Joint Report ECHA and UBA

Expert Workshop on the potential regulatory application of the Fish Embryo Acute Toxicity (FET) Test under REACH, CLP and the BPR

3-4 May 2017, Helsinki

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I. Introduction

This report summarises the outcome of the expert workshop on the potential regulatory application of OECD Test Guideline 236 Fish Embryo Acute Toxicity Test (FET, OECD TG 236) under the REACH Regulation (No 1907/2006)[1], the Regulation on CLP (No 1272/2008)[2] and the BPR (528/2012)[3] held by European Chemical Agency (ECHA) and the German Environment Agency (Umweltbundesamt, UBA) at the premises of ECHA in Helsinki on 3rd and 4th May 2017.

The workshop participants were composed of experts from academia, industry and governmental organizations as well as non-governmental organizations (NGOs). The list of participants is provided in Annex 1.

The workshop was jointly organised by ECHA and UBA, with support of UBA Austria and EURL ECVAM[4] in the steering committee.

The aim of the workshop was to exchange views on the potential regulatory application of the FET and explore possibilities on how the FET might be used as a part of weight of evidence approaches in the EU regulatory context (REACH, BPR and CLP) to adapt standard information requirements for acute fish toxicity. Under REACH, information on short-term toxicity fish is standard requirement for all substances manufactured or imported in the EU in quantities of 10 tonnes or more. This information is used in risk assessment and identification of Persistent, Bioaccumulative and Toxic substances (PBT assessment) under the REACH Regulation and for hazard classification under the Regulation on CLP.

Background to the workshop is the OECD TG 236 Fish Embryo Acute Toxicity (FET, OECD TG 236) developed under the lead of Germany and approved in 2013. OECD TG 236 was developed and validated (*OECD Series on Testing and Assessment No. 157 and 179 Validation Reports Phase 1 and 2 for the Zebrafish Embryo Toxicity Test*) with the goal to determine acute toxicity of chemicals on embryonic fish on the basis of a positive outcome in any of the four indicators of lethality recorded, and to calculate the LC50. The acute embryo toxicity has been shown to correlate well with the acute adult fish toxicity and therefore the FET test (OECD TG 236) may be a promising alternative to standard Fish Acute Toxicity (AFT) Test (OECD TG 203).

[1] Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)
[2] Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP Regulation)
[3] Biocidal Product Regulation (BPR, Regulation (EU) 528/2012)
[4] European Union Reference Laboratory for Alternatives to Animal Testing hosted by European Commission Joint Research Centre, Directorate F

As the FET test is not set out as an alternative method to adapt standard information requirement for short-term toxicity to fish in the REACH Regulation, in 2015 ECHA contracted out the analysis of relevance and adequateness of the new test (OECD TG 236) under the REACH Regulation. The results of this project were discussed with experts from the Member State competent authorities during 2015. The report of this work was published on ECHA's website as 'Analysis of the relevance and adequateness of using Fish Embryo Acute Toxicity test (FET) Test Guideline (OECD TG 236) to fulfil the information requirements and addressing concerns under REACH' (link) together with official recommendation of ECHA on how to use the method under REACH. From the report ECHA concluded that the FET could not be considered as a stand-alone information for adapting the information requirement for the acute fish toxicity test under the REACH Regulation. Based on current knowledge, ECHA considered that OECD TG 236 may be used within a weight of evidence approach (Annex XI, Section 1.2 to the REACH Regulation) together with other independent, adequate, relevant and reliable sources of information leading to the conclusion that the substance has or does not have a particular dangerous property.

Moreover, it has to be noted that at OECD level, Austria and ICAPO (International Council for Animal Protection in OECD Programmes) are leading the OECD project no. 2.54 on inclusion of the FET into OECD Guidance Document No. 126 "Short Guidance on the Threshold Approach for Acute Fish Toxicity" (OECD GD 126). Discussion on the revision is ongoing.

The workshop programme is attached in Annex 2.

All participants were provided the following material as background information for the workshop.

- ECHA Report: Analysis of the relevance and adequateness of using Fish Embryo Acute Toxicity (FET) Test Guidance (OECD TG 236) to fulfil the information requirements and addressing concerns under REACH (14.04.2016); Link: https://echa.europa.eu/documents/10162/13639/fet_report_en.pdf
- ECHA Read-Across Assessment Framework (RAAF) (2017); Link: https://echa.europa.eu/documents/10162/13628/raaf_en.pdf
- ECHA Practical Guide: How to use alternatives to animal testing to fulfil your information requirements for REACH registration (July 2016), Link: https://echa.europa.eu/documents/10162/13655/practical_guide_how_to_use_alternatives_en.pdf
- ECHA Guidance on Information Requirements and Chemical Safety Assessment Chapter R.7b: Endpoint specific guidance (February 2016); Link: https://echa.europa.eu/documents/10162/13632/information_requirements_r7b_en.pdf
- ECHA Practical Guide - How to use and report (Q)SARs (July 2016); Link: https://echa.europa.eu/documents/10162/13655/pg_report_qsars_en.pdf
- ECHA Guidance on Information Requirements and Chemical Safety Assessment Chapter R.6: QSARs and grouping of chemicals, (May 2008);

Link: https://echa.europa.eu/documents/10162/13632/information_requirements_r6_en.pdf

- OECD QSAR Toolbox where also tutorials for training purposes are provided Link: http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm
- OECD project update of OECD GD 126 (Discussion Paper VMGeco[5], VMGeco RCOM1, VMGeco RCOM2, Proposed Update of Threshold Approach, Background Paper to be updated according to the results with regard to the discussion paper).

II. Presentations

The workshop started with 9 presentations on various subjects related to data requirements for acute fish testing and rules for their general adaptation as well as the use of fish toxicity data in different regulatory frameworks with focus on fish embryo toxicity data.

The presentations are included in Annex 3.

After each presentation there was the possibility for the audience to ask questions. The content of the presentations and following discussions can be summarized as follows:

It was pointed out by ECHA in the introductory presentation that information on short-term fish (i.e. not fish embryo) toxicity is the standard information required by the REACH Regulation for substances manufactured or imported in the EU in quantities of 10 tonnes per annum or more. This information requirement is normally addressed by the use of the standard OECD Test Guideline (TG) 203. Data on aquatic toxicity (including fish short-term toxicity) are used under REACH for Chemical Safety Assessment (CSA) of a registered substance. More specifically, these data are used for classification and labelling (C&L) and derivation of predicted no-effect concentrations (PNEC) of a substance as well as for estimating the toxicity threshold in the persistent, bioaccumulative and toxic (PBT) assessment. For proper CSA the information on aquatic toxicity should at least cover species of three trophic levels: algae/aquatic plants, invertebrates (*Daphnia* preferred), and fish. The OECD TG 236 Fish Embryo Acute Toxicity (FET) Test under REACH might be considered as an alternative to the standard method if for a specific substance it can address adequately the fish short-term toxicity (e.g. as part of Weight of Evidence - WoE) and the results of such a prediction of acute fish toxicity would be adequate for the purpose of C&L and/or risk assessment, i.e. have adequate and reliable coverage of the key parameters covered by the standard test.

In contrast to REACH, for Biocides most of the ecotoxicity data for active substances (including information on the acute fish toxicity performed mostly according to the OECD

[5] OECD Validation and Management Group for Ecotoxicity Testing (VMG eco)

203) have been already submitted by industry. Currently, the use of FET would fall into adaptation of data requirements and would need to follow the indications given in Annex IV of the BPR. In general terms ECHA highlighted that the conclusions obtained for REACH in relation to the applicability of FET, should be applicable for Biocides as well.

The fish embryo for acute fish toxicity testing is already used in other regulatory fields such as the testing of effluents (EN ISO 15088 – T6) according to German law. Cosmetic companies use the FET (OECD 236) for assessing cosmetic ingredients for environmental properties in the development phase of their products. Fish embryo testing is furthermore also a well-established screening method for human health endpoints. In Europe the FET is considered a vertebrate animal test but it does not fall under the scope of directive 2010/63/EU on the protection of animals used for scientific purposes meaning that fish embryos are not "protected" and the provisions of the directive do not apply. Contrary to EU, in the US law the FET is considered a vertebrate animal test which limits their interest for its regulatory application. Furthermore, it was noted that under risk assessment scheme applied in the US information on acute fish toxicity is necessary and FET test is not used for this purpose.

When testing the toxicity of substances in the fish embryo it is important to follow the guideline specifically to ensure that the test substance concentrations are maintained during the test by using appropriate analytical techniques. If the substances are difficult-to-test the respective Guidance Document on aquatic toxicity testing of difficult substances and mixtures (OECD GD 23) should be followed as much as possible. This holds true for any other aquatic toxicity testing. Before the adoption of the OECD TG 236, protocols for performing the FET test have varied. One major deficiency in the data produced with some of the former protocols is related to the lack of verification that the exposure concentrations have been maintained constant/stable during the course of the test. In these cases the nominal concentration could deviate from the real exposure concentrations and consequently produce false positive deviation from the LC50 values derived with the AFT. This aspect is of a special importance as the FET test (before adoption of OECD TG 236) were normally performed using plastic microtiter plates which can affect the test substance concentration throughout the test especially for adsorptive compounds.

Triggered by the ECHA report on the regulatory applicability of the FET, further studies have been conducted investigating the large differences between the FET and AFT for some of the narcotic substances and some preliminary results were presented during the workshop. These preliminary results for some selected substances revealed a similar sensitivity of the FET to AFT. Therefore, the participants of the workshop agreed that further analysis of these narcotic compounds could help to better understand applicability domain and regulatory application of FET.

Furthermore, it was suggested in one presentation that it is possible to assess a variety of sub-lethal endpoints with the FET and to adapt the test guideline to assess also neurotoxic substances (e.g. via the touch evoked response or the distance moved). But for inclusion of these new endpoints to the test protocol, revision of the FET test guideline would be necessary.

After the presentation of the ECHA report, it was questioned that the AFT test results used in the ECHA analysis may have been of limited reliability despite the high scrutiny applied in curing the data set. Published AFT data (OECD TG 203) could have been invalid and incorrect results reported, therefore AFT studies should also be subject to a further critical evaluation. Nevertheless, it was clarified that for the analysis performed for ECHA, only AFT data following the OECD 203 TG conditions, conducted according to GLP, with reliability of 1 or 2 (according to Klimish score) were used for the comparison.

Further aspects presented were considerations to decide on the regulatory acceptance of new approaches such as the update of the threshold approach which is currently discussed under OECD project 2.54.

Furthermore, other alternative methods, e.g. the use of fish cell lines for aquatic toxicity/bioaccumulation testing and in particular, the validation status of the RTgill-W1 assay for acute fish toxicity testing were presented. Recent progress in using fish embryos to gain mechanistic insight and for Adverse Outcome Pathways (AOP)/ Integrated Approaches to Testing an Assessment (IATA) development were shown and discussed.

There were differing opinions amongst workshop attendees on certain issues. One group was of the opinion that there is already a large database available of FET and AFT toxicity studies that is adequate and sufficient to decide on the applicability domain of the FET and does not see a need to show FET-AFT data correlations for all groups of substances for which the reliability of the data cannot be demonstrated. Hence, this group questioned the necessity to produce further FET data for establishing the applicability domain.

In contrast, another group of attendees considered that the available studies are insufficient to draw definitive conclusions on whether the FET can be used as a direct replacement for the AFT, nor the applicability domain of the FET test could be established. In particular, as recorded in the ECHA study, some of the available FET studies showed toxic effects above the water solubility or the concentration of the test substance was not measured. Studies conducted without determination of exposure concentrations are likely to underestimate the real LC50 values, especially for unstable substances. Indeed the ECHA study showed that also some narcotic substances were more toxic to adults than to embryos which may as well be caused by nominal versus measured exposure concentrations. Therefore applicability even for narcotics cannot be confirmed using the

current databases. In addition many of the existing FET studies were of a lower duration than required under OECD TG 236. This group of attendees highlighted the importance of data quality and the need of using only reliable data to derive the applicability domain of FET test.

III. Lead Questions discussed in World Café

A World Café was set up as a structured conversational process in which each participant of the workshop discussed the three key questions with different people.

The lead questions were:

1. **Usability of FET for regulatory purposes under REACH, CLP and the BPR** For which type(s) of substances the FET can/cannot be used for risk assessment or classification purposes (e.g. considerations on testing substance properties related to mode of action (MoA), physico-chemical properties)? Research needs and areas for further developments to improve usability of FET for regulatory purposes (e.g. data robustness for OECD TG 203 and 236).
2. **Building weight of evidence approach (WoE) to fulfil regulatory data requirements for aquatic fish toxicity**: What are pieces of evidence to support that the FET can be used for a given substance: Evidence on MoA, metabolic activation…? How should the various lines of evidence (including the FET study) be combined to produce the overall WoE approach? Reporting needs for the individual lines of evidence? Research needs and areas for further developments to enable building of effective WoE approaches with the use of FET test.
3. **Use of FET in Environmental Hazard and PBT Assessment (innovative approaches).** The use within Risk assessment (PNEC setting), CLP and PBT assessment.

As similar topics were addressed in all discussion groups and for each topic the following summary is provided.

1. Use of the FET: For which type(s) of substances the FET can/cannot be used for risk assessment or classification purposes? What are pieces of evidence that are needed to support that the FET can be used for a given substance?

OECD 236 is a standardised robust study protocol for zebrafish embryos especially with regard to the tested life stage, species, replicates, internal control and positive control in each test.

The general requirements for regulatory acceptance of FET were discussed and it was stated that OECD TG 236 method must have been followed, including controlled test conditions and measurements of exposure concentrations, and the test performed in compliance with the principles of Good Laboratory Practice (GLP).

Several participants supported the view that only a systematic deviation of the FET to AFT data correlation towards a lower sensitivity of the FET should result in exclusion of particular types of substances from the applicability domain of the FET (e.g. neurotoxicity, bioactivation). Therefore systematic outliers for FET and AFT need be looked at in more detail.

Another group of participants were of the view that the data quality of many of the available studies was inadequate to draw definitive conclusions on the applicability domain. In particular, some narcotic substances have shown large deviations in toxicity between the embryo and adult fish. However, UFZ[6] presented new FET data for some of the 9 substances, which were identified in the ECHA report as narcotic compounds indicating higher toxicity to adult fish than for fish embryo: For four substances selected for re-analysis the new data showed that they may not be genuine outliers (publication by UFZ in preparation). Therefore further data and analysis is needed to further assess the applicability domain of the FET test.

With regard to difficult-to-test substances there was overall consensus that testing issues are not specific for FET and are addressed in the OECD GD 23 on Aquatic Toxicity Testing of Difficult substances and mixtures. It should be considered that passive dosing can be used for FET which is an advantage when testing highly adsorptive substances.

For highly lipophilic and/or poorly water soluble substances it was proposed that neither the FET (OECD 236) nor the AFT (OECD 203) seem suitable as these substances will not reach steady state conditions during a short-term test due to slower uptake into the organism. Therefore, the general recommendation would be to test the substance in a long-term test, e.g. OECD TG 210 Early Life Stage Test.

Neurotoxicity of compounds leading to respiratory failure in adult fish cannot be predicted by the current test design of OECD TG 236. However, this might not hold true for other MoAs leading to neurotoxicity. In addition, it was discussed that new data showing that *Daphnia* are likely to be most sensitive to neurotoxins and narcotics are in the process of being published (see also question 2 on the defined Approach discussion).

Concern was raised whether the FET can predict substances requiring metabolic bioactivation to elicit fish toxicity. To be able to understand this better insight into

[6] Helmholtz Centre for Environmental Research - UFZ

predicting metabolism would be needed, which in principle may be gained from mammalian toxicity data, mutagenicity tests (showing a difference according to metabolic activation tests (+/-S9[7])), expert judgment, structural alerts, read-across, or *in vitro* assays. However, it was also communicated that new data informing on the metabolic capacity of zebrafish embryos are in the process of being developed and published.

With regard to the insight into assigning likely MoA it was proposed to classify substances "into bins", based on knowledge from QSARs, expert judgment, other toxicology data. Mode of action is also an important parameter for weight of evidence approaches, adverse outcome pathways (AOP) development, molecular initiating event (MIE) identification, chronic toxicity, etc.

It was further highlighted that the ECHA report does not include further detailed analysis of those compounds which show higher toxicity in FET than in AFT. These compounds could have an effect on the embryonic life stage which would be seen in FET but not in AFT.

2. WoE Approach and Guidance: Recommendations? How should the various lines of evidence be combined to produce the overall WoE approach?

As prerequisites for a WoE approach it was discussed that all information needs to be relevant and sufficiently reliable and the approach must be fit for purpose to deliver adequate information to support regulatory decisions. As WoE approach by its nature requires the use of scientific judgement, it is therefore necessary to provide adequate and reliable documentation leading to the conclusion that the substance has or does not have a particular dangerous property (for further information see Annex XI, 1.2 to the REACH Regulation).

The discussions showed a preference for a structured decision tree (e.g. an Integrated Testing Strategy, ITS or a defined approach, DA). It should include a list of elements to consider (criteria and other supporting evidence) when deciding whether the FET test is applicable for the substance and if yes, how it is used in a WoE approach. The different pieces of evidence need to be consistent. The ECHA Read Across Assessment Framework (RAAF) could support the DA/ITS development. The participants agreed to keep the approach as simple as possible while fulfilling the regulatory requirements and weighing the lines of evidence in appropriate and scientifically robust manner (Annex XI to the

[7] Supernatant fraction obtained from an organ (usually liver) homogenate by centrifuging at 9000 g for 20 minutes in a suitable medium; this fraction contains cytosol and microsomes.

REACH Regulation, Guidance on information requirements and chemical safety assessment, Chapter R.6: QSARs and grouping of chemicals).

With regard to evaluating the short-term aquatic toxicity in a context of risk assessment (e.g. PNEC derivation) or classification the fish toxicity data should also be seen in relation to the other trophic levels (*Daphnia* and algae). In REACH the information requirement for acute fish toxicity is currently needed and current data does not allow concluding that the AFT can be conservatively predicted by the FET alone for all types of substances. Other lines of evidence in a WoE approach need to be provided as a support for the prediction of the toxicity from embryonic fish life stage to juvenile/adult stage. Additionally other lines of evidence may include a plausible grouping approach or/and reliable QSAR prediction.

The development of the OECD IATA for environment and/or the revision of the OECD Fish Testing Framework (OECD Series of Testing an Assessment No. 171) could also be an option to give guidance on the use of the FET in relation to other OECD Test Guidelines using fish.

In the discussion it was recommended to encourage registrants to include available FET data in the weight of evidence approach(es) in their registrations. Such case studies may then be used as best practice examples.

Another important point raised by representatives from industry would be a reduction of costs for FET studies. Costs might be reduced when the FET is more frequently used and the quality of new FET data carried out in line with the present OECD TG would be better. Moreover it was argued that cost differences between AFT and FET are small especially when compared to costs for toxicological studies for human health endpoints. Still the economic incentives for registrants to prefer the FET over the AFT are currently not big enough.

The issue that the level of confidence is context dependent, i.e. more precision/less uncertainty is needed near regulatory thresholds (e.g. for CLP an LC50 of 0,9 mg/L or 1,1 mg/L would eventually result in a different classification), was raised and should be kept in mind in the discussion of any testing strategy for regulatory decision making. This is true for test data of all test studies used for regulatory purposes.

3. Research Needs: Research needs and areas for further developments to improve usability of FET for regulatory purposes? Research needs to enable building an effective WoE approach? Innovative approaches?

This section addresses all research issues that were raised during the world café as well as the general discussions. For a better overview the research issues were ordered with regard to short-term, medium-term or long-term research, if possible. It seems obvious that all research issues raised that are needed to advance the regulatory use of OECD TG 236 as alternative to the AFT are of high priority and should be tackled soon. Still, no prioritization was done during the workshop.

Short-term activities should focus on the enhancement of the FET data base used for the ECHA study: good quality data (especially with analytical verification to confirm test concentrations for difficult-to-test substances) are needed to improve its regulatory acceptability and determination of the applicability domain. AFT data used for comparison with FET need to be scrutinized with a similar level of caution. Industry and also regulators are asked to check whether relevant FET and AFT data is available, e.g. for cosmetics, pharmaceuticals, biocides and substances registered under the REACH Regulation. Further research could be done to investigate the reliability of data in FET/AFT data base and to better understand why some weaker FET toxicity could be found, e.g. for narcotics. Research is needed to understand whether there is a systematic bias due to the lack of metabolic activation or specific MoAs (e.g. neurotoxic effects) that are not covered by the FET. In addition the mechanisms for lower sensitivity of FET (biological plausibility) resulting from the ECHA report should be explained for narcotic outliers as well as other substances with unknown MoA. More knowledge on the biotransformation capacity and other kinetic processes in fish embryos is in the process of being published and needs to be reviewed also with a view to potential AFT to FET differences for pharmaco-/toxicokinetics. Therefore the analysis of internal concentrations is needed for screening the applicability domain (kinetics of substances with different physical-chemical properties).

It was proposed to quantitatively analyse the uncertainties of FET versus AFT (e.g. in light of providing sufficient protective potential towards the aquatic environment) and use this information to discuss and finally decide on regulatory acceptability of new approaches. Potential decision criteria were proposed, i.e. the variability and relevance for the protection of aquatic environment must be at least as good when using the FET instead of the AFT. However, neither these criteria nor regulatory consequences were discussed in further detail.

Adaption of the FET to other species, e.g. medaka, fathead minnow and stickleback are underway. Their regulatory utility may be for AOP and IATA development beyond standard acute aquatic toxicity testing. As stickleback embryos take longer in their development there is more time for bioaccumulation during their development.

OECD project 2.54 aiming to integrate the FET into the OECD Threshold Approach will focus on the ability of *Daphnia* and algae data to compensate potential weaknesses of FET-AFT correlations (e.g. for neurotoxic substances). Correlations of QSAR predictions & good-quality FET may also be analysed.

An additional useful way forward for regulatory science may be the validation of the tests using fish cell lines to predict acute aquatic toxicity and its adaptation for regulatory use (e.g. as part of integrated testing strategies).

It was questioned whether probabilistic hazard assessment within current approaches could make the regulatory uncertainty transparent and thereby help transition to improved approaches. Any point estimate, e.g. PNEC, should in scientific terms be accompanied with a confidence interval and a list of qualitative uncertainties with regard to the protection-target, i.e. aquatic environment. Regulatory acknowledgment of this perspective may support recognition that standard approaches can and should be continuously improved. From the current regulatory perspective such probabilistic approaches seem to be far from reality, especially since the large majority of substances that are being assessed (e.g. under REACH) only contain limited information on intrinsic hazards.

Several WS participants were of the opinion that the above mentioned scientific activities might already provide sufficient confidence for regulatory use of FET within a testing strategy. However even in case this will be achieved in short term, a continuous improvement of regulatory approaches shall be envisaged.

It was recognized that there is an urgent need to develop methodologies to screen for MoA and for metabolism which may inter alia be helpful to understand if the FET is not applicable for their particular substance. This would also need to include answers to the question: How to deal with substances having an unknown MoA?

Medium-term research was seen in retrospective analysis of FET data when more regulatory experience and more data may be gained using the FET (also in the REACH registration process).

In the long-term perspective the FET could be enhanced with additional endpoints such as touch evoque response (TER) or locomotive response (LMR) covering neurotoxicity or with vitellogenin (VTG) measurement screening for endocrine disruption. Research is also going on with regard to molecular endpoints like gene expression. Altogether further research with the FET will help to better understand embryotoxic effects in general.

Studies with (zebra-)fish embryos might be adapted to high-throughput screening (HTS) for hazard identification. The ongoing (zebra-)fish embryo research with regard to informing adverse outcome pathways (AOP) that may be the basis for future in vitro approaches is also very promising. For the novel approaches more mechanistic understanding and related data would be needed.

With regard to general environmental risk assessment further research questions were raised beyond the FET. A suggestion was to use animals in a more intelligent way and implement more endpoints in animal tests. The integration of other species to derive a predicted no effect concentration (PNEC), e.g. oysters, was another proposal.

As a further step into the future the hypothesis was raised that novel risk assessment methods may be developed not needing any (acute) fish toxicity testing.

Beyond the environmental risk assessment the zebrafish embryo model is already used to screen for human diseases (e.g. cardiac diseases) or for teratogenicity.

IV. Conclusions

The FET is a promising method to predict acute fish toxicity. All participants believe in the potential of the FET.

Current knowledge gaps that arose during validation of OECD TG 236 and within the current ECHA report 'Analysis of the relevance and adequateness of using Fish Embryo Acute Toxicity test (FET) Test Guideline (OECD TG 236) to fulfil the information requirements and addressing concerns under REACH' show that the FET could not be considered as a stand-alone information for adapting the information requirement for the acute fish toxicity test under the REACH Regulation. One of the reasons is insufficient knowledge on the metabolic capacity of zebrafish embryos, the full understanding of the applicability domain of FET and the uncertainties of the FET and the AFT. However new data (e.g. regarding narcotic outliers, neurotoxicity, metabolic capacity, comparison of sensitivity of FET daphnia and algae, uncertainties of the AFT) are in the process of being developed and published and should be further reviewed in a regulatory context in order to better understand the applicability domain of FET for the regulatory use and eventually refine its regulatory use.

An important message from the workshop is that data from a FET study (performed according to OECD TG 236) can be used for REACH registration dossiers within a weight of evidence approach together with other independent, adequate, relevant and reliable sources of information (for further information see Annex XI, 1.2 to the REACH Regulation). This fact is addressed in the official recommendation on ECHA website and in the Practical

Guide for SME managers and REACH coordinators – How to fulfil your information requirements at tonnages 1-10 and 10-100 tonnes per year (July 2016, Link). Under REACH the burden of proof is on side of the registrants.

With regard to the fulfilment of the information requirements under REACH within a WoE approach there was agreement among the participants to keep the approach as simple as possible while fulfilling the regulatory requirements and weighing the lines of evidence in appropriate and scientifically robust manner (Annex XI to the REACH Regulation, Guidance on information requirements and chemical safety assessment, Chapter R.6: QSARs and grouping of chemicals).

One of the way forward was seen in development of more descriptive approaches such as, e.g. Integrated Approach to Testing an Assessment for aquatic toxicity (IATA), Defined Approaches (DA) under certain circumstances and incorporation of FET into the threshold approach (OECD project 2.45). A further option would also be a revision of the current OECD Fish Testing Framework (OECD Series Testing and Assessment No. 171).

Industry was invited to prepare case studies which could be revised by ECHA and could become the basis for an in depth discussion of defined approaches at a further expert workshop, e.g. to be held at RIVM within the project on the FET as intermediate between human health and environment risk assessment.

On the following issues agreement could be reached among workshop participants:

- OECD TG 236 – like other studies – must be performed under GLP compliance and analytical verification of the exposure concentrations is essential when testing substances in the FET (or other aquatic toxicity tests) for regulatory use.
- In case of difficult-to-test substances (e.g. volatile, lipophilic) the respective OECD GD 23 on aquatic toxicity testing of difficult substances and mixtures should be followed. Difficulties in testing these substances are not a mere problem of the FET.
- Preliminary results show that most of the narcotic substances analysed in the ECHA report are in the applicability domain of the FET. Preliminary data on 4 out of 9 substances, which were identified in the ECHA report as narcotic compounds indicating higher toxicity to adult fish than for fish embryo, show that 4 substances selected for re-analysis may not be genuine outliers – detailed results need to be discussed after data is published by UFZ.
- Lipophilic substances should neither be tested in AFT nor in FET as these substances will not reach steady state conditions during a short-term test due to slower uptake into the organism. For lipophilic and/or poorly water soluble substances a long-term fish test is already recommended under REACH (REACH Endpoint specific guidance, chapter R.7b).

With regard to open issues, more research work is on-going or should be initiated:

- to further curate and re-construct the data base of both FET and AFT with regard to inclusion of reliable data only to address the real predictive power of the test, general uncertainties and systematic bias (short-term activity)
- to clarify the biotransformation capacity in embryos to understand differences of FET and AFT (short-term activity)
- to continue discussions at the OECD level on how to integrate the FET into the OECD Threshold Approach (project 2.54)
- to analyse the overall uncertainty of acute aquatic toxicity testing and assessment approaches (for CLP and limit value derivation) including AFT versus FET
- to develop methodologies to screen for MoA and metabolism (urgent need)
- after clarifying and defining future specific regulatory needs, to enhance the FET (longer-term activities)
    - with additional endpoints (e.g. behavioural, molecular, biomarker) to cover neurotoxicity, endocrine disruption, etc.
    - as high-throughput screen for hazard identification
    - for other species (e.g. medaka, fathead minnow, stickleback)
    - as model for human disease (eventually this will not be an OECD TG 236)

ECHA suggests to prepare a summary document on the most important regulatory research needs to further improve the regulatory applicability of the FET test. This summary could be brought to the attention of e.g. the European Commission to clarify needs for future research funding.

Annex 1 – List of Participants

1	Altmann, Dominik	AT (UBA)
2	Belanger, Scott	P&G
3	Braunbeck., Thomas	University of Heidelberg
4	Cesnaitis, Romanas	ECHA
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8	de Wolf, Watze	ECHA
9	Embry, Michelle	HESI
10	Faßbender, Christopher	PETA
11	Gellatly, Nikki	UK NC3R
12	Greiner, Petra	DE (UBA)
13	Gutierrez Alonso, Simon	ECHA
14	Halder, Marlies	JRC
15	Hassold, Enken	DE (UBA)
16	Hoy, Simon	UK (Envrionment Agency)
17	Jose Tarrazona or Jean Lou Dorne (via Webex on 03.05)	EFSA
18	Katsiadaki, Ioanna	UK (Cefas)
19	Kehrer, Anja	DE (UBA)
20	Knight, Derek	ECHA
21	Léonard, Marc	L'Oreal
22	Lillicrap, Adam	NO (NIVA)
23	Lundbergh, Ivar	SE (KEMI)
24	Noberg-King, Teresa	US (EPA)
25	Nyman, Anna Maija	ECHA
26	Paparella, Martin	AT (UBA)
27	Priha, Maarit	FI (Tukes)
28	Salinas, Edward	BASF/ECETOC
29	Schirmer, Kristin	EAWAG
30	Scholz, Stefan	UFZ
31	Sobanska, Marta	ECHA
32	Stoddart, Gilly	PETA
33	Teigeler, Matthias	Fraunhofer IME
34	Tyle, Henrik	DK (EPA)
35	Walter-Rohde, Susanne	DE (UBA)

Thank you to all participants for their contribution and active participation in the discussions.

Annex 2 – The workshop programme

Workshop on a role and applicability of the Fish Embryo Acute Toxicity Test for European Regulation and beyond

Day 1 – 03.05.2017

Moderation: Wim De Coen (ECHA)

09:00 – 09:10	Welcome & Background (ECHA & UBA)
09:10 – 09:30	Data requirements for acute fish toxicity in perspective of using this data for RA, CLP and PBT under REACH, CLP and the BPR (standard and non-standard). Romanas Cesnaitis (ECHA) & Simon Gutierrez Alonso (ECHA)
9:30 – 10:10	Rules for general adaptations for acute aquatic fish toxicity (WoE, Read Across and grouping approach, QSARs) Henrik Tyle (DK CA) & Anna-Maija Nyman (ECHA)
10:10 – 10:30	Using fish cells in culture to predict the impact of chemicals to fish Kristin Schirmer (EAWAG)
10:30 – 10:50	FET interspecies differences and AOP/IATA development for acute aquatic toxicity Ioanna Katsiadaki (CEFAS)
10:50 – 11:10	Coffee Break
11:10 – 11:40	Outcome of ECHA Study: Analysis of the relevance and adequateness of using the Fish Embryo Acute Toxicity (FET) Test Guideline (OECD 236) to fulfil the information requirements and addressing concerns under REACH Marta Sobanska (ECHA) & Stefan Scholz (UFZ Leipzig)
11:40 - 12:00	Industry's view on the use of the FET test – benefits and challenges with regards to costs, practicability and acceptability Marc Leonard, (L'Oreal)
12:00 – 12:15	Uncertainties of reference-data and what they mean for the validation of alternative approaches in ecotoxicology. Martin Paparella (AT)

12:15–12:45	Broader considerations: OECD VMG Eco/FDG project on the use of the FET within the threshold approach (update of OECD GD 126 FET) (a) Summary of open discussion points of the OECD VMG Eco/FDG expert group in the context of received comments. (b) New data on daphnia and algae Stefan Scholz (UFZ Leipzig), Scott Belanger (P&G),
12:45 – 13:00	US perspective on the use of the FET Teresa Norberg-King (US EPA)
13:00 – 14:00	Lunch, e.g. in the ECHA canteen*
14:00 – 17:30	World Café (with Coffee break in between) Starting with 15 min introduction
17:30 – 17:45	Closing of the day

Day 2 – 04.05.2017

Moderation: Wim De Coen (ECHA)

9:00 -	Summary of discussions of the World Café (per each World café question) Plenary discussion (Conclusion by each question) Coffee break Recommendations
- 13:00	Closing remarks ECHA/UBA

Annex 3 – The workshop presentations

1. Data requirements for acute fish toxicity under REACH, CLP and the BPR (standard and non-standard).

2. Rules for adapting the REACH standard information requirements for short-term toxicity to fish.

3. Using fish cells in culture to predict the impact of chemicals to fish.

4. FET interspecies differences and AOP/IATA development for acute aquatic toxicity.

5. Outcome of ECHA Study: Analysis of the relevance and adequateness of using the Fish Embryo Acute Toxicity (FET) Test Guideline (OECD 236) to fulfil the information requirements and addressing concerns under REACH.

6. Industry's view on the use of the FET test – benefits and challenges with regards to costs, practicability and acceptability.

7. Uncertainties of reference-data and what they mean for the validation of alternative approaches in ecotoxicology.

8. (a) Possibilities for Using Fish Embryo Tests in place of Fish Acute Toxicity – Threshold Approach Strategies for Ecotoxicity Hazard Determination.

8. (b) Summary of the major open discussion points for potential limitations of the FET.
9. Perspectives on the Regulatory Use of the Fish Embryo Acute Toxicity (FET)Test.

Data requirements for acute fish toxicity under REACH, CLP and the BPR (standard and non-standard)

3 May 2017

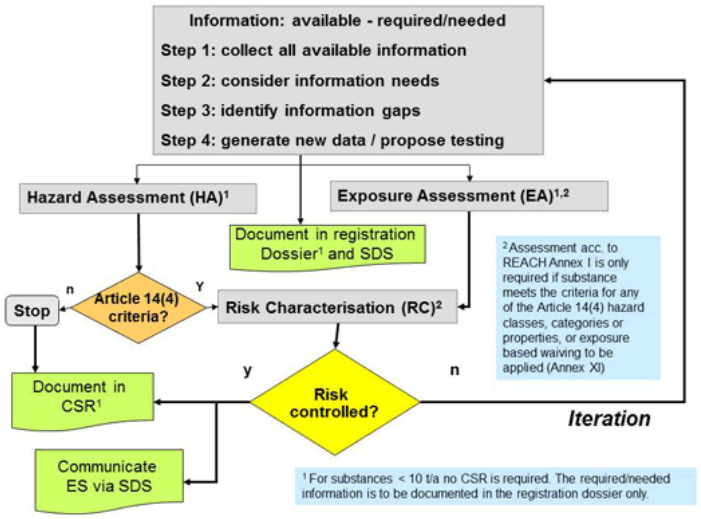
Romanas Cesnaitis
Simón Gutiérrez Alonso

European Chemicals Agency

Outline

- Environmental risk and hazard assessments under REACH regulation
- Standard information requirements for aquatic toxicity and their use for CSA
- Summary

Information requirements and chemical safety assessment under REACH



Chemical Safety Assessment

1. Human health hazard assessment
2. Human health hazard assessment of physico chemical properties
3. Environmental hazard assessment
4. PBT and vPvB assessment

when triggered

5. Exposure assessment
6. Risk characterisation

- CSA should be based on the information contained in the technical dossier and other available/relevant information
- Standard (minimum) requirements for generation of information on intrinsic properties of a substance are specified in Annexes VII-X of REACH

Environmental hazard assessment

Collection and evaluation of information

To determine classification

To identify PNECs

Consider:

1) *Aquatic (including sediment) compartment;*
2) *Terrestrial compartment;*
3) *Atmospheric compartment;*
4) *Accumulation via food-chain; and*
5) *Microbiological activity of sewage treatment systems.*

PBT and vPvB assessment

- Step 1: comparison with the criteria given in Annex XIII
  - T criteria: the long-term NOEC or EC10 for marine/freshwater organisms is less than 0.01 mg/l …
  - P/vP and B/vB criteria
- Step 2: Emission characterisation

Standard information requirements (1)

- Standard (minimum) requirements for generation of information on aquatic toxicity
- Section 9.1. Aquatic toxicity
  - Annex VII
    - 9.1.1. Short-term toxicity testing on invertebrates (preferred species *Daphnia*)
    - 9.1.2. Growth inhibition study aquatic plants (algae preferred)
  - Annex VIII
    - 9.1.3. Short-term toxicity testing on fish (long-term testing can be considered instead)
    - 9.1.4. Activated sludge respiration inhibition testing
  - Annex IX
    - 9.1.5. Long-term toxicity testing on invertebrates (preferred species *Daphnia*)
    - 9.1.6. Long-term toxicity testing on fish (three test types are noted)

Standard information requirements (2)

- Testing can be adapted by using:
  - Specific rules for adaptation listed in column 2 in Annexes VII-X
  - General rules contained in Annex XI (addressed by another presentation)
- Specific rules for adaptation for short-term toxicity testing on fish:
  - There are mitigating factors indicating that aquatic toxicity is unlikely to occur
  - A long-term aquatic toxicity study on fish is available
- For fish short-term toxicity testing test method EU C.1./OECD TG 203 is the preferred test to cover the standard information requirement of Annex VIII, Section 9.1.3.

CSA under REACH (1)

- For PNEC derivation
  - The information should at least cover species of three trophic levels: algae/aquatic plants, invertebrates (*Daphnia* preferred), and fish (Guidance, Chapter R.7b).
  - If there is compelling evidence to suggest that the fish value is likely to be at least a factor of about 10 less sensitive than invertebrates or algae there are no further requirements for acute fish testing.
  - Threshold approach for *in vivo* fish short-term toxicity testing is noted in the Guidance, Chapter R.7b.

CSA under REACH (2)

- For PBT/vPvB assessment
  - Screening threshold value for T: Short-term aquatic toxicity (algae, daphnia, fish) - EC50 or LC50 < 0.1 mg/L
- For classification into acute (short-term) and long-term aquatic hazard (when adequate chronic toxicity data are not available) categories following short-term toxicity information is used
  - 96 h LC50 (for fish)
  - 48 h EC50 (for crustacea)
  - 72 or 96 h ErC50 (for algae or other aquatic plants)

Summary

- Fish acute toxicity test (test method EU C.1. / OECD TG 203) is the preferred test to cover the standard information requirement for short-term toxicity testing on fish.
- This information is needed for CSA.
- Any alternative tests/methods should be adequate for purpose of C&L and/or risk assessment, and have adequate and reliable coverage of the key parameters covered by the preferred test.

Biocides

Legal text

REGULATION (EU) No 528/2012 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 May 2012

concerning the making available on the market and use of biocidal products

Status biocides

- Aprox 300 active substances in total
- 125 active substances already assessed
- around 600 active substance product type combinations still under evaluation in Review Programe → Review Programme to be finished in 2024
- biocidal products on the EU market: around 20,000

Annex II BPR

Information requirements for active substance

- 9.1.1. Short-term toxicity testing on fish (core data set)

When short-term fish toxicity data is required the threshold approach (tiered strategy) should be applied

The study does not need to be conducted if: — a valid long-term aquatic toxicity study on fish is available

Annex II BPR

Information requirements for active substance

- 9.1.6.1. Long term toxicity testing on Fish (additional data set)

Annex IV BPR

GENERAL RULES FOR THE ADAPTATION OF THE DATA REQUIREMENTS (Similar to REACH)

- Sets out rules to be followed when the applicant proposes to adapt the data requirements set out in Annexes II and III
- WoE, QSAR, available information, Read-across, in-vitro methods, etc…
- Testing is technically not possible

Biocides

Guidance on data requirements (Vol IV Part A)

9.1 Toxicity to Aquatic Organisms

- Concentrations up to 100 mg/L should be tested. A limit test at 100 mg/L may be performed when results of a range-finding test indicate that no effects are expected.
- If the data from the base set (algae, daphnids and fish) shows that one trophic level is more sensitive, and this is also corroborated by the mode of action of the substance, additional ecotoxicity studies that are required because of exposure to the marine or brackish environment may only need to be supplied for the most sensitive trophic level. To contribute to reduction of the uncertainty in the PNEC derivation any such additional studies should be long term. at no effects are expected

Biocides

Guidance on data requirements (Vol IV Part A)

9.1.1 Short term toxicity testing on fish

- *The study does not need to be conducted if a valid long-term aquatic toxicity study on fish is available.*

- The threshold approach (tiered strategy) according to the OECD Guidance Document must be considered: essentially the approach uses a limit test at a single threshold concentration determined by the results of *Daphnia magna* and algae tests

PBT and CLH

- Same obligations and guidance as the ones used for REACH apply

Differences to REACH

- Biocides are meant to kill so toxicity is generally higher than industrial chemicals (<0.1 mg/L)
- Complete data packages available (including Mode of toxic Action)
- Most of active substances have been already submitted so data package (including acute fish mostly according to OECD 203) is already available

Experiences so far

- So far no cases presented with FET data
- We intend to use the same outcome as used for REACH if possible
- Our experts at MS level are being informed of the latest news on FET and it's usability

Thank You.

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Environmental Protection Agency
Denmark

Rules for adapting the REACH standard information requirement of short-term toxicity to fish

ECHA/UBA WS on role & applicability of The FET test for EU Regulation and beyond

Anna-Maija Nyman & Henrik Tyle

European Chemicals Agency &
Danish Environmental Protection Agency

3-4 May 2017

Disclaimer

The content of this presentation reflects the views of the authors and not necessarily the position of ECHA or the Danish Environmental Protection Agency

Outline – introduction - REACH – specific rules for adaptation

Henrik

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Outline – Rules for adaptation in REACH short-term toxicity to fish

- Introduction to general and specific rules for adaptation
- Specific rules / column 2 (Annex VIII)
- General rules (Annex XI)
  - Technical feasibility
  - Exposure considerations
  - Use of existing data
  - Read-across and grouping
  - QSARs
  - *In vitro* methods
  - Weight of evidence

Introduction

- Acceptability of alternative data is generally context dependent:
  - Is value close to /far away from "regulatory decision cut off "? The closer the more precision needed
  - Will acceptance mean:
    - No (or "soft") regulatory decision: no further info/ test requirement, no classification, no risk , not T, no further RMM?
    - Significant regulatory decision : further info/ test required, classification, (potential) risk, T , further RMM?
  - If acceptance means greater uncertainty: protection of humans/ wild animals (e.g. fish) vs. lab.animals (fish)

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REACH:

"*The purpose of this Regulation is* ***to ensure a high level of protection of human health and the environment****,* ***including the promotion of alternative methods for assessment of hazards of substances****, as well as the free circulation of substances on the internal market while enhancing competitiveness and innovation*."

Introduction to general and specific rules for adaptation

Standard information requirement for short-term toxicity fish – production volumes >10 tonnes/year (Annex VIII to REACH)

1. **Specific rules:** Column 2 for each information requirement
2. **General rules**: provided that the conditions set out in Annex XI are met

Introduction: background

- Annex VIII **(>10tpa EU manufacturer or importer**)
  - **Short-term fish tox data** required
  - or alternatively long-term fish tox. ("to be considered") if the substance has low water solubility (< 1mg/L)
  - Already available if not waived:
    - EC50 & EC10 short-term Daphnia & algae
    - Log Kow , Sw, VP
    - RBT data

Specific rules of adaptation (Annex VIII column 2)

Short term fish tox data **not needed** if aquatic toxicity not likely e.g. if the substance is :

- "*Highly insoluble in water*" (Sw < 1 ug/L ?)
  - However: ESG: no science based trigger can generally be set
- "*Unlikely to cross biological membranes*"
  - However:
    - Experience PBT gr.: no Dmax or other simple descriptor alone can identify if BCF < 2000. => same for "crossing membranes"
    - Acute toxicity may not only be caused by systemic exposure (e.g. metals causes tox at BL e.g. on gill membrane related structures)
- **WoE** (case by case)

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Specific rules of adaptation

Long-term fish toxicity testing shall be considered:

- For poorly water soluble substances (Sw < 1 mg/L)

or

- If the CSA indicates the need (i.e. RCR > 1 or for T-ass. of PB-substances)
  - However normally first long-term tox testing on Daphnids

General rules for adaptation

Anna-Maija

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General rules to omit testing - provided that the conditions set out in Annex XI are met

1. TESTING DOES NOT APPEAR SCIENTIFICALLY NECESSARY
   - **Use of existing data**
   - **Weight of evidence**
   - **Qualitative or Quantitative structure-activity relationship ((Q)SAR)**
   - ***In vitro* methods**
   - **Grouping of substances and read-across approach**
2. TESTING IS TECHNICALLY NOT POSSIBLE
3. SUBSTANCE-TAILORED EXPOSURE-DRIVEN TESTING

General rules to omit testing

Short-term toxicity to fish (ENV)		
	No. ESR	% ESR
ES	2 368	38.8
TP	0	0.0
RA	2 154	35.3
FO	131	2.1
WE	1 094	17.9
QS	120	2.0
MS	237	3.9
Total	6 104	100

Ref: The Use of Alternatives to Testing on Animals for the REACH Regulation (Article 117(3) report), 2014:

ES = Experimental study
TP = Testing proposal
RA = Read-across
FO = IUCLID flags to omit the study
WE = Weight of evidence
QS = QSAR
MS = Miscellaneous

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General rules for adaptation (Annex XI) - Technical feasibility

- Testing can be omitted if testing technically not possible
  - E.g. highly unstable or reactive, very volatile substances
  - Mixing with water cause danger of fire or explosion
- Guidance given in specific test methods (technical limitations of a test)
- Case by case / WoE

General rules for adaptation (Annex XI) – Exposure driven testing

- Testing can be omitted based on exposure scenarios in CSR, if:
  - ✓ absence or no significant exposure throughout the life-cycle (all identified uses)
  - ✓ PNEC can be derived from available test data, taking into account the increased uncertainty from omission of the SIR
  - ✓ Exposure assessment: exposure well below the PNEC
  - ✓ Substances not in articles: Whole life-cycle strictly controlled (transported isolated intermediates)
  - ✓ Substances (permanently embedded) in articles: No release during whole life cycle & likely exposure negligible
- justification and documentation to be provided

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General rules for adaptation (Annex XI) – Use of existing data

Data from tests not according to GLP or OECD TG / EU TMs

1. Adequacy for C&L and Risk Ass. (PBT ass. not mentioned)
2. Key parameters covered
3. Test duration similar or longer
4. Reliable & adequate documentation

Such acute fish toxicity data are often used and acceptable (if points 1-4 fulfilled)

Grouping of substances

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Grouping of substances and read-across approach

Annex XI to the REACH Regulation, Section 1.5:

Substances whose physicochemical, toxicological and ecotoxicological properties are likely to be similar or follow a regular pattern as a result of structural similarity may be considered as a group, or 'category' of substances. Application of the group concept requires that physicochemical properties, human health effects and environmental effects or environmental fate may be predicted from data for reference substance(s)

Grouping of substances and read-across approach

1. Structural similarity
   - A starting point for prediction
   - But not sufficient alone to predict
     - Structurally similar substances can still have very different environmental fate and/or hazards

2. Physicochemical, toxicological and ecotoxicological properties are likely to be similar or follow a regular pattern
   - Mechanistic explanation: how the structural difference influence properties (bioavailability, physico-chemical properties, degradation, bioaccumulation, mechanism of action) + **Supporting evidence**

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Grouping of substances and read-across approach

- Wide spectrum of possible scientific arguments and different types of data to justify read-across
- Assessment needs to be **consistent**

  => ECHA published a Read-Across Assessment Framework, designed as internal assessment tool (Human Health only in May 2015, updated with environmental aspects in Feb 2017)

Read-Across Assessment Framework

- Hypothesis on grouping/read-across is associated with particular aspects **(assessment elements, AEs**) that are deemed crucial

- Each AE poses questions which lead an assessing expert to select pre-defined conclusions **(assessment options, AOs)**

5 = high confidence - 1 = not acceptable

Read-Across Assessment Framework – Assessment elements

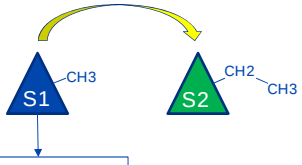
Does the hypothesis provide a scientific explanation why prediction is possible despite the structural difference?

Substance identity
E.g. Impurities, constituents?

Degradation&Fate
E.g. Hydrolysis

Bioaccumulation
E.g. LogKow (organic substances), bioavailability

Toxicity
E.g. Mode of action, consistency of effects in other toxicity data



Substance identity
E.g. Impurities, constituents?

Degradation&Fate
E.g. Hydrolysis

Bioaccumulation
E.g. LogKow (organic substances), bioavailability

Toxicity
E.g. Mode of action, consistency of effects in other toxicity data

Conclusions: Grouping of substances

If the group concept is applied, the results should…

- be adequate for the purpose of classification and labelling and/or risk assessment,
- have adequate and reliable coverage of the key parameters,
- cover an exposure duration comparable to or longer than the corresponding test method, and
- be supported by an adequate & reliable documentation.

In short: The result of read-across should be good enough to be used in the same way as the result of the standard test.

QSARs

Henrik

Background for use of QSARs

Quantitative relationship between chemical structure (and / or expressed by molecular descriptors) and activity (property or effect)

Structure has to be defined (2D) !

- Mono-constituent organic substances
- Multi-constituent substances if:
  - Possible to apply concentration addition approach if all significant constituents are quantitatively known –
- Multi-constituent substances and UVCBs if
  - Representative and close analog structures can be chosen – conc. addition can also be applied

Rules for adaptation Annex XI (Q)SARs

Annex refers to presence / absence of dangerous property

- The scientific **validity of the model** has to be established
  - OECD 5 QSAR validation principles,
  - QMRF
- Model prediction within the **Applicability Domain** of the model (QPRF)
- Reliable and adequate **documentation** (QMRF & QPRF if possible)

Reference to ECHA Technical Guidance R6 (2008), ECHA´s Practical Guidance 2016.

OECDs 5 QSAR validation principles*

1. Defined endpoint
2. Unambiguous algorithm
3. Defined Applicability domain *
4. Statistical validation
5. Mechanistic interpretation, if possible

Point 1 & 3 defines the purpose & scope, 2 defines the method , 4 & 5 concerns the validation and the reliability of individual predictions

EU: *:**Q**SAR **M**odel **R**eporting **F**ormat (QMRF)
*: **Q**SAR **P**rediction **R**eporting **F**ormat (QPRF)

Defined endpoint

- Endpoint : property
- Test method e.g.
  - Subject, species, strain, origin,
  - Experimental conditions such as
    - Media,
    - temperature,
    - pH,
    - hardness, etc
  - duration
  - Response variable
- Data from one or more labs ?

Unambiguous algorithm

- **Model description** & version
- Availability of **training set** data ?
- **Transparency of algorithm** including descriptors (how they were measured or calculated)
- How to take **ionization** into account
- **Global models**: large training set and A.D. Often complex modelling systems with sophisticated approaches for derivation of multiple descriptors, use of sub-molecular fragments and statistical methods employed. Avoid “over-fitting”.
- **Local models**: small training set & A.D . Typically models of for smaller group of structural analogs (“congeneric series”) with simpler approaches

Applicability domain

For which substances can the model make reliable predictions ?

AD is defined by the training set and the model:

- Sub-structure domain
- Descriptor-range domain

And if relevant e.g. in relation to (eco)tox endpoints:

- Mechanistic domain (MoA, chemical class/property)
- ADME-domain
- No single and absolute AD exist for any model – AD definition/description is an active R&D field
- Trade off between AD size and model performance/ prediction accuracy
- Clear AD definition is warranted

Statistical validation

- **Internal performance** (goodness of fit for "training set", R^2)
- **Robustness**
- **External performance**; predictivity for "test set" – has to be within AD & representative (i.e. sufficiently large and diverse); compare MSE for test and predicted data
- **X-validation**: DK QSAR gr. experience:

  LMO(e.g. 10, 20 or 50%) generally provides comparable or more "conservative" results as good external validations

Mechanistic interpretation, if possible

- Try to explain the plausibility of a mechanistic / physical-chemical/biological association between model descriptors / sub-structures and the endpoint
  - E.g. log Kow is a measure of bioavailability due to its relevance for partitioning into /across cell membranes & accumulation in fatty tissues and hence relevant for systemic toxicity..

Acute fish tox QSAR models

- Global / local models
- JRC QSAR DB: 13 acute fish tox LC50 models, 3 long-term tox models First level style
- Hundreds of local models
- Freely available global QSAR Tools : **ECOSAR, VEGA, (T.E.S.T), DK QSAR web tools,**
- Build your own QSAR models by use of the OECD QSAR Appl. TB – more tutorials are available

ECOSAR

Downloadable programme

Input: SMILES, CAS No., name

- **Endpoint:** AFT, species not specified
- **Algorithm**:
  - linear reg. log Kow vs. LC_{50}
  - Chemical class.(6 classes > 10 subst; 105 classes)
  - Training sets available
- **A.D.**: Not really defined
- Warnings: if LC50 > Sw
- **ECHA GD**: disregard prediction if training set too small etc.

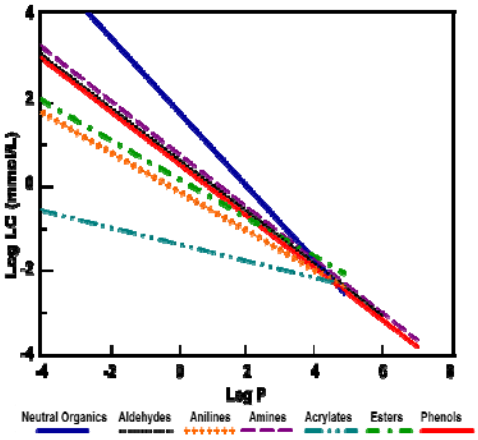


Figure 1. Octanol-Water Partition Coefficient (log P) Cut-Offs and Predicted Magnitude of Fish Acute Toxicity (expressed as median lethal concentration, LC_{50}) for Several Chemical Classes Using Equations from: Clements, R.G., Nabholz, J.V., *ECOSAR: A Computer Program for Estimating the Ecotoxicity of Industrial Chemicals Based on Structure Activity Relationships*, U.S. EPA, OPPT (7403), Technical Publication, 748-R-93-002, 1994.

VEGA

Downloadable program

Input: SMILES

Endpoint: 96 hrs. FHMLC$_{50}$ (USEPA ECOTOX DB)

Training set: 652 substances

Algorithm: LR model with 21 descriptors

A.D.: Global AD Index from 6 sub-indices: fragments, descriptor range, sensitivity analysis of descriptors, concordance with similar subst. with test data, a.o.=> GAD: >0.85=>IN; <0.70=>OUT

Validation: Internal: R^2= 0.69 (RMSE=0.69);
external (164 subst.):Q^2 = 0.64 (RMSE= 0.79)
Structural similar substances with test data displayed

T.E.S.T.: Same training set, 5 models+consensus,
Structural similar substances with test data displayed

DK QSAR website

Web tool:

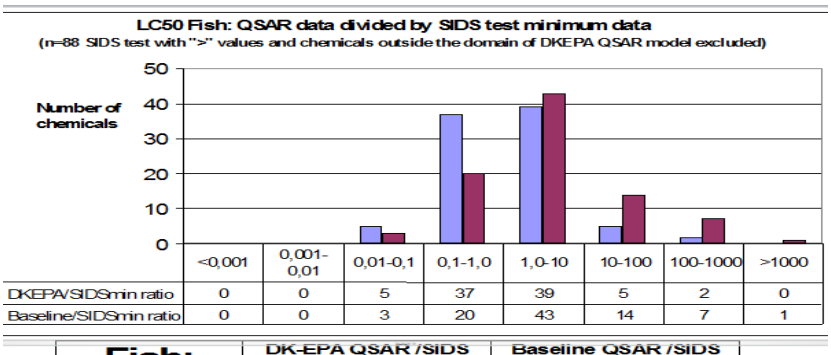
Input: SMILES, CAS no, EC no, PubChem no. (CID), name to structure look up, 2D-structure. DB now: approx. 640.000 entries; Leadscope based web tool with on-the fly-prediction generation for user submitted structure is soon coming !

- Predictions from DTU models by use of SciQSAR™ & Leadscope™
- **Endpoint:** Training set available; 565 org. substances in EUEPS MED-Duluth FMDB; 96 hrs. LC$_{50}$
- **Algorithms**: cf. QMRFs; complex global models
- **Validation**: see QMRFs
  - R^2 = 0,75 & 0.74; Q^2(LMO:5x(2*50%))=0.73 & (LOO)=0.72
- **A.D**: IN/OUT: cf. QMRF :
  - clear algorithm and description
  - but some details from commercial modelling platform not available

Note: structural similarity tool also available

How well do the global models for AFT seem to work ? (I)

- Mcase model predictions on 88 SIDS substances (2004)



	<0,001	0,001-0,01	0,01-0,1	0,1-1,0	1,0-10	10-100	100-1000	>1000
DKEPA/SIDSmin ratio	0	0	5	37	39	5	2	0
Baseline/SIDSmin ratio	0	0	3	20	43	14	7	1

Fish:	DK-EPA QSAR /SIDS test data min.-ratio	Baseline QSAR /SIDS test data min.-ratio
Within a factor of:	%	%
10	86 (80)	72
100	98 (93)	91
1000	100 (100)	99

How well do the global models for AFT seem to work ? (II)

- Note: comparison between QSAR model predictions based on USEPA FM DB and conclusions on ADT in the OECD SIDS Program across all species with test data
- **Predicted L(E)C_{50} values were within one order of magnitude relative to SIDS test data for 4 out of 5 of the fish LC_{50}-values**
- DK EPA QSAR model performed better than the (ESR TGD) QSAR model for non-polar narcosis
- The QSAR acute fish tox model for non-polar narcosis more often under-estimated than over-estimated toxicity
- DK EPA fish QSAR model made approx. similar number of over- and under- estimations of toxicity, when compared with SIDS min. L(E)C_{50} data

How well do the global QSAR AFT models seem to work ? (III)

R^2 for RBT(N=268-351)	ECOSAR	TEST	VEGA
	0.16	0.40	0.35
For FHM DB (N=567):			
• Non-polar narcotics	**0.84**	**0.86**	**0.80**
• Polar narcotics	0.56	**0.89**	**0.72**
• Reactive subst.	0.47	0.50	0.28
• Specifically acting:	0.58	0.72	0.64
• Not classified:	0.66	0.81	0.71

General uncertainty of analysis: extent of overlap between training set and comparison data set

Commercial TerraTox & ADMET Predictor & ACD Tox Suite similar or better. DEMETRA poorer on FHM but better on RBT(but large training set overlap). R^2 varies much according to chemical class

Ref.: Capelli et al: SAR QSAR Env. Res. 26, 977, 2015

Ministry of Environment and Food
Environmental Protection Agency
Denmark

My recommendations on use of QSAR predictions for AFT

- **READ the ECHA** QSAR **GD** (R6) & Practical GD !
- **Freely available global QSAR models** seems to perform pretty well (within one order of magnitude) **for narcotics. Careful if other MoAs !**

=> **Combine use of global model predictions with**

- **Read across/ grouping** e.g. find similar substances with test data, use also OECD QSAR TB.
- Check also whether the these test data compare well with those predicted by the global model
- Make local trend analysis / read across for LC50(fish) by use of **OECD QSAR Tool Box**

In vitro methods

Anna-Maija

ECHA
EUROPEAN CHEMICALS AGENCY

Ministry of Environment and Food
Environmental Protection Agency
Denmark

In vitro methods

Annex XI to the REACH Regulation, Section 1.4:

Results obtained from suitable in vitro methods ***may indicate the presence of a certain dangerous property*** *or may be important in relation to a mechanistic understanding, which may be important for the assessment.*

If the results obtained from the use of such in vitro methods ***do not indicate a certain dangerous property, the relevant test shall nevertheless be carried out at the appropriate tonnage level to confirm the negative result****, unless testing is not required in accordance with Annexes VII to X or the other rules in this Annex.*

In vitro methods

Confirmation with the standard test may be omitted, if the following conditions are met:

1. scientifically validated method (validation according to internationally agreed validation principles);
2. results are adequate for the purpose of classification and labelling and/or risk assessment; and
3. adequate and reliable documentation of the applied method is provided.

ECHA Guidance on IR&CSA, R.7b (2016):

- Currently not enough information available for the extrapolation from in vitro data to in vivo data
- Information from *in vitro* studies might be considered in a *Weight of Evidence* approach

Weight of evidence

Anna-Maija

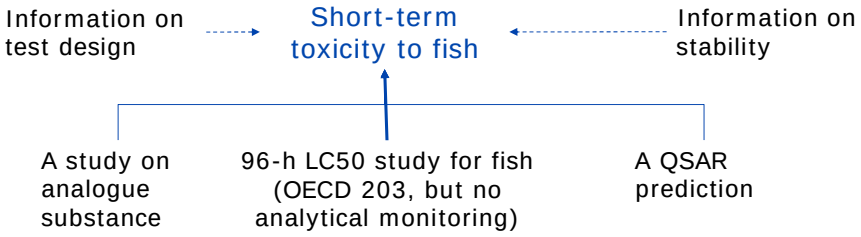
Weight of evidence

Annex XI to the REACH Regulation, Section 1.2:

There may be sufficient weight of evidence from several independent sources of information leading to the assumption/conclusion that a substance has or has not a particular dangerous property, while the information from each single source alone is regarded insufficient to support this notion.

Weight of evidence

1. gathering of information,
2. evaluation of the quality of a distinct piece of information, e.g. a test report or a QSAR result,
3. overall assessment of all available information



Weight of evidence

WoE / integrated testing strategy (ITS) on concluding aquatic pelagic toxicity (REACH Guidance R.7b)

- Step 1 – Characterization of the substance
- Step 2 – Analysis of mode of action
- Step 3 – Identification and evaluation of possible analogues
- Step 4 – Evaluation of existing *in vivo* testing data
- Step 4a – Evaluation of QSAR results
- Step 4b – Evaluation of *in vitro* testing data + FET?
- Step 5 – *Weight-of-Evidence* assessment
- Step 6 – Evaluation of factors relevant for waiving
  - Mitigating factors (intrinsic properties) indicating that aquatic toxicity is unlikely to occur
  - Possibility for test modification, e.g. fish threshold approach

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Environmental Protection Agency
Denmark

Weight of evidence

WoE / integrated testing strategy (ITS) on concluding aquatic pelagic toxicity (REACH Guidance R.7b)

Step 6: Evaluation of factors relevant for waiving

- Intrinsic physico-chemical properties
  - unlikelihood to cross biological membranes
  - very low water solubility (but long-term study needed instead)
- Threshold approach for toxicity testing in fish
  - only the lowest L/EC50 value for species in three trophic levels considered for regulatory purposes
  - lowest of the two EC50 for algae and Daphnia -> a limit test according to OECD TG 203 carried out
  - if no mortality is observed, no further tests needed (LC50 fish reported as greater than –value)
  - if mortality is observed, a full LC50 test to be performed

Weight of evidence

WoE / integrated testing strategy (ITS) on concluding aquatic pelagic toxicity (REACH Guidance R.7b)

Step 5: *Weight-of-Evidence* assessment

- At the end all available information (test data and non-testing information) should be used for a comprehensive conclusion on the endpoint
- Amount of information necessary to draw such conclusions will be different dependent on the regulatory endpoint
  - For C&L, in certain cases limit tests may be sufficient (for a decision whether the toxicity is below a certain trigger value)
  - Derivation of the PNEC: a quantitative figure has normally to be provided (all available relevant information to be used)

Conclusions

Summary / conclusions

- Several possibilities available to adapt REACH information requirements (specific and general rules)
  - most commonly used: read-across and grouping
  - less than 40% study records provide experimental result on the registered substance (short-term fish toxicity)
- All adaptations to be justified and documented
- All adaptations should be equally protective for the purpose of:
  - Classification and labelling
  - Risk assessment
  - Identification of **P**ersistent, **B**ioaccumulative and **T**oxic (PBT) substances, and very **P**ersistent and very **B**ioaccumulative (vPvB) substances (Annex XIII)

Using fish cells in culture to predict the impact of chemicals to fish

Kristin Schirmer & Team; Department of Environmental Toxicology

Kristin.Schirmer@eawag.ch

Eawag: Swiss Federal Institute of Aquatic Science and Technology

Fish = dominant vertebrate species

≥ 10 t/a: fish acute toxicity test obligatory
- Fish acute toxicity test – OECD 203

death after short exposure

≥ 100 t/a: bioconcentration test
- Fish bioconcentration test - OECD 305

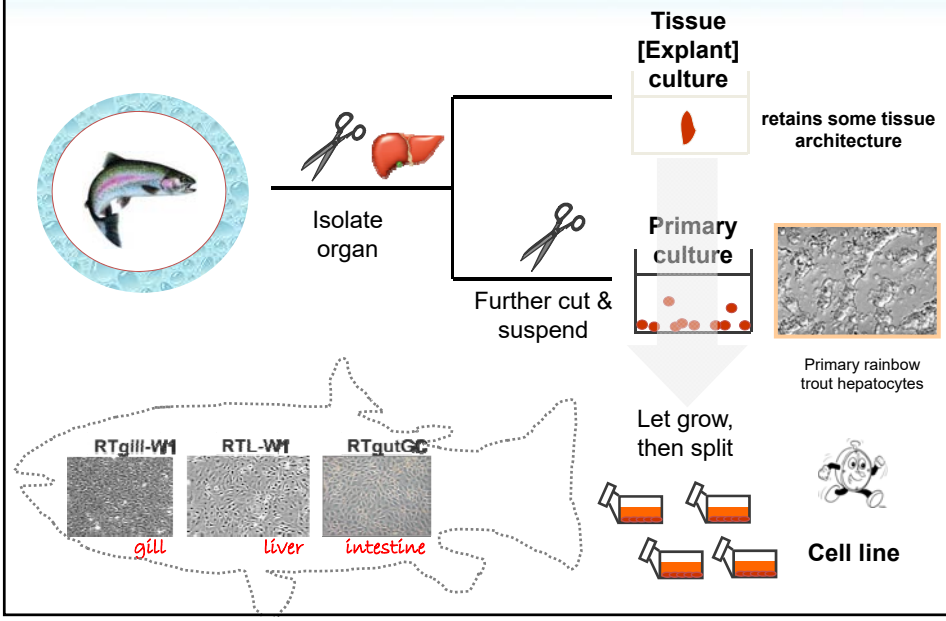
uptake and elimination

≥ 100 t/a: long term tests maybe required
- Fish early life stage test – OECD 210

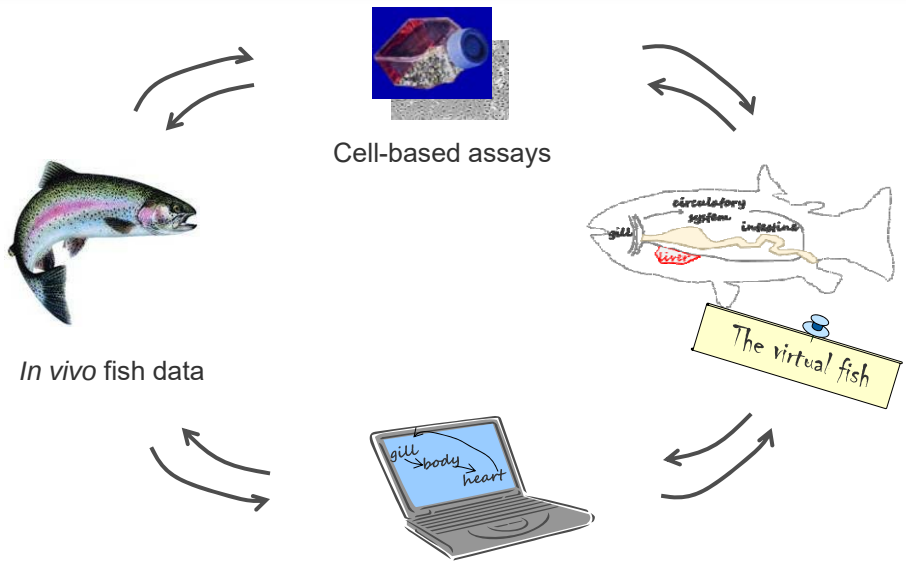
altered growth and/or death over time (1-several months)

REACH Annex 7, 8, 9 und 10

Fish cells in culture



Approaches to predict impact on fish

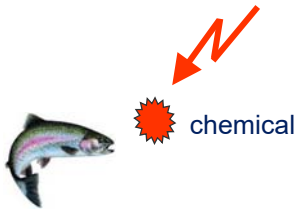


Cell-based systems in action to predict…

1. …acute toxicity

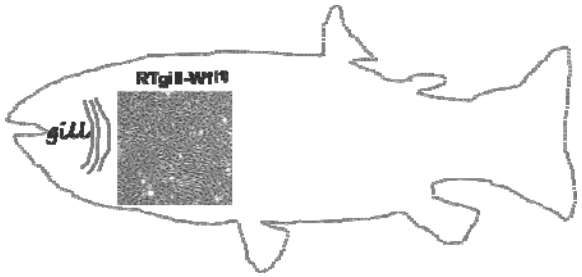
2. …bioaccumulation

3. …impact on growth



Predict acute toxicity to fish (OECD203)

Assumption: Gills as primary site of toxic action



1) Bols et al., 1994, J. Fish Dis 17, 601-611.

Can RTgill-W1 cells predict fish acute toxicity?

- Systematic selection of test chemicals

  → Schirmer et al., 2008, Aquatic Toxicology, 90:128-137.

- Role of physico-chemical properties of test chemicals

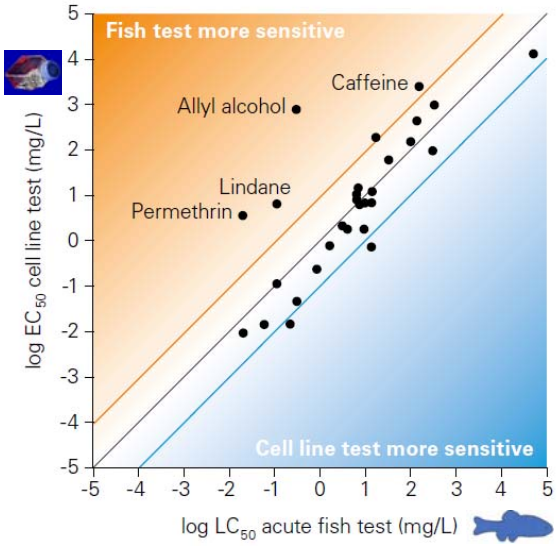
  → Kramer et al., 2009, Toxicology *in Vitro* 23, 1372-1379.

- Improved dosing and exposure

  → Tanneberger et al., 2010, ES&T 44: 4775-4781.

CEllSens Eco8 - supported by

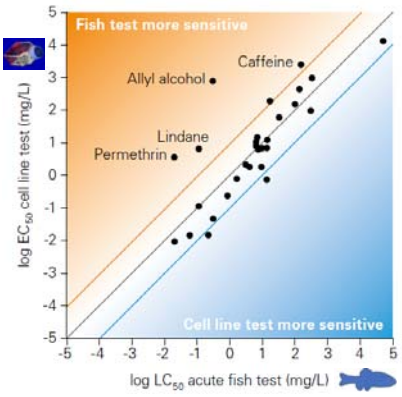
Can RTgill-W1 cells predict fish acute toxicity?



Tanneberger et al, Environ. Sci. Technol. 2013, 47, 1110−1119.

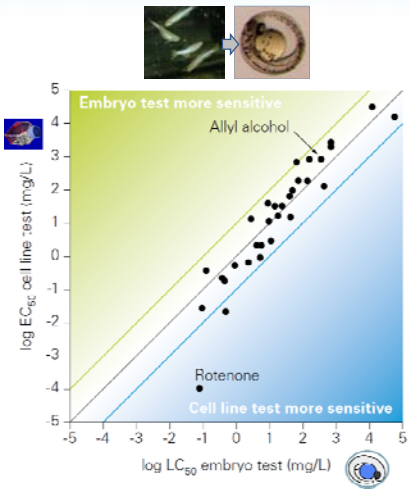
Schirmer et al, Eawag News 2013, 02/Oct

Can RTgill-W1 cells predict fish acute toxicity?



Tanneberger et al, Environ. Sci. Technol. 2013, 47, 1110−1119.

Schirmer et al, Eawag News 2013, 02/Oct

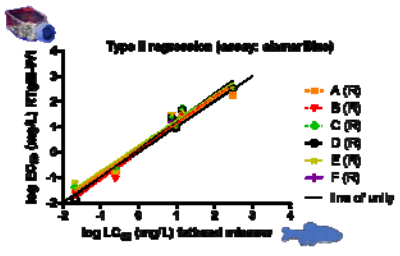


Knöbel et al, Environ. Sci. Technol. 2012, 46, 9690-9700.

Schirmer et al, Eawag News 2013, 02/Oct

Cells in culture to predict fish acute toxicity

- Strong correlation of *in vivo* and *in vitro* data
- Protocol robust and easily transferable



Knöbel et al., in preparation

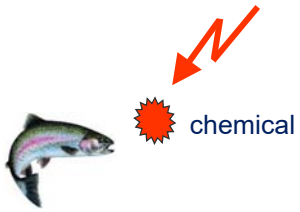
- Seeking internationally accepted guideline (ISO; OECD)

Cell-based systems in action to predict…

1. …acute toxicity ✓

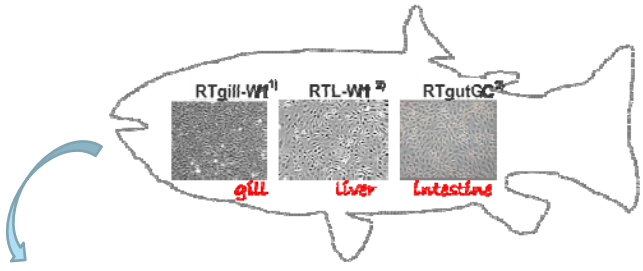
2. …bioaccumulation

3. …impact on growth



Predict bioaccumulation in fish (OECD305)

Assumption: Elimination of chemicals in different tissues

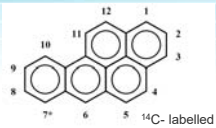


Incorporate into physiologically-based toxicokinetic models (PBTK)

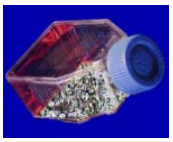
BCF = Bioconcentration factor

1) Bols et al., 1994, J. Fish Dis 17, 601-611.
2) Lee et al., 1993, Cell Biol Toxicol 9(3), 279-294.
3) Kawano et al., 2011, Aquacult. Nutr. 17, e241-252.

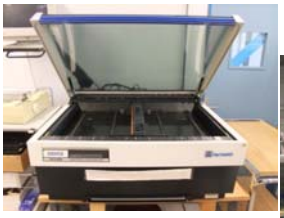
Case study: Benzo(a)pyrene



^{14}C- labelled



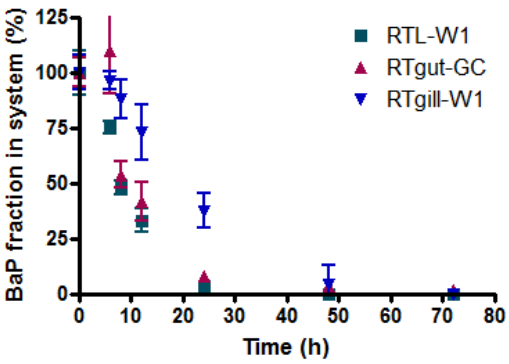
- Radiolabelled BaP
- 1.6 µM BaP (non-cytotoxic)
- 6 time points
- BaP measured in cells, plastic and exposure medium

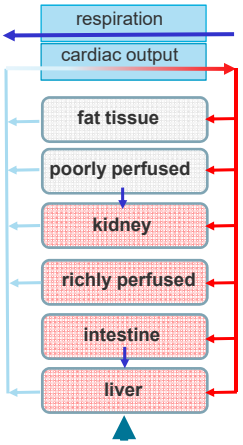

Liquid scintillation counter


Radio-HPLC

Stadnicka et al., in preparation

Disappearance of BaP over time

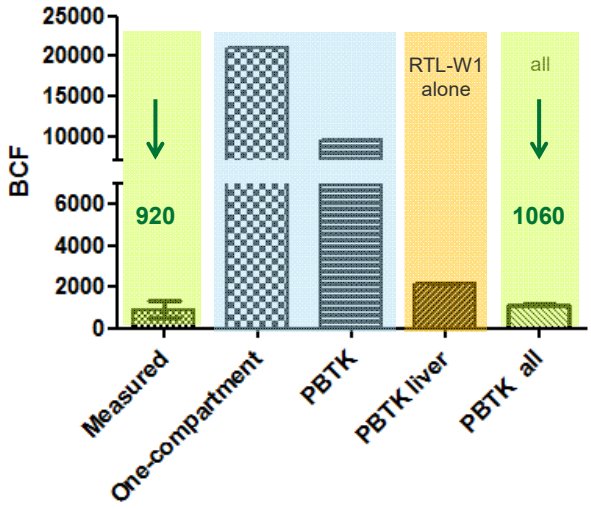




in vitro intrinsic clearance
(ml/h*10^{6}cells)

Stadnicka et al., in preparation

Bioconcentration factor based on cell lines



Stadnicka et al., in preparation

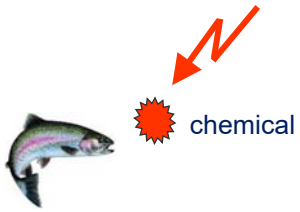
Cells in culture to predict bioaccumulation in fish

- All three cell lines biotransformed BaP
- All-cell line's BCF very close to measured BCF
- More chemicals need to be tested

→ CEFIC-LRI Eco34 project:
http://cefic-lri.org/projects/eco34-a-tiered-testing-strategy-for-rapid-estimation-of-bioaccumulation-by-a-combined-modelling-in-vitro-testing-approach/

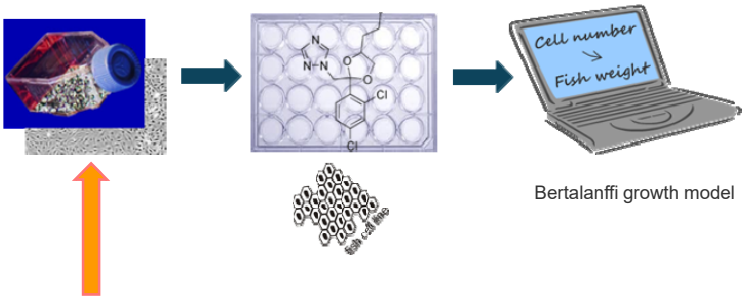
Cell-based systems in action to predict…

1. …acute toxicity ✓
2. …bioaccumulation ✓
3. **…impact on growth**



Predict impact on fish growth (OECD210)

Assumption: less growth means fewer cells in the fish body



Bertalanffi growth model

use PBTK to predict in vitro exposure concentration

Stadnicka-Michalak et al., 2015, Science Advances, 1, 7

Predict impact on fish growth

CYPROCONAZOLE – 62 days of exposure

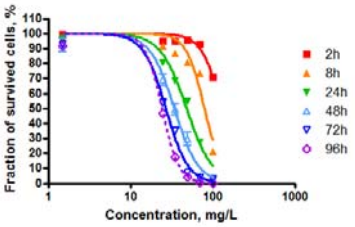
Rainbow trout

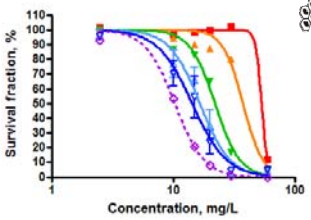
PROPICONAZOLE – 31 days of exposure

Fathead minnow

cell survival

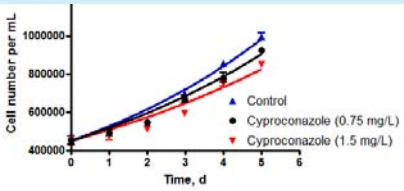
RTgill-W1





cell proliferation

RTgill-W1



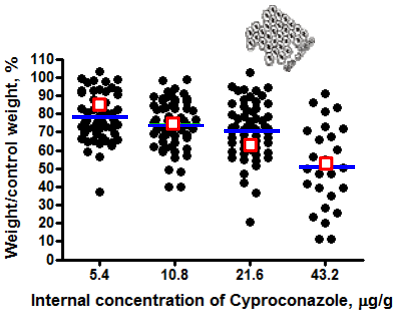
Predict impact on fish growth

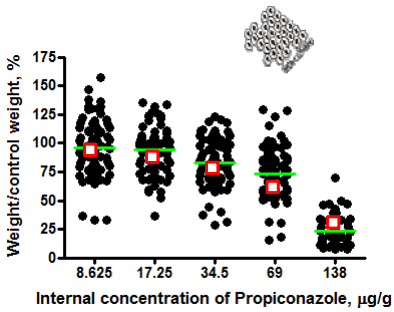
CYPROCONAZOLE – 62 days of exposure

Rainbow trout

PROPICONAZOLE – 31 days of exposure

Fathead minnow

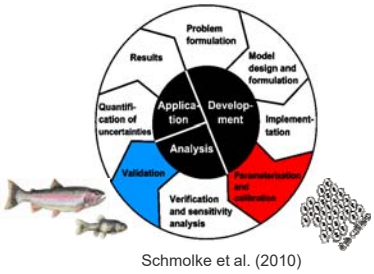




Stadnicka-Michalak et al., 2015, Science Advances, 1, 7

Cells in culture to predict impact on fish growth

- Fish cell line can quantitatively predict impact on growth
- Only in vitro data needed for model calibration
- More work needed to generalize this approach



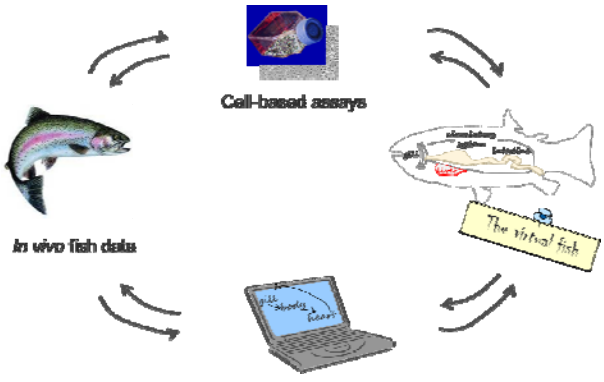
Schmolke et al. (2010)

→ 3R foundation Switzerland project:

http://www.forschung3r.ch/en/projects/pr_145_15.html

Fish cell culture to predict impact of chemicals to fish

- acute toxicity
- bioaccumulation
- impact on growth



Thank you!

Melanie Knöbel
Eawag

Katrin Tanneberger, Eawag, now EcoSense (CH)

Frederik Weiss
Eawag/ETH

Julita Stadnicka
Eawag/EPFL

Roman Ashauer
Eawag, now Uni York, UK

Funding Agencies

3R Foundation Switzerland

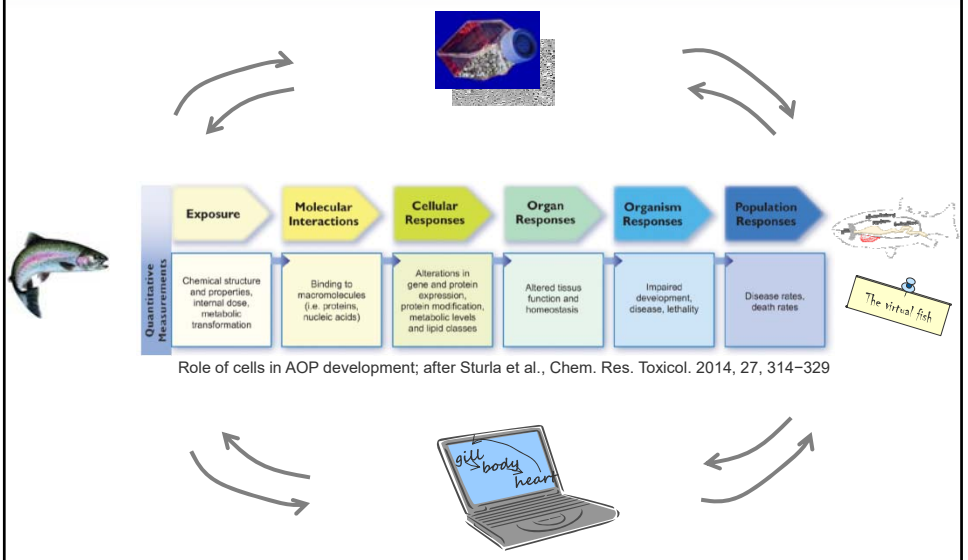
EU Marie Curie Actions

Niels Bols
University of Waterloo

Lucy Lee
University of the Fraser Valley

…and all partners in round-robin study

Fish cell culture to predict impact of chemicals to fish



Role of cells in AOP development; after Sturla et al., Chem. Res. Toxicol. 2014, 27, 314−329

FET interspecies differences and AOP/IATA development for acute aquatic toxicity

Ioanna Katsiadaki and Philipp Antczak

3rd May 2017
ECHA, Helsinki

Centre for Environment Fisheries & Aquaculture Science

World Class Science for the Marine and Freshwater Environment

Cefas

The team

defra

Me

- MRCVS
- Research into fish physiology (not acute toxicity)
- Stickleback queen (spiggin)
- OECD GD 148 (AFSS)
- TG230; TG234
- FET drafting
- Reviewed AOPs (HPG) for OECD
- SETAC Pellston workshops (2016, RvH; 2017, AOPs)

Colleagues, Collaborators and Students

- Cefas, UK: Marion Sebire, Tim Bean
- NIES, Japan: Haruna Watanabe
- University of Birmingham, UK: Tim Williams
- University of Liverpool: Philipp Antczak
- Bournemouth University, UK: David Hartnell
- University of Messina, Italy: Maria Maisano

Cefas

National Institute for Environmental Studies, Japan

University of Birmingham

University of Liverpool

Bournemouth University

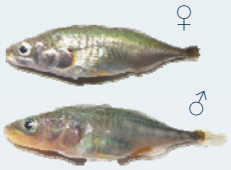
Centre for Environment Fisheries & Aquaculture Science

Cefas

The stickleback FET: species characteristics

- Widespread worldwide (indigenous and sentinel fish in UK)
- Annual reproductive cycle (April-July)-similar to 95% fish species
- Unique biomarker for androgenic xenobiotics (Spiggin)

- Spiggin induction in females detect androgenic action of chemicals (Katsiadaki et al, 2002)
- Spiggin inhibition in the Androgenised female stickleback (AFSS; OECD guidance document 148) can detect anti-androgenic action

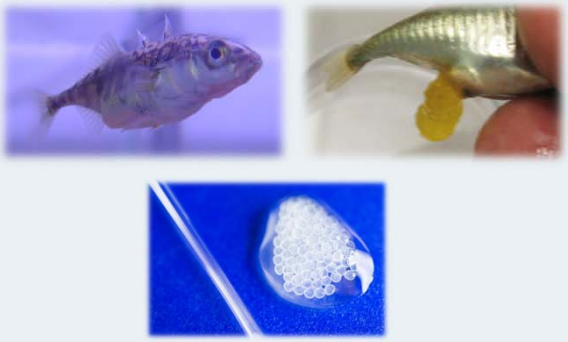


- Substantial molecular, physiological, ecological and behavioural resources available
- High quality, annotated genome sequence is available.

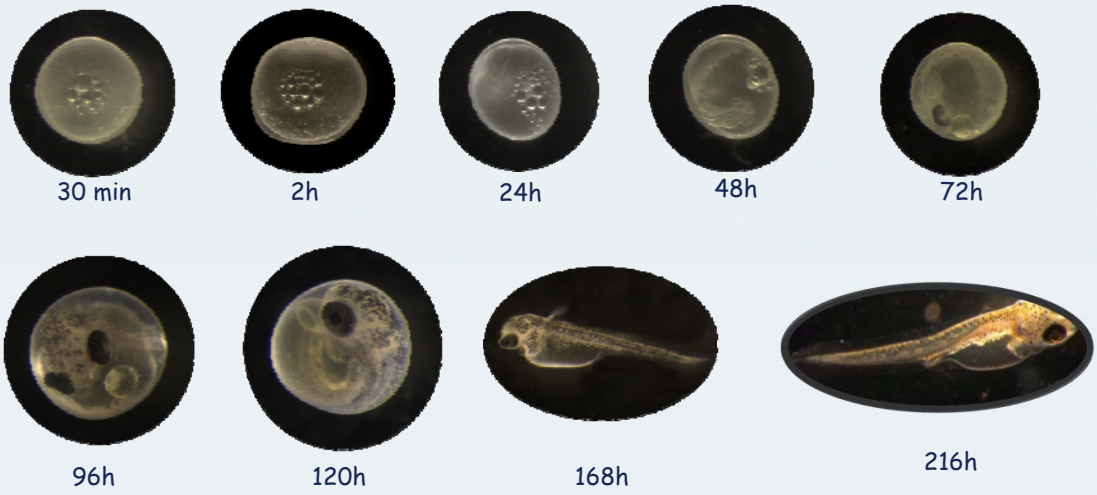
The stickleback FET: Collection of gametes

Natural spawning

In vitro fertilisation



Stickleback development stages at 17-18°C



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Summary of similarities and differences between sFET and zFET

Test species	 Three-spined stickleback	 Zebrafish
Fertilisation	In vitro fertilisation	Natural spawning (in tank)
Test temperature (°C)	17.5±1	26±1
Test duration	9 dpf (2 dph)	4 dpf (1 dph)
Age of test fish: 16-cell stage	<3.75 h after fertilisation	< 1.5 h after fertilisation
Test design	1 egg/well In 24-well plates: 20 eggs/treatment + 4 eggs water control (TG236)	
Exposure type	Semi-static (every 48h)	Static or Semi-static
Water control	Reconstituted water (OECD TG203, hardness: 250 CaCO3mg/L)	
Embryo sex determination	Feasible	Not feasible

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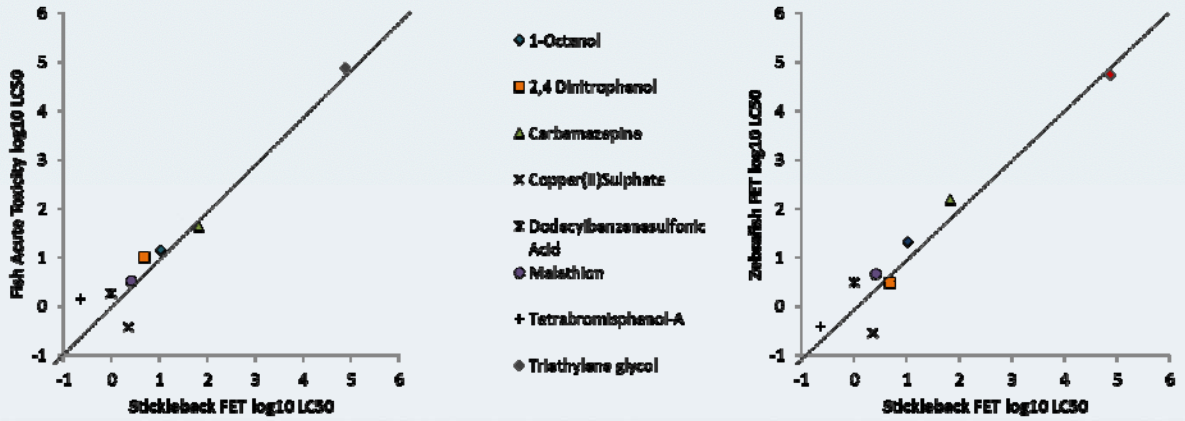
Cefas

Comparative responses: sFET & TG203 and sFET & zFET



Enhanced FET test

FET test

- Survival
- Hatching
- Morphological effects

Behaviour analysis

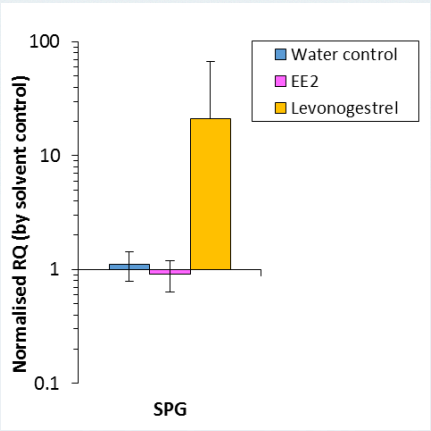
Molecular assays

- DNA damage
- Transgenic animals
- Methylation marks
- Gene expression
- HTP Sequencing
- Metabolomics

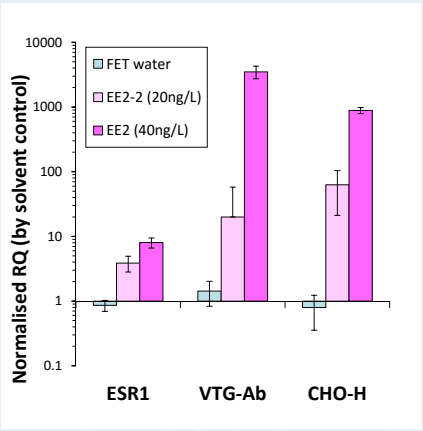
Alternative platform not only for AFT but also developmental toxicity, teratogenicity, genotoxicity, endocrine disruption, and more….

Diagnostic genes for EDCs are responsive in sFET (qPCR)

EE_2 at 20 ng/L and Levonorgestrel at 50 ng/L



EE_2 at 20 ng/L and at 40 ng/L



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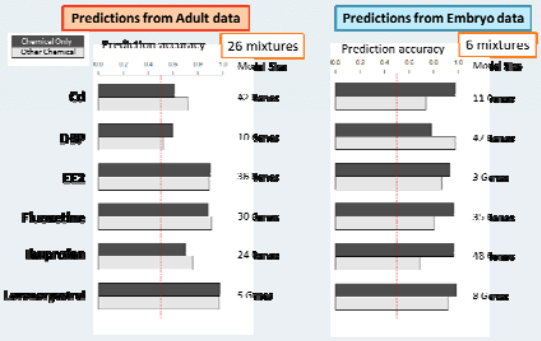
Comparative predictive responses between adult and embryo omics

Single chemicals

Chemical	Adult		Embryo	
	Size	Genes	Size	Genes
Cd	2	ARG2, CD207	3	NOP56, APCS, CHKA
DBP	2	SUPT5H, WSB1	9	VMA1, N4BP1, ZBTB1, CTNNB2, PIP5K2A, SSR1, TTC23L, EFR3A
EE2	2	FAM20C1, VTG2	12	VTG2, VTG3, TWISTNB, CTSE, CYP3A43, CCT2, CCT6A, ASRGL, ABHD14B, GALNTL1, ZP4, TIMM8A
Fluoxetine	2	HGF, PPM1H	3	HGF, SFRS2IP, HYOU1
Ibuprofen	2	ASPDH, CCDC47	9	FES, LPIN3, NOP56, ECE1, KIF15, CXorf41, C1orf51, METTL10
Levo	3	SLC13A3, ESR1, SULT1A3	2	SLC13A3, DDC

Mixtures



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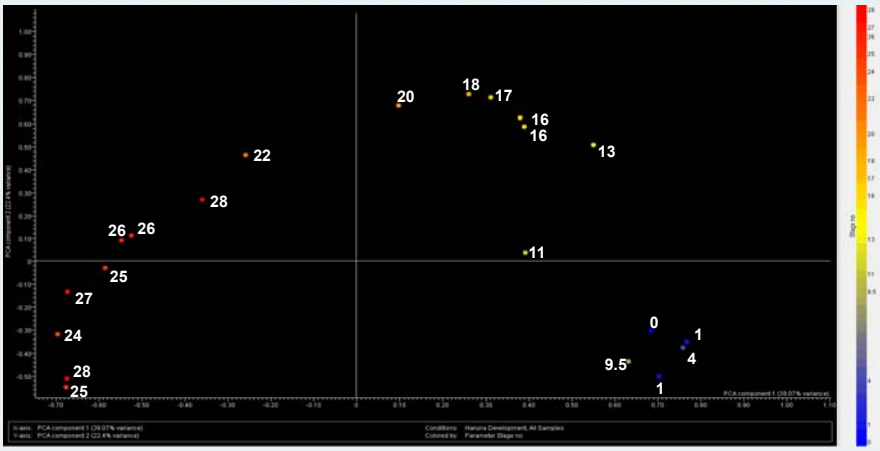
Gene expression changes during embryonic development



PCA all genes, all samples, annotated with development stage

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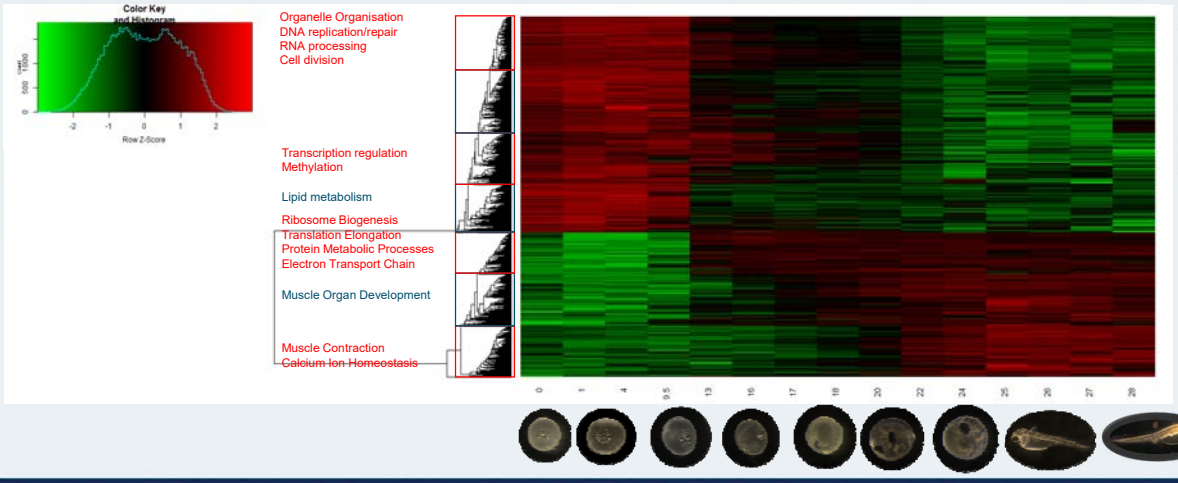
KEGG pathways during stickleback embryonic development



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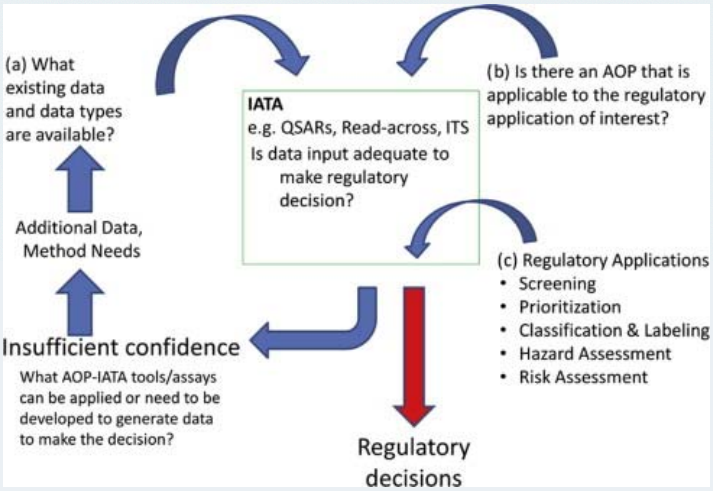
Adverse outcome pathways and IATA



Information and knowledge organisation and management

Tollefsen et al, 2014. Regul Toxicol Pharmacol. 70(3):629-40. doi: 10.1016/j.yrtph.2014.09.009.

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Developing the Ecotoxicological – Predictiv Information – Connectivity Map (EPIC-map)

Dr. Philipp Antczak – University of Liverpool

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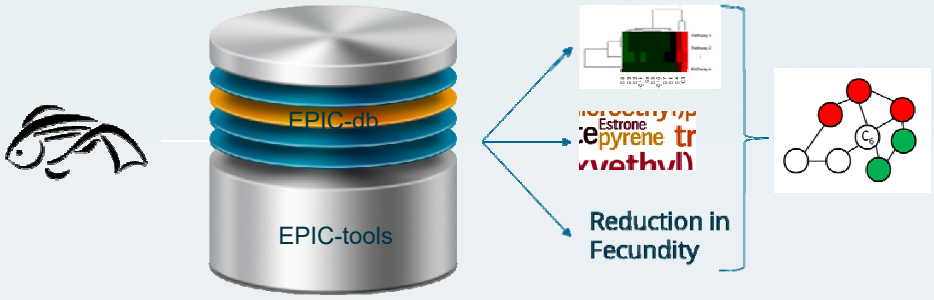
EPIC-map to accelerate AOP development

- Integrate concepts in computational biology to develop Adverse Outcome Pathways
- Provide a platform for the wider community to engage with these concepts



EPIC-map

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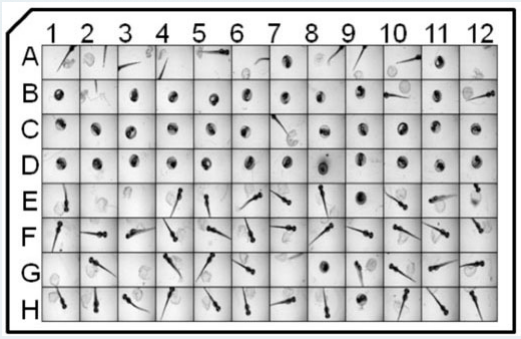
Whole animal bioassay (zFET)



Underlying Data in EPIC

- TG236 compatible data
- Whole animal exposure
- Full Genome molecular response at multiple concentrations

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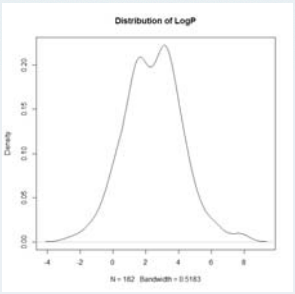
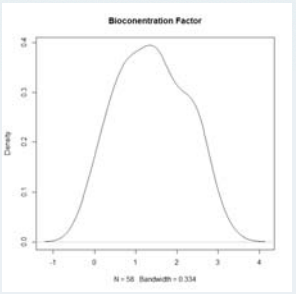
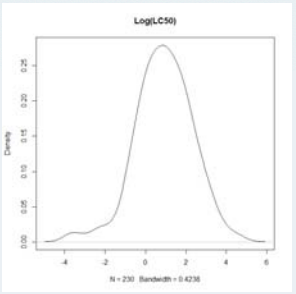
Cefas Cefas

Selection of Compounds

200 compounds – all of environmental concern/emerging contaminants







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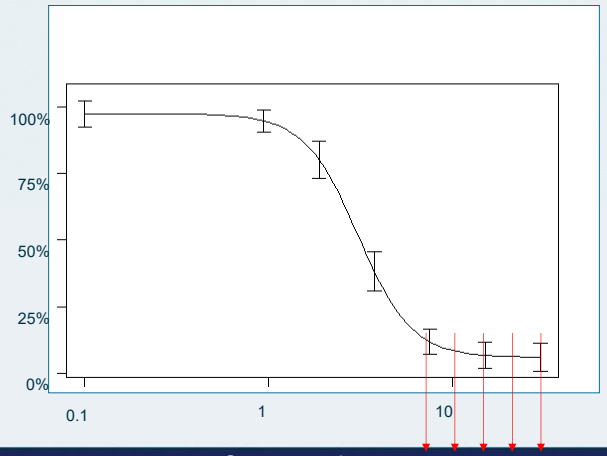
Assessment of Compounds



Concentration

Transcriptomics

Mortality
Heart Rate
Developmental Delay
Hatching Rate
Phenotypic Changes
Reaction to stimuli
etc.

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EPIC summary thus far

- Used Fathead Minnow predicted LC50 as a guide
  - For ~60% of the compounds this was spot on
  - For ~8 compounds it seems the predicted value was too high
    - Methyl Carbamate, o-Phenylenediamine, 1-Butanol, Michler's ketone, Retinoic Acid, Benzophenone, Propachlor, Propoxur
  - For the remaining 30% compounds the predicted LC50 was too low but in most cases multiplying the LC50 by 50-100 was sufficient to observe acute toxicity.
    - Few compounds could not be tested (~10-15) due to solubility issues.
- All information about the project and results will be made available on epic.liverpool.ac.uk in due course.

Summary (sFET versus zFET)

- Longer test: 9 versus 4 days

- Only 7 months (March to August); sticklebacks have annual reproduction like 95% of fish species

- Full control of fertilisation time (IVF)

- Genetic sex determination by PCR

- Fresh, brackish and sea water

- Temperature relevant to most aquatic bodies (10-20°C); no heating of effluent/chemicals required!

Summary (personal thoughts on FET application)

- The FET does not give identical results to AFT but data are too similar to be dismissed as not a good alternative
- Not all genes and pathways are active at embryonic stages, neither static in their expression highlighting the importance of prior knowledge on expression patterns during development
- Efforts should be placed into understanding the principles governing the difference in responses and manage them under AOP frameworks
- Fish embryo OMICs data demonstrate a high potential as screening tool for detecting specific modes of action (e.g. EDCs)
- Fish embryos are at least as important ecologically as adult fish; FET data along algae and daphnia toxicity data could be highly informative and significantly reduce the need for AFT testing

Outcome of ECHA Study:

Analysis of the relevance and adequateness of using the Fish Embryo Acute Toxicity (FET) Test Guideline (OECD 236) to fulfil the information requirements and addressing concerns under REACH

Stefan Scholz (UFZ Leipzig) &
Marta Sobanska (ECHA)

3-4 May 2017, FET WS

Disclaimer

The content of this presentation reflects the views of the authors and not necessarily the position of ECHA or the Helmholtz Centre for Environmental Research - UFZ

OECD guideline 236: Fish Embryo Acute Aquatic Toxicity (FET) Test

- 96-h exposure
- E.g. 24-well plates (glass or polysterene, presaturation)
- Semi-static (24-h renewal) or flow-through
- pH, O_2 control, hardness, conductivity
- 26° C
- If solvents used: maximum 0.01 % (v/v)
- Mortality in conctrols ≤ 10 %
- Positive control: 3,4-dichloroaniline (4 mg/L)
- 20 embryos per concentrations, dilution 2.2x
- 1 embryo/2 ml
- Exposure start: at latest by the 16-cell stage
- Analytical chemistry recommended

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ECHA project

The ECHA project conducted in 2015 by the UFZ as an external contractor for consultancy service.

Aims:

gathering, comparing and analysis of the available data to:

- ✓ determine boundaries and limitations of the test,
- ✓ suggest applicability domain in terms of chemical structure and physico-chemical characteristics (molecular size, lipophilicity, polarity and others), and regarding metabolism, bioavailability, reactivity,
- ✓ assess impact of complex compositions (UVCBs, multi-constituents, complex reaction products) on the test performance and results.

Initial findings:

The majority of the FET studies available in the literature are of various quality, non-guideline studies conducted before the OECD TG 236 was developed

- ✓ complicated selection of the relevant data points for further analysis.

Obtaining a high quality, reliable FET database! - **crucial** for ECHA

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Retrospective data analysis

- Update of an existing and published FET database (Scholz et al. 2014)
- Identification of corresponding acute fish toxicity data and their variability
- Application of quality criteria
- Correlation and enrichment analysis
- Identification of potential limiting factors

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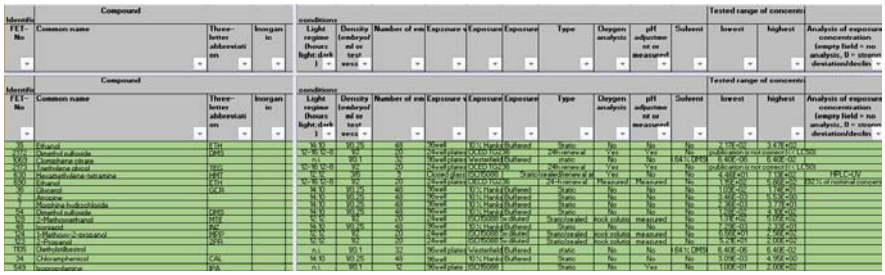
Updated database (~July 2015)

- 2065 study entries = 1415 chemicals
- Detailed documentation of each study (> 60 columns with compound information, protocol used, results, etc.)



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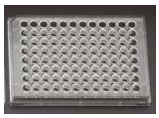
Issues with available FET data

1. Heterogeneous protocols

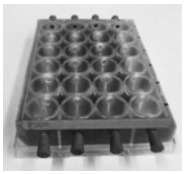


100-2000 µl per embryo

0-8 24 48 72 96 120 144 hpf

Static
Semi-static
Flow-through



Lammer et al. 2009

25-28.5 ° C

Buffered/non-buffered exposure medium

Issues with available FET data

2. Quality limitations

E.g.

- Exposure concentrations rarely confirmed
- Static exposure
- Water quality parameters not recorded
- Water solubility ignored
- pH, O_2 not controlled/measured
- Inappropriate exposure range (below baseline acute fish toxicity)

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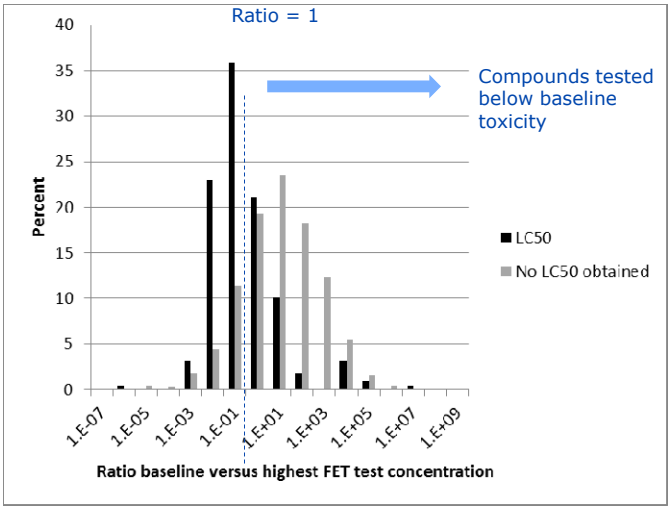
Examples for quality limitations

Inappropriate concentration range (≤ baseline toxicity)



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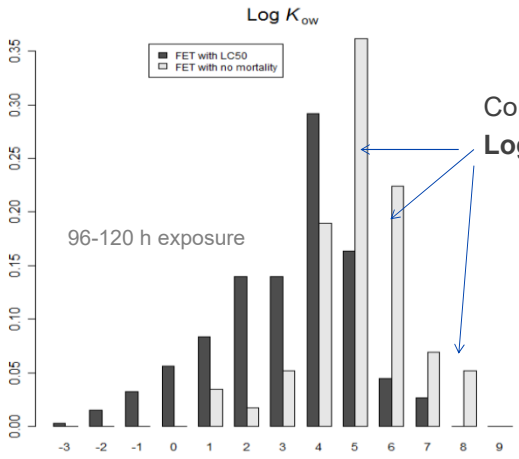
Examples for quality limitations

Inappropriate exposure protocols for hydrophobic compounds



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Results

Data set

Initial FET data set
2065 study entries
1415 chemicals

Quality filters

Final dataset for comparative analysis
156 study entries
123 chemicals

Filters:

- Organic compounds (not enough data for other groups)
- ≥ 96 h exposure
- Water solubility
- Tested at ~neutral pH
- Low control variability
- Baseline toxicity included for non-toxic compounds
- Log K_{ow} ≤ 4 and log K_{aw} <-4 (if no chemical analytics)
- Corresponding 96 h acute fish toxicity data available (Rainbow trout, fathead minnow, bluegill, zebrafish)

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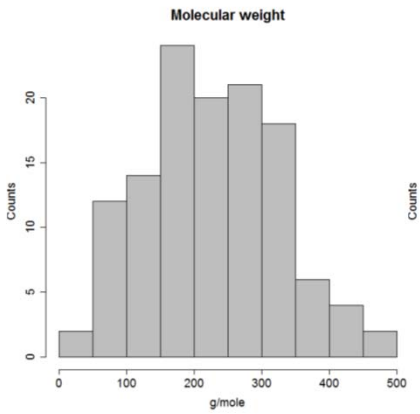
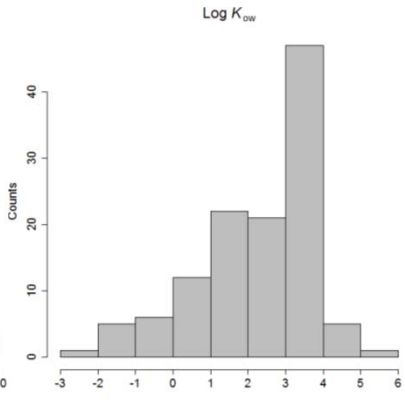
Distribution of physico-chemical characteristics in final FET dataset (examples)



n=123



n=120

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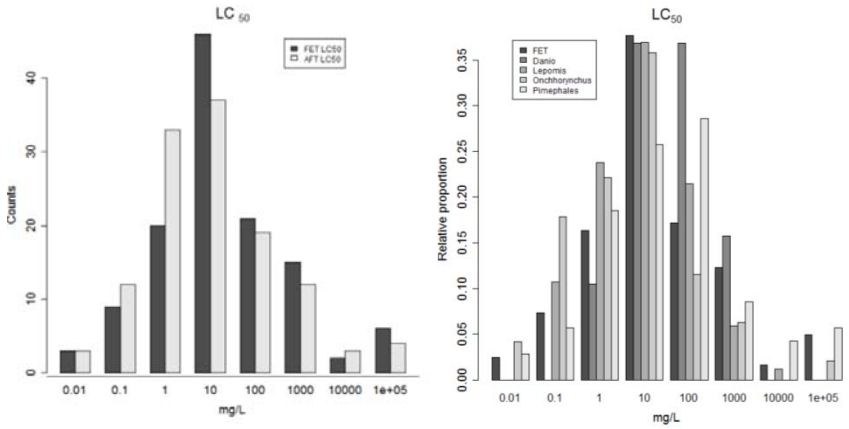
Distribution of toxicities



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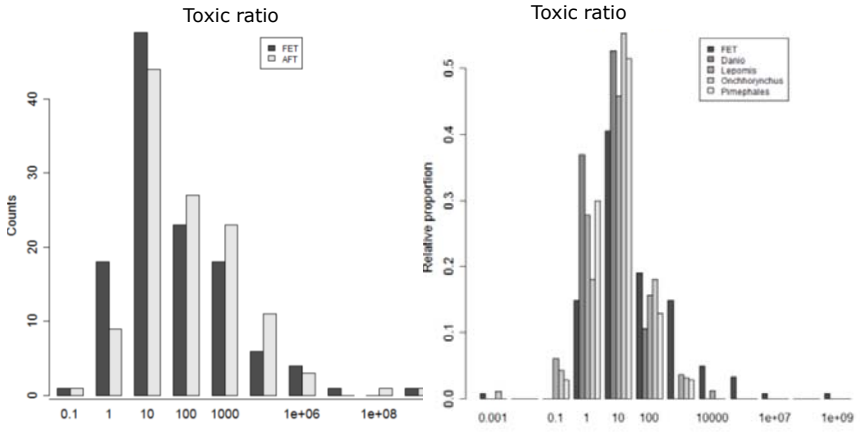
Distribution of toxic ratios



Toxic ratio = Baseline/LC_{50}

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Modes of action in final FET dataset

Mode of action	Number of compounds	Percent
Narcosis	47	38.0
Out of QSAR domain	28	23.0
Neurotoxicity	14	11.0
Mitochondrial electron transport inhibition/uncoupling of oxidative phosphorylation	11	8.9
Reactive	7	5.7
Other	6	4.9
Methemoglobin formation or protoporphyrinogen inhibition	4	3.3
COX inhibitor	2	1.6
Endocrine disruption	2	1.6
Extracellular matrix formation inhibition	2	1.6
Sum	**123**	**100**

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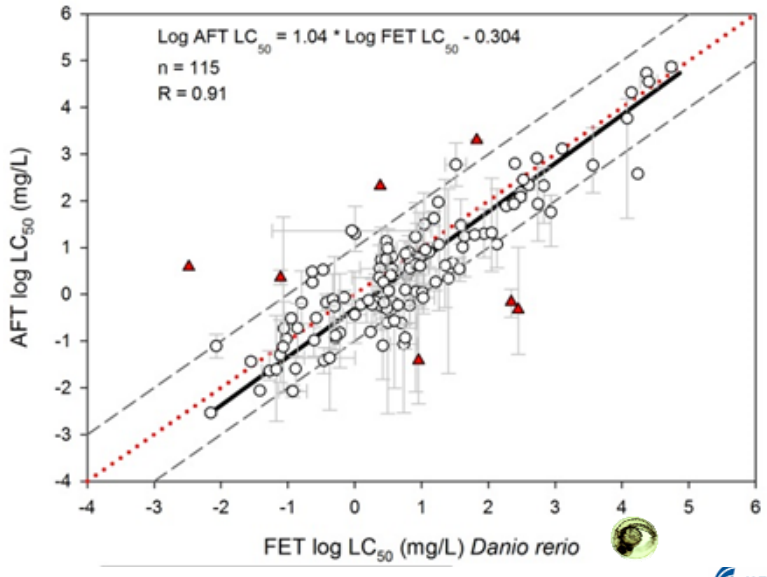
Correlation FET - AFT



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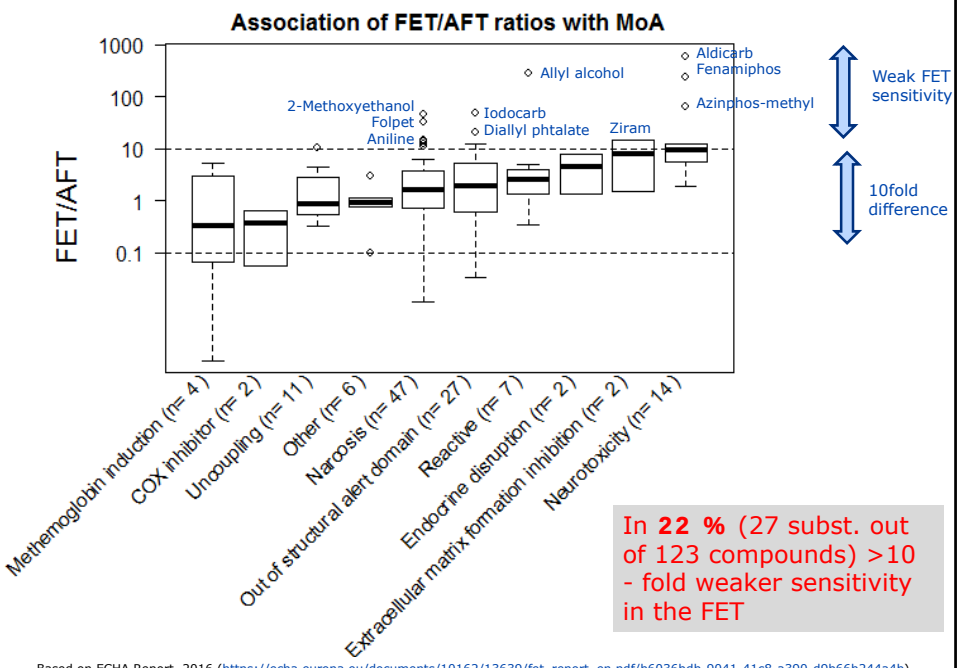

In **22 %** (27 subst. out of 123 compounds) >10 - fold weaker sensitivity in the FET

Based on ECHA Report, 2016 (https://echa.europa.eu/documents/10162/13639/fet_report_en.pdf/b6036bdb-9041-41c8-a390-d9b66b244a4b)

FET / AFT for different MoA

Percent distribution of modes of action in relation to the relative FET/AFT – categories of MoAs within 3 toxicity groups

Mode of action	FET/AFT		
	< 10 (n=95)	10-100 (n=23)	100 (n=4)
Out of structural alert domain	22.1	26.1	
Neurotoxicity	5.26	26.1	75.0
Reactive	6.32		25.0
Extracellular matrix formation inhibition	1.05	4.35	
Mitochondrial electron transport inhibition/uncoupling of oxidative phosphorylation	10.5	4.35	
Narcosis	40.0	39.1	
COX inhibitor	2.11		
Endocrine disruption	2.11		
Methemoglobin formation or Protoporphyrinogen synthesis inhibition	4.21		
Other	6.32		

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From the ECHA Report, 2016 (https://echa.europa.eu/documents/10162/13639/fet_report_en.pdf/b6036bdb-9041-41c8-a390-d9b66b244a4b)

Reasons for weaker sensitivity in the FET

MoA	Hypothesis
Neurotoxicity	Lack of key event „respiratory failure"
Allyl alcohol, 2-methoxyethanol (?)	Insufficient metabolic activation by ADH
Narcosis and out of structural alert domain	Unknown

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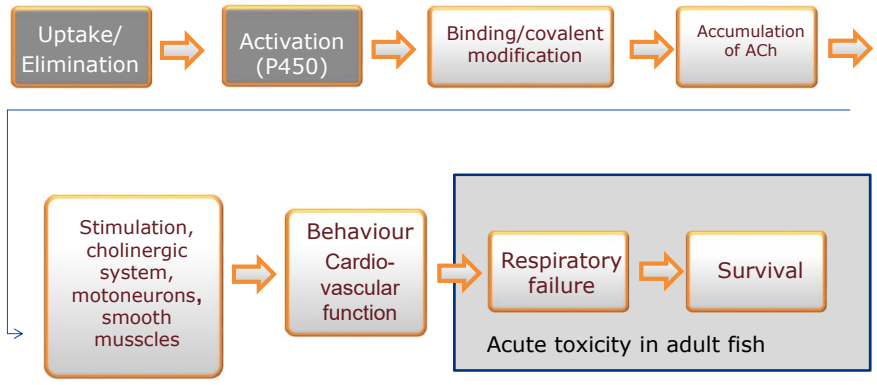
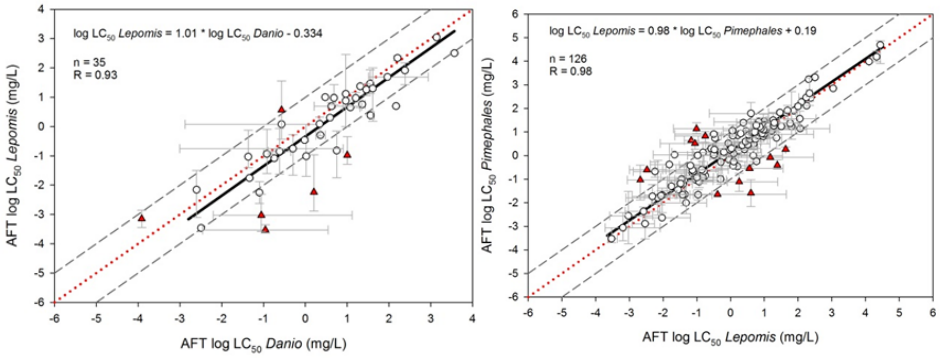
ACHE inhibition leading to respiratory failure and acutetoxicity in fish



Embryos are probably insensitive due to oxygen supply by diffusion

Simplified from Russom et al. 2014, Jacob et al. 2002, Rombough et al. 2002

Interspecies analysis of AFT



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Research needs and areas for further developments

- Better understanding of mechanistic differences among fish species which leads to different sensitivities.
- Mechanistic understanding for compounds with weaker FET toxicity.
- Improve sensitivity/predictivity of FET by additional endpoints (may require validation).
- Increase database (more high quality FET results using OECD 236).
- Chemical analytics as a key requirement for the FET (assuring that endpoints are referring to actual exposure concentrations).
- Guidance for difficult compounds should not be ignored.
- Explore the possibility of using FET within a weight of evidence or IATA.
- Study the consequences of using FET for classification and labelling (-> Daphnia and algae).

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Some final findings

- Lack of quality data makes it challenging to conclude on several aspects of the applicability domain of FET.
- OECD TG 236 protocol should be strictly followed and all deviations should be soundly justified.
- OECD TG 236 could be used as one of lines of evidence within the weight of evidence approach for organic substances. FET results should also be accompanied by information on the substance chemistry and other relevant additional evidence as well as other supporting information (for further information see Annex XI, 1.2 to the REACH Regulation).

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ECHA recommendations

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The reasons for ECHA analysis

- In OECD TG 236 no clear link to OECD 203.
- In JRC Recommendation on the ZFET for Acute Aquatic Toxicity Testing (2014):

*"**Where appropriate**, the ZFET (OECD TG236) should be used for generating information on acute fish toxicity."*

- The following potential limitations indicated:
  - ***metabolism*** - it is not clear whether the metabolic capacity of fish embryo is in the same range as that of adult fish,
  - ***possible barrier function of the chorion***,
  - ***high molecular weight*** (≥3kD) or ***bulky structure*** that may not pass the chorion and/or delay hatching; the reduced bioavailability over a full exposure time may also result in lower toxicity,
  - ***highly hydrophobic substance*** - potential for adsorption.

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OECD Validation study (1)

- ***exposure duration***: In validation study 48 vs. 96 h exposure was compared for 20 chemicals:
  - For 13 substances a slight increase of toxicity after 96 h of exp. was observed (up to 2 fold),
  - For 2 chemicals, due to high molecular weight, an impact of exp. duration has been concluded (see next slide),
  - For 3 substances the duration did not impact toxicity,
  - 1 substance (prochloraz) slightly less toxic at 96h,
  - 1 volatile substance - variable toxicity results (see next slide).
- ***stable test concentration***: It was also especially considered in validation study (e.g. all test vessels were pre-saturated and test solutions were daily renewed during test) - importance of analytical verification!

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OECD Validation study (2)

- ***chorion permeability***: In validation study for 2 out of 5 substances with molecular weight above 300 g/mol, no 48h-LC50 values could be derived (the barrier function of chorion).
- ***high volatility***: For 6-methyl-5-heptane-2-one due to high volatility a high variability in the results was observed in test between different laboratories.
- ***hydrophobicity***: Some problems with maintaining test concentration due to high log Kow,

  e.g. dibutyl maleate where the measured concentrations were significantly (30-40%) lower than nominal concentrations.

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Test conditions in adopted TG

- Based on recommendations from Validation Group:
  - ✓ exposure duration extended from 48 to 96hrs,
  - ✓ 20 embryos/concentration are to be used instead of 10 (to increase statistical power of the results),
  - ✓ a positive control included (96hrs exposure to 3,4-dichloroaniline should result in a minimum mortality of 30%),
  - ✓ test solutions/controls should be renewed on a daily basis (semi-static exposure),
  - ✓ predictive capacity promising but needs to be underpinned with additional data.
- Substance specific properties to be known before testing:

  structural formula, MW, stability in water/light, pKa, Kow, WS, VP, ready biodegrability, calculated Henry's law constant to account for losses due to evaporation.
- Exposure concentration to be verified throughout the test!

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Outcome of ECHA project

The analysis shown that there is not enough data to understand the potential limitations of the test and to properly establish its applicability domain in the regulatory context.

Applicability domain

- More information on applicability of FET for hydrophobic or volatile substances is needed.
- Assessment of the activation capacity of fish embryos would require additional experimental analyses – not enough information to conclude.
- Analytical verification of test concentration is very important!
- No conclusion on inorganic chemicals, multi-constituent or UVCBs could be derived at this stage due to the lack of data.

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FET / AFT comparison

- In **22 %** of the substances in the final dataset (27 subst. out of 123), the FET deviated >10 -fold from the AFT, producing weaker toxicity in fish embryos.

FET / AFT for different MoA

- The analysis of the distribution of the FET/AFT ratios among different MoAs confirmed the previous observation on a weaker toxicity in FET for neurotoxic compounds.
- It must be also noted, that a weaker toxicity in FET was also observed for narcotic compounds, mitochondrial electron transfer substances and for substances that could not be classified to any MoA.
- Therefore, except for the weak sensitivity to a neurotoxic mode of action - no other conclusion on the applicability domain regarding MoA could be drawn based on this study.

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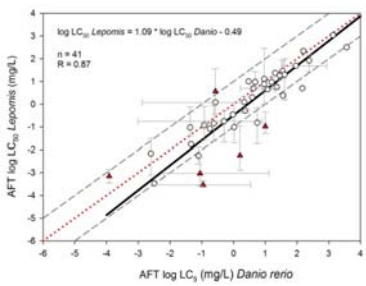
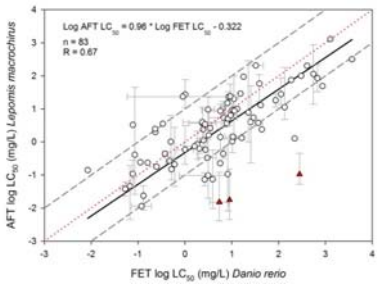
Interspecies analysis of AFT

- The comparison of the zebrafish FET with LC50s of different species did not indicate that the observation of a weaker toxicity in the FET was dependent on the species used in the AFT (FET showed weaker toxicity to all different fish species in AFT).
- A preliminary comparison of AFT data indicated that some variability was observed but a systematic analysis was hindered by the limited number of AFT data available for all four selected species.





Conclusions after the FET project

- It is still not clear how to deal with uncertainties raised from testing of fish embryos which could lead to different regulatory decisions (if FET is used).
- The predictive power of FET comparing to AFT not fully clear.
- A 'life-stage element' of the FET tests adds on uncertainty to the inter-species variability of AFT.
- As fish short-term tox test is an important element of REACH testing strategy, the use of less sensitive test such as e.g. FET may affect long-term testing, C&L and PBT assessment.
- For regulatory purpose, where the protection of human health and the environment is at stake, there is a need to consider not only overall correlations but also the absolute deviations of the FET result from AFT with reliable data in order to verify that the risks and hazards are not underestimated.

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Regulatory application of FET test

- In ECHA's opinion, the current available information demonstrates that the results of the TG 236 would usually not be sufficient alone to meet the information requirement of Annex VIII, 9.1.3.
- Based on current knowledge, ECHA considers that OECD TG 236 might be used within a weight of evidence approach together with other independent, adequate, relevant and reliable sources of information in order to conclude on the acute fish endpoint.

ECHA
EUROPEAN CHEMICALS AGENCY

Thank you!

ECHA
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RESEARCH – UFZ

"INDUSTRY'S VIEW ON THE USE OF THE FET TEST – Benefits & Challenges with regards to costs, practicability and acceptability"

Marc Léonard
Environmental Research Department

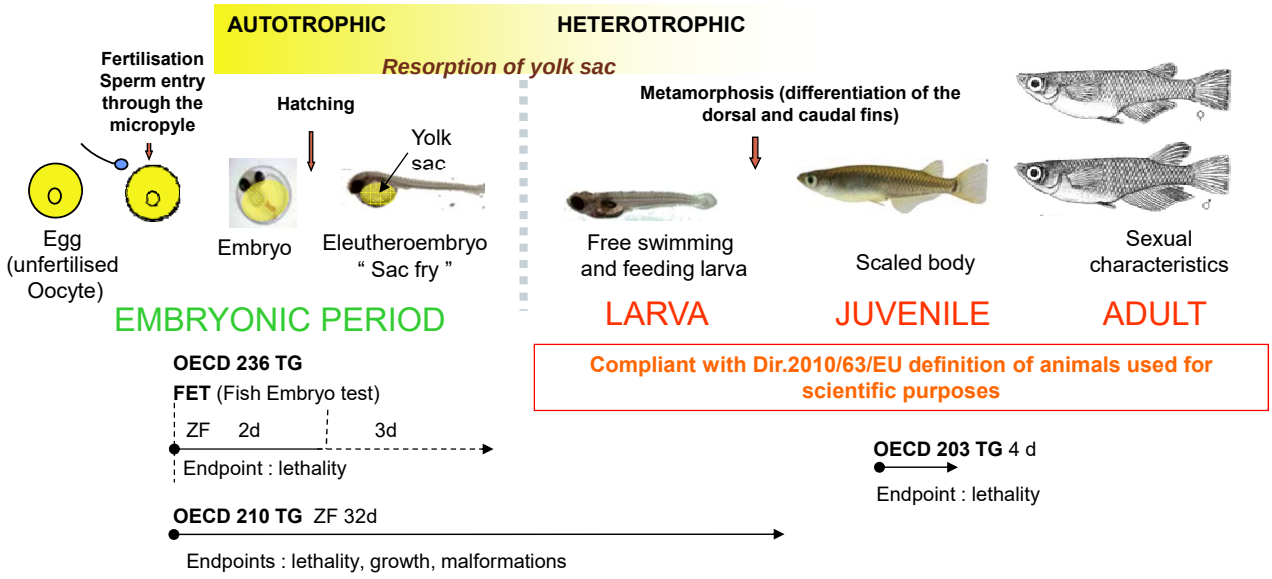
ECHA – UBA Workshop
3rd – 4th May 2017

INDUSTRY'S CONCERNS

PLAN

- Sharing societal concern for animal welfare
- Fulfilling regulatory requirements (chemicals ; effluents)
  - Environmental Hazard and Risk assessment (EC No 1907/2006)
  - Animal testing ban (Cosmetics - EC No 1223/2009)
- Screening for early detection of chemicals with poor environmental profiles
- Potential screening for Human toxicity prediction
  - Systemic toxicity
  - Developmental toxicity

FISH DEVELOPMENT STAGES – Concerns for welfare



OECD 236 TG

FET (Fish Embryo test)

ZF 2d 3d

Endpoint : lethality

OECD 210 TG ZF 32d

Endpoints : lethality, growth, malformations

OECD 203 TG 4 d

Endpoint : lethality

Compliant with Dir.2010/63/EU definition of animals used for scientific purposes

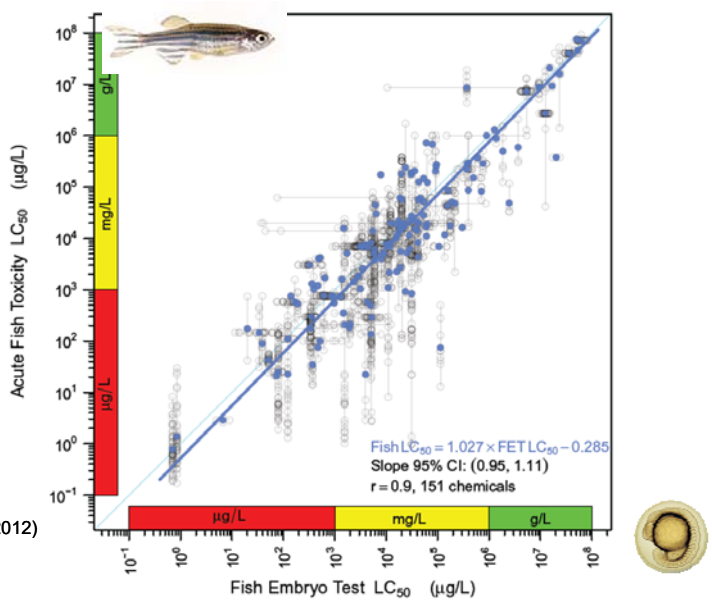
REGULATORY REQUIREMENTS
Environmental Hazard and Risk assessment

- Correlation between FET OECD 236 / OECD 203

Lammer *et al.* (2009),
Knobel *et al*. (2012)
Belanger *et al*. (2013)

Lethality endpoints

- Coagulation of the embryo
- Non-detachment of the tail
- Non-detection of heartbeat



r = 0.91 (n = 151)

S. Belanger & G. Carr (2012)

OECD 203 / OECD 236 Comparison

Few contract labs propose OECD 236

	OECD TG n° 203	OECD TG n° 236
Costs (GLP) - Standard Static : 5 conc. + control	3000 – 4000 €	~ 40 - 50 % more costly
Duration	96 h	120 h.
Work load:		
• Fish pre-culturing	12 days acclimation	Permanent fish breeding for the provision of eggs
• Preparation	More or less equal (~ 4 hours/test)	
• Application	~1 hour/test	Selection of eggs and transfer of test solutions to well plates: ~ 2 – 2.5 hours/test
• Daily evaluation (biology)	15 -30 min/test and day	~ 1 – 2.5 hours/test and day (depending on effects)
• Test termination	Depending on analytical samples etc., more or less equal ~1 - 4 hours	
• Data evaluation	1.5 – 2 hours	2.5 – 5 hours

L'ORÉAL Research & Innovation

SCREENING: FET for Early detection of chemicals with poor environmental profiles

- Acute fish toxicity
- Endocrine activity
- (Chronic fish toxicity prediction)

SCREENING Acute Fish Toxicity

FEET as sensitive as OECD 203

			L'OREAL Aulnay				INERIS	Safety Data Sheet	
			Médaka LC50 (mg/L)	Danio LC50 (mg/L)	Médaka LC50 (mg/L)	Danio LC50 (mg/L)	Danio OCDE 203 (4d)	OECD 203	Fish species
		Code	Egg (5 d)	Egg (2d)	Alevin (2d)	Alevin (2d)	LC50 (mg/L)	LC50 (mg/L	
QUATERNARY AMMONIUMS	Amphoteric polymer	QA1	> 100	~100	2,52	2,11	1,27	3,2	minnow
	Cationic polymer	QA2	> 100	~100	0,76	0,27	0,46	0,6	minnow
		QA3	> 100	100	1,58	1,29	0,55	0,56	
	Cationic	QA4	> 100	> 100	4,16	2,98	2,97	24,8	
		QA5	> 100	> 100	> 100	> 100	>100	> 1000	minnow
		QA6	> 100	5,54	1,21	1,31	0,99	0,7	leuciscus
		QA7	> 100	> 100	29,08	7,24	7,6	0,44	
		QA8	> 100	> 100	> 100	> 100	>100		
		QA9	> 100	> 100	> 100	> 100	>100	4,47	trout
		QA10	> 100	> 100	7,79	1,36	3,25	23,81	trout
SURFACTANTS	Non ionic	S1	> 100	107,5	97,88	>100	40,1		
		S2	9,69	17,50	9,18	4,26	3,8		
		S3	> 100	> 100	> 100	>100	> 100		
		S4	> 100	> 100	> 100	>100	> 100		
		S5	27,82	15,42	24,78	17,38	33,81		
	Cationic	S6	1,31	6,52	1,53	1,95	0,29		
		S7	2,69	1,43	1,46	1,47	1,88		
		S8	2,21	5,82	1,91	3,06	0,37		
	Amphoteric	S9	11,91	15,22	7,23	27,97	10,5		
		S10	7,84	9,32	9,57	7,57	6,34		
		S11	7,87	5,98	3,90	4,72	2,62		
	Anionic	S12	35,39	22,87	32,20	15,07	51,37		
		S13	10,70	< 5	8,78	4,72	18,79		
		S14	11,14	4,38	12,20	7,57	7,51		
		S15	8,62	10,07	7,36	8,98	6,82		

SCREENING Acute Fish Toxicity

Species differences

			L'OREAL Aulnay				INERIS	Safety Data Sheet	
			Médaka LC50 (mg/L)	Danio LC50 (mg/L)	Médaka LC50 (mg/L)	Danio LC50 (mg/L)	Danio OCDE 203 (4d)	OECD 203	Fish species
		Code	Egg (5d)	Egg (2d)	Alevin (2d)	Alevin (2d)	LC50 (mg/L)	LC50 (mg/L	
QUATERNARY AMMONIUMS	Amphoteric polymer	QA1	> 100	~100	2,52	2,11	1,27	3,2	minnow
	Cationic polymer	QA2	> 100	~100	0,76	0,27	0,46	0,6	minnow
		QA3	> 100	100	1,58	1,29	0,55	0,56	
	Cationic	QA4	> 100	> 100	4,16	2,98	2,97	24,8	
		QA5	> 100	> 100	> 100	> 100	>100	> 1000	minnow
		QA6	> 100	5,54	1,21	1,31	0,99	0,7	leuciscus
		QA7	> 100	> 100	29,08	7,24	7,6	0,44	
		QA8	> 100	> 100	> 100	> 100	>100		
		QA9	> 100	> 100	> 100	> 100	>100	4,47	trout
		QA10	> 100	> 100	7,79	1,36	3,25	23,81	trout
SURFACTANTS	Non ionic	S1	> 100	107,5	97,88	>100	40,1		
		S2	9,69	17,50	9,18	4,26	3,8		
		S3	> 100	> 100	> 100	>100	> 100		
		S4	> 100	> 100	> 100	>100	> 100		
		S5	27,82	15,42	24,78	17,38	33,81		
	Cationic	S6	1,31	6,52	1,53	1,95	0,29		
		S7	2,69	1,43	1,46	1,47	1,88		
		S8	2,21	5,82	1,91	3,06	0,37		
	Amphoteric	S9	11,91	15,22	7,23	27,97	10,5		
		S10	7,84	9,32	9,57	7,57	6,34		
		S11	7,87	5,98	3,90	4,72	2,62		
	Anionic	S12	35,39	22,87	32,20	15,07	51,37		
		S13	10,70	< 5	8,78	4,72	18,79		
		S14	11,14	4,38	12,20	7,57	7,51		
		S15	8,62	10,07	7,36	8,98	6,82		

SCREENING Acute Fish Toxicity

Thresholds
1 mg/L
10 mg/L

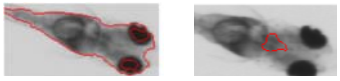
			L'OREAL Aulnay				INERIS	Safety Data Sheet		L'OREAL Aulnay	
			Médaka LC50 (mg/L)	Danio LC50 (mg/L)	Médaka LC50 (mg/L)	Danio LC50 (mg/L)	Danio OCDE 203 (4d)	OECD 203 LC50 (mg/L	Fish species	Daphnia EC50 (mg/L)	Algae IC50 (mg/L)
		Code	Egg (5 d)	Egg (2d)	Alevin (2d)	Alevin (2d)	LC50 (mg/L)			(2d)	(3d)
QUATERNARY AMMONIUMS	Amphoteric polymer	QA1	> 100	~100	2,52	2,11	1,27	3,2	minnow	0,34	0,10
	Cationic polymer	QA2	> 100	~100	0,76	0,27	0,46	0,6	minnow	0,16	0,08
		QA3	> 100	100	1,58	1,29	0,55	0,56		0,68	0,47
	Cationic	QA4	> 100	> 100	4,16	2,98	2,97	24,8		1,59	0,27
		QA5	> 100	> 100	> 100	> 100	>100	> 1000	minnow	> 100	> 200
		QA6	> 100	5,54	1,21	1,31	0,99	0,7	leuciscus	0,40	0,05
		QA7	> 100	> 100	29,08	7,24	7,6	0,44		68,85	0,19
		QA8	> 100	> 100	> 100	> 100	>100			>100	>200
		QA9	> 100	> 100	> 100	> 100	>100	4,47	trout	>100	0,25
		QA10	> 100	> 100	7,79	1,36	3,25	23,81	trout	30,60	0,10
SURFACTANTS	Non ionic	S1	> 100	107,5	97,88	>100	40,1			> 100	> 100
		S2	9,69	17,50	9,18	4,26	3,8			1,55	4,90
		S3	> 100	> 100	> 100	>100	> 100			>100	>100
		S4	> 100	> 100	> 100	>100	> 100			> 100	1,34
		S5	27,82	15,42	24,78	17,38	33,81			22,51	14,00
	Cationic	S6	1,31	6,52	1,53	1,95	0,29			0,06	0,08
		S7	2,69	1,43	1,46	1,47	1,88			0,08	0,01
		S8	2,21	5,82	1,91	3,06	0,37			0,21	0,20
	Amphoteric	S9	11,91	15,22	7,23	27,97	10,5			39,04	5,70
		S10	7,84	9,32	9,57	7,57	6,34			14,59	2,10
		S11	7,87	5,98	3,90	4,72	2,62			34,82	18,00
	Anionic	S12	35,39	22,87	32,20	15,07	51,37			10,18	25,50
		S13	10,70	< 5	8,78	4,72	18,79			6,66	11,81
		S14	11,14	4,38	12,20	7,57	7,51			9,43	41,00
		S15	8,62	10,07	7,36	8,98	6,82			9,86	> 100

SCREENING Acute Fish Toxicity FEET (post hatch)

- Automated assessment of cardiac arrest

Localization of the embryo

Segmentation of the inner parts



Motion detection

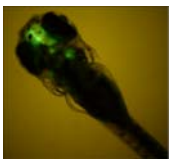
Puybareau *et al.* « An automated assay for the assessment of cardiac arrest in fish embryo »
Computers in Biology and Medicine 81 (2017) 32–44

SCREENING — *Endocrine modulation*

Sexual steroids axis (estrogens - androgens)

- **<u>Médaka</u>** – **Choriogénine H** (ChgH-GFP) – FEET (2d)
  - WATCHFROG (France)
    Oryzias latipes -
  - VITARGENT (Hong Kong)
    Oryzias melastigma

- **<u>Zebrafish</u> Cyp19a1b - Aromatase** – FET (5d)
  - INERIS
    OECD Phase II intercalibration



Brion et al. 2012 PLoS ONE
May | Volume 7 | Issue 5 |

SCREENING: — *Mamalian toxicity*

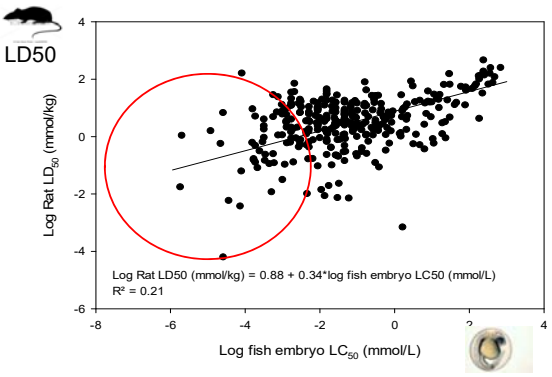
- Systemic toxicity
- (Developmental toxicity)

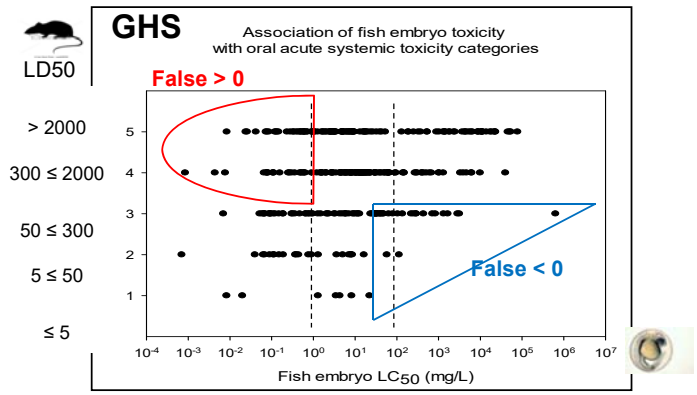
FET LC50 Extrapolation to Mammalian acute toxicity ?

HELMHOLTZ CENTRE FOR ENVIRONMENTAL RESEARCH – UFZ

n= 364 chemicals

Correlation of fish embryo LC_{50} and rat LD_{50}





Scholz *et al.* (2014)

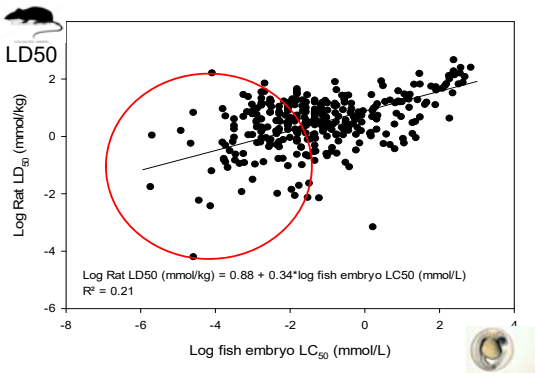
Poor prediction of the most acutely toxic chemicals in rodents

L'ORÉAL Research & Innovation

DON'T THROW OUT THE FISH EMBRYO WITH THE BATH WATER ! LET'S FACE THE OUTLIERS

Correlation of fish embryo LC_{50} and rat LD_{50}

n= 364 chemicals



1- internal concentrations unknown

L'ORÉAL Research & Innovation

DON'T THROW OUT THE FISH EMBRYO WITH THE BATH WATER ! LET'S FACE THE OUTLIERS

Correlation of fish embryo LC_{50} and rat LD_{50}

n= 364 chemicals

LD50

1- internal concentrations unknown ?
2- Species physiologic differences ?

L'ORÉAL Research & Innovation

Chemicals classified H400 or H410

LD50

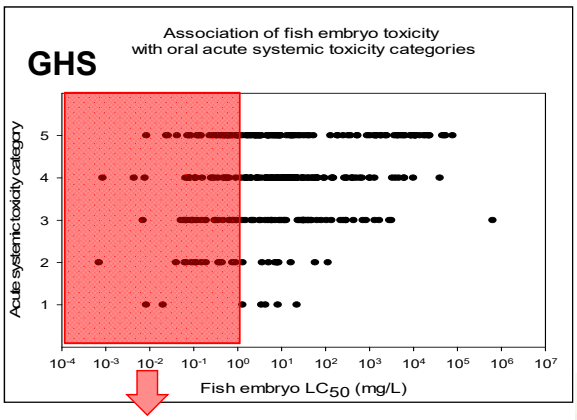
> 2000 mg/Kg

300 ≤ 2000 mg/kg

50 ≤ 300 mg/kg

5 ≤ 50 mg/kg

≤ 5 mg/kg



LC50 < 1 mg/L
H400
&
(H410 , H411)

Calculated Risk for the environment ?

= Cost of
additional testing and monitoring

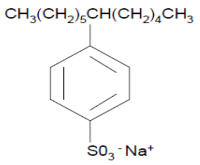
L'ORÉAL Research & Innovation

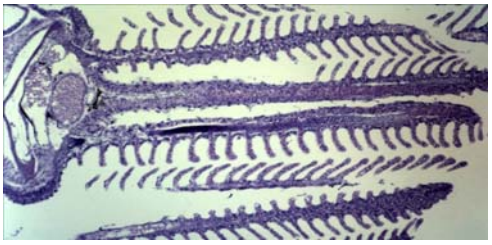
TOXICOKINETICS - INTERNAL CONCENTRATIONS

Nom INCI	CAS	Medaka FEET CL50 (mg/L)	Rodent PO DL50 (mg/Kg)
COCO-BETAINE	68424-94-2	9,57	> 2000
TEA-LAURYL SULFATE	139-96-8	7,36	> 2000
LAURETH-11 CARBOXYLIC ACID	27306-90-7	32,2	> 2000
LAURETH-5 CARBOXYLIC ACID	21127-45-7	8,78	> 2000
SODIUM LAURETH SULFATE	3088-31-1	12,2	4100
LAURETH-12	3056-00-6	9,18	1000
POLYGLYCERYL-3 HYDROXY-LAURYL ETHER	158112-80-2	24,78	> 3000
OLETH-30	9004-98-2	97,88	> 2000
POLYSORBATE 20	9005-64-5	> 100	> 2000
POLYGLYCERYL-2 OLEYL ETHER	71032-90-1	> 100	> 2000

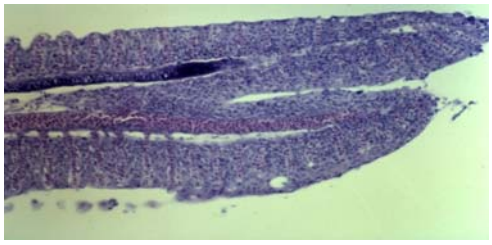
Gill damage induced toxicity / Extrapolation to mammals ?

- LAS - linear alkylbenzene sulfonates - Anionic surfactant - mixture (C9-C14)
- Rat oral acute : LD50 > 500 – 2,000 mg/Kg – GHS cat. 4 - Irritating (eye & skin)
- Fish : LC50 96h: 1,67 mg/L (Echa)





Courtesy of Scott Belanger (P&G)



- Metaplasia
- Fusion of lamellae
- Apoptopic bodies

Consistent with respiratory failure

INTERNAL CONCENTRATIONS Microinjection

KATHOLIEKE UNIVERSITEIT LEUVEN

Laboratory for Molecular Biodiscovery.

Toxicity Endpoints

- Edema
- Impaired circulation
- Change in heart rate
- Hemorrhage
- Loss of posture
- Impaired motility
- Swim bladder defects

Nom INCI	CAS	Medaka FEET CL50 (mg/L)	Rodent PO DL50 (mg/Kg)	Zebrafish eleutheroembryo 0,5 nL IV injection			
				200 mg/ml 400 mg / kg 48h	100 mg/ml 200 mg / kg 48h	50 mg/ml 100 mg / kg 48h	25 mg/ml 50 mg/kg 48h
COCO-BETAINE	68424-94-2	9,57	> 2000	Tox Leth	N	N	N
TEA-LAURYL SULFATE	139-96-8	7,36	> 2000	Tox	Tox	N	N
LAURETH-11 CARBOXYLIC ACID	27306-90-7	32,2	> 2000	Tox Leth	Tox	N	N
LAURETH-5 CARBOXYLIC ACID	21127-45-7	8,78	> 2000	Tox Leth	Tox	N	N
SODIUM LAURETH SULFATE	3088-31-1	12,2	4100	N	N	N	N
LAURETH-12	3056-00-6	9,18	1000	Leth	N	N	N
POLYGLYCERYL-3 HYDROXY-LAURYL ETHER	158112-80-2	24,78	> 3000	N	N	N	N
OLETH-30	9004-98-2	97,88	> 2000		N	N	N
POLYSORBATE 20	9005-64-5	> 100	> 2000	N	N	N	N
POLYGLYCERYL-2 OLEYL ETHER	71032-90-1	> 100	> 2000			N	N

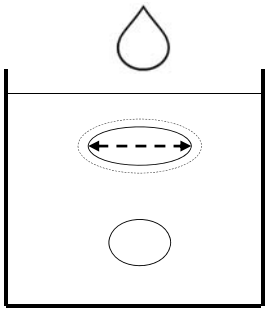
N = symptoms < 20% **Tox =** 20% < symptoms < 90% **Leth =** lethality > 90%

CONTROL OF THE INJECTED VOLUME

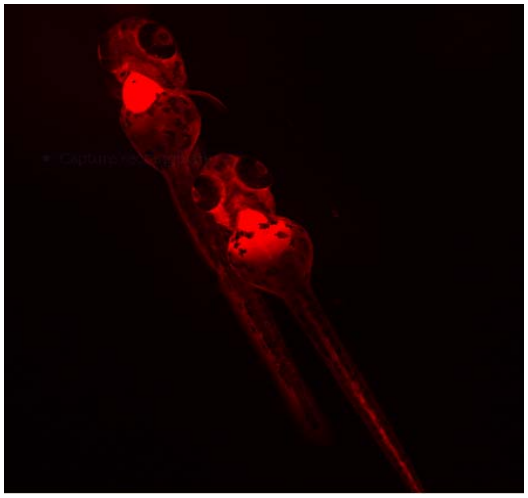
- 0,5 nL in Zebrafish Eleutheroembryo

Mineral oil bath

1 nano L = Diameter 0.125 mm (+/- 20%)



Injection bolus to 0.1mm = 0,5 nanoL



CONCLUSIONS / PERSPECTIVES

- OECD 236 requested to suppliers of cosmetic ingredients in place of OECD 203
- FET or FEET adapted to HT ecotoxicity screening
- Development of fish embryo models for parallel ecological and Mammalian toxicity screening - to be continued

AKNOWLEDGMENTS

L'ORÉAL Research & Innovation
Environmental Research Dept.
M.VANPOUCKE
JP. FEL
N.de CROZE
D. GENEST
JC.CHENEBLE

HELMHOLTZ CENTRE FOR ENVIRONMENTAL RESEARCH – UFZ
S. SCHOLZ
N. KLUVER

ESIEE PARIS
H. TALBOT
E. PUYBAREAU

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INERIS
JM. PORCHER
F. BRION
E. THYBAUD

P&G
S. BELANGER

BASF The Chemical Company
E. SALINAS

HESI
M. EMBRY

eurofins agroscience services
J. BAUMANN


L'ORÉAL
Recherche & Innovation

umweltbundesamt

uncertainties of reference-data

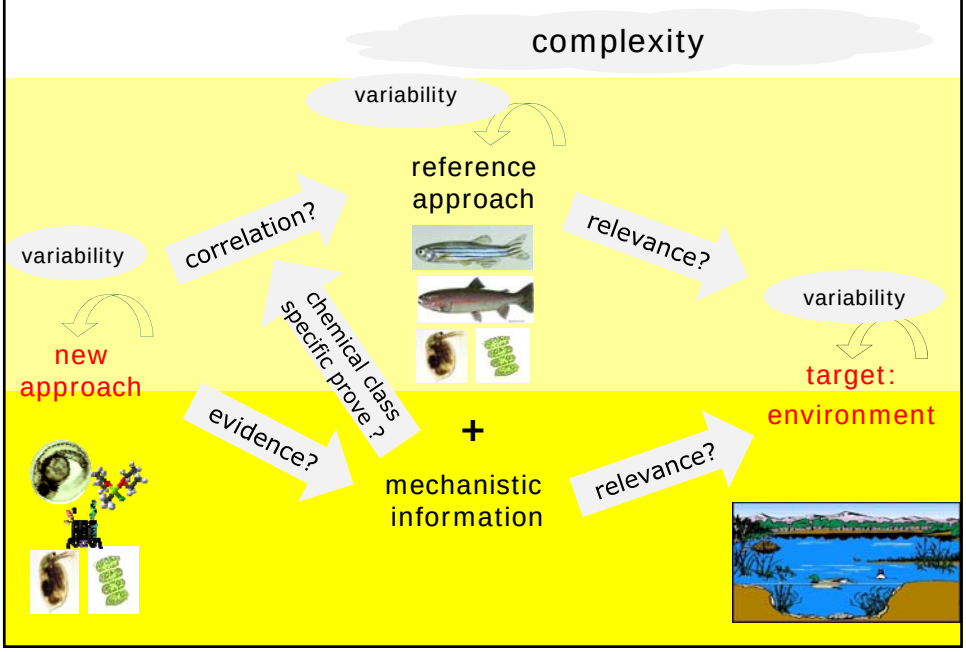
and

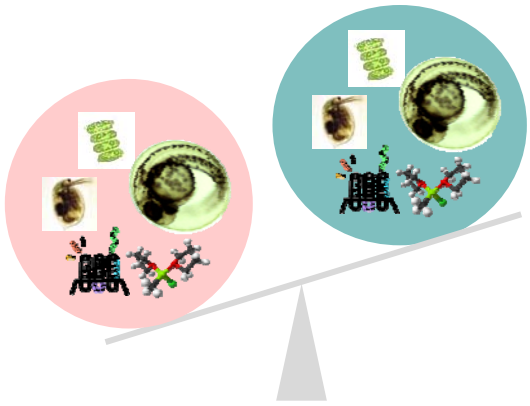
what they mean for the validation of alternative approaches in ecotoxicology

Martin Paparella
Environment Agency Austria

Helsinki, May 3, 2017

validation





consider hazard assessment as comparative

requires ↓variability in testing & assessment

↓exposure, change market wo predicting safety precisely

ANNEX

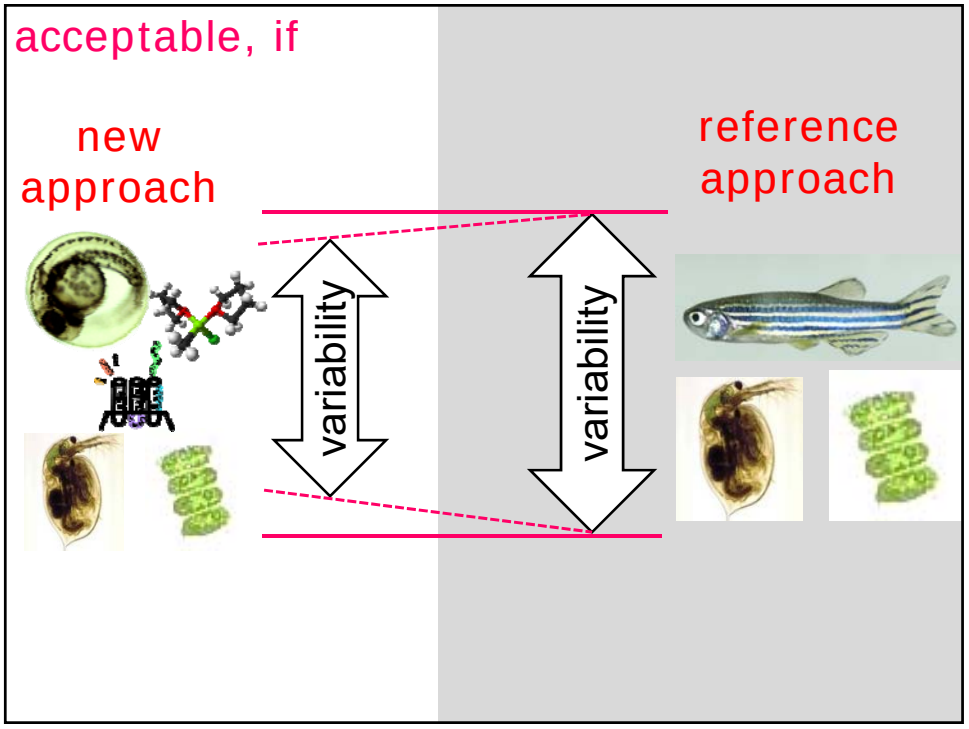
uncertainties of reference-data

and

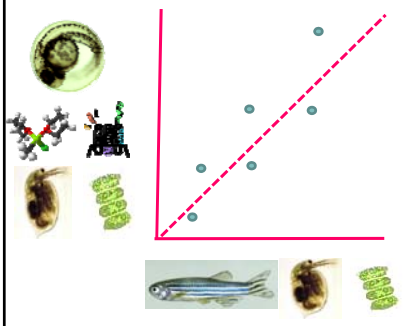
what they mean for the validation of alternative approaches in ecotoxicology

Martin Paparella
Environment Agency Austria

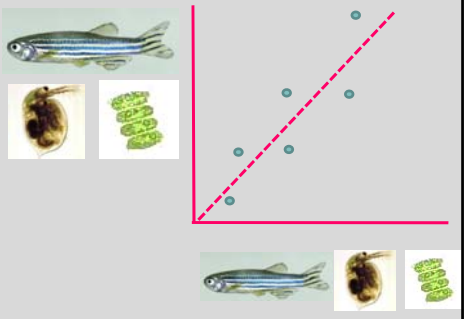
Helsinki, May 3, 2017



acceptable, if

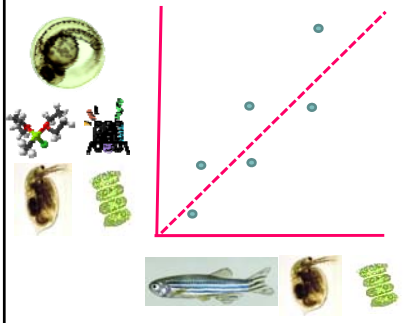


≈



✓ not better than correlation of reference approach with itself, i.e. reproducibility

acceptable, if



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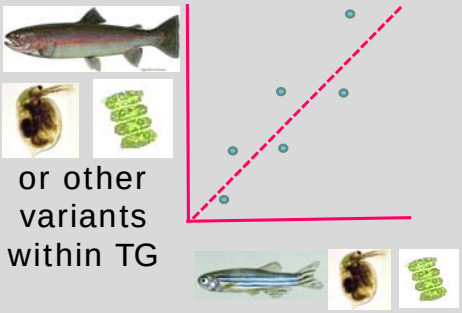
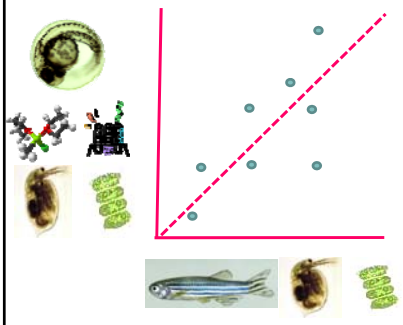


or other variants within TG

✓ within range of correlation of acceptable variants of reference approaches

acceptable, if



✓ similar number and size of deviations towards higher or lower sensitivity

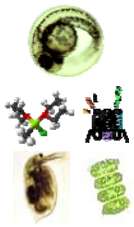
... where sufficient knowledge for building chemicals groups, test groups with more sensitive approach

... where insufficient knowledge, use new approach, since this means equal chance of using the higher or lower sensitive approach

LC50

relevance?

⇒ **1. "additional route for validation": assess mechanisitc relevance**



✓intact organism

✓ relevant life stage

✓ high surface/volume ratio

✓ uptake mechanisms?

✓ gills?

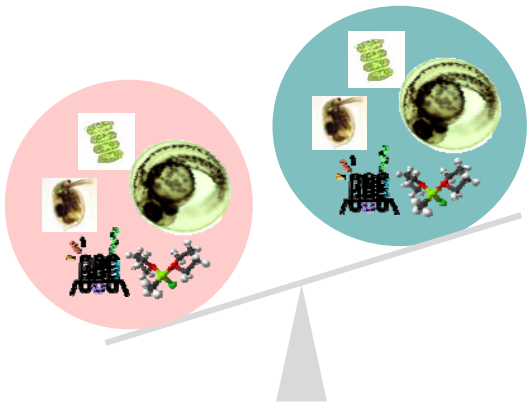
✓chorion ?

✓ metabolism ?

✓neurotox/respiration ?

✓ ...

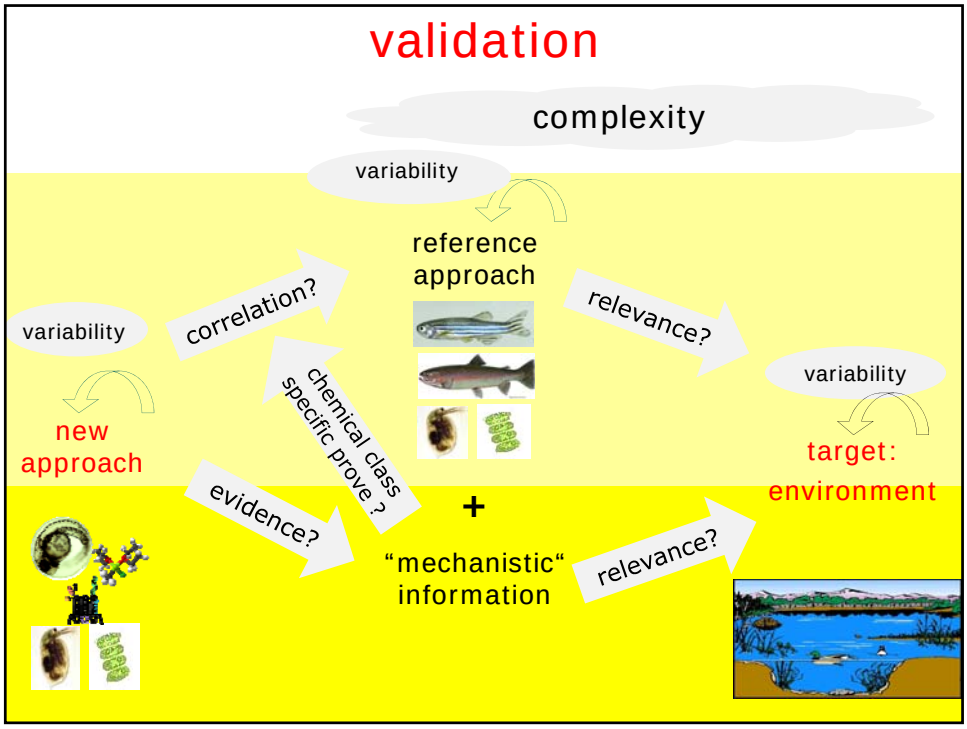
2. define for which class of compounds there is the burden of prove on the new approach?



3. consider hazard assessment as comparative

requires ↓variability in testing & assessment

↓exposure, change market wo predicting safety precisely


validation
complexity
variability
reference approach
correlation?
relevance?
variability
variability
new approach
chemical class specific prove ?
evidence?
+
"mechanistic" information
relevance?
target: environment

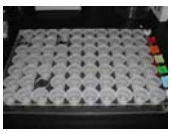
Possibilities for Using Fish Embryo Tests in place of Fish Acute Toxicity – Threshold Approach Strategies for Ecotoxicity Hazard Determination

S. E. Belanger[1], J. M. Rawlings[1], K. A. Connors[1], G. Stoddart[2], P. Bishop[2], C. Fassbender[2], D. Altmann[3], M. Paparella[3], S. Walter-Rhode[4], T. Braunbeck[5]

[1]Procter & Gamble
[2]PETA International Science Consortium
[3]Environment Agency of Austria
[4]Federal Environment Agency of Germany
[5]University of Heidelberg

Overview

- What is the Threshold Approach?
  - Previous evidence
  - Why change it
- Structuring a new assessment
  - Methods
  - Assumptions
- Results
  - Chemical Coverage
  - Order of sensitivity
  - Interspecies relationships
  - Impact on Classification and Labelling
  - Impact on Environmental Risk Assessment

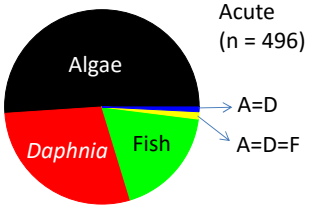


Threshold Approach

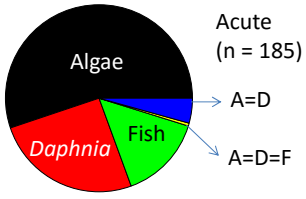
- A smart way to prioritize aquatic toxicity testing using information on non-vertebrate test species
- Takes advantage of the known frequency of greater sensitivity of algae and daphnids versus fish
- Reduces the use of fish overall (animal welfare), increases speed and reduces cost of studies

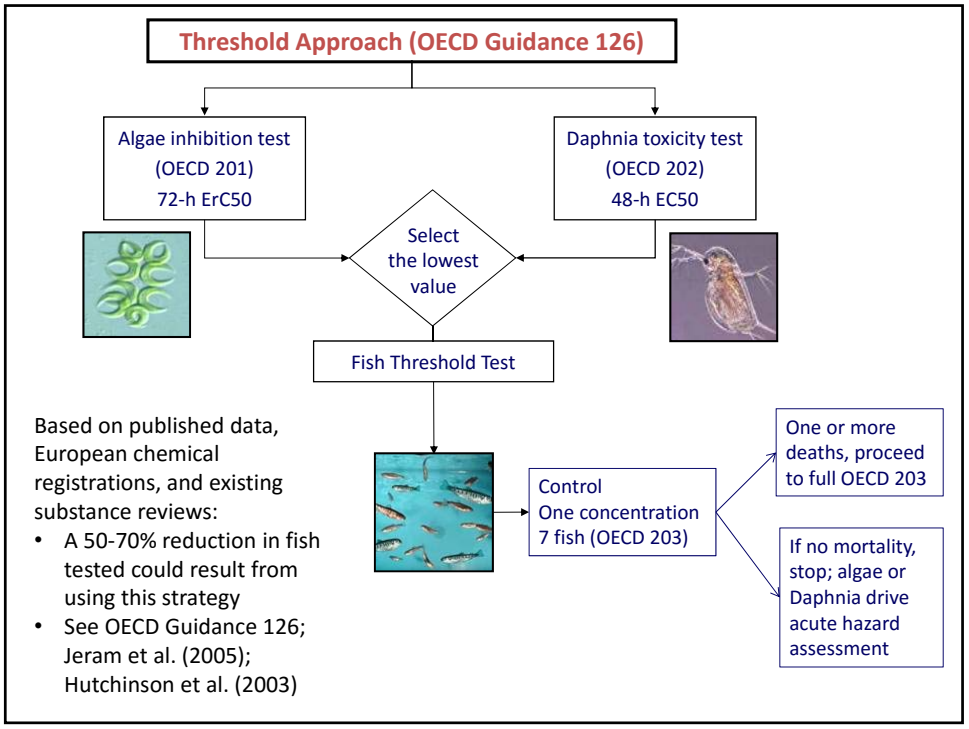
How often are fish the most sensitive taxon?

- Jeram et al. (2005)
  - Most sensitive taxon
  - All chemicals



- Creton et al. (2014)
  - Most sensitive taxon
  - Pharmaceuticals





A New (Updated) Fish, FET, Algal, Daphnid Database was constructed to address several areas:

1. Distributions of most sensitive acute endpoint
   a) Algae-Daphnia-Fish/FET (assumes a mix of Fish/FET)
   b) Algae-Daphnia-Fish
   c) Algae-Daphnia-FET
2. GHS classification outcomes
   a) Do classifications change when using FET or fish?
   b) Are changes associated with a specific chemical group?
3. Apparent inter-taxonomic toxicity relationships
4. Distributions of modes of action, K_{ow}, solubility

Data gathering process

- Began with databases from Lammer et al. (2009) and Belanger et al. (2013)
- Updated with FET data derived from
  - REACH portal
  - Published literature
  - Selected industry studies we are aware of
- Chemicals for which FET data exist, accrue algal *Daphnia magna* acute data as well
- Confirmed adequacy of studies based on sound ecotoxicological principles
  - Allowed nominal exposure data (although the more recent work is trending towards measurement when in support of ERAs)
  - Solubility within 10X of predicted solubility for upper concentrations (likelihood of exposure near the LC50 being below solubility)
  - Sound LC50 supported by the source information

Data base overview

Group	n	CASNOs
Fish Embryo [a]	542	237
Acute Fish	1465	165
Algae [b]	264	88
Daphnia	1164	130

[a] 96-hr data given precedence; shorter tests (48-72 hr were used if no other data was available for the CAS)

[b] 72 and 96 h data

Group	Taxon	Total occurrences	Percentage
FET	Zebrafish	524	96.7
	African sharptooth catfish	2	0.4
	Fathead minnow	13	2.4
	Medaka	3	0.6
Acute Fish	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

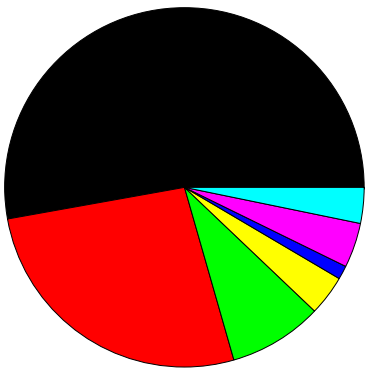
Distribution of chemical functional categories by test type

Chemical functional category	FET %	OECD 203 %	Daphnia %	Algal %
Biocide	3.8	4.8	4.6	2.3
Flame retardent	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
Total	100.0	100.0	100.0	100.0

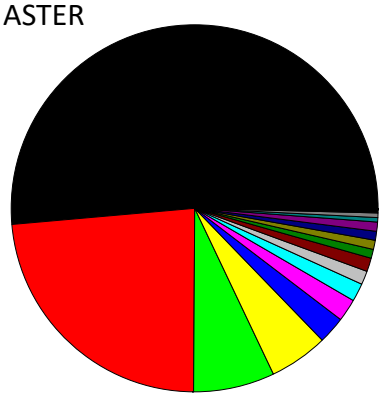
- Coverage is similar across taxa
- Algal data was most difficult to obtain
- Given these similar chemical domains, distributions of solubility and Kow are nearly identical

Distribution of MoA Assignments

Verhaar



ASTER



Most sensitive taxon based on geometric mean value available for each

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

Observations

- When all data are available, fish or FET are most sensitive an equal amount of time
- When considered separately, fish and FET identify different chemicals when they are the most sensitive
- When no Algae or *Daphnia* are available, some indication that fish are more sensitive; but not remarkably different (drill into fish/FET quantitative ratios)

Probing the Fish/FET Relationship

- For 165 possible comparisons (recall we lack algal and/or *Daphnia* data for about ½ of these)

Band for Fish/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

Additional Considerations: if a test has a CV of around 25-30%, the ratio of low to high values within the raw data is about a factor of 3-4; here we have both assays varying (minimally) in that range

We also probed if these differences would result in a changed view of both the driver for GHS Classification and if the Classification itself would change

Complex: Many scenarios

- Use of fish to replace FET and vice versa
- Depends on use of minimum versus mean values (for this part of the exercise we will only show results when geometric mean values are considered)
- Changes in classification result
  - Note that this is a pretty special outcome – the fish/FET value has to be lower at the outset (already hovering around 10% of the time), or
  - The initial classification is at or near a critical value (e.g., 0.1, 1, 10 mg/L)

Influence of FET or Fish in GHS Acute Classifications

Substances whose classification would change dependent on using fish vs FET

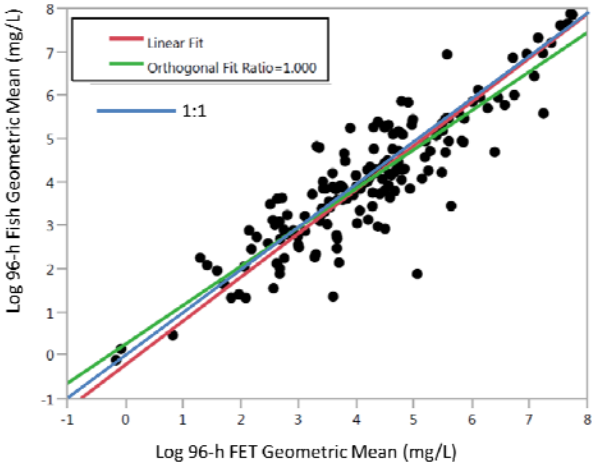
A-D-F	Fish most sensitive	Substance	FET (mg/L)	Fish (mg/L)	Daphnia (mg/L)	Algal (mg/L)	GHS with A-D-F	GHS with A-D-FET	GHS Classification Change
		Tetrachloroethylene	35.7	**8.30**	11.3	504	2	3	Fish lowers GHS

A-D-FET	FET most sensitive	Substance	FET (mg/L)	Fish (mg/L)	Daphnia (mg/L)	Algal (mg/L)	GHS with A-D-FET	GHS with A-D-F	GHS Classification Change
		4-Nitrophenol	**9.84**	13.9	10.6	32.0	2	3	FET lowers GHS
		Diclofenac	**2.06**	65.4	56.2	71.9	2	3	FET lowers GHS
		Ibuprofen	**7.85**	173	82.5	328	2	3	FET lowers GHS

Of 81 substances:

- 16 substances had fish as the most sensitive (ignoring FET)
- 12 substances had the FET as the most sensitive (ignoring fish)
- 4 substances had potential classification changes; 3 of these were lowered by FET replacing fish

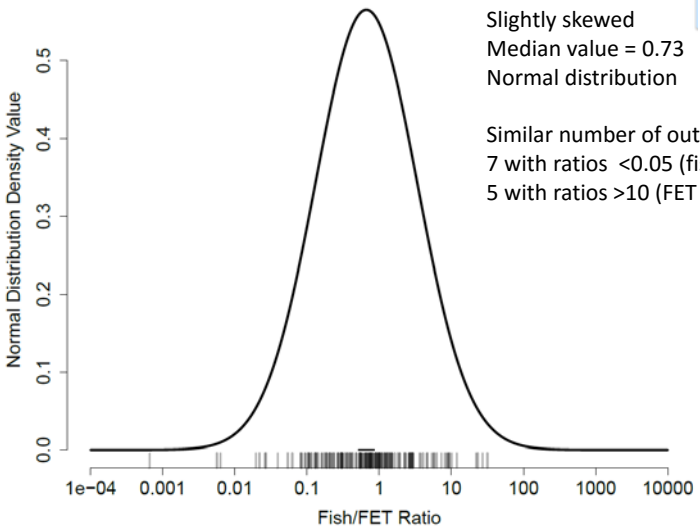
Fish Embryo Test – Fish comparison



- Same trends as Lammer et al. (2009) and Belanger et al. (2013)
- All other inter-taxonomic comparisons are poorer
- Regressions suffer when built from minimum versus geometric mean values
- Orthogonal regressions are more correct in a statistical sense

Log 96-hr FET LC50 (mg/L) = 1.0129*Log 96-hr Fish LC50 (mg/L) – 0.2321
R^2 = 0.94

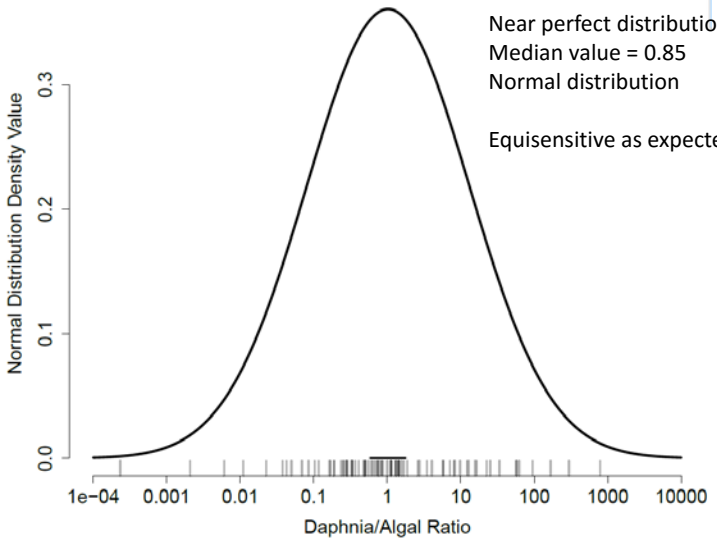
Ratios/distributions



Slightly skewed
Median value = 0.73
Normal distribution

Similar number of outliers
7 with ratios <0.05 (fish sens)
5 with ratios >10 (FET sens)

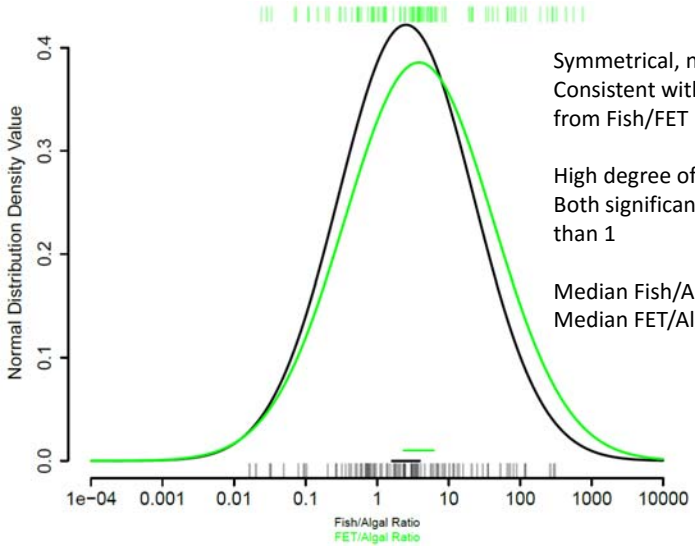
Ratios/distributions



Near perfect distribution
Median value = 0.85
Normal distribution

Equisensitive as expected

Ratios/distributions

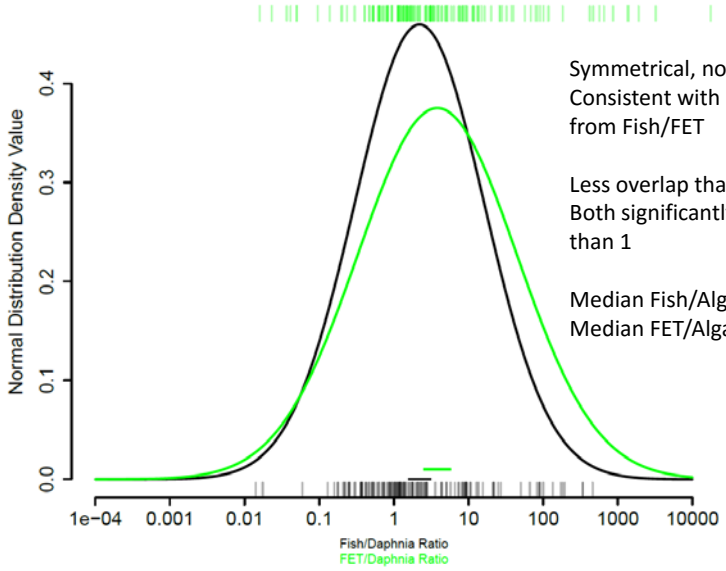


Symmetrical, normal
Consistent with expectation from Fish/FET

High degree of overlap
Both significantly greater than 1

Median Fish/Algae = 2.4
Median FET/Algae = 3.6

Ratios/distributions

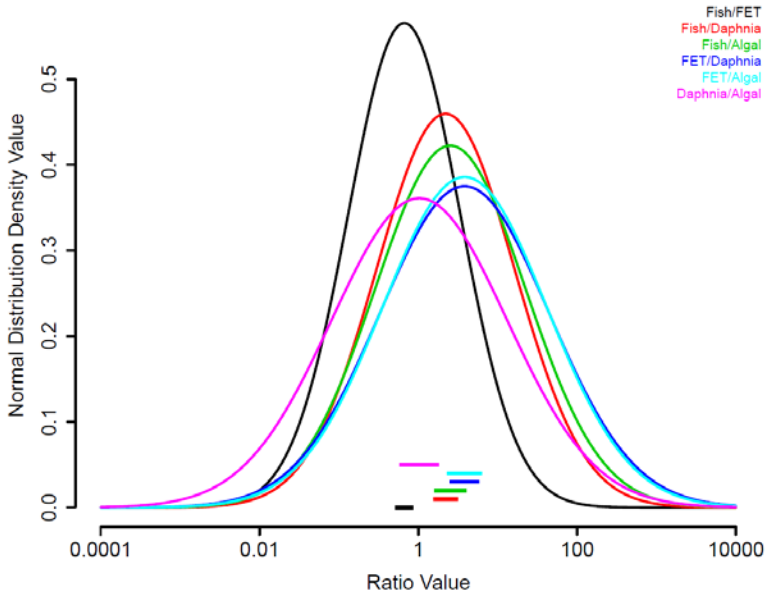


Symmetrical, normal
Consistent with expectation from Fish/FET

Less overlap than algae
Both significantly greater than 1

Median Fish/Algae = 1.4
Median FET/Algae = 2.7

Ratios/distributions

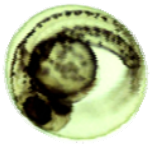


Conclusions

- The FET could reasonably replace fish in a Threshold Approach to improve animal welfare which can further reduce the use of fish in hazard assessment
- The data review is quantitatively consistent with previous observations (order of sensitivity and inter-taxonomic relationships)
- Algae and *Daphnia* indicate the relative importance we should place on the fish/FET data (important, but not the most)
- Few classification decisions are affected by the choice of fish or FET
- Risk assessment decisions based on input source (FET or AFT) will not be altered appreciably.
  - Fish or FET were most sensitive only 26% of the time (21/81)
  - Within this subgroup FET/AFT ratio was within 3-fold 57% of the time and 81% were within a factor of 10
- The predictions are likely best for polar and non-polar narcotics and inorganics and worst (perhaps) for neurotoxicants based on Scholz et al. (2016)
  - however the latter requires much deeper probing in order to draw a substantiated conclusion.

Comments from the **OECD VMG Eco/FDG expert group** in the context of a document on the use of the **FET in the threshold approach**

Summary of major open discussion points for potential limitations of the FET



Stefan Scholz
Helmholtz Centre for Environmental Research - UFZ

Background

- **Discussion Paper for the application of the FET within the threshold approach →** OECD VMGeco, FDG
- Responses by VMGeco and FDG
- Summary of responses and comments
- Additional data

10/2016

12/2016

04/2017

OECD VMGeco: OECD Validation and Management Group for ecotoxicological testing

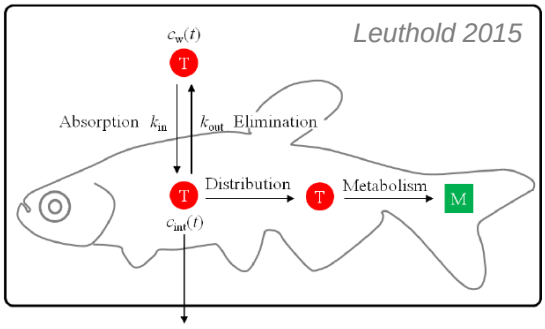
FDG: Fish drafting group

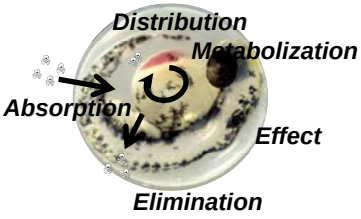
1. Limitations of both the AFT and FET

- **All TGs have limitations**: Is a critical assessment of AFT data required?
- The **burden of proof** is on the FET?
- **FET data from other species** required in order to demonstrate potential species sensitivity differences in the FET?
- Concern on the FET should be only when there is a **systematic bias between AFT and FET?**

2. Gill surface and chorion

Systematic bias for the FET?

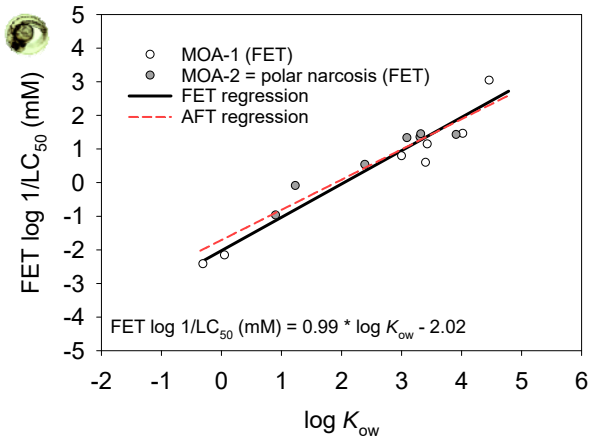




Uptake path:	Gills	Surface & Chorion (≠ membrane) (≤ 2 dpf !!!)

- **Partition equilibrium** is driving toxicity (similar baseline toxicity!)
- Manual dechorionation may be considered for high MW compounds (but eleutheroembryo phase already included in TG 236!)

Baseline toxicity FET and AFT



Klüver et al. 2016

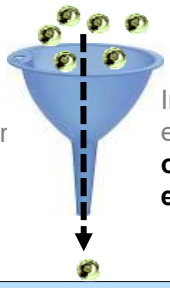
AFT baseline QSAR: $1/LC_{50} = 0.90 * \log K_{ow} - 1.71$

3. Domain of applicability of the FET

Physico-chemical properties

Historic FET data with quality limitations

Filters were applied to avoid bias for AFT-FET comparative analysis



Inappropriate exposure design led to **overestimation of effect** concentrations

Removal of hydrophobic/volatile compounds from data set

Applicability restricted to domains included after filter application?

4. Neurotoxicity

- Mechanistic evidence, that FET has a low sensitivity for mortality of neurotoxic compounds (sytematic bias)

  Knöbel et al. 2012, Klüver et al. 2015, ECHA report 2015, Glaberman et al. 2016

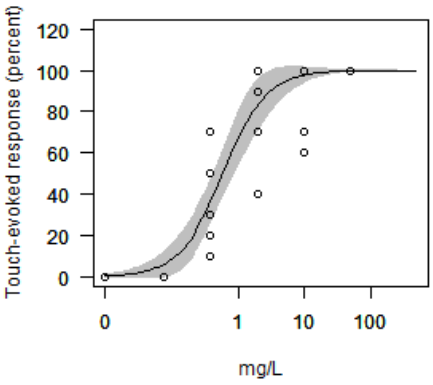
- For the AFT, highest species variability observed for neurotoxic compounds!

  ECHA report Table 4.16.3

- Additional endpoints to be considered?

Simple endpoint: touch-evoked response

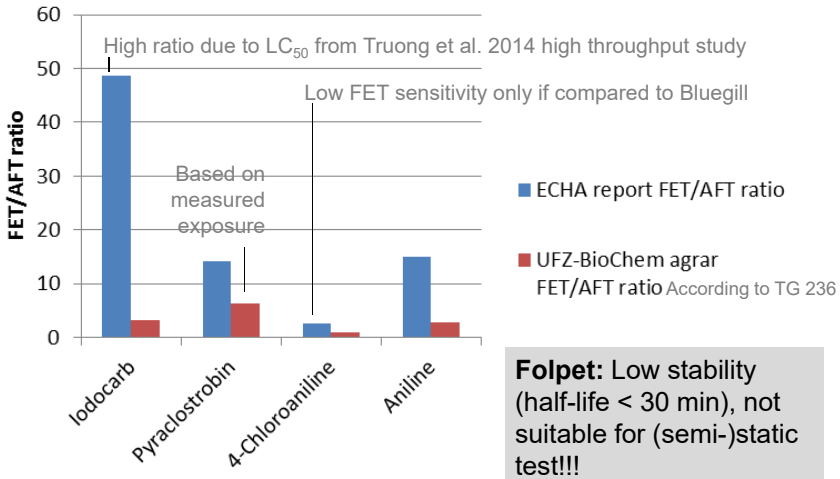
Example: aldicarb tested in a student course at the UFZ



Endpoint	EC50 (mg/L)
AFT (mortality)	1.1
FET (mortality)	280
FET (distance moved)	0.43
FET (touch-evoked response)	**0.59**

5. Narcotic / out of structural alert outliers

could not be confirmed experimtally in most cases



Folpet: Low stability (half-life < 30 min), not suitable for (semi-)static test!!!

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BioChem agrar

Anita Birke, unpuplished results

6. Biotransformation capacity

- **Biotransformation demonstrated for FET** (e.g. Kühnert et al. 2013, Brox et al. 2016) but the quantitative difference to AFT has not been investigated systematically.
- Has been shown to be the reason for the **weak sensitivity in the FET for one compound** (allyl alcohol), are there others?
- Is the potential weaker biotransformation for individual compounds in the FET **balanced by AFT species differences**?

7. Multiconstituent, UVCBs and inorganic/metals

What distinguishes multiconstituents and UVCBs from other (organic) compounds?
→ if single organic chemical tests are considered as valid in the FET, can we conclude that the FET is valid for mixtures as well?

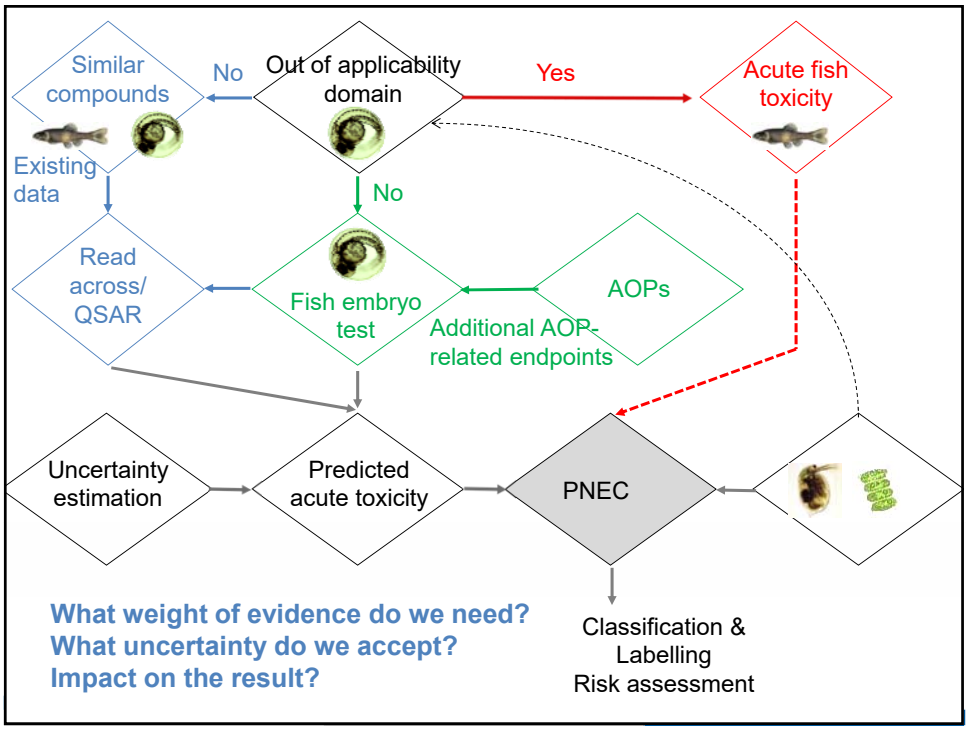
Inorganic: Requires further investigation, but may not represent major focus due to the expected low number of registrations with inorganics in the future?

8. Potential additional research

- Systematic analysis on the impact of using the FET on classification is lacking
- AFT variability
- Biotransformation capacity
- FET species differences
- Increase database by e.g. also considering tests < 96 h (if evidence that toxicity is established completely), or are experimental results required?

9. Use of the FET in a Weight of Evidence

- Use of the FET within an integrated testing strategy/IATA **seems to be the way forward**, where stakeholders, the scientific and regulatory community may agree
- What could the WoE/IATA approach look like?
- See a **draft proposal** next slide (first proposed and discussed at EUSAAT Linz 2016)



ANNEX

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Difficult compounds: OECD guidance No 23

Variability of neurotoxic compounds in AFT (for compounds with FET/AFT>10)

Common name	CAS	MoA (animals, preferably vertebrates)	AFT/AFT ratio					
			DR/LM	DR/OM	DR/PP	LM/OM	LM/PP	OM/PP
Aldicarb	116-06-3	AChE inhibition	95*					
Azinphos-methyl	86-50-0	AChE inhibition						0.017*
Allyl alcohol	107-18-6	*Reactive				1.2	4	3.4
Fenamiphos	22224-92-6	AChE inhibtion				0.13		
Disulfoton	298-04-4	AChE inhibition				0.01*	0.027*	
Ziram	137-30-4	Inhibition of lysyl oxidase/extracellular matrix				0.11*		
Malathion	121-75-5	ACHE inhibtion	11	11	0.077	1.01	0.007*	0.007*
Trichlorfon	52-68-6	ACHE inhibtion				3.77	0.066	0.0174*

ECHA report Table 4.16.3

Analysis of exposure concentration

- …are routinely conducted for the AFT (TG 203)
- Lacking in the majority of FETs conducted prior to adoption of the TG 236
- One of the major source for limited confidence in existing FET data
- Similar as for AFT, verification of exposure concentration is a major quality control parameter

Perspectives on the Regulatory Use of the Fish Embryo Acute Toxicity (FET) Test

Teresa Norberg-King
USEPA, Mid-Continent Ecology Division

Workshop on a role and applicability of the fish embryo acute toxicity test for European regulation and beyond
ECHA, May 2017, Helsinki

Environmental Laws

- In the U.S., EPA is charged with administering all or a part of various laws and executive orders and develops and enforces regulations to help to protect human health and the environment.

Legislation	Goal of the Legislation
Clean Water Act (CWA)	• Regulating discharges of pollutants into the waters of U.S. and regulating quality standards for surface waters. • EPA's Office of Water manages the programs.
Toxic Substances Control Act (amended by Frank R. Lautenberg Chemical Safety for the 21st Century Act)	• EPA evaluates new and existing chemicals for potential risks. These laws require reporting, record-keeping and testing requirements, and restrictions relating to chemical substances and/or mixtures (not food, drugs, cosmetics and pesticides). • EPA's Office of Pollution Prevention and Toxics (OPPT) manages programs under Amended-TSCA and the PPA.
Federal Insecticide, Fungicide, & Rodenticide Act (FIFRA)	• Regulation of pesticide distribution, sale, and use. All pesticides distributed or sold in U.S. must be registered / licensed by EPA. • EPA's Office of Pesticide Programs (OPP) manages this program.

Animal Welfare Act

- All vertebrates, including fish, birds, and amphibians, are regulated organisms beginning at birth / hatch according to U.S. Animal Welfare Act and Health Research Extension Act of 1985.
  - Fish fall within the animal kingdom and are biologically considered vertebrates regardless of their life-stage.
  - Recognize that it is essential to keep the number of organisms used for testing low while keeping precision and power of the test.

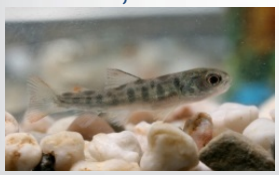
Fish Species Used for Testing

In the U.S., the species chosen typically are those that provide ability to determine the acute to chronic ratio and protect the environment.

- Acute lethality fish test typically use
  - freshwater: fathead minnows, trout, bluegills
  - marine: sheepshead minnow, inland silverside, topsmelt

- Longer term fish exposures in early life stage tests evaluate sublethal effects and range from 28 to 60 day with fathead minnows, sheepshead minnow, bluegills, and trout. Typically the embryo and/or newly hatched larval life-stage is exposed during the first part of the test.

brown trout *Salvelinus fontinalis*



rainbow trout *Oncorhynchus mykiss*

bluegill *Lepomis macrochirus*

fathead minnow *Pimephales promelas*

topsmelt *Atherinops affinis*

sheepshead minnow *Cyprinodon variegatus*

silverside minnows *Menidia beryllina, M. menidia, M. peninsulae*

EPA Toxicity Testing Guidelines

- EPA's test guidelines for pesticides and toxic substances (specified as OCSPP or OPPTS Test Guidelines) provide EPA-recommended methods to generate data that are submitted to EPA to support:
  - Registration of a pesticide under FIFRA.
  - Decision-making process supporting potential regulation of an industrial chemical under TSCA.
  - Setting of a tolerance or tolerance exemption for pesticide residues under section 408 the Federal Food, Drug and Cosmetic Act (FFDCA) (21 U.S.C. 346a).
  - EPA participates heavily in development of Organization of Economic Cooperation and Development (OECD) Test Guidelines; hence, OCSPP/OPPTS Test Guidelines are often identical to or vary only slightly from OECD Test Guidelines.
- EPA's test guidelines for wastewater/effluent specify EPA-promulgated test methods for compliance.

EPA Ecological Test Guidelines

EPA's Ecological Effects Test Guidelines are generally intended to meet toxicity testing requirements for terrestrial and aquatic organisms under TSCA, FIFRA and FFDCA.

OCSPP TG: Group A - Aquatic and Sediment-dwelling Fauna and Aquatic Microcosms

- 850.1000 - Background and Special Considerations-Tests with Aquatic and Sediment-Dwelling Fauna and Aquatic Microcosms
- 850.1010 - Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids
- 850.1020 - Gammarid Amphipod Acute Toxicity Test
- 850.1025 - Oyster Acute Toxicity Test (Shell Deposition)
- 850.1035 - Mysid Acute Toxicity Test
- 850.1045 - Penaeid Acute Toxicity Test
- 850.1055 - Bivalve Acute Toxicity Test (Embryo-Larval)
- 850.1075 - Freshwater and Saltwater Fish Acute Toxicity Test
- 850.1300 - Daphnid Chronic Toxicity Test
- 850.1400 - Fish Early Life Stage Toxicity Test
- 850.1710 - Oyster Bioconcentration Factor (BCF)
- 850.1730 - Fish Bioconcentration Factor (BCF)
- 850.1735 - Spiked Whole Sediment 10-Day Toxicity Test , Freshwater Invertebrates
- 850.1740 - Spiked Whole Sediment 10-Day Toxicity Test, Saltwater Invertebrates

(documents are dated December 2016)

Data Considerations

- EPA relies on the use of scientifically valid test methods and strategies to support regulatory decisions
  - long-standing methods are used for continuity
  - consider means to reduce or replace the use of vertebrate animals while providing information of equivalent or better scientific quality and relevance to support decisions.
- Recognize that it is essential to keep the number of organisms used for animal testing low while keeping precision and power of the test

Data Considerations

- The U.S. regulatory community uses single-chemical (individual) data points for risk assessment purposes.
  - Uncertainty lies with the individual data points
  - Assessment of test methods to adequately determine the testing of certain modes of action (*e.g.*, pesticides with very specific modes of action and which are highly toxic), but also for unknown or baseline (narcosis) modes of action (*e.g.*, commercial chemicals with unknown modes of action).
  - Need to assess whether pesticides, effluents, and commercial chemicals are represented for determining the relationship between FET and the standard *in vivo* juvenile fish testing results with typical test species.

Use of the FET

- EPA does not consider the use of OECD FET Test Guideline 236 as an animal replacement approach (just a different fish life-stage)
- Number of animals (fish) being used:
  - OECD FET TG 236 uses ≥10 fish /test concentration
  - OECD Fish Acute TG 203 uses 7 fish/test concentration
- EPA may consider data from tests conducted with OECD FET TG 236, as with any other available data, as part of the weight-of-evidence for ecological risk assessment for some programs within EPA.
- However, the FET test would not be a 1:1 replacement for the juvenile acute fish toxicity test
- In the U.S., use of FET has been minimal to date. Any modification to include the FET as an acute test endpoint for wastewater evaluations is not being currently considered.

Further Evaluations

- In standard ecotoxicity tests, fish (*e.g.*, rainbow trout) are used as surrogates for aquatic-phase amphibians as well as for endangered and threatened species.
- Current fish test species and methods have been shown to be representative of these taxa, and a need for other acute test procedures has been low.
- Further evaluation is needed to determine if the FET (OECD TG 236) is protective of aquatic-phase amphibians and endangered and threatened species.

Questions?

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