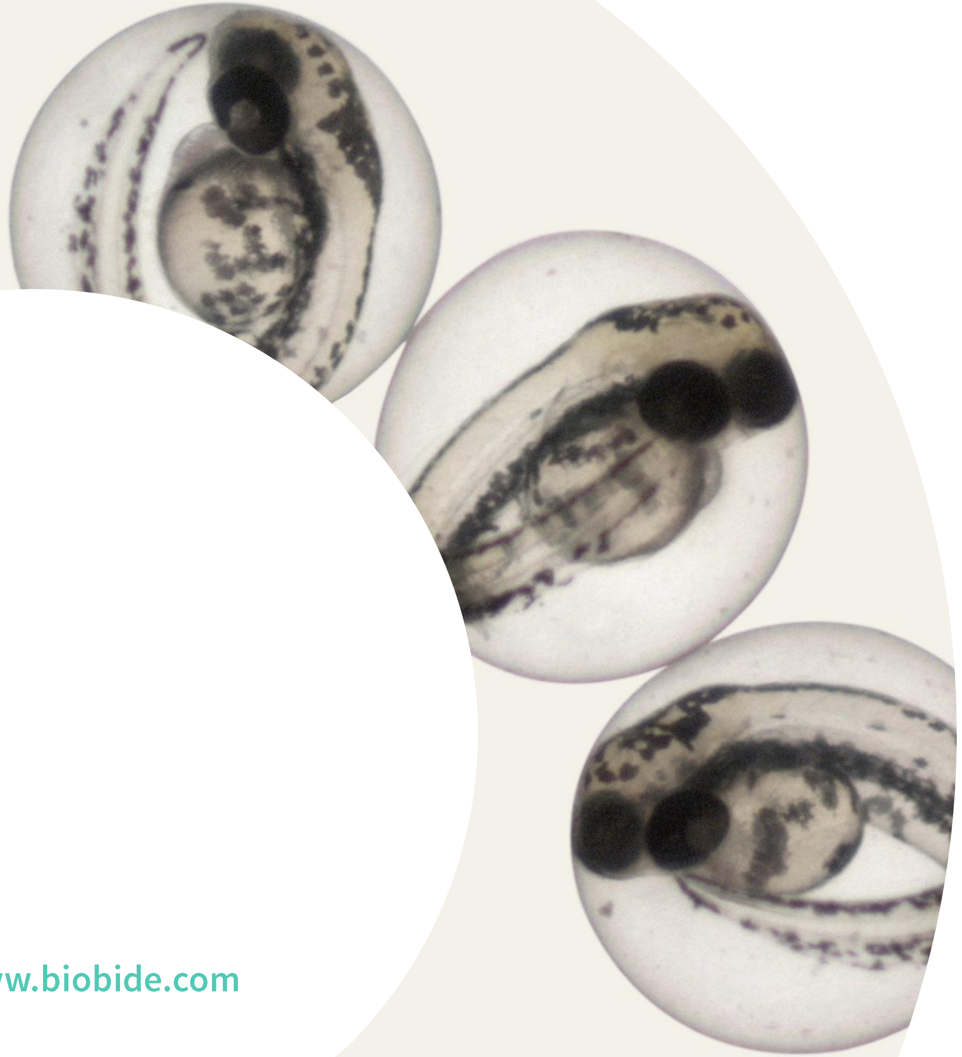

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An Inter-laboratory Case Study to Harmonize Zebrafish Light-Dark Transition Test to Predict Developmental Neurotoxicity

YOUR
ZEBRAFISH
PARTNER

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The Problem: Regulatory need for DNT

Current test guidelines: OECD-426 and 443

- Very expensive and time consuming
- Human relevance unknown
- Only 150 compounds tested

Need for integrated testing battery for DNT

- Screening more chemicals
- As a first screening step for prioritisation
- Human relevance is essential
- Based on mechanistic knowledge and AOPs

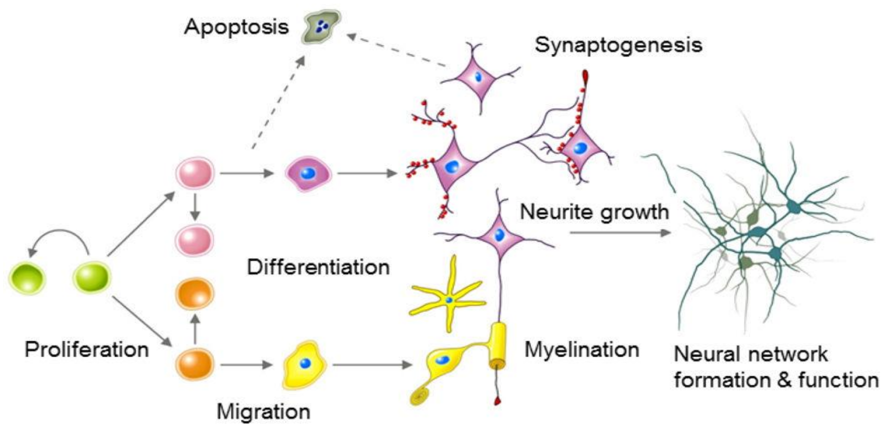
OECD develops a guidance document for DNT

Background OECD guidance document (GD) - IATA

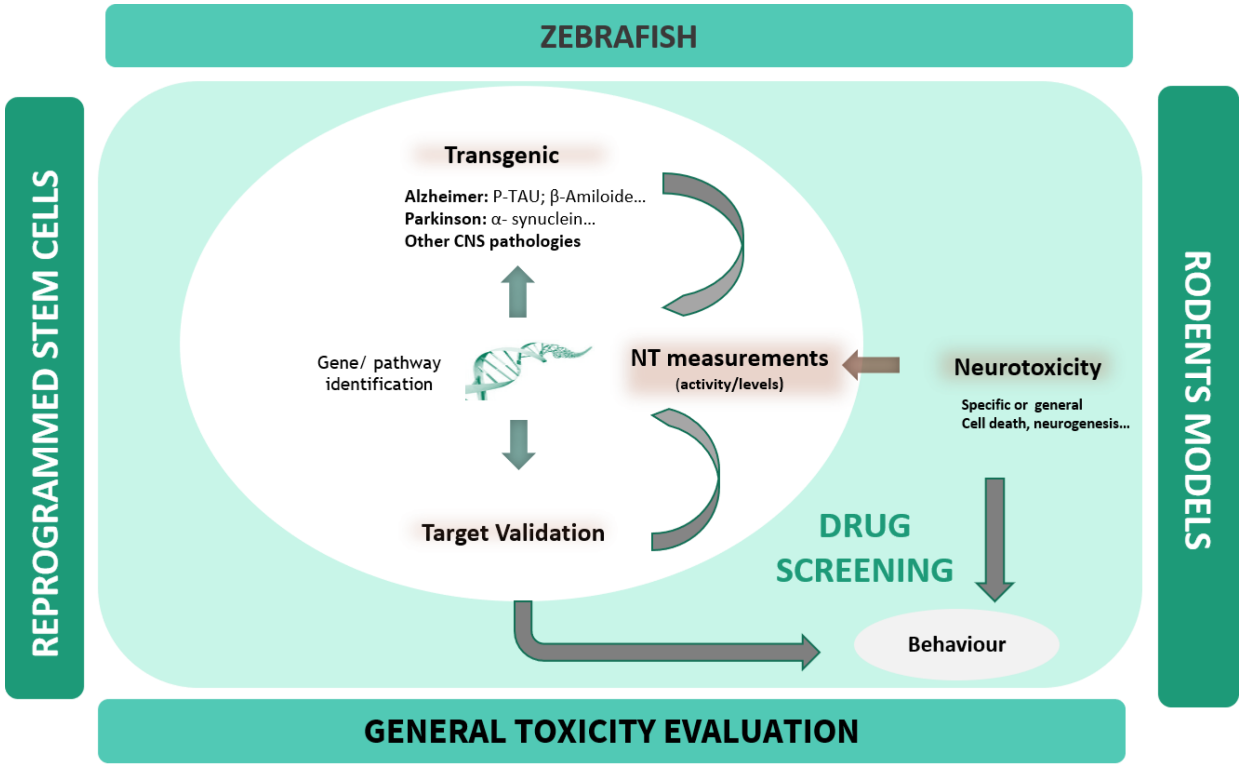
- Aim: description of an *in vitro test battery* for developmental neurotoxicity based on key processes:

How data could be interpreted and used to assess DNT

- Outline an integrated approach to testing and assessment (IATA) for the purposes for screening and prioritisation or for hazard assessment.
- The Zebrafish behavioral assay could be one of the *tests.*



Aschner *et al.*, 2017



OECD Consortium Goals

- Determine the **critical points** of Zebrafish behavioral assay
- Design a **harmonized protocol**
- Re-evaluate the variables affecting the **inter-laboratory reproducibility**
- Establish a definitive Developmental Neurotoxicity **(DNT) assessing protocol**
- Determine the added value of the golden standard Light/Dark (L/D)Transition Assay in Zebrafish inside the in vitro battery of assays (IVB) for the OECD Guidance document.
- Same procedure for other Zebrafish assays

DNT IATA CASE Test Method for Zebrafish-Inter-laboratory Validation		
PHASE 1 **Dose-range finding**	**PHASE 2** **Behavioral Assay: test and optimization**	**PHASE 3** **Definitive study**

Chemical groups tested:
19 Pesticide
1 Drug
9 Flame Retardant

Added value of zebrafish to Guidance document /IATA

- High genetic homology with human > 80%

- Fast development / organogenesis
- Small size
- External fertilization and embryogenesis
- High productivity: 100-300 eggs/couple + week
- Direct administration of compounds to the medium of embryos
- Transparent embryos

- Low cost
- Fewer ethical impediments

Suitability of the model for human assays:

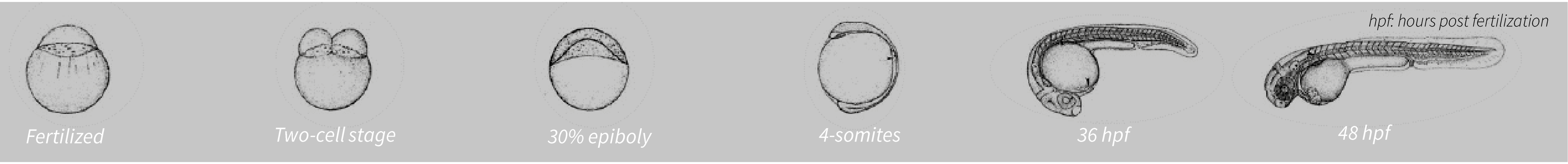
- Suitable screening for human drugs
- Models for efficacy and organ specific toxicity screens

Easy manipulation for assay development:

- Easily sourced model
- Statistically significant result, with small quantity of drug
- Visualization of results by dyes (fluorescence, antibodies, etc.)
- Suitable for applying automation, integration and image analysis for Phenotypic Screening

Wide utility:

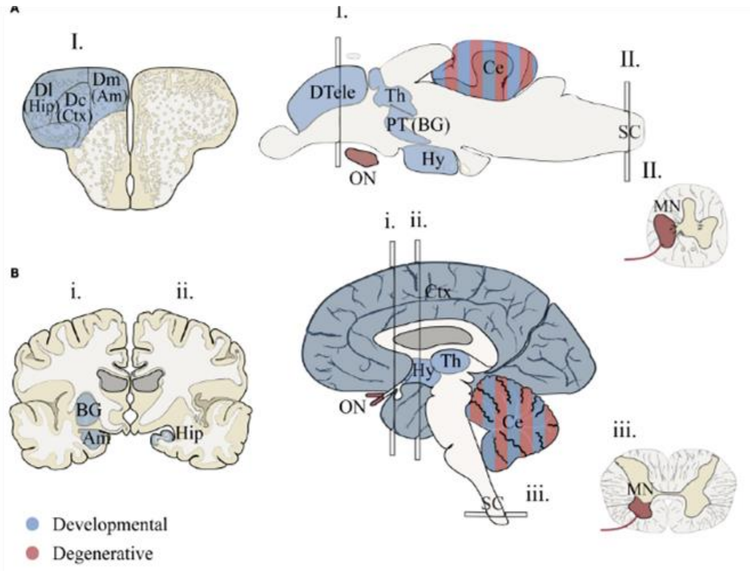
- Cost / time efficiency
- Highly informative results



Stages of embryonic development of the zebrafish. *Kimmel et al. 1995*

Added value of zebrafish for DNT

- Complete and complex neuronal network (brain)
- Occurs within 3 days post-fertilization (dpf)
- Brain regions are well-conserved
- Neuronal subtypes well-conserved
- Zebrafish develops a Blood Brain Barrier (BBB)
- Thyroid axis is present and affects brain development

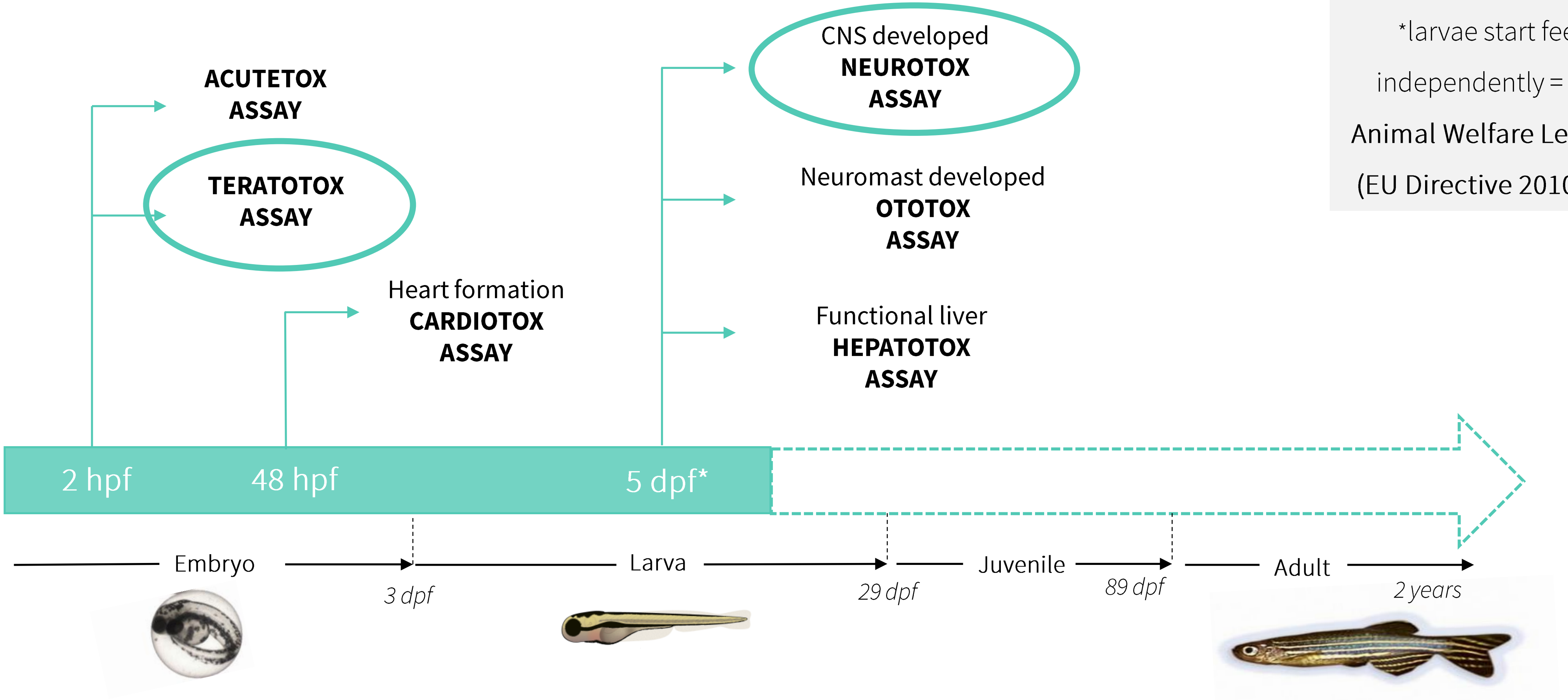


Zebrafish

Human

Kozol *et al.*, 2016

Zebrafish Developmental Process



*larvae start feeding independently = animal Animal Welfare Legislation (EU Directive 2010/63/EU)

hpf: hours post fertilization; dpf: days post fertilization

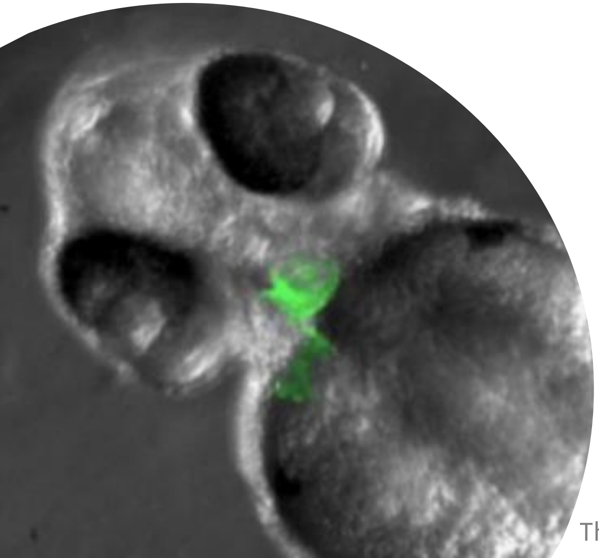
Zebrafish Developmental Toxicity Assay

METHOD DESCRIPTION

✓ Experimental model

Zebrafish embryos strain expressing a **green fluorescent protein** in the heart

The study of the potential induction of developmental defects is desigend in two phases:



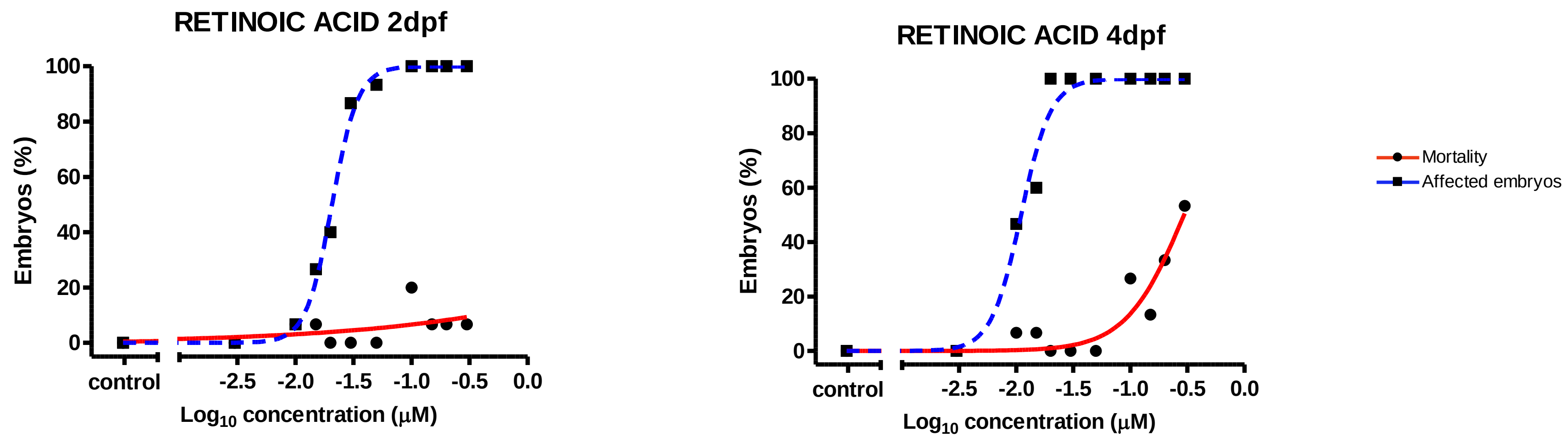
1. Dose Range Finding (DRF)
2. Developmental Toxicity Assay

Zebrafish Developmental Toxicity Assay

- Specific endpoints analyzed at 2 dpf and 4dpf in the Developmental Toxicity Assay

		2dpf	4dpf
Malformation of the head	Jaw morphology		X
	Microcephaly or abnormal head shape	X	X
	Microphthalmia/Cyclopia	X	X
	Edema	X	X
Malformation of the otoliths			X
Malformation of the heart	Edema/irregular shape	X	X
	Abnormal heartbeat	X	X
Deformed body shape	Length	X	X
	Curved/curled	X	X
	Notochord morphology	X	X
	Somite morphology	X	X
Malformation of the tail (including tail fins)		X	X
Yolk deformation	Edema	X	X
	Yolk opacity	X	X
Other		X	X

Zebrafish Developmental Toxicity Assay



EC50 and LC50 is calculated and a Teratogenic Index (TI) established (ratio between LC50 and EC50) at 2 and 4 dpf to conclude about the teratogenic potential of each compound:

- Likely teratogenic: T(2) ≥ 2
- Toxic but not teratogenic: TI(2) < 2
- Not toxic in zebrafish embryos

→ *The higher the TI(2) value obtained, the higher probability of teratogenic effect*

Zebrafish Developmental Toxicity Assay

INTERNAL VALIDATION DATA

REFERENCE PRODUCTS	Classification*	Biobide Classification
Difenoconazole	Not teratogenic	NEGATIVE
Tebuconazole	Teratogenic	NEGATIVE
Penconazole	Teratogenic	NEGATIVE
Epoxiconazole	Teratogenic	POSITIVE
Flusilazole	Teratogenic	POSITIVE
Cyclopamine	Teratogenic	POSITIVE
Diniconazole	Teratogenic	POSITIVE
Voriconazole	Teratogenic	POSITIVE
Triadimenol	Teratogenic	POSITIVE
Myclobutanil	Teratogenic	POSITIVE
Metconazole	Teratogenic	POSITIVE
Propiconazole	Teratogenic	POSITIVE
Ipconazole	Teratogenic	POSITIVE

**based on ECHA, CHP and other sources classification*

Internal validation testing agrochemicals tested in the Developmental Toxicity Assay:

- Sensitivity: 83%
- Specificity : 100%

TRUE POSITIVE

TRUE NEGATIVE

FALSE POSITIVE

FALSE NEGATIVE

Zebrafish Developmental Toxicity Assay

REFERRAL DATA

- Case Study 1: NIH/NIEH Pilot trial of 10 compounds for the evaluation of developmental toxicity, behavior alteration and other toxicities in zebrafish embryos.

  ✓ Summary of the evaluation of developmental toxicity:

Test item identification	NOAEL (µM)		EC50		LC50		TI	
	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf
2-Ethylexhyl diphenyl phosphate	>20	3	-	5.06 (4.89 to 5.24)	-	9.78 (very wide)	-	1.93
2,2'4,4'-Tetrabromodiphenyl ether	>25	2	-	12.01 (8.44 to 17.11)	-	>25	-	>2.08
3,3',5,5'-Tetrabromobisphenol A	1.5	1	1.81 (1.76 to 1.86)	1.48 (very wide)	3.26 (very wide)	1.9 (1.88 to 1.92)	1.8	1.28
Isodecyl diphenyl phosphate	300	20	474.3 (408.3 to 550.8)	77.23 (57.77 to 103.2)	>600	>600	>1.26	>7.77
Phenol, isopropylated, phosphate (3:1)	1	<1	4.3 (3.66 to 5.05)	1.8 (1.31 to 2.47)	>100	12.82 (11.97 to 13.73)	>23.26	7.12
tert-Butylphenyl diphenyl phosphate	8	4	11.45 (10.56 to 12.42)	4.75 (0.086 to 263.1)	84.15 (80.72 to 87.72)	15.24 (12.33 to 18.84)	7.35	3.21
Tricresyl phosphate	8	2	11.48 (11.4 to 11.56)	3 (2.78 to 3.24)	143.8 (107.2 to 192.9)	9.52 (9.46 to 9.57)	12.53	3.17
Triphenyl phosphate	2	1	3.84 (3.41 to 4.33)	1.72 (1.61 to 1.84)	15.11 (very wide)	5.15 (interrupted)	3.93	2.99
Tris(2-chloroethyl) phosphate	400	400	521.2 (462.8 to 587)	415.2 (very wide)	>1000	977.6 (very wide)	>1.92	2.35
Tris(1,3-dichloro-2-propul) phosphate	3	2	4.11 (3.68 to 4.58)	3.08 (2.79 to 3.4)	8.29 (7.15 to 9.61)	6.53 (5.07 to 8.4)	2.02	2.12

Alzualde A., et al. 2018

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Zebrafish Developmental Toxicity Assay

REFERRAL DATA

- Case Study 2: screening of 32 compounds of interest to the National Toxicology Program (NTP) with known or hypothesized developmental toxicity or neurotoxicity for an overall assessment of systems toxicity in zebrafish embryos

  ✓ Compounds with suspected developmental toxicity

LogP	COMPOUND NAME	DEVELOPMENTAL TOXICITY				CARDIOTOXICITY		BEHAVIOR		HEPATOTOXICITY		OTOTOXICITY	
		2 dpf EC_{50}/LC_{50}	4 dpf EC_{50}/LC_{50}	TI class.	Internal dose	Effect	Conc	Effect	Conc	Effect	Conc	Effect	Conc
4.68/5.08	Methoxyclor	9.34/>100	1.01/3.28	1	>100%	texto	*3*	neuroactive	from 0.1	-	*10*	-	*1*
4.1/5.14	Vinpocetine	0.42/8.70	0.36/4.96	1	>100%	cardiotoxic	from 1	hypoactivity	from 1	-	*10*	-	*1*
4.50/4.72	Dibutyl phthalate	2.09/5.29	1.81/2.93	1	>100%	-	*30*	neuroactive	10	-	*10*	-	*10*
4.47	Bisphenol AF	10.44/27.97	4.12/8.53	1	>100%	bradycardia	from 10	hypoactivity	from 3	-	*10*	-	*10*
5.8	Di-n-Pentyl phthalate	7.54/18.14	4.27/4.75	1	13-18%	cardiotoxic	from 30	-	*10*	hepatotoxic	10	ototoxic	100
4.9	HPTE	20.01/42.41	7.97/15.43	1	>100%	bradycardia	from 10	hypoactivity	10	-	*10*	-	*10*
3.2	Linuron	18.23/41.77	15.05/29.16	1	>100%	bradycardia	from 10	-	*10*	-	*10*	-	*30*
2.9*	Trypan blue	*(2000)*	1064/1558	2	N.D.	-	*100*	hypoactivity	from 100	-	*1000*	-	*100*
-0.07	Caffeine	318.6/>2000	126.2/>2000	1	~100%	-	*100*	hypoactivity	from 300	-	*1000*	-	*100*
2.75/2.8	Valproate	289.8/1961	173.2/744.6	1	4-10%	-	*100*	-	*300*	-	*300*	-	*100*
1.46	Prednisone	*(1000)*	*(750)*	-	0.1%	-	*100*	neuroactive	200	-	*2000*	-	*100*
0.23	4-Methylimidazole	*(2000)*	1518/>2000	3	~100%	-	*100*	neuroactive	2000	hepatotoxic	1000	-	*100*
1.19	Aspirin	1656/>2000	1555/1726	2	1-2%	-	*100*	-	*2000*	hepatotoxic	2000	-	*100*
0.175/-1.9	Boric acid	*(2000)*	*(2000)*	-	>100%	-	*100*	-	*2000*	-	*2000*	-	*100*
0	Trimethadione	*(2000)*	*(2000)*	-	1%	-	*100*	-	*2000*	-	*2000*	-	*100*
-1.1	Gabapentin	*(2000)*	*(2000)*	-	0.5%	-	*100*	-	*2000*	-	*2000*	-	*100*
0.1	Dimethadione	*(2000)*	*(2000)*	-	3%	-	*100*	-	*2000*	-	*2000*	-	*100*
-0.31/-0.32	Ethanol	*(2000)*	*(2000)*	-	N.D.	-	*100*	-	*2000*	-	*2000*	-	*100*

N.D.: not detected; Italic numbers indicate the maximum concentration tested without effect

Effect described in other animal models or humans

Quevedo C. et al. 2018.

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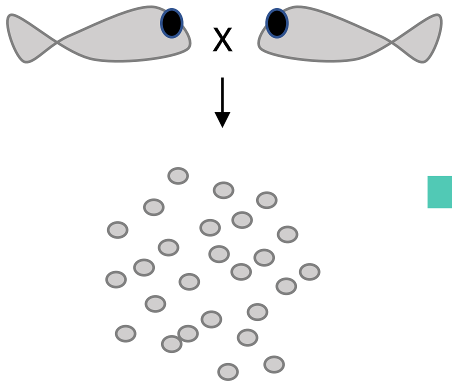
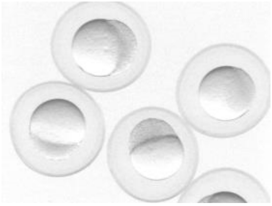
Behaviour Alteration Assay

Crossing adults

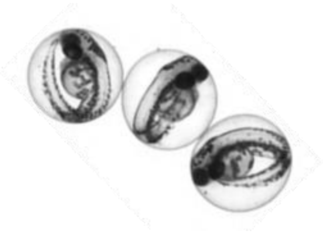
Embryo growing

Treatment





3 – 5 hpf

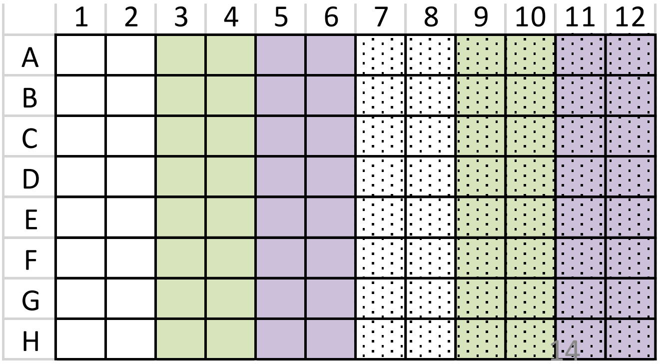


24 hpf

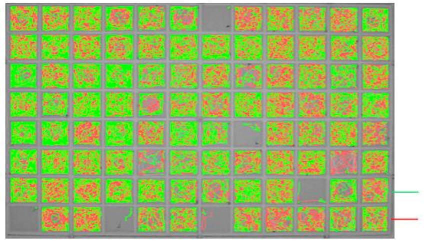
96 hpf

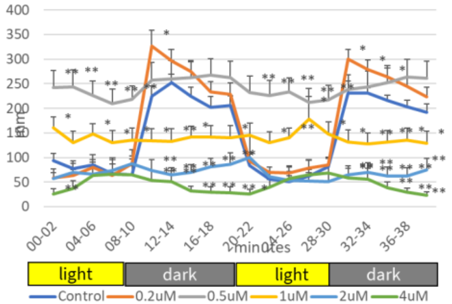
120 hpf

5 dpf



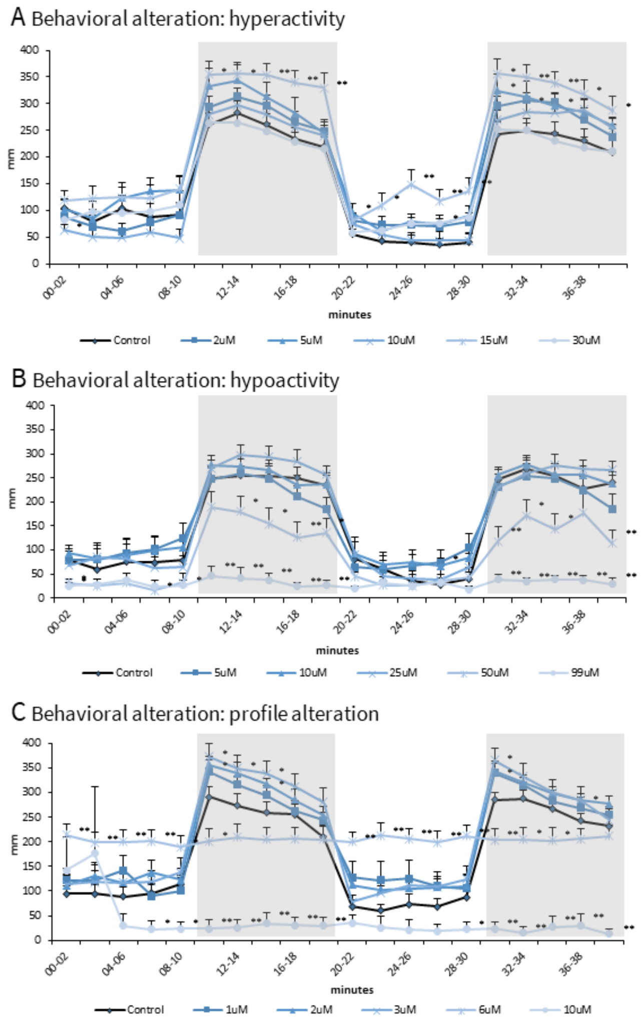
Automated tracking





Behaviour Alteration Assay

- Zebrafish are a diurnal specie
- Regulated by Homeostatic and circadian mechanisms
- Highly conserved circadian system
- Melatonin: principal hormone involved
  - 1dpf → *aanat2* gen expression and melatonin synthesis.
  - 2dpf → circadian clock-controlled rhythms.
  - 4dpf → stable diurnal rhythm of locomotor activity.
- Detectable effects in treated embryos:
  - Basal activity with light and ↑ activity when lights off, but habituation to darkness.
  - Hypoactivity mainly in dark periods
  - Hyperactivity mainly in light periods
  - Reaction to environmental changes (lighting): hypoactivity / hyperactivity



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Neurotoxicity Assay

INTERNAL VALIDATION DATA

Internal validation testing chemicals in the Standard Neurotoxicity Assay:

- SENSITIVITY : 95%
- SPECIFICITY: 100%

COMPOUND	Therapeutic Classification	Adverse effects in human CNS	Results
5-Fluorouracil	Antineoplasic-cytotoxic	-	TN
Acetaminophen	Analgesic-Antipyretic	-	TN
Acetylcysteine	Mucolytic	-	TN
Artemisinin	Antimalaric	+	TP
Ascorbic Acid	Antioxidant	-	TN
Carbamazepine	GABA enhancing anxiolytic	+	TP
Chlorambucil	Alkylating antineoplastic	+	TP
Chloroquine	Antimalaric	+	TP
Dexamethasone	Anti-inflammatory	+	FN
Dieldrin	GABA receptors antagonist	+	TP
Disopyramide	Anti-arrhythmic	+	TP
Dopamine	Neurotransmitter	-	TN
Fluoxetine	SSRI antidepressant	+	TP
Foscarnet	Antiviral	+	TP
Halofantrine	Antimalaric	+	TP
Haloperidol	Antipsychotic	+	TP
Indirubin-3'-oxime	CDKs and GSK3B inhibitors	+	TP
Mefloquine	Antimalaric	+	TP
MPTP	Neurotoxin	+	TP
Norepinephrine	Hormone / Neurotransmitter	-	TN
PTZ	GABA antagonist	+	TP
Sotalol	Anti-arrhythmic	-	TN
Sucrose	Negative control	-	TN
Tacrine	Anticholinesterase	+	TP
Tetracycline	Antibiotic	+	TP
Thalidomide	Immunomodulatory	+	TP
Valproic Acid	Anticonvulsant	+	TP
Warfarin	Anticonvulsant	-	TN

TRUE POSITIVE

TRUE NEGATIVE

FALSE POSITIVE

FALSE NEGATIVE

Zebrafish Developmental Toxicity Assay

REFERRAL DATA

- Case Study 3: Developmental toxicity and Neurotoxicity of 91 compounds from NTP assessed blinded using zebrafish embryos.

Number of chemicals		Classification		
		Toxic	Neurotoxic	Not detected toxicity
Suspected to be neurodevelopmental toxic	29*	**89,6%** *(26/29)*	**48,3%** *(14/29)*	10,4% *(3/29)*
Chemicals with unknown effect	35*	**94,3%** *(33/35)*	**25,79%** *(9/35)*	5,7% *(2/35)*
Negative controls	5*	0%	0%	**100%** *(5/5)*

Alzualde A. et al. 2018.

Zebrafish Developmental Toxicity Assay

REFERRAL DATA

- <u>Case Study 3:</u>
- Developmental toxicity detected in n = 35/ 91, three in duplicates.
- 37.1% of test items also neuroactive (one in duplicate) or neurotoxic.

Test item identification			Neurotoxicity		Developmental toxicity			
			Effect	LOAEL (μM)	Classi-fication	LOAEL treat conc	LOAEL embryo conc	
1937	PE-2028	Benzo(k)fluoranthene	neuroactive	0.5	1	0.1	2.67	
1814	PE-2067	Permethrin	neuroactive	0.5	1	2	14.26	
1109	PE-2014	Acenaphthene	neuroactive	15	1	30	60.78	
1324	PE-2061	Lindane	neuroactive	0.5	1	4	504.7	
1092	PE-2050	Dieldrin	neuroactive	0.05	1	0.5	567.8	
1401	PE-2049	Dichlorodiphenyltrichloroethane (DDT)	neuroactive	0.5	1	2	1052	
1994	PE-2066	Parathion	neuroactive	8	1	8	1531	
1453	PE-2055	Heptachlor	neuroactive	0.5	1	8	2068	
1918	PE-2040	Chlorpyrifos (Dursban)	neuroactive	1	1	5	2262	
1445	PE-2044	Deltamethrin	neuroactive	0.025	1	0.05	ND	x2
1174	PE-2086	Deltamethrin	neuroactive	0.005	1	0.1	ND	
1705	PE-2046	Diazepam	toxic	15	1	8	47.73	
1262	PE-2075	Tebuconazole	toxic	10	1	20	556.4	
1215	PE-2077	Tetraethylthiuram disulfide	toxic	0.3	1	0.3	ND	
1701	PE-2060	Lead (II) acetate trihydrate	toxic	5	1	2	32.33	
1798	PE-2053 -1	Firemaster 550	toxic	2	1	1	175.2	
	PE-2053 -2						275.8	
	PE-2053 -3						107.8	
1664	PE-2063	Methyl mercuric (II) chloride	toxic	1	1	0.3	114.4	
1365	PE-2089	Triphenyl phosphate	toxic	2	1	1	125.5	x2
1241	PE-2081	Triphenyl phosphate	toxic	1	1	2	143.4	
1966	PE-2009	3,3',5,5'-Tetrabromobisphenol A	toxic	1.5	1	1.5	201.4	
1121	PE-2080	Tricresyl phosphate	toxic	10	1	10	622.5	
1910	PE-2076	tert-Butylphenyl diphenyl phosphate	toxic	2	1	4	1275	
1766	PE-2072	Pyrene	toxic	2	1	2	1643	
1864	PE-2032	Bis(tributyltin)oxide	toxic	0.1	1	0.05	ND	x2
1578	PE-2039	Carbaryl	toxic	4	1	4	ND	
1647	PE-2071-1	Phenol, isopropylated, phosphate (3:1)	no toxic	>0.5	1	0.5	105.2	
	PE-2071-2						83.83	
	PE-2071-3						19.14	
1116	PE-2051	Diethylstilbestrol	no toxic	>1	1	1	35.34	
1205	PE-2034	Bisphenol AF	no toxic	>3	1	2	62.91	
1118	PE-2087	Methyl mercuric (II) chloride	no toxic	>0.5	1	0.2	75.98	
1424	PE-2052	Estradiol	no toxic	>10	1	10	472.5	
1614	PE-2015	Acenaphthylene	no toxic	>15	1	15	887.8	
1713	PE-2033	Bisphenol A	no toxic	>20	1	15	1247	
1532	PE-2068	Phenanthrene	no toxic	>15	1	10	2270	
1427	PE-2010	4-H-Cyclopenta(d,e,f)phenanthrene	no toxic	>10	1	8	2403	
1831	PE-2007	2,3,7,8-Tetrachlorodibenzo-p-dioxin	no toxic	>0.0003	1	0.0003	ND	
1231	PE-2020	Aldicarb	no toxic	>1	1	1	ND	
1090	PE-2047	Dibenz(a,h)anthracene	no toxic	>2	1	0.3	ND	
1051	PE-2023	Auramine O	no toxic	>5	1/2	3	87.85	

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Zebrafish Developmental Toxicity Assay

REFERRAL DATA

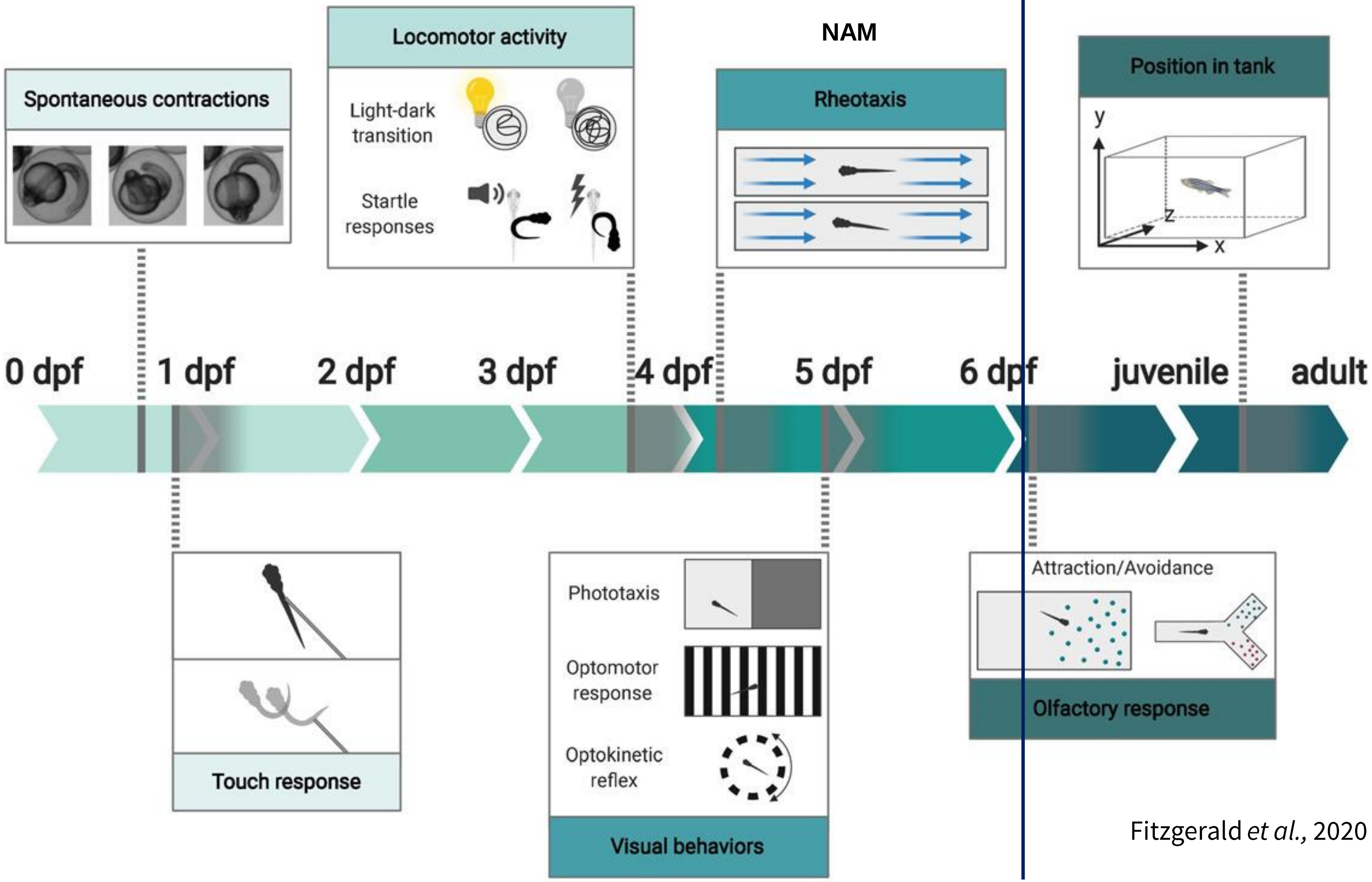
- Case Study 3:

- 9/ 91 were toxic but not teratogenic compounds.
- 33.3% were neuroactive compounds
- Limited uptake except neuroactive compounds and TCPP
- 6/91 were inconclusive for developmental toxicity, due to precipitation event.

Test item identification			Neurotoxicity		Developmental toxicity		
			Effect	LOAEL (µM)	Classi-fication	LOAEL treat conc	LOAEL embryo conc
1881	PE-2054	Fluorene	neuroactive	4	2	20	1418
1196	PE-2001	2-Ethylhexyl diphenyl phosphate (EHDP)	neuroactive	3	2	10	852.3
1786	PE-2042	Colchicine	toxic	50	2	100	1.05
1208	PE-2037	Captan	no toxic	>50	2	75	ND
1707	PE-2038	Carbamic acid, butyl-, 3-iodo-2-propynyl ester	no toxic	>1	2	1	ND
1718	PE-2073	Rotenone	no toxic	>0.1	2	0.1	ND
1938	PE-2083	Valinomycin	no toxic	>0.075	2	0.05	ND
1499	PE-2085	tris(Chloropropyl) phosphate, TCPP	toxic	75	2	75	87.15
1501	PE-2056	Hexachlorophene	toxic	0.5	2	0.3	ND

Test item identification			Neurotoxicity		Developmental toxicity		
			Effect	LOAEL (µM)	Classi-fication	LOAEL treat conc	LOAEL embryo conc
1965	PE-2084	Valproic acid sodium salt	neuroactive	50	4	75	154.7
1367	PE-2006	2,2',4,4'-Tetrabromodiphenyl ether	neuroactive	2	4	20	780.3
1024	PE-2065	Naphthalene	no toxic	>100	4	100	5.16
1784	PE-2026	Benzo(b)fluoranthene	no toxic	>10	4	60	29.24
1655	PE-2024	Benz(a)anthracene	no toxic	>4	4	4	51.20
1301	PE-2058	Isodecyl diphenyl phosphate	toxic	50	4	50	229.8

Zebrafish tests - Endpoints



Fitzgerald *et al.*, 2020

Study Design for Neurodevelopmental Toxicity

We propose to assess the potential Developmental NeuroToxicity (DNT) effect of a number of products from the NTP's chemicals list

WORK FLOW

1. **DRF**
   Test concentrations 5 concentration range
   (Optional)

2. **Developmental toxicity**
   Test concentrations based on DRF or on previous data

3. **Neurotoxicity**
   Test concentrations based on Developmental toxicity

4. **Internal dosing**
   Test concentrations based on neurotoxicity

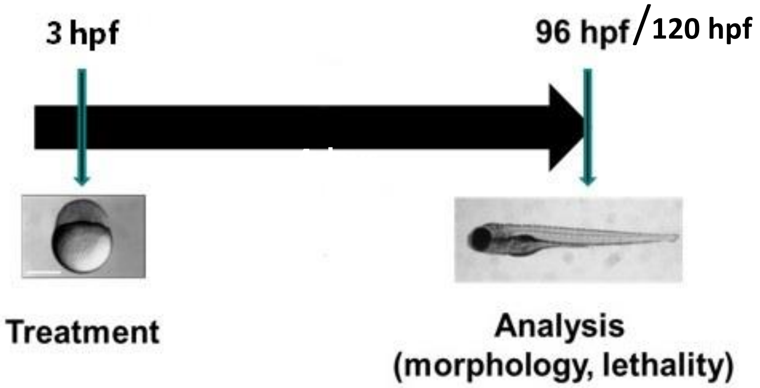
DNT Study Design

1 **DRF:** with ZF AB strain

5 embryos/well, 2 wells/conc., 5 conc/compound in P24,
n=10, DMSO=0.5% – 1%

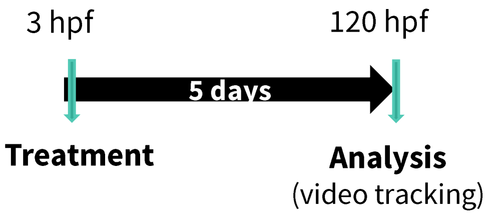
2 **Developmental toxicity**

3 hpf zebrafish embryos treated with 5-8 concentrations per compound, 15 embryos/condition in 96 well format
Static exposure and chorionated embryos – Fish Embryo Toxicity Test (FET) -----LOAEL identification



3 **Neurotoxicity**

Embryos at 3 hpf exposed to 5 concentrations/compound,
16 embryos/condition in 96 well plates, at28°C, 10hr:10hr (light:dark)



DanioVision

→ Locomotor activity + Gross morphology

4 **(Internal dosing)**

Determination of internal exposure to the chemicals by HPLC-MS or another appropriate technique.

Phenotypic Evaluation

2 Protocols

- FET - Fish Embryo Toxicity test (Phase 1)
- Light-dark transition test (Phase 2)

FET Assay
6 -120 hpf

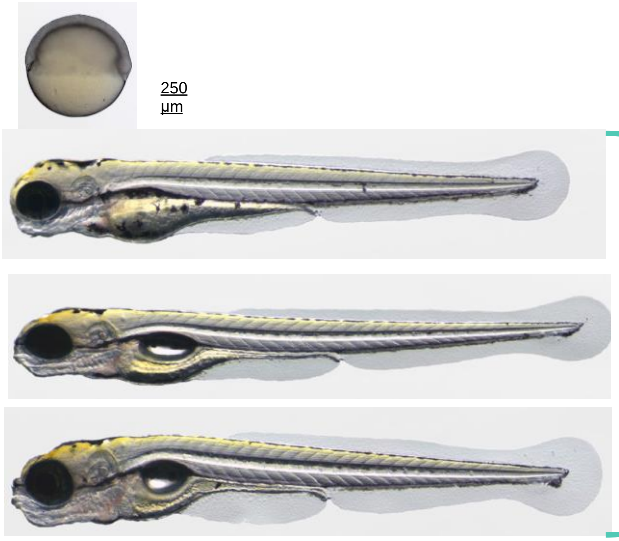
Phenotypic
observations 120 hpf

6 hpf

72 hpf

96 hpf

120 hpf



Phenotypic
observations 120 hpf

Behavioural 120 hpf
distance travelled in light dark “startle” response

- Danio rerio AB strain
- 96 well forma round vs squared
- **Chorionated** embryos - FET
- Buffered media
  (10 mM pH7.2 HEPES E3 media)
- **Static** exposure
- 28̊C, 14hr:10hr (light:dark)

The DNT ZF Group Members: International Experts

INSTITUTE/COMPANY	COUNTRY	PEOPLE
Biobide	Spain	Ainhoa Alzualde; Arantza Muriana
Division of the National Toxicology Program, NIH	USA	Jui-Hua Hsieh; Kristen Ryan; Mamta Behl
European Food Safety Authority (EFSA)	Italy	Andrea Terron (General coordinator)
Health Canada / Government of Canada	Canada	Cindy Woodland
Helmholtz Centre for Environmental Research GmbH - UFZ	Germany	Nils Klüver
National Institute for Public Health and the Environment (RIVM)	Netherland	Ellen Hessel (Coordinator Zebrafish Group)
National Research Council of Canada	Canada	Lee Ellis
Oregon State University	USA	Lisa Truong; Robyn Tanguay
Organization for Economic Co-operation and Development (OECD)	France	Magdalini Sachana
U.S. Environmental Protection Agency	USA	Bridgett Hill; Stephanie Padilla; Tim Shafer
VU University Amsterdam	Netherland	Jessica Legradi
ZeClinics	Spain	Valentina Schiavone; Davide Rubbini; Vincenzo Di Donato; Javier Terriente

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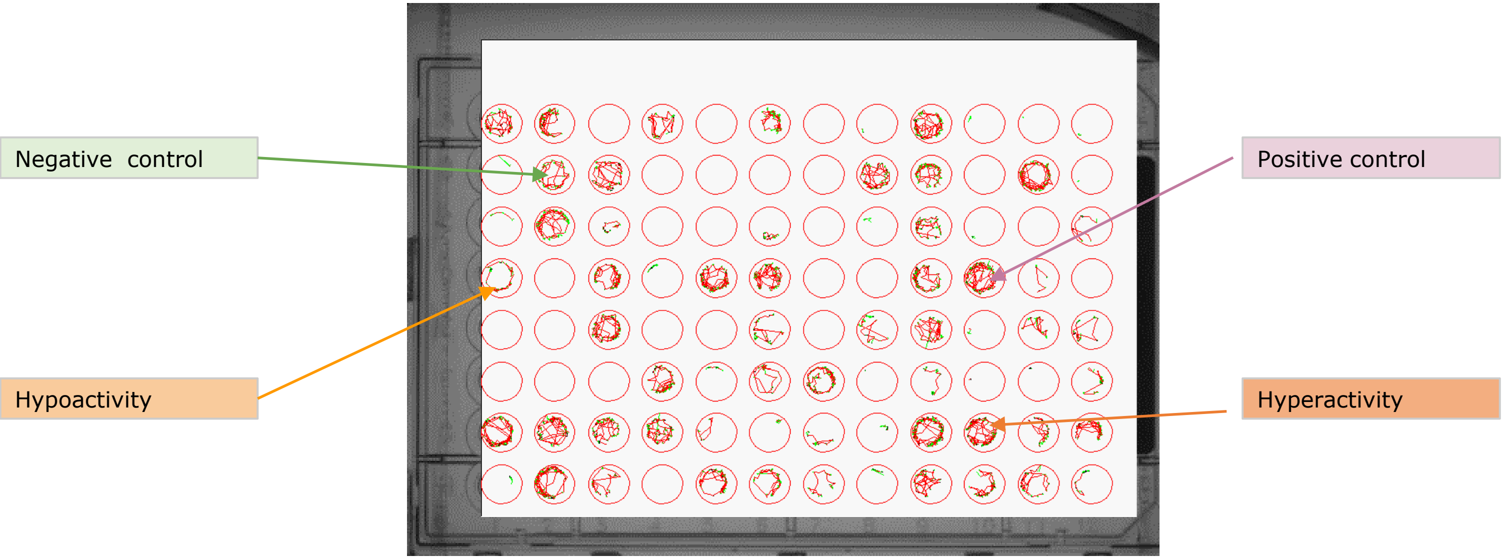
Phenotypic Evaluation

Experimental Task	Breeding	Drug Exposure		Malformation Evaluation & Behavioral Assay
Hours Post fertilization		6-8	24, 48, 72, 96	120
Critical Points		**Well-plate** 96 wells squared vs round 48 wells group/concentration Sealed vs Unsealed	**Exposure** Single dose vs Repeated dose	**Light/Dark Transition Test** 10′ on/10′ off; 10 min on; 20 min off Some labs additional 40′on/40′off

Readout



From Lisa Truong – Oregan State

Protocol: 28 Compounds

Chemical Name	CAS	Chemical Name	CAS
3,3',5,5'-Tetrabromobisphenol A	79-94-7	tert-Butylphenyl diphenyl phosphate	56803-37-3
Acetamiprid	135410-20-7	2-Ethylhexyl diphenyl phosphate	1241-94-7
Allethrin	584-79-2	Aldicarb	116-06-3
Benomyl	17804-35-2	Chloramben	133-90-4
Diazinon	333-41-5	Chlorpyrifos	2921-88-2
Heptachlor	76-44-8	Cypermethrin	52315-07-8
Nicotine	54-11-5	Deltamethrin	52918-63-5
Parathion	56-38-2	Dieldrin	60-57-1
Permethrin	52645-53-1	Dimethoate	60-51-5
Trichlorfon	52-68-6	Kepone	143-50-0
Triphenyl phosphates isopropylated	68937-41-7	Methyl parathion	298-00-0
Tris(1,3-dichloro-2-propyl) phosphate	13674-87-8	Thiacloprid	111988-49-9
Tris(2-chloroethyl) phosphate	115-96-8	Tri-o-cresyl phosphate	78-30-8
Tris(2-chloroisopropyl)phosphate	13674-84-5	Tris(methylphenyl) phosphate	1330-78-5

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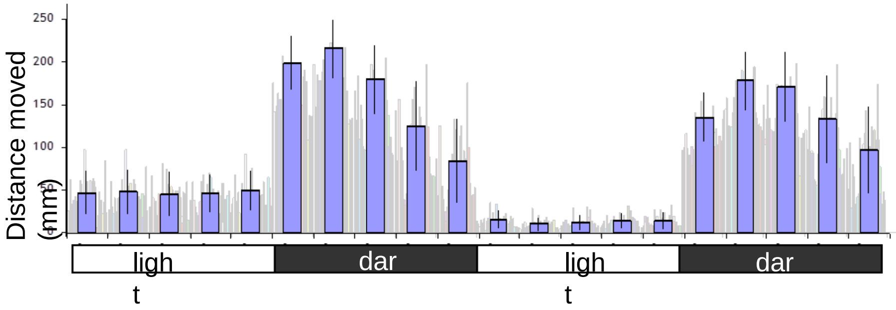
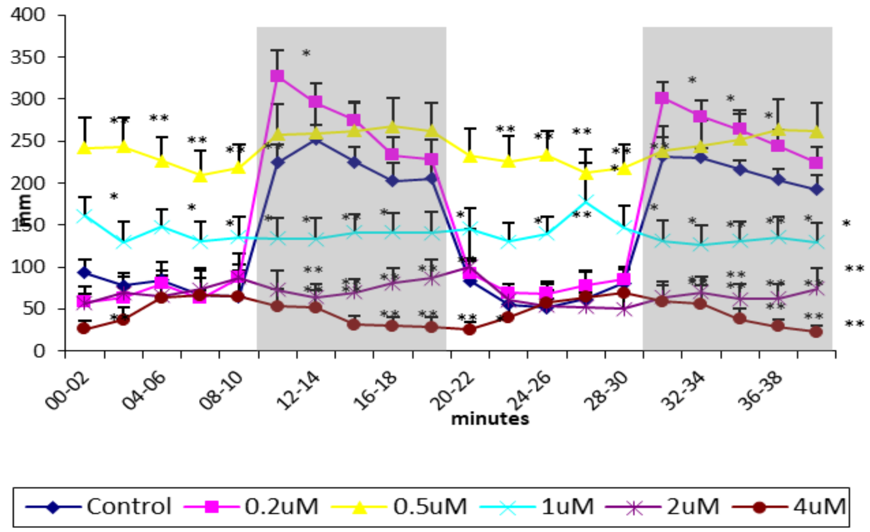
Example of the data obtained

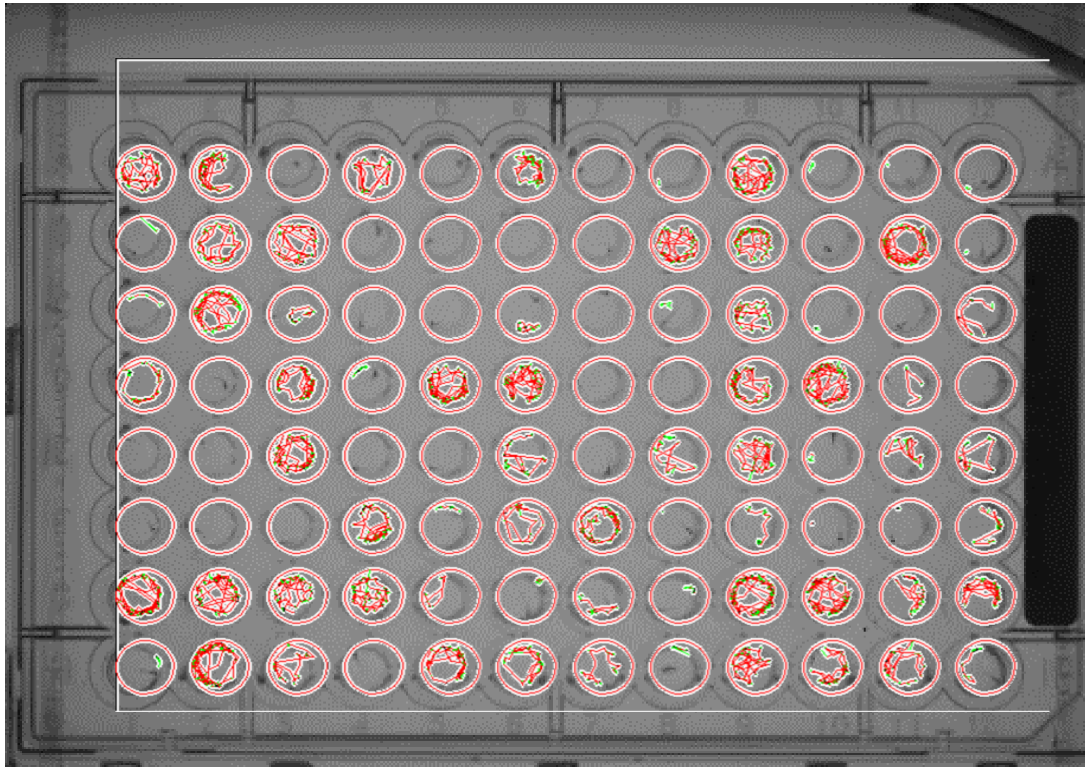
- **Morphology data**
  - Morphological Change/Dead observed
  - Swim-Bladder Issue
  - Larvae included Y/N
- **DNT data**
  - Distance moved (every minute)
  - Velocity (every minute)



(*) On 750 wild type embryos from 50 different plates

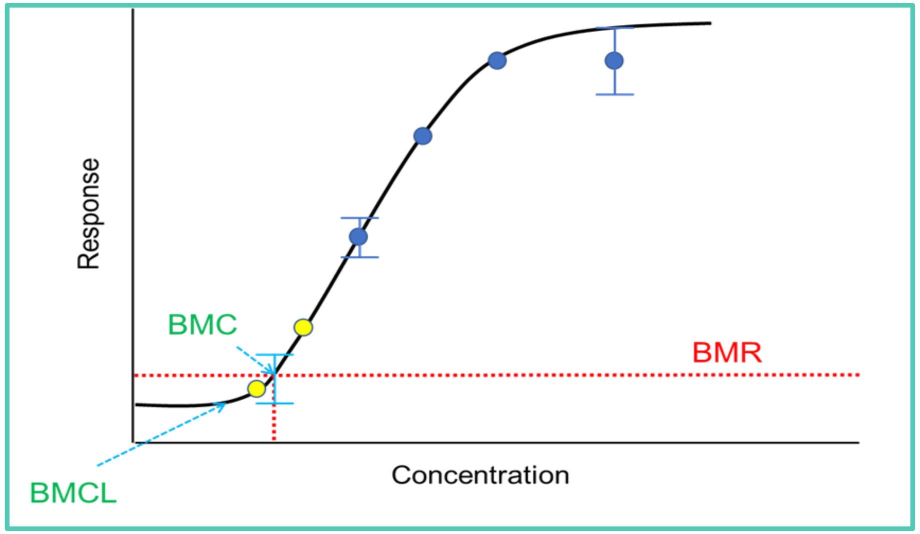


Example of the data obtained



Data Analysis

- Application of **Bench Mark Dose (BMD) modeling**
  - Focus on dose-response trend and onset of the response
  - Used in quantitative risk assessment
- Evaluate the direction and amount of movement during light/dark stimulation (vs vehicle control)
- Evaluate the similarity of the movement pattern across experiment (vs vehicle control)

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SOT Society of Toxicology
www.toxsci.oxfordjournals.org

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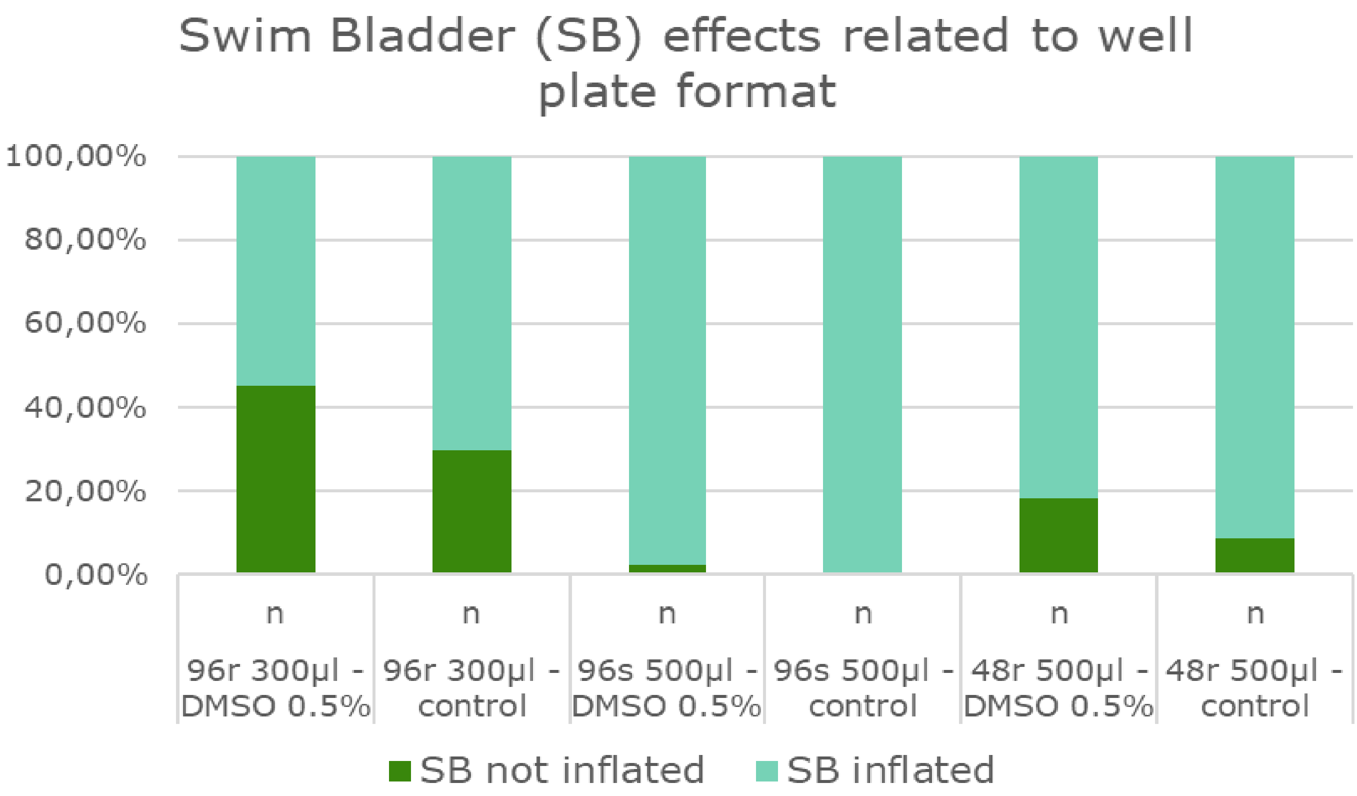
Dryad Digital Repository DOI: doi:10.5061/dryad.rt7f0b0
Advance Access Publication Date: October 13, 2018
Research Article

Application of Benchmark Concentration (BMC) Analysis on Zebrafish Data: A New Perspective for Quantifying Toxicity in Alternative Animal Models

Jui-Hua Hsieh,[*,1] Kristen Ryan,[†] Alexander Sedykh,[‡] Ja-An Lin,[§] Andrew J. Shapiro,[†] Frederick Parham,[†] and Mamta Behl[†]

Data analysis Jui-Hua Hsieh (NTP)

Swim Bladder (SB) issue affected by well volume



r = round
s= squared
Total 12 / group

Data UFZ Nils Klüver

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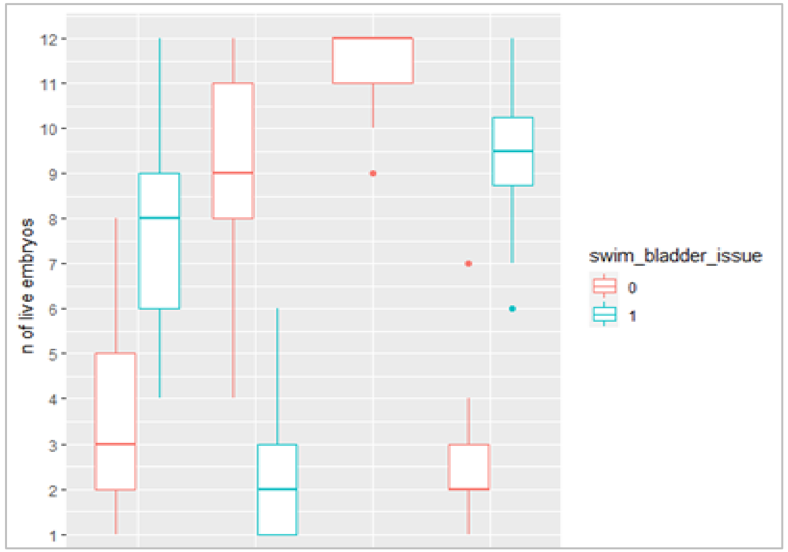
Towards Protocol Harmonization: Swimming Bladder

1. Inter-lab difference for proper swim bladder inflation

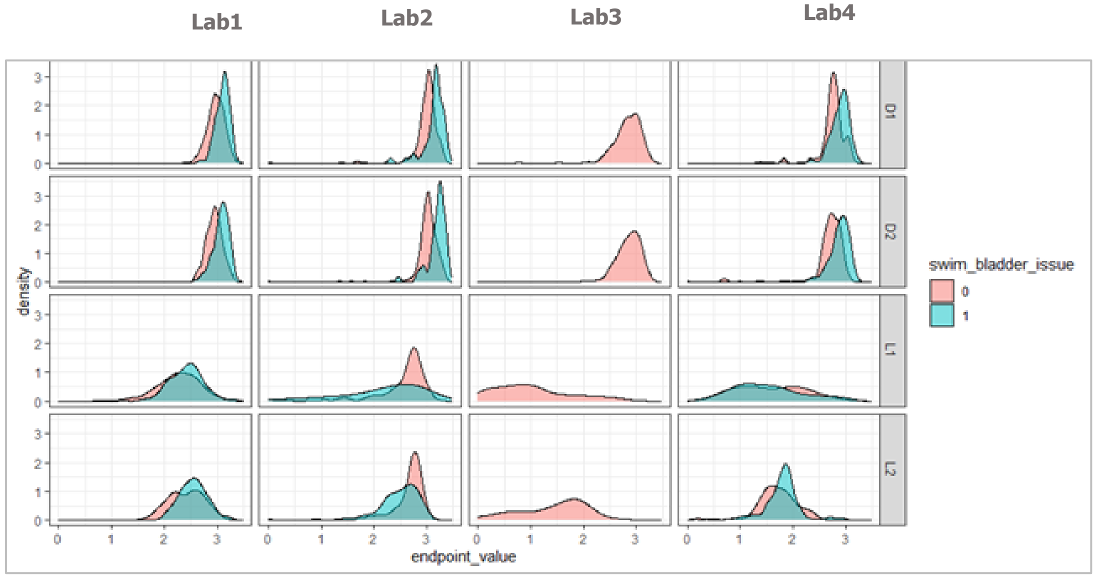


Legend:

- normal swim bladder (0 - orange bars)
- uninflated swim bladder (1 - blue bars)

Lab1 (3/12), Lab2 (9/12), Lab3 (12/12), Lab4 (2/12) (normal / total)

2. Significant *(Wilcoxon test)* distance moved difference between embryos with and without swim bladder issue



Embryos with **swim bladder** issue present:

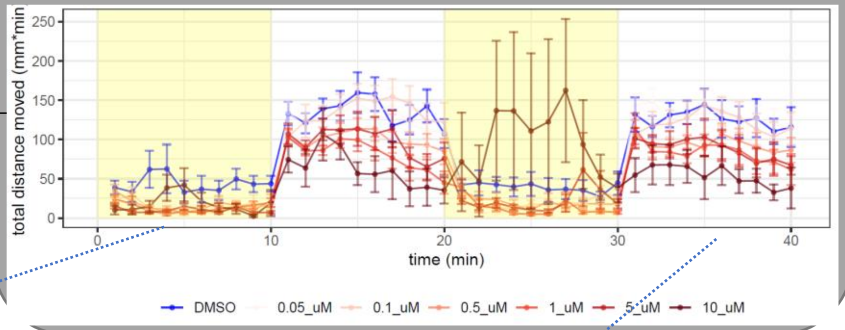
- Increased distance moved in D phases
- Larger movement variation in L phases

Data Analysis Jui-Hua Hsieh (NTP)

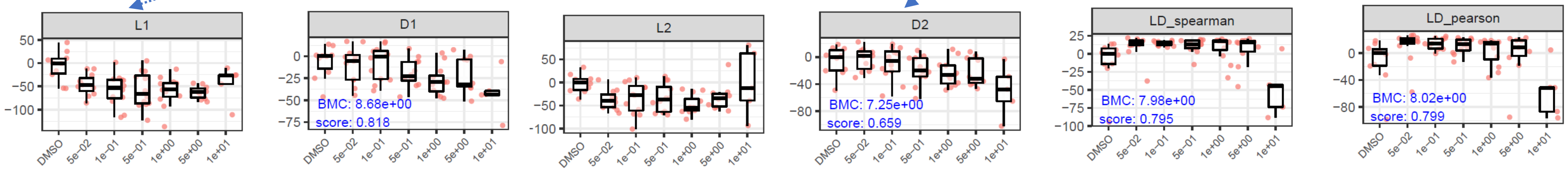
A unified data processing Pipeline

1. Biobide
2. NRC
3. OSU
4. ZeClinics

Raw data (B3, Biobide)



Convert raw data to endpoints for BMC modeling



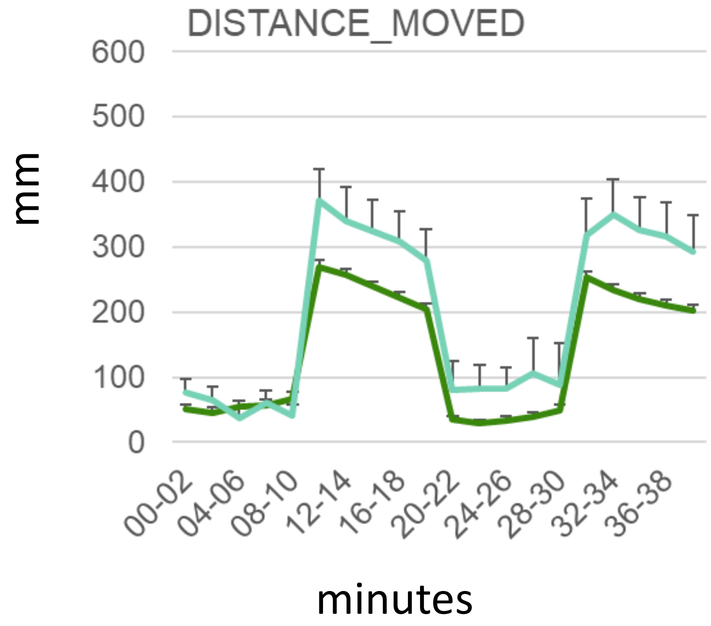
Evaluate **direction** and **amount** of movement

Evaluate **pattern** of movement

A unified data processing Pipeline

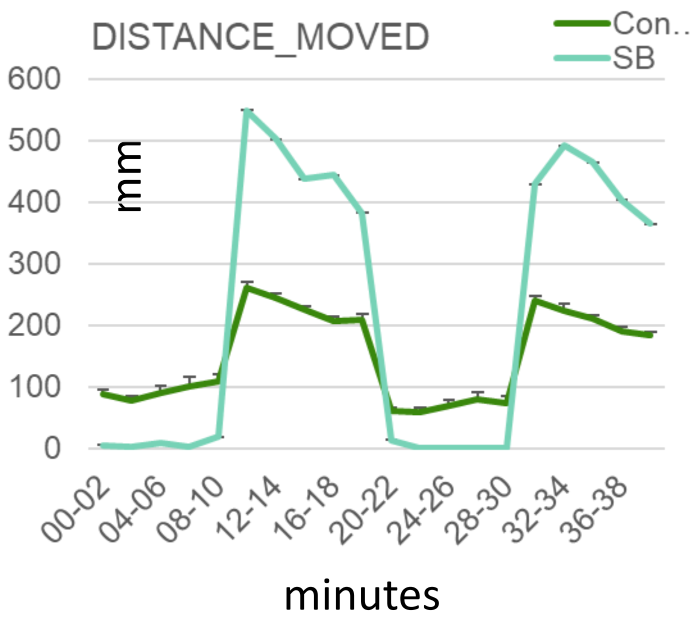
EXPERIMENT #1

Round 96 well plate – 300 uL



(Control) Swim bladder present:71
(SB) Swim bladder absent: 15

Square 96 well plate – 600 uL

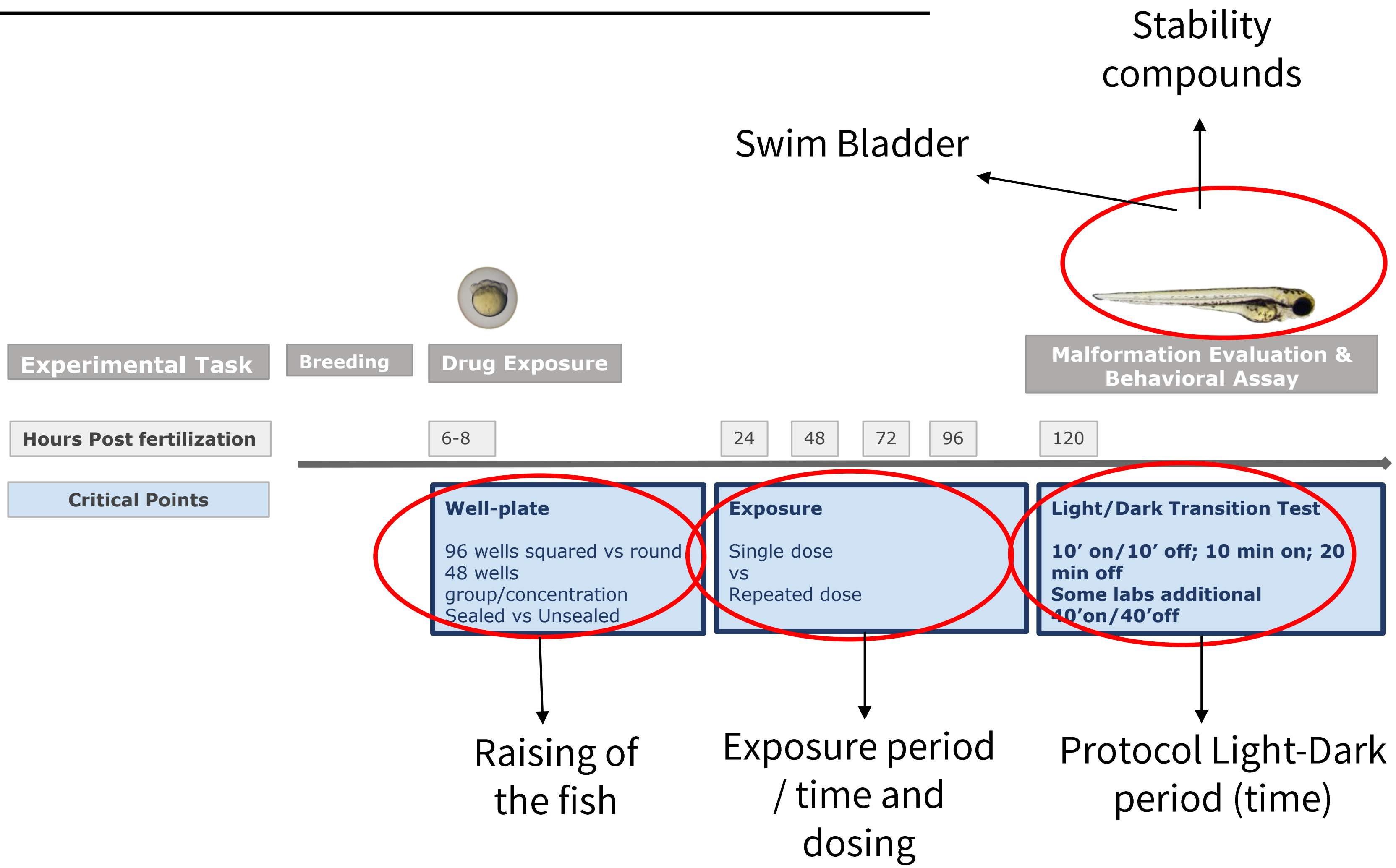


(Control) Swim bladder present:74
(SB) Swim bladder absent: 1

* The plates were not sealed

Questions answered

PROTOCOL



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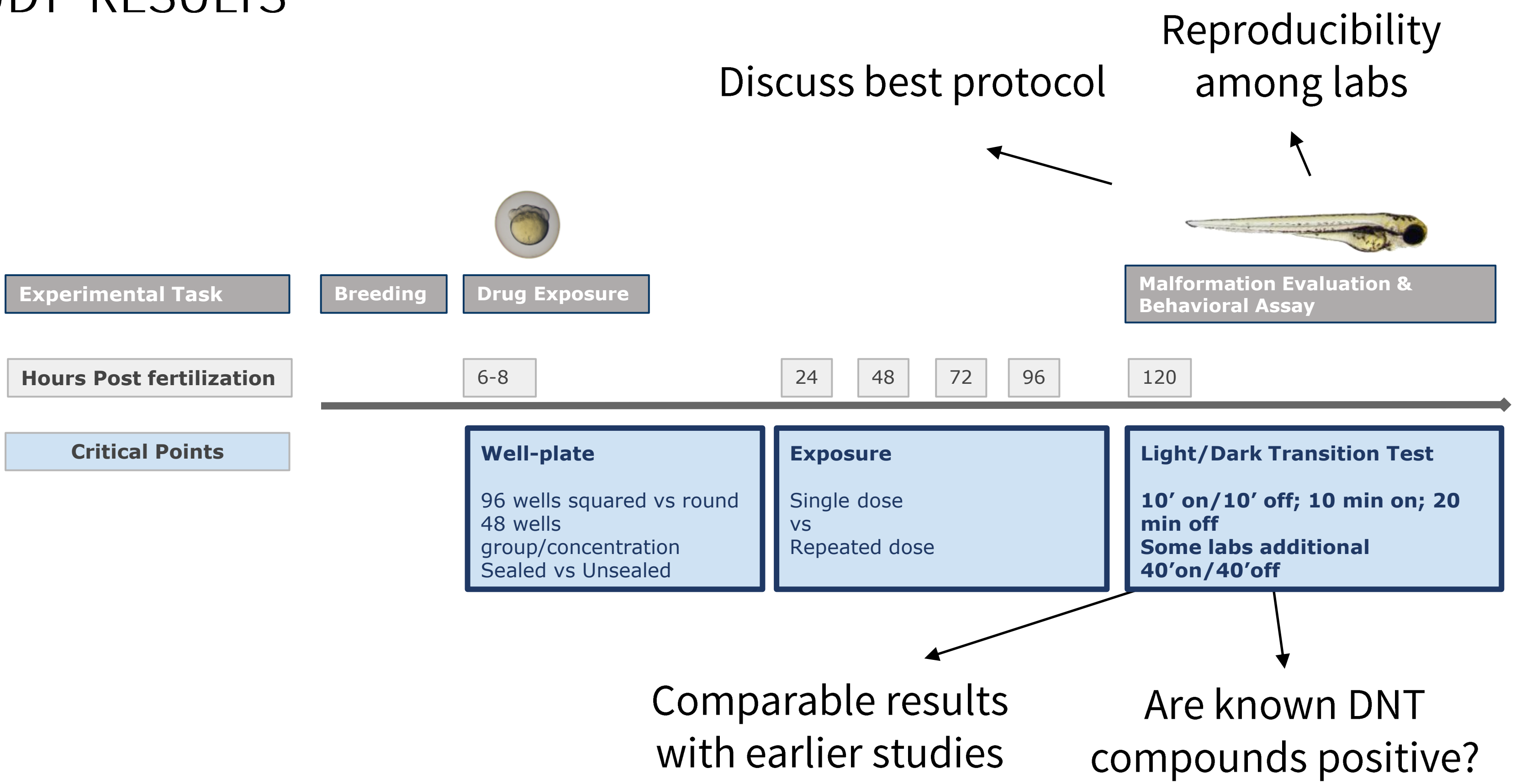
Questions answered

PILOT STUDY RESULTS



Conclusions of the Pilot study

- Zebrafish behavior models can have an added value to the OECD guidance document for DNT.
- Volume in well plate affects swim bladder inflation.
- Harmonization of the Protocol is essential.
- Inter-laboratory replication is a challenge.
- Key players in the field working together to develop a harmonized protocol for the Light-Dark transition test.
- In future Protocols for other zebrafish tests for DNT can be also harmonized.

Planning case study, data analysis and discussion

- Discussion where in the Guidance Document (GD) does the zebrafish assay fit
  - General description of the zebrafish in the GD
    - ✓ Human relevance
    - ✓ Endpoints measured
  - Protocol as addendum
- Discuss draft GD zebrafish and what is further needed
- 4 laboratories – 4 finished
- Data analysis done
- Extra studies in 2 different labs can be done if needed
- Planning to finish after summer and have a final Protocol
- Start with other zebrafish assays (if needed)

Phase 3 – Definitive Study

DNT IATA CASE Test Method for Zebrafish-Inter-laboratory Validation		
PHASE 1 Dose-range finding	PHASE 2 Behavioral Assay: test and optimization	PHASE 3 Definitive study

- Detailed data analysis
- Compare data among the labs and published data
- Concordance with other model systems for DNT
- Recommendations future studies
- Final Protocol for OECD Guidance Document

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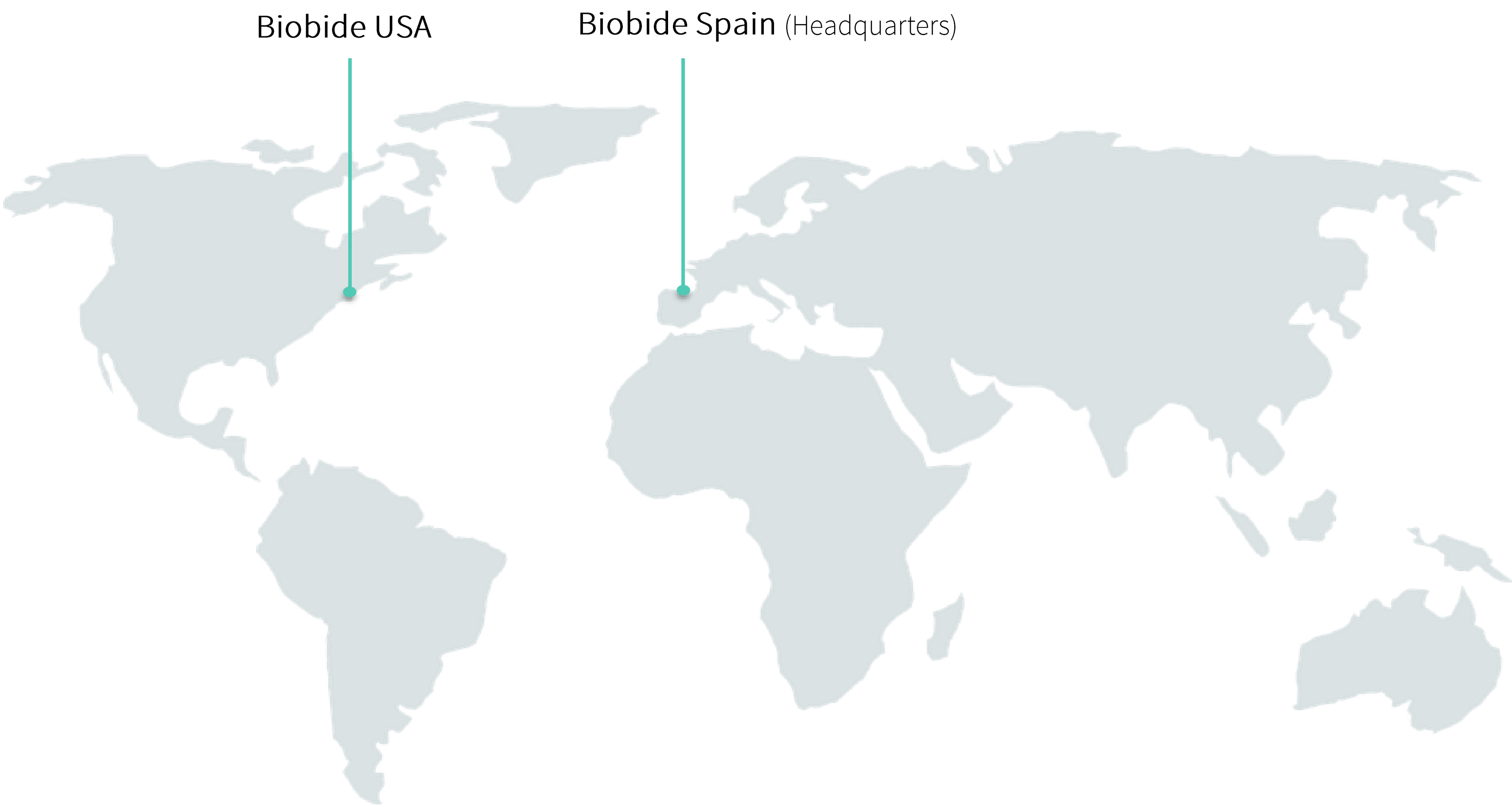

TARGET VALIDATION

DISEASE MODELS GENERATION

EFFICACY ASSAYS

ONCOLOGY
METABOLISM
CARDIOVASCULAR
MUSCULAR DISEASES
CNS
RARE DISEASES

TOXICITY ASSAYS

ACUTETOX
DEVELOPMENTAL TOX
NEUROTOX
CARDIOTOX
HEPATOTOX
OTOTOX
IMMUNOTOX
NEPHROTOX
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Services tailored to your needs

THANK YOU VERY MUCH

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