Reducing the number of fish used in acute toxicity testing: Incorporation of the Fish Embryo Acute Toxicity test into the threshold approach

PETA INTERNATIONAL SCIENCE CONSORTIUM LTD.

PISC@piscltd.org.uk

Christopher Faßbender,[1] Dominik Altmann,[2] Martin Paparella,[2] Susanne Walter-Rohde,[3] Jane Rawlings,[4] Scott Belanger,[4] Thomas Braunbeck,[5] Stefan Scholz,[6] Gilly Stoddart[1]

umweltbundesamt ENVIRONMENT AGENCY AUSTRIA

HELMHOLTZ CENTRE FOR ENVIRONMENTAL RESEARCH – UFZ

[1]PETA International Science Consortium Ltd., United Kingdom, [2]Environment Agency, Austria, [3]Environment Agency, Germany, [4]Procter & Gamble, USA, [5]University of Heidelberg, Germany, [6]Helmholtz Centre for Environmental Research GmbH, Germany

Umwelt Bundesamt

P&G

Introduction and background information

In 2011, nearly 180,000 fish were used for toxicological and other safety assessments in Europe.[1] Assessment of aquatic toxicity is required in various regulatory frameworks, so strategies to reduce the number of animals used are urgently needed.

The acute fish toxicity test (AFT; OECD Test Guideline [TG] 203) is one of the most frequently used aquatic toxicity tests and, as death is the endpoint, animal welfare is a significant concern.

Applying the threshold approach (OECD Guidance Document 126), in which an initial fish test is conducted at one concentration derived from test responses in Daphnia and algae and continued testing is triggered only if mortality is observed at this threshold concentration, can substantially reduce the number of fish used in the AFT. Furthermore, as embryos are used, the Fish Embryo Acute Toxicity Test (FET; OECD TG 236) provides a significant refinement to the AFT.

Aim: Incorporate the FET into the threshold approach for acute fish toxicity

Strategy: A strategy for incorporating the FET into the threshold approach is being developed which builds on extensive earlier work and three individual efforts:

1. Development of a new database containing acute toxicity data for adult/juvenile fish, embryos, Daphnia and algae to analyse how the FET can be incorporated into the threshold approach
2. Clarification of the applicability domain, reliability and relevance of the FET and comparison with the uncertainties of the AFT for the protection of aquatic ecosystem, including
   - Reproducibility of FET and AFT
   - Correlation of FET to AFT and AFT to AFT
3. Consideration of the European Chemicals Agency's report on the use of the FET for REACH.[2]

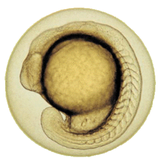
On this basis, a concept for defining acceptance criteria for the new approach will be proposed.

When deciding on the acceptability of the new approach, conceptually:

1. The new approach should be at least as reproducible as the AFT-based reference data.
2. If inclusion of the FET into the threshold approach leads to higher or lower sensitivity for approximately the same number of chemicals, it should be acceptable. Improving the approach by informed triggers for AFT or FET is a subsequent step.
3. The correlation of the new approach with reference data should not be expected to be better than the variability of reference data that are usually accepted for regulatory purposes, i.e. including interspecies variability and study designs.
4. Variable environmental conditions and biological diversity affect toxicity and therefore the ability of any standardised test, including the AFT, to predict environmental toxicity is limited. The higher the uncertainty regarding the relevance of the reference data to the target of evaluation, i.e. aquatic environment, the lower the need for a tight correlation of the new approach with the reference data, and more weight should be given to mechanistic considerations. For example, is the FET based on an environmentally relevant life-stage? Is this life-stage expected to be less sensitive than later stages? How do the chorion and metabolic competence of embryos contribute to these considerations? Would the use of embryos from one species at a clearly defined stage lead to more consistent classification and points of departure for a predicted no effect concentration (PNEC) derivation?

This poster presents analysis of the database and considers the applicability domain of the FET.

Proposal for the FET threshold approach



*The requirement for TG 203 may be based on the applicability domain of the FET or legislative requirements

Database

- Based on Lammer *et al.* [3] and Belanger *et al.* [4]
- Updated with FET data from the REACH portal, literature and selected industry studies
- New acute aquatic toxicity database includes the following:
  - FET (96h)
  - AFT (OECD 203)
  - Daphnia acute immobilization test (OECD 202)
  - Algal growth inhibition test (USEPA 850 or OECD TG 201)
- Database includes substances with a broad range of physicochemical properties and functional groups
  - Log $K_{o/w}$: -5 to 7
  - Solubility: 0.001 to 1000000 mg/L
- Adequacy of studies was confirmed based on ecotoxicological principles:
  - Nominal exposure data included
  - Solubility within 10X of predicted solubility for upper concentrations
  - Sound LC_{50} supported by source information
  - Data from Truong *et al.*[5] and Padilla *et al.*[6] excluded because of issues with concentration spacing and replication

Table 1. Data distribution

Group	Taxon	Occurrences	%
FET	Zebrafish	524	96.7
	African sharptooth catfish	2	0.4
	Fathead minnow	13	2.4
	Medaka	3	0.6
AFT	Zebrafish	87	5.9
	Bluegill	361	24.6
	Fathead minnow	492	33.6
	Rainbow trout	424	28.9
	Medaka	101	6.9
Algae	*Pseudokirchneriella subcapitata*	140	53.0
	Desmodesmus subspicatus	76	28.8
	Anabaena flos-aquae	3	1.1
	Chlorella pyrenoidosa	12	4.5
	Chlorella vulgaris	15	5.7
	Microcystis aeruginosa	2	0.8
	Skeletonema costatum	16	6.1
Daphnia	*Daphnia magna*	1041	89.4
	Daphnia pulex	123	10.6

Table 2. Data distribution by functional category

Functional category	FET %	TG 203 %	Daphnia %	Algal %
Biocide	3.8	4.8	4.6	2.3
Flame retardant	0.4	0.6	0.8	1.1
Food additive/Vitamin	1.3	0.0	0.0	0.0
Hair dye	3.8	1.8	0.8	1.1
Industrial organic	52.3	53.3	55.4	58.0
Inorganic	0.8	1.2	1.5	1.1
Metal	3.0	4.2	5.4	8.0
Natural/Botanical	1.7	0.6	0.0	0.0
Organometal	0.4	0.6	0.8	0.0
Perfume	0.4	0.6	0.8	1.1
Pesticide	10.5	12.7	15.4	12.5
Petrochemical	0.4	0.6	0.0	0.0
Pharmaceutical	9.3	6.1	6.2	4.5
Polymer	1.3	1.2	0.8	0.0
Surfactant	10.5	11.5	7.7	10.2

Evaluation of data to support FET threshold approach

Table 3. Most sensitive taxon based on geometric mean

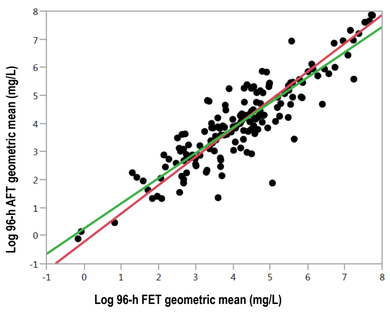
Comparison	n	Algae	Daphnia	Fish	FET
A-D-AFT-FET	81	31 (38%)	29 (36%)	12 (15%)	9 (11%)
A-D-AFT	81	32 (40%)	33 (41%)	16 (20%)	
A-D-FET	81	35 (43%)	34 (42%)		12 (15%)
A-D	81	39 (48%)	42 (52%)		
AFT-FET	81			52 (64%)	29 (36%)
AFT-FET	165			102 (62%)	63 (38%)

Observations

- When all data are available, AFT or FET are the most sensitive an equal number of times.
- When considered separately, AFT and FET identify different chemicals when they are the most sensitive.
- When algae and Daphnia are unavailable, there is some indication that fish are more sensitive

Table 4 and Figure 1. AFT/FET Relationship

AFT/FET ratio	n	%
Within a factor of 2	68	41
Within a factor of 3	93	56
Within a factor of 10	144	87
Greater than 10	21	13



Influence of FET or AFT on GHS acute toxicity classification

Of 81 substances based on geometric means:

- 16 substances had fish as the most sensitive (ignoring FET)
- 12 substances had the FET as the most sensitive (ignoring fish)
- Potential GHS classification was lowered from 3 to 2 when using FET instead of AFT for 4-nitrophenol, diclofenac and ibuprofen. Using the AFT instead of the FET, GHS classification was lowered from 3 to 2 only for tetrachloroethylene.

Summary

- Results are quantitatively consistent with previous observations.
- Inclusion of algae and Daphnia indicate the relative importance we should place on the fish/FET data (important, but not the most).
- Very few GHS classifications are affected by the choice of AFT or FET.
- Risk assessment decisions based on input source (FET or AFT) will not be altered appreciably. Fish or FET were the most sensitive only 26% of the time (21/81); within this subgroup the FET/AFT ratio was within 3-fold 57% of the time, and 81% were within a factor of 10.
- The predictions are best for polar and non-polar narcotics as well as inorganics and somewhat less robust for neurotoxicants indicating further work is needed to support predictions for neurotoxicants.

Applicability domain of FET: ongoing considerations

Lipophilicity and volatility	• The principles for testing of difficult substances outlined in the OECD Guidance Document 23 apply also to the FET. There is no additional limitation in testing volatile or hydrophobic compounds in the FET compared to the AFT.
Inorganic compounds	• Further data are needed to validate the potential of the FET to predict acute fish toxicity of metals.
Molecular weight	• MW (eg > 3 kD), 3D structure and charge influence transfer across the chorion; consider removing the chorion.
Neurotoxicity	• Additional endpoints such as behaviour analysis may indicate neurotoxicity and be used as an indicator of concentrations that would cause lethality in later life-stages or may trigger the AFT.
Biotransformation	• There is evidence of expression of biotransformation enzymes and metabolism of chemicals in early development. • Read-across could be used to determine applicability of the FET.
Multi-constituent compounds	• No evidence that the FET is not applicable to multi-constituent compounds.

The views, conclusions and recommendations are those of the authors and do not necessarily represent the policies or positions of the organisations to which the authors are affiliated.

Next steps

- Data analysis:
  - Refine regression outputs
  - Deeper analysis of taxonomic inclusion/exclusion (refined species-specific analysis; multiple algae, multiple fishes)
  - Multivariate analyses to explore physicochemical and biological properties
  - Publication
- Further clarification of FET applicability domain
- Consideration of how the FET can be used in a weight of evidence approach to predict acute fish toxicity

References and abbreviations

[1]EU (2013) Seventh Report from the Commission to the Council and the European Parliament on the Statistics on the number of animals used for experimental and other scientific purposes in the member states of the European Union COM(2013)859/final.

[2]Scholz S, Klüver N, Kühne R. 2016. Report ECHA-UFZ contract ECHA/2014/341: Analysis of the relevance and adequateness of using Fish Embryo Acute Toxicity (FET) Test Guidance (OECD 236) to fulfil the information requirements and addressing concerns under REACH.

[3]Lammer E, Carr GJ, Wendler K, Rawlings, JM, Belanger SE, Braunbeck TH. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp Biochem Phys, Part C, 2009;149:196-209.

[4]Belanger SE, Rawlings JM, Carr GJ. Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. *Env Toxicol Chem*, 2013; 32(8):1768-1783.

[5]Truong L, Reif DM, St Mary L, Geier MC, Truong HD, Tanguay RL. Multidimensional In Vivo Hazard Assessment Using Zebrafish. *Toxicol Sci*, 2014; 137:212-233.

[6]Padilla S, Corum D, Padnos B, Hunter DL, Beam A, Houck KA, Sipes N, Kleinstreuer N, Knudsen T, Dix DJ, Reif DM. Zebrafish developmental screening of the ToxCastTM Phase I chemical library. *Reprod Toxicol*, 2012;33:174-187.

AFT	Acute fish toxicity test	PNEC	Predicted no-effect concentration
EC_{50}	Median effective concentration	REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
FET	Fish embryo toxicity test	TC	Threshold concentration
GHS	*Globally Harmonized System* of Classification and Labelling of Chemicals	TG	Test guideline
LC_{50}	Median lethal concentration		