Alternative Approaches for Fish Testing

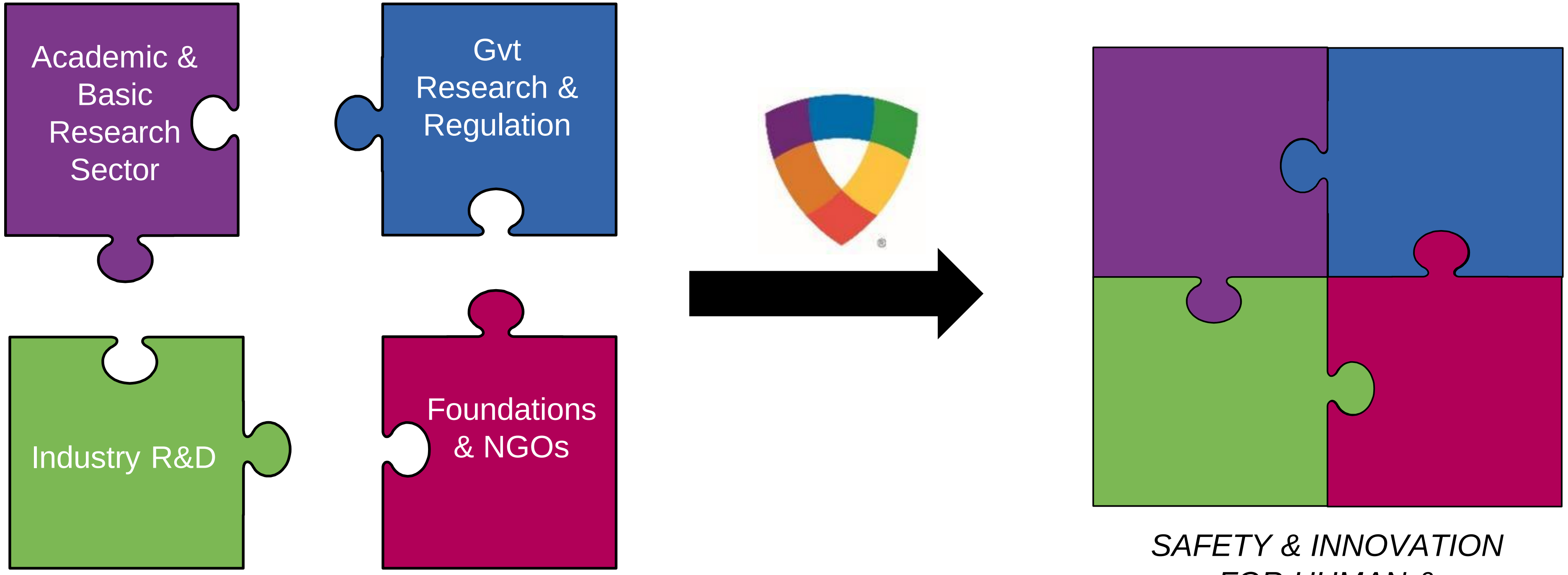
Michelle Embry, PhD
Associate Director, Environmental Science
Health and Environmental Sciences Institute (HESI)

Webinar Series on the Use of New Approach Methodologies (NAMs) in Risk Assessment
13 November 2019

Outline

- Brief background on HESI
- Context for animal alternatives in ecotoxicology
- NAMs for fish acute toxicity
    - Ecotoxicological threshold of concern (ecoTTC)
    - Fish cell lines (RTG-W1)
    - Fish embryo toxicity (FET) test
    - Threshold approach
- Fish NAMs in other contexts
    - Bioaccumulation
    - Effluent Assessment
- Validation & regulatory acceptance
- Ongoing work and future directions

The HESI Model



SAFETY & INNOVATION FOR HUMAN & ENVIRONMENTAL HEALTH

Mission of the HESI Animal Alternatives in ERA Committee *(est. 2007)*

- Develop a sound technical basis for 3Rs-based ecotoxicity tests around the globe
- Serve as a forum / community of practice
- Address needs for hazard assessment, effluent assessment, risk assessment, classification and labeling, and other regulatory needs.

Reduction

Refinement

Replacement

Ecological vs. Human Health Risk Assessment

Human Health

- One Species
- Protection of individual
- Effects / toxicity on target organ

Ecological RA

- All taxa; many more species
- Protection of population
- Effects / toxicity on growth and reproduction

Challenges:

- Protection target = all species, all ecosystems
- Many compounds lack data and are not well-studied
- Limited resources for testing
- Regulatory restrictions on vertebrate use
- Need tools for prioritization



Ecological Risk Assessment – General Strategy

Tier I: a) phys-chem analyses, literature search, read across

b) *in silico* methods

↓

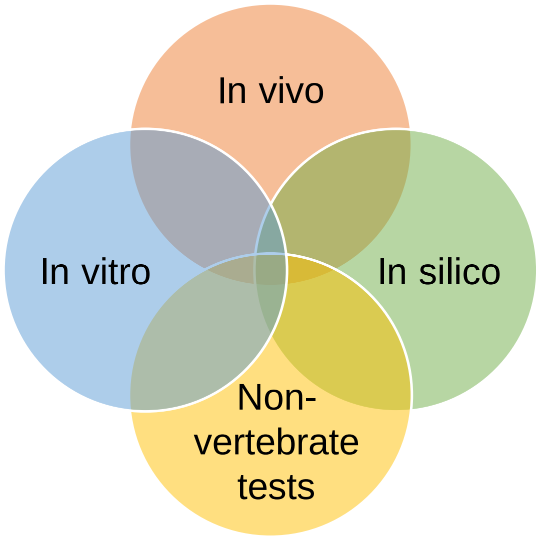
Tier II: non-vertebrate or *in vitro* assays to evaluate toxicity / effect

↓

Tier III: Refined *in vivo* tests to measure toxicity

↓

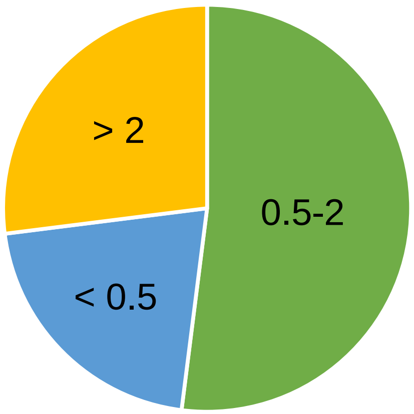
Tier IV: Standard *in vivo* tests



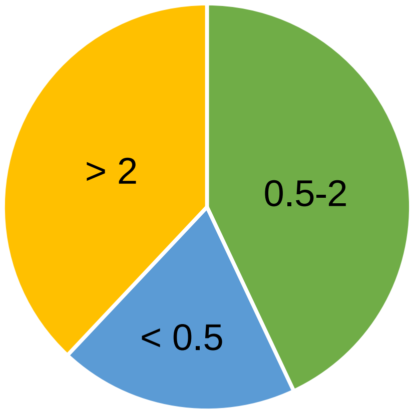
Motivations and Background

- Acute fish toxicity testing remains an integral part most chemical management programs and whole effluent toxicity (WET) testing
- Acute fish toxicity testing is the most common endpoint required
- More fish are used for a standard acute fish toxicity test (e.g., OECD 203) than any other aquatic vertebrate assay

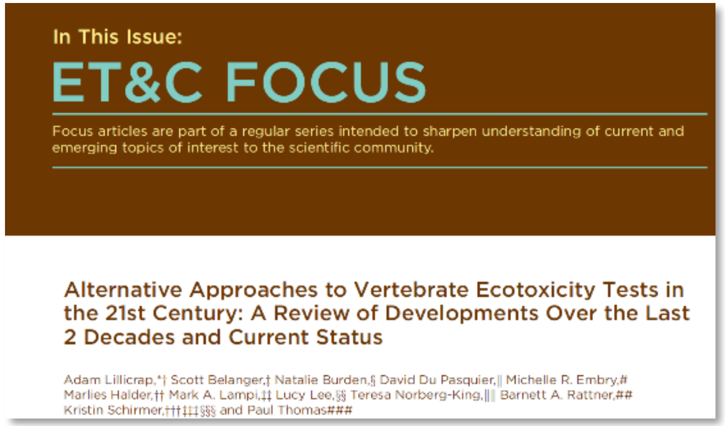
Acute Toxicity
Fish : Invertebrate ratio



Chronic Toxicity
Fish : Invertebrate ratio



Fish 2X more sensitive than invertebrates ~20% of the time


In This Issue:
ET&C FOCUS
Focus articles are part of a regular series intended to sharpen understanding of current and emerging topics of interest to the scientific community.

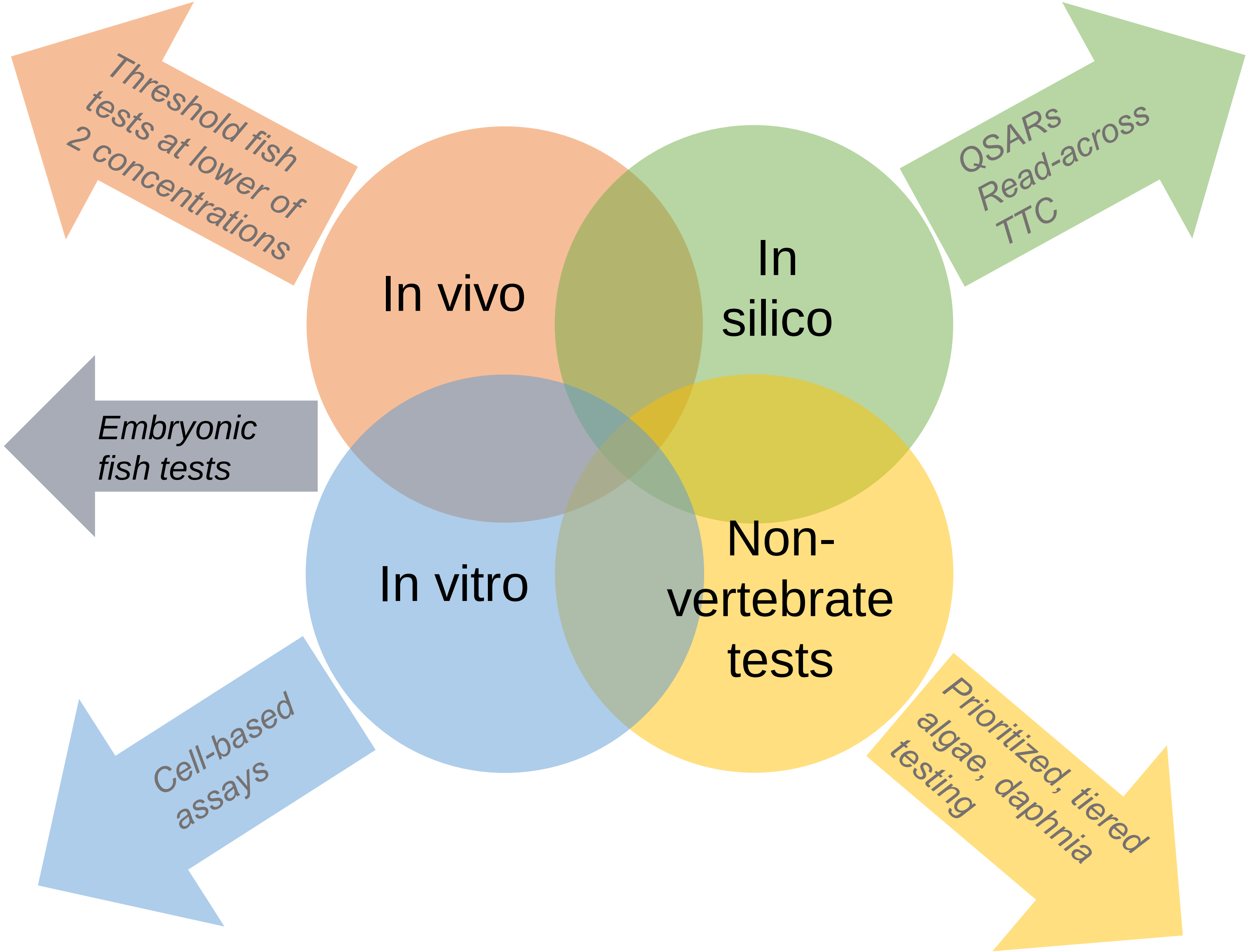
Alternative Approaches to Vertebrate Ecotoxicity Tests in the 21st Century: A Review of Developments Over the Last 2 Decades and Current Status

Adam Lillicrap,*† Scott Belanger,‡ Natalie Burden,§ David Du Pasquier,|| Michelle R. Embry,# Marlies Halder,†† Mark A. Lampi,‡‡ Lucy Lee,§§ Teresa Norberg-King,||| Barnett A. Rattner,## Kristin Schirmer,†††‡‡‡§§§ and Paul Thomas###

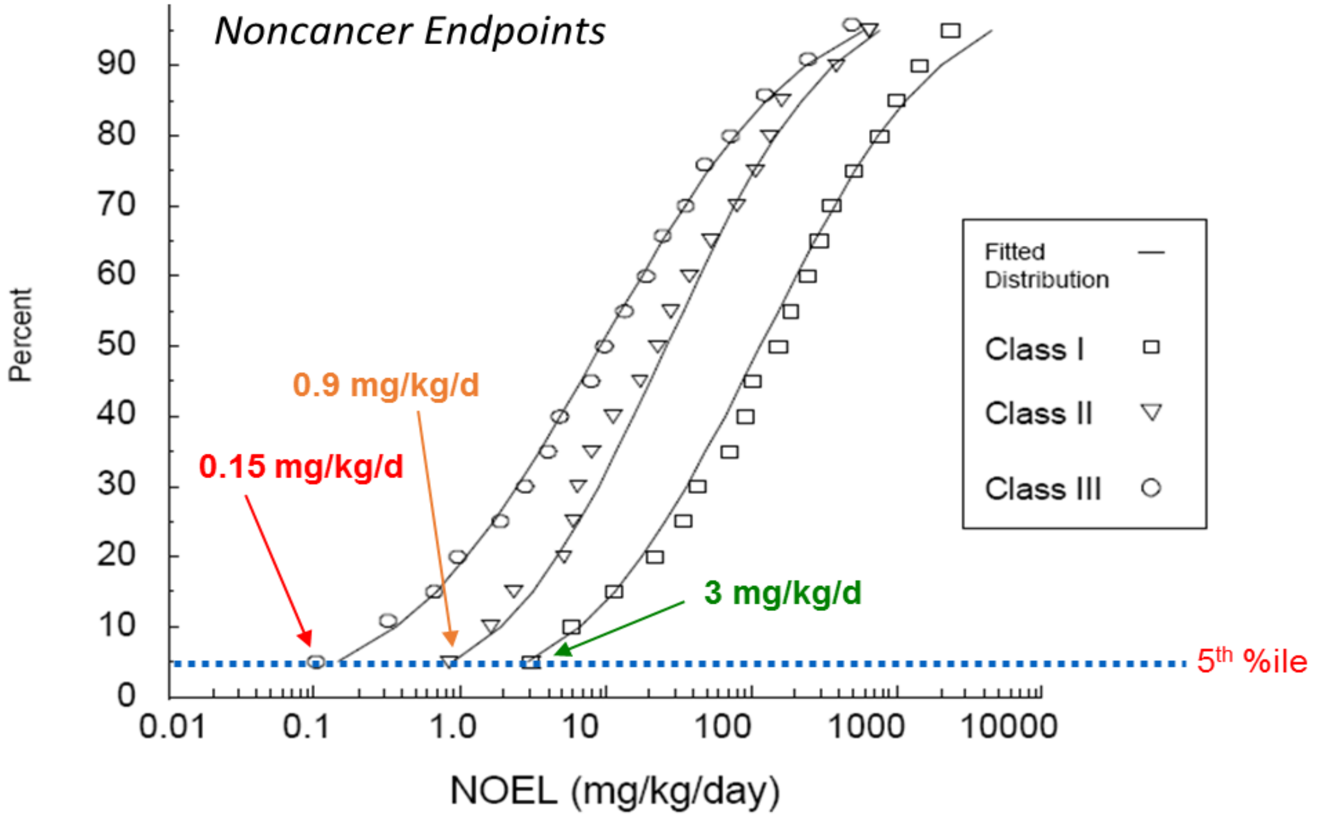
ECETOC, 2003. Technical Report No. 91

ET&C 35(11): 2637-2646

Lead Alternatives: Fish Acute Toxicity



Threshold of Toxicological Concern



- Can identify *de minimis* values for many chemicals, including those of unknown toxicity
- Originally applied to assess chemicals in food contact materials, flavorings, impurities
- Successfully used for a variety of human health endpoints for decades

Kroes et al., 2004

Eco-TTC & EnviroTox Platform

www.EnviroToxDatabase.org

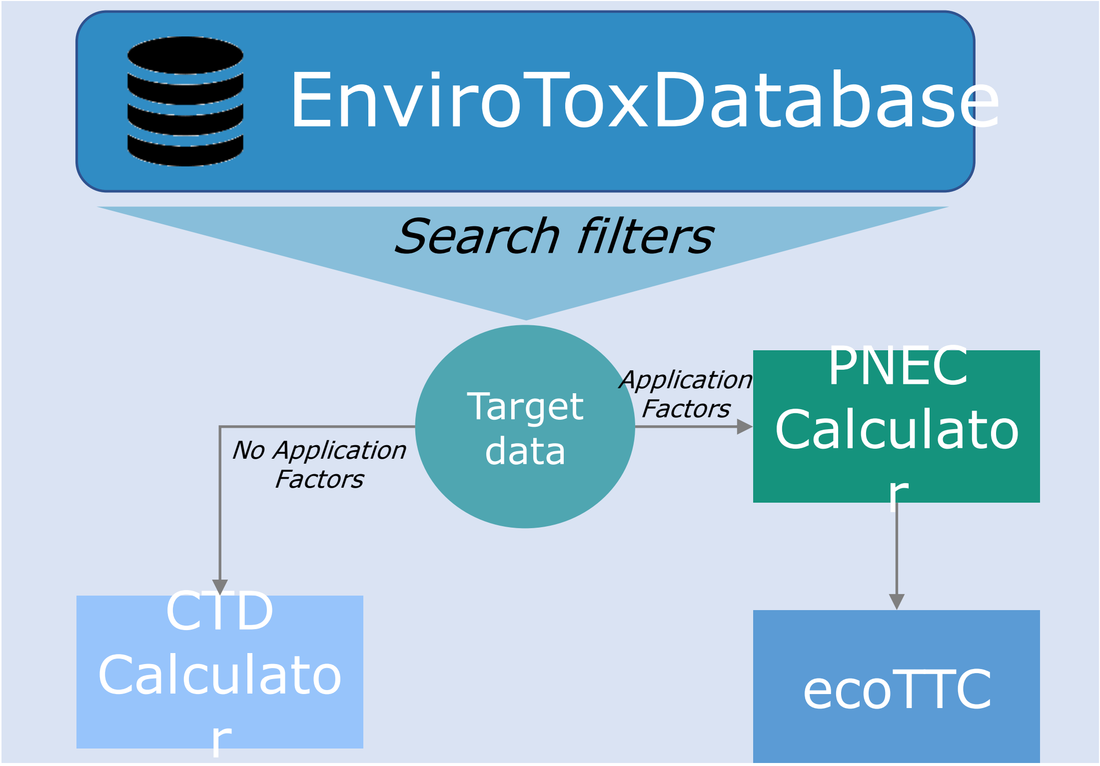
Database of ~91K curated aquatic toxicity records

User-friendly database filtering interface

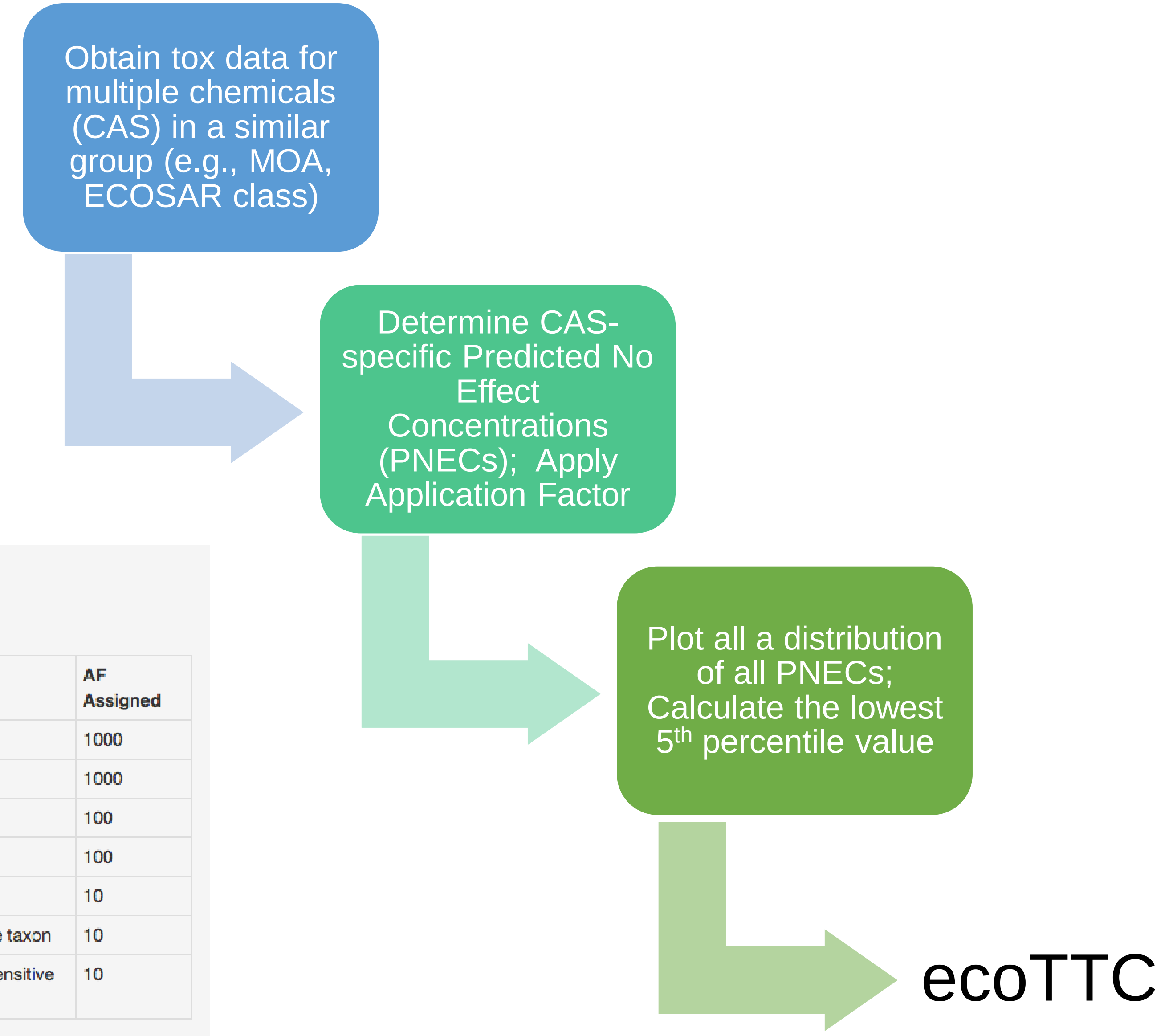
Freely available analysis tools:

- PNEC calculator (US & Europe)
- ecoTTC distribution tool
- Chemical Toxicity Distribution (CTD) tool

Developed via a global, collaborative partnership with government, academia, and industry, managed by HESI



ecoTTC Calculation

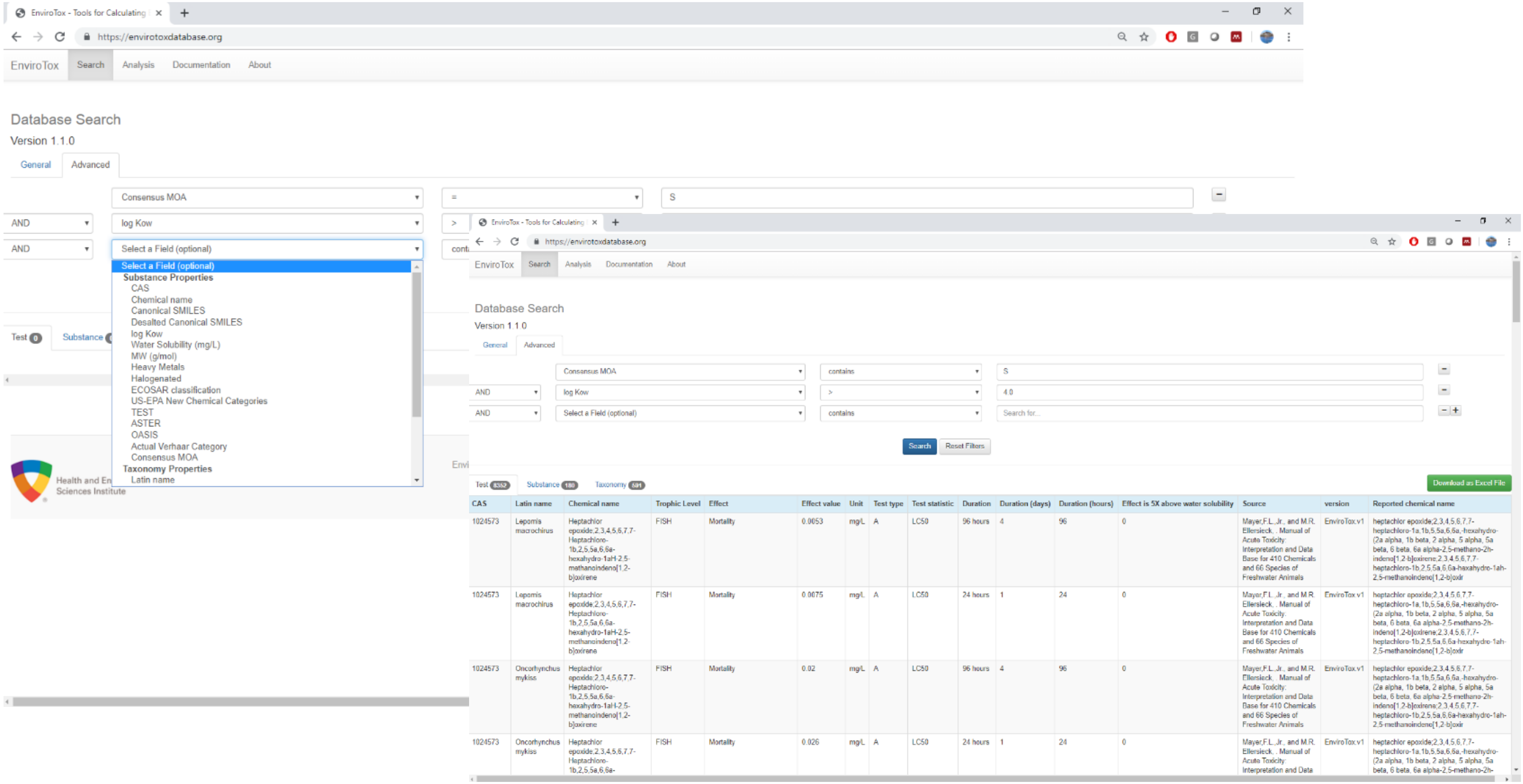


PNEC Group Definitions

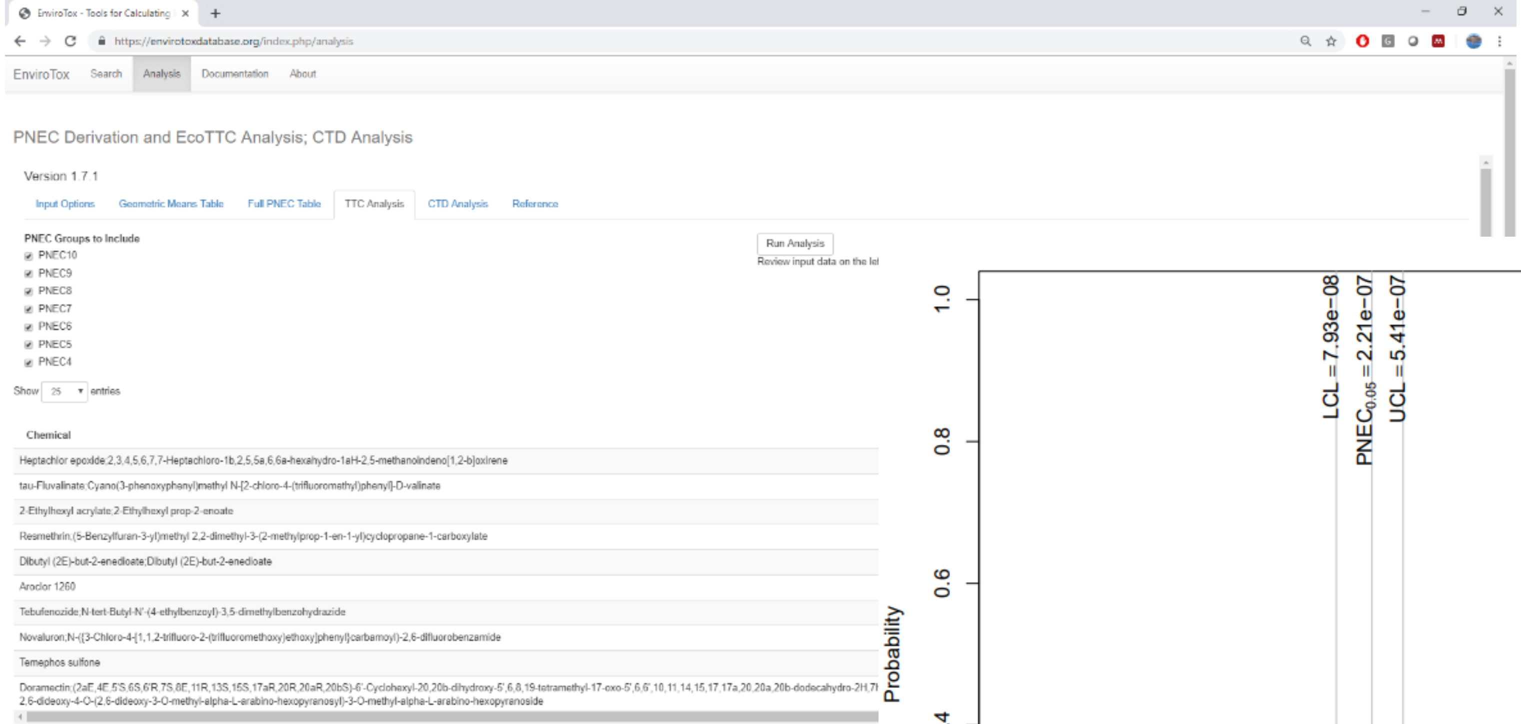
US Region

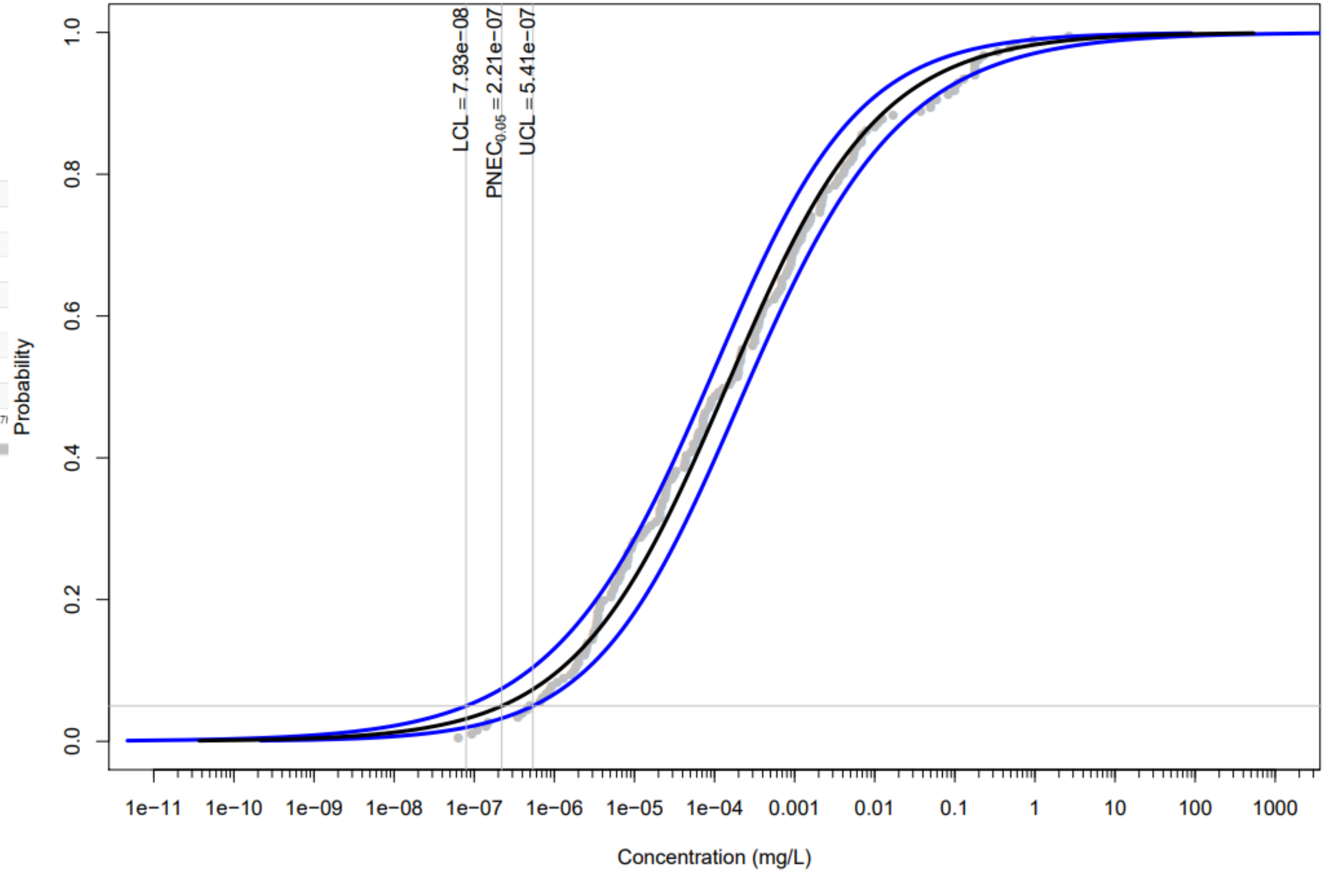
	PNEC Group	Data Combination	AF Assigned
1	PNEC4	1 trophic level acute	1000
2	PNEC5	2 trophic levels acute; use most sensitive taxon	1000
3	PNEC6	3 trophic levels acute; use most sensitive taxon	100
4	PNEC7	3 trophic level acutes; 1 chronic on less sensitive acute taxon	100
5	PNEC8	3 trophic level acutes; 1 chronic on most sensitive acute taxon	10
6	PNEC9	3 trophic level acutes; 2 chronics including most sensitive acute taxon	10
7	PNEC10	3 trophic level acutes; 3 trophic level chronics including most sensitive acute taxon	10

Database searching / filtering

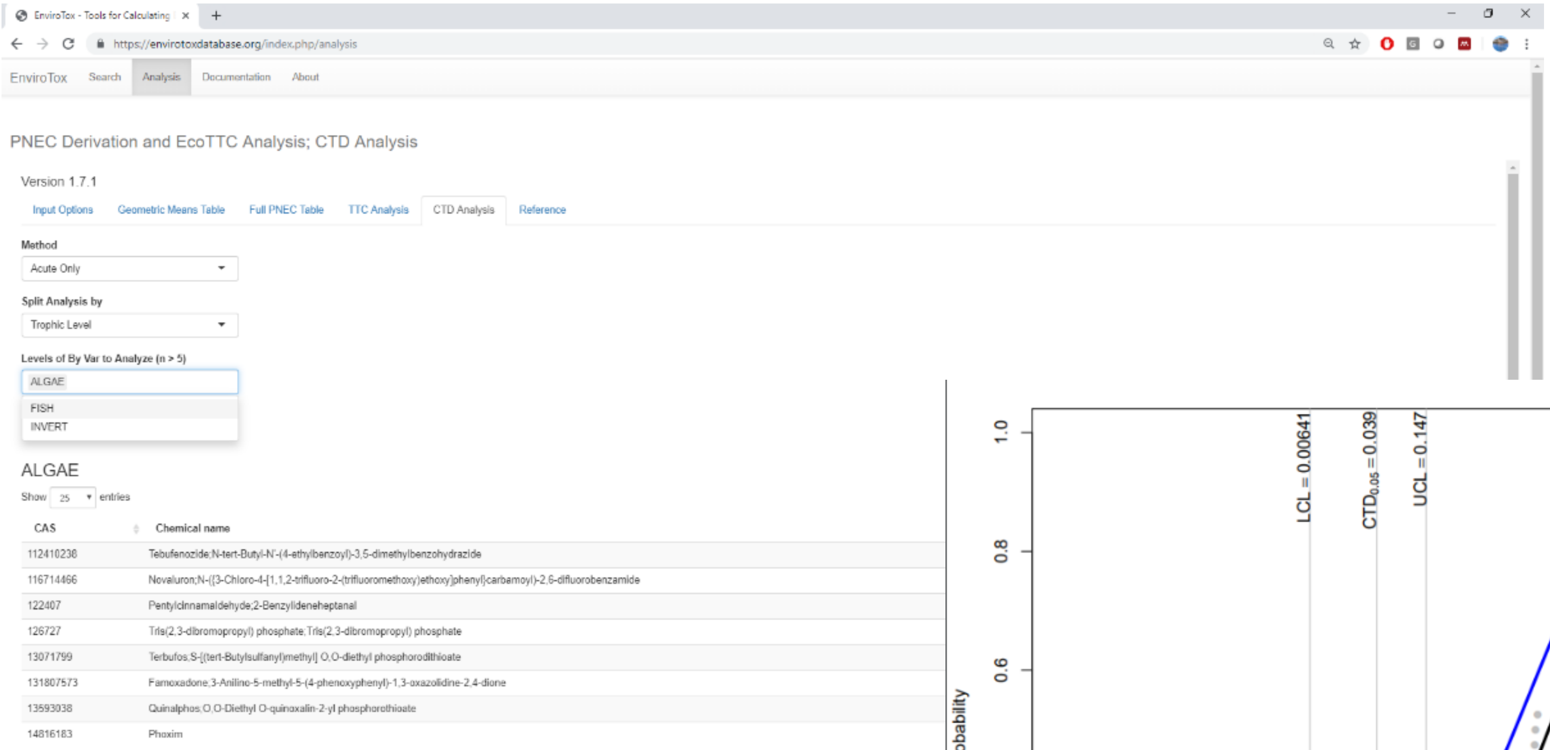


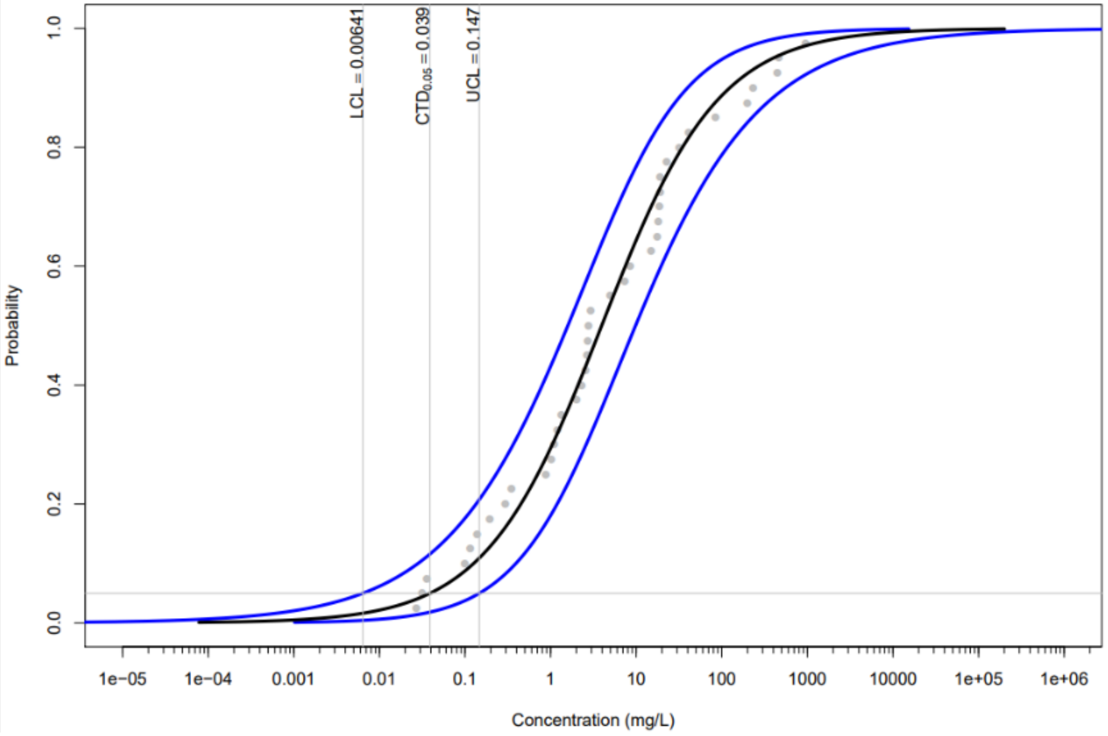
PNECs and ecoTTC Distribution

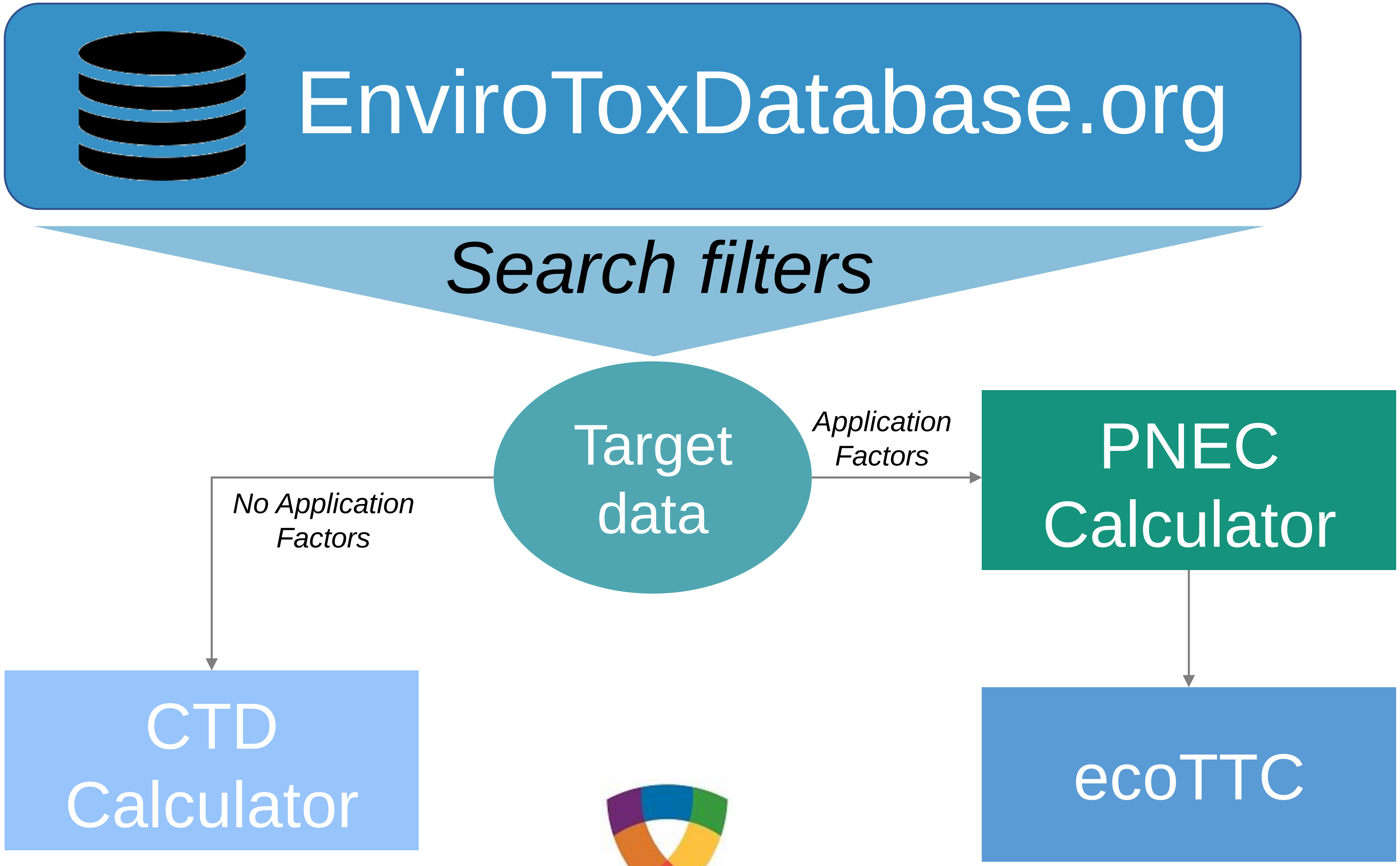




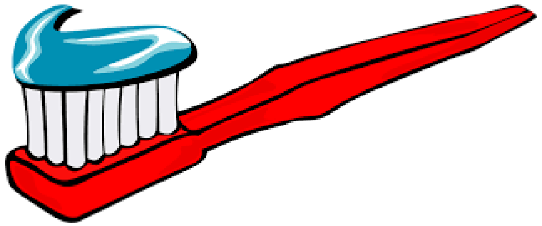
Chemical Toxicity Distribution






EnviroToxDatabase.org
Search filters
Target data
Application Factors
PNEC Calculator
No Application Factors
CTD Calculator
ecoTTC

Framework for eco-TTC?



Problem Formulation

↓

Exposure assessment

↓

Application of eco-TTC

↓

Preliminary risk characterization:
Is there a concern at estimated levels of exposure?

↓ Exemption of further consideration at current levels of exposure → Risk management

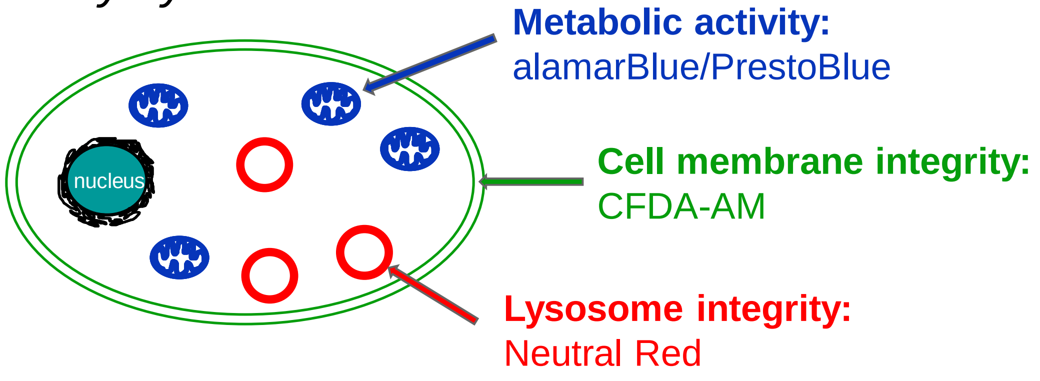
↓ Hazard identification & classification → Full risk characterization

Modified from Kroes et al., 2005

Fish Cell Line Work: Rainbow trout gill cell line

- RTgill-W1
  - Cell viability as a surrogate for fish survival
  - Acute toxicity to water samples & chemicals
  - New ISO Guideline 21115:2019
  - Full test submission to EURL ECVAM Sept 2018 (TM2014-01)
  - Proposed to OECD Fall 2018 as a new Test Guideline
  - Bols et al., 1994, J. Fish Dis 17, 601-611
  - American Type Culture Collection ATCC® CRL-2523™
  - Assumption for fish acute toxicity:
    - Gill as primary site of interaction

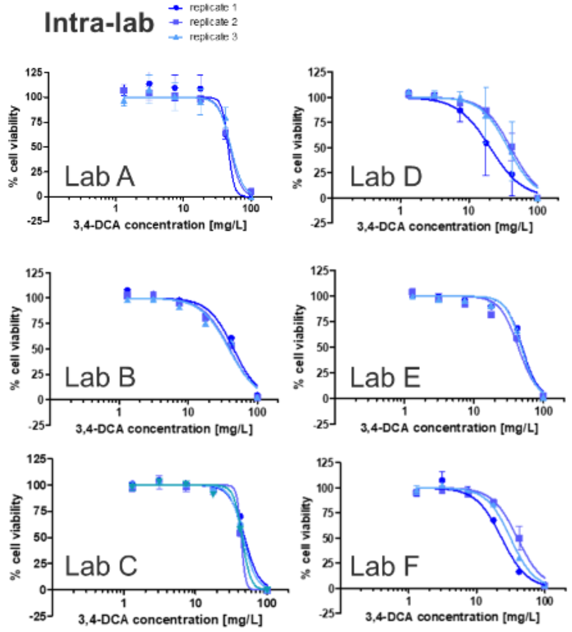
Toxicity assessed with a combination of cell viability dyes

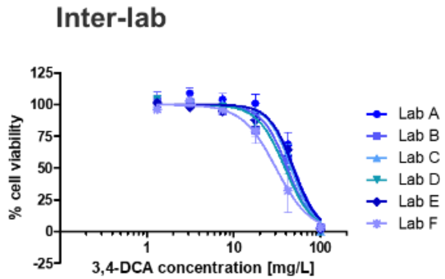


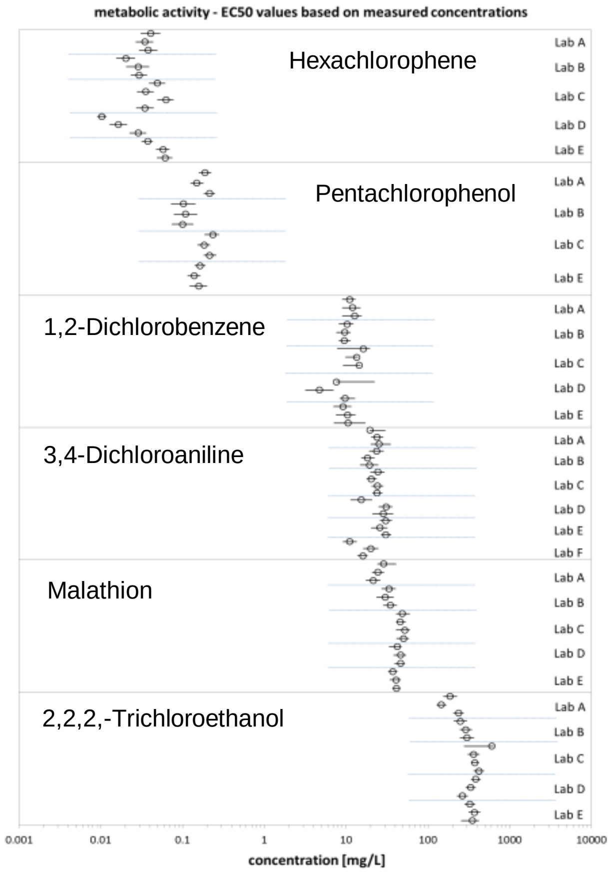
Slide modified from K. Schirmer

Fish Cell Line Work: Rainbow trout gill cell line

- Well developed, optimized and widely tested SOP
    - Transferability and robustness evaluated via ring-trial
    - One chemical tested per 24-well plate
    - 24 hours from start of exposure to test result
    - Sampling for chemical analysis integrated
    - Unambiguous quantification of effect
    - Multiple sample types: chemicals, product mixtures, effluents, extracts



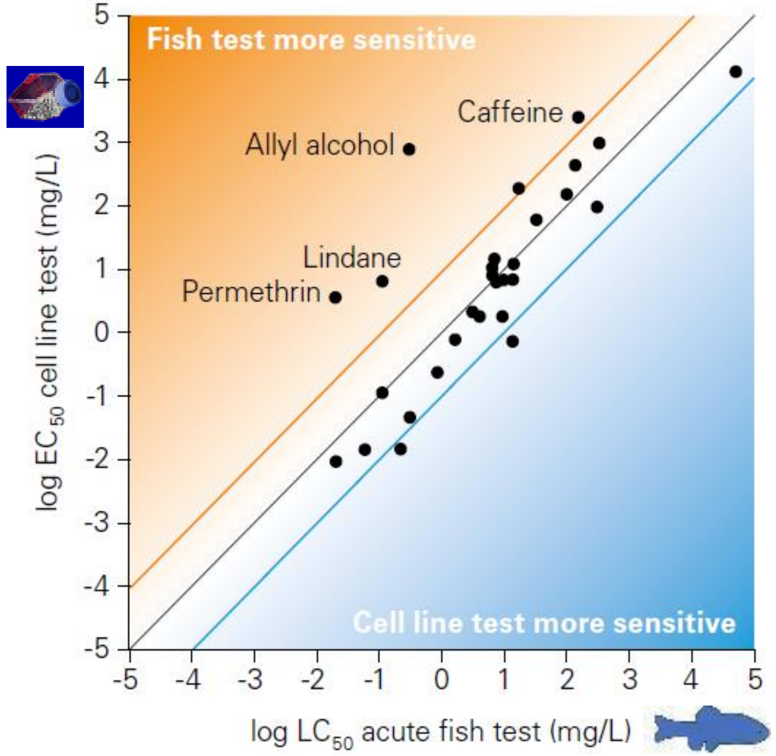




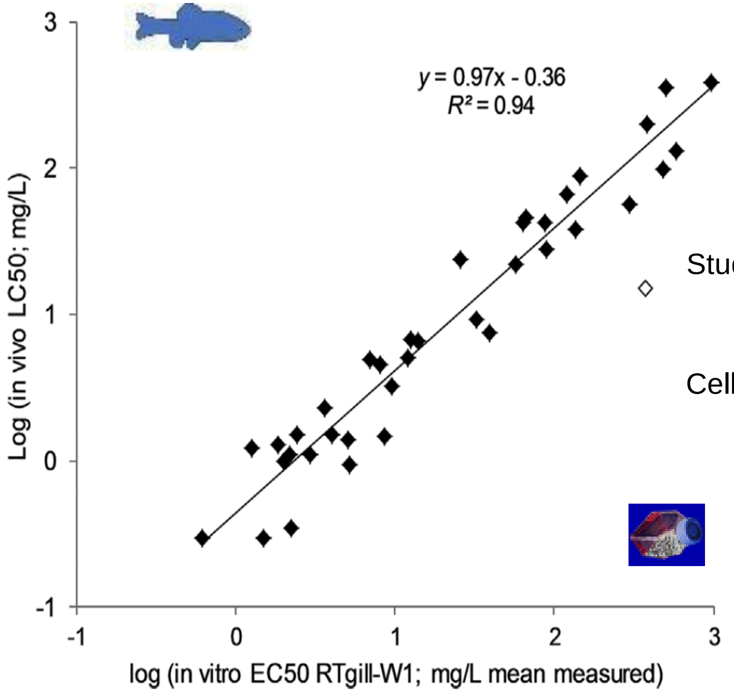
Slide modified from K. Schirmer

Fisher et al., 2019

Fish Cell Line Work: Rainbow trout gill cell line



Tanneberger et al, ES&T 2013, 47, 1110−1119
& Schirmer et al Eawag News 2013, 02/Oct



Study on Fragrances

Givaudan

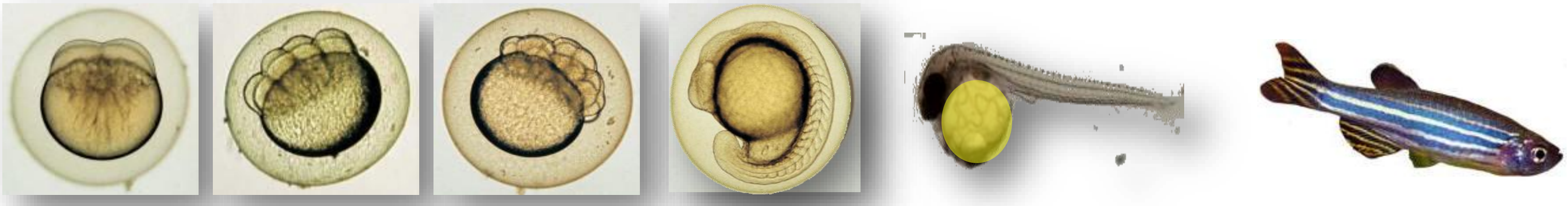
Cell line assay superior to QSAR

Natsch et al., 2018, ET&C 37(3): 931-941

Slide modified from K. Schirmer

Fish Embryo Toxicity (FET) Test

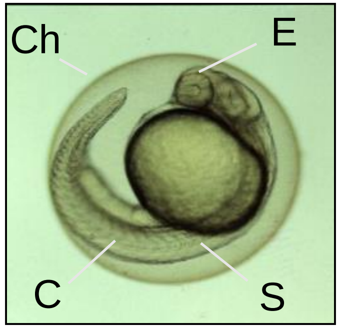
- Adopted as OECD TG236 in 2016
- Pioneered by scientists in Germany; mandatory for sewage effluent testing in Germany since 2005
- Basics of the method:
  - *Newly fertilized embryos*
  - *20 embryos / concentration / control*
  - *5 test concentrations*
  - *96h exposure with semi-static renewal*
  - *4 endpoints for acute lethality (24, 48, 72, and 96h)*
  - *LC50 calculation at 48 and 96h*

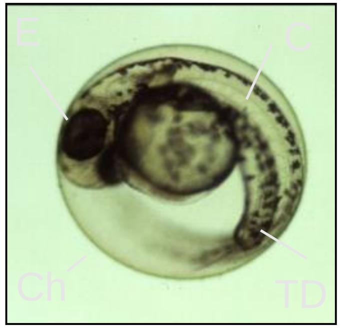


Fish Embryo Toxicity (FET) Test

1 h | 24 h | 48 h

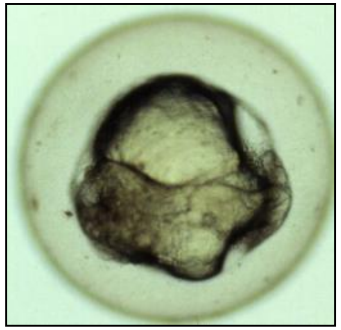
Normal





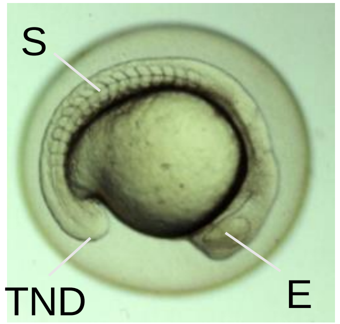
Lethal effects

Beginning coagulation



Malformation due to lack of somites

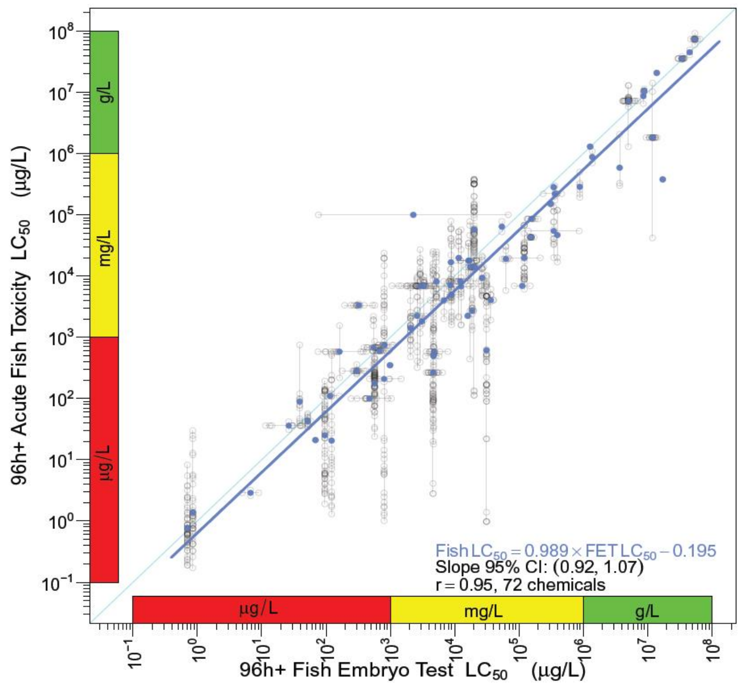
Tail bud not detached



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

photos provided by J. Bachmann, UBA, Berlin

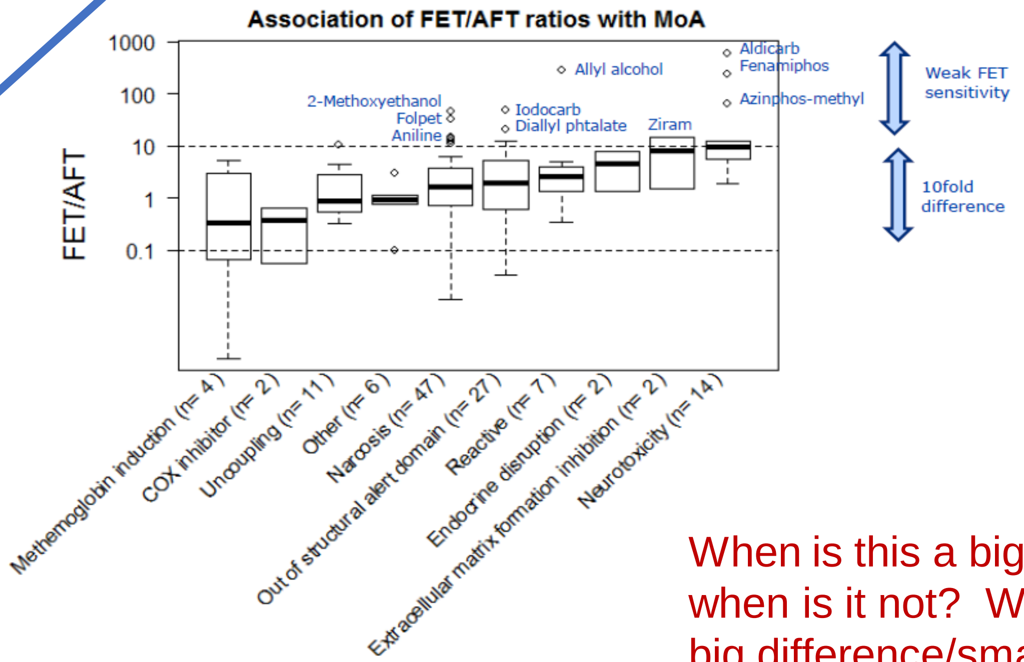
FET / Acute Fish Test Comparisons



Belanger et al. 2013. ET&C.

- High apparent r
- Scatter for some chemicals
- Distribution/chemical coverage

- Some MoA seem to be underpredicted by FET
- Different process of data compilation/filtering
- AFT issues abound



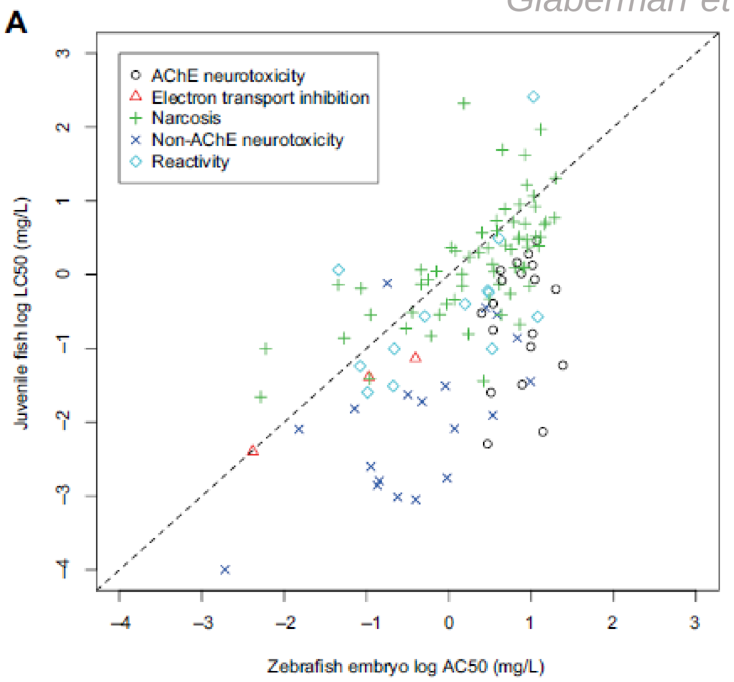
When is this a big deal, when is it not? What is a big difference/small difference?

Slide modified from S. Belanger

Sobanska et al. 2018. ET&C

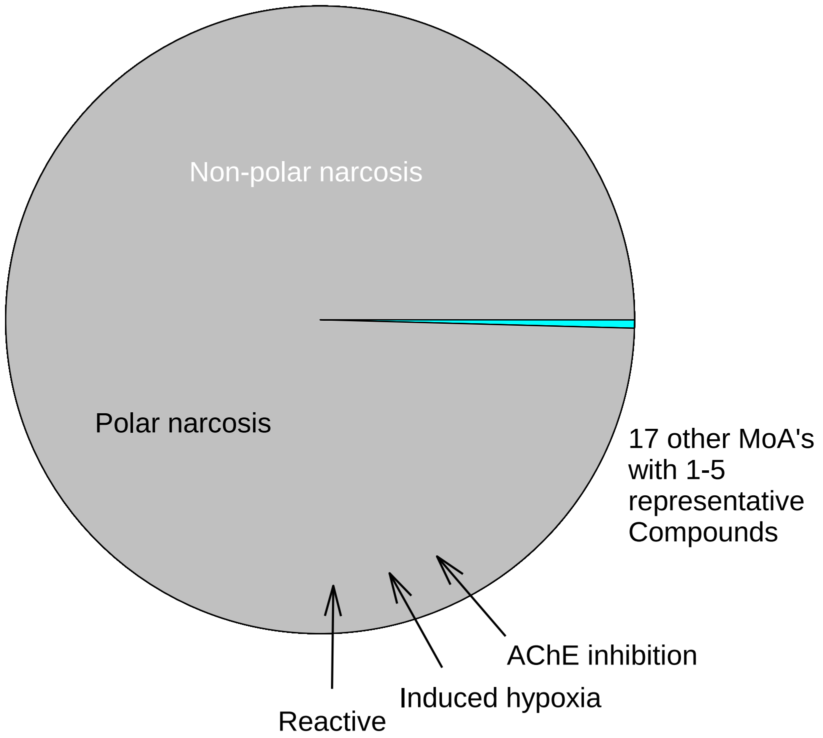
Mode of Action Impact

Glaberman et al., 2017 (ET&C 36: 1221)



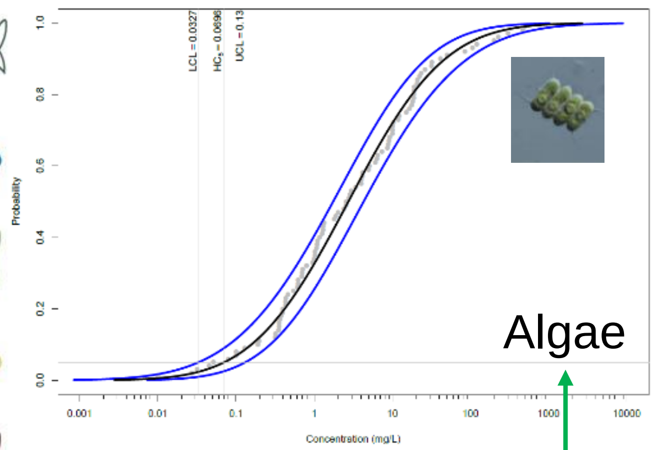
- >20 MoA represented in published FET-AFT comparisons
- Dominated by narcotic MoA (also dominant chemical group in commerce)
- ~15% of the FET database are reactive and neurotoxicants

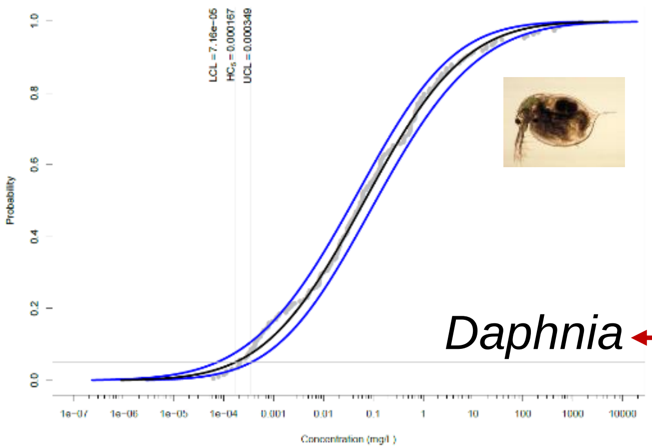
- Recent analysis (Glaberman et al. 2017; Sobanska et al. 2018) indicate that embryos are somewhat less sensitive to neuorotoxic compounds than juvenile fish
- Some uncertainty on this conclusion due to mixing of many types of embryo assays

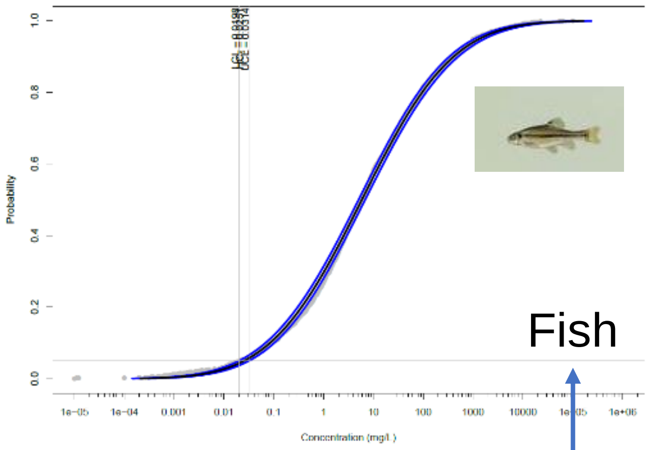


Slide modified from S. Belanger

Trophic Sensitivity to Specific MOAs







Algae least studied

Daphnia most sensitive

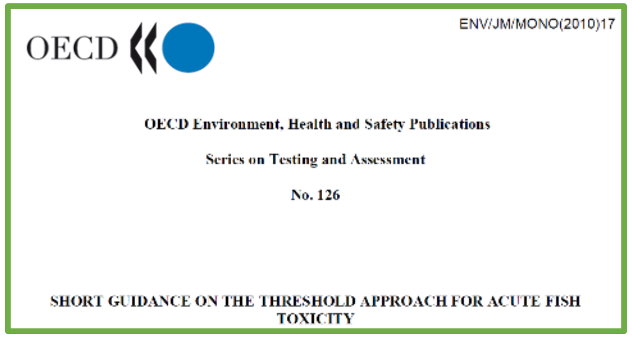
Fish – relatively invariable

Group	5th percentile (mg/L)	95% LCL (mg/L)	95% UCL (mg/L)
Algae	0.0696	0.0326	0.1298
Daphnia	0.000166	7.1583×10^{-5}	0.000349
Fish	0.025072	0.019807	0.031419

- Trophic group tolerance/sensitivity curves for compounds with specific modes of action (from www.EnviroToxdatabase.org)

Slide modified from S. Belanger

Threshold Approach


ENV/JM/MONO(2010)17

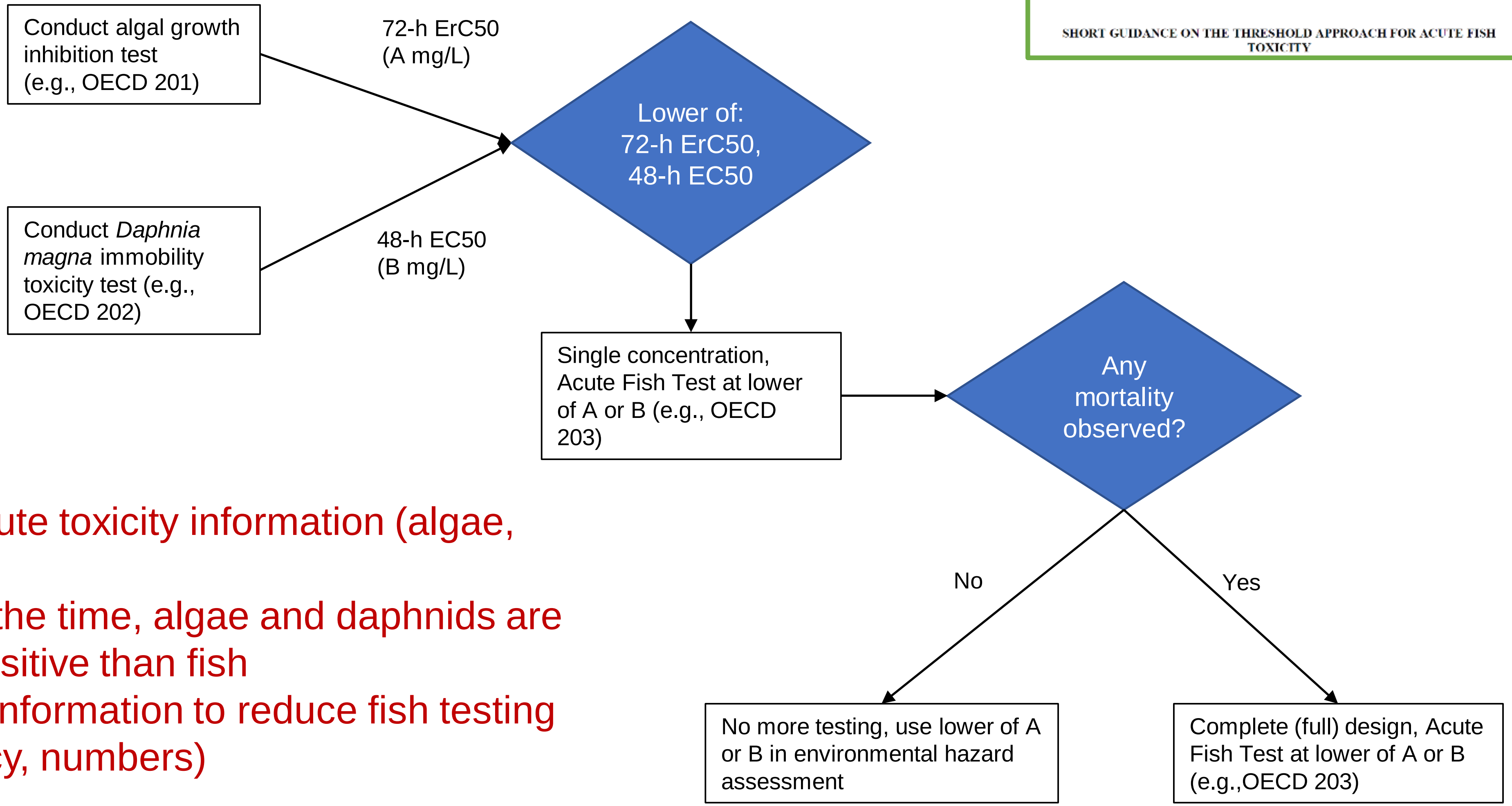
OECD

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 126

SHORT GUIDANCE ON THE THRESHOLD APPROACH FOR ACUTE FISH TOXICITY

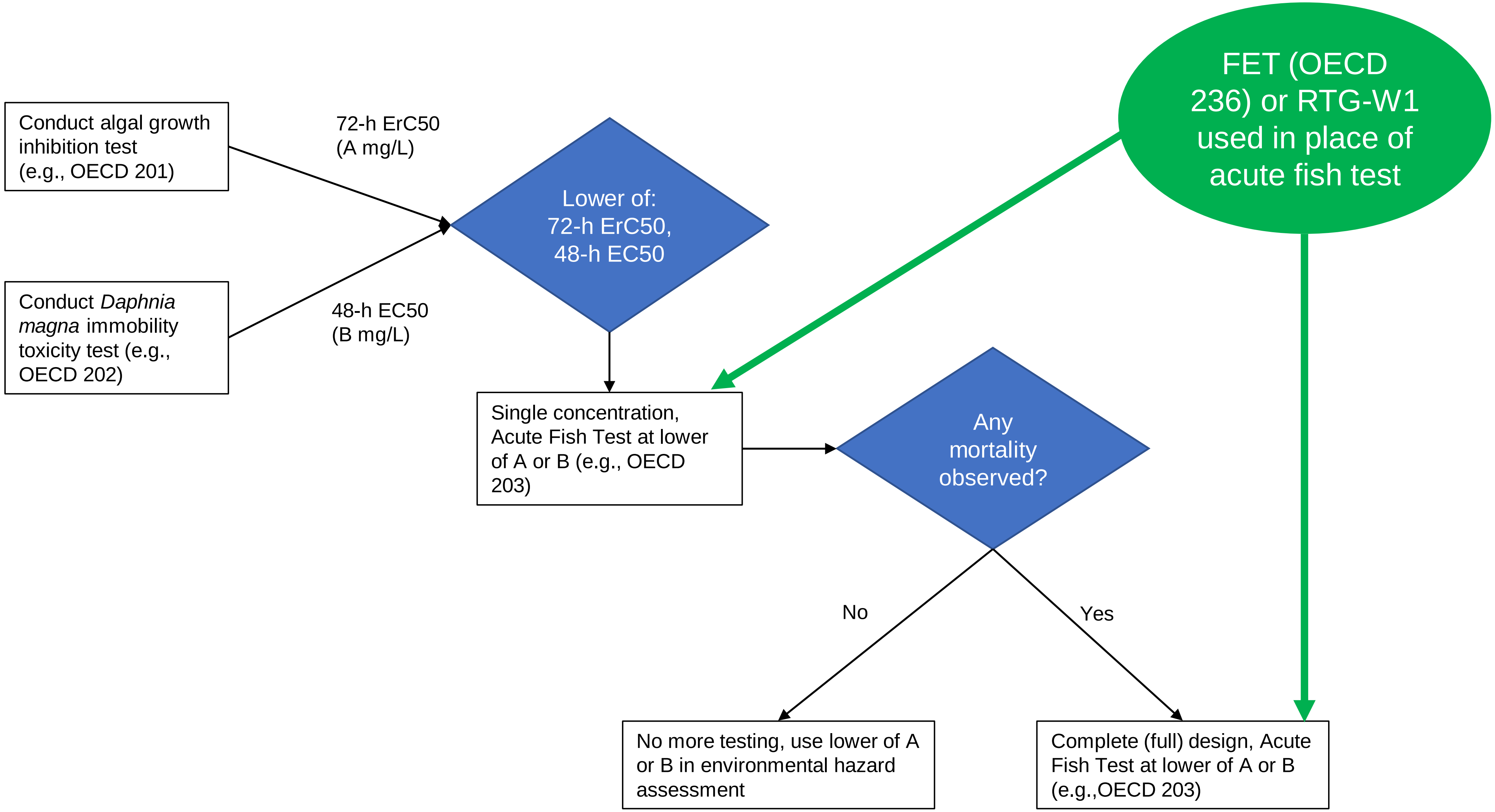


Leverages all acute toxicity information (algae, daphnid, fish)

- ~80% of the time, algae and daphnids are more sensitive than fish
- Use this information to reduce fish testing (frequency, numbers)

Slide modified from S. Belanger

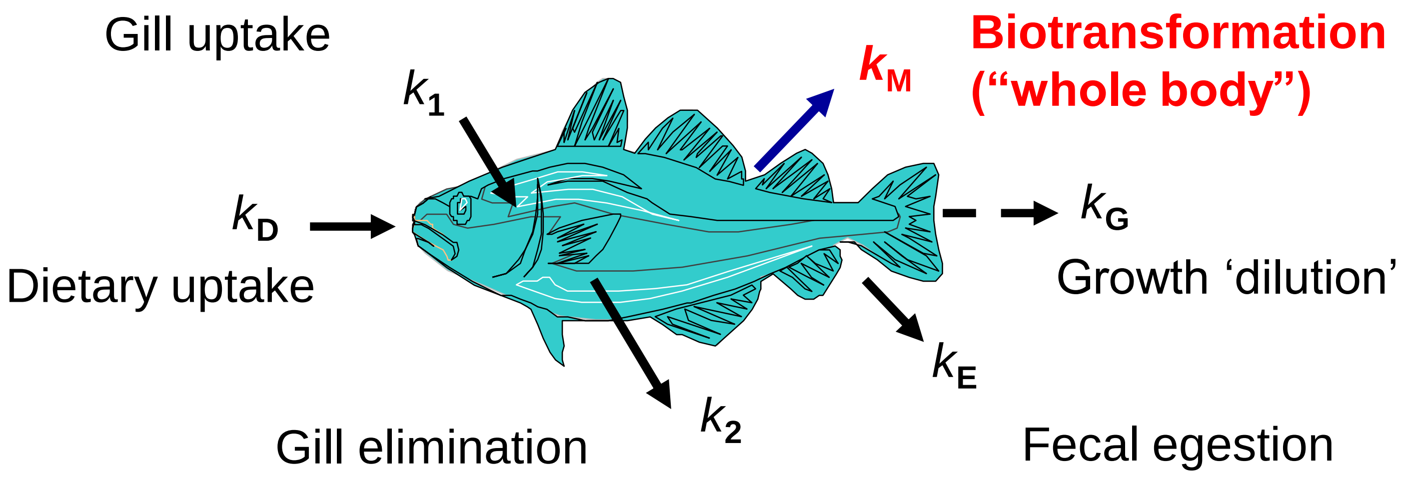
Threshold Approach – Role of NAMs



Slide modified from S. Belanger

Bioaccumulation Concepts

- Mathematical models (QSARs) using K_{OW}-based relationships predict bioaccumulation correctly for many compounds if they are not metabolized
- Metabolism tends to reduce bioaccumulation
- Metabolism is the most critical uncertainty in bioaccumulation assessments



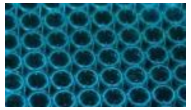
Mass Balance Equation

$$C_{Fish} = \frac{[(k_1 C_W) + (k_D C_D)]}{(k_2 + k_E + \mathbf{k_M} + k_G)}$$

$$\boxed{\frac{\text{Uptake}}{\text{Elimination}}}$$

Bioaccumulation Concepts

In vitro



Rainbow Trout Liver:

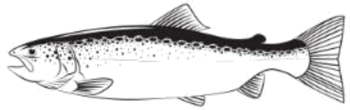
- S9 subcellular fractions
- Cryopreserved hepatocytes

$CL_{IN\ VITRO,INT}$
(ml/h/mg protein or ml/h/10^6 cells)

In vitro – in vivo extrapolation (IVIVE)

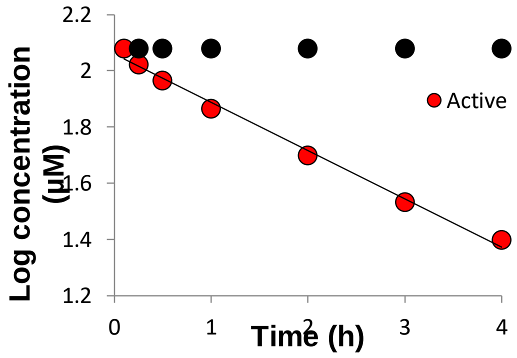
$CL_{IN\ VIVO,INT}$	CL_H	k_M
(ml/h/kg BW)	*(L/day/kg liver)*	*(1/day)*

In vivo



Combine k_M with estimates of k_1, k_2 and k_E to simulate C_{Fish} and predict BCF (L/kg)

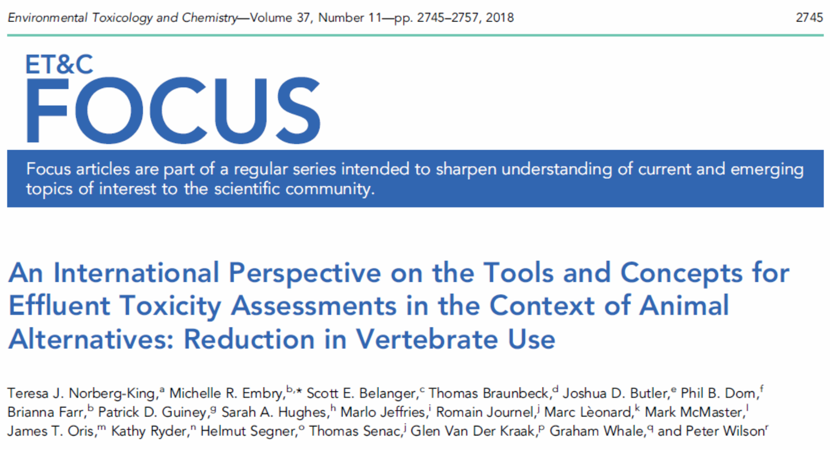
Comparison of predicted BCFs based on in vitro – in vivo extrapolation to in vivo BCFs and BCFs predicted with QSARs (e.g., EpiSuite)



Two new OECD Test Guidelines!
OECD TG 319A&B

Effluent Assessment

- Whole effluent testing (WET) is a common assessment tool for aquatic environmental protection
- Toxicity testing for effluent requires use of fish (acute & chronic)
- Effluents are variable and require recurrent testing
  - Weekly, monthly, quarterly, annually
- 3 – 6 million fish used annually in the US alone



Environmental Toxicology and Chemistry—Volume 37, Number 11—pp. 2745–2757, 2018 2745

ET&C

FOCUS

Focus articles are part of a regular series intended to sharpen understanding of current and emerging topics of interest to the scientific community.

An International Perspective on the Tools and Concepts for Effluent Toxicity Assessments in the Context of Animal Alternatives: Reduction in Vertebrate Use

Teresa J. Norberg-King,[a] Michelle R. Embry,[b,*] Scott E. Belanger,[c] Thomas Braunbeck,[d] Joshua D. Butler,[e] Phil B. Dom,[f] Brianna Farr,[b] Patrick D. Guiney,[g] Sarah A. Hughes,[h] Marlo Jeffries,[i] Romain Journel,[j] Marc Lèonard,[k] Mark McMaster,[l] James T. Oris,[m] Kathy Ryder,[n] Helmut Segner,[o] Thomas Senac,[j] Glen Van Der Kraak,[p] Graham Whale,[q] and Peter Wilson[r]

Effluent Assessment - Alternatives

- Several promising alternative approaches for effluent assessment:
  - RTgill-W1
  - Algal growth test
  - MicroTox assay (bacteria)
  - LumiMARA (bacteria)
  - Daphnid acute toxicity test
  - DART assay (nematode)
  - FET
  - Whole effluent assessment (WEA; MicroTox paired with biodegradation assessment)

Regulatory Acceptance

- NAMs often challenge the status quo
  - *New expertise / skills needed to evaluate*
  - *Ability to place NAMs in the right context (e.g., IATA, fit for purpose)*
- Many regulatory environments are heavily codified and change is difficult
- Mutual Acceptance of Data (MAD) within OECD Member Countries; but implementation occurs at the federal level
- Method validation bodies have different strategies and interests

Suggestions on a path forward…..

Separate & specific guidance on implementation of NAMs
Participation by regulators / governments in validation programs
Early discussion on use and implementation (and scope / domain of applicability)
Databases to facilitate comparisons / interpretations (e.g., EnviroTox, others)

Next Steps

- Ongoing OECD Project 2.54: Guidance Document on IATA for Fish Acute Toxicity Testing
  - *Will include revision of the threshold approach Guidance Document (GD 126)*
  - *Combines QSARs, fish embryo test, RTgill-W1 assay in a threshold context*

- HESI Animal Alternatives in ERA Committee
  - *ecoTTC / CTD case study work*
  - *Alternatives to effluent assessment*
  - *Continued work on the FET analyses*

- HESI Bioaccumulation Committee
  - *Follow-up from recent expert workshop on in vitro methods – October 2019*
  - *Ongoing outreach to stakeholders on weight of evidence & integration of NAMs*
  - *Ongoing projects on bird & invertebrate biotransformation*

- RTgill-W1 approved for OECD TG development in April 2019 (Part of workplan)

Conclusions

- Several new ‘ecoNAMs’ relatively recently advanced:
  - *OECD 236 (FET)*
  - *OECD 319 A&B (Bioaccumulation)*
  - *ISO and hopefully OECD TG (RTgill-W1)*

- Integrated / tiered approaches are already in place (e.g., Threshold Approach) – need to be modified to allow for NAMs

- Use of existing information is key for NAM / existing method comparisons as well as development of in silico tools:
  - ecoTTC
  - QSAR advancement

- More work is (still) needed!
  - Effluent assessment NAMs could have a very big impact

Want to get involved?

HESI Committees working in this space

Ecological & human health NAMs: www.hesiglobal.org

Contact Michelle Embry (membry@hesiglobal.org)

SETAC Animal Alternatives in ERA Interest Group:
https://www.setac.org/group/IGAnimalAlternatives

- Sessions being held at SETAC NA, Europe, and World Congress
- SETAC Europe (Dublin, May 2020) abstracts due Nov 27th!
  - https://dublin.setac.org/
    - *Alternative Approaches to Animal Testing for Aquatic Ecotoxicity Assessments and Environmental Risk Assessments*

Thank you!

Michelle Embry
membry@hesiglobal.org
202-659-3306 x183

References

Animal Alternatives in ERA

- Lillicrap et al., 2016. Alternative approaches to vertebrate ecotoxicity tests in the 21st century: A review of developments over the last 2 decades and current status. ET&C 35(11):2637-2646. https://doi.org/10.1002/etc.3603

- Scholz et al., 2013. A European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. RTP: 67 506-530. https://doi.org/10.1016/j.yrtph.2013.10.003

- ECETOC, 2003. TR No. 91. Aquatic Hazard Assessment II. http://www.ecetoc.org/wp-content/uploads/2014/08/ECETOC-TR-091.pdf

OECD Threshold approach

- https://www.oecd.org/chemicalsafety/testing/40985084.pdf

Effluent Assessment

- Norberg-King et al., 2018. An International Perspective on the Tools and Concepts for Effluent Toxicity Assessments in the Context of Animal Alternatives: Reduction in Vertebrate Use. ET&C 37(11)L2745-2757. https://doi.org/10.1002/etc.4259

References

ecoTTC / EnviroTox: www.envirotoxdatabase.org

- Belanger et al., 2015. It is time to develop ecological thresholds of toxicological concern to assist environmental hazard assessment. ET&C 34(12):2864-2869. https://doi.org/10.1002/etc.3132

- Connors et al., 2019. Creation of a Curated Aquatic Toxicology Database: EnviroTox. ET&C 38(5):1062-1073. https://doi.org/10.1002/etc.4382

- Kienzler et al., 2019. Mode of Action Classifications in the EnviroTox Database: Development and Implementation of a Consensus MOA Classification. ET&C 38(10): 2294-2304. https://doi.org/10.1002/etc.4531

- Kienzler et al., 2017. Mode of Action (MOA) Assignment Classifications for Ecotoxicology: An Evaluation of Approaches. ES&T 51(17):10203-10211. https://doi.org/10.1021/acs.est.7b02337

- Kroes et al., 2004. Structure-based thresholds of toxicological concern (TTC): Guidance for application to substances present at low levels in the diet. Tox Sci 42:65-83. https://doi.org/10.1016/j.fct.2003.08.006

- Kroes et al., 2005. The threshold of toxicological concern concept in risk assessment. ToxSci 86(2):226-30. https://doi.org/10.1093/toxsci/kfi169

Gill Cell Line

- Bols et al., 1994. Development of a cell-line from primary cultures of rainbow-trout Oncorhynchus mykiss (Walbaum), gills. J of Fish Dis 17(6):601-611. https://doi.org/10.1111/j.1365-2761.1994.tb00258.x

- Fisher et al., 2019. Repeatability and Reproducibility of the RTgill-W1 Cell Line Assay for Predicting Fish Acute Toxicity. Tox Sci. https://doi.org/10.1093/toxsci/kfz057

- Natsch et al., 2018. Accurate prediction of acute fish toxicity of fragrance chemicals with the RTgill-W1 cell assay. ET&C 37:931–941. https://doi.org/10.1002/etc.4027

- Tanneberger et al., 2013. Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. ES&T 47:1110–1119. https://doi.org/10.1021/es303505z

References

Bioaccumulation in vitro method:

- Nichols et al., 2007. In vitro-in vivo extrapolation of quantitative hepatic biotransformation data for fish. II. Modeled effects on chemical bioaccumulation. ET&C 26: 1304-1319. https://doi.org/10.1016/j.aquatox.2006.01.017

- Nichols et al., 2013. Towards improved models for predicting bioconcentration of well-metabolized compounds by rainbow trout using measured rates of in vitro intrinsic clearance. ET&C 32: 1611-1622. https://doi.org/10.1002/etc.2219

- Nichols et al., 2018. Reliability of In Vitro Methods Used to Measure Intrinsic Clearance of Hydrophobic Organic Chemicals by Rainbow Trout: Results of an International Ring Trial. Tox Sci 164(2):563-575. https://doi.org/10.1093/toxsci/kfy113

- OECD Test Guidelines (319 A&B)
  - https://www.oecd-ilibrary.org/environment/test-no-319a-determination-of-in-vitro-intrinsic-clearance-using-cryopreserved-rainbow-trout-hepatocytes-rt-hep_9789264303218-en
  - http://www.oecd.org/publications/test-no-319b-determination-of-in-vitro-intrinsic-clearance-using-rainbow-trout-liver-s9-sub-cellular-fraction-rt-s9-9789264303232-en.htm

- OECD Guidance Document
  - http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2018)12&doclanguage=en

FET References

FET

- Belanger et al., 2013. Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. ET&C 32: 1768-1783. https://doi.org/10.1002/etc.2244

- Belanger et al., 2015. It is time to develop ecological thresholds of toxicological concern to assist environmental hazard assessment. ET&C 34(12):2864-2869. https://doi.org/10.1002/etc.3132

- Busquet et al., 2014. OECD validation study to assess intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test for acute aquatic toxicity testing. RTP 69:496-511. https://doi.org/10.1016/j.yrtph.2014.05.018

- Embry et al., 2010. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. Aquat Tox 97(2):79-87. https://doi.org/10.1016/j.aquatox.2009.12.008

- Glaberman et al., 2017. Evaluating the zebrafish embryo toxicity test for pesticide hazard screening. ET&C 36(5):1221-1226. https://doi.org/10.1002/etc.3641

- Halder et al., 2010. Regulatory aspects on the use of fish embryos in environmental toxicology. IEAM 6(3):484-491. https://doi.org/10.1002/ieam.48

- OECD, 2013. OECD Guideline for Testing of Chemicals, 236. Fish Embryo Acute Toxicity (FET) Test. https://www.oecd-ilibrary.org/environment/test-no-236-fish-embryo-acute-toxicity-fet-test_9789264203709-en

- Sobanska et al., 2018. Applicability of the fish embryo acute toxicity (FET) test (OECD 236) in the regulatory context of Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH). ET&C 37(3):657-670. https://doi.org/10.1002/etc.4055

Acknowledgments

- Scott Belanger & Kristin Connors (P&G)
- Thomas Braunbeck (U of Heidelberg)
- Marlies Halder (EC JRC)
- Kristin Schirmer (Eawag)

- HESI Bioaccumulation Committee
- HESI Animal Alternatives in ERA Committee
- SETAC Animal Alternatives Interest Group