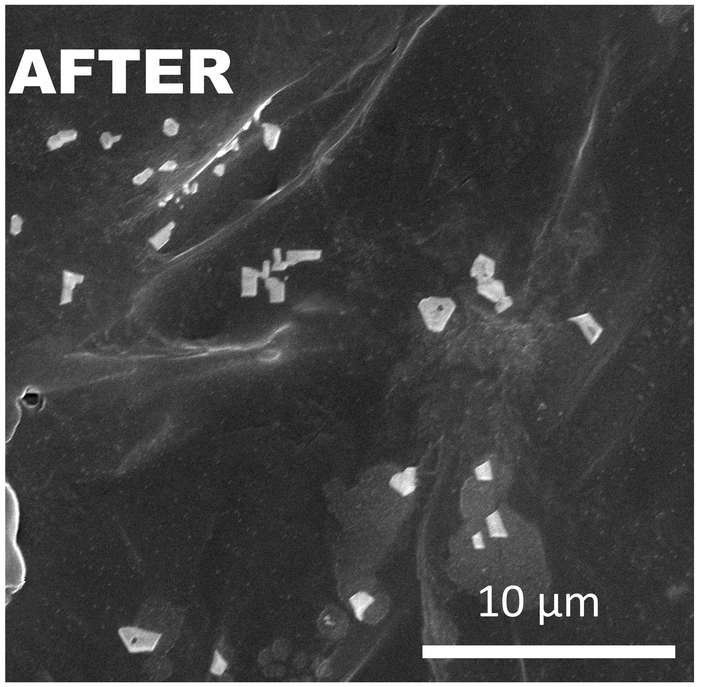
Enzymatic Gastric Digestion

Changes the surface texture of microplastics









Study 1: Cellulose Nanocrystals

Ede JD, Ong KJ, Mulenos MR, Pradhan S, Gibb M, Sayes CM*, Shatkin JA. Physical, chemical, and toxicological characterization of sulfated cellulose nanocrystals for food-related applications using in vivo and in vitro strategies. Toxicol Res (Camb). 2020 Dec 3;9(6):808-822.

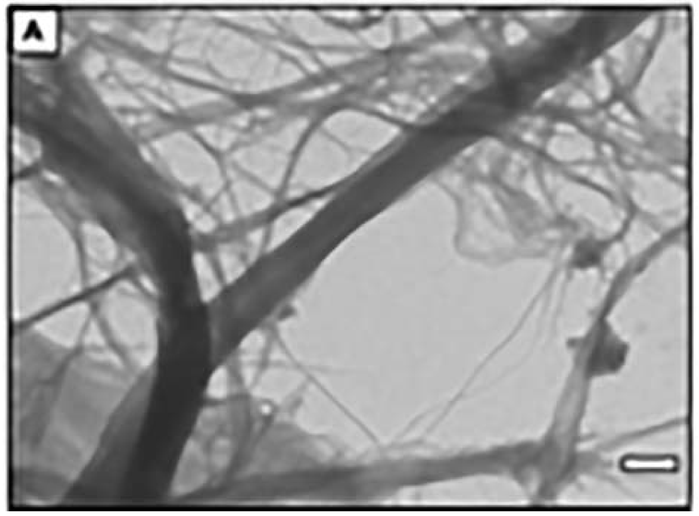
Pradhan SH, Mulenos MR, Steele LR, Gibb M, Ede JD, Ong KJ, Shatkin JA, Sayes CM*. Physical, chemical, and toxicological characterization of fibrillated forms of cellulose using an in vitro gastrointestinal digestion and co-culture model. Toxicol Res. 2020 May 20;9(3):290-301.

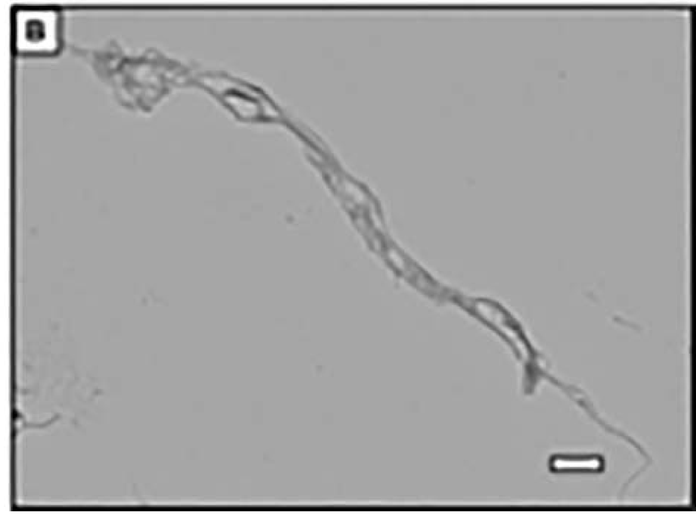
Ong KJ, Ede JD, Pomeroy-Carter CA, Sayes CM, Mulenos MR, Shatkin JA*. A 90-day dietary study with fibrillated cellulose in Sprague-Dawley rats. Toxicol Rep. 2020 Jan 20;7:174-182.

Mulenos MR, Zechmann B, Sayes CM*. Sample preparation utilizing sputter coating increases contrast of cellulose nanocrystals in the transmission electron microscope. Microscopy (Oxf). 2019 Dec 3;68(6):471-474.

Cellulose Nanocrystals
Simulated Gastric Fluid Digestion

CNF

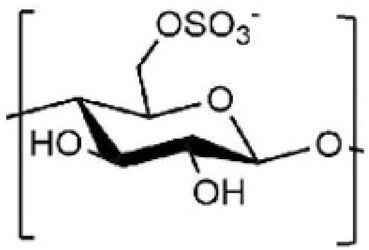


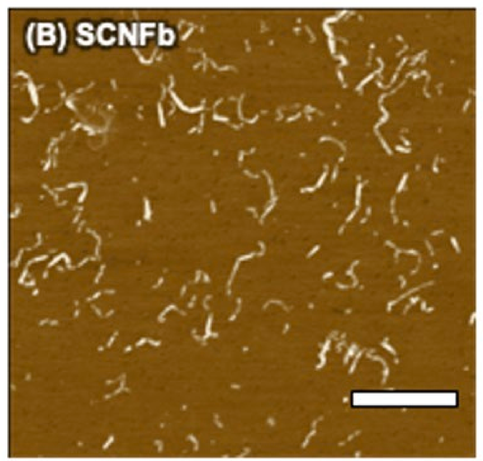


Sample ID	In ultrapure water			After simulated digestion		
	HDD (nm)	DI (unitless)	ZP (mV)	HDD (nm)	DI (unitless)	ZP (mV)
CNF	14,410	0.827	-33.0	977	0.750	-25.2
Sulfonated CNF	846	0.965	-61.2	436	0.453	-39.1
TEMPO oxidized CNF	1,448	0.980	-60.9	522	0.526	-38.8

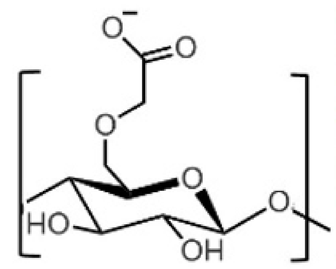
TEMPO = a method to oxidize cellulose using 2,2,6,6-tetramethylpiperidine-1-oxyl radical

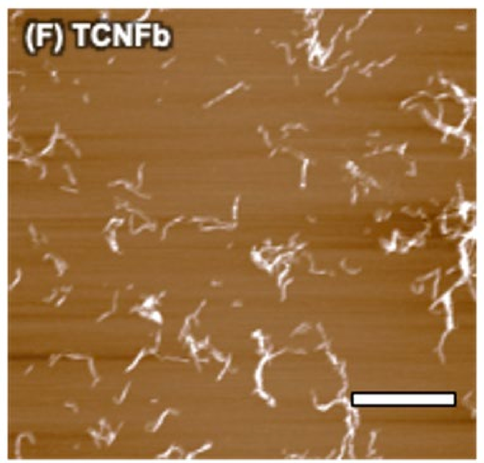
Sulfonate (SCNF)





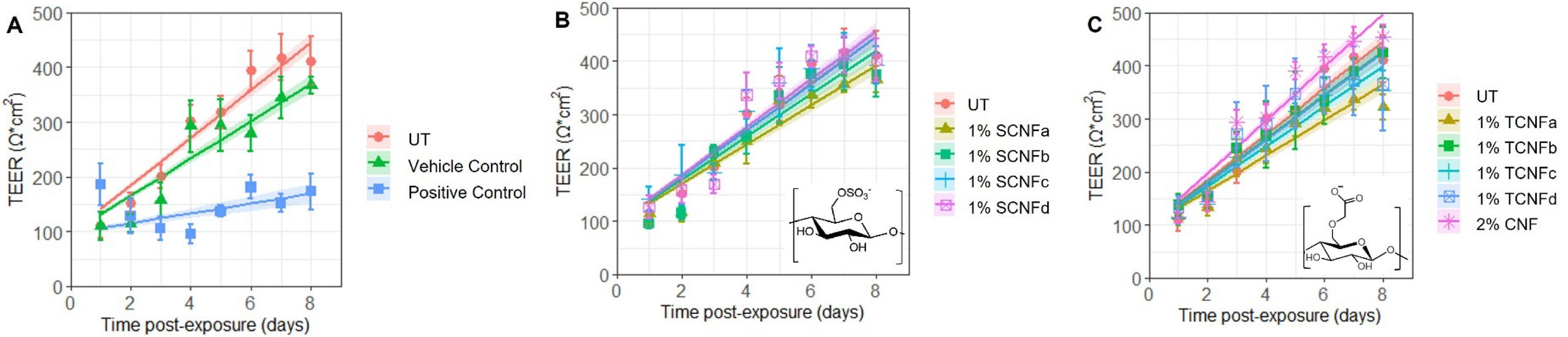
TEMPO (TCNF)





Gut Cell Model Barrier Integrity

Effects of digested functionalized cellulose on co-cultured gastrointestinal model barrier integrity as measured by TEER



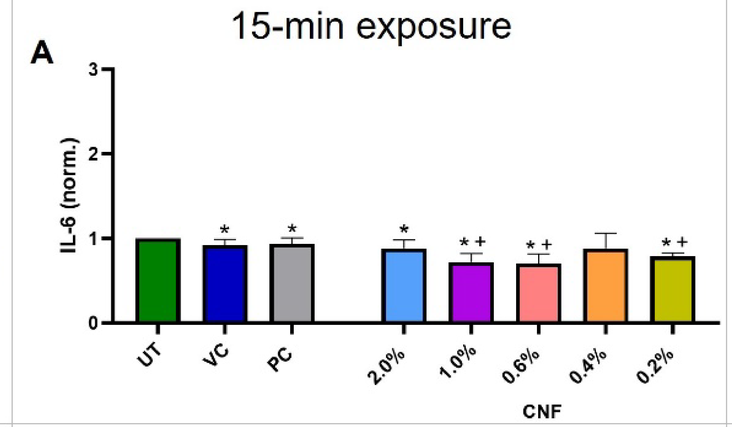
Takeaways:

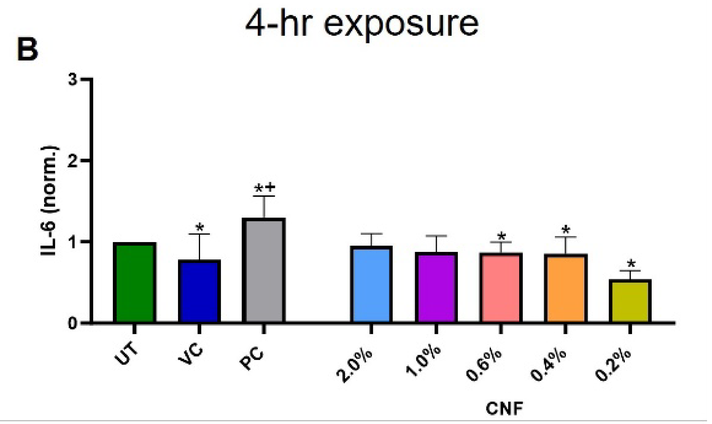
- Decreased resistance is an indication of decreased barrier integrity
- The digested functionalized cellulose materials produced similar resistivity plots
- None of the celluloses used in this study induced detrimental effects in the gastrointestinal cell model used in this study
- The positive control material used in this study was rotenone and it induced the least resistance

Pro-inflammatory response

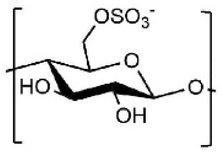
via interleukin 6 (IL-6) expression in gut cells

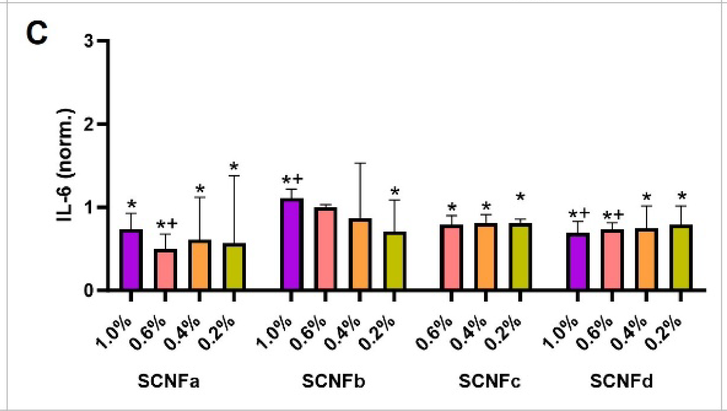
Control Groups and Unmodified CNF

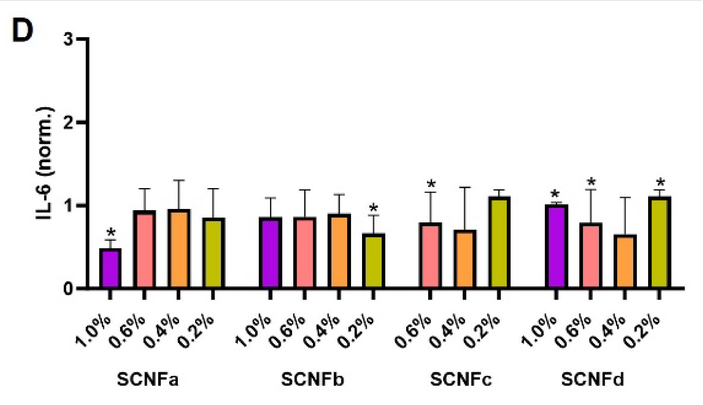




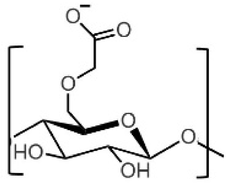
Sulfonate (SCNF)

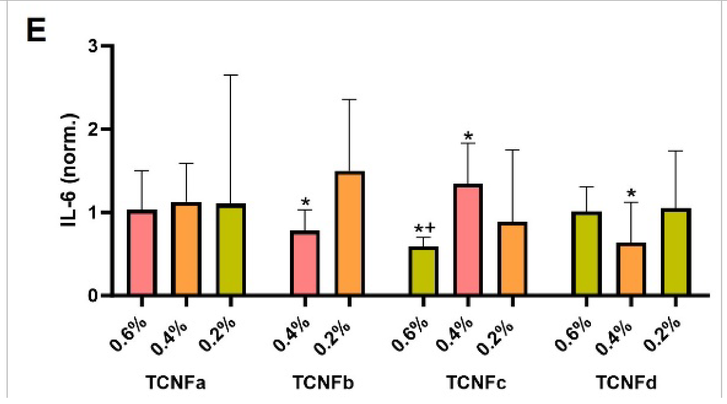


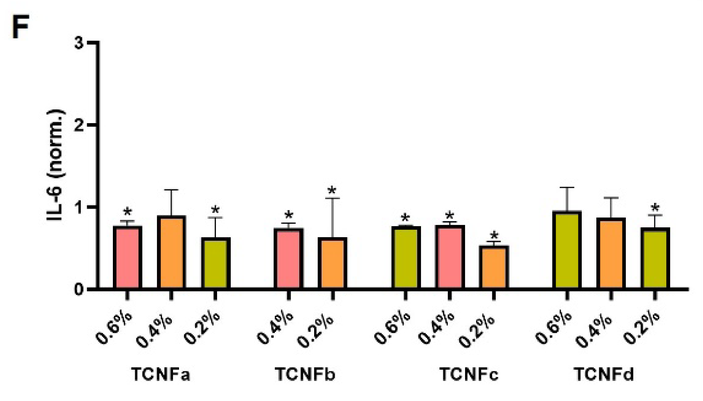




TEMPO (TCNF)







Takeaways:

- Very little cytotoxicity is observed
- Inflammatory response is dose-dependant
- IL-6 diminishes over time

Study 2: Disinfection Byproducts

Jiaqi Liu, Matthew Gibb, Sahar H. Pradhan, Christie M. Sayes. (2022). Synergistic Cytotoxicity of Bromine-Containing Disinfection Byproducts (DBPs) against Human Intestinal and Neuronal Cells. Chemosphere 287(1):131794.

Liu J, Sayes CM, Sharma VK, Li Y, Zhang X*. Addition of lemon before boiling chlorinated tap water: A strategy to control halogenated disinfection byproducts. Chemosphere. 2021 Jan;263:127954.

Liu J, Li Y, Jiang J, Zhang X, Sharma VK, Sayes CM*. Effects of ascorbate and carbonate on the conversion and developmental toxicity of halogenated disinfection byproducts during boiling of tap water. Chemosphere. 2020 Sep;254:126890.

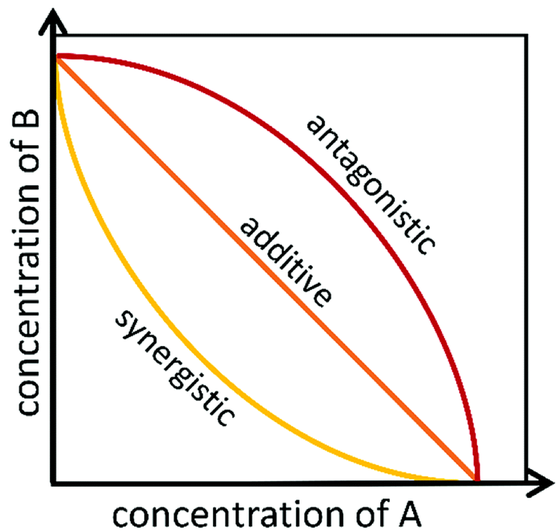
Liu J, Olson C, Qiu N, Sayes CM*. Differential Cytotoxicity of Haloaromatic Disinfection Byproducts and Lead Co-exposures against Human Intestinal and Neuronal Cells. Chem Res Toxicol. 2020 Sep 21;33(9):2401-2407.

Mixture Toxicity Background

and the case for using in vitro models for rapid screening assessment

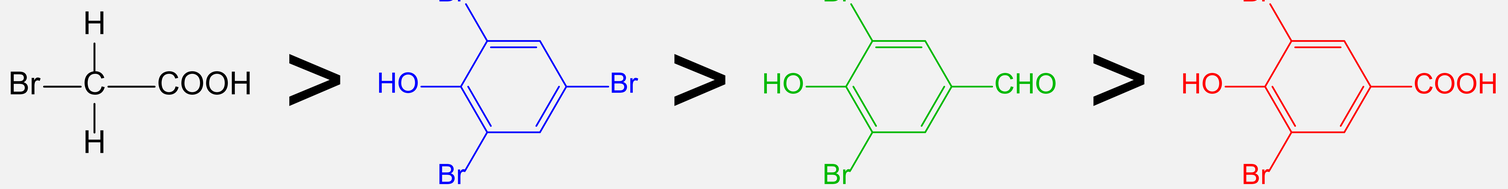
- Mixture toxicity is a function of a combination of chemicals to an individual
- Exposures need not be concurrent; the effect of one chemical may persist and only be expressed after a secondary later exposure to a second chemical
- Humans and ecological receptors are exposed to large numbers of complex mixtures everyday
- The combination of exposures from all sources form an individual's **exposome**
- Difference in individuals' exposomes have been shown to have significant impacts on **human health**

Type of Interaction	Toxic Effect Chemical A	Toxic Effect Chemical B	Combined Effect Chemicals A+B
Additivity	20 %	30 %	50 %
Antagonism	20 %	30 %	5 %
Potentiation	0 %	20 %	50 %
Synergism	5 %	10 %	100 %



There are no standards established for the study of exposures to mixtures related to drinking water hazards

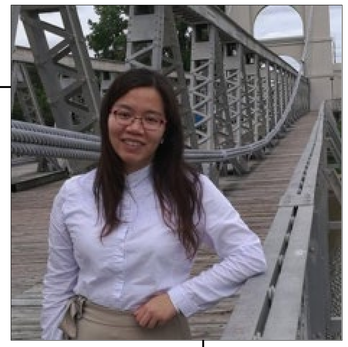
Toxicity ranking (based on LC_{50}):



Regulated | **Not Regulated**

But the mixtures produce different rankings

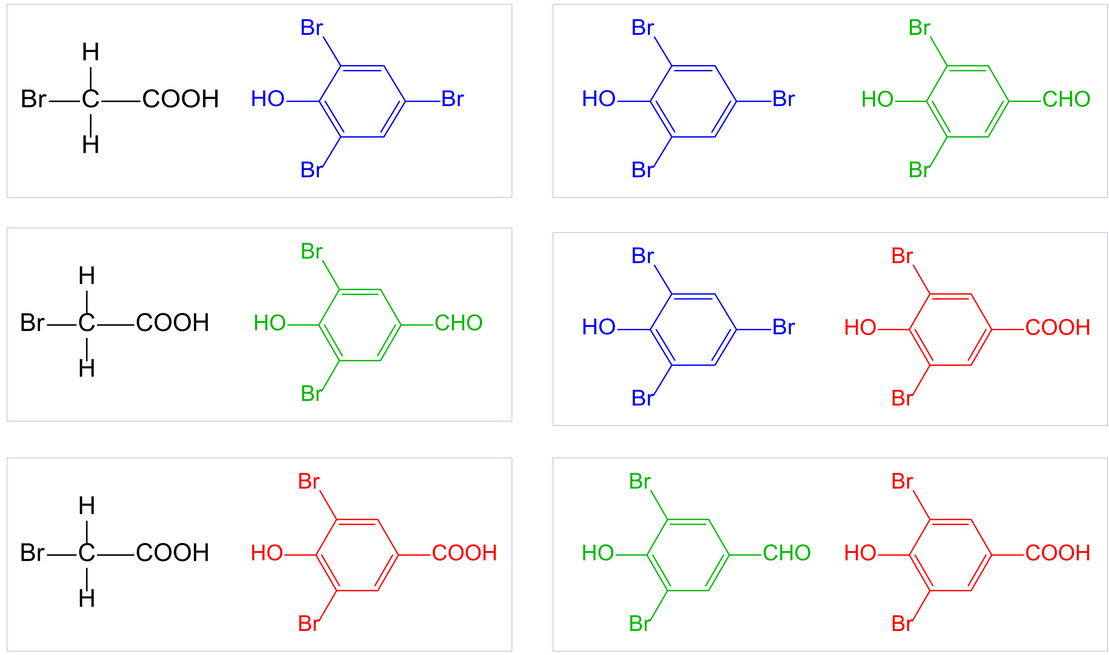
Dr. Vicky Liu



Differential Cytotoxic Effects

Synergism of Bromo-DBPs Mixtures

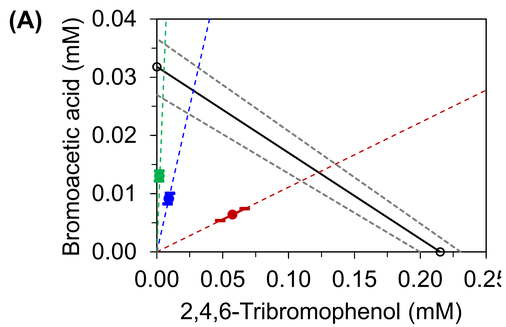
Six (6) tests for four bromo-DBPs

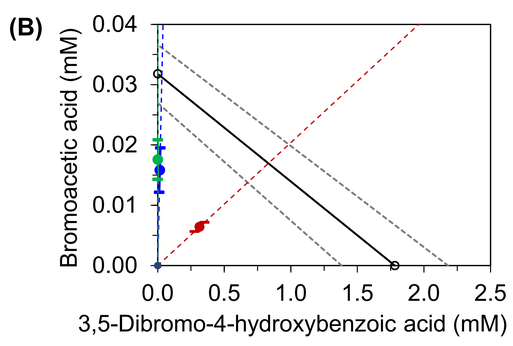


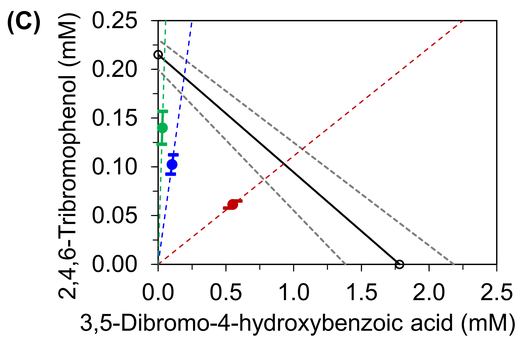
Combinatorial Effect of Bromo-DBPs Against Human Gut Model

Bromoacetic acid (BAA); 2,4,6-tribromophenol (TBP); and 3,5-dibromo-4-hydroxybenzoic acid (DBHBAC) produced synergistic effects, but each of these three DBPs had an additive effect with 3,5-dibromo-4-hydroxybenzaldehyde (DBHBAD)

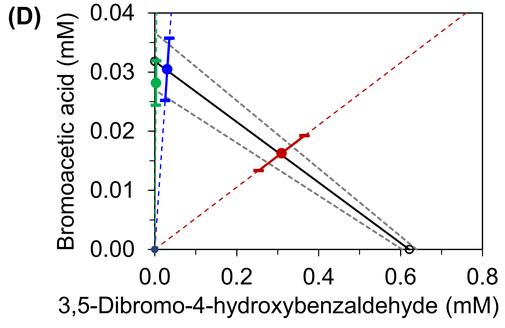
Synergism

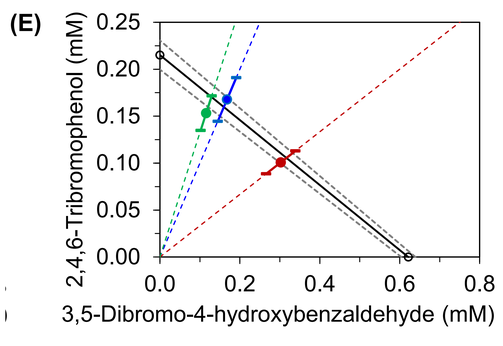


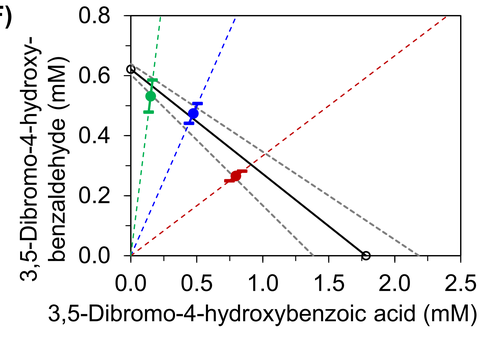


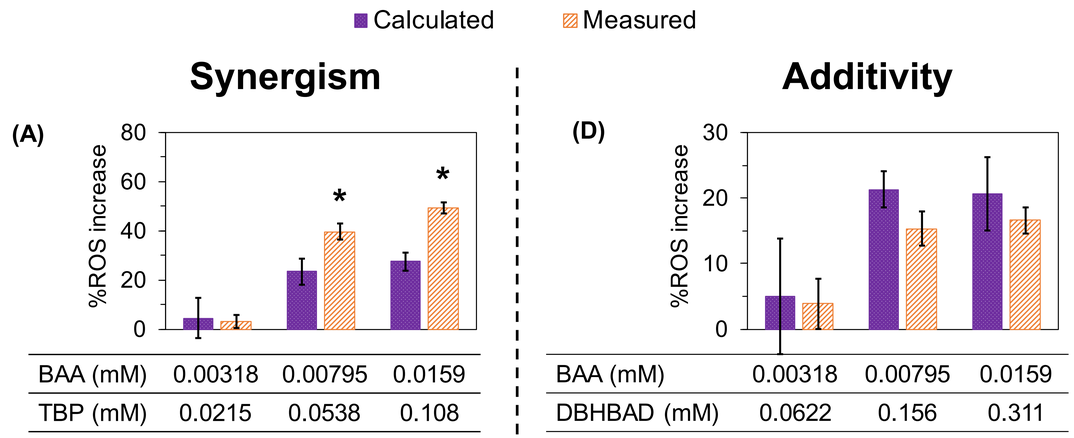


Additivity



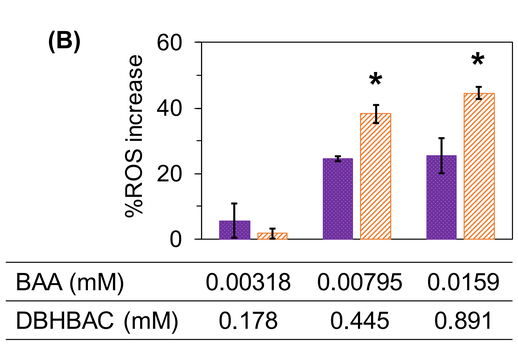




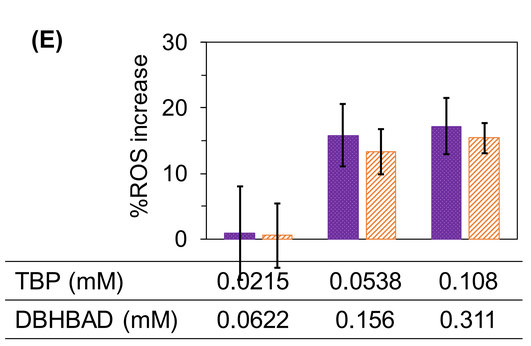


BAA (mM)	0.00318	0.00795	0.0159
TBP (mM)	0.0215	0.0538	0.108

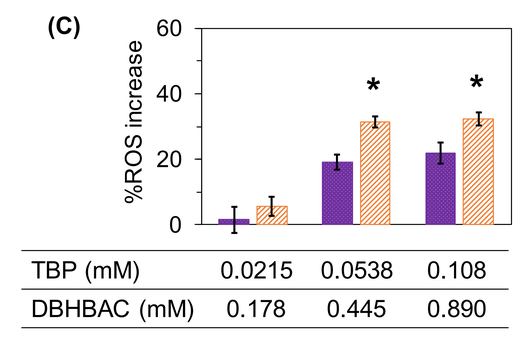
BAA (mM)	0.00318	0.00795	0.0159
DBHBAD (mM)	0.0622	0.156	0.311



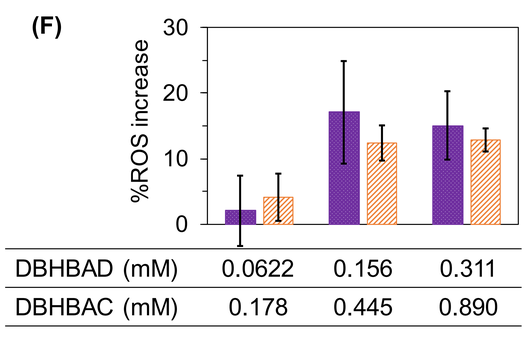
BAA (mM)	0.00318	0.00795	0.0159
DBHBAC (mM)	0.178	0.445	0.891



TBP (mM)	0.0215	0.0538	0.108
DBHBAD (mM)	0.0622	0.156	0.311



TBP (mM)	0.0215	0.0538	0.108
DBHBAC (mM)	0.178	0.445	0.890



DBHBAD (mM)	0.0622	0.156	0.311
DBHBAC (mM)	0.178	0.445	0.890

Study 3: Glyphosate Mixtures

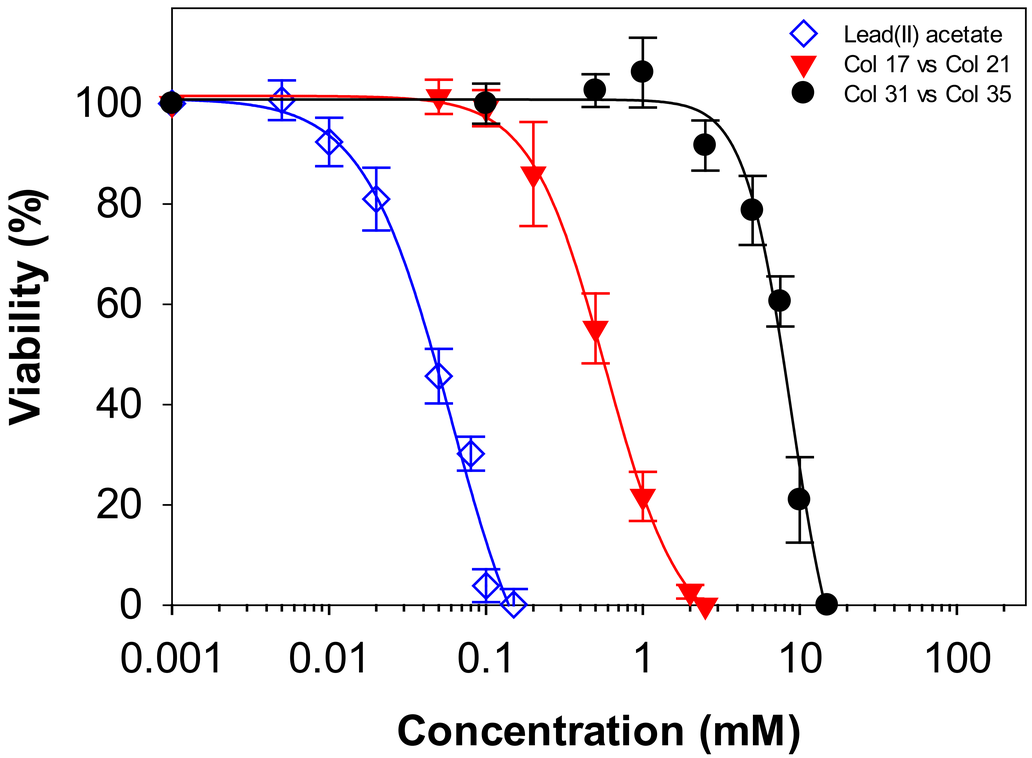
Kramer AT, Stevens MD, Norton J, Coogan MA, and Sayes CM. (2022). Developmental effects of zebrafish (Danio rerio) embryos after exposure to glyphosate and lead acetate mixtures. Toxicology In Vitro (under review).

Pradhan SH, Cruz G, Sayes CM*. (2022). Impact of mitochondria dysfunction neuronal cell death. Toxicology (under review).

Collom C, Pradhan SH, Liu JY, Liu J, Sharma V, Sayes CM. Toxicity of binary mixtures of copper, lead, and glyphosate on neuronal cells. Journal of Hazardous Materials Advances. 2023 Aug 1;11:100355.

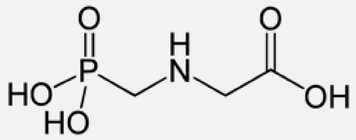
Dose-response curves related to glyphosate, copper nitrate, and lead acetate

using our gut in vitro model

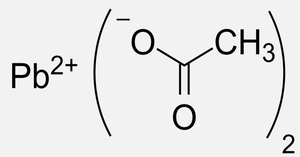


Undergraduate Research Project

Glyphosate



Lead acetate



GLY Exposure

PbAc Exposure

Post-exposure timepoints = 3, 4, 5, & 6 DPF

Zebrafish Embryo (ZFE)

LC_{50} values:

Lead (II) acetate = 0.0460 ± 0.0028 mM

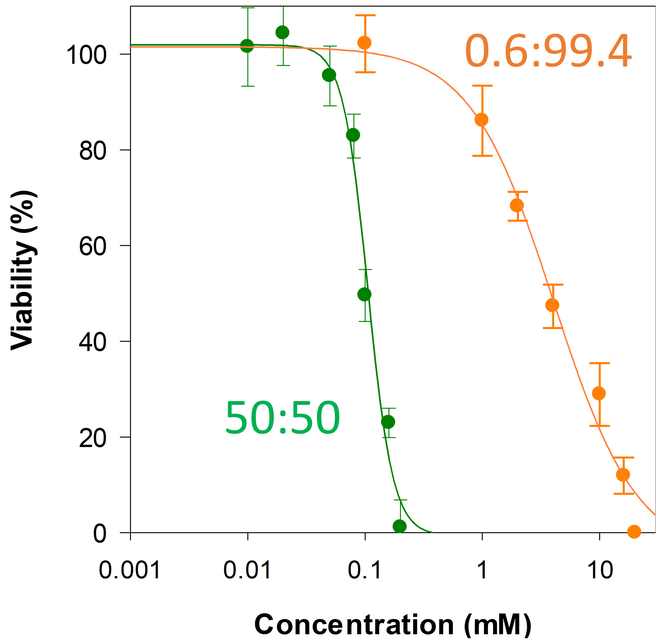
Copper (II) nitrate = 0.542 ± 0.044 mM

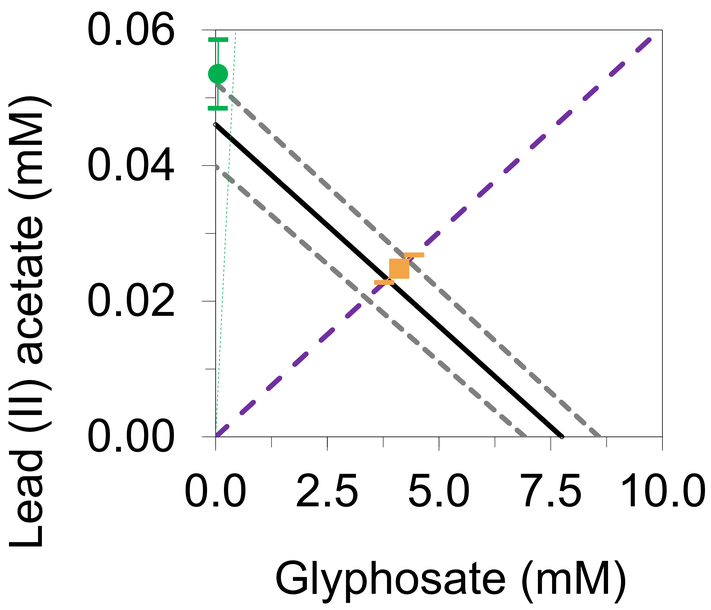
Glyphosate = 7.75 ± 0.64 mM

Cytotoxicity was measured 24 hours after inoculation

- Lead acetate is more cytotoxic than copper nitrate by an order of magnitude
- Glyphosate is less cytotoxic than lead acetate by over two order of magnitudes and less cytotoxic than copper by over an order of magnitude

Summary of Glyphosate Mixture Results





Mixture		Tested fraction (%) [a]		LC_{50} (95% confidence limit) (mM)		Interaction	Interact. index
Chemical$_1$	*Chemical$_2$*	*P_1*	*P_2*	*Predicted* [b]	*Measured*		
Copper	Lead	50	50	0.0848 (0.0732−0.0965)	0.143 (0.132−0.153) [c]	Antagonism	1.68
		92	8	0.291 (0.245−0.377)	0.623 (0.562−0.685) [c]	Antagonism	2.14
Copper	Glyphosate	50	50	1.01 (0.834−1.19)	1.61 (1.47−1.75) [c]	Antagonism	1.59
		7	93	4.01 (3.42−4.59)	7.26 (6.41−8.12) [c]	Antagonism	1.81
Lead	Glyphosate	50	50	0.0915 (0.0793−0.104)	0.107 (0.0969−0.117) [d]	Additivity	1.17
		0.6	99.4	3.88 (3.39−4.33)	4.13 (3.79−4.47) [d]	Additivity	1.07

Takeaways

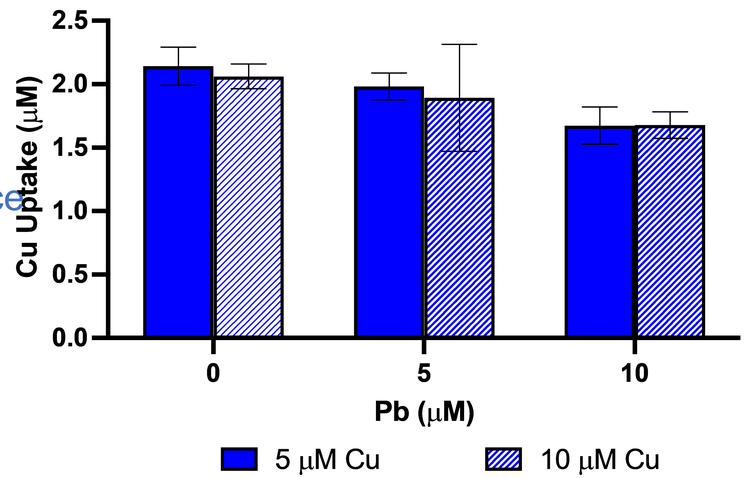
- The copper + lead mixtures and the copper + GLY mixtures show antagonistic responses
  - In both mixtures, equimolar is more toxic than equipotent
- The lead + glyphosate mixture shows additive responses
  - In this case, equipotent is more toxic than equimolar

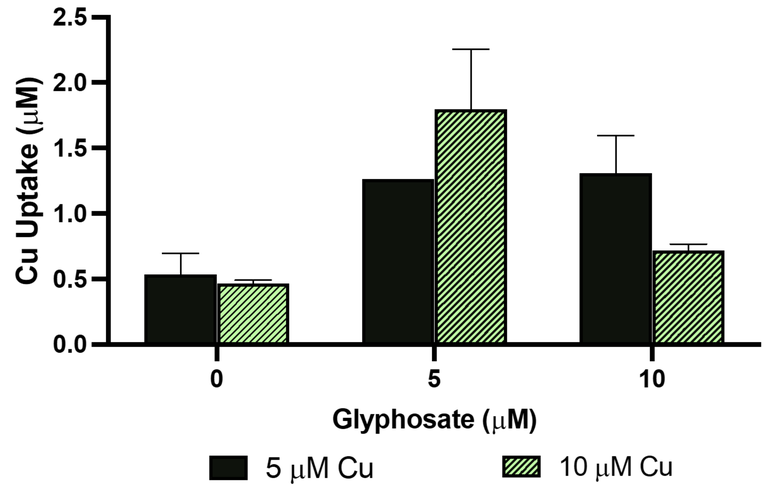
Mechanistic Analyses

Cellular Uptake of Contaminant May Be Driver for Observed Toxicological Response

Lead's effect on copper uptake:

- Cu uptake slightly decreases in the presence of Pb
- Uptake of Cu may not be dependent on concentration



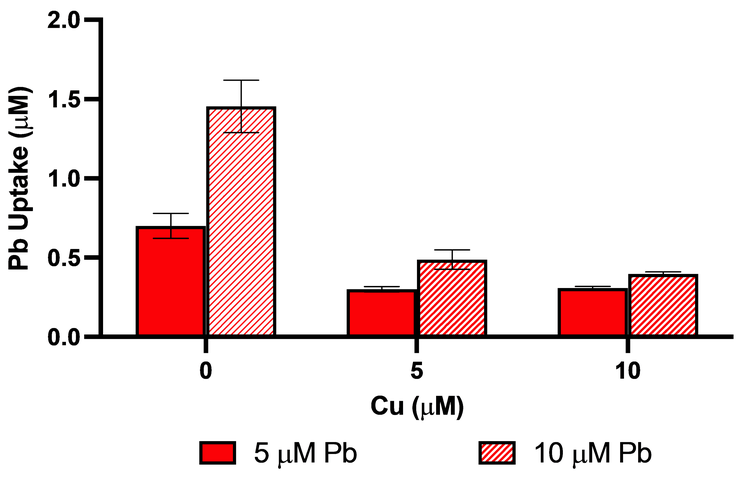


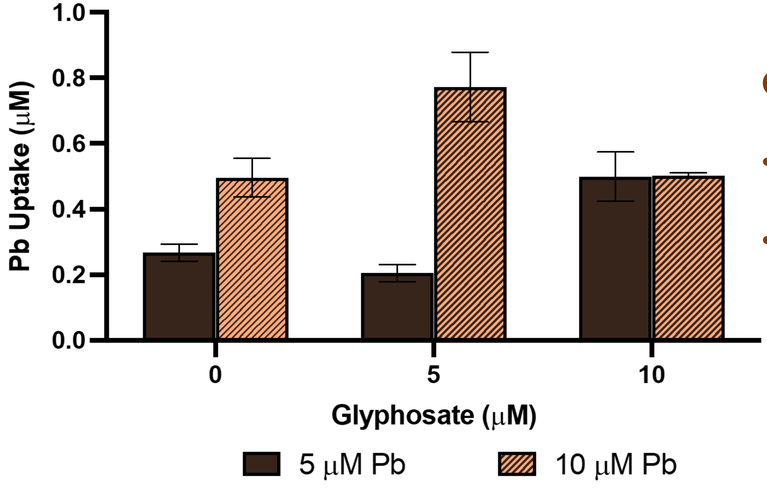
Glyphosate's effect on copper uptake:

- GLY increases Cu uptake
- Increase in GLY has no effect low Cu concentration
- Increase in GLY decreases Cu uptake in high concentration

Copper's effect on lead uptake:

- Cu reduces Pb uptake
- Increase in Cu concentration does not reduce Pb uptake





Glyphosate's effect on lead uptake:

- GLY increases Pb uptake
- Pb uptake is greatest at low GLY concentration with high Pb concentration

Conclusions

- Advanced materials are currently used in consumer and industrial processes
- There is a need to understand the underpinned mechanisms of altered metabolism after environmental exposures
    - **Simple and complex toxicological models can aid in understanding mechanistic analyses**
- Humans and ecological receptors are exposed to large numbers of complex mixtures everyday
    - Mixtures can induce synergistic, additive, or antagonistic responses when compared to single chemical exposures
- These studies may help establish a standard of toxicity studies for co-exposure testing of binary mixtures of metal and organic toxicants which is more representative of real-world exposure