

Le modèle «**Zebrafish**», un outil innovant pour l'évaluation de la toxicité humaine et l'écotoxicité

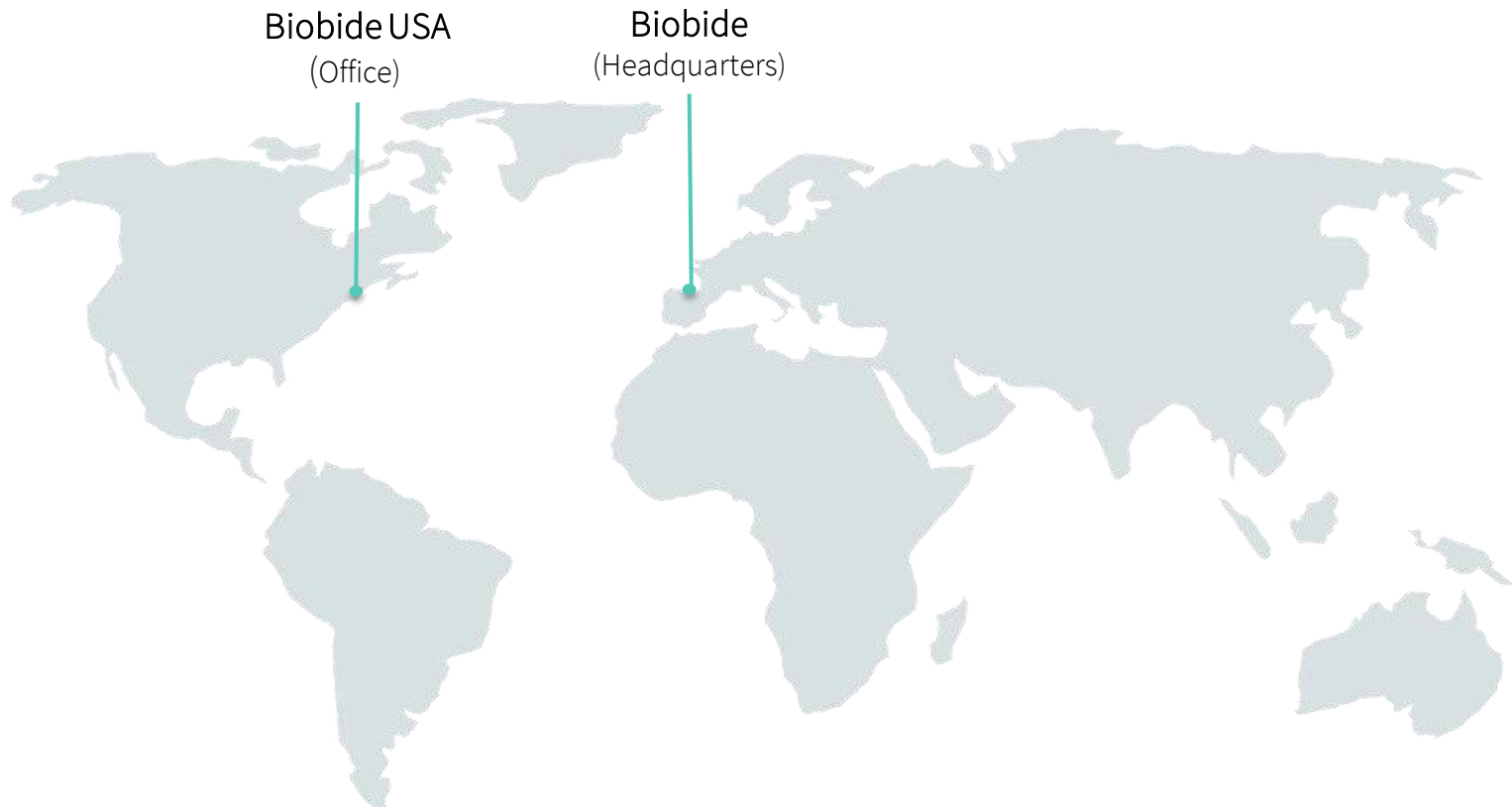
Iñaki Iturria, Ph.D.



Congrès Annuel
de la Société Française de Toxicologie

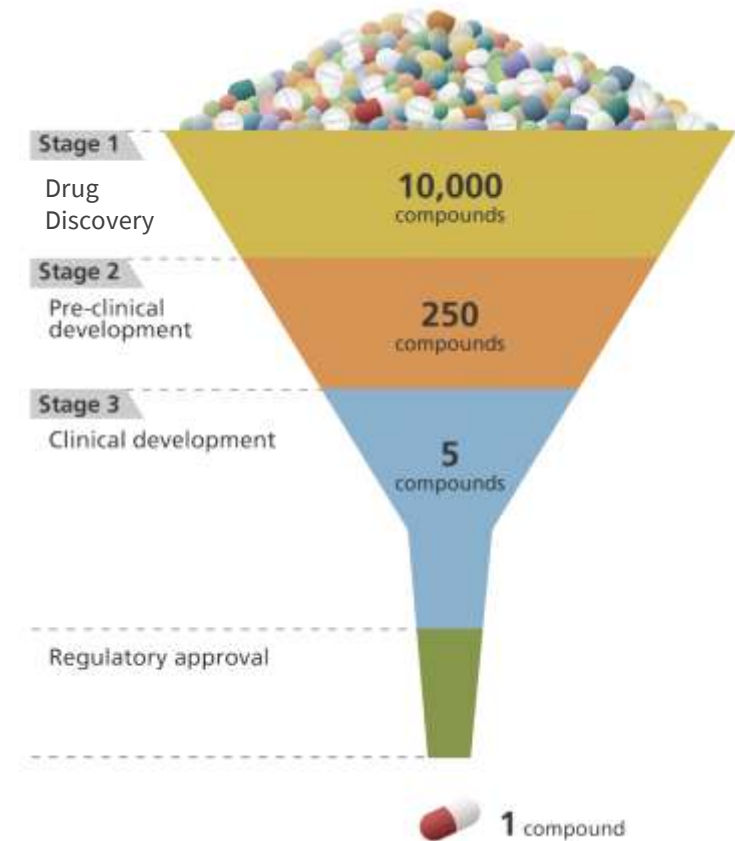
Lille, 27 & 28 Novembre 2018

Biobide is a Contract Research Organization (CRO) with more than 13 years of experience specialized in the **zebrafish** animal model, offering **TAILOR-MADE** pre-clinical services to Pharmaceutical, Biotech, Chemical, Cosmetic and Nutraceutical companies worldwide under Good Laboratory Practices (GLPs) environment and 3Rs principles (Refinement, Reduction and Replacement of animals.)



Pourquoi le Poisson Zèbre?

- The number of new compounds in pharma, chemical and agrochemical industries' early discovery is constantly increasing as is increasing the need for risk reducing time- and cost-effective toxicity screening methods.
- There is a need to reduce the number of animals and time required to screen this large number of chemicals.



Experimental models



In vitro



Invertebrates



Mammals



Clinical Trials

Cost

€

€€

€€€€

€€€€€

Capacity

+++++

+++

++

+

Biologic
Relevance

+

+

++++

+++++

Experimental models



In vitro



Invertebrates



Zebrafish



Mammals



Clinical Trials

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Capacity

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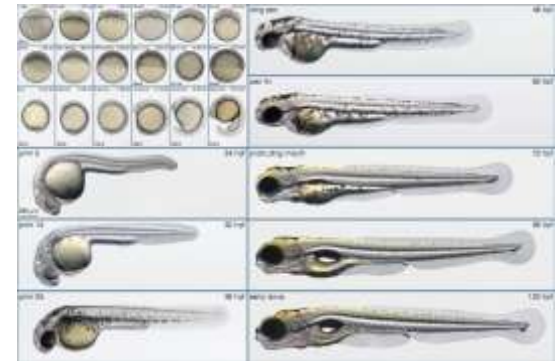
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3Rs: Replacement, Reduction, Refinement

Zebrafish

Name:	<i>Danio rerio</i>
Family:	Cyprinidae
Origin:	Southeast Asia
Diet:	Omnivorous
Size:	4 cm long
Lifespan:	Up to 3 years



- First studies in **developmental biology and genetic** research in the 1970's by Dr. Streisinger (University of Oregon).
- The **Zebrafish reference genome** was published in 2013.
- Nowadays, this **vertebrate model** is widely used in many research fields such as **pharmacology, toxicology, and environmental sciences**.

Zebrafish Biological Model

Cost-efficient

- Small size
- High fecundity
- Rapid Development
- Low Housing cost

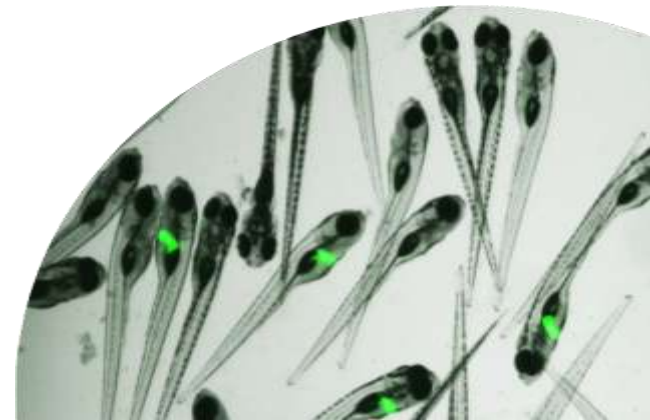


Vertebrate Model

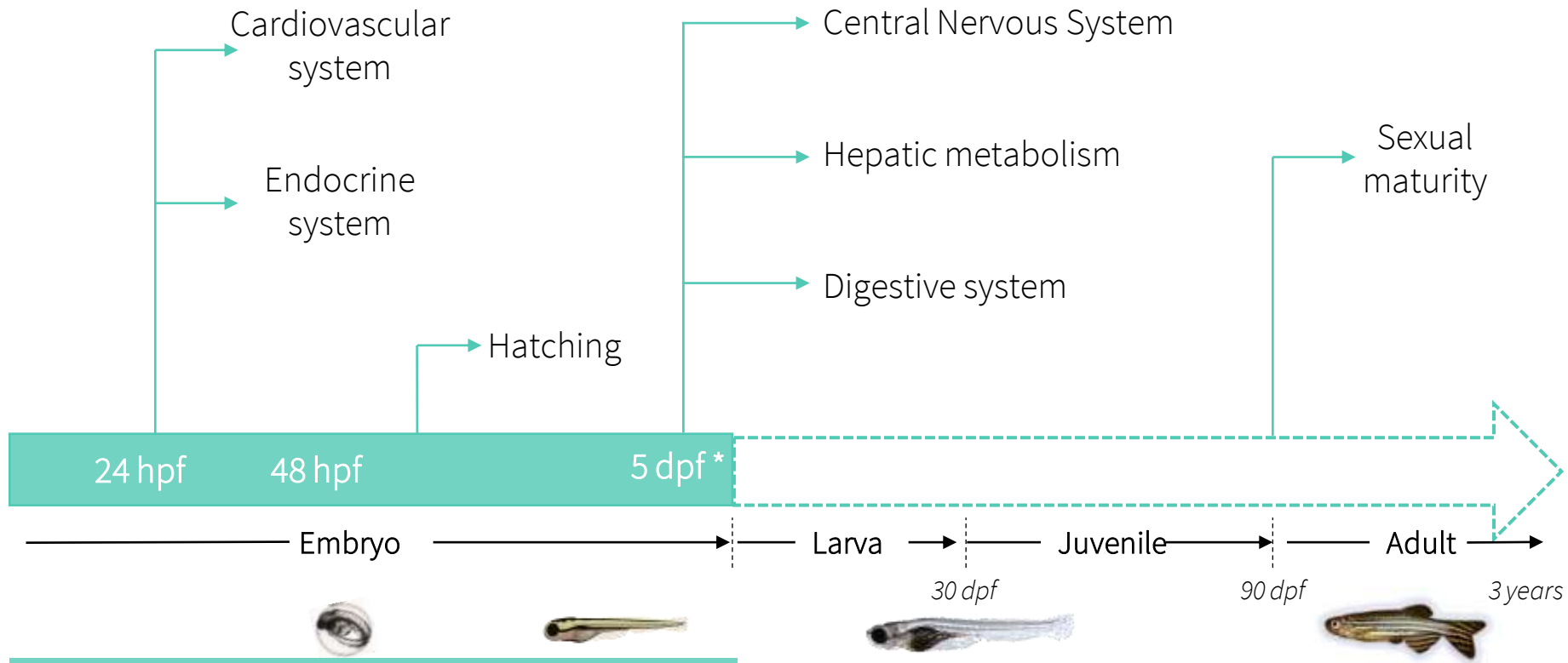
- Similarities in major organs and tissues
- Genome sequenced
- High genetic homology
- Transgenic disease models

High-throughput High-content

- Transparent embryos
- Fluorescent reporters
- Phenotypic screening
- Multi-well format



Zebrafish Developmental Process



* ALTERNATIVE ANIMAL MODEL
(EU 2010/63/EU)

hpf: hours post fertilization
dpf: days post fertilization

Zebrafish Embryo Biotransformation Capacity

- 86 CYP genes grouped in 17 families¹
- Metabolic activity starts in early fish stages²
 - **Phase I:** *CYP1*, esterase, *Adh* and *Aldh* have activity from 2.5 hpf
 - **Phase II:** *Gst* and *Nat* have activity from 48 hpf
- Biotransformation of environmental pollutant
 - ✓ Polycyclic aromatic hydrocarbons (PAH): *CYP1A*, *CYP1B* & *CYP1C* induction through PAH binding to aryl hydrocarbon receptors³
 - ✓ Endocrine disruptors: Phase II metabolization of Bisphenol and Benzophenone-2⁴
 - ✓ Pesticides: Transformation of chlorothalonil to more toxic metabolites⁵
 - ✓ Solvents: Transformation of 2-methoxyethanol to methoxyacetic acid²

¹ Genome Reference consortium, 2015 ⁴ Le Fol et al. 2017

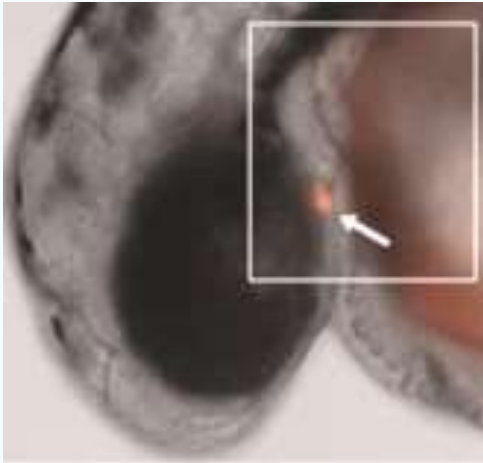
² Otte et al. 2017

⁵ Zhang et al. 2016

³ Kühnert et al. 2017

Zebrafish Transgenic Models

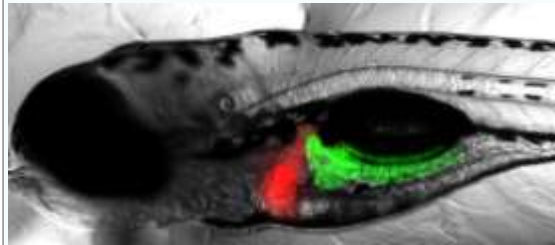
Transgenic induced gene alterations: ZFN, CRISP/Cas9 and TALENs technologies applied in zebrafish model generation.



tg(tg:mCherry)

Opitz et al.2012

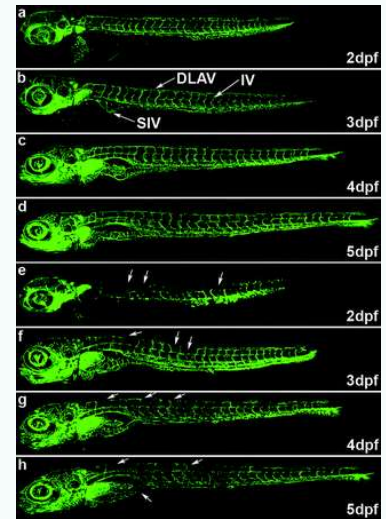
- mCherry production coupled to thyroglobulin gene expression.
- Concentration-dependent response to thyroid disrupting substances.



tg(fabp10a:DsRed;elaA:gfp)

Zhang et al.2014

- DsRed expression in the liver.
- GFP expression in pancreas.
- Sensitive to hepatotoxicants.



tg(fli1:EGFP)

Lawson et al.2002

- GFP expression in endothelial cells of blood vessels.
- Useful to angiogenesis studies.

Regulation - Zebrafish as alternative model

- Several **OECD guidelines** include zebrafish as a recommended biological model in the risk assessment of chemical compounds:

OECD 203, 210, 212, 229, 236



- **EURLS-ECVAM** *European Union Reference Laboratory for Alternatives to Animal Testing:*



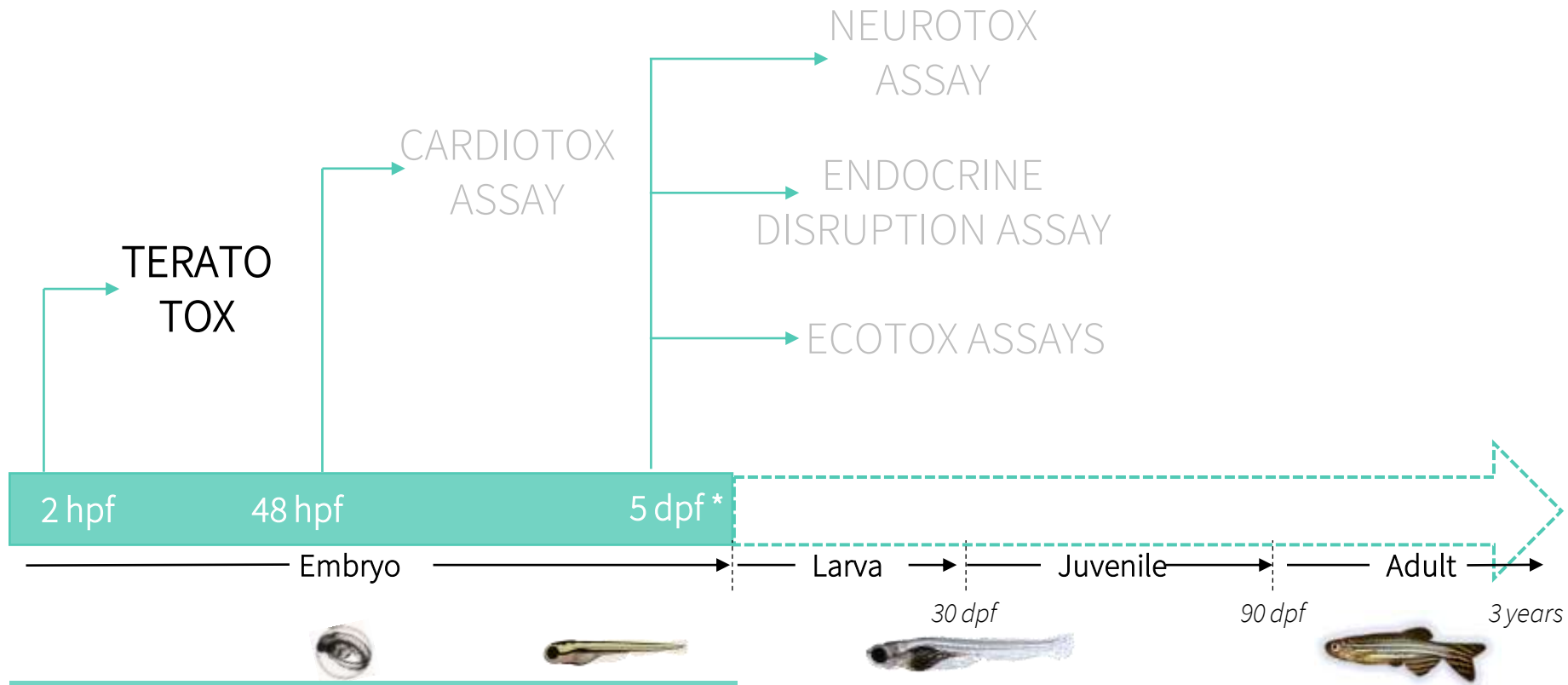
Recommendation on the Zebrafish Embryo Acute Toxicity Test Method (ZFET) for Acute Aquatic Toxicity Testing- *July 2014*

Directive 2010/63/EU

Zebrafish embryos are not considered animals until 5 dpf



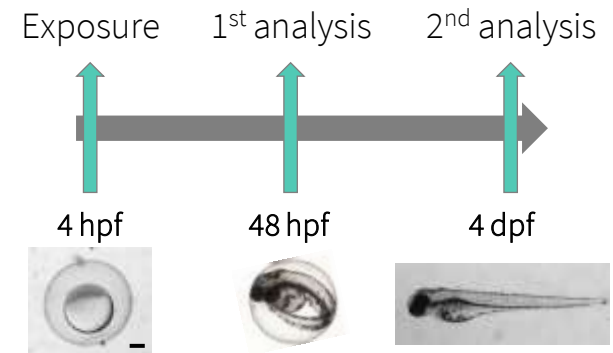
Zebrafish Toxicity Assays



* ALTERNATIVE ANIMAL MODEL
(EU 2010/63/EU)

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dpf: days post fertilization

Systemic toxicity Teratotox Assay



Phenotypic-based screening assay for the evaluation of developmental toxicity.

Morphological endpoints related to the development of major organs and body structures.

EXPERIMENTAL SETUP

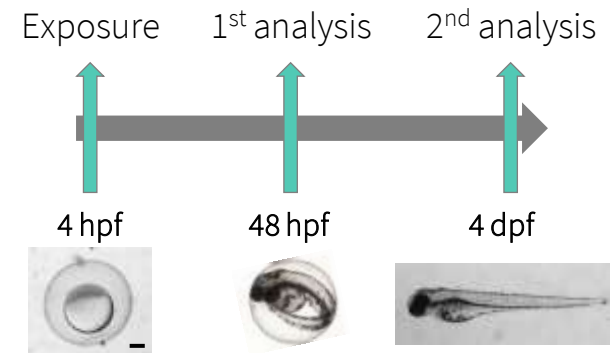
- 24 or 96 wells microplate
- 5 embryos per well, 3 wells per condition
- 8 test concentrations per compound
- Visual observation at 48 and 96 hpf

LC_{50} , EC_{50} and a Teratogenic Index (TI) (LC_{50}/EC_{50}) are calculated at 2 and 4 dpf

		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

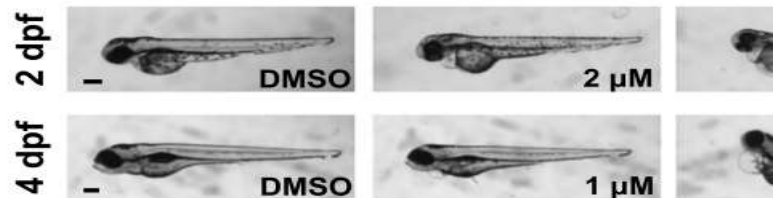
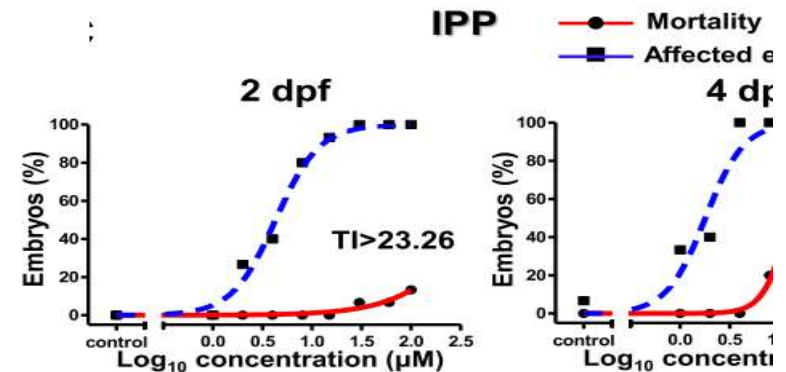
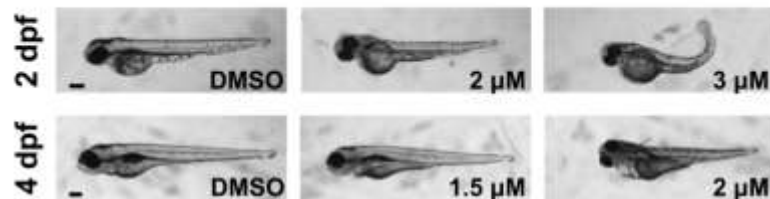
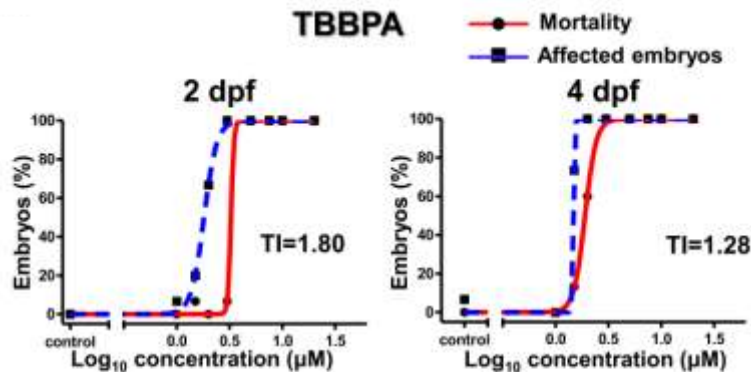
Systemic toxicity

Teratotox Assay



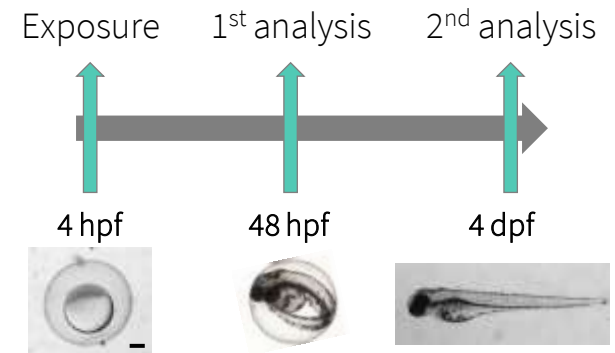
Phenotypic-based screening assay for the evaluation of developmental toxicity.

Morphological endpoints related to the development of major organs and body structures.



Systemic toxicity

Teratotox Assay



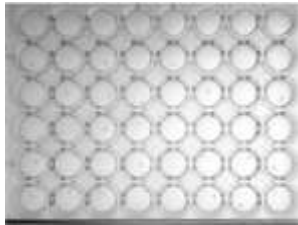
Phenotypic-based screening assay for the evaluation of developmental toxicity.

Morphological endpoints related to the development of major organs and body structures.

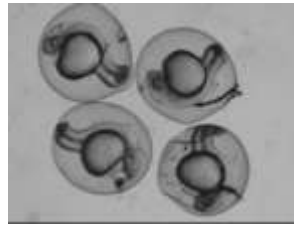
Test item	NOAEL (μM)		EC50 (μM)		LC50 (μM)		TI		Internal concentration	LEL (μM)	
	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf	2 dpf	4 dpf		Nominal	Internal
BDE47*	>25	2	—	12.01 (8.44 to 17.11)	—	>25	—	>2.08	Internal concentration	4	1040
BPDP	8	4	11.45 (10.56 to 12.42)	4.75 (0.086 to 263.1)	84.15 (80.72 to 87.72)	15.24 (12.33 to 18.84)	7.35	3.21		8	1222
EHDP	>20	3	—	5.06 (4.89 to 5.24)	—	9.78 (Very wide)	—	1.93		5	2880
IDDP*	>150	20	—	77.23 (57.77 to 103.2)	—	>150	—	>1.94		40	665.1
IPP	1	<1	4.30 (3.66 to 5.05)	1.80 (1.31 to 2.47)	>100	12.82 (11.97 to 13.73)	>23.26	7.12		1	4.21
TMPP	8	2	11.48 (11.40 to 11.56)	3.00 (2.78 to 3.24)	143.8 (107.2 to 192.9)	9.52 (9.46 to 9.57)	12.53	3.17		4	1078
TPHP	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		1.5	335.2
TBBPA	1.5	1	1.81 (1.76 to 1.86)	1.48 (Very wide)	3.26 (Very wide)	1.90 (1.88 to 1.92)	1.80	1.28		1.5	20.68
TCEP	400	400	521.2 (462.8 to 587.0)	415.2 (Very wide)	>1000	977.6 (Very wide)	>1.92	2.35		600	342.7
TDCIPP	3	2	4.11 (3.68 to 4.58)	3.08 (2.79 to 3.40)	8.29 (7.15 to 9.61)	6.53 (5.07 to 8.40)	2.02	2.12		3	76.68

Exposure concentration assessment

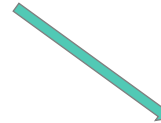
The **internal concentration** can be **determined** with the appropriate analytical methods in order to **discard false negatives/positives**.



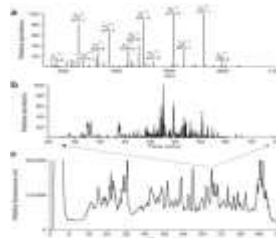
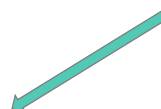
Exposed zebrafish embryos



Homogenization



Analytical technique
(HPLC-MS/MS, GC/MS,
HS/GC...)



Concentration
assessment



Real exposure to
the substance

This analysis can be done after
any toxicity or efficacy assay

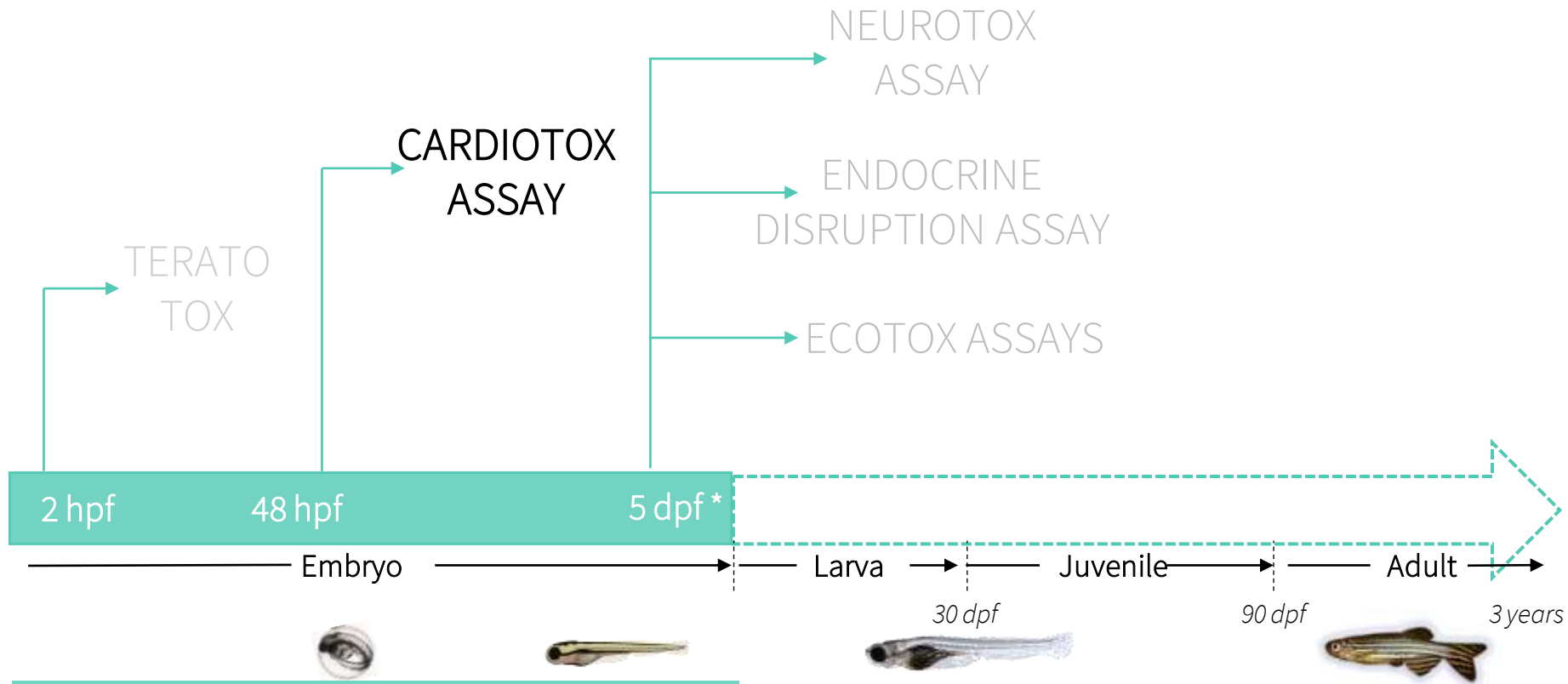
Substance	Classification	BIOBIDE Classification
Aflatoxin A	Teratogenic in rodents	POSITIVE
Acetaminophen	Moderate risk teratogen	NEGATIVE
Dexamethasone	Teratogenic	POSITIVE
Tetracycline	Teratogenic	POSITIVE
Penicillin G	Non-Teratogenic	NEGATIVE
Warfarin	Teratogenic	POSITIVE
Chlorambucil	Teratogenic	POSITIVE
5-Fluorouracil	Teratogenic	NEGATIVE
Thalidomide	Teratogenic	NEGATIVE
Hydroxyurea	Teratogenic	NEGATIVE
Amiodarone	Teratogenic	POSITIVE
Sotalol	Non-Teratogenic	NEGATIVE
Acebutolol	Non-Teratogenic	NEGATIVE
Carbamazepine	Teratogenic	POSITIVE
Valproic Acid	Teratogenic	POSITIVE
Pilocarpine	Non-Teratogenic	NEGATIVE
Tacrine	Teratogenic	POSITIVE
Testosterone	Teratogenic	POSITIVE
Norepinephrine	Teratogenic	POSITIVE
Hydrocortisone	Teratogenic	POSITIVE
Ascorbic acid	Non-Teratogenic	NEGATIVE
Retinol	Teratogenic	POSITIVE
N-Acetyl-Cysteine	Non-Teratogenic	NEGATIVE
Sucrose	Non-Teratogenic	NEGATIVE
Retinoic Acid	Teratogenic	POSITIVE

Substance	Classification	BIOBIDE Classification
Difenoconazole	Non-teratogenic in animal experiments	NEGATIVE
Epoxiconazole	Teratogenic	POSITIVE
Flusiloazole	Teratogenic	POSITIVE
Cyclopamine	Teratogenic in animal experiments	POSITIVE
Myclobutanil	Teratogenic	POSITIVE
Metconazole	Teratogenic	POSITIVE
Propiconazole	Developmental toxicity in rats	POSITIVE
Ipconazole	Developmental toxicity in rat and rabbit	POSITIVE
Penconazole	Not development toxicity in rat and rabbit	NEGATIVE
Diniconazole	Development toxicity in rats and not in rabbits	POSITIVE
Voriconazole	Teratogenic in rats (not in rabbits)	POSITIVE
Glycolic Acid	Teratogenic	NEGATIVE
Camphor	Non-teratogenic	NEGATIVE
Dimethyl phthalate	Teratogenic	POSITIVE
Levothyroxine	Non-Teratogenic	POSITIVE
Metoclopramide	Non-teratogenic	NEGATIVE
Saccharin	Non-teratogenic	NEGATIVE
Tetrabromobispheno IA	Non-teratogenic	NEGATIVE
Caffeine	Teratogenic	POSITIVE
Ramelton	Teratogenic	POSITIVE

Drugs with different human therapeutic indications were tested for the **validation study**

POSITIVE
NEGATIVE
FALSE NEGATIVE
FALSE POSITIVE

Zebrafish Toxicity Assays



* ALTERNATIVE ANIMAL MODEL

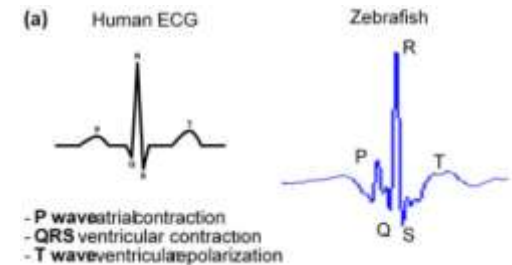
(EU 2010/63/EU)

hpf: hours post fertilization
dpf: days post fertilization

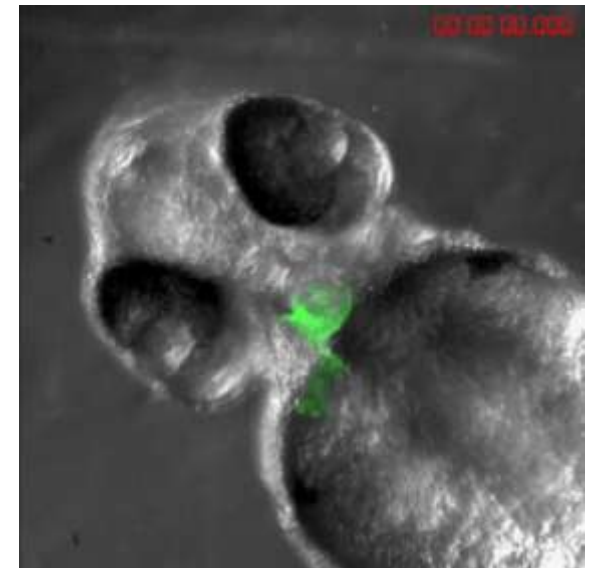
Organ-specific assays

Cardiotox

- Bicameral heart (one atrium, one ventricle)
- ECG pattern similar to human
- Voltage-gated Na channels
- L&T-type Ca channels
- K Channels
- Embryos can survive up to 5 pdf days without circulation
- Zebrafish tg(mlc2a::copGFP) strain expressing green fluorescent protein (GFP) in the heart



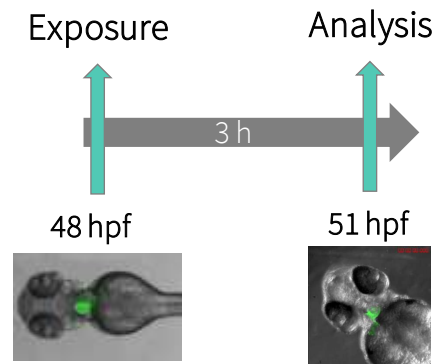
Yu et al. 2011. Biomedical Microdevices.



72 hpf zebrafish embryo. Biobide

Organ-specific assays

Cardiotox



Microscopy video recording and automatic assessment of cardiac movements.

Automatic detection of heartrate, arrhythmia, bradycardia, and cardiac arrest caused by cardiotoxicity of the test substances

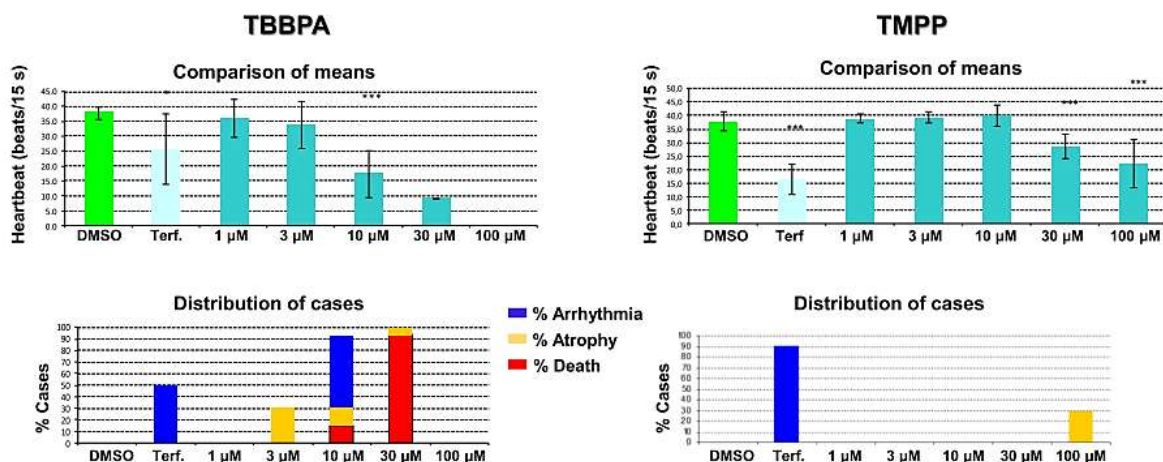
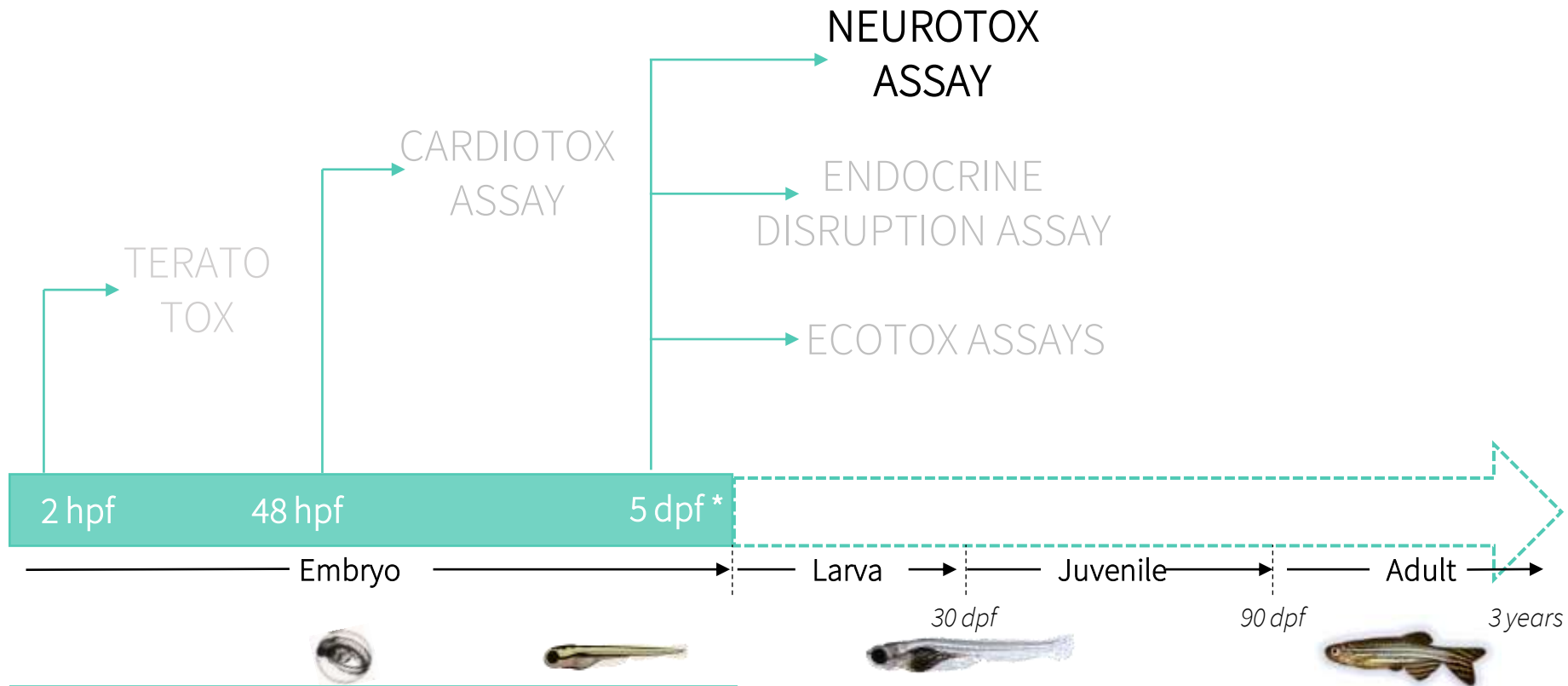


Fig. 3. Examples of cardiotoxicity results obtained for the BFR TBBPA and the OPFR TMPP (Experiment 1). (A) Schematic depicting a summary of the experimental design. B, C Upper graphs show the mean \pm S.D. of the heartbeat rate obtained for TBBPA (B) and TMPP(C) (** p < 0.001, Mann-Whitney U test). In the lower graphs, bars represent the percentage of embryos with altered heartbeat rhythmicity, atrophy, or death. Terf.: 5 μ M Terfenadine. Scale bar represents 200 μ M.

Test item	Cardiotoxicity	
	Effect	LEL (μ M)
BDE47	Not detected (30 μ M)	–
BPDP	Bradycardia/ Atrial failure	10
EHDP	Bradycardia	30
IDDP	Bradycardia	100
IPP	Bradycardia/ Atrial failure	100
TMPP	Bradycardia/ Atrial failure	30
TPHP	Bradycardia/ Atrial failure	10
TBBPA	Arrhythmia/ Ventricular failure	3
TCEP	Not detected	–
TDCIPP	Not specific	–

Zebrafish Toxicity Assays



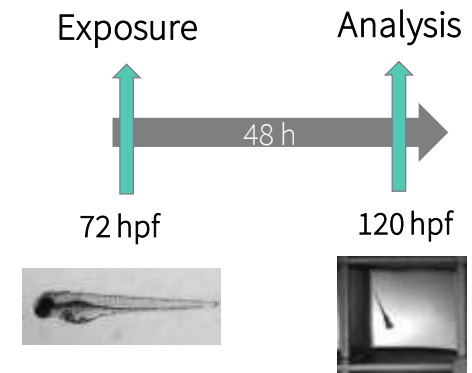
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Organ-specific assays

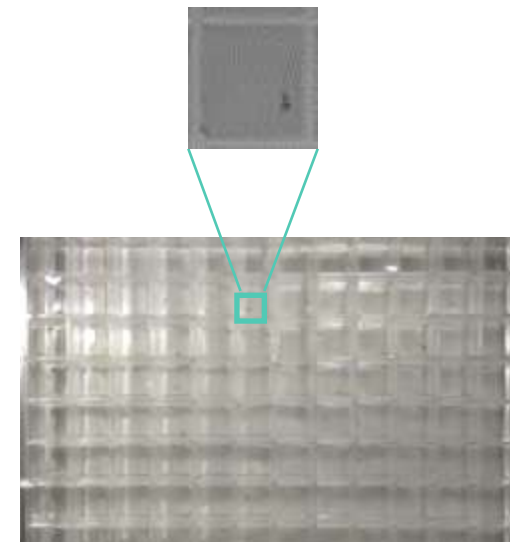
Neurotox



Behavior tracking and analysis of zebrafish embryos alternating light/dark periods.
Assessment of neuroactive/neurotoxic activity of test compounds.

Measured variables:

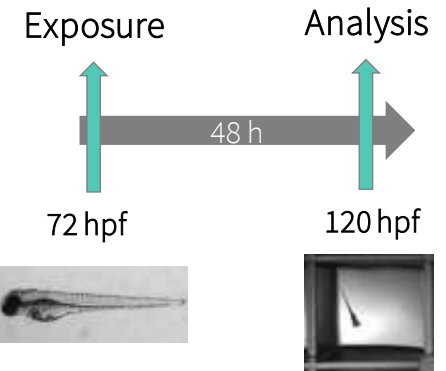
- ✓ Distance
- ✓ Rotation
- ✓ Maximum and mean velocity
- ✓ Movement Duration & Frequency
- ✓ Distance moved at high velocity
- ✓ Mobile and Highly mobile Duration & Frequency
- ✓ Ratio of peripheric distance vs. total distance moved in the well



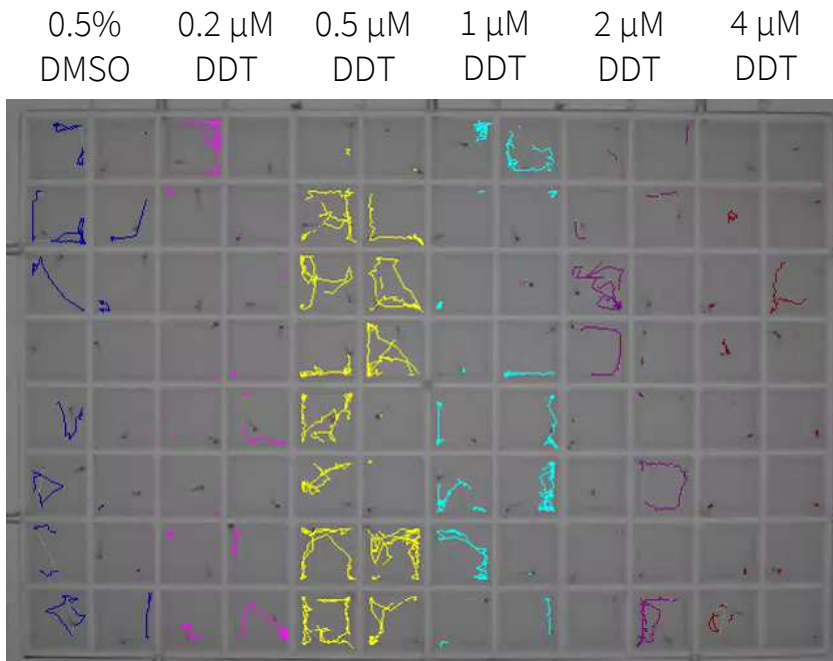
Screening on 96 well-plates

Organ-specific assays

Neurotox

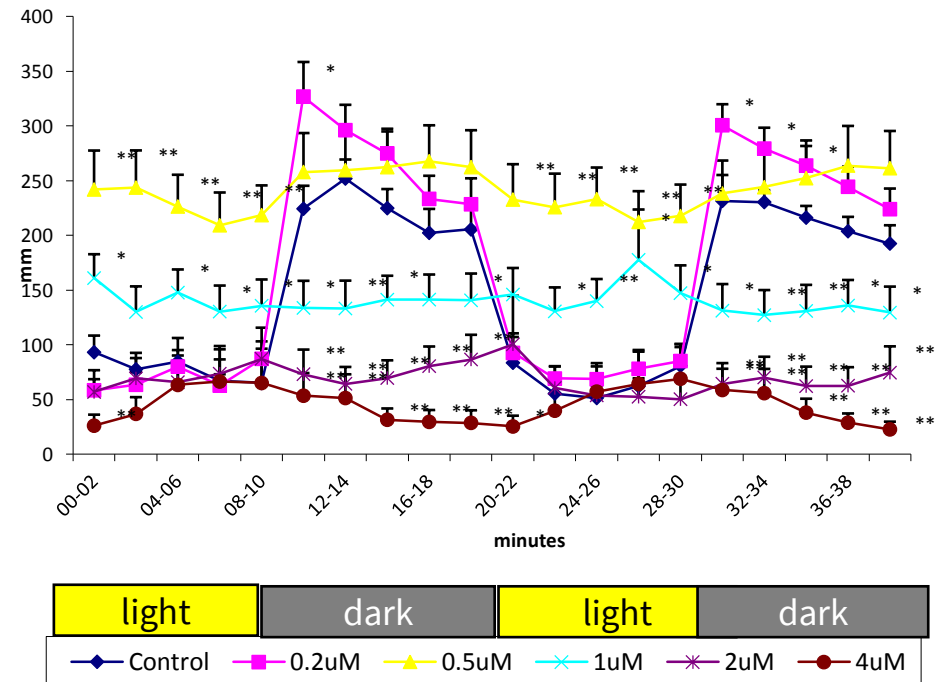


Tracking of embryo exposed to DDT



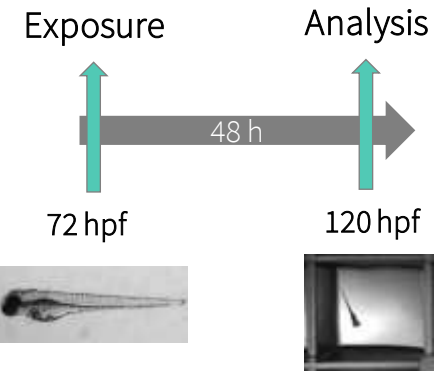
Concentration dependent effects:

- Higher concentration, lower locomotor activity



Organ-specific assays

Neurotox

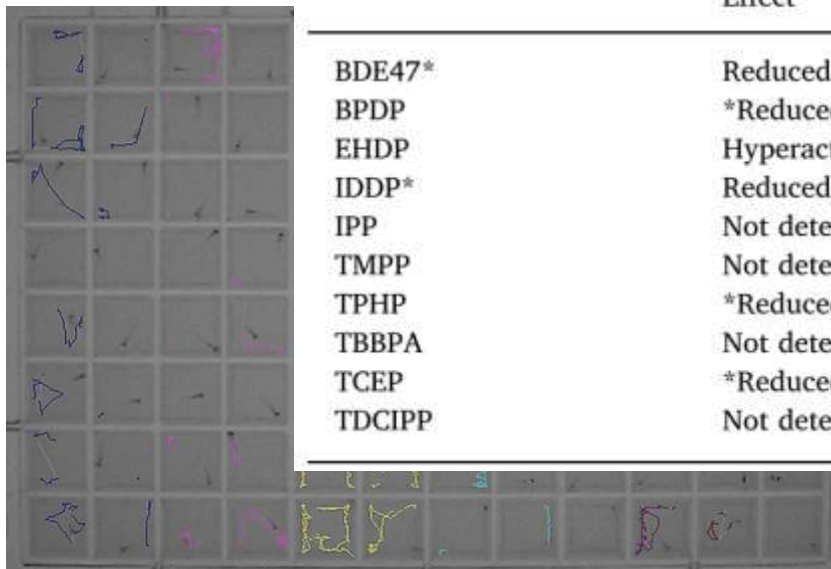


Tracking of embryo exposed to DDT

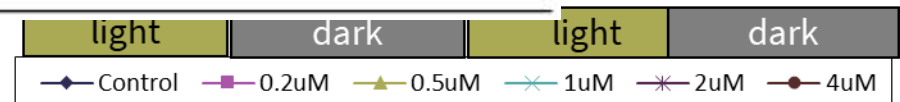
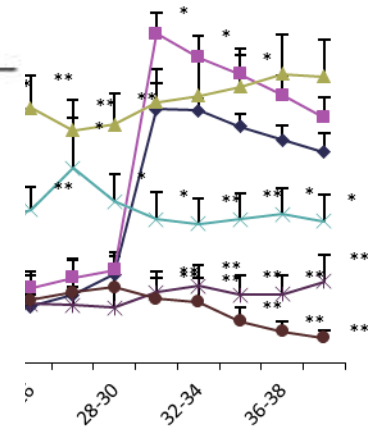
Concentration dependent effects:

- Higher concentration, lower locomotor activity

0.5% DMSO 0.2 μ M DDT



Test item	Behavior alteration
Effect	LEL (μ M)
BDE47*	Reduced activity
BPDP	*Reduced activity/toxic
EHDP	Hyperactivity
IDDP*	Reduced activity
IPP	Not detected
TMPP	Not detected
TPHP	*Reduced activity/toxic
TBBPA	Not detected
TCEP	*Reduced activity/toxic
TDCIPP	Not detected



Organ-specific assays

Neurotox

TP: true positive

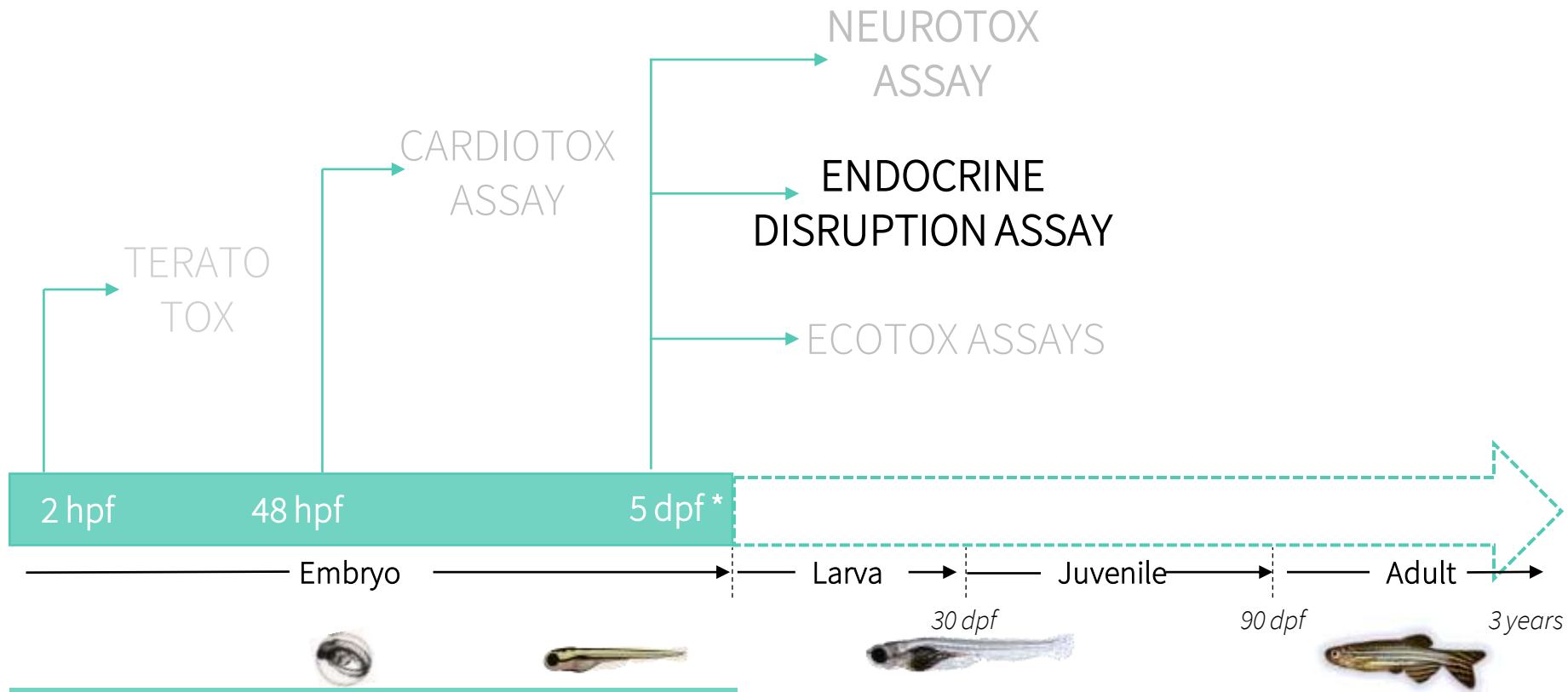
TN: true negative

FP: false positive

FN: false negative

COMPOUND	Therapeutic Classification	Adverse effects in human CNS	Results
5-Fluorouracil	Antineoplastic-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 GSK3 β 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

Zebrafish Toxicity Assays



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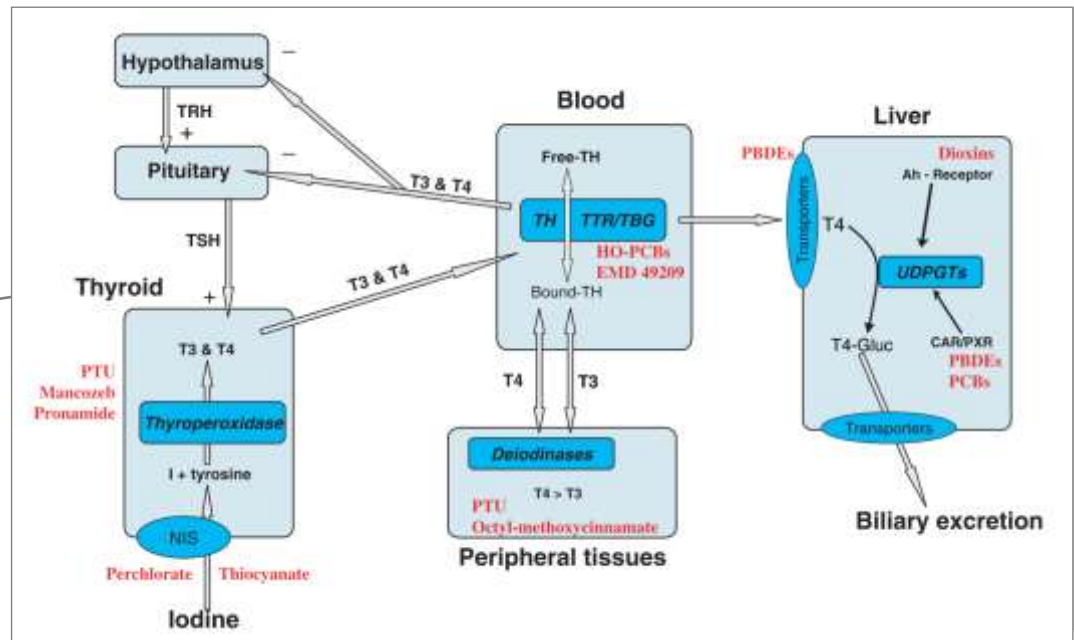
Endocrine Disruption Assay

Thyroid Pathway

Endocrine disruptors are one of the most significant hazards in environmental risk assessment of chemicals.

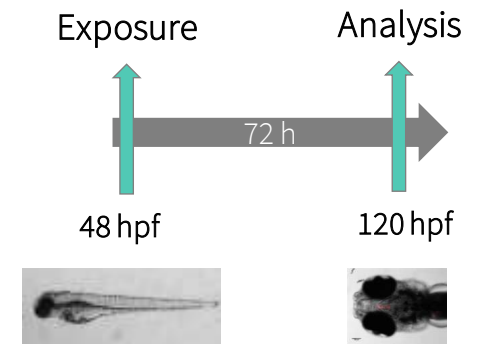
Main Endocrine pathways:

- Estrogen
- Androgen
- Thyroid
- Steroidogenesis



Endocrine Disruption Assay

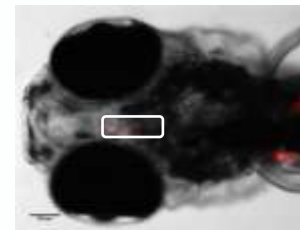
Thyroid Pathway



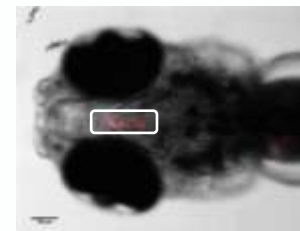
Tg(tg:mcherry) transgenic line: mCherry fluorescence expression driven by the promoter of thyroglobulin (*tg*) gene.

EXPERIMENTAL DESIGN

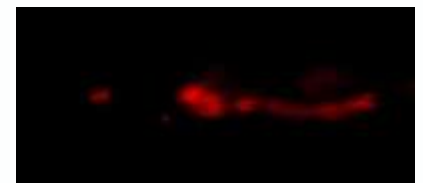
- Exposure from 48 hpf to 120 hpf.
- 5 concentrations per compound.
- 20 embryos per condition.
- Fluorescence intensity assessment by image analysis.



tg(tg:mcherry) Control embryo



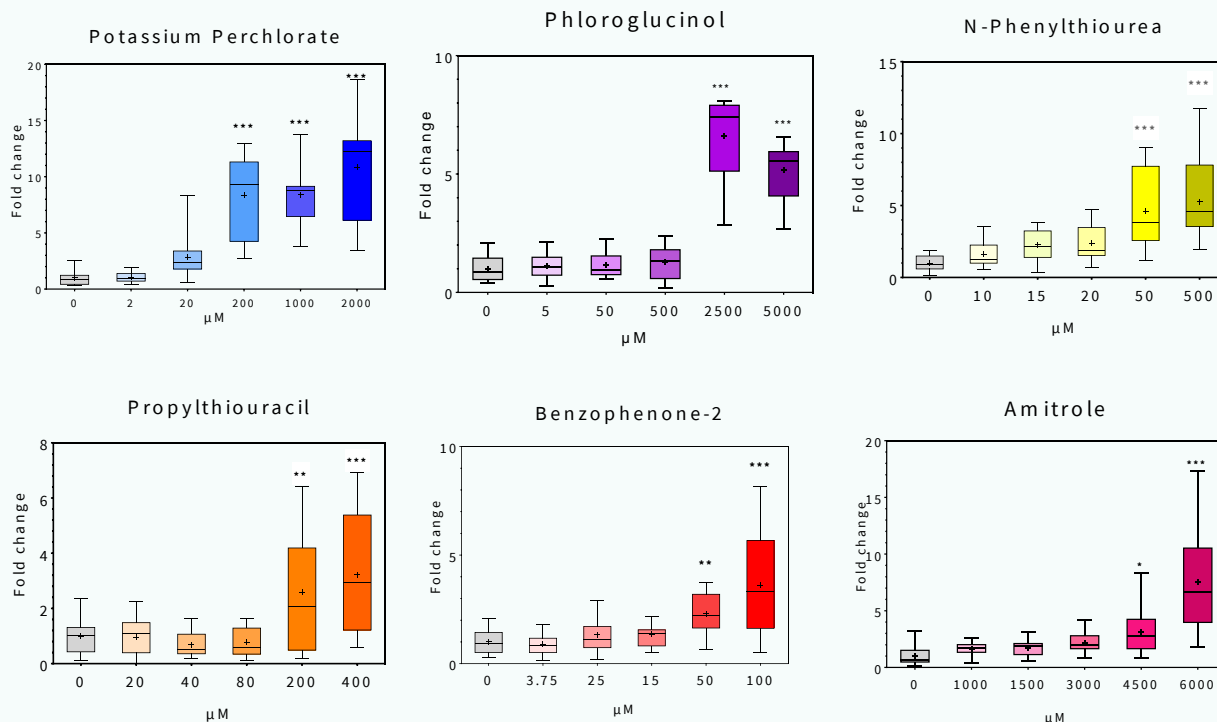
tg(tg:mcherry) embryo exposed to 200 μM KClO₄



Thyroid Pathway

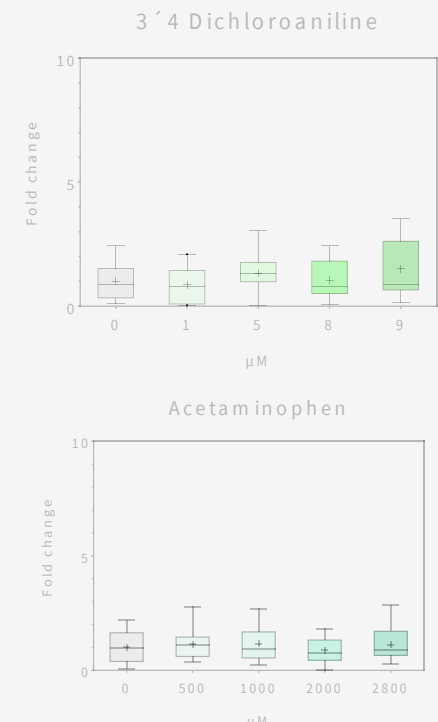
Reference TD chemicals (+ controls)

- Concentration-dependent fluorescence increase.
- = Induction of thyroglobulin expression

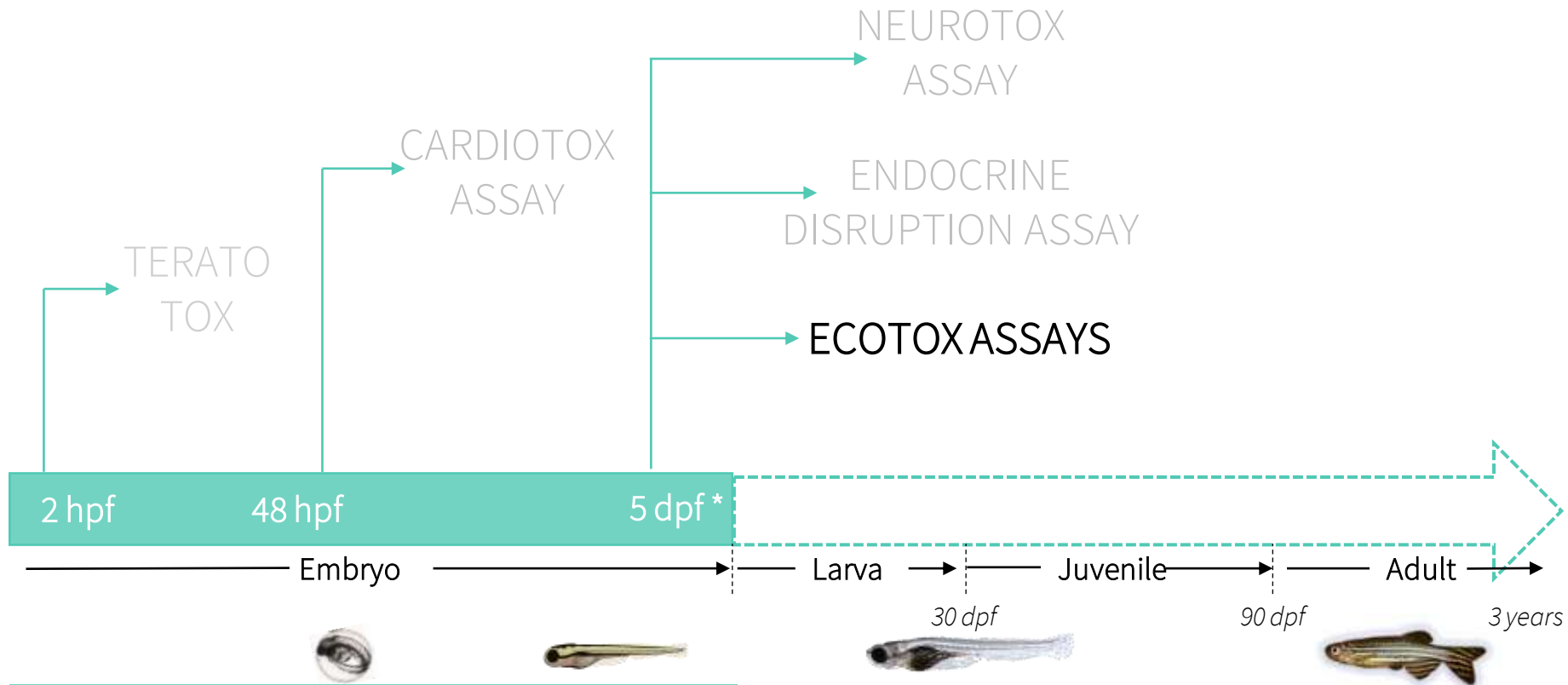


Non-TD chemicals (- controls)

No fluorescence induction at any concentration.



Zebrafish Toxicity Assays



* ALTERNATIVE ANIMAL MODEL

(EU 2010/63/EU)

hpf: hours post fertilization
dpf: days post fertilization

Ecotox Assays

Regulatory Assays

- ✓ OECD TG203: Fish Acute Toxicity Test in adults
- ✓ OECD TG204: Fish 14-Day Prolonged Toxicity Test
- ✓ OECD TG210: Fish, Early-life Stage Toxicity Test
- ✓ OECD TG215: Fish Juvenile Growth Test
- ✓ OECD TG212: Fish Short-term Toxicity Test on Embryo and Sac-fry Stages
- ✓ OECD TG229: Fish Short Term Reproduction Assay
- ✓ OECD TG236: Fish Embryo Acute Toxicity (FET) Test



Early-Phase Assays

- ✓ Zebrafish Larvae Acute Toxicity Test
- ✓ Sperm Quality Assessment in Adult Zebrafish
- ✓ Algae Toxicity Test in Microplate Format
- ✓ Daphnia magna Immobilization Assay



Conclusions

- The **rapid development**, **cost-efficiency** and **high homology** with higher vertebrates makes the **zebrafish** a **suitable biological model** for **toxicologic and environmental risk assessment studies**.
- The use of **zebrafish model** in the process of toxicological profile analysis of new candidates could **speed up the toxicity screening process** while **decreasing the cost**.



Merci beaucoup
Muchas gracias
Thank you




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