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EURL ECVAM Recommendation on the Zebrafish Embryo Acute Toxicity Test Method (ZFET) for Acute Aquatic Toxicity Testing

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Abstract

Acute fish toxicity testing is an important component of the environmental hazard assessment of chemicals. Since many years, (zebra-)fish embryo-based methods have been proposed as alternatives to the acute fish toxicity test carried out with juvenile or adult fish. On behalf of the Organisation for Economic Cooperation and Development (OECD), the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) coordinated during 2008-2012 the validation of the zebrafish embryo acute toxicity test method (ZFET) to evaluate its reproducibility in support to the development of an OECD Test Guideline. In parallel to this study, Belanger and colleagues continued to collect acute fish embryo toxicity and acute fish toxicity data to assess the relevance, predictive capacity and applicability of the ZFET and submitted their report to EURL ECVAM in July 2012. Following independent scientific peer review by EURL ECVAM's Scientific Advisory Committee (ESAC) of both studies and having considered input from regulators, stakeholders, international partners and the general public, EURL ECVAM concluded that the ZFET - being available as OECD TG236 since 2013 – should be used for generating information on acute fish toxicity, where appropriate. Its use would result in an overall reduction of the numbers of juvenile and adult fish for aquatic toxicity testing. It is recognised that further guidance on the use of OECD TG236 across the various regulatory frameworks and regions should be developed addressing in particular the possible use of the ZFET to generate information on acute fish toxicity and its potential limitations.

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

EURL ECVAM RECOMMENDATION

on the Zebrafish Embryo Acute Toxicity Test Method (ZFET) for Acute Aquatic Toxicity Testing

BACKGROUND TO EURL ECVAM RECOMMENDATIONS

The aim of a EURL ECVAM Recommendation is to provide EURL ECVAM views on the validity of the test method in question, to advise on possible regulatory applicability, limitations and proper scientific use of the test method, and to suggest possible follow-up activities in view of addressing knowledge gaps.

During the development of a Recommendation, EURL ECVAM consults with its Scientific Advisory Committee (ESAC), its advisory body for Preliminary Assessment of Regulatory Relevance (PARERE) and its EURL ECVAM Stakeholder Forum (ESTAF). Moreover, EURL ECVAM consults with other Commission services and partner organisations of the International Collaboration on Alternative Test Methods (ICATM). Before finalising its Recommendation, EURL ECVAM invites comments from the general public and, if applicable, from the test method submitter.

ACKNOWLEDGEMENTS

This Recommendation was prepared by the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), part of the Institute for Health and Consumer Protection (IHCP), Directorate-General Joint Research Centre (DG JRC) of the European Commission. The Recommendation was drafted on the basis of the ESAC Opinion and ESAC Working Group Report outlining the detailed Scientific Peer Review of the EURL ECVAM-coordinated OECD validation study of the Zebrafish Embryo Acute Toxicity (ZFET) test method and the retrospective analysis of fish embryo and acute fish toxicity data carried out by Belanger et al (2012). The Recommendation further benefitted from comments and suggestions received from members of PARERE (EURL ECVAM's advisory body for Preliminary Assessment of Regulatory Relevance that brings together representatives of Member State regulatory bodies as well as EU agencies including ECHA, EFSA and EMA), and ESTAF (EURL ECVAM's Stakeholder Forum). Input was also provided by partner organisations of EURL ECVAM in the framework of the International Collaboration on Alternative Test Methods (ICATM), and by the general public.

Marlies Halder coordinated the drafting of this Recommendation. Patric Amcoff and Claudius Griesinger coordinated the Peer Review conducted by ESAC.

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TABLE OF CONTENTS

EXECUTIVE SUMMARY 4
1. INTRODUCTION 6
2. TEST METHOD DEFINITION 7
3. OVERALL PERFORMANCE OF THE ZEBRAFISH EMBRYO ACUTE TOXICITY TEST 8
4. POTENTIAL LIMITATIONS 9
5. SUGGESTED REGULATORY USE 10
6. FOLLOW-UP ACTIVITIES RECOMMENDED BY EURL ECVAM 11
REFERENCES 12
ANNEX 1 – ESAC OPINION 15
ANNEX 2 – EURL ECVAM REQUEST FOR ESAC ADVICE 26

EXECUTIVE SUMMARY

In 2012, the OECD validation study on the transferability and reproducibility of the zebrafish embryo acute toxicity test method (ZFET) was finalised. The study was coordinated by EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) with the support of a Validation Management Group established by OECD and supervised by the OECD ad hoc Expert Group on the Fish Embryo Toxicity Test. In parallel to this study, Belanger and colleagues (2012) as members of the OECD expert group, evaluated the predictive capacity of (zebrafish) fish embryo acute toxicity tests for acute fish toxicity testing by comparing data from acute fish embryo toxicity tests and juvenile or adult acute fish toxicity tests.

The ZFET is based on the use of newly fertilised eggs from zebrafish (*Danio rerio*). It is a short-term exposure test (96 h) and determines the concentration that is lethal to 50% of the zebrafish embryos (LC_{50}) as an indication of acute fish toxicity. Observation of one of the following apical endpoints indicates the death of the embryo: coagulation of the embryo, lack of somite formation, non-detachment of the tail and lack of heartbeat.

Upon finalisation of these studies, EURL ECVAM requested ESAC to review both the ZFET validation study and the retrospective analysis of Belanger *et al.* (2012). ESAC finalised its review in March 2013. EURL ECVAM fully endorses the ESAC Opinion (Annex 1) on the overall performance of the ZFET and recommends the following:

(1) The OECD validation study showed that the ZFET is transferable and reproducible within and between laboratories. The retrospective analysis demonstrated, on the basis of data on 144 chemicals, a strong correlation (r = 0.9) between fish acute toxicity data (96 h; five freshwater species recommended in OECD TG203) and fish embryo acute toxicity data (24-120 h exposure; mainly zebrafish). Thus, the ZFET can provide information on acute fish toxicity comparable to that derived from standard tests (*e.g.* OECD TG203; OECD 1992). Notably, the chemicals evaluated in the retrospective analysis covered a broad range of physico-chemical properties, toxicological modes of action, and functional use, *e.g.* industrial chemicals (77), plant protection products (21), surfactants (15), pharmaceuticals (8), and biocides (5) thereby indicating a wide applicability domain of the ZFET (Belanger *et al.* 2012; 2013). Following validation, the ZFET was described in OECD Test Guideline 236 "Fish embryo acute toxicity (FET) test" (OECD 2013a).

(2) Where appropriate, the ZFET (OECD TG236) should be used for generating information on acute fish toxicity. In case the ZFET cannot be used, information on acute fish toxicity should be derived with the threshold approach following OECD Guidance Document 126 (OECD 2010). This option is already in place for chemicals (ECHA 2012), biocidal products (EU, 2012a) and plant protection products (EU 2013a, EU 2013b).

    Furthermore, prospective users of the method should consult EURL ECVAM's DataBase for ALternative Methods (DB-ALM) to access the detailed ZFET protocol (see DB-ALM Protocol no. 140 at the address: http://ecvam-dbalm.jrc.ec.europa.eu).

(3) Prior to the use of the ZFET, the following potential limitations should be considered:

    a) Like all developing organisms, zebrafish embryos have metabolic (biotransformation) capacity. Currently however, it is not fully understood how the embryonic metabolism compares to that of juvenile or adult fish. OECD TG236 therefore states that if there are any indications that metabolites or transformation products would be more toxic than the parent chemical, the test should also be carried out with these and the results should be considered when concluding on acute toxicity. Another option would be to perform a test which takes metabolism into account.

    b) There is evidence that chemicals with high molecular weight (≥3kD) or a bulky structure do not pass the chorion while some chemicals may delay hatching. The reduced bioavailability of

such chemicals over the full exposure period may result in lower toxicity; therefore OECD TG236 states that other toxicity tests might be more appropriate.

(4) To support the use of the ZFET (OECD TG236), it should be included into the respective regulations and associated guidance documents, e.g. industrial chemicals (REACH Regulation 1907/2006; EC 2006 and endpoint specific guidance, ECHA 2012), biocidal products (Regulation 528/2012, EU 2012a), plant protection products (Regulation 1107/2009, EC 2009a and associated data requirements outlined in Regulation 283/2013, EU 2013a; Regulation 284/2013; EU 2013b), veterinary pharmaceuticals (CVMP/VICH/790/03; EMA 2004), and feed additives (Regulation 429/2008, EC 2008; further specified in EFSA 2008).

In case of the REACH guidance on aquatic toxicity (ECHA 2012), the testing strategy for chemical safety assessment (e.g. of Predicted No-Effect Concentration (PNEC) derivation) includes a placeholder for validated alternative methods. In this context the ZFET is mentioned as a possible alternative to the acute fish toxicity test provided that it is fully validated and available as a standardised method (e.g. OECD test guideline). This requirement can now be considered satisfied due to the availability of OECD TG236 "Fish embryo acute (FET) toxicity test" and thus the REACH guidance document should be updated accordingly.

(5) An OECD guidance document on the use of OECD TG236 across the various regulatory frameworks and regions should be developed. It should particularly address the possible use of the ZFET to generate information on acute fish toxicity and its potential limitations.

(6) The use of the ZFET will result in an overall reduction of the numbers of juvenile and adult fish required for aquatic toxicity testing. Notably, since Directive 2010/63/EU (EU 2010) on the protection of animals used for scientific purposes covers larval forms of non-human vertebrate animals once they are independently feeding, the ZFET as used in OECD TG236 is outside the Directive's scope: zebrafish start to feed independently not before 5 days post-fertilisation and the method uses zebrafish embryos only up to 4 days (= 96 h) post-fertilisation.

(7) The database containing fish embryo acute toxicity data and fish acute toxicity data (Belanger *et al.* 2012; 2013) should be maintained and updated on a regular basis. This would provide additional insight into the practical use of the ZFET and enhance confidence in the applicability domain. As noted by Belanger *et al.* (2012; 2013), it was not possible to find acute fish toxicity data for all chemicals for which fish embryo toxicity data were available. For example, for only eight out of the 22 pharmaceuticals, acute fish data could be retrieved. Therefore, industry and regulators are encouraged to make existing data available where possible.

(8) Further effort should be invested in the development and validation of methods that avoid the use of fish for environmental hazard and risk assessment. The use of Adverse Outcome Pathways to aid in the design of integrated approaches and the development of new methods based on fish cells or fish embryo tests are some of the areas that deserve investigation in the context of fish toxicity testing.

1. Introduction

Methods using (zebra-)fish embryos have been proposed as alternatives to the acute fish toxicity test in juvenile or adult fish for many years (Schulte and Nagel 1994; Nagel 2002; Braunbeck *et al.* 2005; Scholz *et al.* 2008). In fact, the so-called "zebra fish egg test" was validated in Germany for waste water testing and has replaced the test in adult fish in 2005 (DIN38415-6, DIN 2001; German Federal Law Gazette 2005; ISO 2007).

Within the framework of the Organisation for Economic Cooperation and Development (OECD) Test Guidelines Programme, the German Federal Environment Agency (UBA) proposed the development of a test guideline to assess the acute aquatic toxicity of chemicals using fish embryos as a potential alternative to the acute fish toxicity test (OECD TG203) and in 2005 submitted the draft test guideline "Fish Embryo Toxicity (FET) Test" (OECD 2006) together with a supporting Background Paper (Braunbeck and Lammer 2006) to the OECD.

In order to follow up on the comments received from the National Coordinators of the Test Guidelines Programme (WNT), the OECD established an ad hoc Expert Group on the Fish Embryo Toxicity Test (AHEG FET) to review the submitted documents taking into consideration the scientific basis, reproducibility, predictive capacity and potential applicability of the FET. The AHEG FET noted that most data had been generated with zebrafish embryos and the thorough re-evaluation of existing data demonstrated that acute fish embryo toxicity data (LC_{50}) correlated well with LC_{50} values derived from acute fish toxicity tests (Lammer *et al.* 2009). However, since data providing sufficient evidence for the reproducibility of the method as described in the draft OECD test guideline were lacking, OECD launched a study to assess the transferability, within and between laboratory reproducibility of the zebrafish embryo acute toxicity test method (ZFET). The study was coordinated by EURL ECVAM with the support of the validation management group (VMG) established by OECD in November 2008. A total of 20 chemicals were tested in five concentrations with respective controls and three independent runs in at least three laboratories. A secondary aim was to establish the appropriate exposure duration (48 h *versus* 96 h) and number of embryos per concentration (10 *versus* 20). Therefore, all experiments were carried out with 96 h exposure and 20 embryos.

Building on the dataset of Lammer *et al.* (2009), Belanger and colleagues continued to collect acute fish embryo toxicity and acute fish toxicity data to assess the relevance, predictive capacity and applicability of the ZFET and other fish embryo tests as a possible alternative to the acute fish toxicity test (OECD TG203). The report of this retrospective data analysis was submitted to EURL ECVAM in July 2012 (Belanger *et al.* 2012) and recently published in peer reviewed literature (Belanger *et al.* 2013).

Validation Study Reports of the prospective study were approved by the OECD WNT and published by OECD (Part 1: OECD 2011 and Part 2: OECD 2012). Based on the outcome of the validation study, the draft OECD test guideline was finalised and recently adopted as OECD TG236 "Fish embryo acute toxicity (FET) test" (OECD 2013a).

EURL ECVAM requested the EURL ECVAM Scientific Advisory Committee (ESAC) at its 36th meeting on 21 March 2012 to provide an ESAC Opinion on the study (ECVAM Request for ESAC Advice, Annex 2). An ESAC Working Group (WG) was established to review the results of the prospective study compiled in the Validation Study Reports and of the retrospective study provided by Belanger *et al.* (2012). Both the ESAC Opinion (EURL ECVAM 2013a) (see Annex 1) and the ESAC WG report (EURL ECVAM 2013b) were adopted by ESAC on 15 March 2013.

2. Test method definition

The ZFET is based on the use of newly fertilised eggs from zebrafish (*Danio rerio*). It is designed to determine acute lethal effects (LC_{50}) of chemicals on zebrafish embryos as indication of acute fish toxicity.

Biological and mechanistic relevance of the test method

The current standard test for acute fish toxicity (OECD TG203) is carried out with juvenile or adult fish. It is a short-term exposure test (96 h) and determines the concentration that is lethal to 50% of the fish (LC_{50}).

Zebrafish is a well-established model organism in basic research and toxicology and one of the fish species recommended in OECD test guidelines for acute (TG203) and chronic (*e.g.* TG210) fish toxicity testing. Under appropriate conditions, zebrafish produce a large number of non-adherent, fully transparent eggs. Normal embryonic development is well-described (*e.g.* by Kimmel *et al.* 1995) and the transparency of the live zebrafish embryo permits observation of developmental parameters and their possible disturbance using light microscopy.

During the development of the ZFET a number of parameters indicating disturbance of the embryonal development were proposed as possible toxicological endpoints (Schulte and Nagel 1994; Lange *et al.* 1995; Nagel 2002). Out of the initially 12 parameters, four indicate that the embryo will neither hatch nor survive, *i.e.* observation of at least one indicates the death of the embryo. During the validation of the "zebrafish egg test" for effluent testing these four parameters were confirmed to predict acute fish toxicity and included in the guideline (DIN38415-6; DIN 2001). They are also used in the present ZFET protocol as lethal endpoints:

a) *coagulation of the embryo* may occur within a few hours after start of exposure or throughout the exposure period and indicates general acute toxic effects;
b) *lack of somite formation* – somites should be visible from 12 h post-fertilisation onwards; in their absence the embryo will not further develop and die;
c) *non-detachment of the tail* – the tail should be detached from the yolk at 24 h post-fertilisation indicating normal growth of the embryo; and
d) *lack of heartbeat* – the heartbeat is easily detectable from 30 h post-fertilisation onwards and its absence indicates death of the embryo.

The percentage of dead zebrafish embryos per concentration is used for calculation of the LC_{50} value, *i.e.* the concentration of a chemical that is lethal to 50% of the zebrafish embryos.

Until hatching (48-72 h post-fertilisation), the zebrafish embryo is surrounded by the chorion, a non-cellular membrane, which may act as a barrier for high molecular weight chemicals and hence reduce their exposure to the embryo. Therefore, the exposure time in the current ZFET protocol (OECD TG236) was set to 96 h (see also Potential Limitations).

Several studies related to the use of zebrafish embryos for human developmental toxicity testing and early drug screening provide evidence that zebrafish embryos have metabolic competence with Phase I and Phase II biotransformation (*e.g.* Jones *et al.* 2010; Goldstone *et al.* 2010; Weigt *et al.* 2011; Kubota *et al.* 2011; Incardano *et al.* 2012; Weigt *et al.* 2012; Mohammed *et al.* 2012). However, it is unclear whether the metabolic competence is in the same range as the one of juvenile or adult fish (see also Possible Limitations).

3. Overall performance of the zebrafish embryo acute toxicity test

Level of standardisation of the test method

The results of the EURL ECVAM coordinated OECD validation study led to a well-described standardised protocol. Compared to the initial draft OECD guideline (2006), the following modifications are most important:

- The exposure duration was set to 96 h to enhance exposure of the embryo to chemicals not readily penetrating the chorion (*e.g.* due to high molecular weight);
- 20 embryos are used per concentration instead of only 10. This increased sample size positively influences the statistical power of the results;
- A new acceptance criterion was established for the positive control; *i.e.* 96 h exposure to 3,4-dichloroaniline (4.0 mg/L) should result in a minimum mortality of 30%;
- Test vessels (*e.g.* 24-well plates) should be conditioned with the respective test concentrations/controls at least 24 h before starting the test;
- Test solutions/controls should be renewed on a daily basis (semi-static exposure).

Following evaluation of the validation study by the AHEG FET and WNT, the ZFET was finally approved in May 2013 at the 25th meeting of the OECD WNT and adopted by the OECD Joint Meeting in July 2013. It is now available as OECD TG236 "Fish embryo acute toxicity (FET) test" on the OECD website.

The protocol of the ZFET is also available via EURL ECVAM's DataBase for ALternative Methods (DB-ALM) as "DB-ALM Protocol n°140" at the address: http://ecvam-dbalm.jrc.ec.europa.eu. It provides a comprehensive description of the method together with all the necessary technical details needed by an end-user laboratory to implement the ZFET in a self-sufficient manner.

Reproducibility based on the 20 chemicals tested in the OECD validation study

a) Within laboratory reproducibility (96 h exposure)

A total of 85 coefficients of variation (CV) were calculated using the LC_{50} values (n = 3) derived for each chemical by each laboratory. In four cases, the CVs could not be calculated since two laboratories provided data for only two runs (3,4-dichloraniline in Phase 1) or LC_{50} values could not be calculated due to low toxicity observed in two laboratories for one chemical (prochloraz tested at its solubility limit in Phase 2). In general, the within laboratory reproducibility was considered good. The CVs range from 1.3 to 38% with the vast majority of CVs (71) below 20%, eight CVs between 20 and 30%, and 6 between 30 and 40% (OECD 2011; OECD 2012).

b) Between laboratory reproducibility (96 h exposure)

Mean LC_{50} values derived for each chemical were compared between laboratories (at least three/chemical) and the calculated CV used as a measure of between laboratory reproducibility for that chemical. The CVs range from 1.8 to 56.3%. In general, the between laboratory reproducibility was considered good with CVs below 30% for 15 chemicals. The lower reproducibility (CV > 30%) observed for five chemicals might be explained with the properties (high volatility, low solubility, high molecular weight) of three chemicals, whereas for two chemicals it might be linked to their high acute toxicity, since relatively small differences in the LC_{50} values close to 0 were magnified and resulted in a larger CV (OECD 2011; OECD 2012).

Hrovat *et al.* (2009) systematically investigated fish acute toxicity data contained in the US EPA ECOTOX database and revealed a significant variability of 96 h LC_{50} values spanning over three orders of magnitude when considering only rainbow trout as test species and over six orders of magnitude when considering other fish species.

Transferability

The ZFET was successfully transferred from the lead laboratory to the 10 laboratories participating in the validation study. Laboratories intending to conduct the test on a routine basis should have expertise in the maintenance and breeding of zebrafish or have immediate access to newly fertilised zebrafish eggs. It is strongly recommended that the standardised protocol is followed. The OECD TG236 and the DB-ALM Protocol n°140 include guidance on the maintenance and breeding[1] of zebrafish and a catalogue of images on normal development of zebrafish and the lethal endpoints.

Predictive capacity

A comparison of fish acute toxicity data (96 h; five freshwater species recommended in OECD TG203) and fish embryo acute toxicity data (24-120 h; mainly zebrafish) collected for 144 chemicals from various sources demonstrated a strong correlation (r = 0.9). An even stronger correlation (r = 0.95) was evident when comparing 96 h acute fish toxicity data to 96 h fish embryo test data available for 72 chemicals. Moreover, fish embryo toxicity data fall within the variability of the fish inter-species comparisons performed on the same dataset (Belanger *et al.* 2012; 2013). This indicates that the ZFET is suitable for predicting effects in species other than zebrafish and can provide information on acute fish toxicity comparable to that of the standard test (OECD TG203).

Notably, the chemicals evaluated in the retrospective analysis covered a broad range of physico-chemical properties, toxicological modes of action, and functional use, *e.g.* industrial chemicals (77), plant protection products (21), surfactants (15), pharmaceuticals (8), and biocides (5) thereby demonstrating the wide applicability domain of the ZFET (Belanger *et al.* 2012; 2013).

4. Potential limitations

There are two potential limitations of the ZFET that are addressed in OECD TG236 under "Initial considerations":

Metabolism: Since it is not entirely clear to what extent the metabolic capacity of the zebrafish embryo corresponds to that of the juvenile and adult fish, *i.e.* whether the metabolic profile is comparable, OECD TG236 states *"Concerning substances that may be activated via metabolism, there is evidence that zebrafish embryos do have biotransformation capacities (19)(20)(21)(22)[2]. However, the metabolic capacity of embryonic fish is not always similar to that of juvenile or adult fish. For instance, the protoxicant allyl alcohol (9)[2] has been missed in the FET. Therefore, if there are any indications that metabolites or other transformation products of relevance may be more toxic than the parent compound, it is also recommended to perform the test with these metabolites / transformation products and to also use these results when concluding on the toxicity of the test chemical, or alternatively perform another test which takes metabolism into further account."* To date, the only case reported in the literature indicating that zebrafish embryos may not have the same levels of enzymes as juvenile or adult fish, is allyl

[1] Support on zebrafish maintenance and breeding as well as health service can be obtained from Zebrafish International Resource Center (ZIRC), University of Oregon, Eugene, OR, USA and the European Zebrafish Resource Center (EZRC), Karlsruhe Institute of Technology, Karlsruhe, Germany.

[2] 19 = Weigt *et al.* 2011; 20 = Weigt *et al.*, 2012; 21 = Incardo *et al.* 2011; 22 = Kubota *et al.* 2011; 9 = Knöbel *et al.* 2012.

alcohol which is transformed to the more toxic acrolein by juvenile or adult fish but not by fish embryos (Knöbel *et al.* 2012; Klüver *et al.* 2014).

Reduced bioavailability: There is evidence that chemicals with a high molecular weight (≥3kD) or bulky structure do not pass the chorion while some chemicals may delay hatching. The reduced bioavailability of such chemicals over the full exposure period may result in lower toxicity. Therefore, OECD TG236 states *"For substances with a molecular weight ≥3kDa, a very bulky molecular structure and substances causing delayed hatch which might preclude or reduce the post-hatch exposure, embryos are not expected to be sensitive because of limited bioavailability of the substance, and other toxicity tests might be more appropriate."*

5. Suggested regulatory use

The validation study showed that the ZFET can be used to determine acute fish toxicity. When considering the use of the ZFET instead of the traditional juvenile/adult fish acute toxicity test (OECD TG203), the potential limitations of the ZFET (section 4) should be taken into account.

In Europe, acute fish toxicity data are required for the hazard assessment of industrial chemicals (>10t/year; REACH Regulation 1907/2006; EC 2006), biocidal products (Regulation 528/2012, EU 2012), plant protection products (Regulation 1107/2009, EC 2009a; data requirements outlined in Regulation 283/2013, EU 2013a; Regulation 284/2013; EU 2013b), veterinary pharmaceuticals (CVMP/VICH/790/03, EMA 2004), and others (*e.g.* feed additives; Regulation 429/2008, EC 2008; further specified in EFSA 2008). Aquatic toxicity is not an endpoint considered in the cosmetics regulation (Regulation 1223/2009, EC 2009b); however, environmental concerns of cosmetics ingredients and products are considered through REACH. Acute fish toxicity is usually determined with OECD TG203 ("Fish, Acute Toxicity Test"; OECD 1992) or OECD GD126 ("Short Guidance on the Threshold Approach for Acute Fish Toxicity"; OECD 2010).

Noteworthy, the REACH guidance on acute aquatic toxicity, first published in 2008 (ECHA 2012), outlines a testing strategy for chemical safety assessment (PNEC derivation), which includes a placeholder for validated alternative methods to the acute fish toxicity test. The ZFET is specifically mentioned as a possible alternative provided that it is fully validated and available as a standardised method (e.g. OECD test guideline), a requirement now met by the availability of OECD TG236 "Fish embryo acute toxicity (FET) test".

Additionally, the ZFET can be used for range-finding tests to determine the appropriate concentration range for higher tier tests, *e.g.* chronic fish toxicity tests (see OECD TG210, OECD 2013b), thus avoiding the use of juvenile or adult fish for this purpose.

Impact on 3Rs of the suggested regulatory use

As per Article 1(3)(a)(i) of Directive 2010/63/EU (EU 2010) on the protection of animals used for scientific purposes, live non-human vertebrate animals including independently feeding larval forms are covered by its scope. According to the description of OECD TG236, the zebrafish embryos are used until 96 h post-fertilisation. Zebrafish is generally not considered as being capable of independent feeding until five days post-fertilisation. This is confirmed by the Commission Implementing Decision 2012/707/EU (EU 2012b) on a common format on collection of information on the use of animals for scientific purposes in the EU states that *"Fish should be counted from the stage of being capable of independent feeding onward. Zebrafish kept in optimal breeding conditions (approximately + 28°C) should be counted 5 days post fertilisation".*

Considering the foregoing, the embryos in question should not be considered as "independently feeding larval forms" within the meaning of the Directive and therefore the procedure, as far as the embryos are concerned, does not fall within its scope.

The use of the ZFET will result in an overall reduction of the numbers of juvenile and adult fish required for aquatic toxicity testing.

6. Follow-up activities recommended by EURL ECVAM

a) Where appropriate, the ZFET (OECD TG236) should be used for generating information on acute fish toxicity. In case the ZFET cannot be used, information on acute fish toxicity should be derived with the threshold approach following OECD Guidance Document 126 (OECD 2010).

b) To support the use of the ZFET (OECD TG236), it should be included into the respective regulations and associated guidance documents, e.g. industrial chemicals (REACH Regulation 1907/2006; EC 2006 and endpoint specific guidance, ECHA 2012), biocidal products (Regulation 528/2012, EU 2012a), plant protection products (Regulation 1107/2009, EC 2009a and associated data requirements outlined in Regulation 283/2013, EU 2013a; Regulation 284/2013; EU 2013b), veterinary pharmaceuticals (CVMP/VICH/790/03; EMA 2004), and feed additives (Regulation 429/2008, EC 2008; further specified in EFSA 2008).

c) In case of the REACH guidance on aquatic toxicity (ECHA 2012), the testing strategy for chemical safety assessment (e.g. derivation of Predicted No-Effect Concentration [PNEC derivation]) includes a placeholder for validated alternative methods. In this context the ZFET is mentioned as a possible alternative to the acute fish toxicity test provided that it is fully validated and available as a standardised method (e.g. OECD test guideline). This requirement can now be considered satisfied due to the availability of OECD TG236 "Fish embryo acute (FET) toxicity test" and thus the REACH guidance document should be updated accordingly.

d) An OECD guidance document on the use of OECD TG236 across the various regulatory frameworks and regions should be developed. It should particularly address the possible use of the ZFET to generate information on acute fish toxicity and its potential limitations.

e) The database containing fish embryo acute toxicity data and fish acute toxicity data (Belanger *et al.* 2012; 2013) should be maintained and updated on a regular basis. This will provide additional insight into the practical use of the ZFET and enhance confidence in the applicability domain. As noted by Belanger *et al.* (2012; 2013), it was not possible to find acute fish toxicity data for all chemicals for which fish embryo acute toxicity data were available. For example, for only eight out of the 22 pharmaceuticals, acute fish data could be retrieved. Therefore, industry and regulators are encouraged to make existing data available where possible.

f) Further effort should be invested in the development and validation of methods that avoid the use of fish for environmental hazard and risk assessment. The use of Adverse Outcome Pathways to aid in the design of integrated approaches, methods based on fish cells or fish embryo tests are some of the areas that deserve investigation in the context of fish toxicity testing.

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Annex 1 - ESAC Opinion

Opinion of the EURL ECVAM Scientific Advisory Committee (ESAC) on the validity (reliability and relevance) of the zebrafish embryo toxicity test for acute aquatic toxicity testing

Ispra, 15 March 2013

Summary of the ESAC opinion

The ESAC was asked to provide an opinion on the transferability and within- and between-laboratory reproducibility of the zebrafish embryo toxicity test (ZFET) for acute aquatic toxicity testing in view of its possible future regulatory use (prospective study). The ESAC was also asked to provide an opinion on the suitability of the ZFET for acute aquatic toxicity testing as a potential alternative to the acute fish toxicity taking into account the data collected by Belanger and colleagues (retrospective study).

Prospective Study

The goal of the prospective study was to evaluate the transferability and within- and between-laboratory reproducibility of the zebrafish embryo toxicity test (ZFET), for which an OECD draft guideline (status May 2008) and a draft standard operating procedure (SOP) already existed. The draft SOP was provided by the lead laboratory, which has significant experience in the ZFET and was instrumental in developing the first draft OECD guideline. Prior to the start of the prospective study, the validation management group (VMG) reviewed the SOP and incorporated the concerns expressed by the OECD ad hoc expert group FET (a group established by OECD to develop the new OECD guideline "Fish embryo test"). Moreover, during Phase 1a the pre-saturation of exposure vessels and daily renewal of test concentrations so as to promote establishment of chemical equilibrium during exposure was introduced into the SOP. In Phase 1a of the prospective study, the SOP was transferred to seven participating laboratories, all testing one chemical (3,4-Dichloroaniline; 3,4-DCA), which served as a positive control throughout. The transfer of the SOP was successful, with promising intra- and inter-laboratory reproducibility. In addition, a test concentration for the positive control was derived for which a minimum of 30% lethality over 96 h can be expected (in contrast to the 10% lethality initially required in the OECD draft guideline, which is difficult to distinguish statistically from a lack of lethality). Then in Phase 1b, six laboratories were involved in testing six additional chemicals, which were selected based on a wide range of toxicity.

Overall, the ESAC has the opinion that the ZFET was successfully transferred to the participating laboratories with very good within- and between-laboratory reproducibility for five of the six chemicals. The lower reproducibility of the sixth chemical was attributed to its high volatility and indeed, significantly lower test concentrations were analytically confirmed. The goal of the second phase of the prospective study was to increase confidence in the very good within- and between-laboratory reproducibility by testing 13 additional chemicals. In Phase 2a, newly joining laboratories went through a training phase using 3,4-DCA as in Phase 1a before. Then, nine laboratories, trained either in Phase 1a or Phase 2a, were involved in testing the 13 additional chemicals. These chemicals were selected based on rational criteria, which included, aside from a wide range of toxicity, different physical-chemical properties and modes of toxic action. As well, a coefficient of variation (CV) below 30% was agreed upon as an acceptable within- and between-laboratory reproducibility. The testing results confirmed that the ZFET SOP could be transferred successfully to new laboratories and that the within- and between-laboratory reproducibility is indeed generally

below 30%. A higher CV for three chemicals was attributed to very steep concentration-response curves. Moreover, analytical measurements were performed in two laboratories, demonstrating that (i) quantitative chemical analysis can be performed despite the small testing volumes, and that (ii) chemicals with a combination of low water solubility, high biodegradability and volatility comprise the most challenging to test.

In summary, the ESAC concludes that the scientific work presented for the prospective study is of very high quality. The rational for the testing design and chemical selection was well described and the result thoroughly evaluated. The conclusions drawn are very well justified and supported by the data. A minor weakness in the report was the inconsistent assignment of toxicity classes to the chosen chemicals, likely due to the utilisation of different sources of information.

Retrospective Study

The retrospective analysis provided by Belanger et al. (2012) comprised an exhaustive update of the correlation of the fish embryo toxicity test and the acute fish toxicity test. It takes into account previously published comparisons (e.g., the review by Lammer et al. (2009) on the ZFET) but adds any information available until the completion of the report (Feb/2012): peer-reviewed and open literature for the ZFET and fish embryo tests (FET) data obtained for other species of fish; and data made available by a number of groups prior to peer-reviewed publication. In fact, the final database for evaluating the relationship between the FET and the acute fish toxicity consisted of 985 FET studies on 229 compounds (dominated by ZFET) and of 1532 fish acute toxicity studies on 151 compounds (dominated by rainbow trout, fathead minnow, bluegill sunfish), presenting more than double the size of the information presented in Lammer et al. (2009). The analysis was largely based on orthogonal regression analysis, which takes uncertainty of both the FET and the fish acute toxicity data account. The analysis showed that (i) the ZFET basically responds to chemical exposure like a fish in the acute fish toxicity test – the regression lines were statistically indistinguishable, meaning that the ZFET data can be taken directly to establish the acute toxicity to fish; (ii) even though data are sparse for FETs with other species, data available thus far look similarly promising; (iii) the very good correlations hold true despite chemicals having a wide range of physical-chemical properties, toxicities, modes of toxic action, and substance classes, thus revealing a broad applicability.

In summary, the ESAC concludes that the scientific work presented for the retrospective study is also of very high quality and that the results strongly support the application of the ZFET as an alternative to the acute fish toxicity test. Embryos of other species may be considered as alternatives as well although more studies should be conducted to confirm this. The presented analysis overall was very robust, partly because of the high number of data points; in general, no class of chemical revealed an exceptional difference to the predictivity of the method.

1. Mandate of the ESAC

On its 36th meeting on 20-21 March 2012, the ESAC was requested by EURL ECVAM to conduct a scientific review on the validity (reliability and relevance) of the zebrafish embryo toxicity test for acute aquatic toxicity testing (see ANNEX 2).

The opinion of the ESAC should provide expert advice to the EURL ECVAM on the study coordinated on behalf of the OECD in view of assessing whether the zebrafish embryo toxicity test is transferable to other laboratories and reproducible within- and between-laboratories and may therefore be fit for future regulatory use (prospective study).

Taking into account the data collected by Belanger and colleagues, the ESAC should further provide an opinion on the suitability of the zebrafish embryo toxicity test for acute aquatic toxicity testing as potential alternative to the acute fish toxicity test (retrospective study).

In particular, ESAC was requested to address the following three questions and issues for achieving the objective of the advice (see EURL ECVAM's request for ESAC advice, Annex 2):

1.) Design and conduct of the study

(a) Clarity of the definition of the study objectives, (b) appropriateness of the study design for the prospective part, (c) appropriateness of the study design for the prospective part, (d) appropriateness of the statistical analysis used in both part of the study.

2.) Conclusions of the study

(a) Is the ZFET protocol sufficiently detailed in view of supporting its standardised use?, (b) Is the ZFET reproducible within- and between laboratories?, (c) Are the conclusions on predictive capacity of the ZFET justified and plausible?, (d) Do the results provide new information on the applicability and possible limitations of the ZFET?, (e) Are there possible gaps between study design and study conclusions which remain to be addressed in view of its proposed use of the test method?

3.) Potential regulatory use of the test method

2. Detailed opinion of the ESAC

Taking into account (a) the detailed review of the ESAC WG, (b) the information made available to ESAC by EURL ECVAM including the VSR and other relevant information, (c) the ECVAM request for ESAC advice outlining review questions (Annex 2) the ESAC has the following opinion:

2.1 Background, regulatory and scientific rationale

Acute aquatic toxicity testing is an essential part of the environmental hazard and risk assessment of chemicals, plant protection products, biocides, pharmaceuticals for veterinary use, feed additives and others. The required tests cover the three trophic levels plants, invertebrates and vertebrates and are carried out on algae, crustaceans (*e.g. Daphnia*) and fish. The acute fish toxicity test (OECD Test Guideline 203; TG 203) is a short-term exposure test (96h) and determines the concentration which is lethal to 50% of the fish (LC50). The fish embryo toxicity test constitutes a potential alternative test method to the acute toxicity tests with juvenile and adult fish, thus providing a reduction in fish usage. In autumn 2005, the German Federal Environment Agency (UBA) submitted a draft TG on "*Fish Embryo Toxicity (FET) Test*" to the OECD. In May 2008, the OECD asked ECVAM to coordinate the "*ZFET Performance Validation Study*" (prospective study) and the validation management group (VMG) was established in November 2008. The objective of the prospective study was to assess the transferability and the within- and between-laboratory reproducibility of the ZFET. All together, 20 chemicals covering specific areas of use (chemicals, pharmaceuticals, pesticides, biocides), a wide range of toxicity and various modes of action were tested.

In 2009, Lammer and co-workers demonstrated that the FET correlates well with acute fish toxicity tests by re-evaluating existing data (Lammer *et al*, 2009). To gain more insight into the suitability of the FET as an alternative model, S. Belanger and colleagues continued to search the scientific literature and other qualified sources to compare FET and acute fish toxicity for as many chemicals as possible. A total of 985 FET studies (229 chemicals) and 1531 acute fish toxicity studies (151 chemicals in common with the FET) were found and analysed (retrospective study). FET-fish acute toxicity regressions were performed to understand the potential relationships or biases based on physical-chemical properties, species choices, duration of exposure, chemical classes, chemical functional uses, and modes of action.

2.2 Design and conduct of the study

2.2.1 Definition of the study objectives

The ESAC judged that the objectives of the study were clearly articulated in the VSR. The study design of the prospective and the retrospective part of the validation study were well thought through and planned accordingly.

The objective of the prospective study was to assess the transferability and the within- and between-laboratory reproducibility of the ZFET. The objective of *the* retrospective study was to assess the suitability of the FET as an alternative model by relating FET and acute fish toxicity (AFT).

2.2.2 Study design for the prospective part

General study design: The ESAC judged the design of the study as appropriate for evaluating transferability, intra-laboratory reproducibility, and inter-laboratory reproducibility. Assessing the predictive capacity of the ZFET was not an objective of the prospective study. Transferability was assessed in two steps in Phase 1. First, the SOP was transferred using one chemical to see if any amendments to the SOP were needed (the chemical was 3,4-DCA, which was used throughout as a

positive control). Subsequently, to further assess the transferability and also to assess intra- and inter-laboratory reproducibility six additional chemicals were tested in Phase 1 followed by 13 chemicals in Phase 2. For each phase acceptance criteria were well defined in the SOPs. Prior to statistical analysis, the data underwent a quality check by the coordinator and by an independent person, who checked whether complete information was provided and whether the runs met the acceptance criteria as described in the SOP.

Chemical selection: For Phase 1 of the prospective study no detailed selection criteria were included in the VSR. Indeed, it was only stated that "Chemicals were selected based on the recommendations of the ad hoc Expert Group (see Minutes of the meeting in May 2008). Nevertheless, though the criteria for chemical selection in Phase 1 were not presented in detail, chemicals appeared to be chosen pragmatically to cover different classes and a range of toxicities, and the ESAC believes that the choices are fully acceptable. The criteria utilised for selection of chemicals for Phase 2 of the study are well documented (see Annex II, Phase 2 report), and the ESAC judged the criteria for selecting the chemical very appropriate for Phase 2 of the study. Overall, 20 chemicals (including the positive control) were tested in eleven participating laboratories covering specific areas of use, a wide range of toxicity and various modes of action. The ESAC considered the number of test items and the number of independent runs/laboratory per chemical (N=3) sufficient to draw conclusions on the reproducibility. The distribution of chemicals ensured that each chemical was tested in at least 4 (Phase 1) or at least 3 (Phase 2) laboratories.

2.2.3 Study design for the retrospective part

The ESAC judged the analysis of the correlation between FET and AFT (provided by the document authored by Belanger et al. 2012) as extremely wide-ranging and thorough. Data inclusions and exclusions were described in detail. Sound rational explanations were always given. In particular, the ESAC judged the design of the database as appropriate to allow conclusions on the suitability of the FET as an alternative model for acute fish toxicity testing. Indeed, the database covered: a large chemical domain, specific areas of use (chemicals, pharmaceuticals, pesticides, biocides), a wide range of toxicity, various modes of action, chemicals with different physical-chemical properties, data from different species of fish (OECD 203-based acute fish toxicity data).

2.2.4 Statistical analysis used in both part of the study

The ESAC judged the chosen statistical methods for evaluating data of both the prospective and retrospective study to be appropriate. For the prospective study, LC50 values were determined for 48 h and 96 h exposure, by logistic regression while confidence intervals were calculated using the delta method (Phase 1a). For Phases 1b, 2a and 2b, LC50 values were calculated by logistic regression (two parameter logistic function or, in some problematic cases, three parameter logistic regression). Confidence intervals were calculated using the profile likelihood method. The reports state that LC50 values were calculated for 48 h and 96 h following the recommendations of the OECD Guidance 54 in the statistical analysis of ecotoxicity data (OECD, 2006). Also for the retrospective study the statistical approach was very well explained and justified. Orthogonal regression was used to fit the linear relationship between the two experimental methods, thus adjusting for measurement errors. By applying this approach, both the variability between the FET and fish acute toxicity from different species and between acute toxicity data of different fish species could be tested.

2.3 Study results and conclusions

2.3.1 Standardised use of the ZFET protocol

The ESAC considers the SOP used in the validation study during the different phases as sufficiently detailed and complete for standardised use. Several modifications of the ZFET protocol which were well documented in the VSR were performed before the start and during the study.

2.3.2 ZFET within- and between laboratory reproducibility

Within-laboratory reproducibility: Within-laboratory reproducibility (WLR) was assessed by the analysis of the coefficient of variation (CV). The ESAC confirms that the conclusions drawn are justified by the data. In agreement with the VMG the ESAC judges that the WLR of the ZFET is generally acceptable at 48 h and 96 h. Most compounds had intra-laboratory coefficients of variations below 30% (acceptance criterion set by the VMG). Plausible reasons were suggested for compounds with CVs above 30% (steep dose-response curves, physical-chemical properties). Overall, the WLR (measured as coefficient of variation) was <30% for 14 compounds. At 48 h the WLR was > 30% for 3 chemicals (6-Methyl-5-hepten-2-one, 4,6-Dinitro-o-cresol, Tetradecylsulfate). For two substances (Merquat 100 and Luviquat HM 552) no CV could be calculated due to insufficient toxicity. At 96 h the WLR was >30% for 6 chemicals (Triclosan, 6-Methyl-5-hepten-2-one, 2,4-Dinitrophneol, Merquat 100, Tetradecylsulfate, Malathion).

Between-laboratory reproducibility: In Phase 2b of the prospective study 9 laboratories tested a sub-selection from 13 compounds. Evaluation of the data is complicated by the fact that only one laboratory tested all the compounds, the other laboratories tested between 3-7 compounds from the 13. Each compound was tested 4 times except for methylmercury chloride which was tested in three independent runs in three labs. The target for acceptability was an inter-laboratory variability (CV) of less than 30%. This was exceeded for four compounds. These compounds were at the higher end of the toxicity spectrum and it is likely that the steepness of the dose-response curve for such compounds makes a reproducible LC50 more challenging. Overall, the ESAC was of the opinion that the between-laboratory reproducibility was acceptable.

2.3.3 Conclusions on the predictive capacity of the ZFET

Prospective part: Assessing the predictive capacity of the ZFET was not an objective of the prospective study. However some conclusions on the predictive capacity were drawn by the VMG Overall, the ESAC agreed that the data are strong enough to justify the conclusion on the predictive capacity of the ZFET. Nevertheless, the ESAC was concerned that use of the chemical classification values as an aid to evaluating predictivity was a source of confusion. As these classification values are bounded by cut-offs, it is important to ensure that they are used consistently. This has not been done in the VSR, Phase 2, and means that there are small inconsistencies between Tables 1 (p. 29) and 20 (p.53). It is debateable whether these regulatory classifications (with their arbitrary boundaries) add anything to the analysis of the data as the choice of "representative" LC50 values can lead to different classifications. Therefore, evaluating the data in terms of correctly predicting the toxicity "class" can be very misleading.

Retrospective part: The retrospective study was designed specifically to look at predictivity and used orthogonal regression analysis which takes into account the variability in the reference data and the experimental data. In this way it was possible to look at the predictivity with 151 chemicals without needing to decide the "correct values" for the reference compounds. From this analysis with all compounds a correlation coefficient of 0.9 was derived with a slope close to 1 (95% C.I. 0.95-1.11) and an intercept close to the origin. Similar comparisons were made between different species

in the fish database and revealed differences in species sensitivity (rainbow trout most sensitive). The zebra fish embryo toxicity data fell within the variability of the fish inter-species comparisons. Correlations were examined between classes of chemicals and, in general, no class of compounds was revealed an exceptional difference to the predictivity of the method. In general, as might be expected, the correlation was less robust for classes of compounds with fewer examples.

2.3.4 Applicability and possible limitations of the ZFET

The ESAC concludes that the data obtained in the prospective as well as retrospective part of the study provides new information on the applicability and the possible limitations of the (Z)FET. The study designs in both the prospective and retrospective study were intended to cover a broad range of chemistry from both the use category and the toxicity perspectives. As a result, there is no obvious gap in the applicability domain, even if some categories may have relatively few examples. It is reasonable to conclude on the basis of this data that the (Z)FET has a broad applicability domain with no identified exclusions, provided that it is run for 96 h and exposure concentrations are verified.

2.3.5 Identified gaps between study design and study conclusions

Inasmuch as the prospective study had to rely on voluntary participation of the testing labs, not all labs tested all the chemicals, leading to a complex matrix of test results. Nevertheless, statistics was appropriately done and the fact that more labs were involved can also be interpreted as strength in that the very good overall results were obtained despite this difficulty. For future validation studies, the ESAC feels that it would be important to provide funding and to establish legal contracts for round-robin tests.

Despite its thoroughness, the retrospective study did not consider volatility (logH) as a parameter (both for the spread in terms of applicability domain and for investigating its influence on fish-fish embryo relationships [as was done for solubility and hydrophobicity (focussing on logKow)]). Impact of test results due to volatility might be mitigated in the testing design used in the prospective study by the pre-saturation of plates and daily exposure solution renewal. Consideration is nevertheless important because neither plate sealers nor completely closed test vessels with a headspace can completely abolish losses due to evaporation (Schreiber et al., 2008; Knoebel, Scholz, Schirmer, personal communication).

2.4 Potential regulatory use of the test method

The FET using zebrafish (i.e., the ZFET) for 96 h constitutes an alternative test method to the acute toxicity tests with juvenile and adult fish, i.e., the OECD Test Guideline 203 (OECD TG 203, 1992) and similar guidelines thus providing a reduction in fish usage. An OECD guideline for the ZFET is currently under development and it would be important to finalise this guideline, which is currently being circulated as draft guideline (updated December 2012), as soon as possible. Once the guideline is finalised, the ZFET would be ready for regulatory use. As the prospective and retrospective studies show, it has been thoroughly evaluated for a wide range of chemicals with different physical-chemical properties and modes of toxic action; as well, transferability has been shown with acceptable within- and between-laboratory variability (see p. 54 VSR, Phase 2). The new OECD guideline could make its way into many testing schemes, including industrial chemicals, agrochemicals, veterinary pharmaceuticals, biocides, effluent testing. With regard to testing effluents for acute toxicity, an ISO guideline (DIN EN ISO 15088, called the fish egg test) already exists. It has been the basis of the first draft for the FET OECD guideline submitted to OECD by the

German Environmental Protection Agency (UBA) in 2006 and is accepted as replacement of the acute fish toxicity test for effluent testing in Germany since 2005.

The application of the FET in a regulatory context should be considered with regard to the OECD fish toxicity testing framework (OECD Fish Toxicity Testing Framework 2012), which suggests reduction of use of fish tests on several levels (e.g., use of invertebrate and algae/aquatic plant EC50 values, application of limit tests). On page 150, reference is given to the FET as an alternative to the fish acute toxicity test: "…There may, furthermore, be scope to use the draft Fish Embryo Test (OECD 2006a), although this test has not yet been fully evaluated."

Thus, with the thorough evaluation now provided by the prospective and retrospective studies in particular for the ZFET, specific areas of regulatory use, for which reference to the FET is already given, include:

- Classification and labelling:

  see Guidance for implementation of REACH: "Guidance on information requirements and chemical safety assessment" Chapter R.7b, page 43, where it is stated that, in case a fish acute toxicity test would be required (i.e., if data on aquatic invertebrates and plants/algae indicate EC50 values > 1 mg/L and a QSAR values for fish acute toxicity cannot be convincingly obtained):

  "…if alternative and adequate test methods are available for the acute fish toxicity they may be considered to be used instead for classification… E.g., a proposal to use the fish embryo test (FET) as an alternative to the acute fish toxicity test has been made and is currently under evaluation in the OECD Guideline program…". [Note: reference is also given in the associated Figure R.7.8-3 on page 45]

- Chemical safety assessment of industrial chemicals (REACH):

  see Guidance for implementation of REACH: "Guidance on information requirements and chemical safety assessment" Chapter R.7b, page 49, where it is stated that:

  "If there is a need to generate new data on the toxicity in fish and an accepted alternative method is available instead of in vivo fish testing perform the alternative test… A possible alternative, the fish embryo toxicity test, is currently under evaluation in the OECD Guideline program…" [Note: reference is also given in the associated Figure R.7.8-4 on page 53]

  One issue requiring discussion in the context of regulatory use is the performance of the embryo test under GLP. It is thus far not common practice to run this test under GLP conditions; however, for regulatory use, compliance to GLP quality control should become standard.

2.5 Recommendations

Analytical confirmation of exposure concentration: The ESAC recommends that analytical confirmation of exposure concentrations should be an explicit requirement, in fact, for both the ZFET as well as the OECD 203 for acute fish toxicity. The ESAC has the opinion that considering measured exposure concentrations is particularly important for aqueous exposure assessments because it will be used to deduce environmentally safe concentrations. The ESAC appreciates that the statements made in the ZFET draft OECD guideline (Version December 2012) regarding chemical concentration verification for the ZFET are much stronger than in the OECD 203 testing guideline. However, the ESAC strongly recommends harmonizing the requirements for chemical concentration verification in both guidelines, the ZFET draft OECD guideline and the OECD 203 guideline for acute fish toxicity. It would be important to have the same level of quality assurance with regard to chemical concentration verification in both guidelines.

Test duration: Based on the results for high molecular weight compounds (which elicited toxicity only if test duration was extended beyond hatch), the ESAC supports the strategy to run the ZFET for 96 hours.

Prediction model and predictive performance: According to the retrospective study, where the correlation of FET and fish acute toxicity is non-discernible from the line of unity, a prediction model is not required, and this is true independent of the fish species used in the acute exposure tests. One has to keep in mind, though that the FET studies are still dominated by zebrafish (*Danio rerio*); however, the retrospective study also shows that a similar level of correlation can be found for, e.g. embryos of fathead minnow (*Pimephales promelas*).

Improvements of SOPS and draft OECD guideline for the ZFET:

The ESAC recommends to keep 3,4-DCA as the positive control, since it has been used in the validation exercise. Otherwise, criteria have to be given for the selection of benchmark chemicals or a list of benchmark chemicals used for positive control needs to be established.

The OECD draft guideline states to use self-adhesive foil to cover the 24-well plates or vapour-dense lids provided with plates; while this is certainly useful, one has to consider that none of these measures really prevents loss due to evaporation of volatile compounds. Similarly, the use of glass instead of plastic "in case adsorption to polystyrene is suspected" can maybe lower losses due to adsorption but not abolish them. It should, however, be noted that these technical issues, i.e. volatility of substances and adsorption of substances to experimental glassware used, are not unique to the ZFET assay but of more general concern and may be applicable to in vivo as well as in vitro test systems.

In the prospective study, certain procedures were implemented from the start of the study based on recommendations by the OECD ad-hoc working group. These concerned the number of embryos per concentrations (20) and the exposure time beyond hatch up to 96 h. More changes were implemented later on during the study based on decisions taken by the VMG during the study, such as pre-saturation of well plates and daily exposure medium renewal. Based on the data and reports provided, the ESAC fully supports these changes of the protocol, which have also been included in the OECD draft guideline for the ZFET (status March 2013).

3. References

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ESAC and ESAC Working Group charged with the scientific review

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Dr. Marlies HALDER (Project Leader (Z)FET Study) and Dr. François BUSQUET

Annex 2 – EURL ECVAM Request for ESAC Advice

EURL ECVAM request for ESAC advice on the validity (reliability and relevance) of the zebrafish embryo toxicity test for acute aquatic toxicity testing

1. TYPE OF REQUEST

Request Type		**Identify request ("YES")**
1. ESAC Peer Review of a Pre-validation Study or Validation Study		**YES**
If 1) applies please specify further:		
Prevalidation Study	NO	
Prospective Validation Study	The prospective part of the study was designed to assess the transferability, within- and between-laboratory reproducibility of the Zebrafish Embryo Toxicity Test (ZFET) in view of supporting the further development of the draft OECD Test Guideline on "Fish Embryo Toxicity Tests"	
Retrospective Validation Study	The retrospective part of the study addresses the predictive capacity of fish embryo tests (incl the ZFET), i.e. their relevance to predict acute fish toxicity. It is based on the collection of fish embryo toxicity and fish toxicity data provided by S. Belanger and colleagues.	
Validation Study based on Performance Standards	NO	
2. Scientific Advice on a test method submitted to ECVAM for validation (e.g. the test method's biological relevance etc.)		NO
3. Other Scientific Advice (e.g. on test methods, me-too tests, performance standards, their use; on technical issues such as cell culturing, stem cells etc.)		NO

2. TITLE OF STUDY OR PROJECT FOR WHICH SCIENTIFIC ADVICE OF THE ESAC IS REQUESTED

The validity (reliability and relevance) of the zebrafish embryo toxicity test for acute aquatic toxicity testing

3. BRIEF DESCRIPTION OF THE STUDY OR PROJECT

Background

Acute aquatic toxicity testing is an essential part of the environmental hazard and risk assessment of chemicals, plant protection products, biocides, pharmaceuticals for veterinary use, feed additives and others. The required tests cover the three trophic levels plants, invertebrates and vertebrates and are carried out on algae, crustaceans (e.g. Daphnia) and fish. The acute fish toxicity test (OECD Test Guideline 203; TG 203) is a short-term exposure test (96h) and determines the concentration which is lethal to 50% of the fish (LC50). In autumn 2005, the German Federal Environment Agency (UBA) submitted a draft TG on "Fish Embryo Toxicity (FET) Test" to the OECD together with a supportive Background Paper. Based on the comments received from the national coordinators of the Test Guidelines Programme (WNT), the OECD decided to establish an ad hoc Expert Group on the Fish Embryo Toxicity Test. During several teleconferences and two face-to-face meetings, the submitted documents were reviewed taking into consideration the scientific basis, reproducibility and predictive capacity of the FET. A thorough re-evaluation of existing data demonstrated that the FET correlates well with acute fish toxicity tests (Lammer et al, 2009). The ad hoc Expert Group noted that most data were available for the ZFET, however, data providing sufficient evidence for the reproducibility of the method were lacking.

Purpose of the study

In May 2008, the OECD asked ECVAM to coordinate the "*ZFET Performance Validation Study*" (prospective study assessing the reliability of the ZFET) and the validation management group (VMG) was established in November 2008. In addition to the prospective study, S. Belanger and colleagues continued to collect FET and fish data to underpin the relevance and predictive capacity of the ZFET as a possible alternative to the acute fish toxicity test (TG 203).

Objective and organisation of the study

Prospective part

The objective of the prospective part of the study was to assess the transferability and the within- and between-laboratory reproducibility of the ZFET. The study design was agreed upon by the VMG and supported by the OECD *ad hoc* expert group. The study was divided into two phases and a total of 20 chemicals were tested, six in Phase 1, 13 in Phase 2, and one in both phases. Each chemical was tested in three independent runs in five different concentrations in at least three laboratories. For a subset of chemicals, confirmatory analytical measurements of stock solutions and test concentrations were performed in two laboratories. The VMG issued for each phase a trial plan, which described the preparation of the chemical stock solutions and stated the

concentrations to be used as a result of the range finding tests carried out in two laboratories. The laboratories returned the reporting templates to the study coordinator, who performed a quality check and compiled the data in an Excel table for statistical analysis. For each chemical, the number of dead embryos/concentration/or control and laboratory, as well as the hatching rates at 24, 48, 72, and 96 h were included.

Retrospective part

To complete the picture of the suitability of the FET as an alternative model, the scientific literature and other qualified sources were searched to compare FET and acute fish toxicity for as many chemicals as possible. A total of 985 FET studies (229 chemicals) and 1531 acute fish toxicity studies (151 chemicals in common with the FET) were found and summarised. FET-fish acute toxicity regressions were performed to understand the potential relationships or biases based on physical-chemical properties, species choices, duration of exposure, chemical classes, chemical functional uses, and modes of action.

Study results and conclusions

Prospective part

Phase 1: The VMG concluded that the ZFET test was successfully transferred from the lead laboratory to the participating laboratories and that the within- and between-laboratory reproducibility of the LC50 values was promising. In general, within-laboratory variability was low while the between-laboratory variability was higher.

Phase 2: In general, the results of Phase 2 confirm the findings of Phase 1. The ZFET was successfully transferred to four new laboratories participating in Phase 2. For nine chemicals, the intra- and inter-laboratory reproducibility of the ZFET is acceptable with coefficients of variation (CV) below 30% regardless of the chemical or the laboratory. For three chemicals, CVs above 30% were calculated. However, a factor contributing to the large CVs is the very high acute toxicity of these three chemicals, since relatively small differences in the LC50 values are magnified and result in a larger CV. As expected, the chorion acted as a barrier for chemicals with high molecular weight, *i.e.* for the two cationic polymers tested with the ZFET some lethality was observed at 48h and LC50s were mostly confined to 96h exposures. It was not possible to find a time-dependant pattern of toxicity for chemical categories other than the above mentioned cationic polymers. For the 13 chemicals tested in Phase 2, the predictive capacity of ZFET for acute fish toxicity is very promising but will need to be underpinned with additional data.

Retrospective part

The FET-fish acute toxicity relationships are very robust with most slopes near 1.0 and intercepts approaching 0 across almost 9 orders of magnitude in potency. A suitable recommendation for the predictive regression relationship is:

log FET LC50 = (0.989*log Fish LC50) -0.195, n = 72 chemicals, r = 0.95, p < 0.001 (LC50 in mg/L)

This regression is restricted to 96h fish and FET data points. A similar and not statistically different regression is available for the entire data set (n=144 chemicals, following deletion of unreliable studies involving 7 compounds). FET-fish regressions were robust for subsets of major chemical classes (neutral organics, aliphatic amines, phenols) for which suitably large data sets were available. Furthermore, regressions were similar to that above for large groups of functional

chemical categories such as pesticides, surfactants, and industrial organics. Pharmaceuticals had a much smaller database (n=8) but trends were directionally correct. FET-acute fish toxicity regressions could not be distinguished from interspecies fish toxicity regressions (fathead minnow, rainbow trout, bluegill sunfish, Japanese medaka, zebrafish) further supporting the predictive nature of the relationship. In summary, the Fish Embryo Test predicts acute fish toxicity exceptionally well. The size of the database encompasses a broad range of chemical classes, modes of action, functional use categories. In relevant statistical aspects, the Fish Embryo Test behaves like the acute fish toxicity test. If regarded as alternative to the acute fish test, the FET will provide nearly equivalent predictions of hazard while improving overall animal welfare.

4. OBJECTIVES, QUESTIONS, TIMELINES

4.1 OBJECTIVE

Objective *Why does EURL ECVAM require advice on the current issue?*	The opinion of the ESAC should provide expert advice to the EURL ECVAM on the study coordinated on behalf of the OECD in view of assessing whether the zebrafish embryo toxicity test is transferable to other laboratories and reproducible within- and between-laboratories and may therefore be fit for future regulatory use. Taking into account the data collected by Belanger and colleagues, the ESAC should further provide an opinion on the suitability of the zebrafish embryo toxicity test for acute aquatic toxicity testing as potential alternative to the acute fish toxicity test.

4.2 QUESTION(S) TO BE ADDRESSED

Questions *What are the questions and issues that should be addressed by the ESAC WG?*	**1) DESIGN & CONDUCT OF THE STUDY** The ESAC is requested to review whether the prospective and retrospective parts of the validation study were conducted appropriately in view of the objectives of the study, i.e. to assess the reliability (transferability, within- and between-laboratory reproducibility) and the relevance (predictive capacity) of the ZFET. In particular the following issues should be addressed: (a) Clarity of the definition of the study objectives. (b) Appropriateness of the study design for the <u>prospective part</u> in view of study objectives, *inter alia*: - Were the criteria for the chemical selection appropriate? - Is the toxicity range of the selected chemicals, their number, the number of independent runs / laboratories per chemical appropriate to allow conclusions on the reproducibility of the ZFET? - Was the execution of the study appropriate (*e.g.* were there pre-defined acceptance criteria for the ZFET, were these respected)? How were exceptions and deviations handled, *e.g.* censoring of values/data, retesting etc? c) Appropriateness of the study design for the <u>retrospective part</u> in view of study objectives, *inter alia*: - Were the criteria for inclusion / exclusion of FET / fish data into the

	comparison appropriate (*e.g.* species, endpoints used in the FET, level of standardisation of the protocols used for FET and fish tests)? - Is the toxicity range of the chemicals, their number, coverage of chemical classes, mode of actions etc appropriate to allow conclusions on the relevance of the ZFET for acute aquatic toxicity testing? d) Appropriateness of the statistical analysis used in both parts of the study. **2) CONCLUSIONS OF THE STUDY** The ESAC is requested to assess whether the conclusions, as presented in the OECD ZFET Validation Study Reports are substantiated by the information generated and are plausible with respect to existing information and current views. Moreover, the ESAC is requested to review whether the information provided by the FET/Fish data compilation supports the potential regulatory use of the ZFET. In particular: (a) Is the ZFET protocol sufficiently detailed in view of supporting its standardised use? (b) Is the ZFET reproducible within- and between laboratories? (c) Are the conclusions on predictive capacity of the ZFET justified and plausible? (d) Do the results provide new information on the applicability and possible limitations of the ZFET? (e) Are there possible gaps between study design and study conclusions which remain to be addressed in view of its proposed use of the test method? **3) POTENTIAL REGULATORY USE OF THE TEST METHOD** The ESAC is requested to advice on the potential regulatory use of the ZFET test method.

4.3 TIMELINES

Timelines concerning this request *When does EURL ECVAM require the advice?*	**Timeline**	**Indication**
	Finalised ESAC Opinion required by:	ESAC 38, 26-27/3 2013
	Request to be presented to ESAC by written procedure (e.g. due to urgency) prior to the next ESAC	**YES**
	Request to be presented to ESAC at ESAC plenary meeting	

5. EURL ECVAM PROPOSALS ON HOW TO ADDRESS THE REQUEST WITHIN ESAC

5.1 EURL ECVAM PROPOSAL REGARDING REQUEST-RELATED STRUCTURES REQUIRED

Specific structures required within ESAC to address the request *Does the advice require an ESAC working group, an ESAC rapporteur etc.?*	**Structure(s) required**	**Required according to EURL ECVAM? (YES/NO)**
	ESAC Rapporteur	NO
	ESAC Working Group	**YES**
	Invited Experts	**YES**
	If yes – list names and affiliations of suggested experts to be invited and specify whether these are member of the EEP	
	If other than above :	

5.2 DELIVERABLES AS PROPOSED BY EURL ECVAM

Deliverables *What deliverables (other than the ESAC opinion) are required for addressing the request?*	**Title of deliverable other than ESAC opinion**	**Required? (YES/NO)**
	ESAC Rapporteur Report and draft opinion	NO
	ESAC Peer Review Report and draft opinion	**YES**
	If other than above :	

6. LIST OF DOCUMENTS TO BE MADE AVAILABLE TO THE ESAC

Count	Description of document	Already available? (YES/NO)	File name
1	OECD monograph 157 – Validation report (Phase 1) for the zebrafish embryo toxicity test, Part 1 (Summary of results; Annexes I-V)	YES	1_OECD ZFET validation Phase 1_part 1.pdf
2	OECD monograph 157 – Validation report (Phase 1) for the zebrafish embryo toxicity test, Part 2 (Annexes VI, VII, VIII and IX)	YES	2_OECD ZFET validation Phase 1_part 2.pdf
3	OECD monograph 179 – Validation	YES	3_OECD ZFET validation Phase 2_part

	report (Phase 2) for the zebrafish embryo toxicity test, Part 1 (Summary of results)		1.pdf
4	OECD monograph 179 – Validation report (Phase 2) for the zebrafish embryo toxicity test, Part 2 (Annexes I-X)	YES	4_OECD ZFET validation Phase 2_part 2.pdf
5	Chemicals tested in the OECD ZFET validation study (Table 1)	YES	5_OECD ZFET validation chemicals.doc
6	OECD ZFET validation tabled results (Table 2 WLR Phase 1; Table 3: WLR Phase 2; Table 4 BLR Phase 1&2)	YES	6_OECD ZFET validation tabled results.xls
7	FET / Fish data comparison (retrospective part as provided by S. Belanger et al)	YES	7_FET-Fish ESAC Peer Review.docx
8	Draft FET TG (version July 2012)	YES	8_2012-07-09_Draft_FET_TG_v8_FINAL.pdf
9	Correction of Annex VI (SOP) in "OECD monograph 179 – Validation report (Phase 2) for the zebrafish embryo toxicity test, Part 2 (Annexes I-X)" (count 4)	YES	9_corr to 4- OECD Phase 2 Annex VI - SOP_ZFET_OECD_V02 10.pdf
10	OECD TG203 – Fish, acute toxicity	YES	10_OECD TG203

7. TERMS OF REFERENCE OF THE ESAC WORKING GROUP

7.1 ESTABLISHMENT OF THE ESAC WORKING GROUP

During its 36th meeting on 21 March 2012 the ESAC plenary unanimously decided to establish an ESAC Working Group charged with the detailed scientific review of a study on the Zebrafish Embryo Toxicity test (ZFET).

7.2 TITLE OF THE STUDY OR PROJECT

Full title: The validity (reliability and relevance) of the zebrafish embryo toxicity test for acute aquatic toxicity testing

Abbreviated title: ZFET

7.3 MANDATE OF THE ESAC WG

The EWG is requested to conduct a scientific peer review of the ZFET test method. The peer review needs to address the questions in Section 4.2 of this request to ESAC by EURL ECVAM. The general template for reporting should be applied.

7.4 REQUESTED DELIVERABLES OF THE ESAC WG

The ESAC WG is requested to deliver to the chair of the ESAC the following two documents:

1) Draft ESAC WG Report detailing its analyses and conclusions

2) Draft ESAC Opinion outlining the key findings and recommendations

The conclusions drawn in the report should be based preferably on consensus. If no consensus can be achieved, the draft Report and Opinion should clearly outline the differences in the appraisals and provide appropriate scientific justifications.

7.5 PROPOSED TIMELINES OF THE ESAC WG

The Secretariat has proposed timelines which should be agreed upon during the first Teleconference (Item 1 in the table):

Item	Proposed date/time	Action	Deliverable
1	Late October 2012	Introductory telephone conference to outline the work needed.	EWG internal work plan and commenting form
2	20-21 November 2012	Meeting of the EWG	Develop EWG Draft Report and Opinion
3	15 February 2013	Approximately 1 month prior to the ESAC38	EWG Draft Report and Opinion

END OF EURL ECVAM RECOMMENDATION

European Commission
EUR 26710 – Joint Research Centre – Institute for Health and Consumer Protection

Title: **EURL ECVAM Recommendation on the Zebrafish Embryo Acute Toxicity Test Method (ZFET) for Acute Fish Testing Toxicity**

Author(s): European Union Reference Laboratory for Alternatives to Animal Testing

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