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Using zebrafish embryo high-content screening to assess complex chemical mixtures in environmental samples from Nigeria

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1. Zebrafish as alternative model

Background

- Zebrafish embryos (Danio rerio) offer an ethical alternative to standard animal testing and align with the 3Rs principle.
- This research uses high-content screening to explore how complex chemicals with diverse modes of action impact on effect patterns.
- Knowledge gained will strengthen the interpretation of environmental sample data and advance effect-based monitoring.

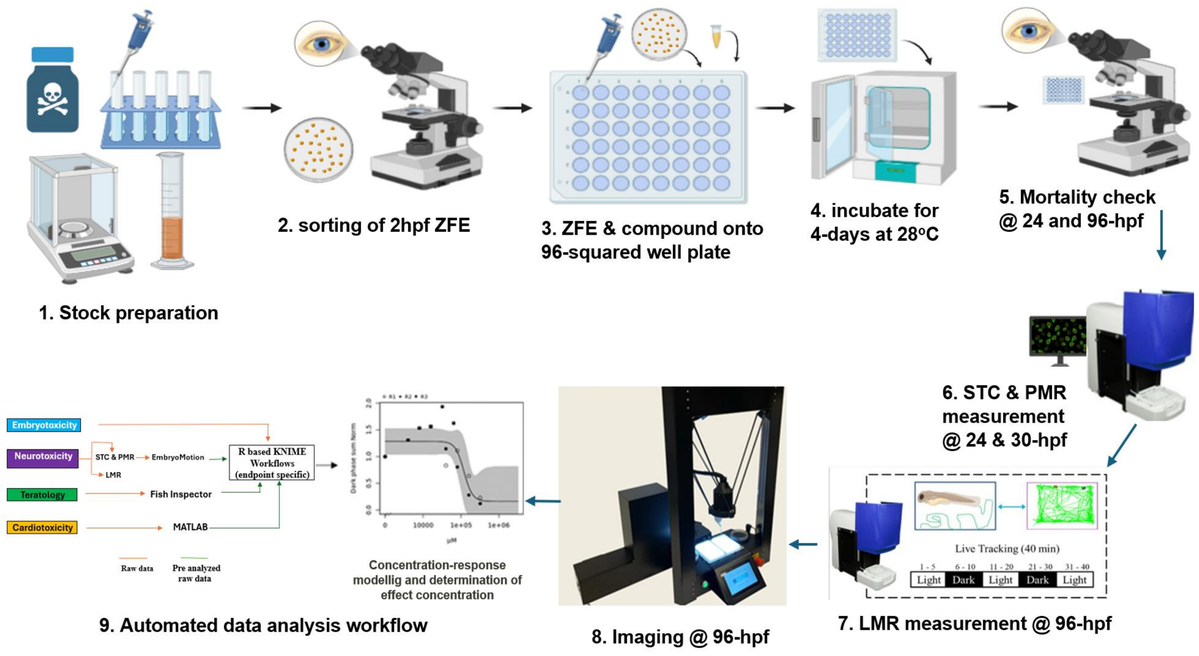
Objectives

Task 1: Artificial model mixtures (known modes of action) → **Task 2:** Prototype environmental mixtures (known composition) → **Task 3:** Environmental samples (Nigeria)

Nigeria case study (Agriculture, Oil, drought)

Global Network Impact (One world initiative)

2. High content screening of chemicals



Studied toxicological endpoints

Toxicological endpoint	Type of observation	Type of assessment	Observation stage (hpf)
Mortality	Coagulation/necrosis	Visual Focal Microscope	24 & 96 hpf
Motor behavior	LMR – Locomotor activity	Automatic video tracking system ZebraBox (View Point)	96 hpf
Phenotype	Measurement of 96hpf ventral and dorsal structural features	Automated imaging platform Automatic Imaging Robot (AIR)	96 hpf
Heart frequency	Measurement of the heart frequency during 5-15 seconds	Automated imaging platform Automatic Imaging Robot (AIR)	96 hpf

3. Assessment of model compounds to identify candidates for mixture assessment.

- Compounds from the PrecisionTox project were analyzed.
- Selection was based on Toxic Ratio (TR) and Sensitivity Ratio ($SR_{basline}$).
- Compounds with TR>20 and/or SR>10 with diverse MOA were prioritized for further mixture design.

Toxic Ratio (TR): **TR is defined as the ratio of the LC50 predicted from a QSAR for baseline toxicity and the experimental LC50 value.**

$$TR = \frac{\text{LC50 predicted baseline toxicity}}{\text{Observed toxicity (LC50)}}$$

$$SR_{mortality} = \frac{LC_{50}}{EC_{50}^{morph}}$$

Where:

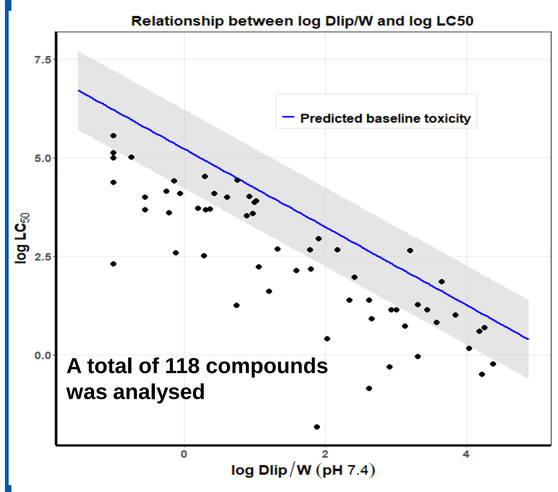
- LC_{50} = concentration causing 50% mortality

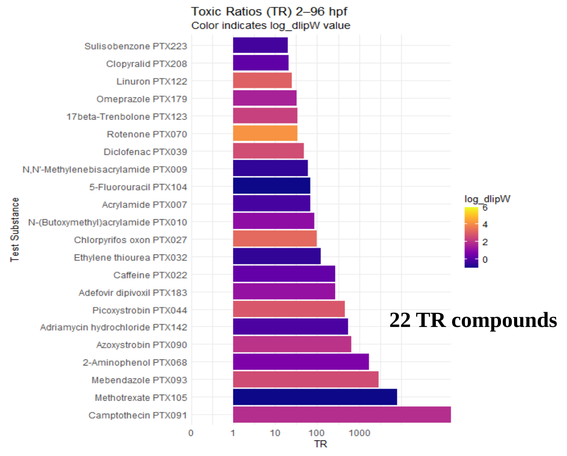
- EC_{50}^{morph} = concentration causing 50% morphological effects

The goal is to design 2 mixtures of several compounds based on

- high TRs
- SR (multiple endpoints)

Toxic Ratio(TR) assessment and selection of compounds





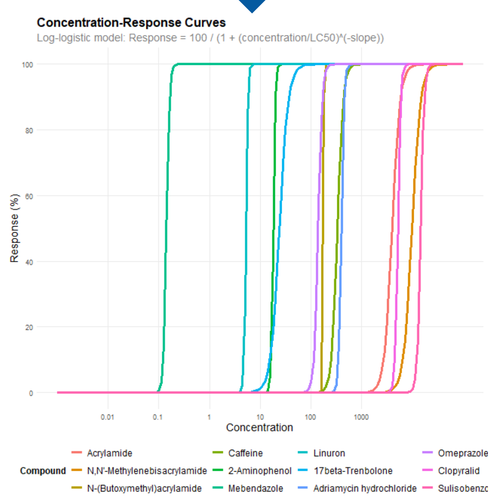
Next after identifying potential candidates from TR assessment?

- design initial TR mixtures for testing
- Concentration Addition (CA) and Independent Action (IA) models comparison
- evaluate applicability of mass balance model

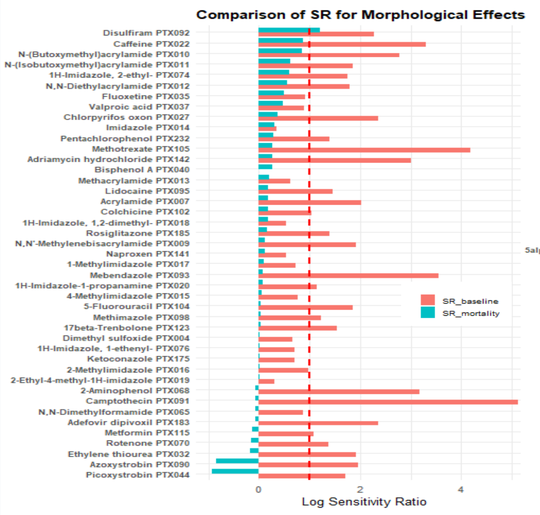
10 Compounds of high TR & diverse MOA

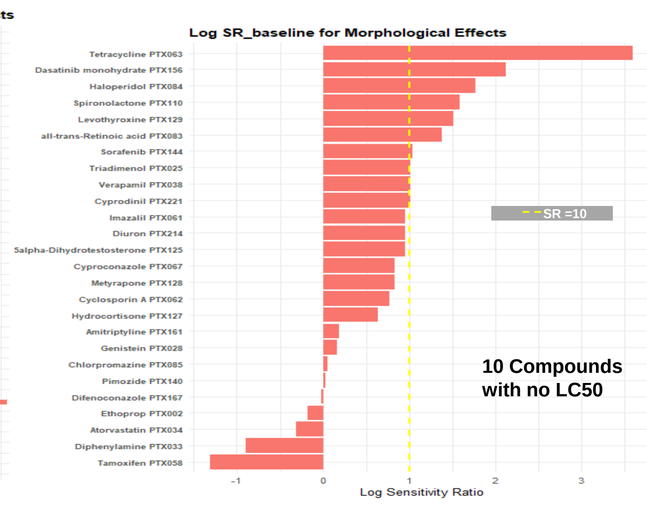
Compounds with similar dose response relationship used for:

- **Equitoxic mixture design**
- **Mixture effect prediction**



Sensitivity Ratio (SR) analysis for designing a mixture to study morphological effects





Total of 11 Compounds have been identified as potential candidate for mixture design base on SR for morphological effects ($SR_{mortality}$) assessment

Next after identifying potential candidates from $SR_{mortality}$ assessment.

- Cumulative assessment to get slopes and design equitoxic mixture.
- Compare individual effect patterns with mixture
- Explore prediction possibilities

4. Outlook

Phase	Key Task	Tool/Metric	Purpose
1	Artificial mixture design	Fixed-ratio, MOA grouping	Reflect complex mixtures with contrasting MoAs
2	Mixture testing	CA/IA Models, SR, MDR	Compare predicted vs actual toxicity
3	Environmental sample analysis	LC-MS/MS, ZF-HCS, SR	Assess real-world mixture effects
4	Data integration	SR, TR, endpoint prioritization	Guide risk assessment and regulation

References

- Nils Klüver, Kai Bittermann, Beate I. Escher. 2019. QSAR for baseline toxicity and classification of specific modes of action of ionizable organic chemicals in the zebrafish embryo toxicity test. Aquatic Toxicology 207 (2019) 110–119
- OECD (2013), Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris
- Teixidó, E., et al. Grouping of chemicals into mode of action classes by automated effect pattern analysis using the zebrafish embryo toxicity test. *Archives of Toxicology (2022), 1-17*

Extending the Diagnostic Capacity for High-Content Analysis in the Zebrafish Embryo Model(ZET)

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Optimized ZET + More Chemical Assessment = Greener Environment & Health

1. Background, Problem And Aim:

- Chemicals are essential to modern life; requires better testing models for safety.
- Over 350,000 chemicals in commerce, only a fraction tested so far.
- Emergence of zebrafish embryo test (ZET) as NAMs alternative.
- Need for ZET enhancement by additional morphological endpoints to:
  - improve diagnostic capacity, for individual compounds and environmental mixtures.
  - establish biomarkers for developmental delay -> required for using oxygen consumption as effect parameter
  - capacity as potential endpoint for chemical effect assessment

2. Methodology:

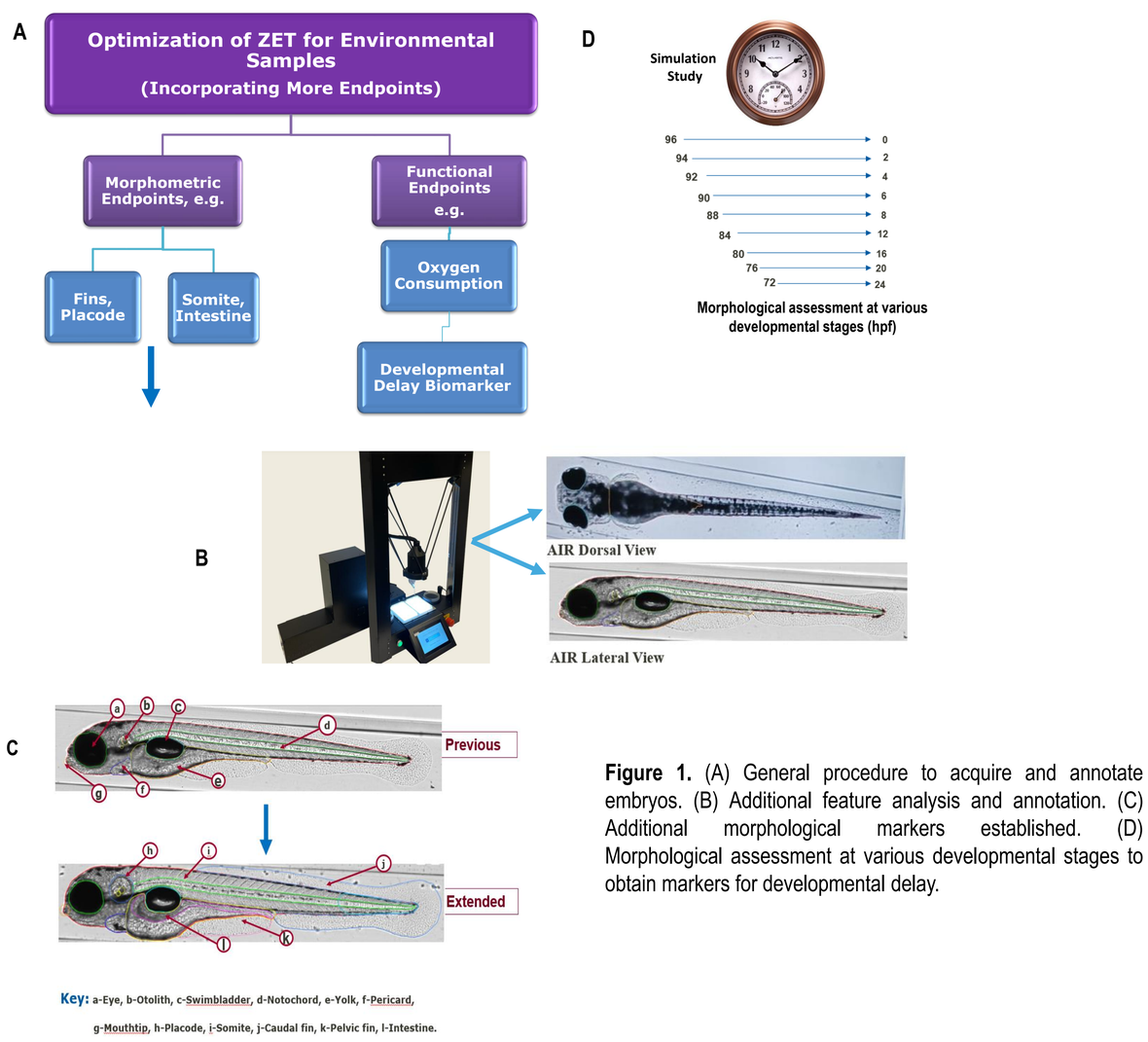


Figure 1. (A) General procedure to acquire and annotate embryos. (B) Additional feature analysis and annotation. (C) Additional morphological markers established. (D) Morphological assessment at various developmental stages to obtain markers for developmental delay.

3. Results:

i. Developmental Delay Marker – comparison of morphological descriptors at different developmental stages

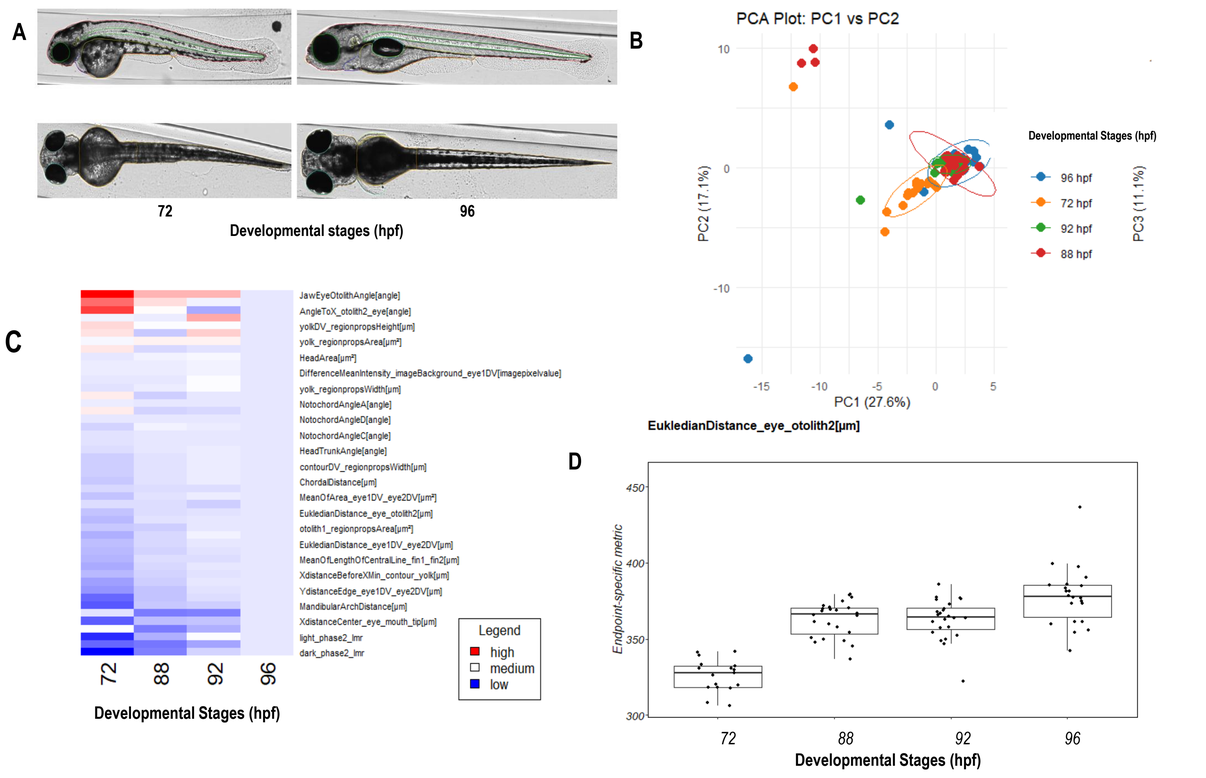


Figure 2. (A) Morphological images depicting developmental delay (72hpf) and controls (96hpf) incubated at 27.7 °C. (B) Dataset of a single replicate with individual embryos analysed by PCA for reducing dimension – 72 hpf group is distinctly separated from the 96 hpf, whereas others overlapped, potentially to variability greater than differences in many endpoints. (C) 48 metric features visualized as a heatmap (marked differences mostly detected at 72 hpf). (D) Euclidean distance of the eye and otolith2 as a selected example, representing one of the key developmental stage biomarkers.

4. Validation Experiment:

ii. Validation of developmental delay biomarkers by reduced incubation temperatures

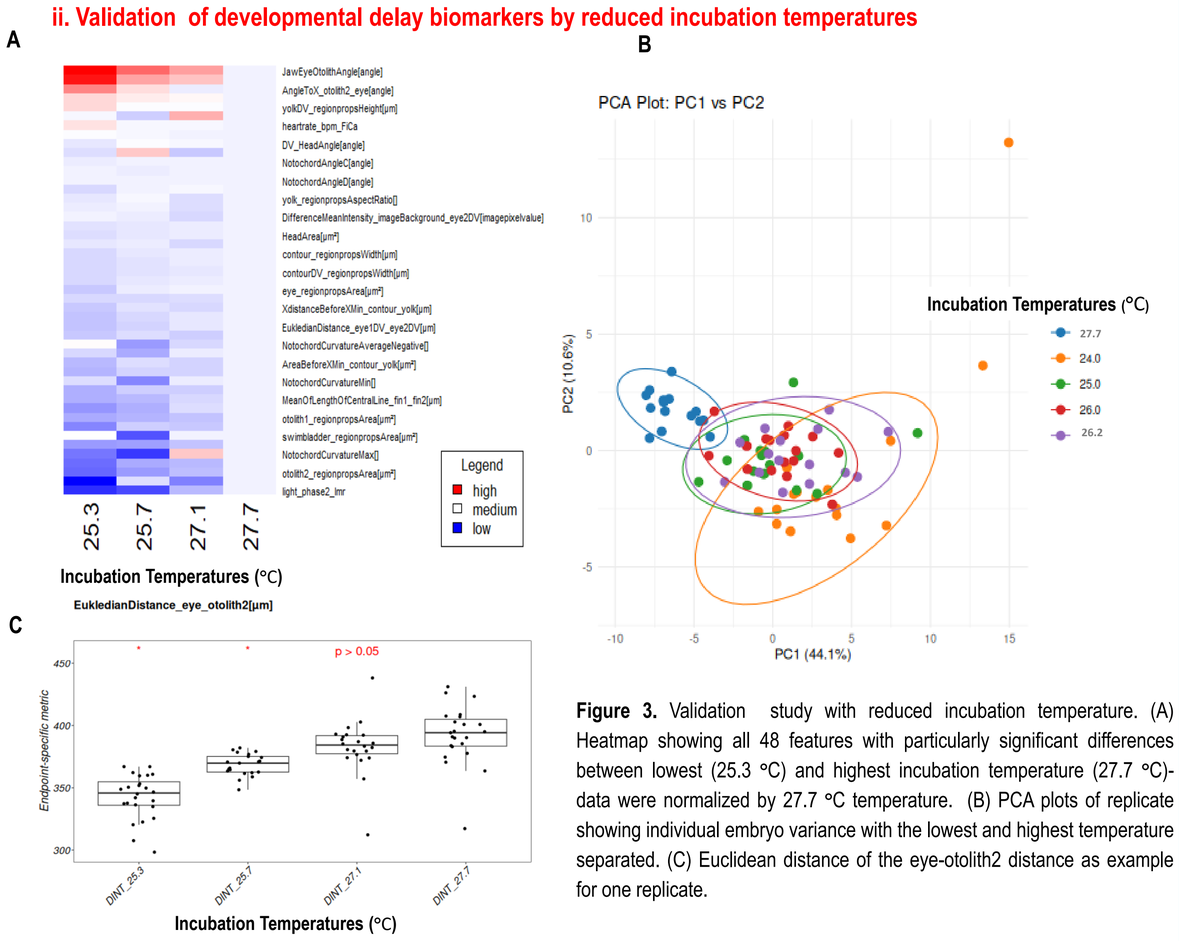


Figure 3. Validation study with reduced incubation temperature. (A) Heatmap showing all 48 features with particularly significant differences between lowest (25.3 °C) and highest incubation temperature (27.7 °C)-data were normalized by 27.7 °C temperature. (B) PCA plots of replicate showing individual embryo variance with the lowest and highest temperature separated. (C) Euclidean distance of the eye-otolith2 distance as example for one replicate.

5. Nine consistently affected endpoints identified:

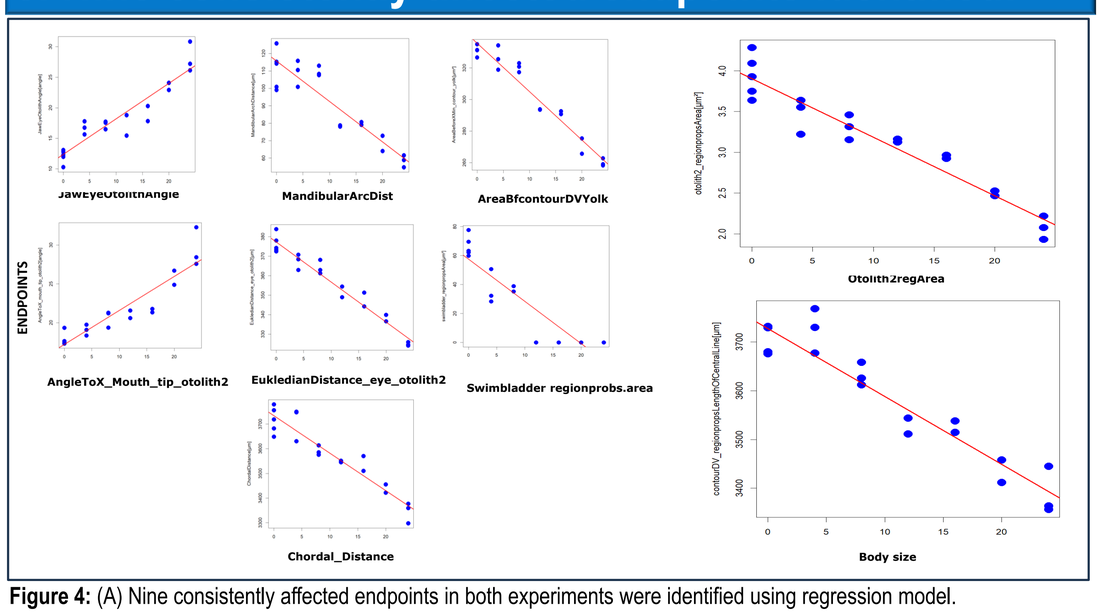
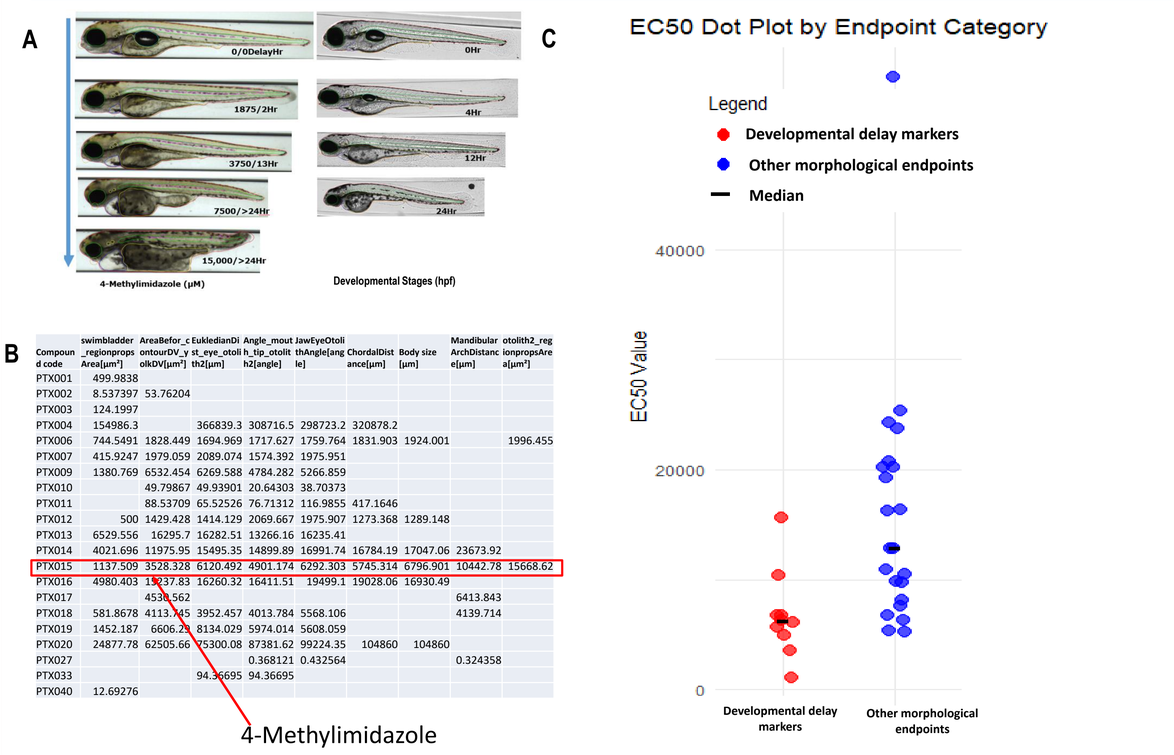


Figure 4: (A) Nine consistently affected endpoints in both experiments were identified using regression model.

6. Search for chemicals with effects on developmental stages:



Compound code	swimbladder_regionpropsArea[µm²]	AreaBefor_contourDV_yolkDV[µm²]	EukledianDist_eye_otolith2[µm]	Angle_mouth_tip_otolith2[angle]	JawEyeOtolithAngle[angle]	ChordalDistance[µm]	Body size [µm]	MandibularArchDistance[µm]	otolith2_regionpropsArea[µm²]
PTX001	499.9838								
PTX002	8.537397	53.76204							
PTX003	124.1997								
PTX004	154986.3		366839.3	308716.5	298723.2	320878.2			
PTX006	744.5491	1828.449	1694.969	1717.627	1759.764	1831.903	1924.001		1996.455
PTX007	415.9247	1979.059	2089.074	1574.392	1975.951				
PTX009	1380.769	6532.454	6269.588	4784.282	5266.859				
PTX010		49.79867	49.93901	20.64303	38.70373				
PTX011		88.53709	65.52526	76.71312	116.9855	417.1646			
PTX012	500	1429.428	1414.129	2069.667	1975.907	1273.368	1289.148		
PTX013	6529.556	16295.7	16282.51	13266.16	16235.41				
PTX014	4021.696	11975.95	15495.35	14899.89	16991.74	16784.19	17047.06	23673.92	
PTX015	1137.509	3528.328	6120.492	4901.174	6292.303	5745.314	6796.901	10442.78	15668.62
PTX016	4980.403	15237.83	16260.32	16411.51	19499.1	19028.06	16930.49		
PTX017		4530.562						6413.843	
PTX018	581.8678	4113.745	3952.457	4013.784	5568.106			4139.714	
PTX019	1452.187	6606.29	8134.029	5974.014	5608.059				
PTX020	24877.78	62505.66	75300.08	87381.62	99224.35	104860	104860		
PTX027				0.368121	0.432564			0.324358	
PTX033			94.36695	94.36695					
PTX040	12.69276								

4-Methylimidazole

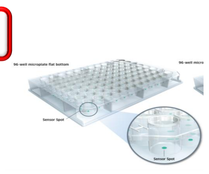
Figure 5. (A) 4-Methylimidazole images showing developmental delay indicated by comparison to different developmental stages. (B) 56 compounds elicited positive responses for at least one developmental delay biomarker (only a fraction is shown). 4-Methylimidazole affected all biomarkers. (C) Dot plot of EC50 values of 4-methylimidazole effects for developmental delay biomarkers and morphological endpoints..

Key Take Away:

- Extended the morphological endpoints of ZET with more structural features.
- Establishment of developmental delay biomarker signatures.
- Validation of the new model using low incubation temperatures.
- Identified 9 endpoints as developmental delay biomarkers
- Correlation between developmental delay and low temperature
- Identified chemicals causing developmental delay

Perspectives:

- Calculation of Developmental Delay Index
- Chemical testing to ascertain their delay severity using delay signature
- Determining oxygen consumptions by chemicals.
- Application of optimized ZET model to environmental chemical sampling



Compact Multi-Channel Oxygen Reader for 96-Well Microplates.

UFZ HELMHOLTZ Zentrum für Umweltforschung

DAAD Deutscher Akademischer Austauschdienst German Academic Exchange Service

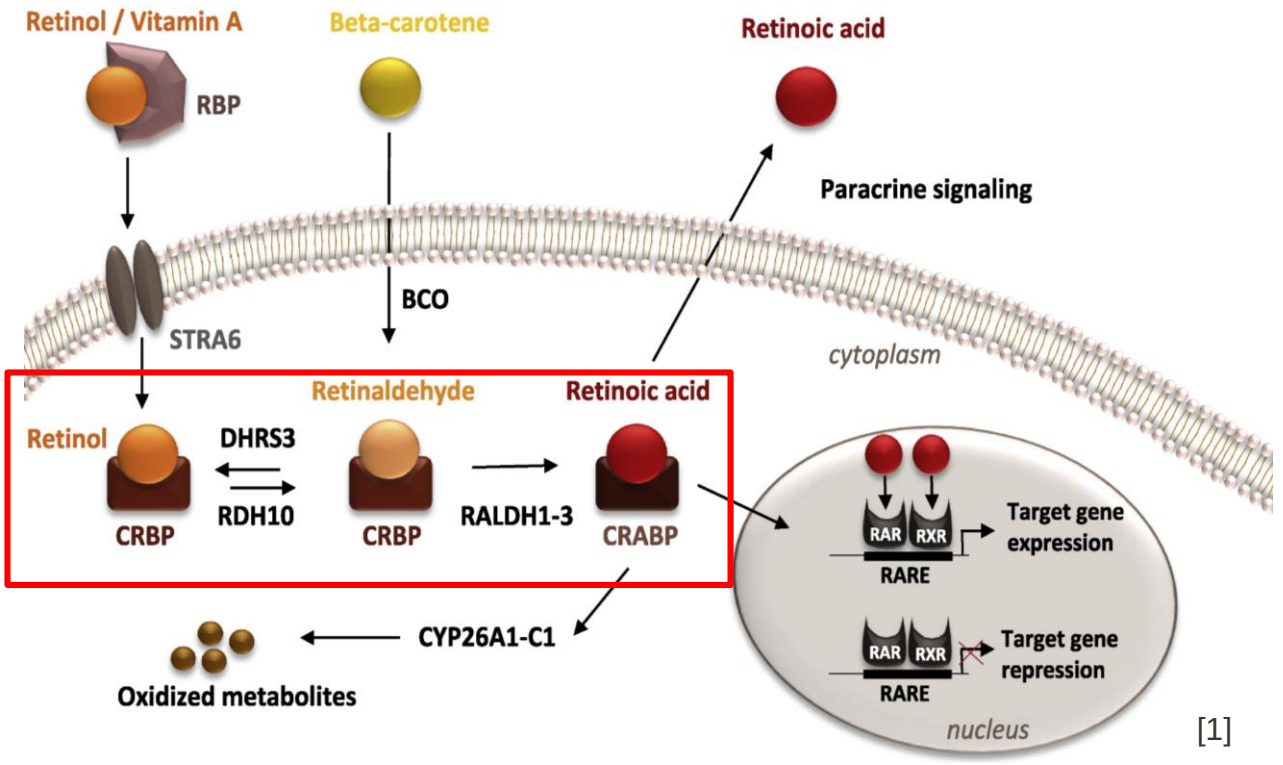
PTDF

Prediction of prenatal developmental toxicity using the zebrafish embryo model - retinoic acid AOPs

Bingxu Chen, Stefan Scholz, Tamara Tal, Wibke Busch

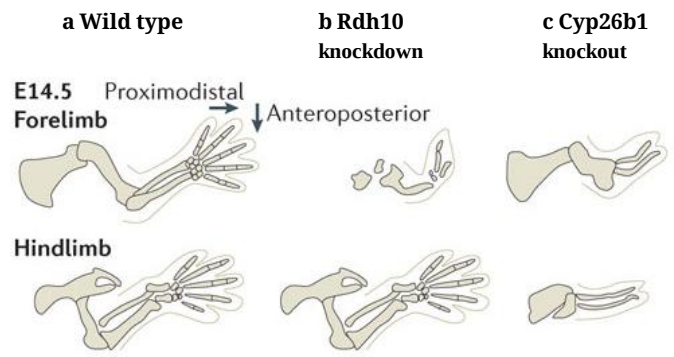
Introduction

- Retinoic acid (RA) regulates **embryonic development** (morphogenesis, organogenesis).
- Balance between **RALDH (synthesis) and CYP26 (metabolism)** is crucial.
- Hypothesis: **Zebrafish embryos exposed to chemicals** interfering with RA homeostasis, as well as **CRISPR/Cas9 F0 crispants**, will provide **biomarkers** that can be integrated into the adverse outcome pathway (AOP) framework to improve toxicity prediction.



[1]

Mouse study – limb defects[2]



Methods

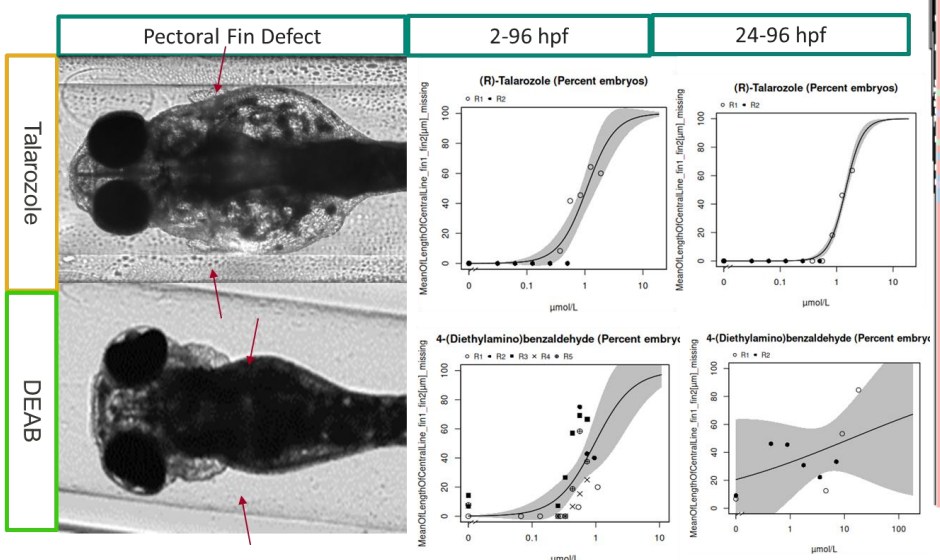
- Chemical exposure
- Gene knockdown
- Transcriptomics analysis

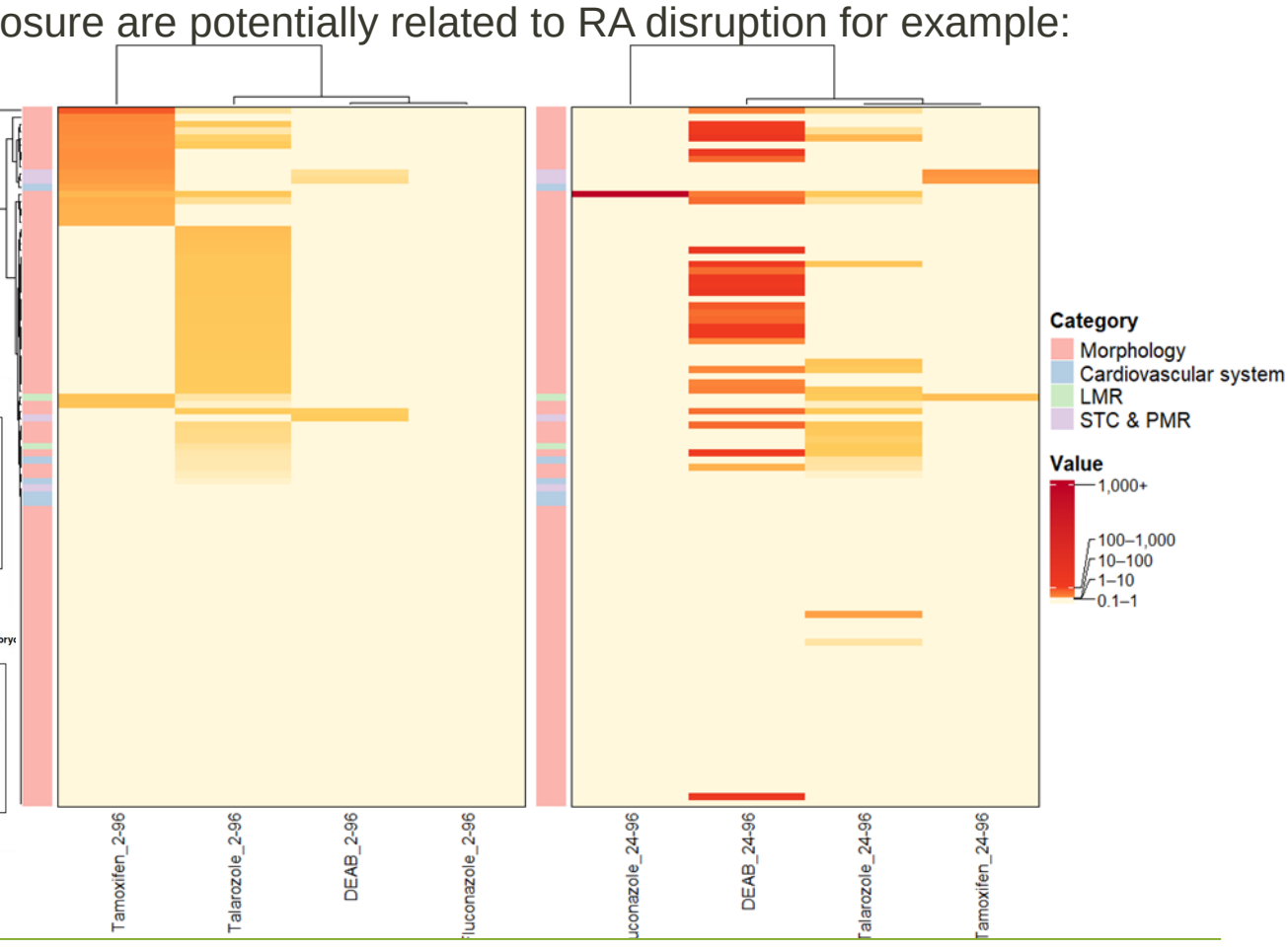
Objectives

- Identification of **model compounds.**
- **Quantitatively assess morphology** caused by model compounds (RALDH & CYP26 inhibitors) exposure.
- Apply **CRISPR/Cas knockdowns** of *aldh* and *cyp26* to build causal links between gene function and phenotypes.
- Characterize **transcriptomic changes** in **exposed zebrafish and CRISPR/Cas9 F0 crispants**.

Current Results

- **DEAB (**RALDH inhibitor**) and Talarozole** (CYP26 inhibitor) were identified as model compounds.
- **Many phenotypes** observed in chemical exposure are potentially related to RA disruption for example:
  - Pectoral fin defects
  - Caudal and ventral fin malformations
  - Curved tail
  - Cornea defects
  - Craniofacial malformations





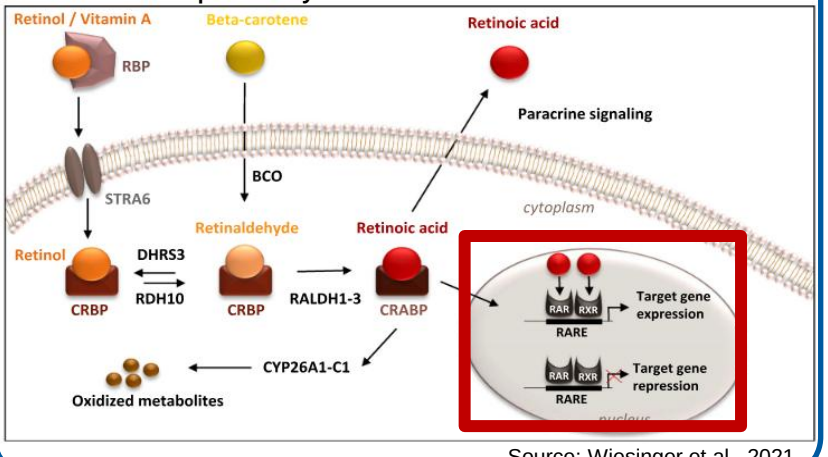
Reference

1. Wiesinger, A., et al., Retinoic acid signaling in heart development: Application in the differentiation of cardiovascular lineages from human pluripotent stem cells. Stem Cell Reports, 2021. 16(11): p. 2589-2606..
2. Cunningham, T.J. and G. Duester, *Mechanisms of retinoic acid signalling and its roles in organ and limb development.* Nature Reviews Molecular Cell Biology, 2015. 16(2): p. 110-123.

RetiNAM:
IDENTIFICATION OF SENSITIVE BIOMARKERS FOR DISRUPTION OF THE RETINOIC ACID SIGNALING PATHWAY

BACKGROUND

The retinoic acid signaling pathway as a target of endocrine disruption has been of growing interest recently highlighting the need for validated assays. Current standardized test methods for endocrine disruption only focus on EATS (estrogen, androgen, thyroid, steroidogenesis) modalities, neglecting the retinoic acid pathway.



Source: Wiesinger et al., 2021

PROJECT

Literature review

Decision on model chemicals & evaluation of the broader ecotoxicological relevance of the retinoic acid signaling pathway

High Content Screening

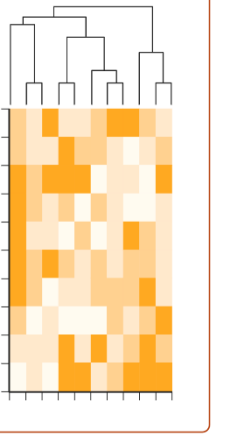
Morphological effects

Behavioral Fingerprinting
Acute neuroactivity & Developmental neurotoxicity (DNT)

Transcriptomics

Changes in gene expression

Quantification & Automated Pattern Analysis



Laboratory experiments:

Model organism:
Zebrafish larvae

Model compounds:
Agonists and antagonists of RAR and RXR, environmentally relevant chemicals

RESULTS

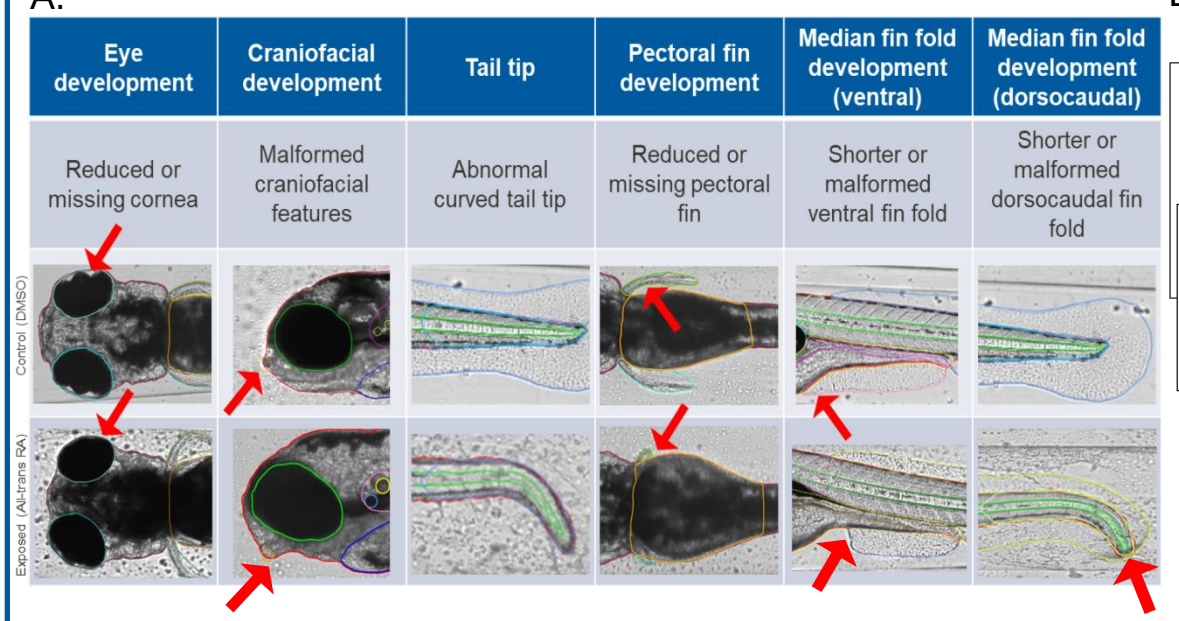
Global evidence for RA pathway disruption in the environment: RA pathway disruption was observed by several classes of chemicals in several species.

RAR and RXR agonists and antagonists cause similar and specific effects on morphology: Exposure causes eye and craniofacial malformations, a bent tailtip and fin defects in zebrafish larvae.

RAR and RXR agonists and antagonists cause similar and specific effect during acute neuroactivity testing: Hyperactivity in quiescent endpoints, effects on habituation and memory, early decrease of motoractivity and decreased startle responses.

A.

Eye development	Craniofacial development	Tail tip	Pectoral fin development	Median fin fold development (ventral)	Median fin fold development (dorsocaudal)
Reduced or missing cornea	Malformed craniofacial features	Abnormal curved tail tip	Reduced or missing pectoral fin	Shorter or malformed ventral fin fold	Shorter or malformed dorsocaudal fin fold



B.

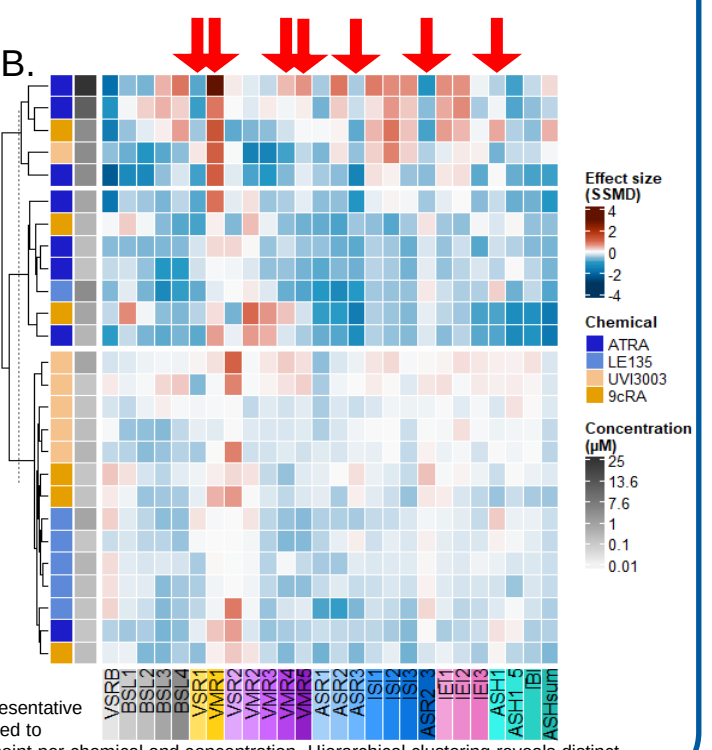


Figure 1: Disruption of the retinoic acid signaling pathway induces morphological and behavioral changes in zebrafish embryos. A. Representative images showing morphological phenotypes in zebrafish embryos. Upper row: control embryo (0.1 % DMSO); lower row: embryos exposed to all-trans retinoic acid. B. Heatmap of visual and acoustic motor response assay (VAMR) endpoints showing SSMD values for each endpoint per chemical and concentration. Hierarchical clustering reveals distinct behavioral profiles.

OUTLOOK

- Testing for **developmental neurotoxicity** using the VAMR assay
- **Additional environmental and reference chemicals** in the VAMR assay
- Concentration-dependent **transcriptome analysis**,
- Data integration & **pattern analysis**

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[2] Teixidó, E.; Kießling, T. R.; Klüver, N.; Scholz, S. (2022): Grouping of chemicals into mode of action classes by automated effect pattern analysis using the zebrafish embryo toxicity test. In: Archives of toxicology 96 (5), S. 1353–1369. DOI: 10.1007/s00204-022-03253-x.

[3] Leuthold, David; Herold, Nadia K.; Nerlich, Jana; Bartmann, Kristina; Scharkin, Ilka; Hallermann, Stefan J. et al. (2024): Multi-behavioral phenotyping in zebrafish identifies a novel disruptor of non-associative learning with environmental and human relevance.