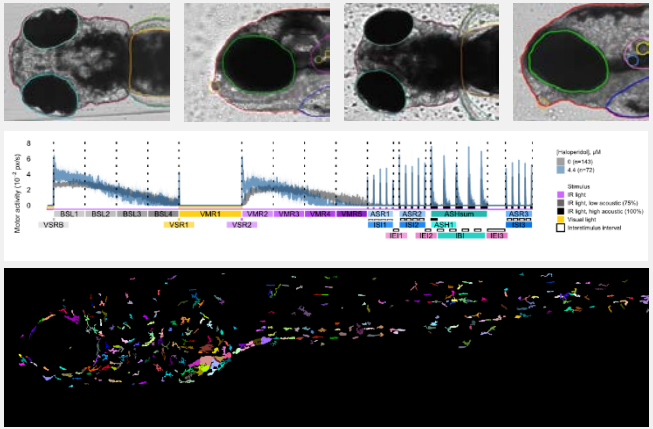
mTOX working group

1. Develop New Approach Methods

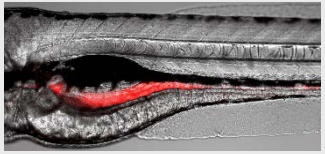
(DevTox, DNT, ANT, gut inflammation)

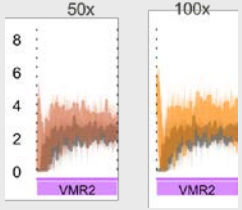


Identify toxic chemicals, mixtures & underlying mechanisms

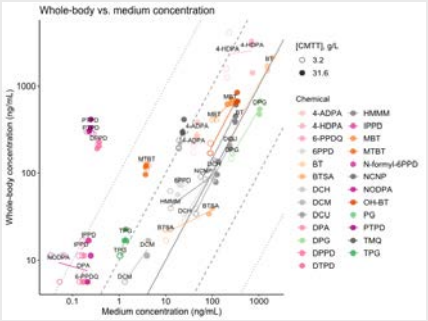
3. Identify risk drivers and modifying factors

Microbiome

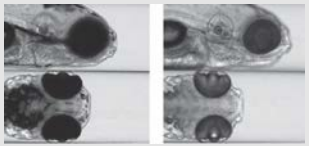


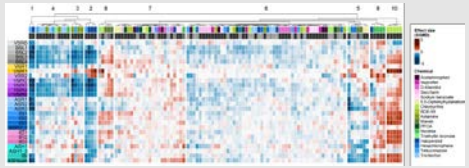


Complex mixtures

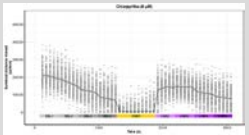


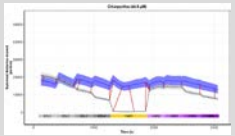
2. Discover mode of action

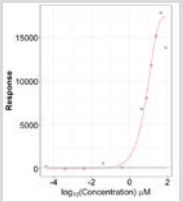




4. Translation to impact



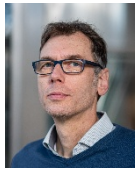




mTOX working group

PIs

T. Tal



S. Scholz

PIs

Postdocs

D. Leuthold

P. Opute

N. Herold

Postdocs

PhD students

S. Gutsfeld

C. Wray

R. Owen

E. Nicolay

V. Saalmann

B. Chen

E. Chukwu

G. Ajugwo

PhD students

Master's, visiting PhD, technicians

J. Spath

J. Raab

T. Silvestrini

N. Schweiger

T. Jonat

Master's, visiting PhD, technicians

mTOX working group
Afternoon session

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- Mechanism discovery
- Complex mixtures

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- Interplay with enteric nervous system

Multi-Behavioral Phenotyping in Early-Life-Stage Zebrafish

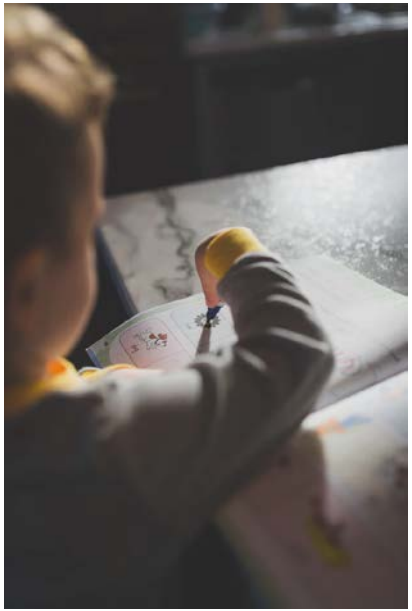
Dr. David Leuthold

1 October 2025

Leipzig | Germany

(Developmental) Neurotoxicity

A Persistent Data Gap in the Context of Expanding Chemical Use



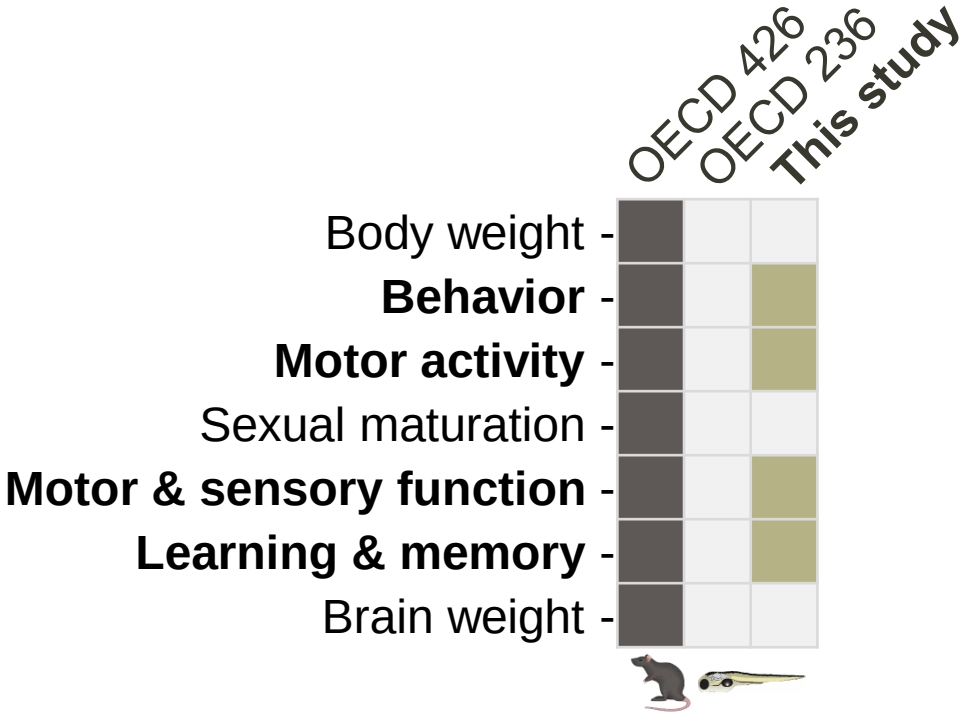
www.shutterstock.com

Current Testing Methods Cannot Keep Pace With Chemical Invention

Zebrafish as an Alternative Model

OECD No. 426:

Developmental Neurotoxicity Study

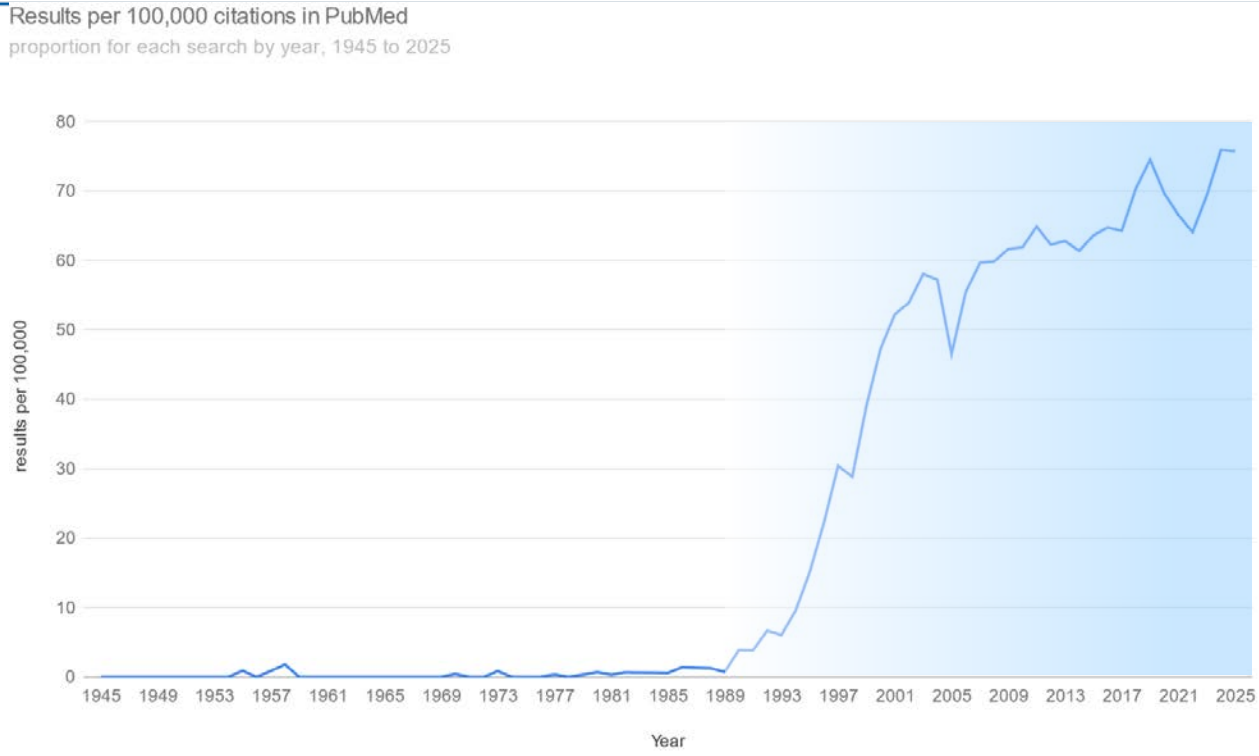


OECD No. 236:

Fish Embryo Acute Toxicity (FET) Test

Rise in Studies Utilizing Zebrafish Behavior

The Behavioral Repertoire of Zebrafish is Underutilized



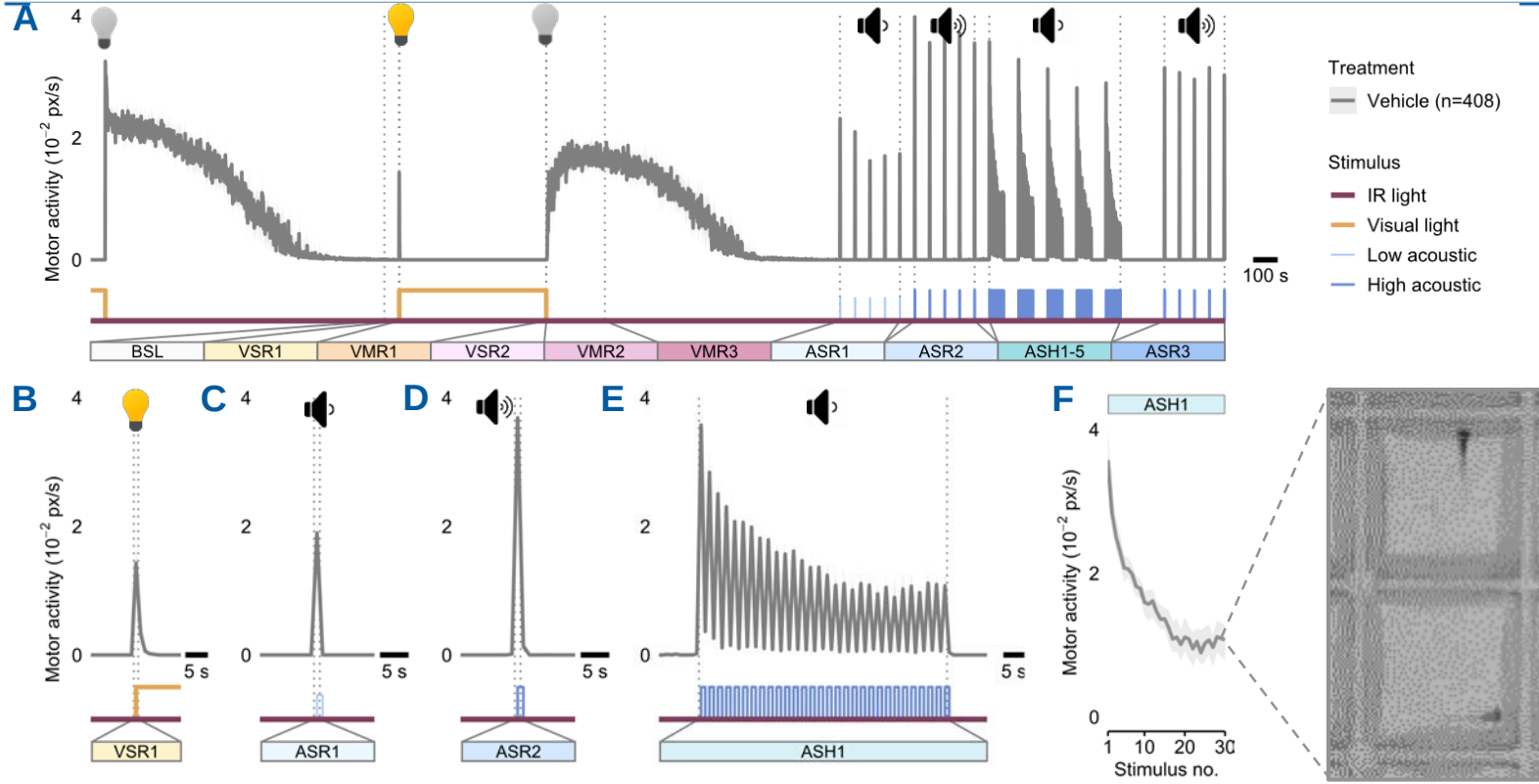
- **Behavior** reflects the **integrated output** of genetic, neural, and environmental influences.
- Neuronal circuit function is **not reflected in a single behavior**, but in diverse behavioral domains.
- *"With sufficiently **rich behavioral phenotyping** and a large sampling of compounds, it should be possible to **identify neuroactive compounds** that have desirable multitarget **mechanisms of action**."*

  Bruni et al., Nat Chem Biol, 2016

Sperr E. PubMed by Year [Internet]. 2016 [cited 2025/09/29]. Available from http://esperr.github.io/pubmed-by-year/

Expanding Phenotypic Space

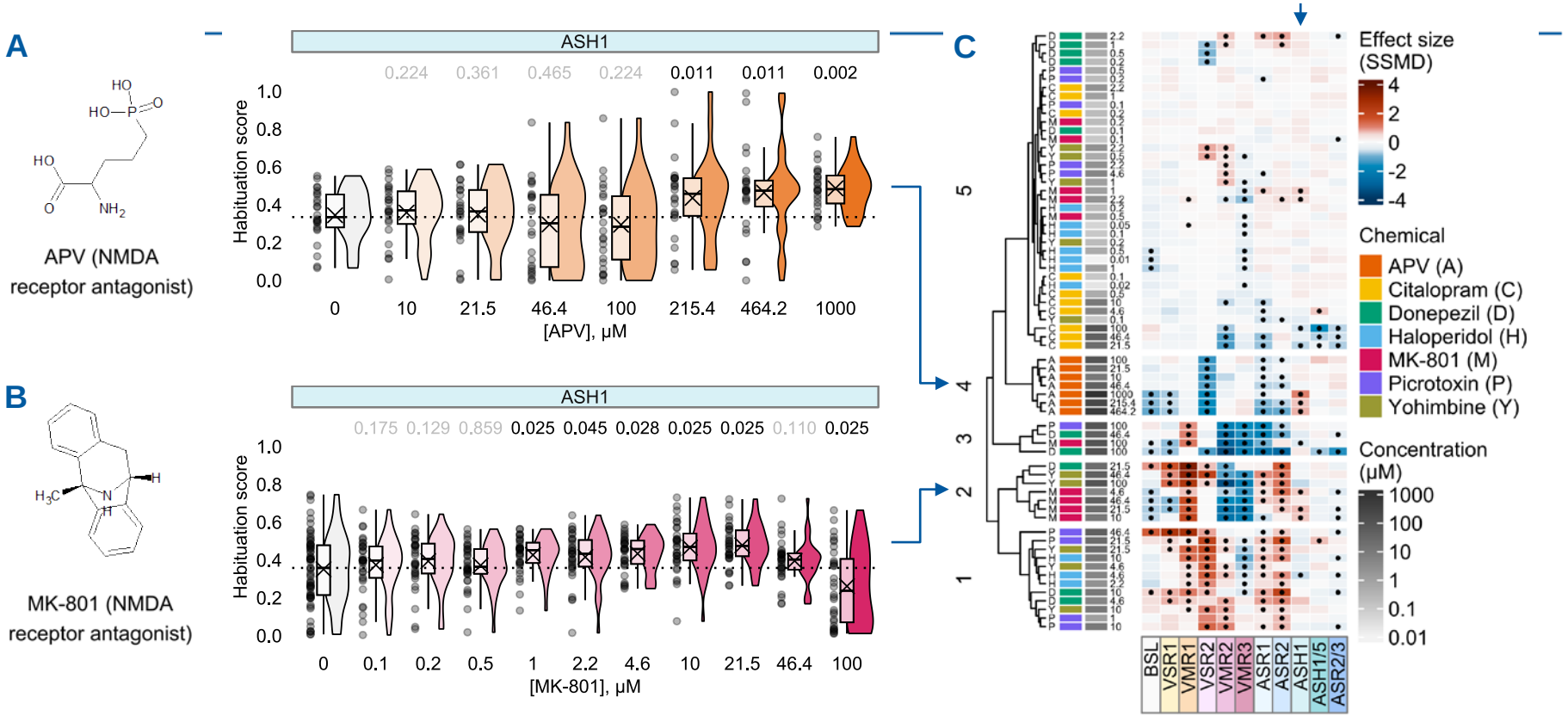
A Multi-Behavioral Phenotyping Approach to Quantify Visual & Acoustic Behaviors



BSL - baseline, VSR - visual startle response, VMR - visual motor response, ASR - acoustic startle response, ASH - acoustic startle habituation

Recapitulating Phenotypes in Pharmacological Space

Known Modulators of Habituation Learning Alter Mutliple Zebrafish Behaviors

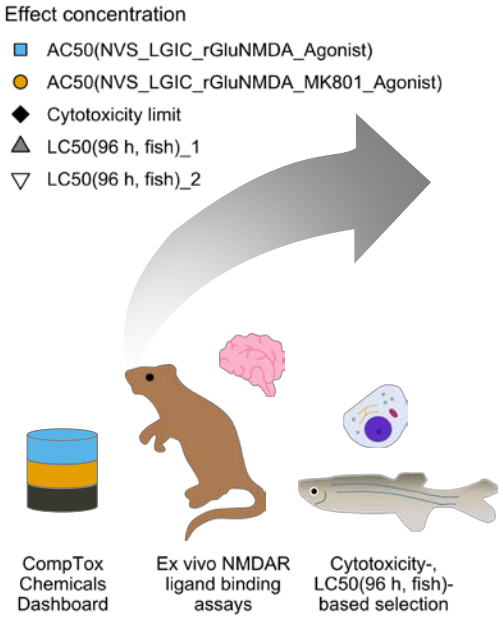


Leuthold et al. (2025), *Environ. Health Perspect.* DOI:10.1289/EHP16568

Integrating *Ex Vivo* & *In Vitro* Data to Validate *In Vivo* Targets

Targeted Screen for Chemicals that Affect Learning & Memory

A



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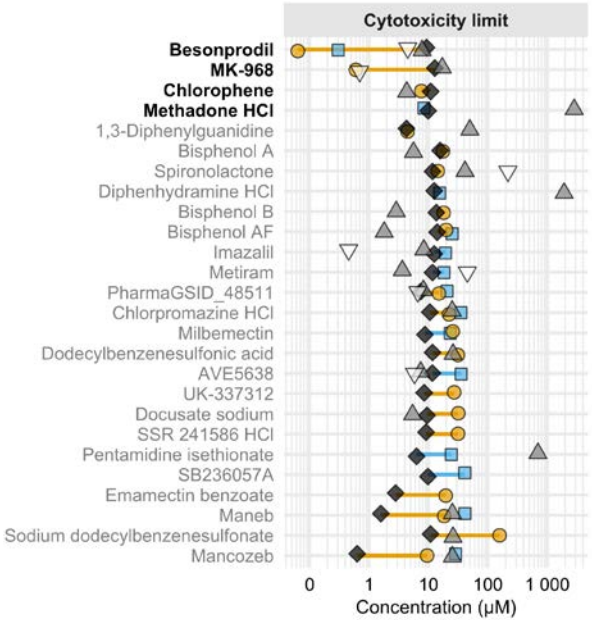
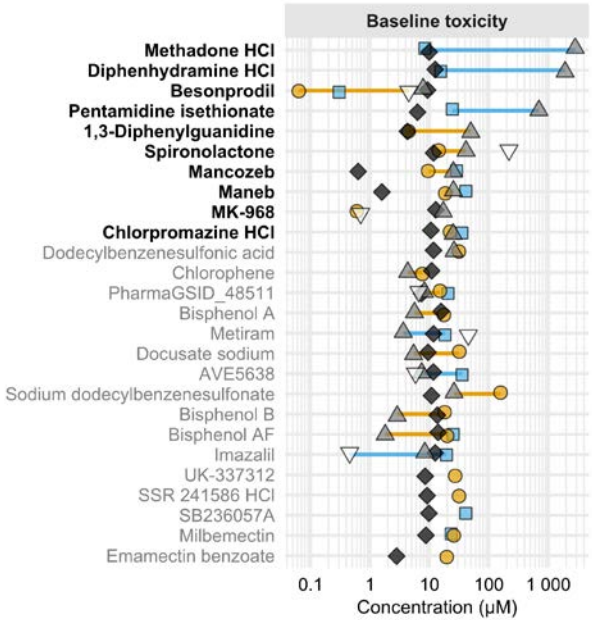


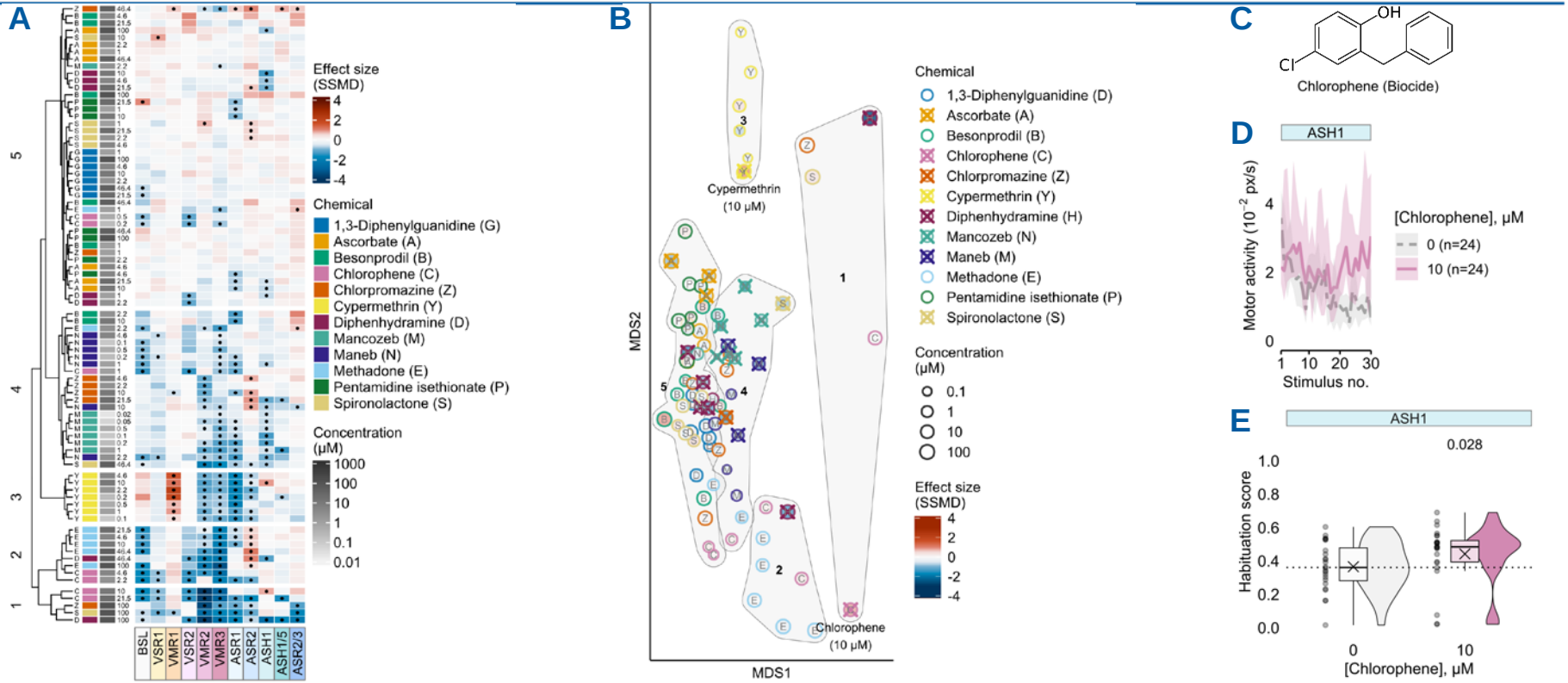
C



Leuthold et al. (2025), *Environ. Health Perspect.* DOI:10.1289/EHP16568

6 of 10 Compounds Impair Habituation in Zebrafish

The Biocide Chlorophene Disrupts Non-Associative Learning



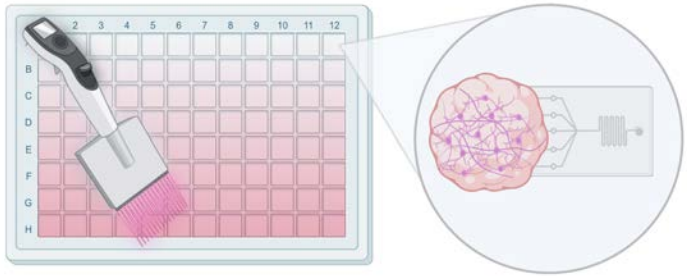
Leuthold et al. (2025), *Environ. Health Perspect.* DOI:10.1289/EHP16568

Unraveling Chlorophene‘s MOA: Across Species – Across Institutes

Chlorophene Does Not Directly Act on NMDARs & Alters Glutamatergic Signaling

UNIVERSITÄT LEIPZIG

IUF
LEIBNIZ-INSTITUT FÜR UMWELT-MEDIZINISCHE FORSCHUNG



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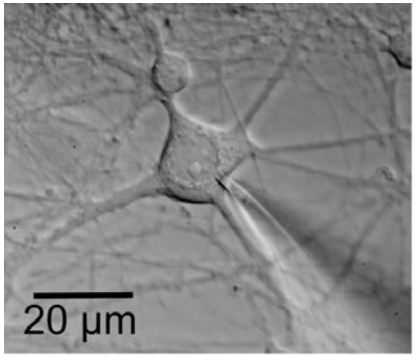

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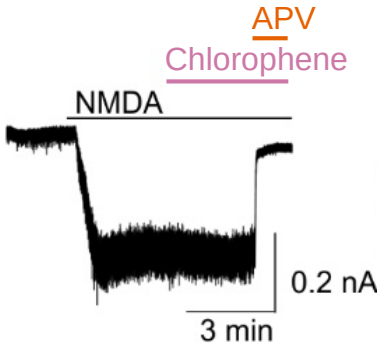

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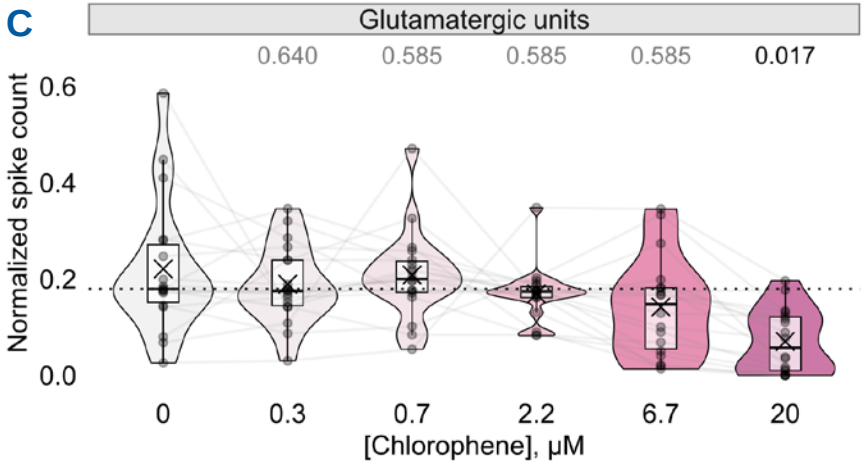
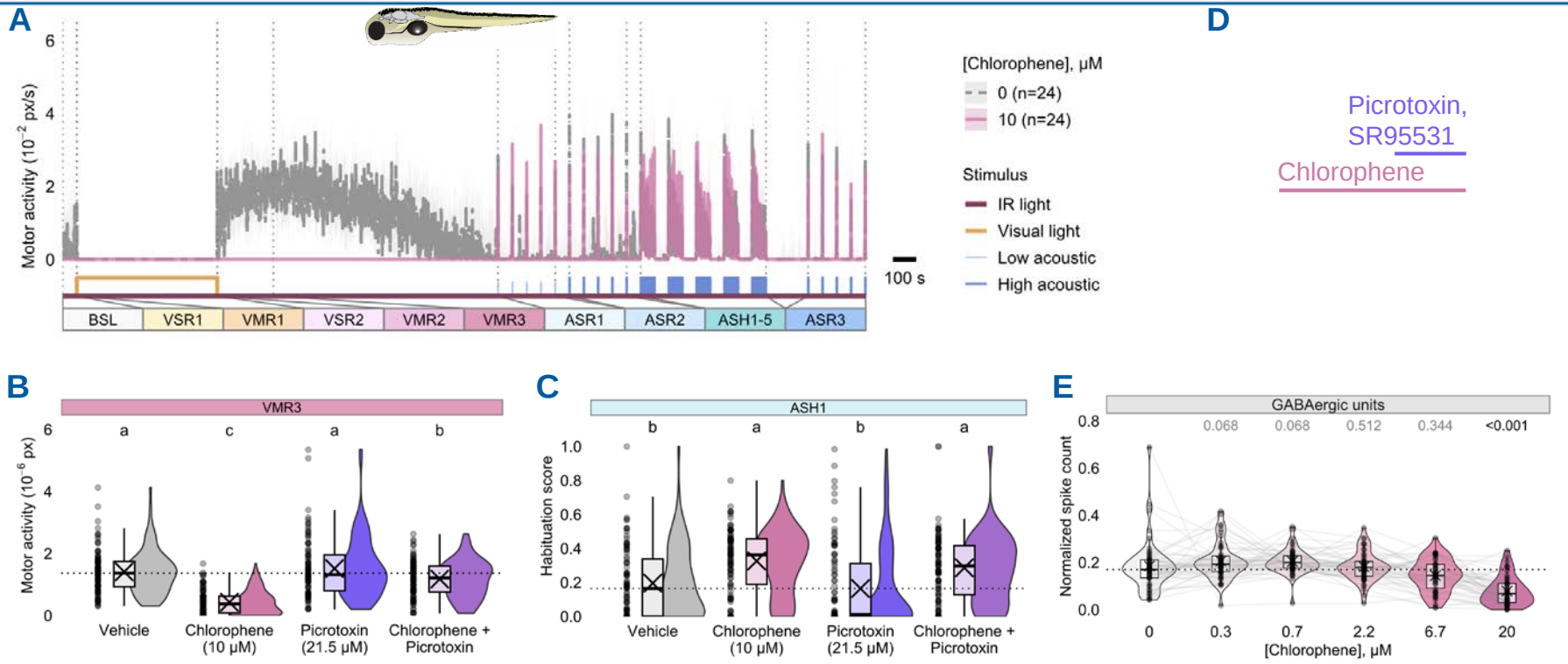


Leuthold et al. (2025), *Environ. Health Perspect.* DOI:10.1289/EHP16568

Paradoxical Excitation

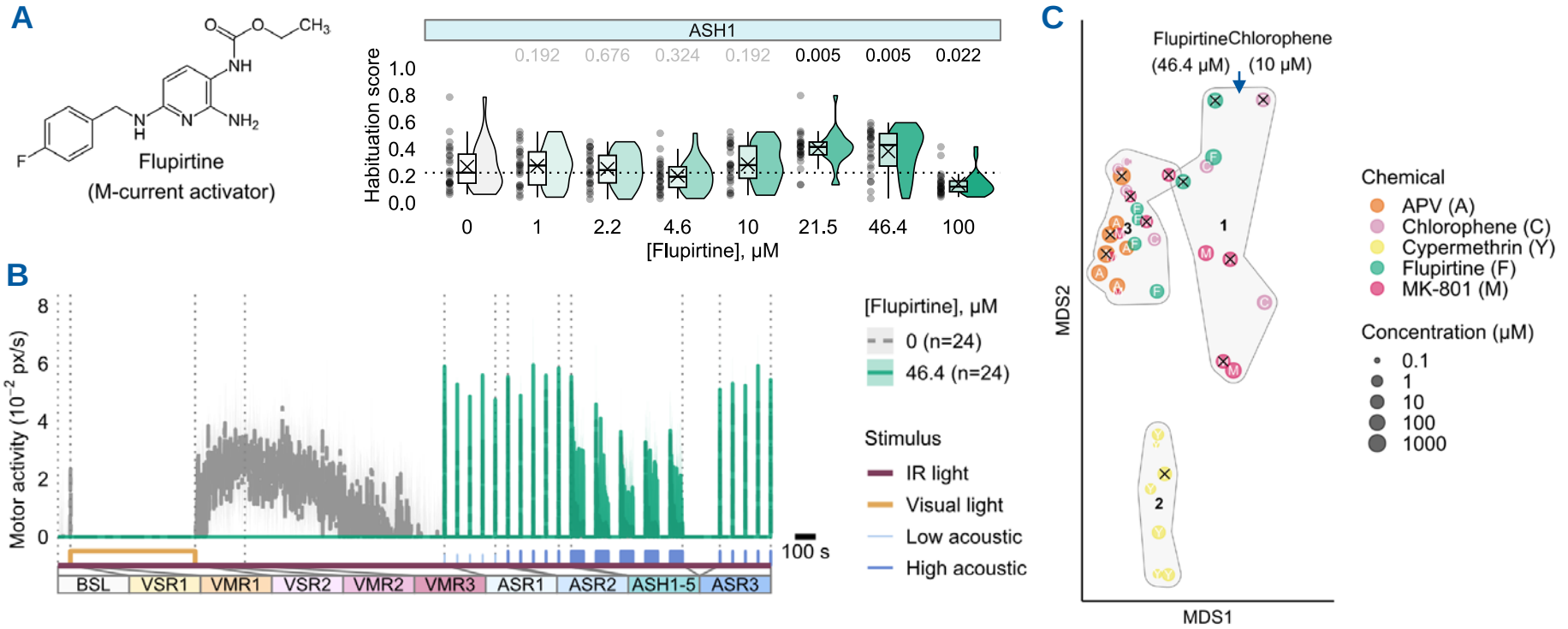
Chlorophene Acts on $GABA_A$ Receptors in Zebrafish, Mouse, and Human



Leuthold et al. (2025), *Environ. Health Perspect.* DOI:10.1289/EHP16568

Phenocopy Apporach Reveals MOA

Chlorophene Indirectly Acts on NMDARs Through Kv7 & $GABA_AR$ Activation



Leuthold et al. (2025), *Environ. Health Perspect.* DOI:10.1289/EHP16568

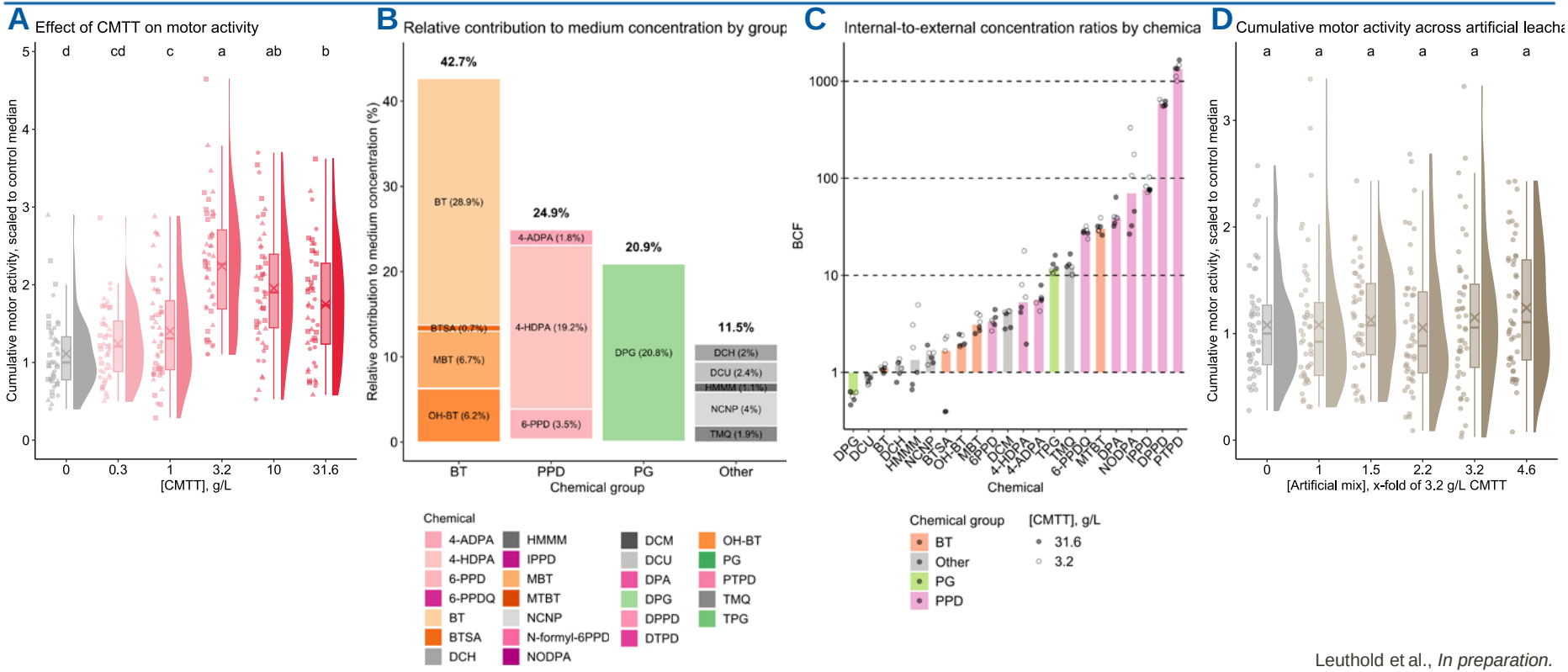
Conlusions

- A 3R-compliant alternative to rodent-based neurotoxicity guideline studies

- Identification and mechanistic elucidation of neurotoxic compounds:
  - Chlorophene, a biocide (cosmetics, disinfectants), inhibits visual behaviors & learning
  - Chlorophene is a functional $GABA_A$ receptor agonist, activates Kv7 channels, and indirectly blocks NMDA receptors
  - Chlorophene's mechanism of action is conserved in rodent and human models

Behavioral Profiling of Environmental Samples (ETOX + EAC)

Identification of Drivers & Mechanisms of Acute Tire Tread Leachate Toxicity

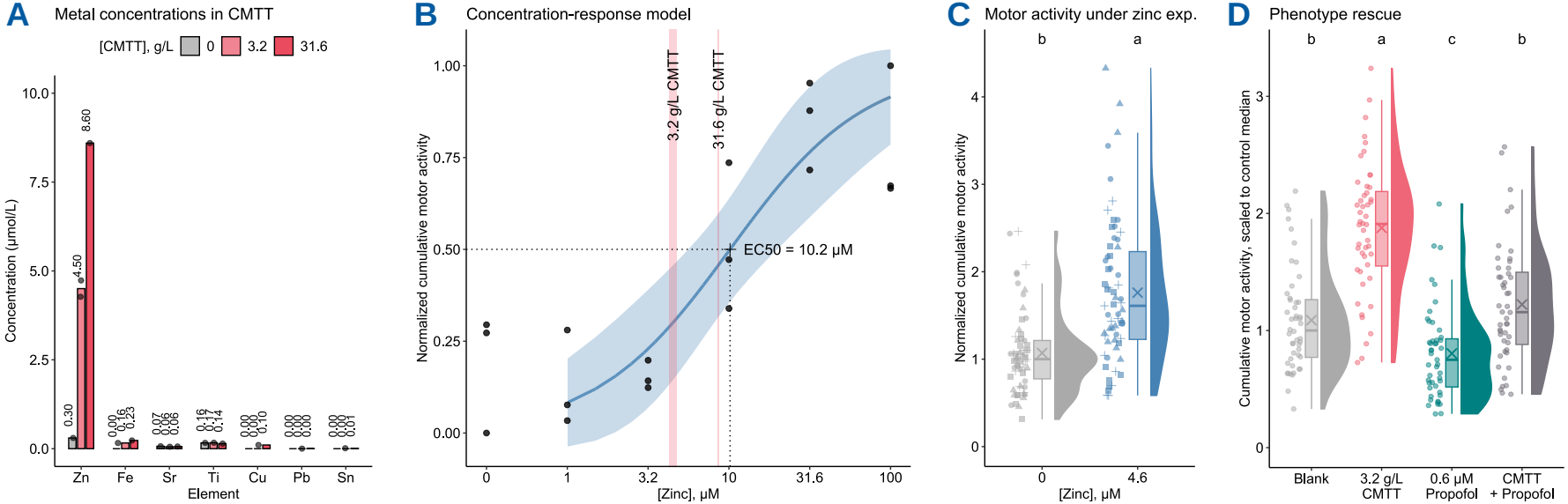


Leuthold et al., *In preparation.*

CMTT – Cryo-milled tire tread, BT – Benzothiazoles, PPD – Para-phenlyenediamines, PG – Phenylguanidines.

Behavioral Profiling of Environmental Samples (ETOX + EAC)

Identification of Drivers & Mechanisms of Acute Tire Tread Leachate Toxicity



Leuthold et al., *In preparation.*

CMTT – Cryo-milled tire tread.

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- Mechanism discovery
- Complex mixtures

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- Interplay with enteric nervous system

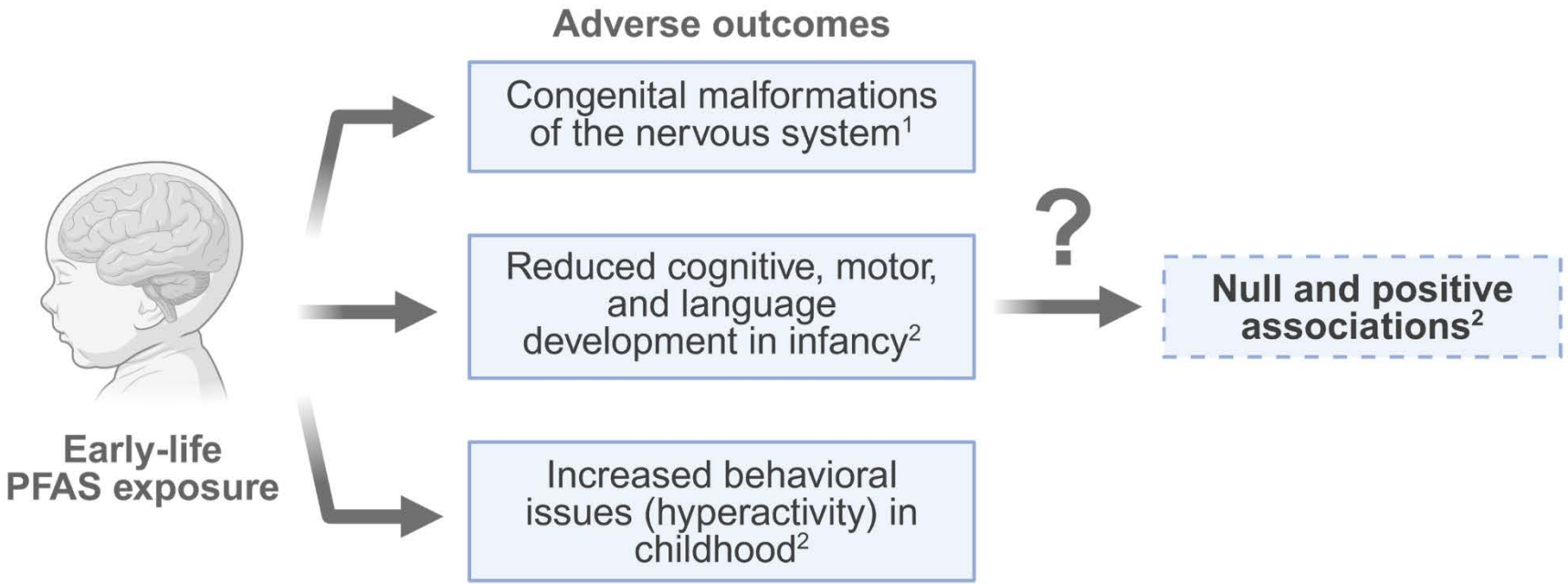
Identifying chemical drivers of mixture effects and unraveling molecular mechanisms:

A per- and polyfluoroalkyl substances (PFAS) case study

Renee Owen

Per- and polyfluoroalkyl substances (PFAS)

Conflicting epidemiological data on developmental neurotoxicity



[1]Säve-Söderbergh et al. Envir Int. 2025. [2]Ames et al. Curr Envir Health Rpt. 2025.

Chemical mixtures
Human-relevant PFAS mixture design

Sulfonic acid PFAS

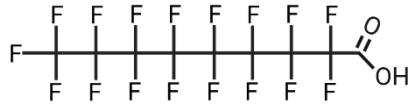
PFHxS

PFOS

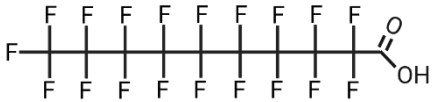
Carboxylic acid PFAS

PFOA

PFNA

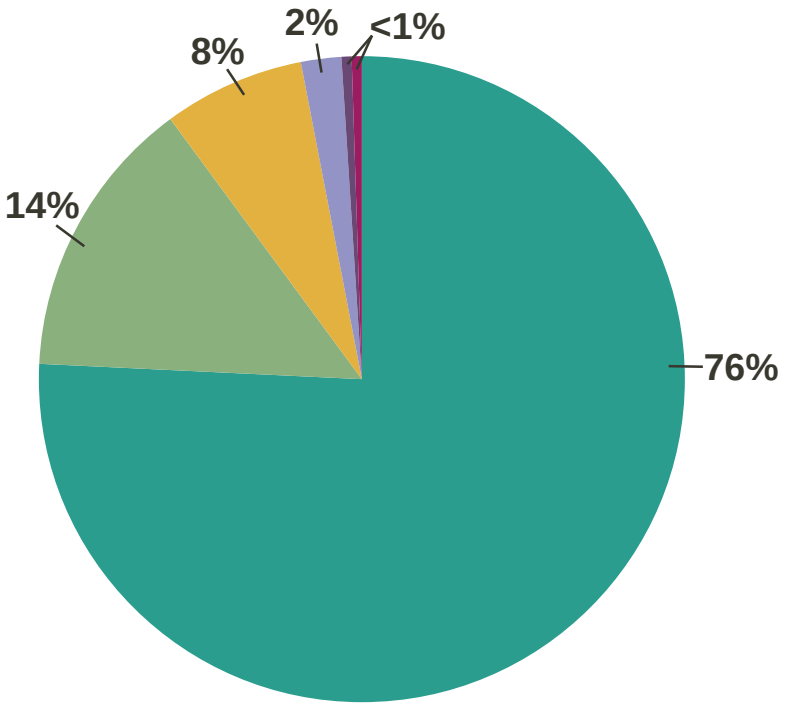


PFDA



PFUnDA

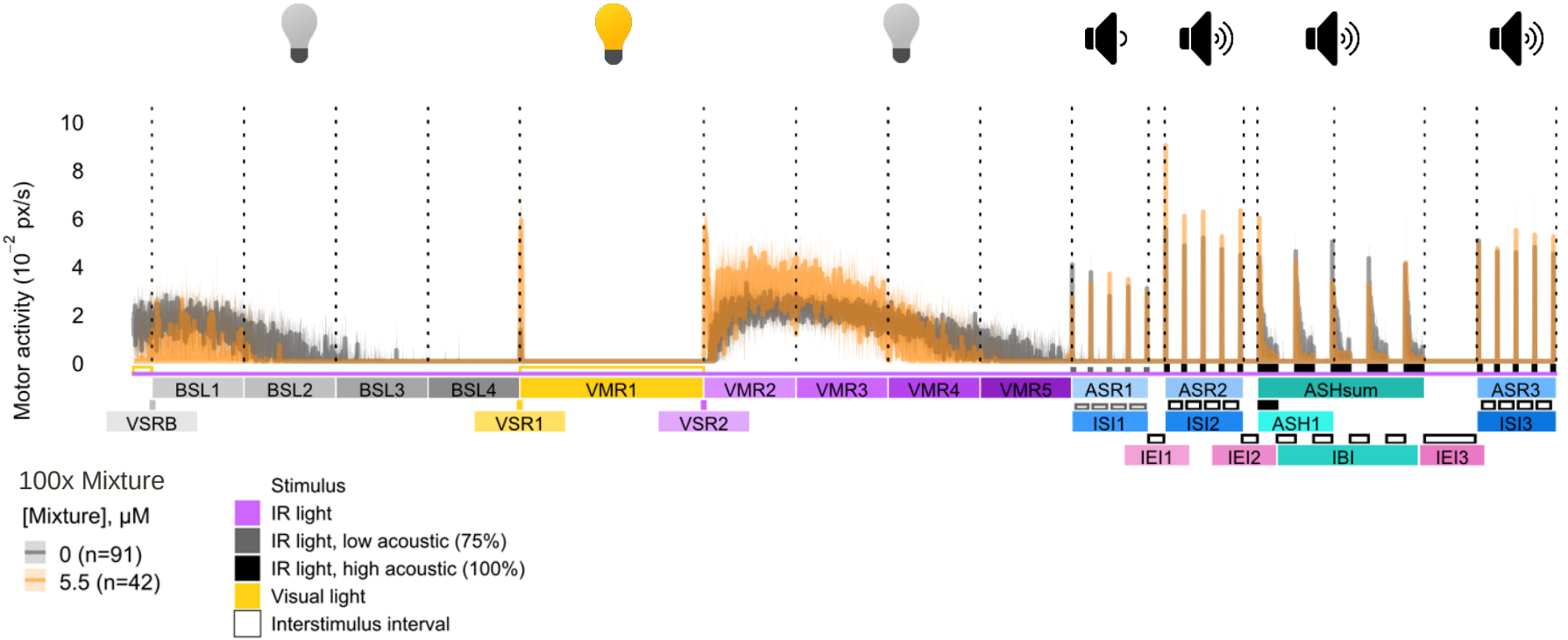
Mixture Composition



Dorte Herzke, Line Småstuen Haug, Oddvar Myhre (NIPH)

Battery of visual and acoustic stimuli

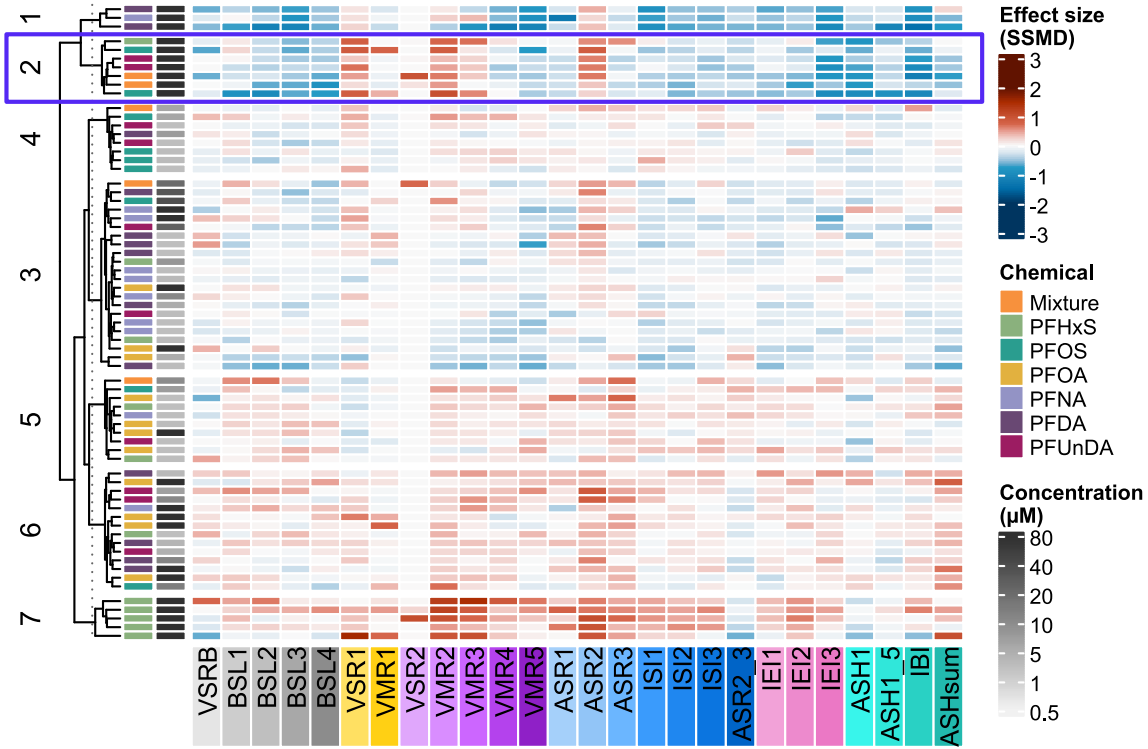
Visual and Acoustic Motor Response (VAMR) assay



BSL (Baseline); VSR (Visual Startle Response); VMR (Visual Motor Response); ASR (Acoustic Startle Response); ASH (Acoustic Startle Habituation); ISI (Inter-stimulus interval); IEI (Inter-endpoint interval)

Potential drivers underlying mixture effects

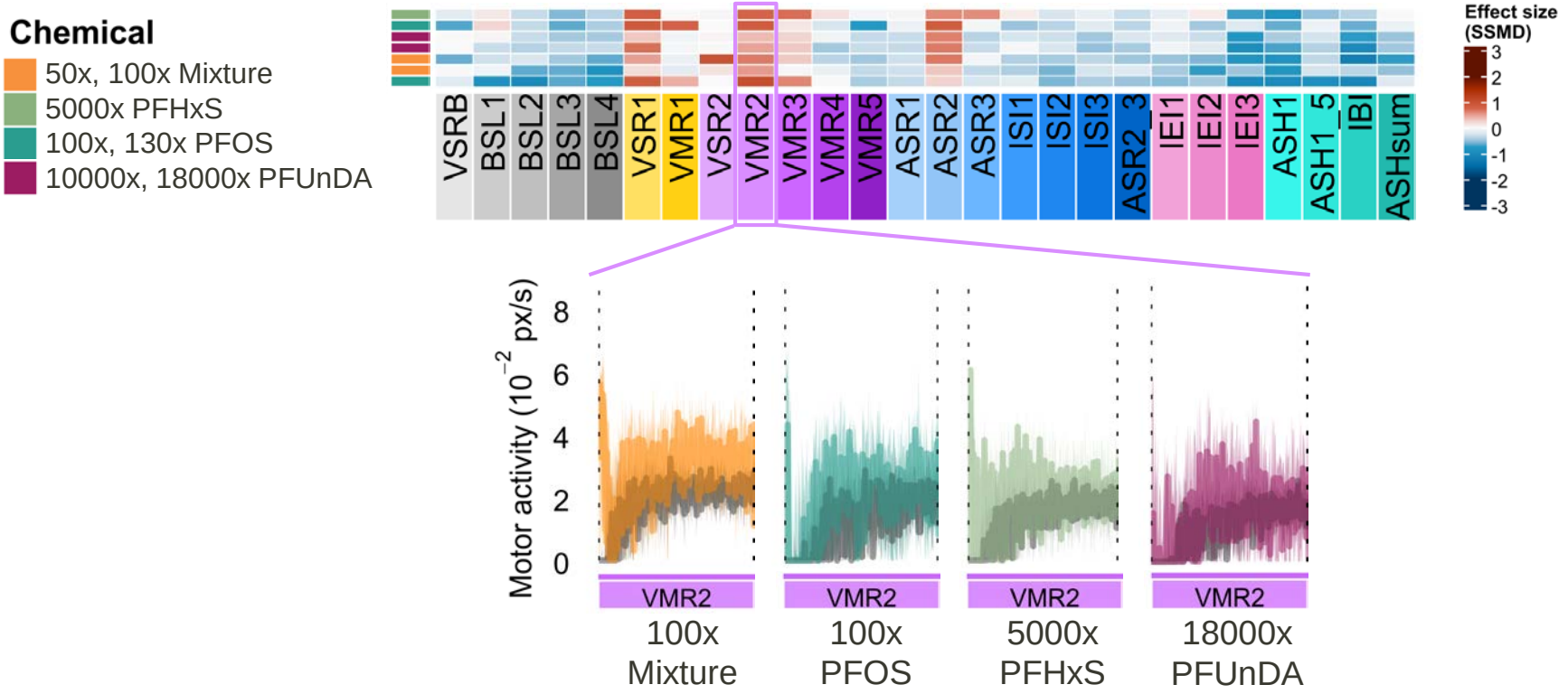
Hierarchical clustering of mixture and six individual substances



SSMD- strictly standardized median difference

Potential drivers underlying mixture effects

PFOS, PFHxS, and PFUnDA cluster with 50-100x the mixture

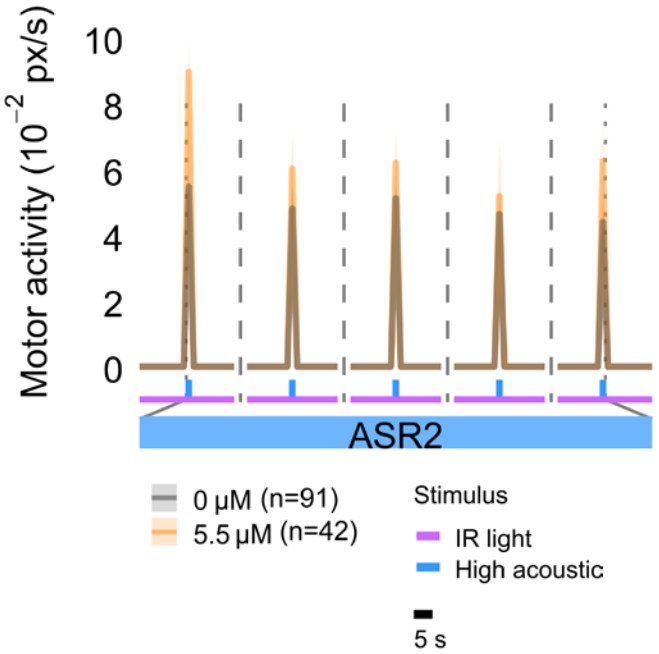


SSMD- strictly standardized median difference; VMR- Visual Motor Response

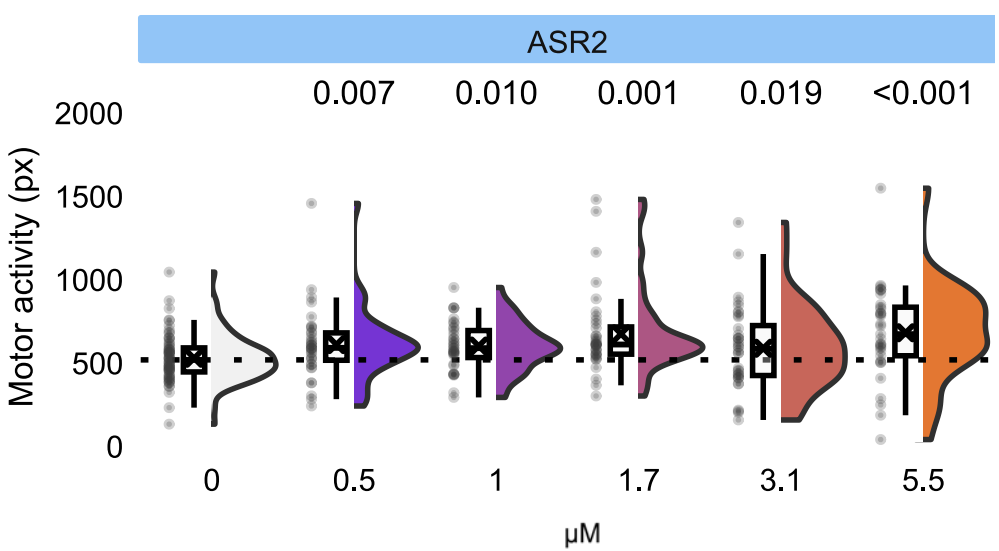
Mixture effects nearing human blood levels

Robust acoustic startle response hyperactivity

[A]



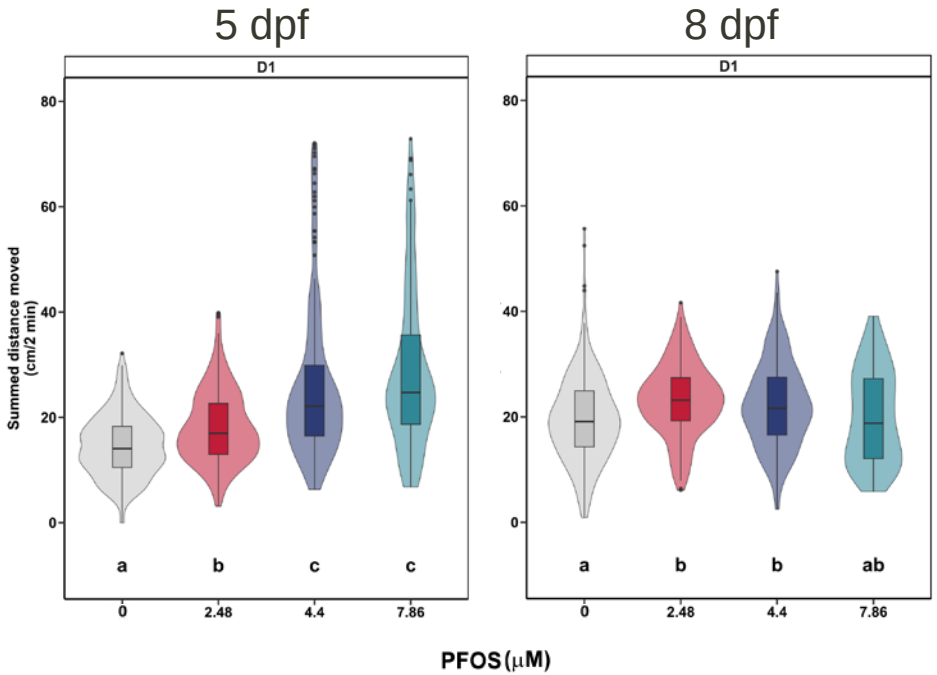
[B]



ASR- acoustic startle response

Unraveling molecular mechanisms

Dark-phase hyperactivity is transient



Gutsfeld et al. EHP. 2024.

dpf- days postfertilization; D- dark phase

Characterization of GABAR modulator behavioral profiles

$GABA_AR$ antagonist phenocopies the behavioral response induced by PFOS

Hypothesis

PFOS antagonizes GABARs in larval zebrafish

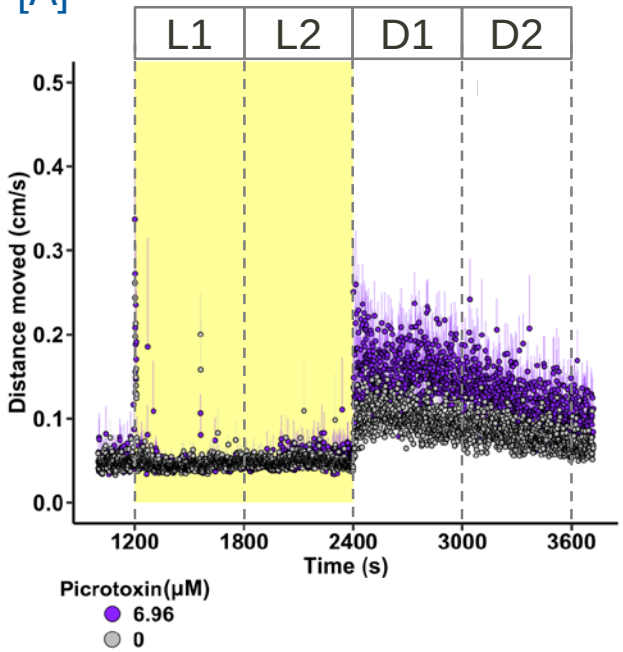
Approach 1

Exposure to a GABAR antagonist

Predicted Outcome

Phenocopy hyperactivity

[A]



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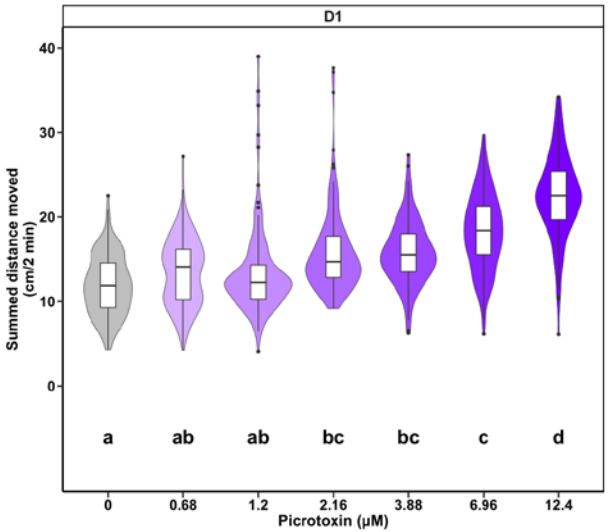


Owen et al. Tox Sci. 2025.

L- light phase; D- dark phase

Pharmacological validation of PFOS as a GABAR antagonist

Co-exposure with a $GABA_AR$ PAM rescues the hyperactivity phenotype

Hypothesis

PFOS antagonizes GABARs in larval zebrafish

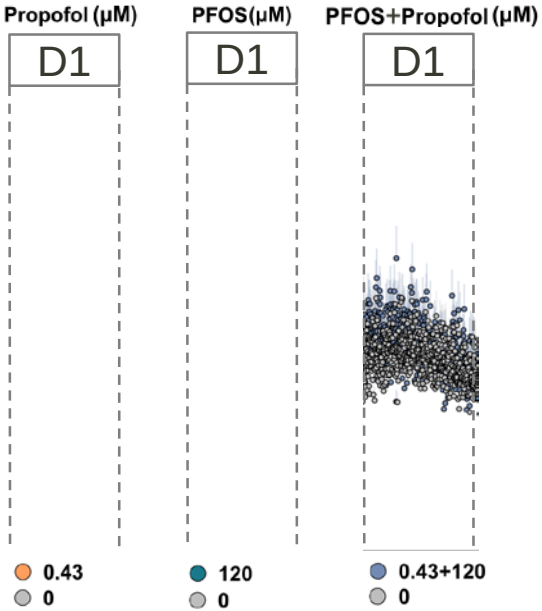
↓ Approach 2

Co-exposure to a GABAR agonist

↓ Predicted Outcome

Blunt hyperactivity phenotype

[A]



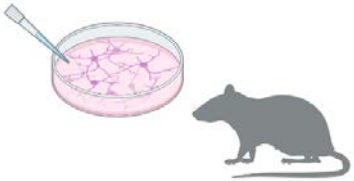
[B]

Owen et al. Tox Sci. 2025.

D- dark phase

Orthogonal evaluation

PFOS antagonizes $GABA_AR$ in mouse and human models



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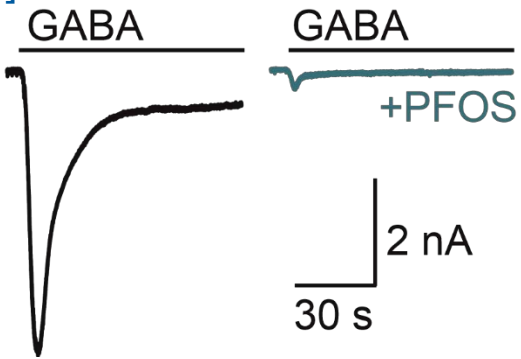

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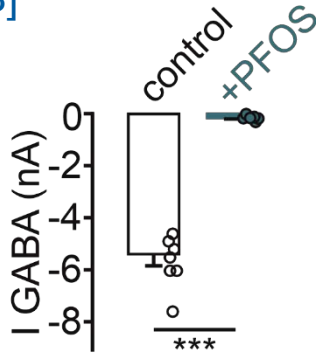

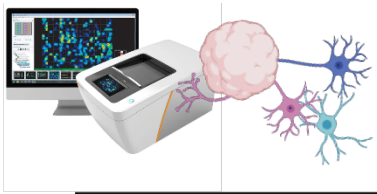

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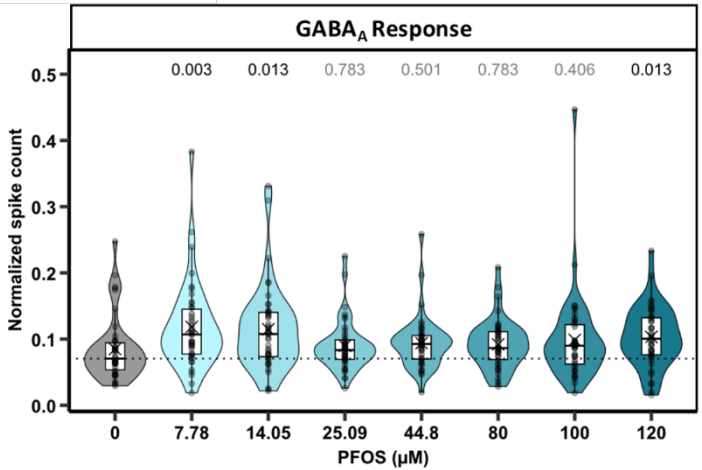


Jana Nerlich (Uni Leipzig)

Ilka Scharkin (IUF)

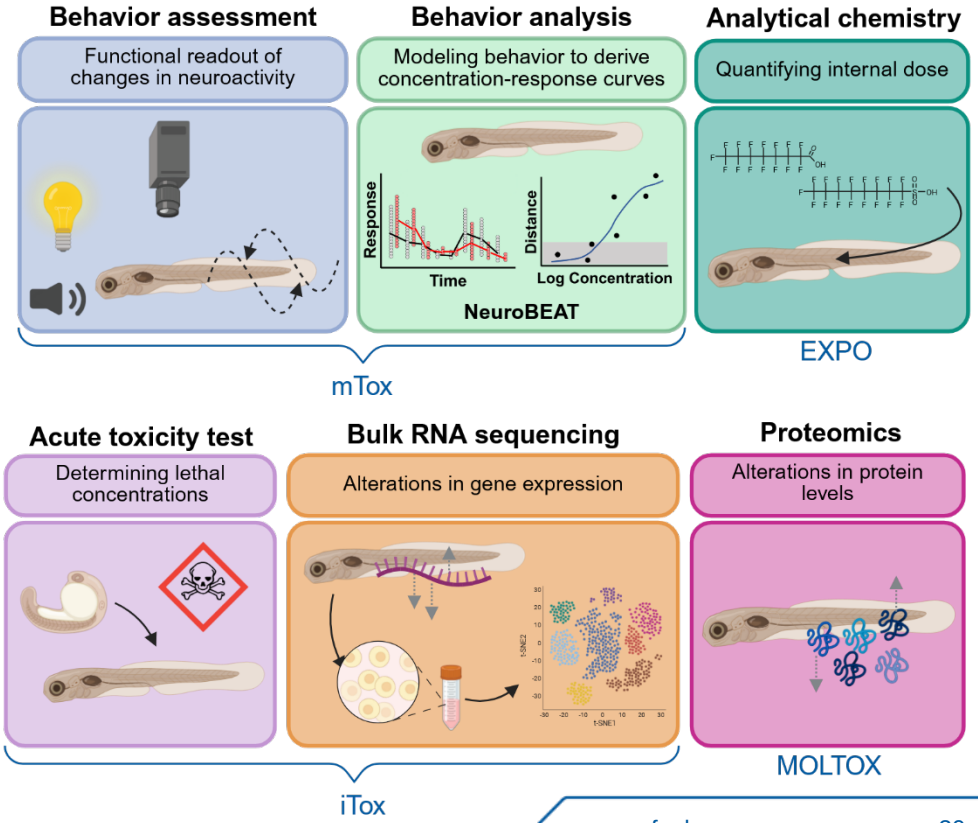
Owen et al. Tox Sci. 2025.

IGABA- $GABA_AR$ mediated current; ***p = 0.001

Take home message

Future directions: untangling PFAS mixture effects

- Identified PFAS induced behavioral phenotypes including dark-phase hyperactivity
- Uncovered a conserved causative mechanism by which PFOS alters neuroactivity, through GABAR interaction
- Overall, building confidence in the use of zebrafish behavior data for human risk assessment



mTOX working group
Afternoon session

David Leuthold
Postdoc
- Novel NAM for neurotoxicity testing
- Mechanism discovery
- Complex mixtures

Renee Owen
PhD Student
- PFAS mechanism discovery
- Human-relevant mixture

Sebastian Gutsfeld and Chloe Wray
PhD Students
- Microbiome as a modifying factor for developmental neurotoxicity effects

Elena Nicolay
PhD Student
- Novel NAM to identify chemical-dependent intestinal inflammation
- Interplay with enteric nervous system

Elucidating the functional relevance of chemical-microbiome interactions and their effects on the host

Sebastian Gutsfeld and Chloe Wray

Microbiota-gut-brain axis

Bidirectional communication between the brain and the gut

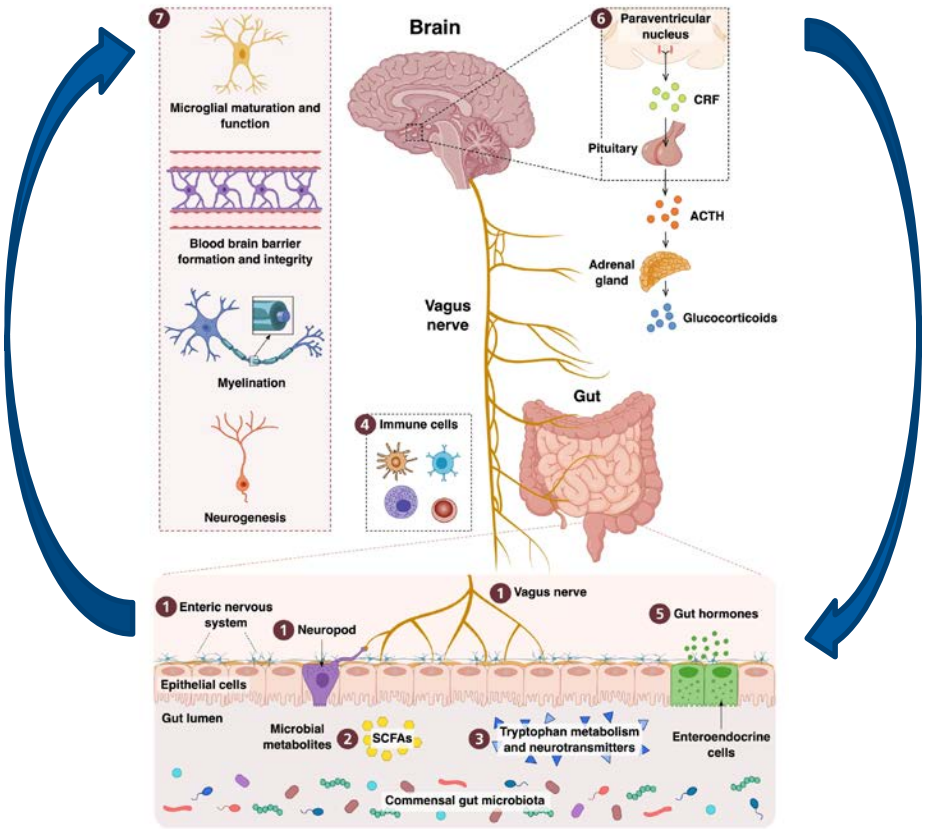
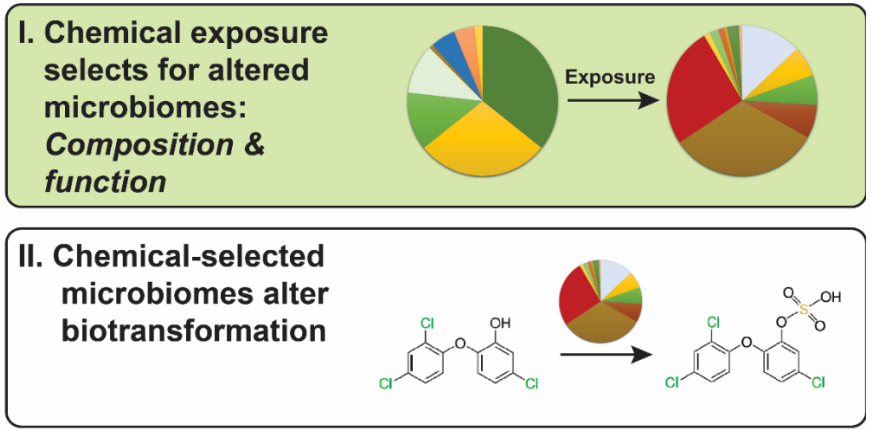


Image: Rochellys Diaz Heijtz

Microbiome toxicology

Chemical-microbiome-host interactions that influence host health

Known:

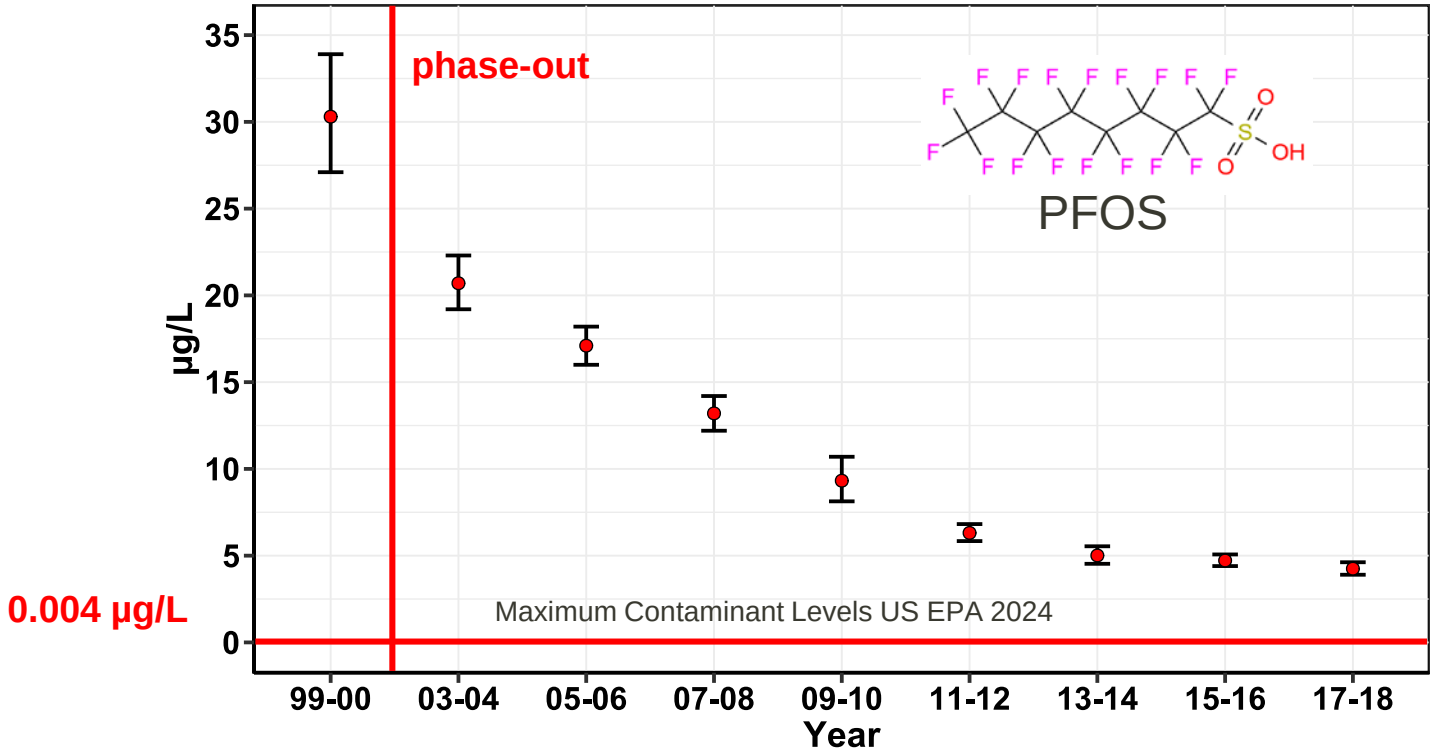


Gap:

- Functional relevance of these interactions to the host

Source: Adapted from Bertotto, Catron, and Tal. Neurotoxicol. 2020.

Perfluorooctanesulfonic acid (PFOS) blood serum levels may be influenced by transformation products of PFOS precursors



NHANES, accessed 2025

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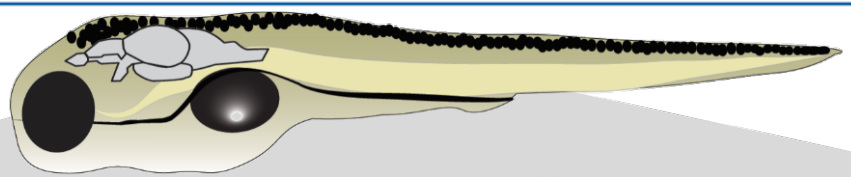
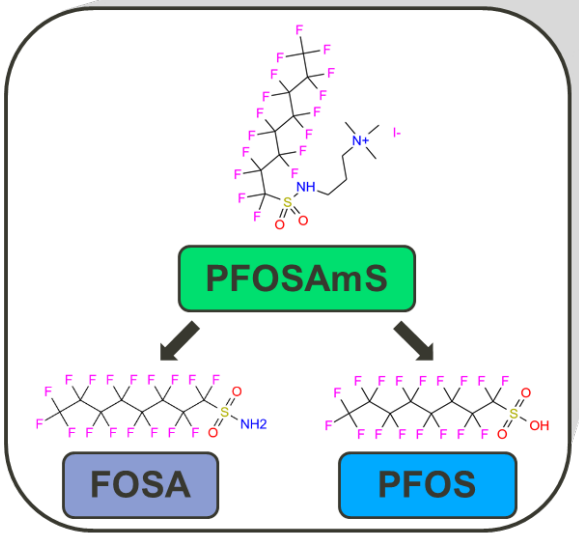
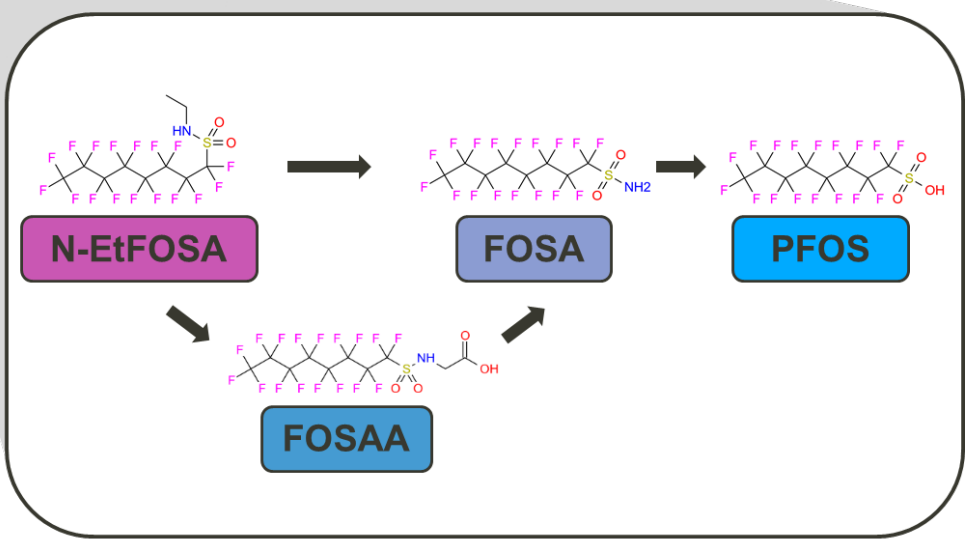


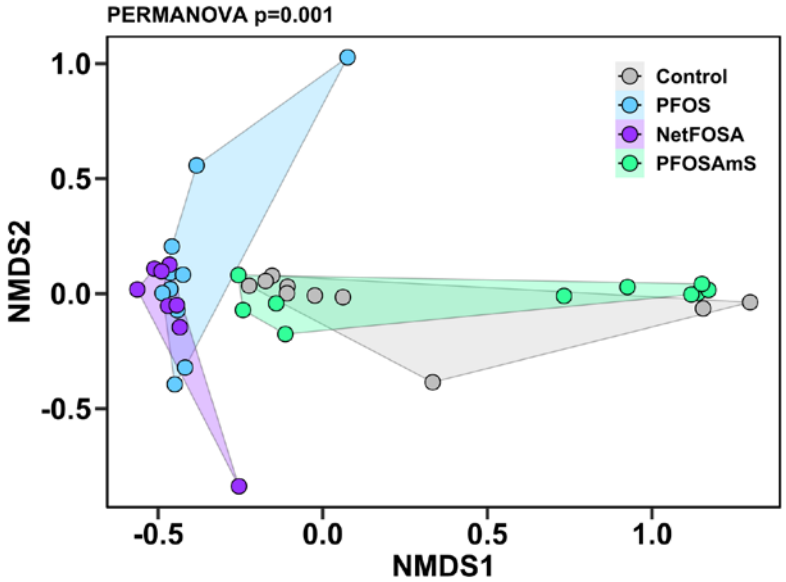


Mod after Han et al. 2021, EHP



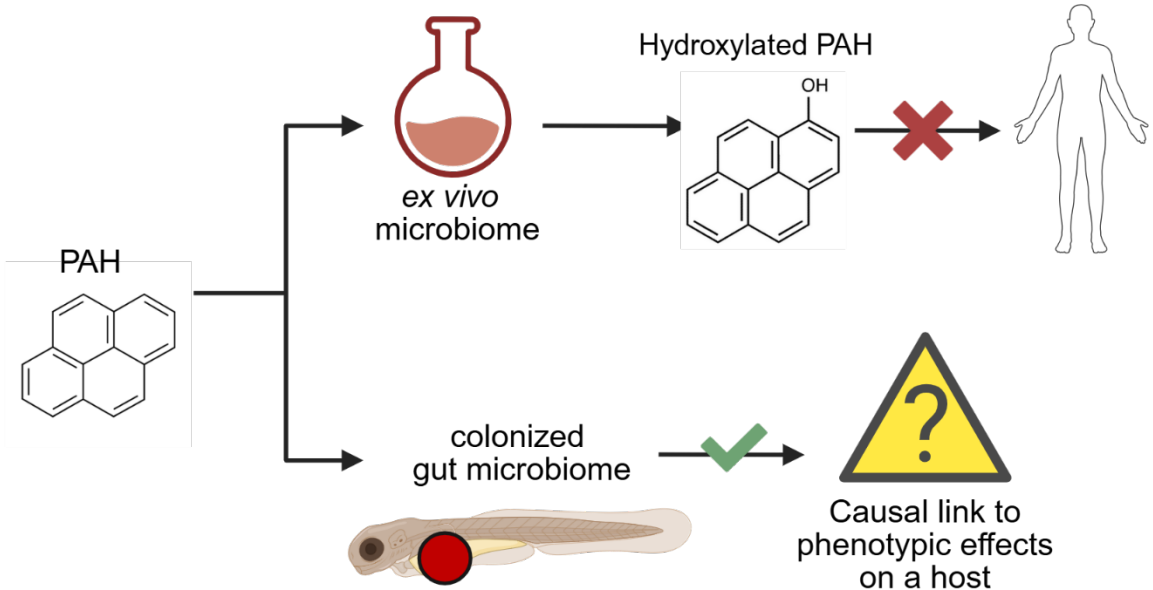
Mod after Glaser et al. 2020, Integr. Assm. And Managm.

Exposure to PFOS or N-EtFOSA alters microbiome composition



Ex vivo human microbiome generates estrogenic metabolites

Defining the functional consequences of chemical-microbiome interactions in vivo

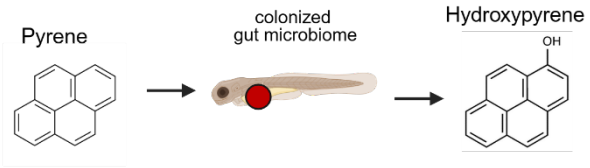


Van de Weile et al 2005

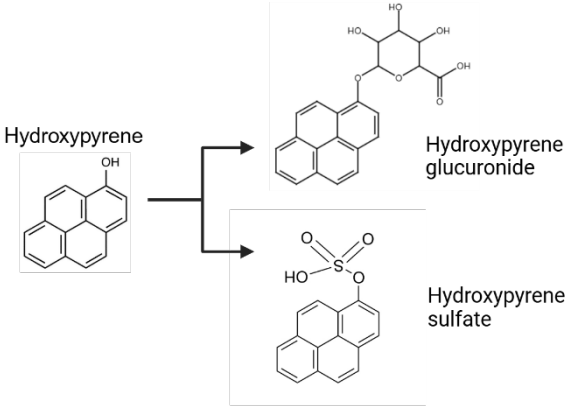
Preliminary Evidence of Pyrene Biotransformation

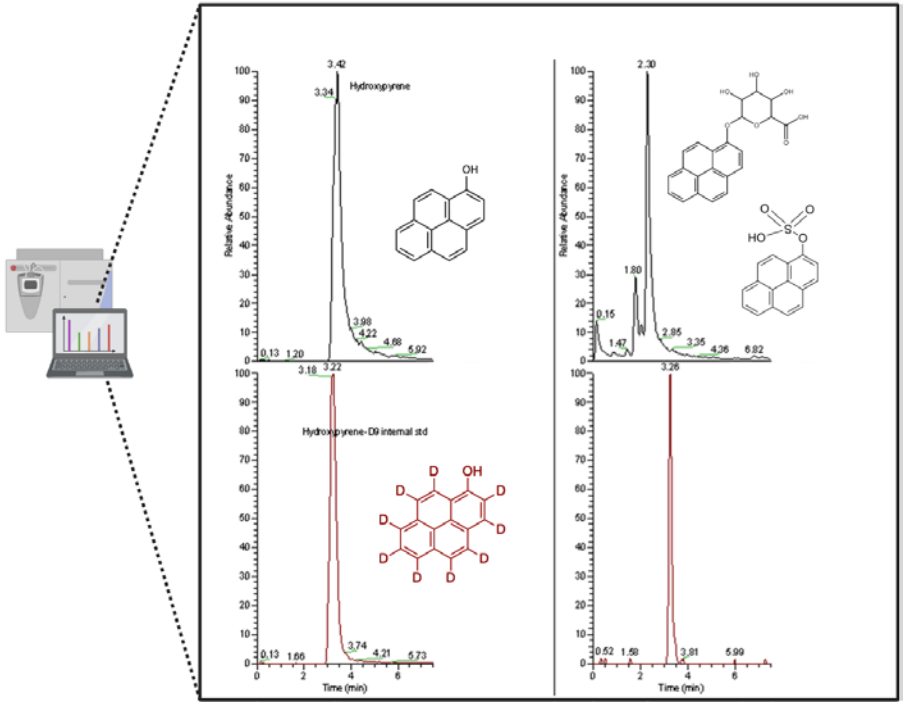
Evidence of Phase II metabolism

Phase I Metabolism: Hydroxylation



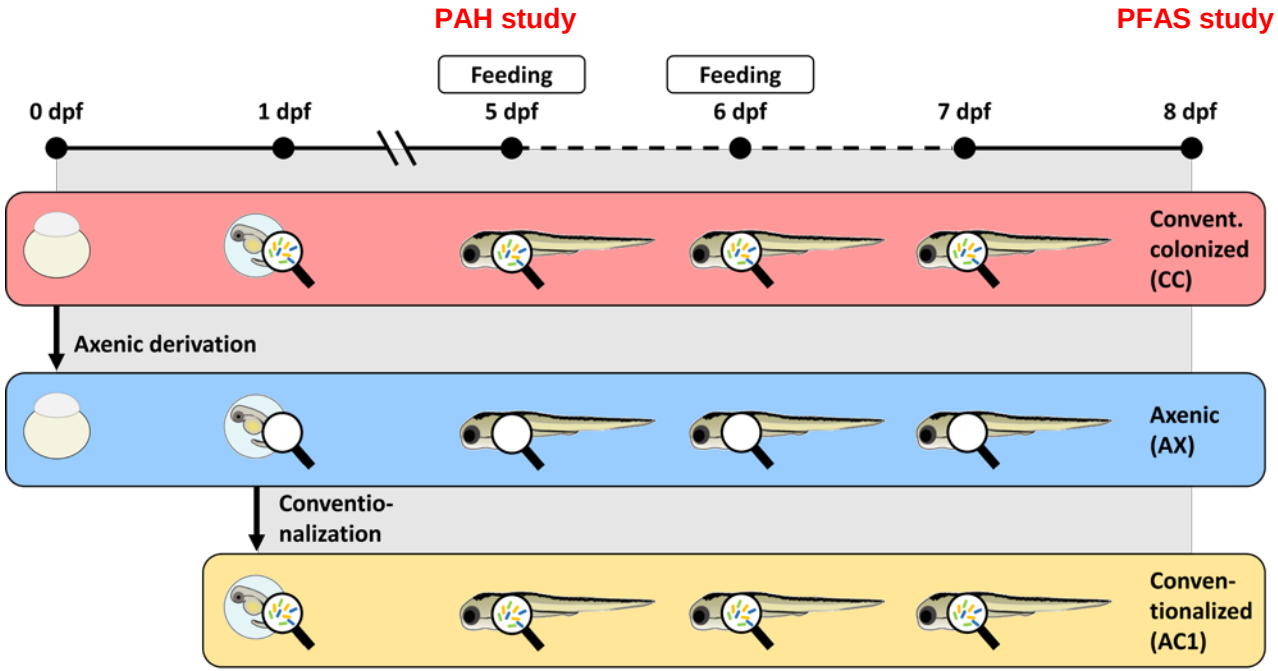
Phase II Metabolism: Conjugation



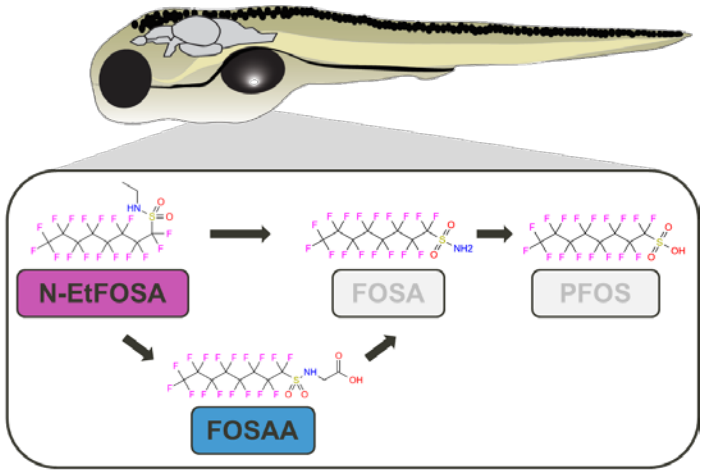


Identify microbiome-dependent host effects

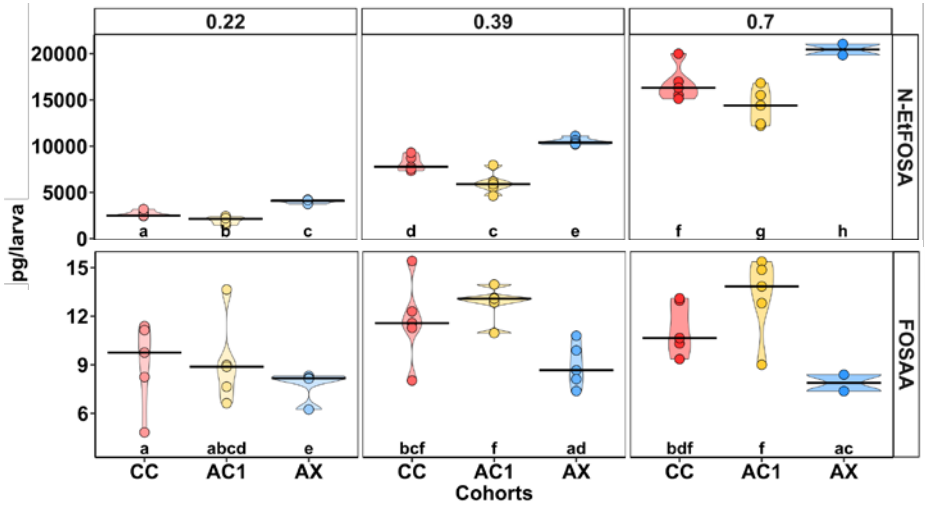
Multi-colonization cohort system



Microbiome status influences precursor transformation

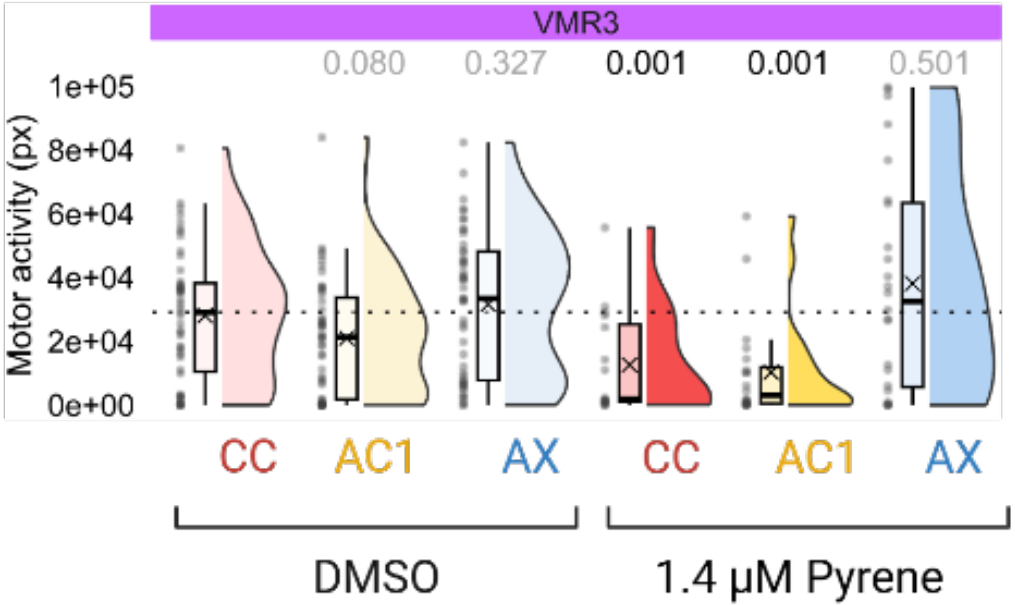


Mod after Glaser et al. 2020, Integr. Assm. And Managm.

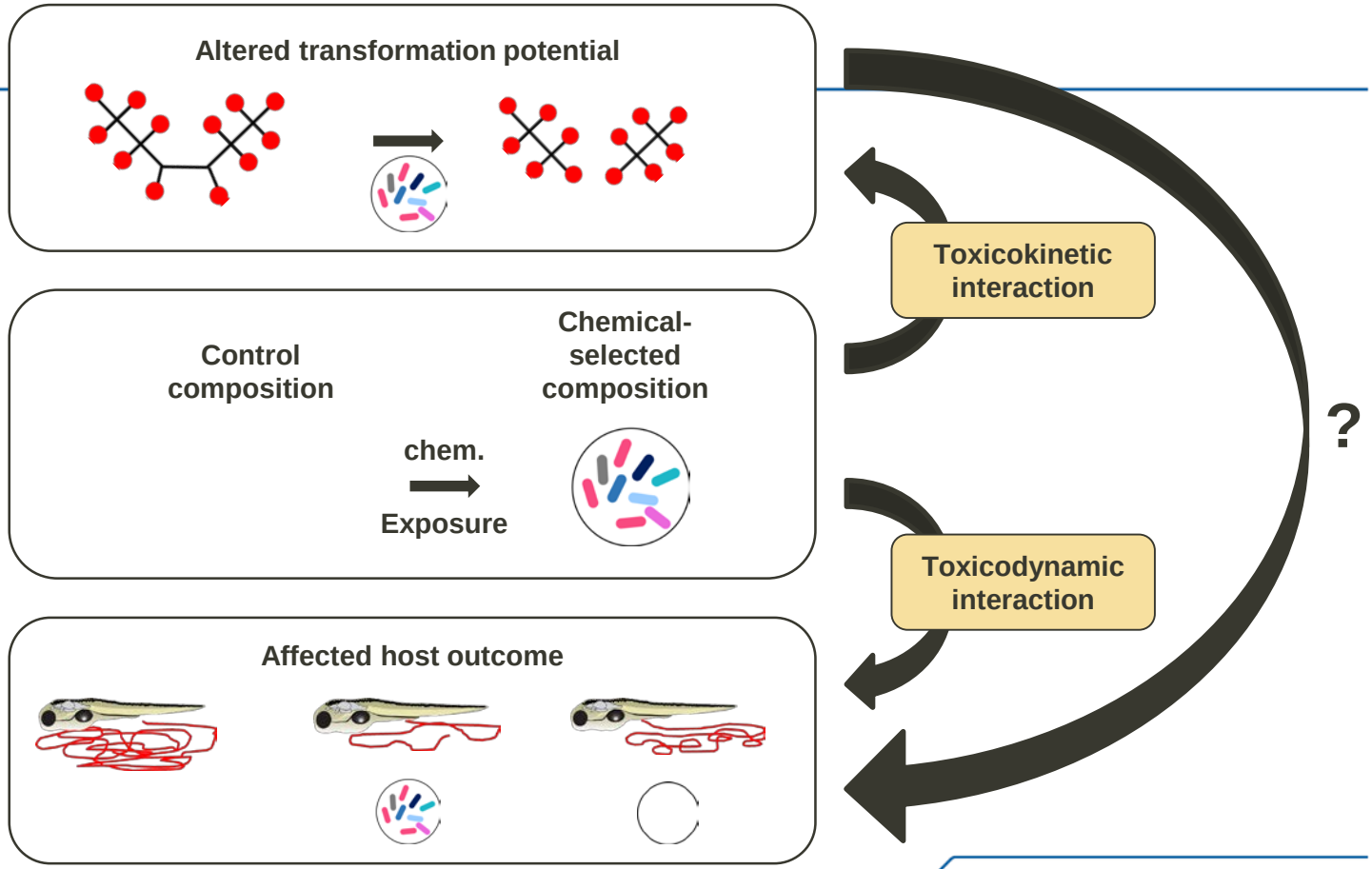


Potential microbiome-mediated bioactivation event

AX status blocks some behavior effects of pyrene



Main findings



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Establishing a high-content imaging workflow

Investigating environmental chemical effects on macrophages and enteric neurons in zebrafish larvae

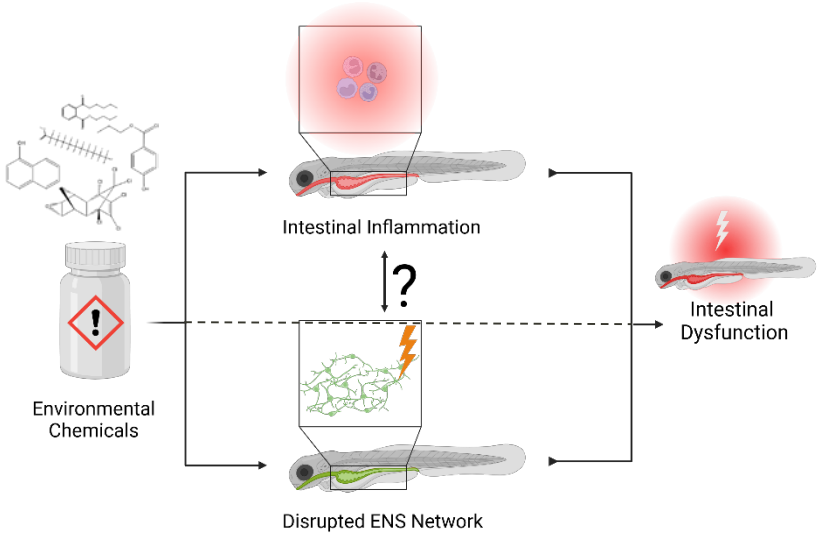
October 1st, 2025

Elena Nicolay

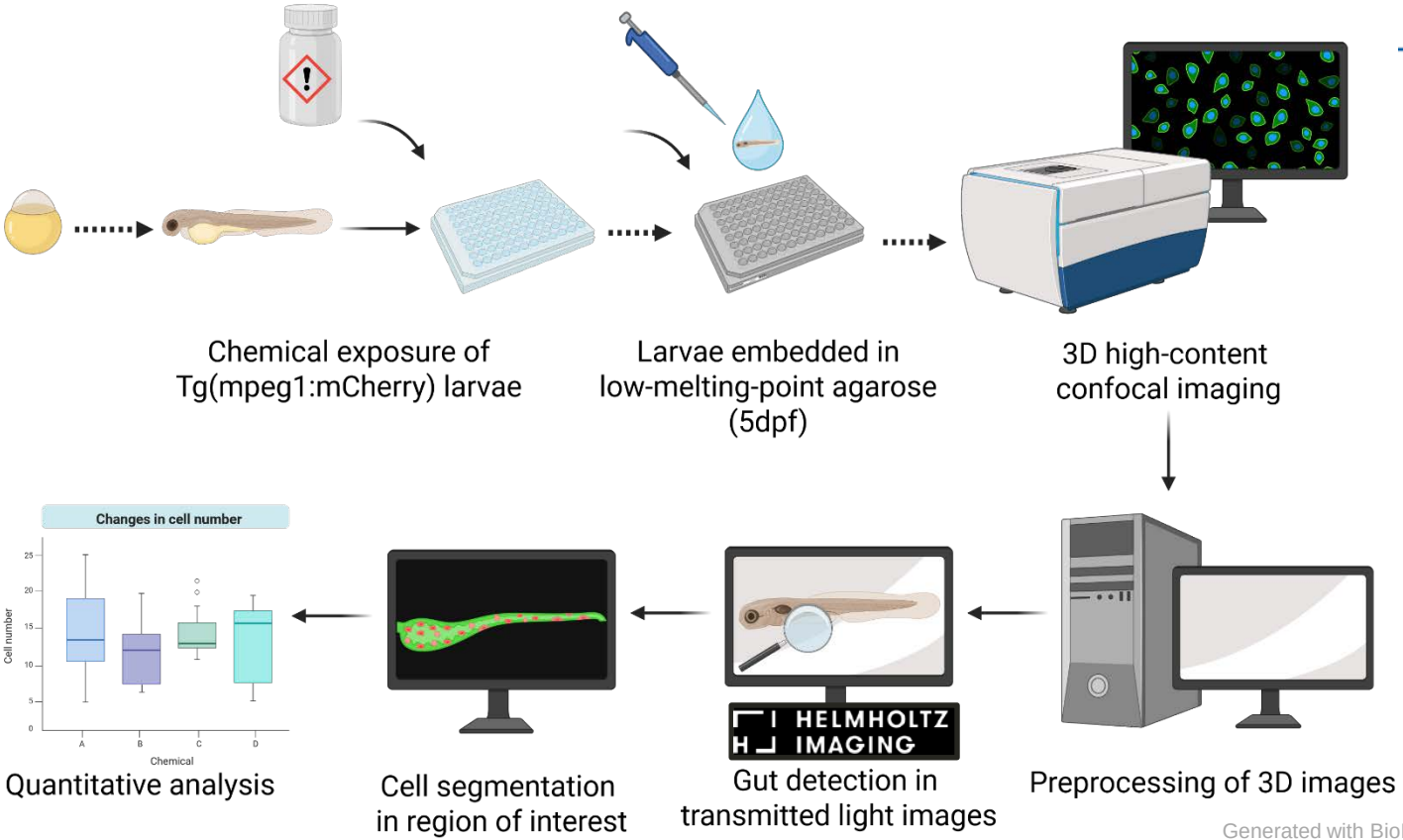
Environmental chemicals enter the body via the gut

Mostly overlooked in neurotoxicology: enteric nervous system (ENS)

- Intestinal tract is a **major route** of chemical exposure
- Exposure may impair **intestinal physiology**
  - Intestinal **inflammation**
  - Disruption of **ENS development** and function?
- <u>Hypothesis:</u> Environmental chemicals impair **innate immunity** and **ENS development**
- <u>Method:</u> Open-source workflow for high-content imaging that addresses the lack of **automation** and precise **region detection** in transgenic zebrafish



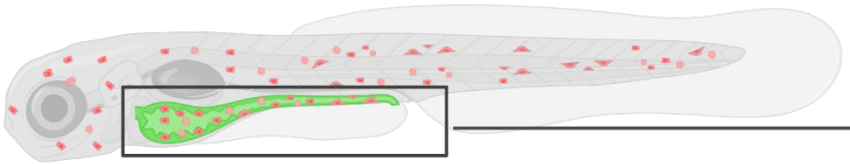
High-content image analysis workflow for macrophages



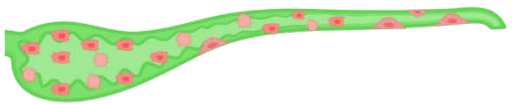
Generated with BioRender

Calculation of ratio to conclude about gut-specific macrophages

Accounting for natural variance and systemic inflammation



Total macrophage counts in fishbody



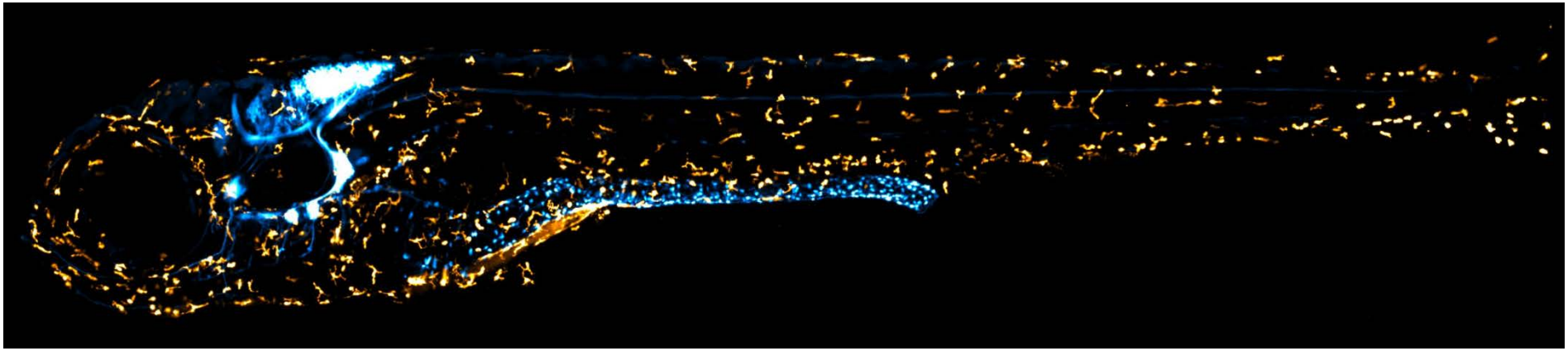
Macrophage counts in intestine

Intestinal counts / fishbody counts

Gut-specific counts of macrophages

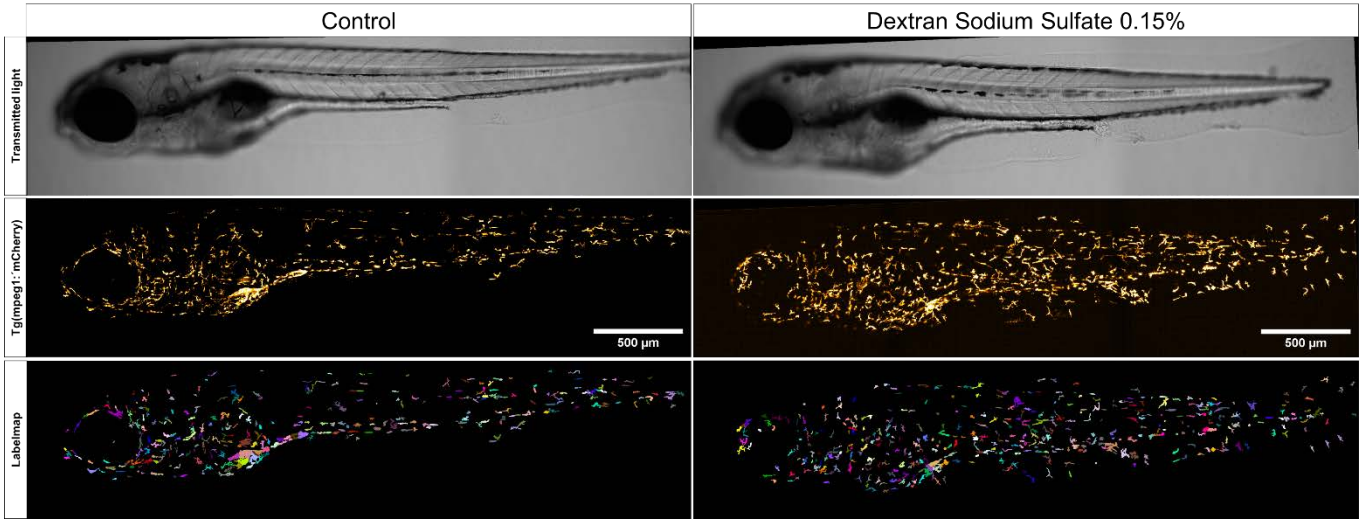
Transgenic lines to detect macrophages and enteric neurons

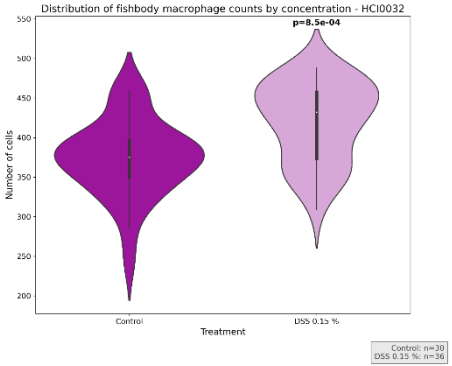
Tg(mpeg1:mCherry) X Tg(phox2bb:GFP)



Dextran Sodium Sulfate (DSS) increases macrophage number in whole body

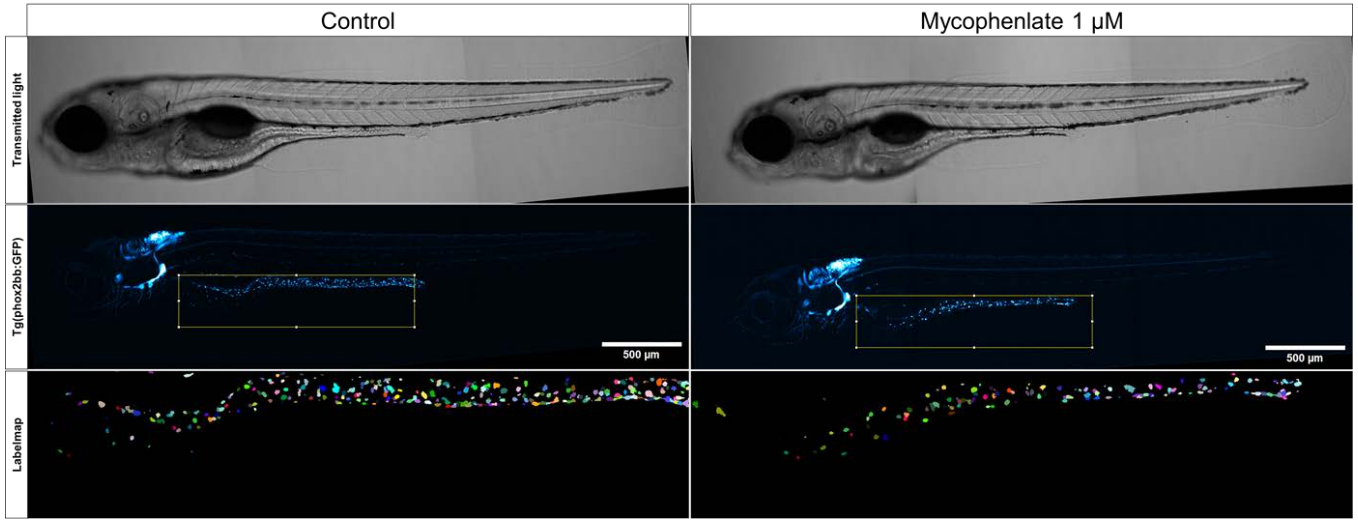
Not specific for intestinal inflammation

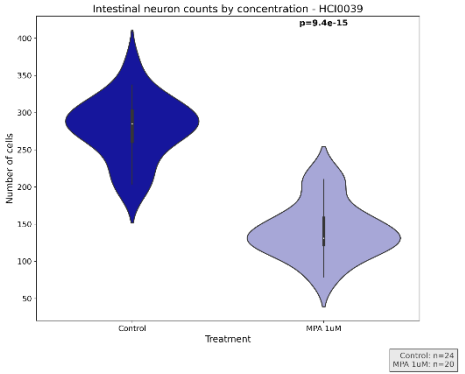




Mycophenolate (MPA) reduces number of enteric neurons in 5dpf embyros

Exposure from 2-3dpf delays neuronal migration to intestinal wall

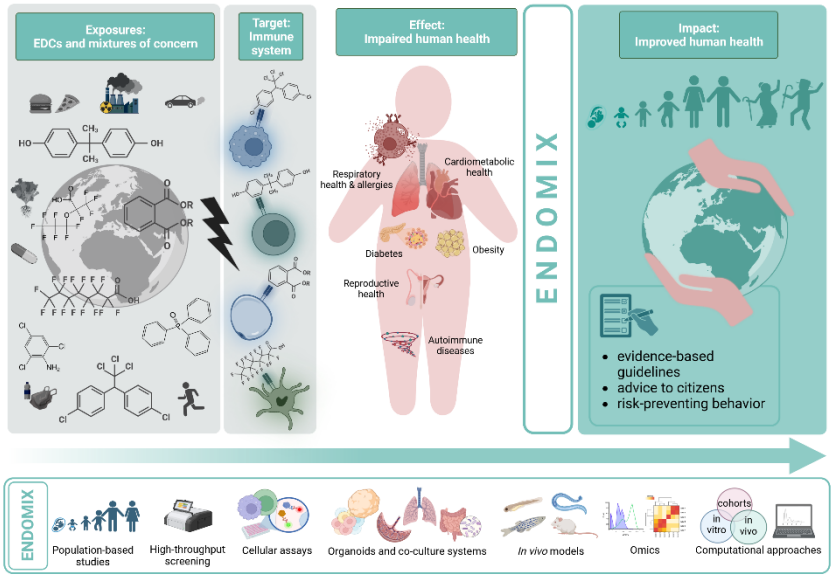




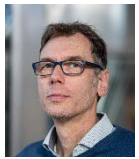
Outlook: ENDOMIX – endocrine disruptors and chemical mixtures

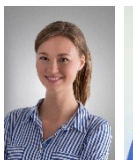
How EDCs impair immunity and drive disease

- Horizon Europe initiative studying how **EDC mixtures** disrupt **immune** function
- Predicted **harmful mixtures** from **literature**, **databases** and human **cohort** sample screening
- Chemical mixture groups:
  - Per- and polyfluoroalkyl substances
  - Phthalates
  - Chlorinated Pesticides
  - Phenols
  - Polycyclic aromatic hydrocarbons



Acknowledgements
People and funding





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PhD College
US EPA Pathfinder Innovation Projects

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