(Zebra)Fish Embryo Acute Toxicity Test to predict short term toxicity to fish (and beyond) webinar

14 April 2015, 4:00pm BST

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Today's webinar aims

- To describe the REACH data requirements for short term toxicity testing on fish and the OECD acute fish toxicity test (OECD TG 203).
- To describe the Fish Embryo Acute Toxicity Test (FET).
- To provide an assessment of the correlation between the OECD acute fish toxicity test and the fish embryo toxicity test.
- To look at the potential use of fish embryos beyond acute fish toxicity testing.

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Speakers

- **Marlies Halder**, *EURL ECVAM*

- **Scott Belanger**, *Procter & Gamble*

- **Thomas Braunbeck**, *University of Heidelberg*

- **Gilly Stoddart**, *PETA International Science Consortium, Ltd.*

- Chair: **Philip Lightowlers,** *Chemical Watch*

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Questions

- **Please submit questions during the webinar using your chat box**

- Any unanswered questions can be raised on our Forum following the webinar: http://forum.chemicalwatch.com/

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(Zebra)Fish Embryo Acute Toxicity Test to predict short term toxicity to fish (and beyond)

Marlies Halder, EC JRC, EURL ECVAM
Scott Belanger, Procter & Gamble
Thomas Braunbeck, University of Heidelberg

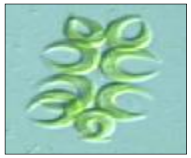
Outline

- Fish embryo acute toxicity (FET) test
  - REACH requirements on "Aquatic toxicity"
  - Standard method :fish acute toxicity test
  - FET background, scheme, lethal endpoints
  - Use of fish embryos & animal welfare legislation
  - EURL ECVAM recommendation on ZFET
- Does the FET predict fish toxicity?
  - Background
  - Data collected, distribution of chemicals
  - FET – Fish comparisons
  - Might QSARs work?
  - Conclusions on FET – Fish comparisons
- Use of fish embryos beyond acute aquatic toxicity
  - Teratogenicity
  - Neurotoxicity
  - Cytochrom P450 induction
  - Endocrine disruption, estrogens, thyroid disruption
  - Genotoxicity
  - Microarrays
- Key references

Fish embryo acute toxicity test

Ecotoxicological information under REACH

Annex VII – Standard information requirements; ≥ 1 t/year
9.1. Aquatic toxicity
9.1.1 Short-term toxicity testing on invertebrates
9.1.2 Growth inhibition on aquatic plants



Annex VIII – Standard information requirements; ≥ 10 t/year

9.1.3 Short-term toxicity testing on fish: the registrant may consider long-term toxicity testing instead of short-term

Note: no need to conduct study if

- *aquatic toxicity is unlikely to occur;*
- *a long-term aquatic toxicity study on fish is available*

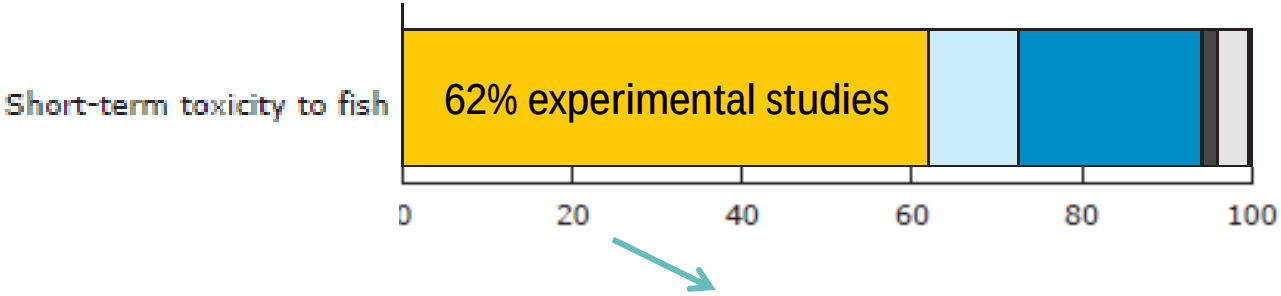
Short-term toxicity testing on fish may also be required by other EU legislations: biocides, plant protection products, veterinary pharmaceuticals, feed and others

Annex IX – Standard information requirements; ≥ 100 t/year
Long-term toxicity testing required if chemical safety assessment indicates the need to further investigate:
9.1.5 Long-term toxicity testing on invertebrates
9.1.6 Long-term toxicity testing on fish

Photos by T. Braunbeck (University of Heidelberg, Germany)

REACH testing needs

- ≥ 100 t/year (Annex X and XI) – 3662 substances registered



- Testing proposal not required for Annex VIII endpoints
- 25 - 50,000 substance registrations expected for 2018 deadline
  http://echa.europa.eu/documents/10162/684852/media_briefing_2014_musset_en.pdf

Standard method for acute fish toxicity

Test guideline	OECD TG 203, Fish, acute toxicity test
Species	zebrafish, fathead minnow, Japanese medaka, rainbow trout, bluegill sunfish, common carp, guppy, and others
Life stages	Juvenile or adult fish
Endpoint	LC50 (concentration lethal to 50% of fish)
Concentrations / controls	At least 5 concentrations, 1 water control, (1 solvent control)
No of animals	7-10 fish / concentration / control


203

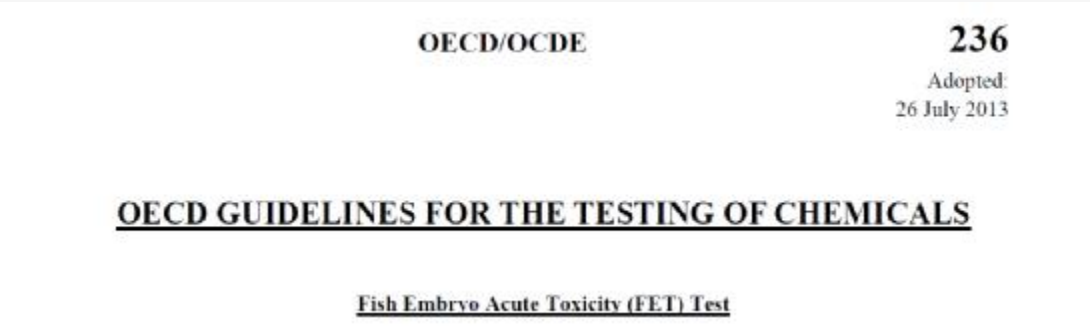
OECD GUIDELINE FOR TESTING OF CHEMICALS

- **Limit test (according to OECD TG 203)**
  - Testing at a single concentration (100 mg/L);
  - If mortality occurs, full TG 203 otherwise LC50 > 100 mg/L

Fish embryo acute toxicity (FET) test

Background

- Included into OECD TG work plan in 2004; lead country: Germany
- 2006 draft TG & supportive background document submitted
  - draft TG based on “fish egg test” (DIN 38415-6, ISO 15088) for effluents testing; zebrafish; 48h exposure
- 2006 OECD ad hoc expert group FET created to address WNT comments
- 2008 – 2012 validation study to assess the reproducibility (within- and between laboratories) of the FET using zebrafish embryos (ZFET)
- 2012 – 2013 Finalisation of TG (incl WNT commenting rounds)
- 2013 adoption by OECD



OECD/OCDE

236

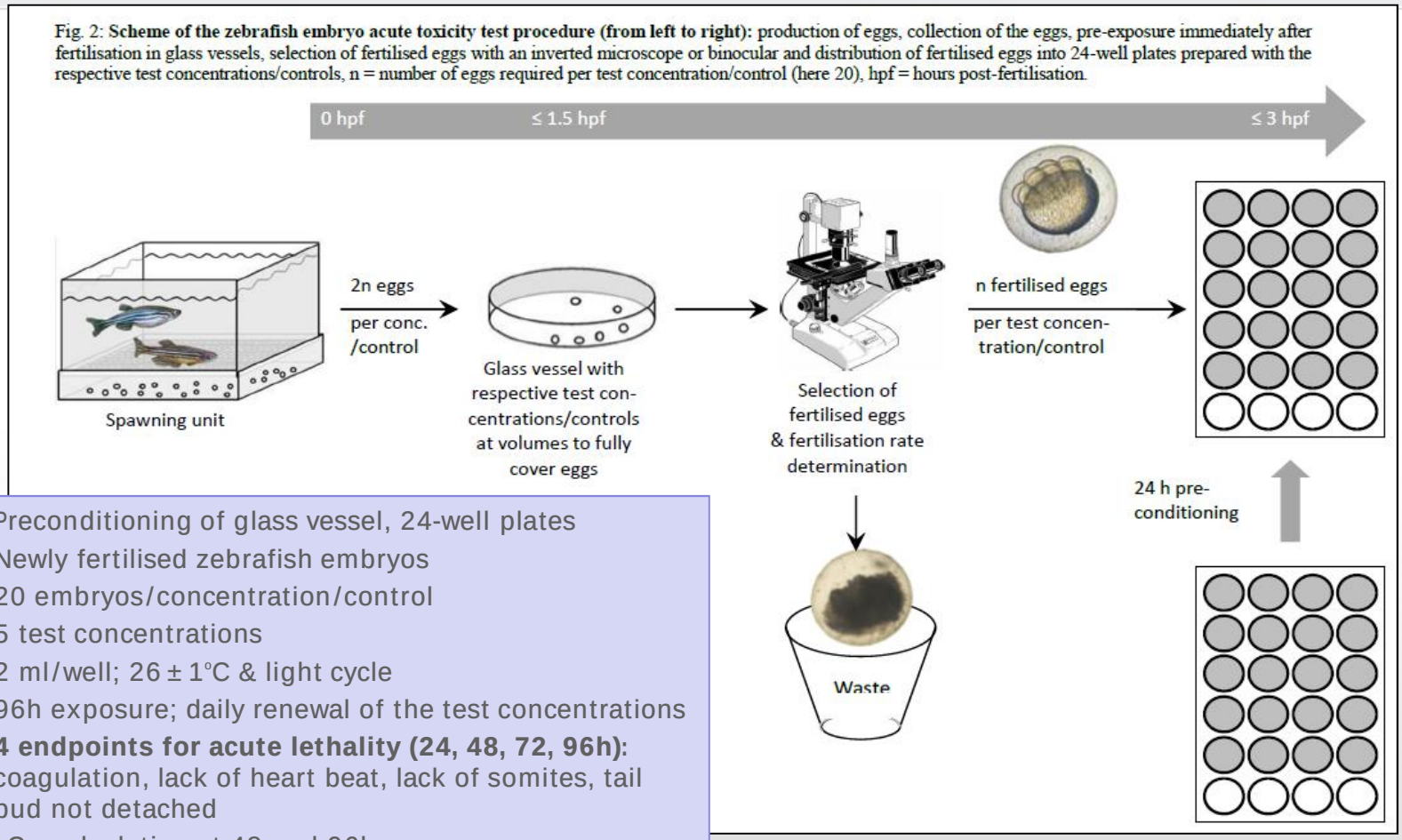
Adopted:
26 July 2013

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Fish Embryo Acute Toxicity (FET) Test

http://www.oecd-ilibrary.org/environment/test-no-236-fish-embryo-acute-toxicity-fet-test_9789264203709-en;jsessionid=247ansnvdfuxm.x-oecd-live-02

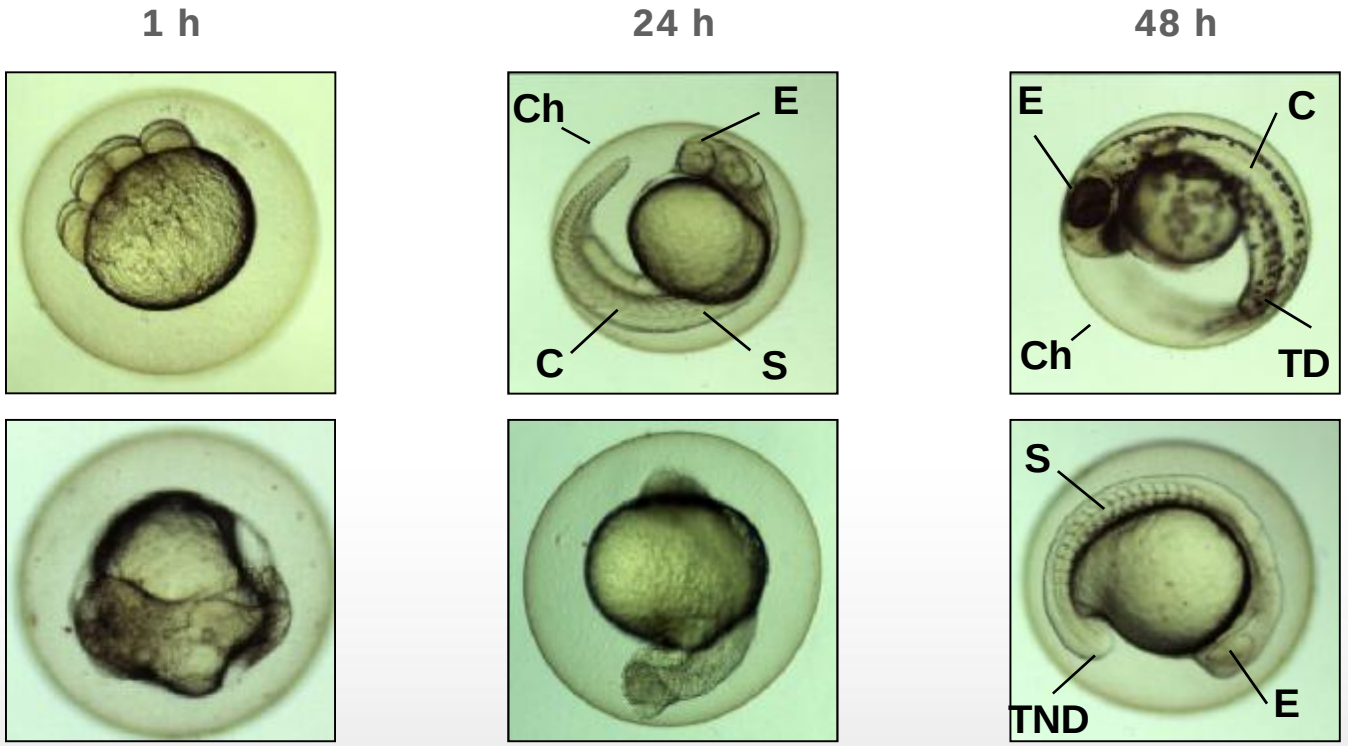
FET scheme as in OECD TG 236



- Preconditioning of glass vessel, 24-well plates
- Newly fertilised zebrafish embryos
- 20 embryos/concentration/control
- 5 test concentrations
- 2 ml/well; 26 ± 1°C & light cycle
- 96h exposure; daily renewal of the test concentrations
- **4 endpoints for acute lethality (24, 48, 72, 96h):** coagulation, lack of heart beat, lack of somites, tail bud not detached
- LC_{50} calculation at 48 and 96h

OECD TG 236 Annex 4

FET apical endpoints



photos provided by J. Bachmann, UBA, Berlin

E = eye; S = somites; Ch = chorion; C = chorda; TD = tail detached; TND = tail not detached

More illustrations of normal zebrafish embryo development and lethal endpoints in TG236 (Annex 3, Annex 5)

Use of FET and animal welfare legislation

DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL

of 22 September 2010

on the protection of animals used for scientific purposes

(Text with EEA relevance)

Article 1(3) This Directive shall apply to
(a)live non-human vertebrate animals including
(i) independently feeding larval forms ...

COMMISSION IMPLEMENTING DECISION

of 14 November 2012

establishing a common format for the submission of the information pursuant to Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes

(notified under document C(2012) 8064)

(Text with EEA relevance)

(2012/707/EU)

"Fish should be counted from the stage of being capable of independent feeding onward. Zebrafish kept in optimal breeding conditions (approximately + 28°C) should be counted 5 days post fertilisation".

- In OECD TG236, the zebrafish embryos are used until 96 h post-fertilisation
- Zebrafish is generally not considered as being capable of independent feeding until 120 h post-fertilisation
- Considering the foregoing, the embryos in question should not be considered as "independently feeding larval forms" within the meaning of the Directive and therefore the procedure, as far as the embryos are concerned, does not fall within its scope.
- The use of the ZFET will result in an overall reduction of the numbers of juvenile and adult fish required for aquatic toxicity testing.

EURL ECVAM recommendation on ZFET

- To provide EURL ECVAM views on the validity of the test method in question, to advise on possible regulatory applicability, limitations and proper scientific use of the test method, and to suggest possible follow-up activities in view of addressing knowledge gaps.
- Consultation with stakeholders & public

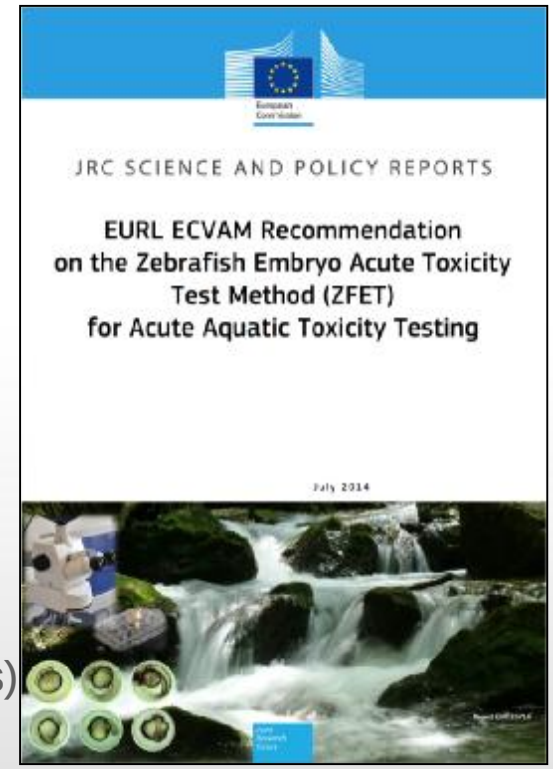
§ reproducible

§ provides information on acute fish toxicity comparable to information derived with OECD TG 203

§ ready for regulatory use
  - use as alternative to OECD TG 203
  - update of relevant legislation & guidance, e.g. REACH guidance, Annex VIII

§ future guidance document
  - address *potential* limitations (metabolic capacity, high molecular weight chemicals, additional endpoints)

§ maintain fish / FET database



Does the FET predict acute fish toxicity?

Does the FET Predict Acute Fish Toxicity?

Investigation separate from the method development of the FET and its validation…yet a key element for acceptance

Seen early on by the OECD FET *ad hoc* expert group and OECD FET VMG as essential to address – perfectly acceptable FET method is of no value if it does offer a prediction of acute fish toxicity

Success looks like:

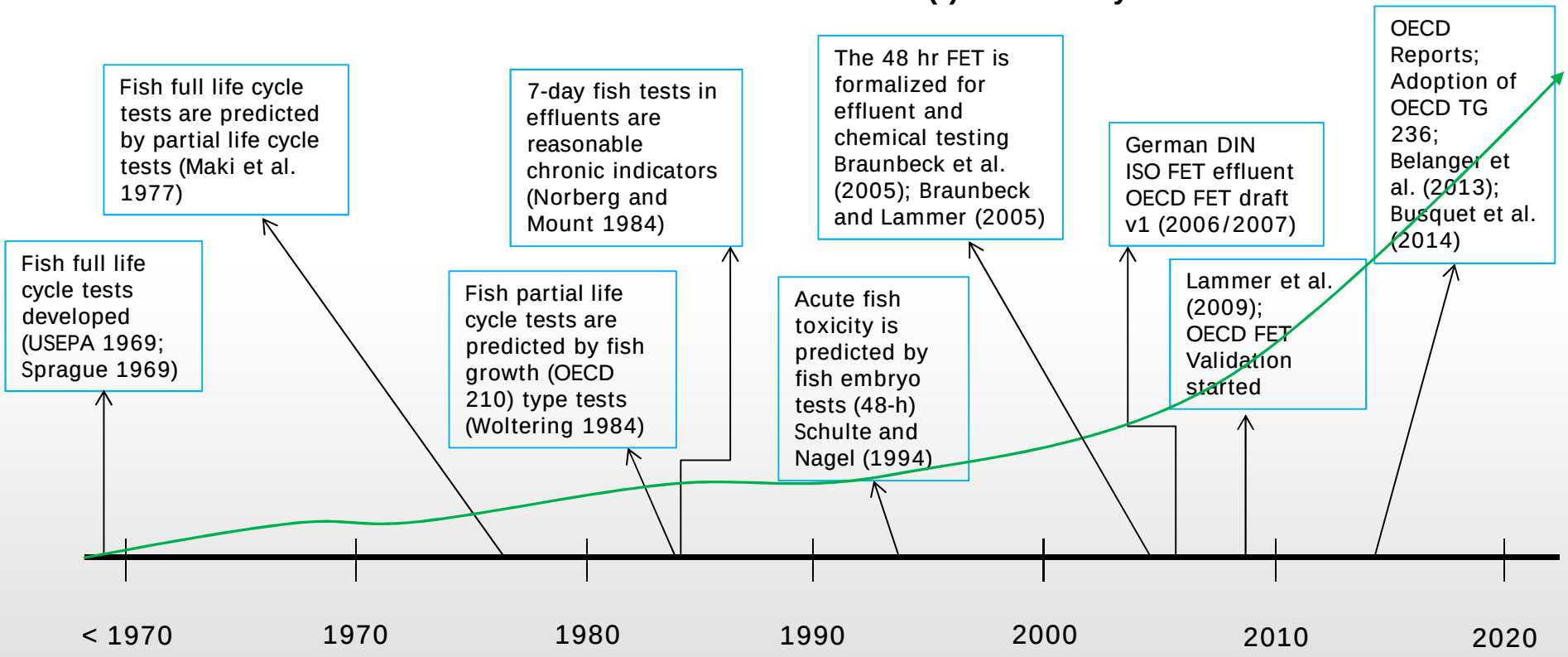
1. **FET result is quantitatively equal to acute fish toxicity test result for the same chemical**
2. **All compounds are predicted equally well with similar or better statistical power**
3. **Chemical coverage (domain of applicability) is very broad**
4. **Results are repeatable**

Led to a data gathering exercise, parallel to the FET validation

History, more or less, repeats itself

Shortening of the time span employed in acute and chronic fish tests for the determination of chemical and effluent hazard has occurred over many decades

Considerations as to animal welfare are more recent and still (r)evolutionary



FET-Fish Comparisons

How much information is there?

What fish dominate the available information?

How many compounds have been covered?

What chemical categories and modes of action do we have data on?

Can we address sources of variability – what are they, are fish and FET subject to the same sources, etc.

Do QSARs developed for fish also work for the FET?

And there is always more…









FET-Fish Comparisons

A very large data collection effort:

1532 fish acute toxicity studies included
985 Fish Embryo Toxicity studies included

203 compounds
17 modes of action
38 chemical categories
>15 broad industrial use categories

MW from 49 to >100,000
Span of –log Kow from -4.1 to 7.9
Solubility from 0.8 µg/L to > 1g/L

Evaluate FET versus fish toxicity

- Influence of chemical class, mode of action, etc.
- Influence of test design, duration, etc.
- QSAR predictions
- Fish-fish toxicity relationships
- Sources of variability

?

=

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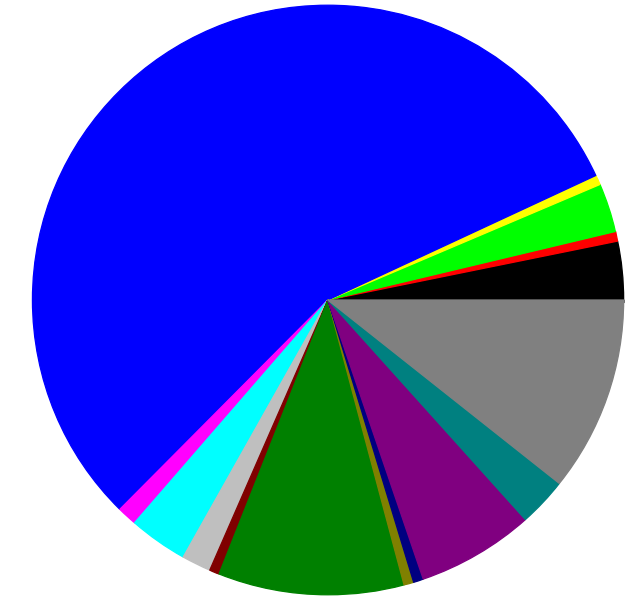
Distribution of Chemicals Tested

- Biocide
- Flame retardant
- Food additive/Vitamin
- Hair dye
- Industrial organic
- Inorganic
- Metal
- Natural/Botanical
- Organometal
- Pesticide
- Perfume
- Petrochemical
- Pharmaceutical
- Polymer
- Surfactant

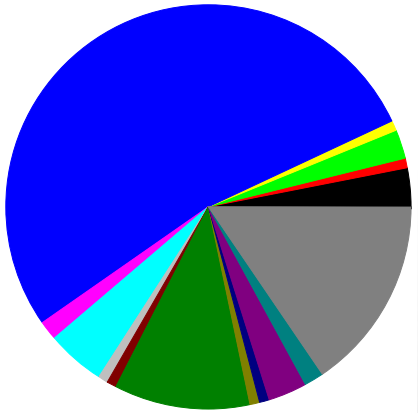
Size of the oval = number of chemicals

FET
N = 229 chemicals



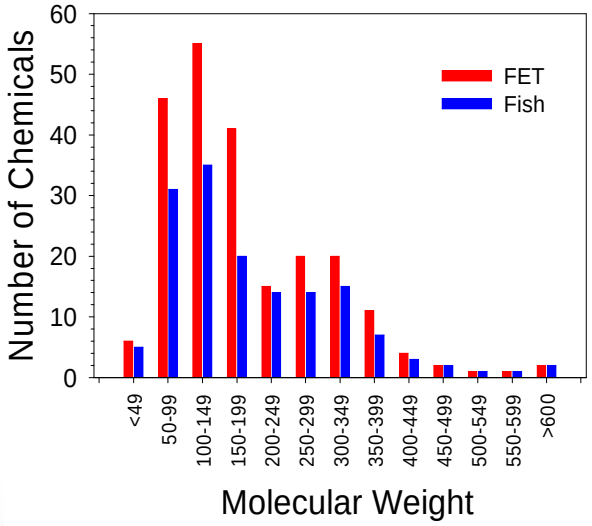
985 studies

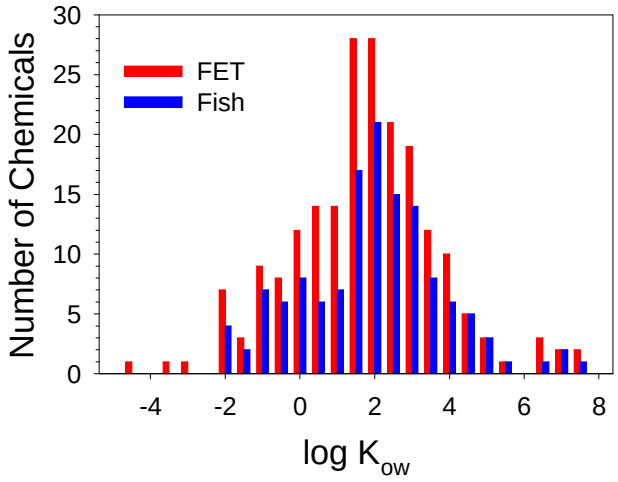
OECD 203
N = 151 chemicals

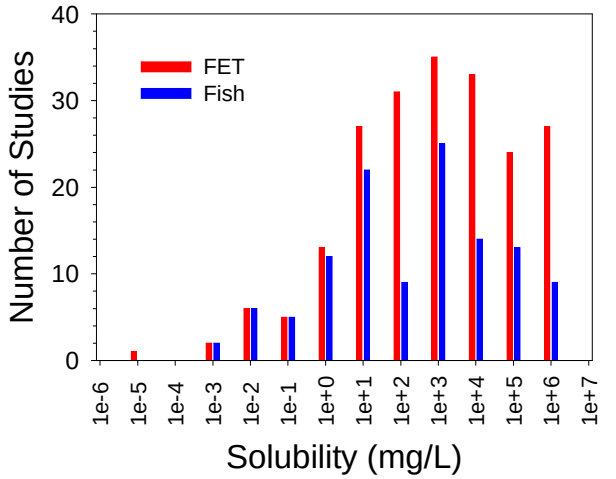


1531 studies

Does the FET Predict Acute Fish Toxicity?



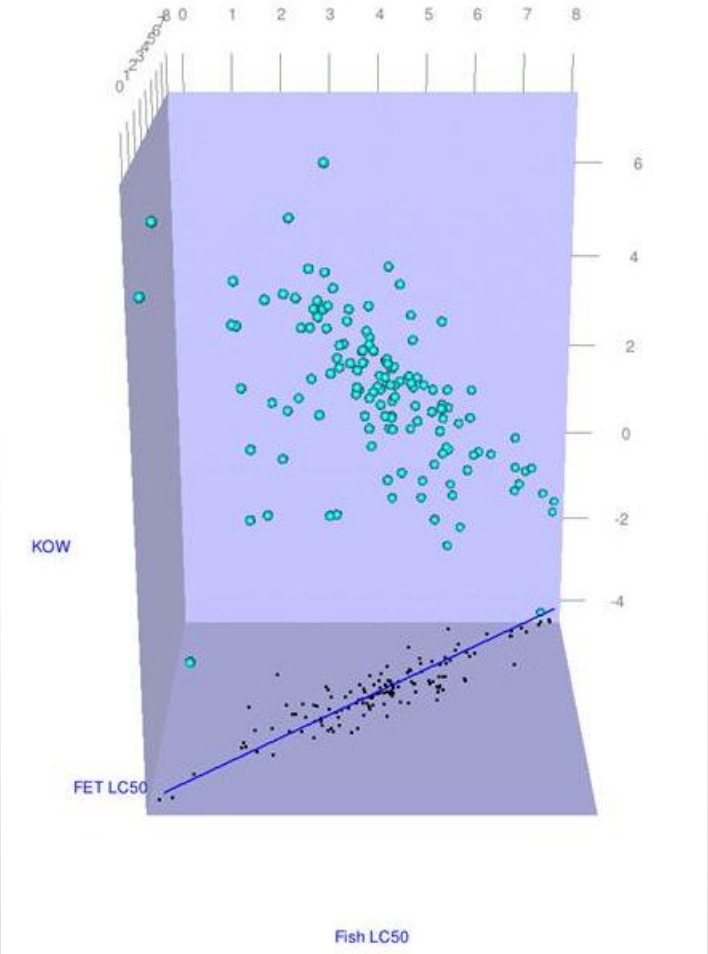




Wide range of chemical space

Validation program touched the edges of these factors

Does the FET Predict Acute Fish Toxicity?



FET-Fish-K_{ow}

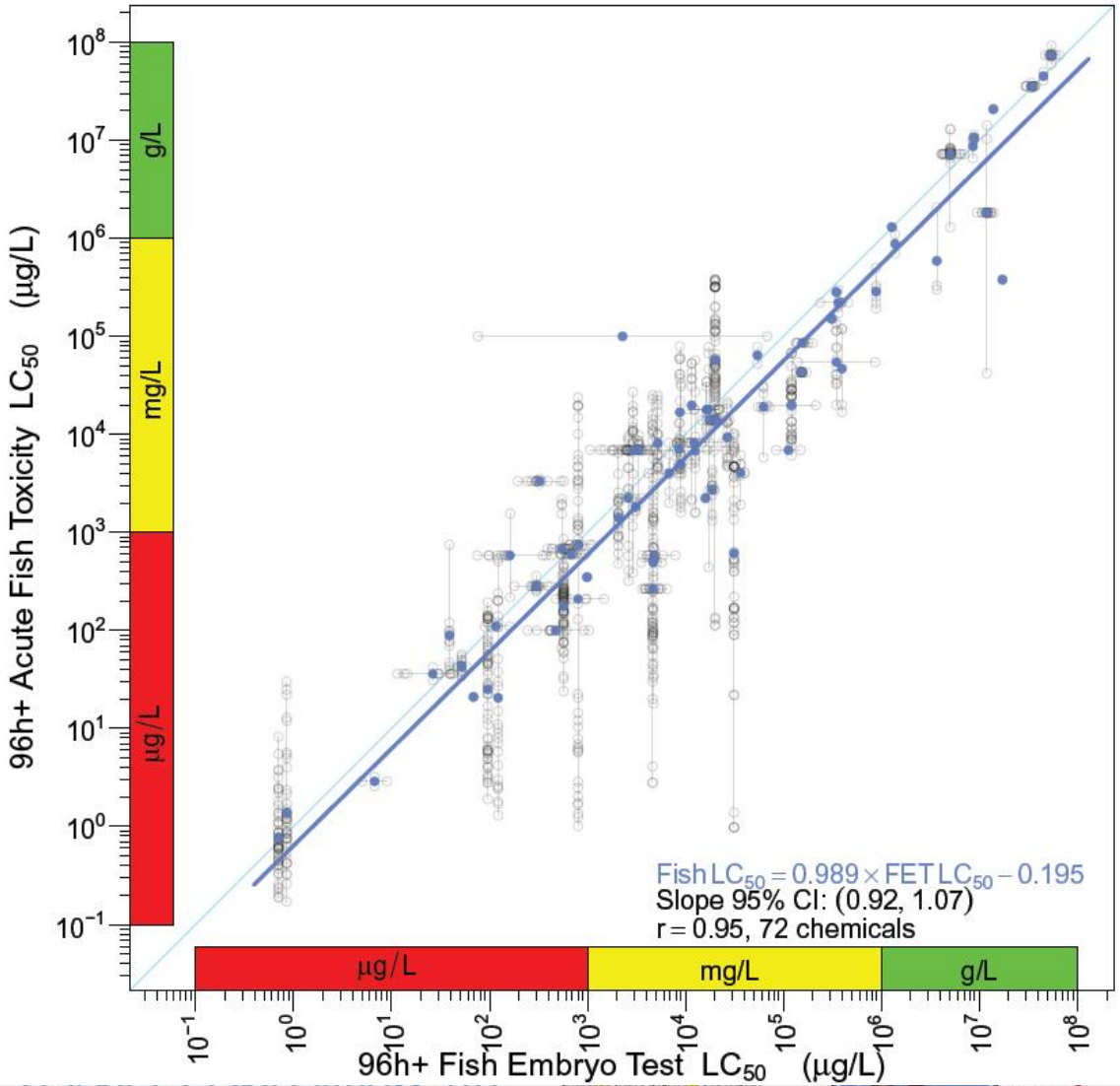
High Kow compounds are plotted for FET and fish toxicity on the floor of the plot

log K_{ow} is presented in the “z-axis” vertically

No pattern of bias appears across the range of compounds

Spread of K_{ow} across potency is highly influenced by chemical class

FET-Fish Comparisons



Parallel to the line of 1:1 correspondence

Large range of potency, database similar in size to that of Lammer et al. (2009)

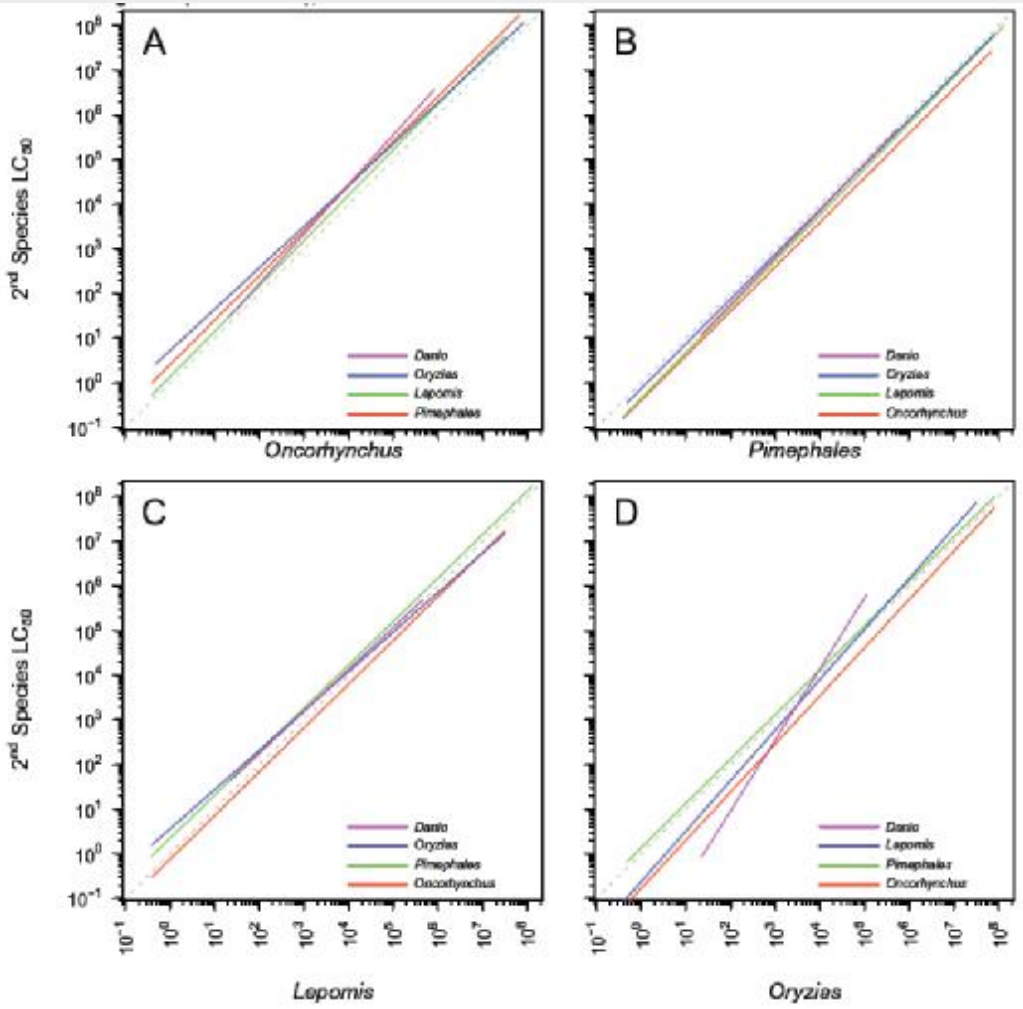
In practical terms:

0.1 mg/L FET = 0.07 mg/L fish

1 mg/L FET = 0.7 mg/L fish

10 mg/L FET = 6.2 mg/L fish

Comparisons of Fish to Fish versus FET to Fish



Inter-species toxicity of chemicals are highly correlated

Line of 1:1 correspondence indicates the fish are equisensitive across all levels of potency

Regressions above the line of equality indicate that the second species is less sensitive

Regressions below the line of equality indicate the second species is more sensitive

Medaka is generally the less sensitive and rainbow trout is more sensitive.

Comparisons of Fish to Fish versus FET to Fish

Fish 1 (X)	Fish 2 (Y)	Slope	Intercept	N	Corr
FET Kow 3+	Fish Kow 3+	0.840	0.240	41	0.840
BG	Medaka	0.883	0.552	23	0.899
RBT	Medaka	0.927	0.719	32	0.961
BG	Zfish	0.942	0.362	22	0.880
BG	FHM	0.973	0.340	56	0.940
BG	RBT	0.982	-0.127	48	0.918
Medaka	FHM	0.996	0.133	37	0.934
FHM	RBT	0.998	-0.396	57	0.929
RBT	FHM	1.002	0.397	57	0.929
FHM	Medaka	1.004	-0.134	37	0.934
RBT	BG	1.019	0.130	48	0.918
FHM	BG	1.028	-0.350	56	0.940
All FET	All fish	1.030	-0.290	151	0.900
All FET	96 h fish	1.030	-0.310	144	0.900
FHM	Zfish	1.048	-0.337	26	0.834
Medaka	RBT	1.079	-0.775	32	0.961
RBT	Zfish	1.110	0.001	25	0.858
Medaka	BG	1.132	-0.625	23	0.899
Medaka	Zfish	1.593	-2.225	16	0.719

Simply put, fish-fish regressions cannot be distinguished from FET-fish regressions

Might QSARs work?

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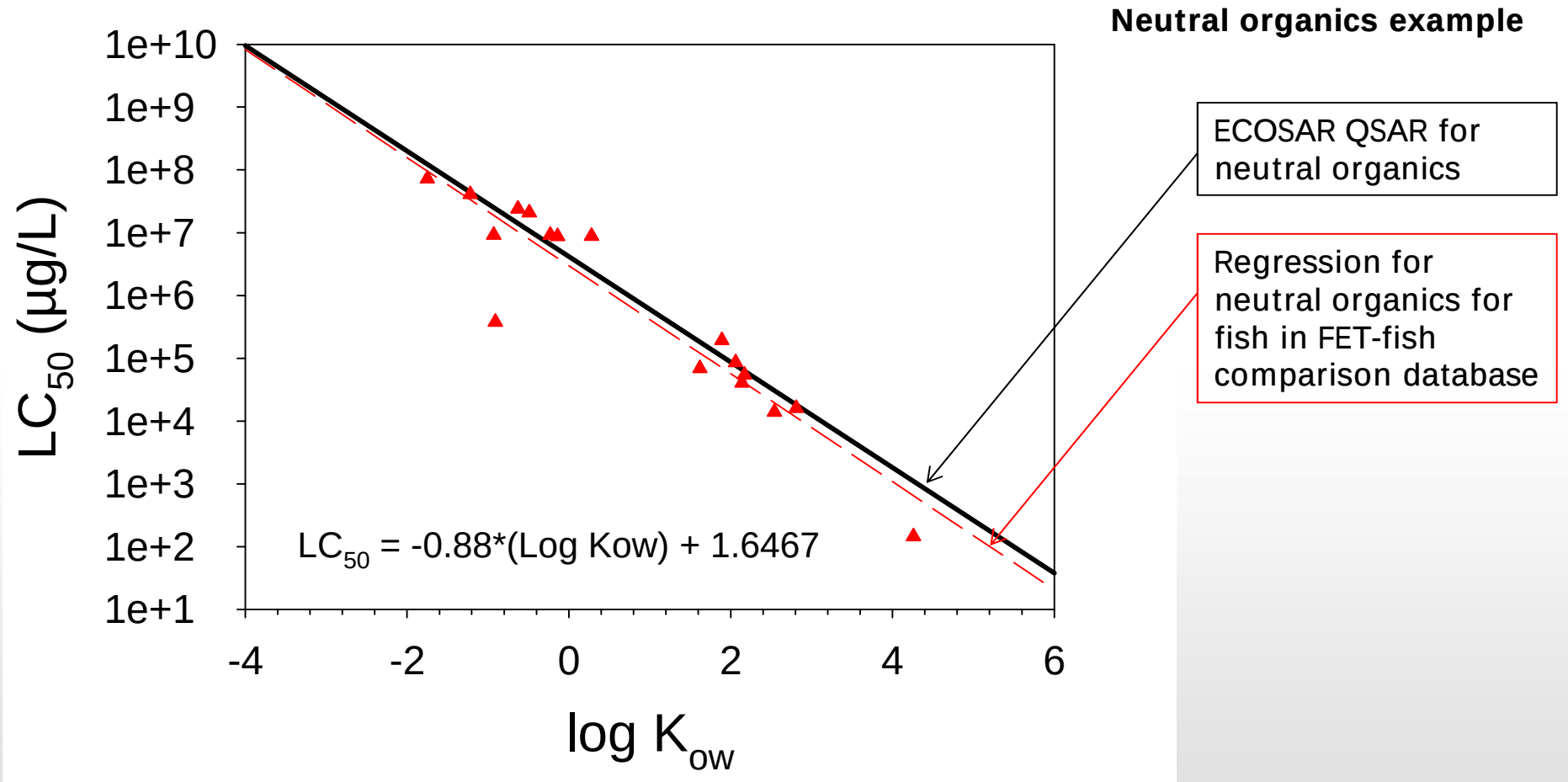
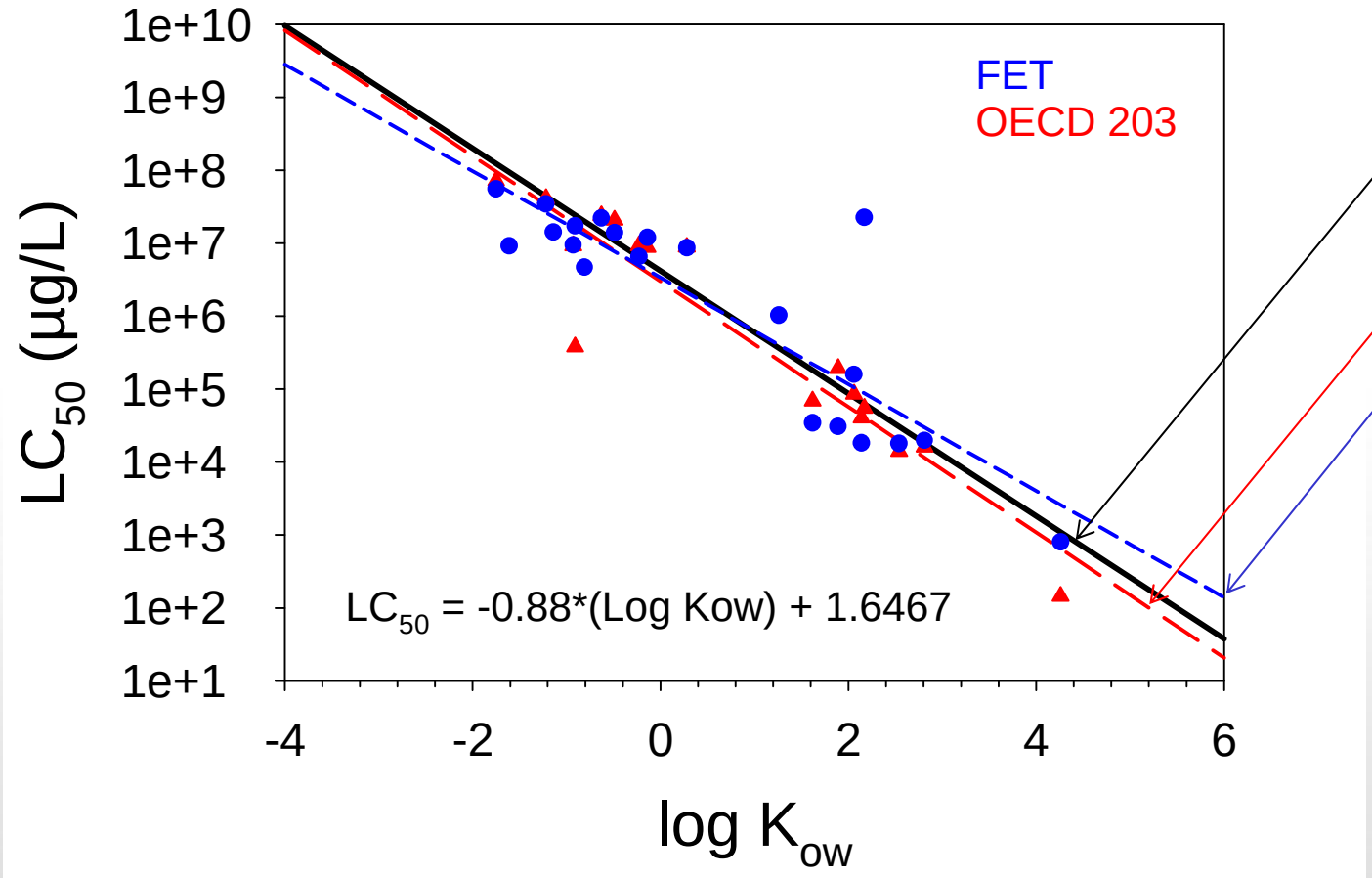


Might QSARs work?



Similar results

-0.75 slope (FET)

-0.84 slope (Fish)

-0.88 slope (ECOSAR)

FET-Fish Overall Conclusions

- **The available data to allow fish and fish embryo comparisons are very rich.**
- **The most commonly tested species are fathead minnow, rainbow trout, zebrafish, bluegill and Medaka, the choices reflect geographic preferences.**
- **Over 200 chemicals have been assessed in the FET and of these 144 had reliable fish data; the highest quality data set consists of nearly 80 96 hr fish data and FET data on those same chemicals.**
- **15 chemical functional and at least 38 modes of action are included across a wide range of potencies, molecular weights and solubilities.**

FET-Fish Overall Conclusions

- **Sources of variability are innumerable; fish and FET are subject to the same influences. FET data is more species homogenous (*Danio*) but this is changing.**
- **QSARs developed for fish also work for the FET; when there are departures it is likely that the fish data on a chemical is not well represented in the QSAR.**
- **Industry scientists are already actively using the FET in chemical hazard assessment and decision-making. Industry is confident in the assay and aware of the animal welfare considerations it represents.**
- **Most major contract laboratories in Europe and the US are already offering the assay in their menu of services.**

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Use of fish embryos

beyond acute fish toxicity

Use of fish embryos beyond acute fish toxicity

- **alternative to OECD TG 203**
- any kind of range-finder for higher-tier testing
  - prolonged fish toxicity: OECD TGs 210, 212, (215)
  - Endocrine disruption: OECD TGs 229, 230, 234
  - future OECD TGs, e.g. Medaka Extended One-Generation Reproduction Test

Use of fish embryos beyond acute fish toxicity

- **alternative to OECD TG 203**
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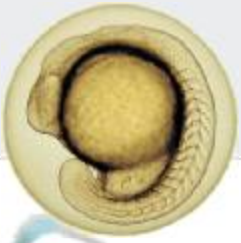
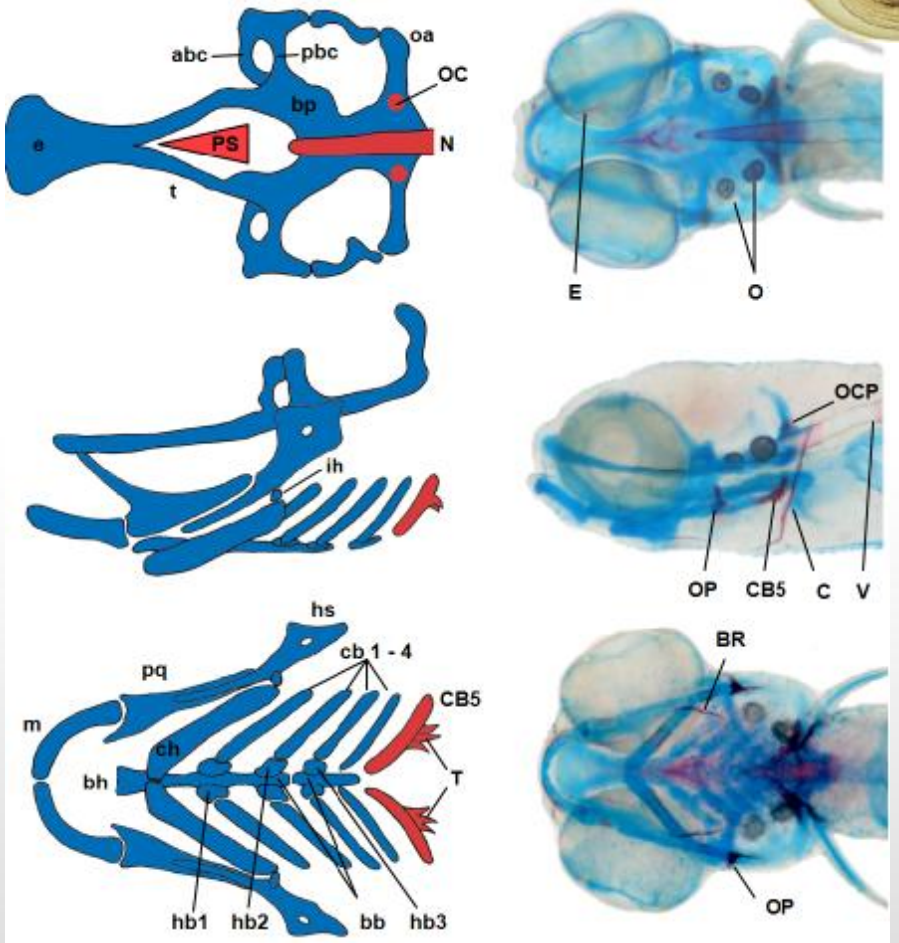
  - Endocrine disruption: OECD TGs 229, 230, 234
  - future OECD TGs, e.g. Medaka Extended One-Generation Reproduction Test
- testing for more specific endpoints
  - teratogenicity
  - neurotoxicity
  - biotransformation induction
  - endocrine disruption
  - genotoxicity
  - gene activation

ZF embryos: Teratogenicity



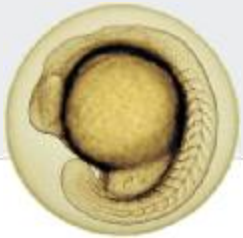
Staining with Alcian Blue (cartilage) and Alizarin Red (bones)

according to Walker and Kimmel (2007)



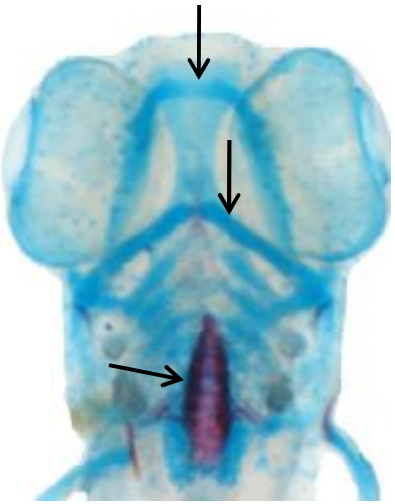
Strecker, R., Weigt, S., Braunbeck, T. (2013) Toxicol. Appl. Pharmacol. 268: 221-231.

ZF embryos: Teratogenicity



Teratogenic effects in the head by dithiocarbamates and hydrazides

Stage 1



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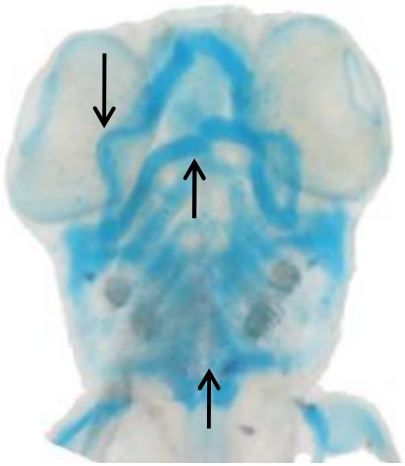
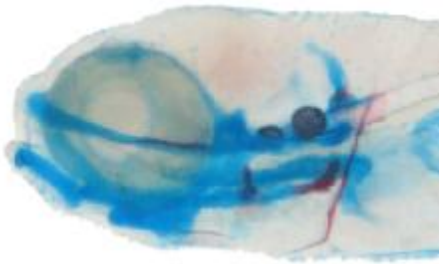
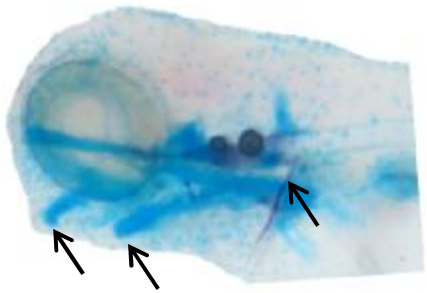
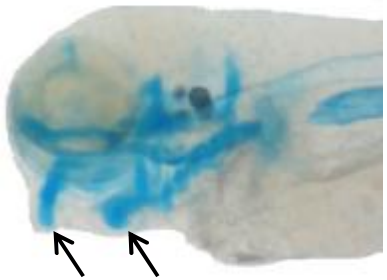


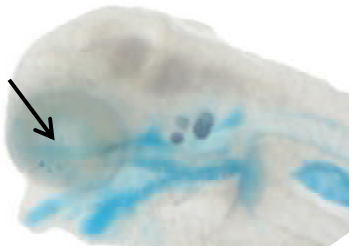
Stage 3

Stage 4









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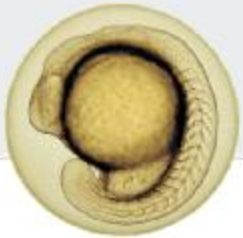
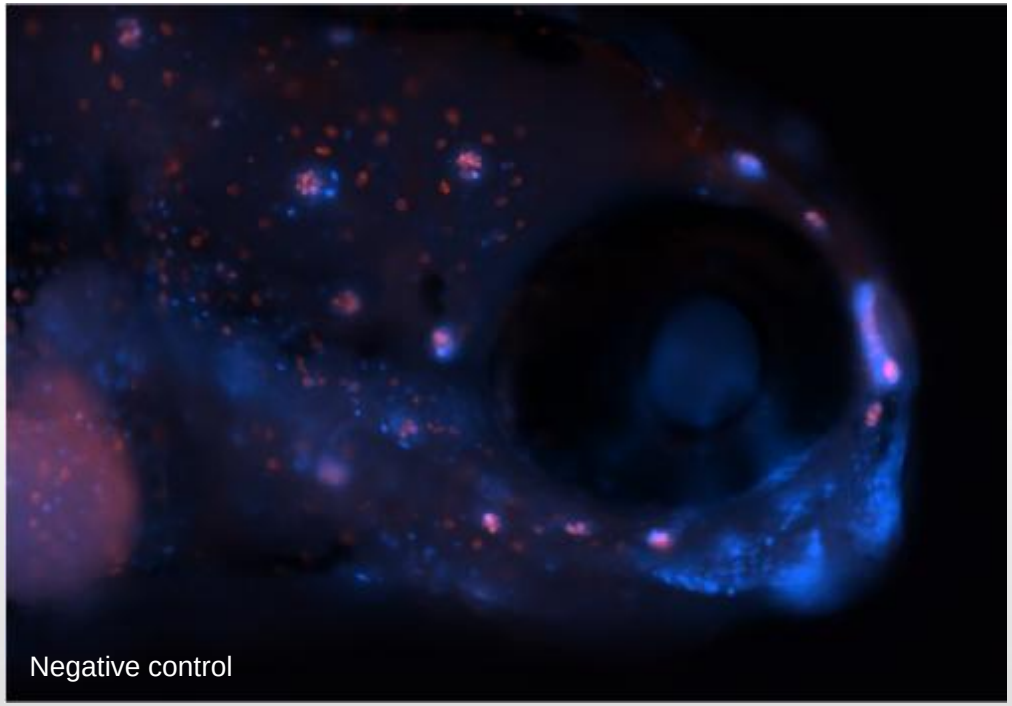
ZF embryos: Neurotoxicity

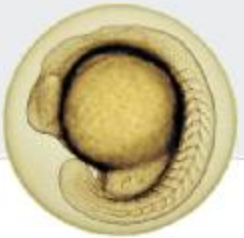


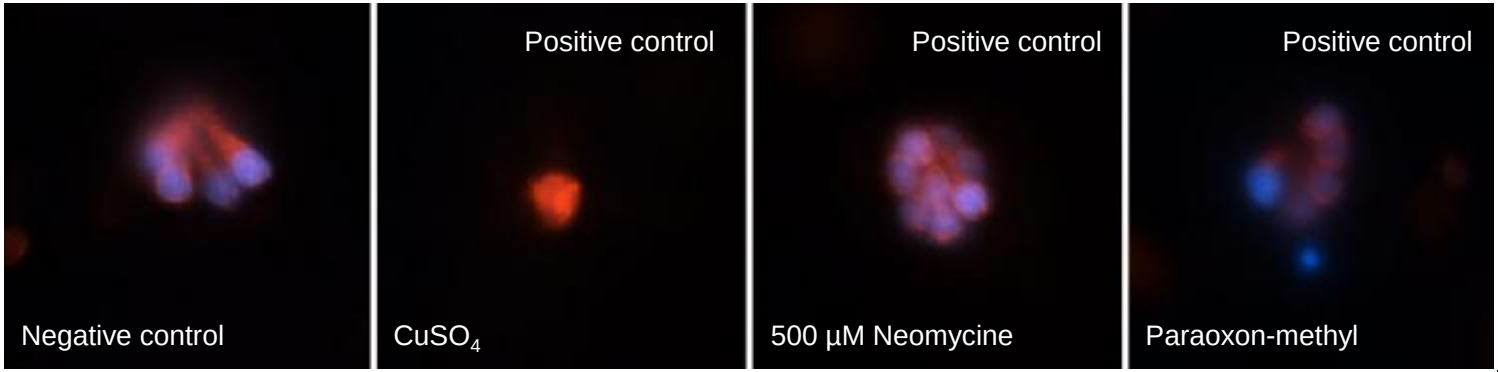
Neuromast staining
in 4 d old ZF



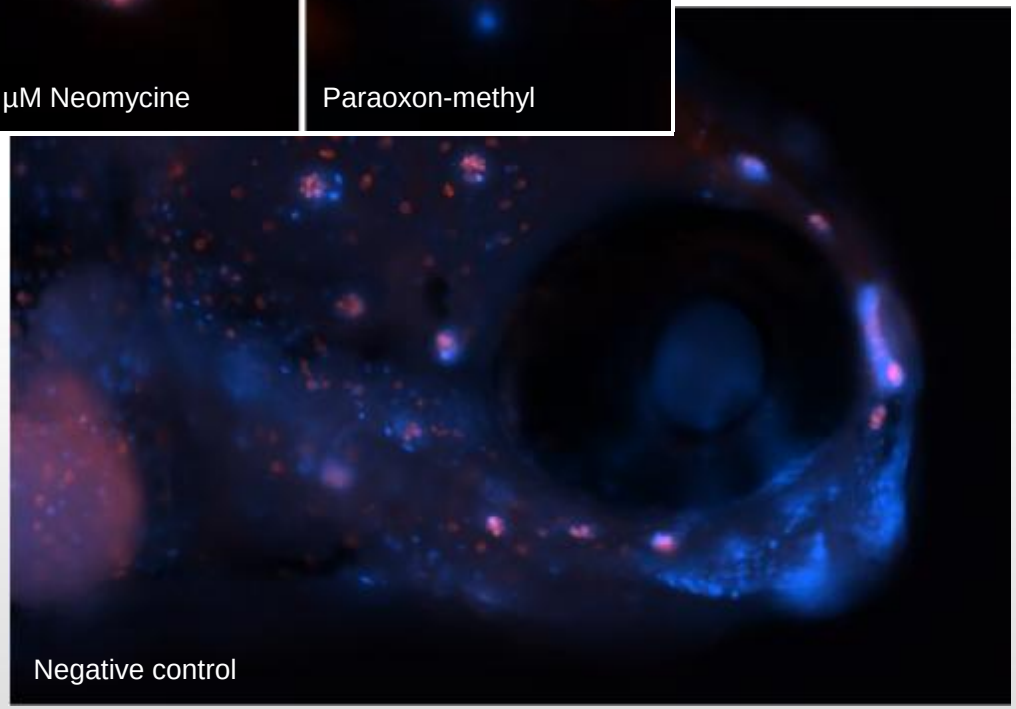
Braunbeck, T., Kais, B., Lammer, E., Otte, J., Schneider, K., Stengel, D., Strecker, R.T. (2015) Environ. Sci. Pollut. Res. DOI 10.1007/s11356-014-3814-7.

ZF embryos: Neurotoxicity





Neuromast staining in 4 d old ZF



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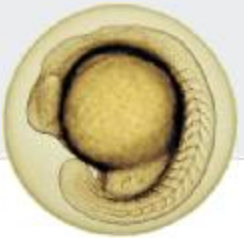
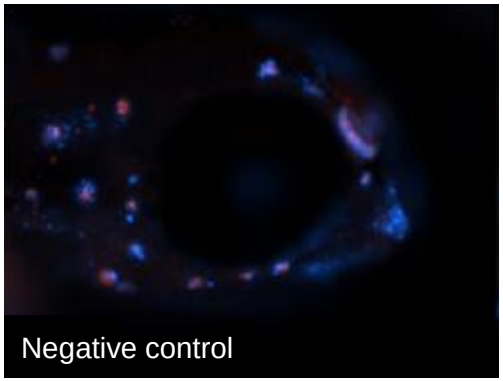
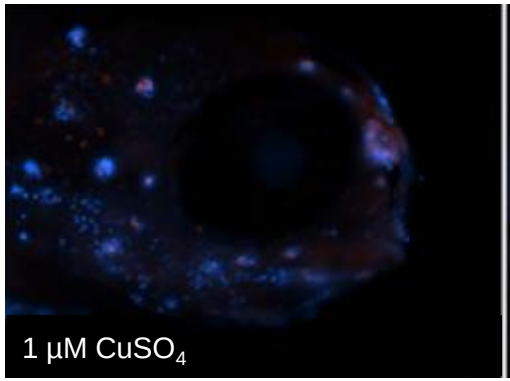
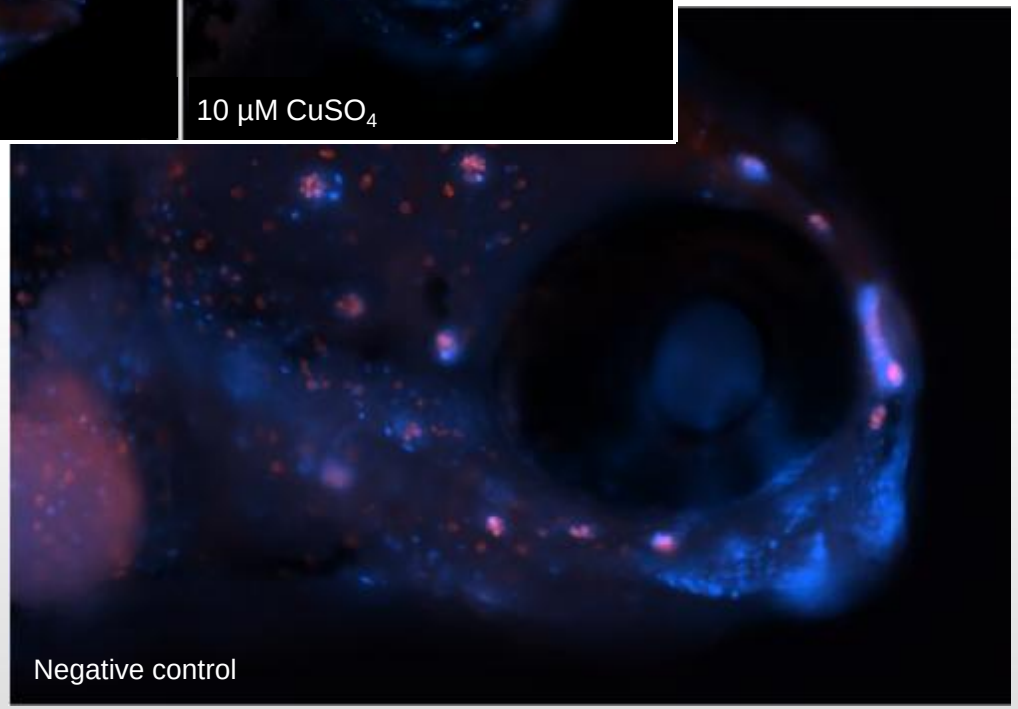
ZF embryos: Neurotoxicity







Neuromast staining in 4 d old ZF



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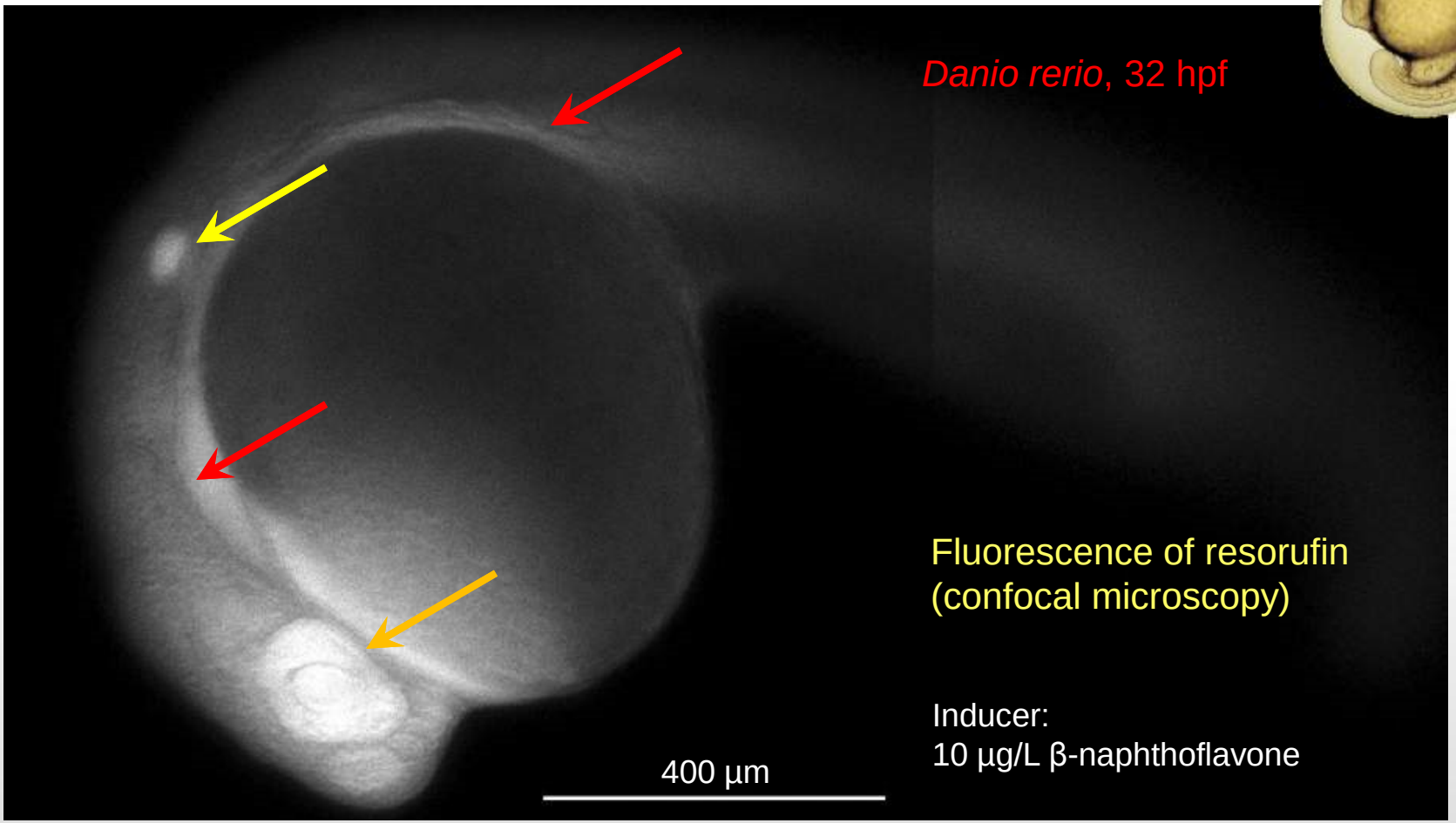
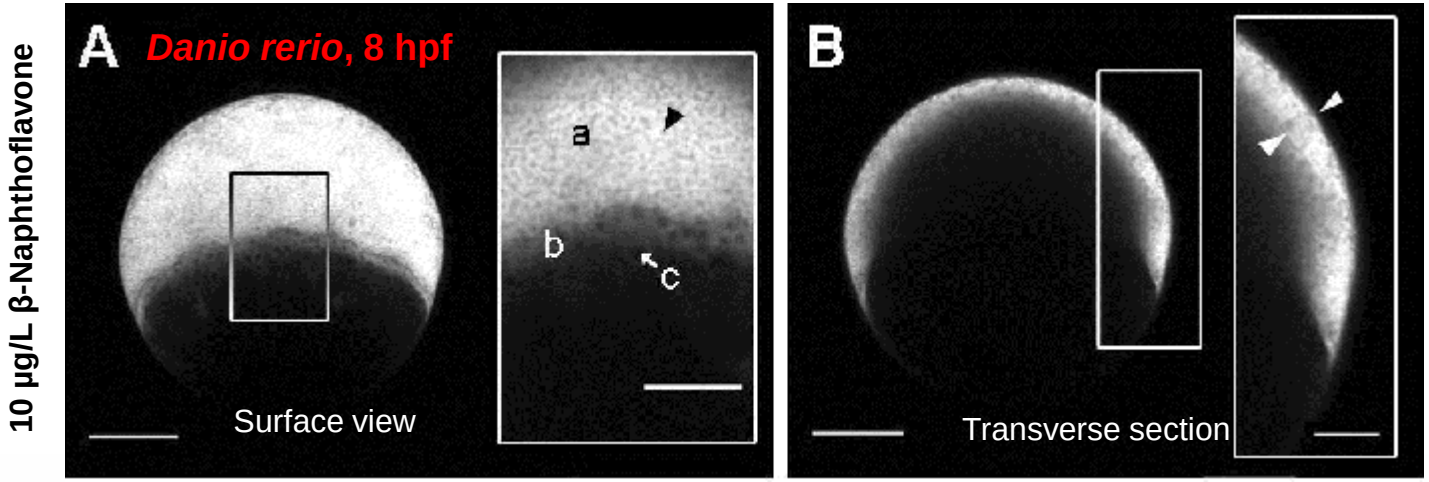
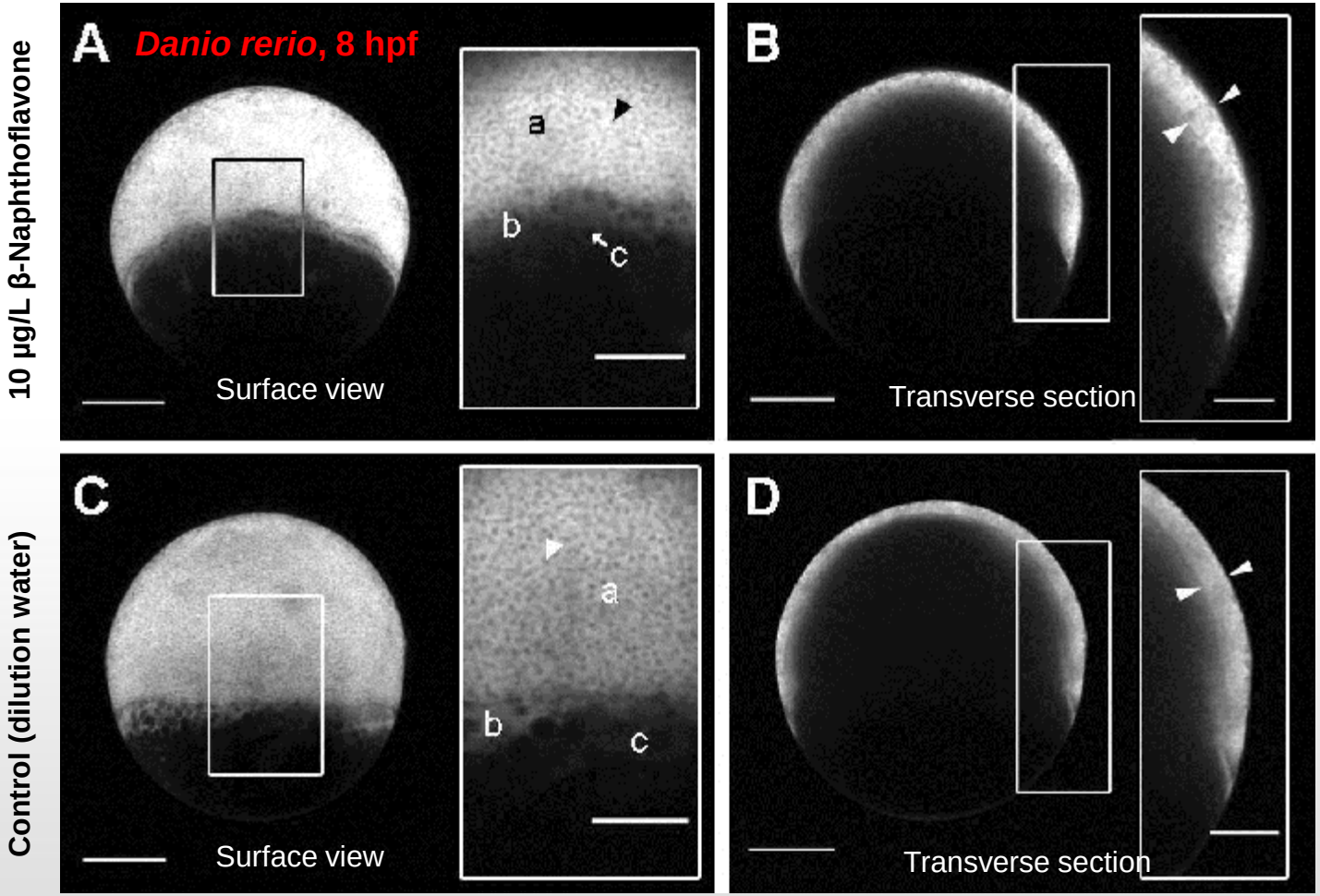
ZF embryos: Cytochrome P450 induction



ZF embryos: Cytochrome P450 induction



ZF embryos: Cytochrome P450 induction



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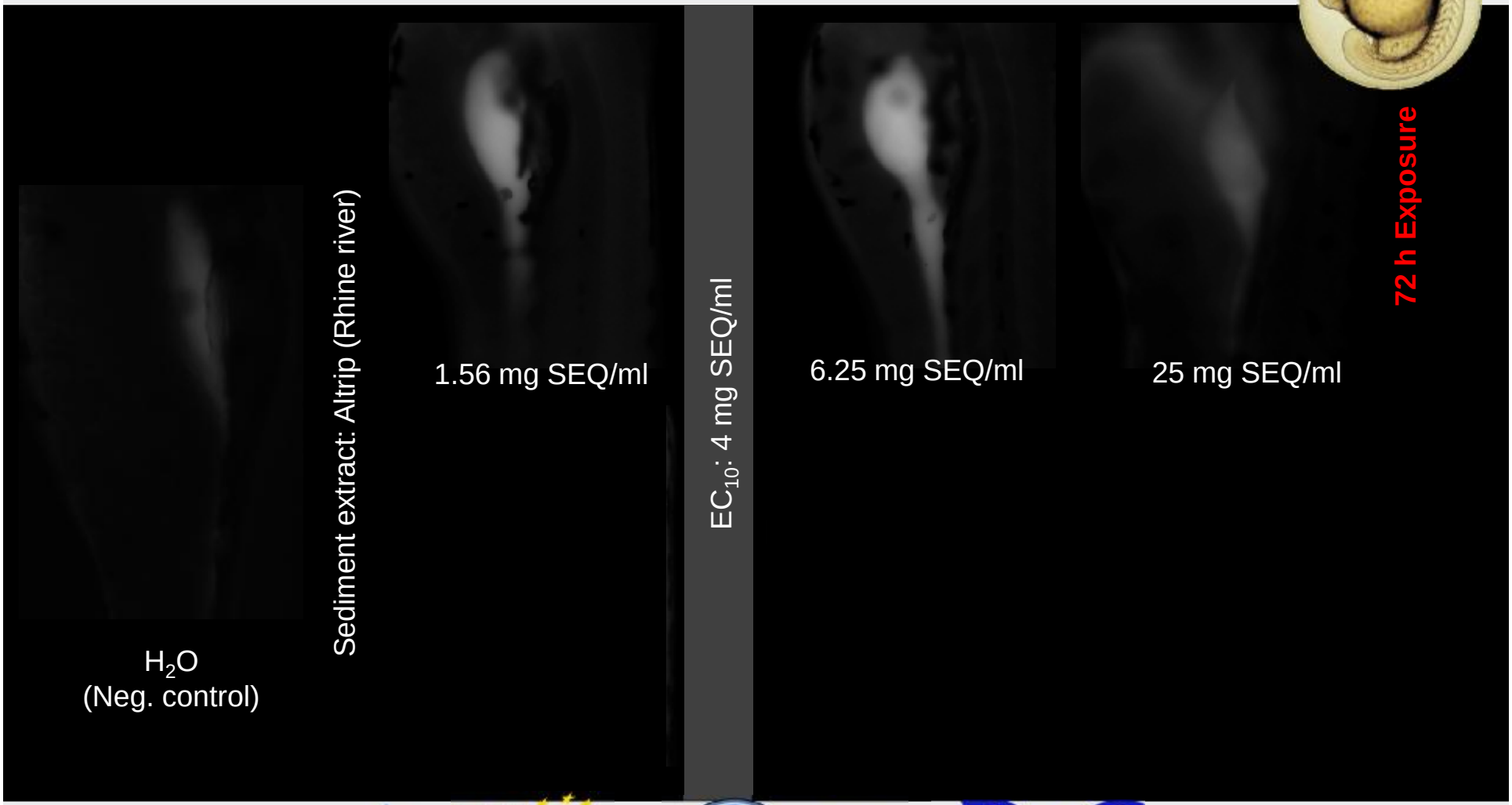
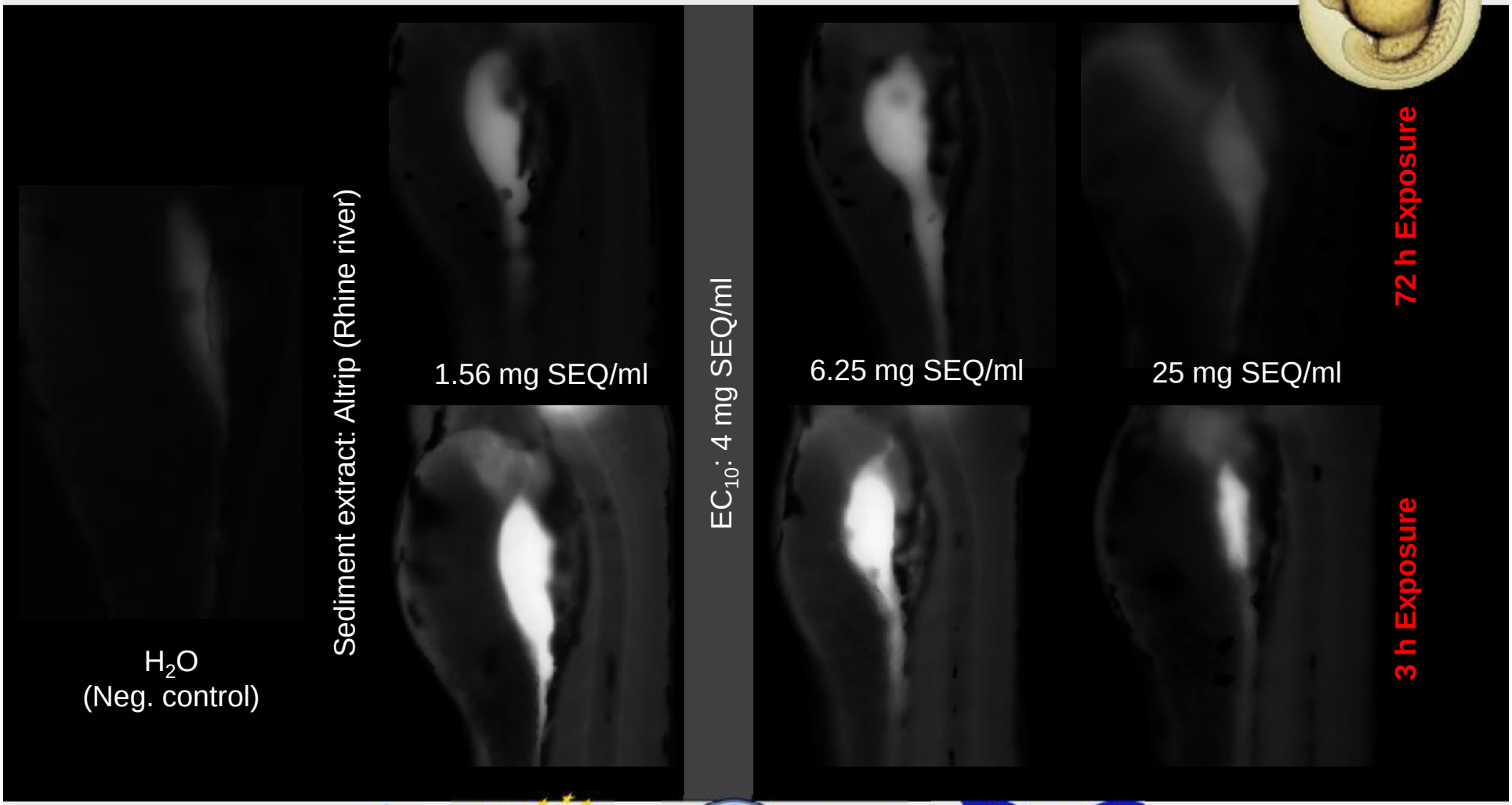
ZF embryos: Cytochrome P450 induction



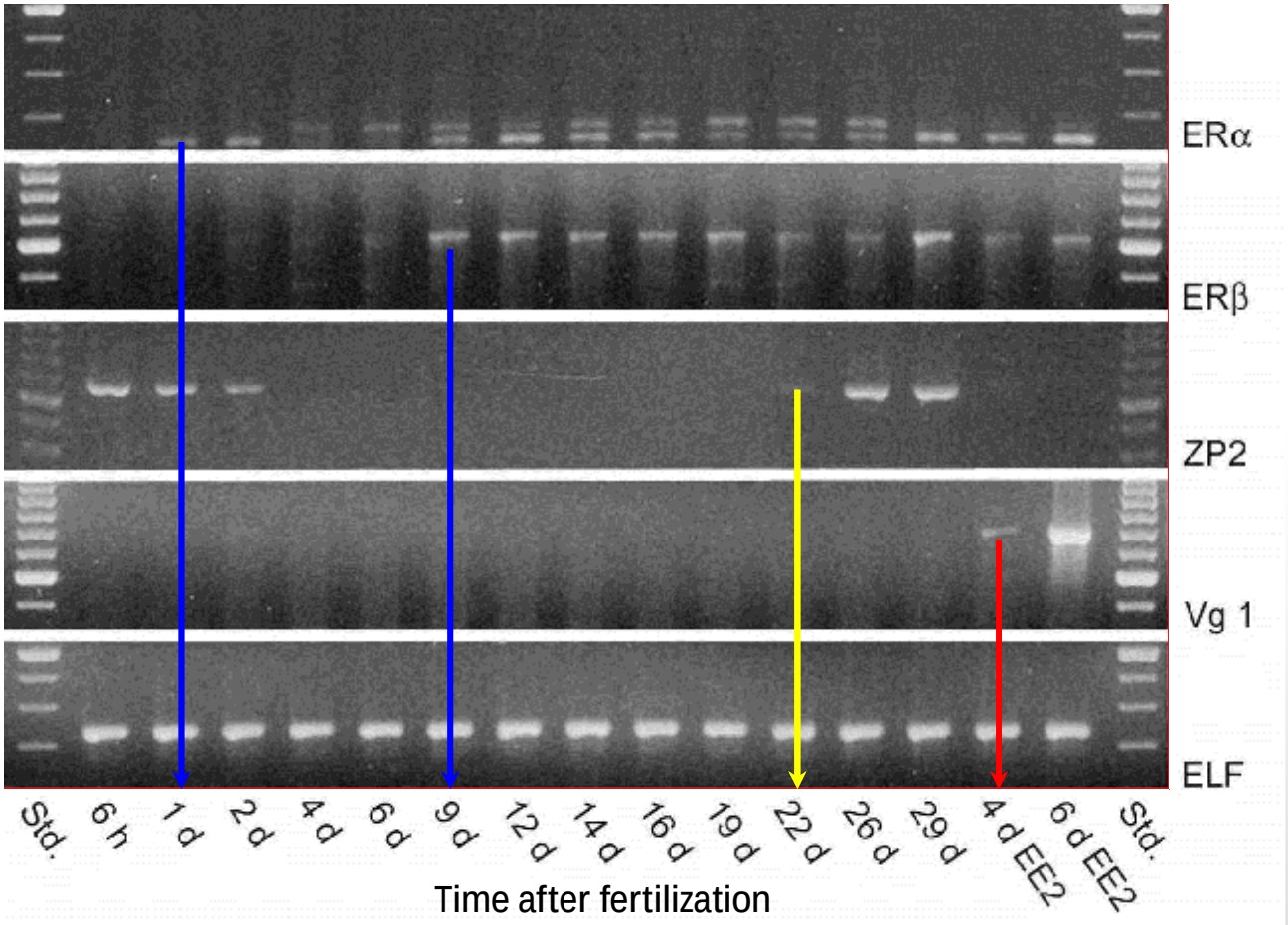
ZF embryos: Cytochrome P450 induction



ZF embryos: Endocrine disruption

ER	Estradiol receptor a, b
ZP	Zona pellucida protein
Vg 1	Zebrafish vitellogenin 1
ELF	Elongation factor 1α
EE2	17α-Ethinylestradiol

Estrogens



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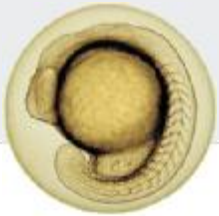
ZF embryos: Thyroid disruption

Goitrogens

ZF embryos: Thyroid disruption



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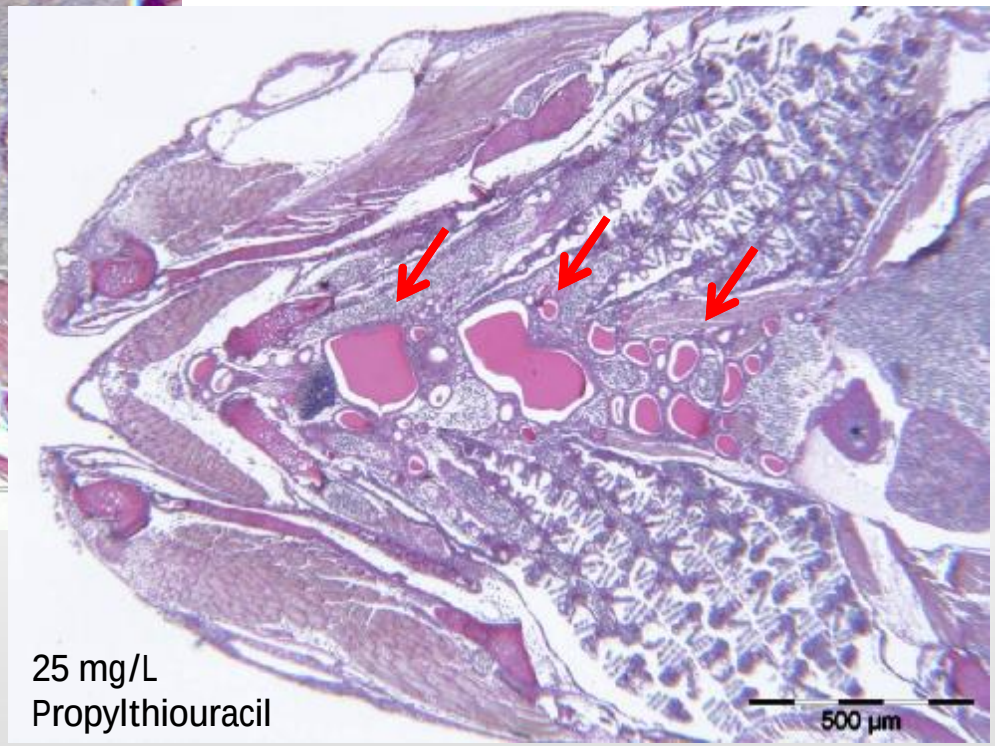


Goitrogens

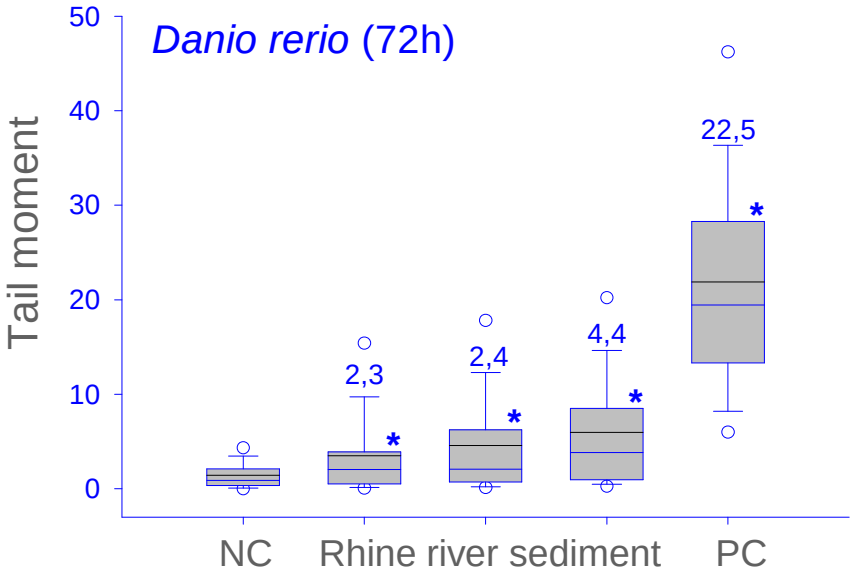
ZF embryos: Genotoxicity testing

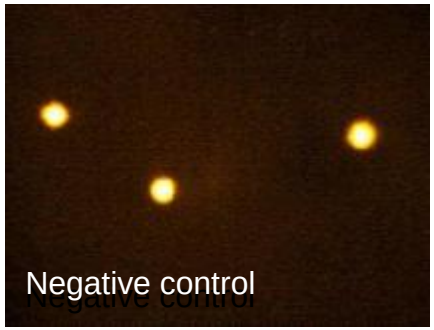
Exposure for 48 h

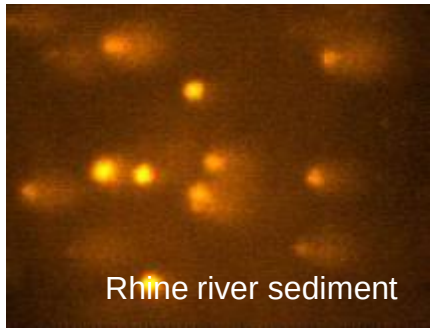
Cell isolation

Comet assay

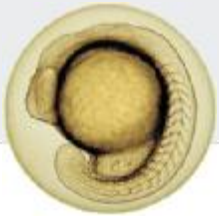
Comet assay in zebrafish embryos

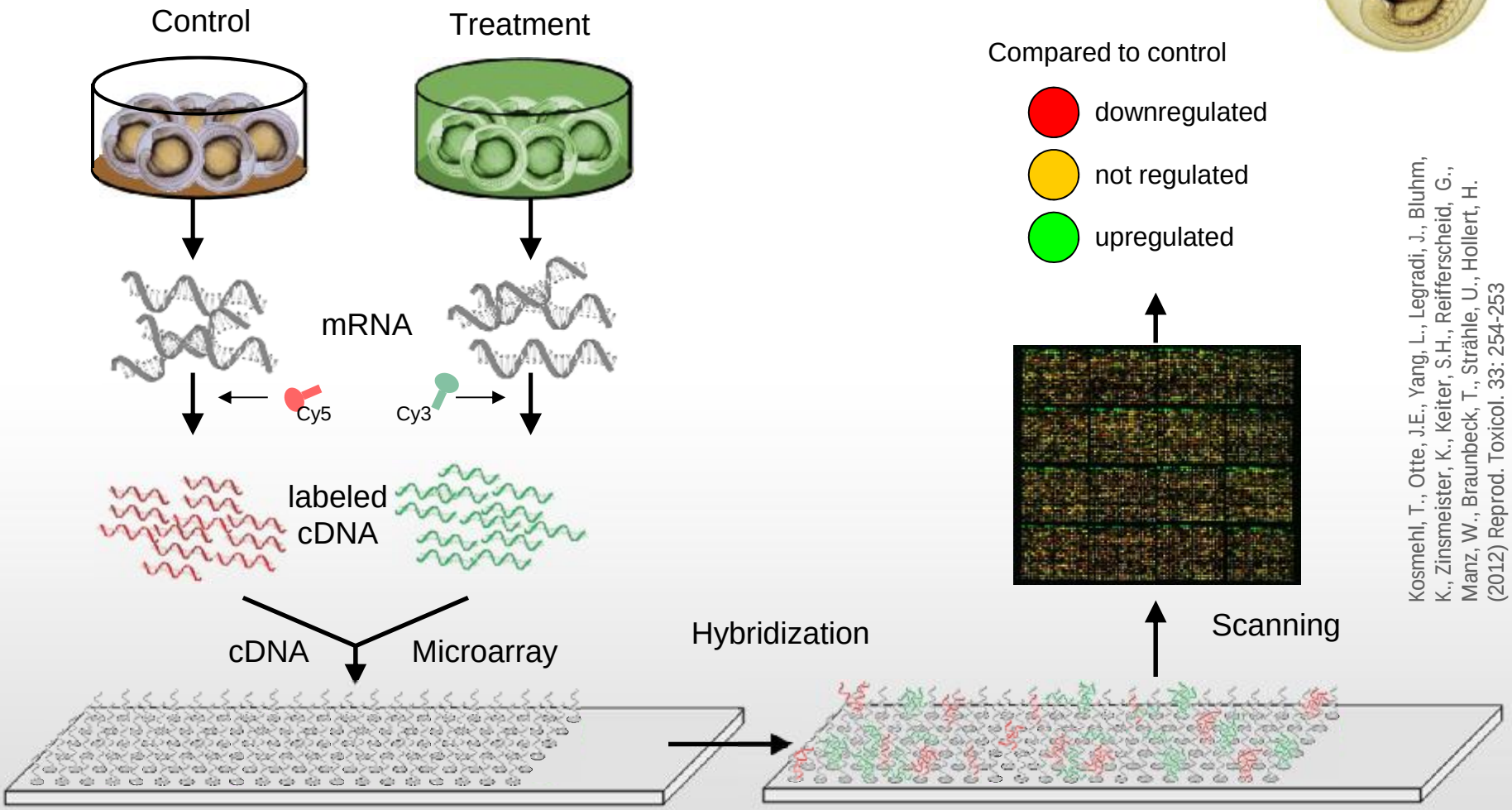






ZF embryos: Microarrays





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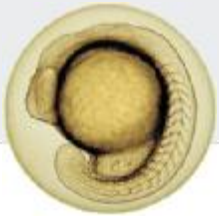
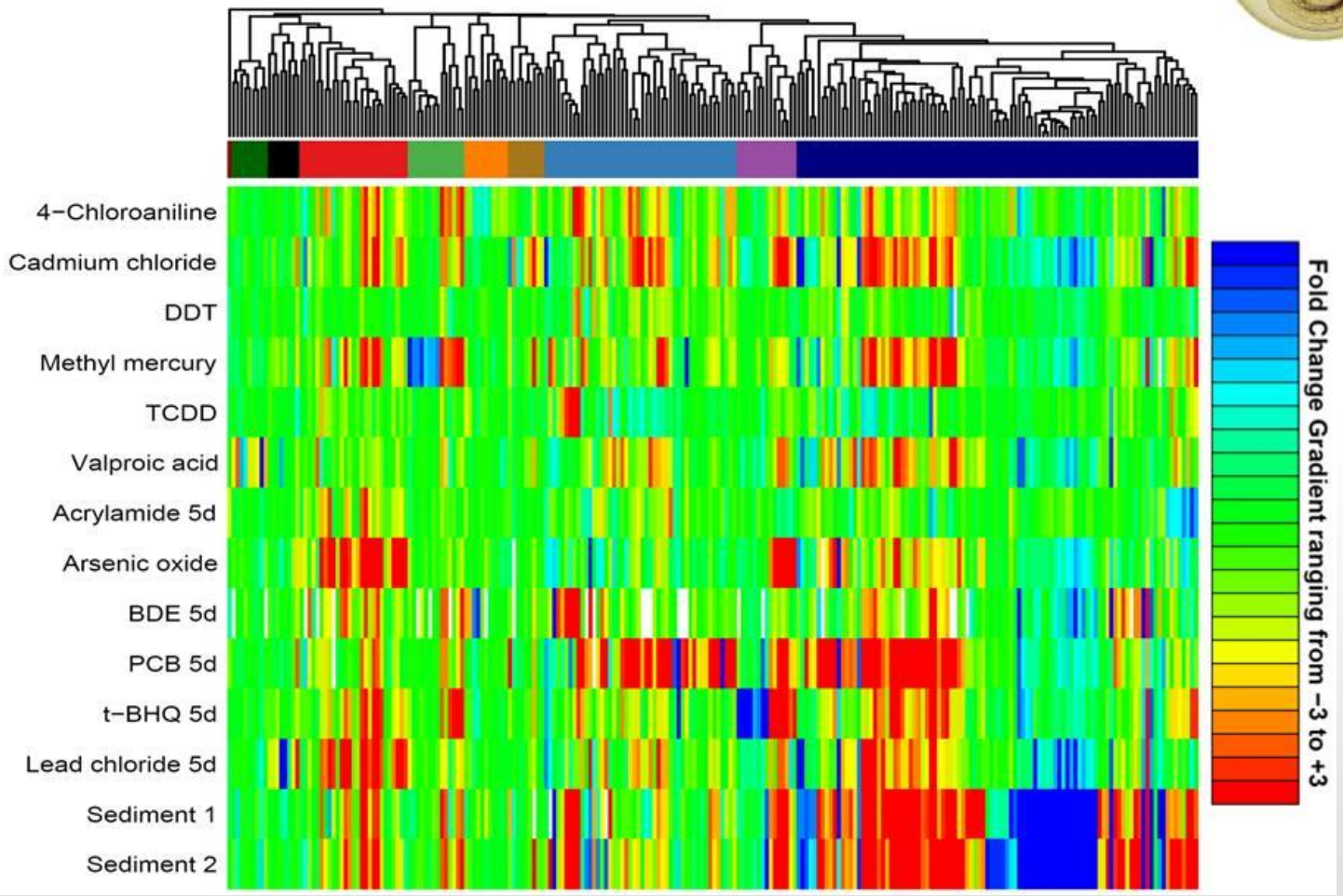
ZF embryos: Microarrays





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