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EURL ECVAM Strategy to replace, reduce and refine the use of fish in aquatic toxicity and bioaccumulation testing

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Abstract

The assessment of aquatic toxicity and bioaccumulation are important components of the environmental hazard and risk assessment of all types of chemicals and are therefore included in several pieces of European Union and international legislation. In this document, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) outlines approaches which will deliver an impact on the replacement, reduction and refinement (3Rs) of fish tests used for aquatic toxicity and bioaccumulation testing. The document is based on an assessment of the regulatory needs for these endpoints, the scientific state-of-the art and recent activities in these areas. It highlights ongoing efforts at research, validation, guideline development and regulatory level. The proposed strategy is also intended to provide a framework for the prioritisation of alternative test methods submitted to EURL ECVAM for validation. Implementation of the strategy will rely on the coordinated efforts of multiple stakeholders.



EUROPEAN COMMISSION
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Systems Toxicology Unit
EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

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Executive summary

The assessment of aquatic toxicity and bioaccumulation are important components of the environmental hazard and risk assessment of all types of chemicals and are therefore included in several pieces of European Union (EU) and international legislation. Aquatic toxicity refers to the effects of chemicals on organisms living in the water and is usually determined by testing on organisms representing the three trophic levels, i.e. plants (or algae), invertebrates (crustaceans) and vertebrates (fish). Information on bioaccumulation in aquatic organisms is important for understanding the behaviour of a compound in the environment.

Whereas acute aquatic toxicity testing is a basic requirement in most pieces of EU chemicals legislation, chronic aquatic toxicity data may be required when the outcome of the acute testing indicates a risk or when long term exposure is expected. In addition, experimental determination of bioaccumulation may not be necessary if it can be deduced by other means (e.g. consideration of physicochemical properties) that a chemical has a low potential to bioaccumulate.

In this document, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) outlines a strategy based on approaches which will deliver an impact on the replacement, reduction and refinement (3Rs) of fish tests used in the assessment of aquatic toxicity and bioaccumulation. The strategy is based on an assessment of the regulatory needs for these endpoints, the scientific state-of-the art and recent activities in these areas. It is also intended to provide a framework for the prioritisation of alternative test methods submitted to EURL ECVAM for validation.

With regard to aquatic toxicity, the strategy proposes the further development of mechanistically-based replacement alternatives for acute and chronic fish toxicity, as well as the need to revise existing test guidelines to reduce and refine fish testing. Furthermore, the development of guidance on the application of integrated approaches is recommended. This includes the use of data-driven approaches such as interspecies extrapolations, acute-to-chronic relationships and the threshold of toxicological concern approach.

Concerning bioaccumulation, efforts are encouraged for the development and application of *in silico* models such as quantitative structure-activity relationships and physiologically based toxicokinetic models, as well as the standardisation of *in vitro* methods for hepatic metabolism in fish.

EURL ECVAM is focusing its own in-house activities on promoting the use of available alternative methods for fish acute toxicity testing, on exploring the usefulness of scientific approaches to support the waiving of chronic fish tests, and on supporting activities at OECD level.

The achievement of the objectives in pursuit of the strategic aims presented here will depend on the proactive and coordinated engagement of multiple stakeholders.

Abbreviations and Glossary terms

3Rs	Replacement, Reduction, Refinement
ADME	Absorption, distribution, metabolism, and excretion
AOP	Adverse Outcome Pathway
AOP KB	AOP Knowledge Base
BCF	Bioconcentration Factor
BAF	Bioaccumulation Factor
CEFIC	European Chemical Industry Council
CEFIC LRI	CEFIC Long-range Research Initiative
CLP	Classification, Labelling and Packaging of Substances and Mixtures
EC50	The concentration of a chemical showing effect in 50% of the test species during a given observation period
ECHA	European Chemicals Agency
ECx	The concentration of a chemical showing effect in x% of the test species during a given observation period
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ETNCaq	Aquatic Exposure Threshold of No Concern
EU	European Union
EURL ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FELS	Fish early life-stage toxicity test
IATA	Integrated Approaches to Testing and Assessment
ICAPO	The International Council on Animal Protection in OECD Programmes
ICE	Interspecies Correlation Estimation
ILSI HESI	International Life Sciences Institute - Health and Environmental Sciences Institute
JRC	Joint Research Centre
LC50	The lethal concentration of a chemical killing 50% of the test species during a given observation period
LOEC	Lowest Observed Effect Concentration
MOA	Mode of Action
NOEC	No Observed Effect Concentration
OECD	Organisation for Economic Co-operation and Development
PBTK	Physiologically-Based Toxicokinetic
PBT	Persistent, bioaccumulative and toxic substances
PNEC	Predicted No Effect Concentration
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RTgill-W1	Rainbow trout gill cell line
TG	Test Guideline
TTC	Threshold of Toxicological Concern
US EPA	US Environmental Protection Agency
US ERDC	US Army Engineer Research and Development Center
WNT	Working Group of National Coordinators for the OECD Test Guideline Program
ZFET	Zebrafish embryo acute toxicity test

Table of contents

1.	Introduction	1
2.	Background information on fish testing and mechanisms of aquatic toxicity	2
2.1	Current fish tests	2
2.2	Mechanistic understanding of aquatic toxicity	2
3.	Background information on fish bioaccumulation testing.....	3
4.	Proposed strategy to replace, reduce and refine the use of fish in aquatic toxicity and bioaccumulation testing.....	5
4.1	Strategic Aim 1: Replacement and reduction of the use of fish in aquatic toxicity testing	6
4.2	Strategic Aim 2: Replacement and reduction of the use of fish in bioaccumulation testing	11
4.3	Strategic Aim 3: Reduction and refinement of current fish tests	12
5.	Indicative timelines	13
6.	Conclusions.....	14
7.	References	15
	Annex I. EU regulatory information requirements for fish toxicity and bioaccumulation.....	21
	Annex II. Test Guidelines covering fish toxicity and bioaccumulation	24

1. Introduction

The assessment of aquatic toxicity and bioaccumulation are important components of the environmental hazard and risk assessment of all types of chemicals, and are therefore included in several pieces of EU chemicals legislation. These include the Regulation concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; EC, 2006), the Biocidal Products Regulation (EU, 2012a), the Plant Protection Products Regulation (EC, 2009a) and data requirements (EU, 2013a & b), pharmaceuticals (EMA, 2004; EMA, 2006), feed additives (EC, 2008a; EFSA, 2008) and others. In addition, the Cosmetics Regulation (EC, 2009b) states that environmental concerns of cosmetic ingredients and products should be addressed through REACH. Information requirements according to the different regulations are summarised in Annex I.

Aquatic toxicity refers to the effects of chemicals on organisms living in the water and is usually determined by testing on organisms representing the three trophic levels, i.e. plants (or algae), invertebrates (crustaceans such as *Daphnia* spp.) and vertebrates (fish). Information on accumulation in aquatic organisms is important for understanding the behaviour of a compound in the environment. The information on aquatic toxicity and bioaccumulation may be used for classification and labelling (EC, 2008b), the derivation of Predicted No Effect Concentration (PNEC) values for use in risk assessment, and for the assessment of PBT substances. In general, the lowest of the available toxicity values (50% effective concentration [EC_{50}] or 50% lethal concentration [LC_{50}] for acute aquatic toxicity; x% effective concentration [EC_x], Lowest Observed Effect Concentration [LOEC], No Observed Effect Concentration [NOEC] for chronic aquatic toxicity) of the different trophic levels (fish, crustacean, algae or aquatic plants) are used to define the hazard category, derive the PNEC or "Toxicity" criterion. Fish is the preferred species for bioaccumulation testing and derivation of a bioconcentration factor (BCF), although data from tests using invertebrates or reliable BCF prediction models can be used.

Whereas acute aquatic toxicity testing is a basic requirement in most pieces of EU chemicals legislation, chronic aquatic toxicity testing may be required when the outcome of the acute testing indicates a risk, or in the case that long term exposure is expected. Moreover, experimental determination of bioaccumulation may not be necessary if it can be demonstrated that a chemical has a low potential to bioaccumulate, by using physicochemical properties (e.g. $\log K_{ow} < 3$) or other evidence.

In the light of the EU Directive on the protection of animals used for scientific purposes (EU, 2010), the EURL ECVAM strategy aims to significantly reduce the overall reliance on animal testing. Vertebrate animals used for testing environmental hazard and risk assessment are fish, amphibians, birds and, on rare occasions, mammals. A recent paper of Scholz et al. (2013) summarises possibilities to reduce the use of vertebrates in environmental risk assessment covering aquatic toxicity, avian toxicity, bioaccumulation and endocrine activity. The EURL ECVAM strategy described here is narrower in scope, focusing specifically on the use of fish for aquatic toxicity and bioaccumulation testing, i.e. does not cover avian toxicity, use of fish for testing of endocrine activity and nanomaterials. It is based on an

analysis of ongoing international efforts in the research and regulatory domains, including method development and validation, test guideline development and regulatory acceptance of alternative approaches. Implementation of the EURL ECVAM strategy will rely on the coordinated efforts of multiple stakeholders.

2. Background information on fish testing and mechanisms of aquatic toxicity

2.1 Current fish tests

The OECD Test Guidelines (TGs) and EU test methods for acute and chronic fish toxicity involve the use of fish at various life stages, e.g. early life stages, juvenile or adult fish (Annex II).

The fish acute toxicity test (OECD TG 203; OECD, 1992) is a short-term exposure test (96 h) and determines the concentration that is lethal to 50% of the fish (LC₅₀).

The fish early life-stage toxicity test (FELS; OECD TG 210; OECD, 2013a) is the most commonly used test to establish chronic fish toxicity, i.e. to determine lethal and sublethal effects of a chemical on early life stages of fish (embryos, larvae, juvenile fish). The endpoints are hatching success, abnormal appearance, abnormal behaviour, survival or mortality, and the weight and length of the fish at the end of the test. By comparison to the values of control fish, the LOEC, NOEC and/or EC_x are determined for each endpoint.

Two further OECD TGs are available for chronic fish toxicity testing, however, they cover fewer developmental stages and are less frequently used: OECD TG 212 (Fish, Short-term Toxicity Tests on Embryo and Sac-fry Stages, OECD 1998) uses fish embryos and sac-fry stages; OECD TG 215 (Fish, Juvenile Growth Test; OECD, 2000) assesses the effects of a chemical on the growth of juvenile fish for 28 days. Some regulatory frameworks recommend the use of OECD TG 212 and OECD TG 215, if it is not possible to carry out an OECD TG 210. For plant protection products (EU, 2013a & b), a fish full life-cycle test (US EPA, 1996) may be required.

2.2 Mechanistic understanding of aquatic toxicity

A number of modes of action that can lead to (acute) aquatic toxicity have been defined and associated with chemical classes. For example, Verhaar et al. (1992) proposed four aquatic Modes of Action (MOA) and assigned them to four classes (Verhaar Class 1-4): MOA1 for chemicals acting by nonpolar narcosis (or baseline toxicity); MOA2 for chemicals acting by polar narcosis (including [substituted] phenols and [substituted] anilines); MOA3 for chemicals with non-specific reactivity (including aldehydes and epoxides); and MOA4 for chemicals with specific reactivity (including pesticides, polychlorinated biphenyls and some polycyclic aromatic hydrocarbon metabolites). Later, a fifth class was defined for chemicals not covered by MOAs 1-4 (Enoch et al., 2008).

Russom et al. (1997) extended the Verhaar classification scheme and distinguished eight modes of action (base-line narcosis or narcosis I, polar narcosis or narcosis II, ester narcosis or narcosis III, oxidative phosphorylation uncoupling, respiratory inhibition, electrophile/proelectrophile reactivity, acetylcholinesterase inhibition, and central nervous system seizure). These structure-based schemes for MOA classification have informed the development of Quantitative Structure-Activity Relationship (QSAR) models and the application of read-across.

More recently, Ankley et al. (2010) discussed how mechanistic considerations could advance ecological risk assessment and proposed the Adverse Outcome Pathway (AOP) as "a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event and an adverse outcome at a biological level of organisation relevant to risk assessment", e.g. survival, development, and reproduction at the population level for ecology. AOPs could be applied to focus toxicity testing in terms of species and endpoint selection, extrapolation between chemicals, the prediction of mixture effects (Ankley et al., 2010) and the development of Integrated Approaches to Testing and Assessment (IATA) and the building of chemical categories (Schultz, 2010).

To coordinate and harmonise various international efforts in AOP development, the OECD launched the AOP Development Programme in January 2013. This programme is managed by the Extended Advisory Group on Molecular Screening and Toxicogenomics that the European Commission (Joint Research Centre - EURL ECVAM) is co-chairing together with the US Environmental Protection Agency (US EPA). In this context an AOP Knowledge Base (AOP KB) is currently being developed within a multi-partner collaborative project led by EURL ECVAM and the US EPA to facilitate the contribution to, and evaluation of, current pathway information collected by a wide range of experts. The first AOP KB module, the AOP wiki, is publicly accessible online (<https://aopkb.org/>).

3. Background information on fish bioaccumulation testing

Assessing whether a chemical accumulates in aquatic organisms is important for understanding its behaviour in the environment. Bioconcentration describes the accumulation of a water-borne chemical by an aquatic organism, whereas bioaccumulation covers the uptake from all environmental sources, e.g. water, food and sediment. The bioconcentration potential of a chemical, expressed as the 'BCF', is either predicted or measured (typically in fish, but if necessary, also in invertebrates). The BCF describes the ratio of the concentration of a chemical in the whole organism to its concentration in the water, under equilibrium conditions.

Accumulation of a chemical in an organism is the result of several physiological processes, namely Absorption, Distribution, Metabolism, and Excretion (ADME). Apart from excretion via fish gills and intestine, metabolism (biotransformation) plays an important role in the elimination (deuration) of a chemical from the fish organism.

OECD TG 305 (*Bioaccumulation in Fish: Aqueous and Dietary Exposure*; OECD, 2012a) includes two exposure routes, namely, aqueous and dietary. The aqueous exposure setup determines the BCF. In general, two concentrations, a water and, if necessary, a solvent control are required and fish are sampled at various time points (five during the uptake phase and four during the depuration phase). For non-polar organic chemicals, OECD TG 305 allows several exposure scenarios; for example, the test is conducted only at one concentration and/or the sampling steps are reduced to four. These simplified testing schemes have the potential to reduce the overall number of fish used (i.e. at least 108 fish are used for a full test). Creton et al. (2013) showed in their analysis of BCF values of 55 plant protection products that there was no statistically significant difference in the BCF values derived with low and high concentrations, and concluded that testing at one concentration would be sufficient.

As stated in the OECD TG 305, a dietary magnification factor is determined with the dietary exposure route, which should be used for strongly hydrophobic substances ($\log K_{ow} > 5$ and solubility below 0.01-0.1 mg/L) where the aqueous exposure methodology is not practicable. The test consists of two phases: uptake (test substance-spiked feed) and depuration (clean, untreated feed). Depending on the test design and sampling scheme, at least 50-120 fish for chemical exposure, 50-110 control fish, and, if needed 15 fish for lipid correction, are used.

Over the last decade, several strategies for fish bioaccumulation testing (or waiving) have been published taking into account existing information, non-testing methods (*in silico*, read-across, weight-of-evidence), and testing methods (biomimetic techniques, *in vitro* methods, reduced fish bioconcentration tests, use of other aquatic species as invertebrates) when deciding on the bioaccumulative potential of a chemical (de Wolf et al., 2007; Grindon et al., 2008; ECHA, 2012a; Lombardo et al., 2014). The further development of these strategies should consider recent advances in the development of physiologically-based toxicokinetic (PBTK) models (see Section 4) that can be used to predict bioaccumulation and toxicologically relevant internal concentrations in test animals (e.g. fish) following defined exposure scenarios.

4. Proposed strategy to replace, reduce and refine the use of fish in aquatic toxicity and bioaccumulation testing

Taking into account the current state-of-the-art, EURL ECVAM proposes that efforts should be directed towards the identification and classification of acute and chronic fish toxicity as well as bioaccumulation using a variety of approaches that minimise or preferably avoid the use of animals. To achieve this goal, the following three key aims should be pursued:

Strategic Aim 1: Replacement and reduction of the need of fish for aquatic toxicity testing

Strategic Aim 2: Replacement and reduction of the need of fish for bioaccumulation testing

Strategic Aim 3: Reduction and refinement of current fish tests

The objectives and related activities to achieve these aims are summarised in Figure 1 and are described below. Although different approaches will be more applicable in different sectors, their collective realisation is expected to have a significant impact on regulatory testing while ensuring an effective level of protection of the environment.

EURL ECVAM Strategy to replace, reduce and refine the use of fish in aquatic toxicity and bioaccumulation testing			
Objectives and activities	Strategic Aim 1: Replacement and reduction of fish for aquatic toxicity testing	Strategic Aim 2: Replacement and reduction of fish for bioaccumulation testing	Strategic Aim 3: Reduction and refinement of current fish tests
	Explore the use of fish cell line-based assays <ul style="list-style-type: none"> possible validation for acute fish toxicity testing 	Explore the use of toxicokinetic models to predict bioaccumulation <ul style="list-style-type: none"> use of <i>in vitro</i> data, embryo data, <i>in vitro</i> metabolism 	Revision of OECD TG 203 Fish Acute Toxicity <ul style="list-style-type: none"> less fish / concentration; use of humane endpoints
	Promotion of the ZFET (OECD TG 236) for acute fish toxicity testing <ul style="list-style-type: none"> regulatory use, guidance document, maintenance of database 	Improvement of QSAR models for BCF prediction <ul style="list-style-type: none"> e.g. by including metabolism 	Revision or deletion of OECD TG 212 Short-term Toxicity Tests on Embryo and Sac-fry Stages <ul style="list-style-type: none"> update to state-of-art or consider deletion
	Promotion of the threshold approach (OECD GD 126) for acute fish test toxicity testing <ul style="list-style-type: none"> e.g. for veterinary pharmaceuticals, feed 	Development of OECD test guideline <i>In vitro</i> fish hepatic metabolism <ul style="list-style-type: none"> Ring trial with two methods (S9, cryopreserved hepatocytes) TG development 	Development of an OECD guidance document on OECD TG 305 <ul style="list-style-type: none"> should include detailed guidance on the use of the minimised aqueous approaches
	Applying the AOP concept <ul style="list-style-type: none"> chronic fish toxicity testing 		Reduction of control animals in fish tests <ul style="list-style-type: none"> explore possibilities of waiving water control when a solvent control is used
	Explore possibilities in supporting the waiving of chronic fish toxicity tests <ul style="list-style-type: none"> <i>In silico</i> methods for chronic fish toxicity Across species extrapolation and acute-to-chronic relationships Threshold of Toxicological Concern for ecotoxicity 		

Figure 1: Summary of EURL ECVAM strategy to replace, reduce and refine the use of fish in aquatic toxicity and bioaccumulation testing

4.1 Strategic Aim 1: Replacement and reduction of the use of fish in aquatic toxicity testing

Objective 1.1: Explore the use of fish cell line-based assays for fish toxicity testing

A vast number of fish cell lines have been established covering a wide range of fish species and tissues. For the purpose of toxicity testing, cell lines deriving from gills or liver are playing an important role, i.e. gills are the primary target and uptake site of aquatic contaminants and are involved in gas exchange, osmoregulation and other critical functions (Lee et al., 2009), while the liver has a high metabolic capacity and detoxification function (Lee et al., 1993; Schirmer, 2006). For many years, fish cell line-based assays have been proposed as alternatives to fish in aquatic toxicity testing (reviewed by e.g. Castaño et al., 2003; Bols et al., 2005; Schirmer, 2006). More recently, they have also been proposed as tools to explore toxicity pathways at the molecular and cellular levels (Ankley et al., 2010; see below) and to predict internal concentrations in fish by using toxicokinetic models (Stadnicka-Michalak et al., 2014).

A recent project funded by the Cefic Long-range Research Initiative (CEFIC-LRI; ECO8 - CELLsens project; <http://www.cefic-lri.org/projects>) aimed at the standardisation of a cytotoxicity assay using the rainbow trout gill cell line (RTgill-W1). Tanneberger et al. (2013) tested 35 organic chemicals with the standardised assay and recommended its use for acute fish toxicity testing. Further evaluation of its reproducibility, predictive capacity and applicability domain is however needed. As a follow-up of the CELLsens project, a ring trial was launched in 2014 under the lead of the Swiss Federal Institute of Aquatic Science and Technology (K. Schirmer), which aims at assessing the transferability and reproducibility of the assay with a restricted number of chemicals. Following the pre-submission of the RTgill-W1 cytotoxicity assay in early 2014, EURL ECVAM has invited the test submitter to provide a full test submission.

Objective 1.2: Promote the use of the zebrafish embryo acute toxicity test as an alternative to the acute fish toxicity test

The zebrafish embryo acute toxicity test (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 (LC₅₀). 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. EURL ECVAM contributed to the validation of the ZFET and the subsequent development of the recently published OECD TG 236 "Fish embryo acute toxicity test" (OECD, 2013b), both as a member of the relevant OECD expert group and as the coordinator of the OECD validation study (OECD, 2011; 2012b, Busquet et al., 2014).

The EURL ECVAM Recommendation on the ZFET was published in July 2014 after consultation with regulators, stakeholders and the public (EURL ECVAM, 2014). It concludes

that the ZFET is transferable and reproducible within and between laboratories as shown in the OECD validation study. The comparison of data on 144 chemicals (Belanger et al., 2013) demonstrated a strong correlation ($r = 0.9$) between fish embryo acute toxicity data (24-120 h exposure; mainly zebrafish) and fish acute toxicity data (96 h; five freshwater species recommended in OECD TG 203). Notably, the chemicals covered a broad range of physicochemical properties, toxicological modes of action, and sectorial use, e.g. industrial chemicals (77), plant protection products (21), biocides (5), and pharmaceuticals (8). The EURL ECVAM Recommendation concluded therefore that the ZFET provides information on acute fish toxicity that can be considered comparable to that derived from standard acute fish toxicity tests (e.g. OECD TG 203; OECD 1992) and thus it should be seriously considered for deriving information of acute fish toxicity whenever possible.

The use of the ZFET will result in an overall refinement and reduction of tests on juvenile and adult fish required for aquatic toxicity testing. Within the provisions of the Directive 2010/63/EU on the protection of animals used for scientific purposes (EU, 2010), the embryos used in the ZFET should not be considered as "independently feeding larval forms" and therefore the procedure, as far as the embryos are concerned, does not fall within its scope. Moreover, 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 Member States instructs 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.*

The EURL ECVAM Recommendation underlines that OECD TG 236 should be used for generating information on acute fish toxicity where appropriate and, accordingly, be included into the respective pieces of legislation and guidance documents. If deemed necessary, an OECD guidance document on the use of OECD TG 236 across the various regulatory frameworks could be developed addressing in particular its use to generate information on acute fish toxicity and its limitations.

The database containing fish embryo acute toxicity data and fish acute toxicity data (Belanger et al., 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. (2013), it was not possible to find acute fish toxicity data for all chemicals for which fish embryo toxicity data were available. For example, acute fish data could be retrieved for only eight out of the 22 pharmaceuticals tested. Therefore, industry and regulatory bodies are encouraged to make existing data available where possible. This would also be a valuable resource for the development of QSARs and AOPs.

Objective 1.3: Promote the use of the threshold approach for acute fish toxicity testing

The Threshold Approach for Acute Fish Toxicity Testing is a tiered testing strategy which has the potential to significantly reduce the number of fish used for acute aquatic toxicity testing. It is based on the fact that the LC₅₀/EC₅₀ value of the most sensitive of the three test species (fish, algae and invertebrates) is commonly used for hazard and risk assessment and that fish is often not the most sensitive test species. This concept was first described for pharmaceuticals as a "threshold/step-down" approach by Hutchinson et al. (2003) and further developed for chemicals by the Joint Research Centre (JRC) (Jeram et al., 2005; ECVAM, 2006).

The OECD Guidance Document (GD) 126 "Short guidance on the use of the threshold approach for acute fish toxicity testing" has been available since 2010 (OECD, 2010). In brief, an acute fish test (following the limit test as given in OECD TG 203) is performed at a single concentration (threshold concentration) corresponding to the lowest LC₅₀/EC₅₀ value derived with daphnids or algae. If no mortality occurs at the threshold concentration, it is concluded that fish are not the most sensitive of the three species and toxicity to fish is reported as greater than the threshold concentration. If mortality occurs, a full OECD TG 203 study is needed.

The threshold approach has been incorporated into various testing strategies and guidance documents, e.g. the REACH guidance on information requirements and chemical safety assessment (Chapter R.7b: Endpoint specific guidance; ECHA, 2012b) and the OECD Fish Toxicity Testing Framework (OECD, 2012c). It is further mentioned as a preferred method for deriving data on acute fish toxicity in the biocidal products regulation (EU, 2012a) and in the Commission regulations on data requirements for plant protection products (2013a & b). The use of the threshold approach in other areas where acute fish toxicity data may be specifically required, e.g. veterinary pharmaceuticals and feed additives, should be explored.

Objective 1.4: Applying the Adverse Outcome Pathway (AOP) concept

The paper of Ankley et al. (2010) triggered many activities focusing on the development of AOPs for environmental toxicity. For example, two workshops organised by the "Animal Alternatives in Environmental Risk Assessment Technical Committee" of the International Life Sciences Institute's Health and Environmental Sciences Institute (ILSI HESI) specifically addressed AOPs relevant for chronic fish toxicity and how they could be discovered and annotated (Volz et al., 2011; Villeneuve et al., 2014).

In 2013, the CEFIC LRI-funded project (LRI-ECO20-UA) *Development of an alternative testing strategy for the fish early life-stage test for predicting chronic toxicity* started. It aims to map FELS-relevant AOPs, develop an *in vitro* toolbox for screening FELS-relevant AOPs (Tier 1) and zebrafish embryo based assays (Tier 2).

At the recent workshop on "Advancing Adverse Outcome Pathways for Integrated Toxicology and Regulatory Applications" (March 2014; Somma Lombardo Italy; co-organised by Environment Canada, US EPA, US ERDA, JRC, as well as academic institutes/organisation from USA, Switzerland, and Norway), one working group specifically addressed AOPs and their use in chronic toxicity testing for environmental hazard and risk assessment. The outcome of the discussions is presented in two papers, one addressing the challenges and research needs (Groh et al., 2014a) and the other focusing on possible AOPs for fish growth impairment (Groh et al., 2014b).

The increasing availability of AOPs for aquatic toxicity endpoints should be used to inform the development of IATA that will serve to replace and reduce the use of fish.

Objective 1.5: Evaluate possibilities for supporting the waiving of chronic fish toxicity tests

Several research groups are working on data-driven approaches, such as those described below, to develop a sound scientific basis to justify the waiving of animal tests.

1.5.1: Use of Quantitative Structure-Activity Relationships

There is an extensive literature on QSARs for fish toxicity, and in particular acute fish toxicity, (Netzeva et al., 2007; 2008; Brooke et al., 2006a; Brooke et al., 2006b; EFSA, 2013). Many of these studies have focused on the development of QSARs for specific modes of action, including polar narcosis, non-polar narcosis, reactivity-based toxicity, and specifically acting mechanisms (involving non-covalent interactions with receptors or enzymes or endocrine active chemicals [e.g. Jacobs, 2004]).

Recently, several QSARs have been developed for chronic fish toxicity (de Haas et al., 2011; Claeys et al., 2013; Austin and Eadsforth, 2014) mainly addressing chemicals acting via narcosis. The development of mechanistically based QSARs for fish toxicity with a view to their potential use in supporting the waiving of chronic fish testing should be supported.

1.5.2: Extrapolating across species and from acute to chronic effects

Regulatory aquatic risk assessment schemes require toxicity testing of chemicals on a limited number of laboratory species; thus, extrapolation from the obtained toxic responses to all species representing that trophic level in the environment is a fundamental tenet of regulatory ecotoxicological risk assessment. To derive the PNEC for aquatic toxicity, safety factors are applied to the laboratory data. These factors are intended to account for interspecies differences in sensitivity, extrapolation from acute to chronic effects, the physicochemical complexity of natural water versus laboratory test media, and the complexity of the ecosystem versus single species laboratory tests. The choice of the factor (10, 100 or 1000) depends on the quality and quantity of the available data.

Differences in species sensitivity to acute aquatic toxicity have been well described (Weyers et al., 2000; Hutchinson et al., 2003, Jeram et al., 2005; Hoekzema et al., 2006; Tebby et al., 2011). To address the question of whether it is possible to predict acute toxicity in fish

from non-vertebrate species, Netzeva et al. (2007) reviewed several Quantitative Activity-Activity Relationships between species, the most relevant and reliable relationship for acute fish toxicity being between *Daphnia (D.) magna* and rainbow trout ($n=360$), with an r^2 value of 0.67. More recent studies have confirmed the good correlation between acute fish and daphnia toxicity data, especially for organothiophosphates ($0.74 < r^2 < 0.94$) and (benzo)triazoles ($r^2=0.87$) (Zvinavashe et al., 2009; Kar et al., 2010; Zhang et al., 2010; Cassani et al., 2013). The correlation depends on both the bio-uptake process and the MOA of the chemical (Zhang et al., 2010), as well as its physicochemical properties (Tebby et al., 2011).

The US EPA has developed the Interspecies Correlation Estimation (ICE) tool (implemented as WebICE; Raimondo et al., 2010) to predict acute toxicity to three relevant fish species (fathead minnow, rainbow trout, and common carp) on the basis of *D. magna* toxicity, with the strongest correlation being evident between *D. magna* and rainbow trout ($r^2=0.51$).

EURL ECVAM is currently exploring whether interspecies extrapolations and acute-to-chronic relationships can be used for supporting the waiving of chronic fish tests. For this purpose, data (LC_{50} , NOEC) for *D. magna* and fish have been extracted from various databases and analysed to identify possible relationships taking into consideration different MOAs (intended for publication).

1.5.3: Threshold of Toxicological Concern in aquatic toxicity assessment

The Threshold of Toxicological Concern (TTC) approach is based on the premise that there is a general exposure limit for chemicals below which no significant risk to human health or the environment is expected. The potential application of the TTC approach in aquatic toxicity assessment has been explored by de Wolf et al. (2005) who collated reliable acute and chronic aquatic toxicity data from several ecotoxicity databases and derived an aquatic exposure threshold of no concern ($ETNC_{aq}$) of 0.1 $\mu\text{g/L}$ for chemicals with MOA1-3 according to the Verhaar scheme. Tolls et al. (2009) proposed using this approach for environmental risk assessment of poorly water soluble ingredients (e.g. as long-chain fatty alcohols, esters, and ethers) of personal care products. They set the $ETNC_{aq}$ of 1.9 $\mu\text{g/L}$ calculated by de Wolf et al. (2005) for chemicals with MOA 1-2 as a limit for water solubility. If the water solubility of a chemical is less than 1.9 $\mu\text{g/L}$, the aquatic exposure level would not exceed the ecotoxicological no-effect concentration level and thus further testing would not be necessary.

The ILSI HESI "Animal Alternatives in Environmental Risk Assessment Project Committee" is currently exploring in collaboration with international partners the applicability and usefulness of the TTC approach for ecotoxicity assessment in view of its potential to reduce testing on vertebrate organisms.

4.2 Strategic Aim 2: Replacement and reduction of the use of fish in bioaccumulation testing

Objective 2.1: Explore the use of toxicokinetic models to predict bioaccumulation

As for the prediction of acute and chronic fish toxicity, there is ongoing research into toxicokinetic models using data derived from a combination of fish cell line based assays (Stadnicka-Michalak, presentation at SETAC EU 2014) or fish embryos (Kühnert et al., 2013) to predict bioaccumulation. However, more research and application experience are necessary before these approaches may be deployable in a broader regulatory context.

Nichols et al. (2013) developed two toxicokinetic models to predict the bioconcentration of well-metabolised chemicals in rainbow trout. Both are 1-compartment models and the whole body biotransformation rate is extrapolated from *in vitro* intrinsic clearance rates derived with S9 trout liver fraction or trout hepatocytes (see below). Laue et al. (2014) used several models including the S9 BCF model of Nichols et al. (2013) to predict the BCF values of nine fragrances ingredients and compared them to measured fish BCF values. This comparison showed that the S9 BCF model (with no correction for potential binding effects on hepatic clearance) provided the most accurate predictions of measured BCFs; i.e. correctly identified the two bioaccumulative and the nine non-bioaccumulative chemicals.

Objective 2.2: Improvement of QSAR models for BCF prediction

QSAR models based on log K_{ow} are widely used to screen chemicals for potential bioaccumulative properties and predict BCFs (reviewed by Pavan et al., 2008). These are mostly linear regression models that do not account for ADME processes. By neglecting the contribution of metabolism as a clearance mechanism, these models might overestimate the bioaccumulative potential of a chemical and consequently trigger unnecessary *in vivo* tests.

More recent research has focused on the development of QSAR models which include metabolism/biotransformation as a contributing factor (Dimitrov et al., 2005). Arnot et al. (2009) developed a QSAR model which predicts metabolic biotransformation rates based on chemical structure. This model is part of the BCF/BAF™ programme of the US EPA Episuite software (US EPA, 2012).

Other authors proposed the use of *in vitro* metabolic transformation rates to refine BCF models and make them more reliable (Han et al., 2007; Han et al., 2009; Crowan-Ellsberry et al., 2008; Dyer et al., 2008; Gomez et al., 2010; Nichols et al., 2013).

Objective 2.3: Development of an OECD test guideline "In vitro fish hepatic metabolism"

In April 2014, the OECD Working Group of National Coordinators for the OECD Test Guideline Program (WNT) approved a project (under the lead of USA and European Commission represented by JRC - EURL ECVAM) on the development of a new OECD TG on *In vitro Fish Hepatic Metabolism*. The project aims at standardising two *in vitro* methods using rainbow trout S9 fraction (Johanning et al., 2012) or cryopreserved rainbow trout hepatocytes (Fay et al., 2014) to determine *in vitro* fish intrinsic hepatic clearance rates. A multi-laboratory ring trial to assess the reliability, transferability, and predictive value of the two *in vitro* systems started in late 2014 and will provide results in 2015. It builds on work carried out within the framework of the ILSI HESI project "Bioaccumulation", which will coordinate the ring trial. The outcome will serve as basis for developing the OECD TG.

Other methods for deriving information on *in vitro* fish metabolism could be based on the use of the fish liver cell line RTL-W1 (Lee et al., 1993) or the 3D cultures of trout hepatocytes (Baron et al., 2012).

4.3 Strategic Aim 3: Reduction and refinement of current fish tests

Objective 3.1: Revision of OECD TG 203 Fish Acute Toxicity

OECD TG 203 is one of the few regulatory required tests using death as an endpoint, which according to Directive 2010/63/EU (Article 13; EU, 2010) should be avoided whenever possible in favour of humane endpoints based on clinical signs.

Switzerland and the UK have initiated an OECD project on the revision of OECD TG 203 aiming at reduction and refinement. The project follows up recommendations of the OECD Fish Toxicity Testing Framework (OECD, 2012c) and is based on the work of Rufli and Springer (2011) and Rufli (2012). The first draft of the revised guideline became available in September 2014 on the OECD website. It proposes that the number of fish per concentration should be reduced from seven to six and that humane endpoints should be applied, i.e. fish showing severe clinical signs as abnormalities in swimming behaviour, equilibrium, respiration, pigmentation, should be humanely killed.

Objective 3.2: Revision or deletion of OECD TG 212 Short-term Toxicity Tests on Embryo and Sac-fry Stages

The OECD Fish Toxicity Testing Framework (OECD, 2012c) recommends the deletion of OECD TG 212 due not only to scientific and animal welfare concerns, but also a lack of regulatory relevance. In Europe, only the REACH legislation refers to OECD TG 212 as a possible test for chronic fish toxicity. According to OECD TG 212, exposure starts with fertilised eggs and should be terminated before the yolk-sac is completely absorbed or *before mortality due to starvation of the fish embryos begins*. The guideline includes recommendations on the duration of the test, which varies depending on the species used.

However, nowadays it is well known that fish embryos may depend on external food supply and start feeding even before the yolk-sac is absorbed. For example, zebrafish embryos start feeding around 48h after hatch and would starve without external food supply if kept for the recommended 8-10 days.

The recommendation of OECD Fish Toxicity Testing Framework (OECD 2012c) should be followed up at OECD level, either by deleting OECD TG 212 or by revising it in the light of its potential use and taking into account animal welfare concerns.

Objective 3.3: Development of an OECD guidance document on OECD TG 305

As mentioned above, OECD TG 305 "Bioaccumulation in Fish: Aqueous and Dietary Exposure" (OECD, 2012a) has recently been revised and now includes two exposure routes - aqueous and dietary. For non-polar organic chemicals, OECD TG 305 allows various exposure scenarios; for example, the test is conducted only at one concentration and/or the sampling steps are reduced to four. Both approaches use significantly fewer fish than a full aqueous or dietary study.

Germany, the Netherlands, and the United Kingdom are leading an OECD project aiming at the development of an OECD guidance document on OECD TG 305. Among other aspects, this document should include detailed guidance on the use of the minimised aqueous approaches.

Objective 3.4: Reduction of control animals in fish tests

The OECD Fish Toxicity Testing Framework (OECD, 2012c) states that there is a need to consider whether both water and solvent controls are needed when a solvent is used. At present this is a requirement for various fish tests. Following up discussions initiated by the International Council on Animal Protection in OECD Programmes (ICAPO) at the 25th WNT meeting in 2013, the European Commission (JRC - EURL ECVAM) and ICAPO have submitted a project proposal to OECD which aims at reviewing historical data and the relevance of running two controls.

5. Indicative timelines

An indication of the timelines related to the achievement of the aims and objectives underpinning the proposed strategy are provided in Figure 2. It should be noted that these are simply estimates of what EURL ECVAM believes might be possible with the concerted and coordinated effort of all stakeholders and the availability of sufficient resources.

EURL ECVAM Strategy – Indicative timelines			
	Strategic Aim 1: Replacement and reduction of fish for aquatic toxicity testing	Strategic Aim 2: Replacement and reduction of fish for bioaccumulation testing	Strategic Aim 3: Reduction and refinement of current fish tests
1–3 years	Promotion of OECD TG 236 for acute fish toxicity testing	Development of OECD test guideline <i>In vitro fish hepatic metabolism</i>	Revision of OECD TG 203 Fish Acute Toxicity
	Promotion of the threshold approach (OECD GD 126) for acute fish test toxicity testing	Explore the use of toxicokinetic models to predict bioaccumulation	Update to state-of-the art or deletion of OECD TG 212
3–5 years	Explore possibilities in supporting the waiving of chronic fish toxicity tests	Improvement of QSAR models for BCF prediction	Development of an OECD guidance document on OECD TG 305
> 5 years	Fish cell line-based assays possible validation for acute fish toxicity testing		Reduction of control animals in fish tests
	Applying the AOP concept		

Figure 2: Indicative timelines of the EURL ECVAM strategy assuming the concerted and coordinated effort of all stakeholders and the availability of sufficient resources.

6. Conclusions

This document presents the EURL ECVAM strategy for achieving significant 3Rs impact in the areas of aquatic toxicity and bioaccumulation. The aims and objectives of the strategy are underpinned by a number of activities that have been identified and involve a variety of actors. Successful implementation of the strategy will deliver alternative approaches that address standard information requirements in many sectors while ensuring that animal testing is only conducted as a last resort. One important near-term impact could be the reduction of animal testing necessary for the implementation of REACH and the 2018 registration deadline.

EURL ECVAM is focusing its current in-house activities on promoting the use of available alternative methods for fish acute toxicity testing, on exploring the usefulness of scientific approaches (e.g. acute-to-chronic relationships) to facilitate the waiving of chronic fish tests, and on supporting activities at OECD level. However, the intention is continually review the EURL ECVAM work programme as the implementation of the overall strategy progresses and in order to complement the work of important European and international actors. EURL ECVAM will also continue evaluating alternative test methods submitted for validation in light of their potential value to contribute to aspects of this strategy.

The ultimate achievement of the aims and objectives comprising this strategy will depend on the proactive and coordinated engagement of multiple stakeholders.

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Annex I. EU regulatory information requirements for fish toxicity and bioaccumulation

Regulatory framework	Endpoint		
	Short-term fish toxicity	Long-term fish toxicity	Bioaccumulation
Industrial chemicals (REACH) Regulation (EC) No. 1907/2006 (EC, 2006)	<p>> 10t/year (Annex VIII)</p> <p>Not to be conducted if:</p> <ul style="list-style-type: none"> - aquatic toxicity is unlikely to occur, e.g. chemical highly insoluble in water or unlikely to cross biological membranes or - a long-term aquatic toxicity study on fish is available <p>In general applicants are invited to consider long-term aquatic toxicity testing:</p> <ul style="list-style-type: none"> - as described in Annex IX <ul style="list-style-type: none"> o shall be considered if the chemical safety assessment according to Annex I indicates the need to investigate further effects on aquatic organisms. o if the substance is poorly water soluble. 	<p>> 100t/year (Annex IX)</p> <ul style="list-style-type: none"> - Long-term toxicity testing shall be proposed by the registrant if the chemical safety assessment according to Annex I indicates the need to investigate further the effects on aquatic organisms. The choice of the appropriate test(s) depends on the results of the chemical safety assessment. <p>Appropriate tests:</p> <ul style="list-style-type: none"> - Fish early-life stage (FELS) toxicity test (OECD TG 210); - Fish short-term toxicity test on embryo and sac-fry stages (OECD TG 212) or - Fish, juvenile growth test (OECD TG 215) 	<p>>1000t/year (Annex X)</p> <p>Not to be conducted if:</p> <ul style="list-style-type: none"> - the substance has a low potential for bioaccumulation (for instance a $\log K_{ow} \leq 3$) and/or a low potential to cross biological membranes, or - direct and indirect exposure of the aquatic compartment is unlikely.
Cosmetic ingredients Regulation (EC) No 1223/2009 (EC, 2009b)	Recital (5): The environmental concerns that substances used in cosmetic products may raise are considered through the application of Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) and establishing a European Chemicals Agency(4) OJ L 396, 30.12.2006, p. 1. (4), which enables the assessment of environmental safety in a cross-sectoral manner.		
Plant protection products data requirements - active substance Regulation (EU) No 283/2013 (EU, 2013a) –	<p><i>Active substance:</i></p> <p>Threshold approach or OECD TG 203 (rainbow trout)</p>	<p><i>Active substance:</i></p> <p>Fish early-life stage (FELS) toxicity test (OECD TG 210)</p> <ul style="list-style-type: none"> - if exposure of surface water likely and the substance stable in water; <p>Fish full life cycle test (USEPA OCSP 850.1500) may be required</p>	<p><i>Active substance:</i></p> <p>To be conducted if</p> <ul style="list-style-type: none"> - $\log K_{ow} > 3$ or other indications that the chemical may bioaccumulate in fish, - the substance is stable in water

Regulatory framework	Endpoint		
	Short-term fish toxicity	Long-term fish toxicity	Bioaccumulation
<p>Plant protection products data requirements - product Regulation (EU) No 284/2013 (EU, 2013b)</p>	<p><i>Product:</i> Only if the toxicity cannot be predicted from the active substance(s) or read-across</p> <p>In case that fish data are needed: Threshold approach or OECD TG 203 (rainbow trout)</p>	<p><i>Product:</i> Only if the toxicity cannot be predicted from the active substance(s)</p> <p>Necessary studies should be discussed with competent authority</p>	
<p>Biocidal products Regulation (EU) No 528/2012 (EU, 2012a)</p> <p>Guidance on data requirements (ECHA, 2013)</p> <p>Only to be carried out if exposure is likely.</p> <p><i>Note for Products:</i> Testing on the product itself only when valid data on components are missing or synergistic effects to be expected</p>	<p><i>Active substance:</i> <i>Core data set</i> Short-term toxicity testing on fish -When short-term fish toxicity data is required the threshold approach (tiered strategy) should be applied.</p> <p>The study does not need to be conducted if a valid long-term aquatic toxicity study on fish is available.</p>	<p><i>Active substance:</i> <i>Additional data set</i> depending on the results of the studies on fate/behaviour and intended use, e.g. risk to the aquatic environment, and long-term exposure: - long-term toxicity studies on fish may be required, one or two tests, OECD TGs 210, 212, 215 or fish full life cycle test</p>	<p><i>Active substance:</i> <i>Core data set:</i> Estimation methods and experimental determination. The experimental determination may not need to be carried out if: - it can be demonstrated on the basis of physico-chemical properties (e.g. $\log K_{ow} < 3$) or other evidence that the substance has a low potential for bioconcentration</p> <p><i>Additional data set:</i> bioaccumulation in an appropriate aquatic species may be required</p>
<p>Veterinary Medicinal Products Stepwise approach with initial screening (Phase I) to identify exposure, bioaccumulation, persistence. If given then studies are performed (Phase II). Phase II: CVMP/VICH/790/03-FINAL; EMEA, 2004) (corresponds to VICH GL38)</p>	<p><i>Phase II:</i> Tier A – aquatic effect study - OECD TG 203</p>	<p><i>Phase II:</i> Tier B - if risk quotient ($PEC_{refined}/PNEC$) > 1 for fish, OECD TG 210 - fish early life stage test</p>	<p><i>Phase II</i> Tier B – if $\log K_{ow} > 4$ and evidence for bioaccumulation from other studies, OECD TG 305 to be carried out</p>

Regulatory framework	Endpoint		
	Short-term fish toxicity	Long-term fish toxicity	Bioaccumulation
<p>Human Medicinal Products Stepwise approach with initial screening (Phase I) to identify exposure, bioaccumulation, persistence. If given then studies are performed (Phase II) Phase II: CHMP/SWP/4447/00 (EMA, 2006)</p>	Not required	<p><i>Phase II:</i> Tier A – base set requirement OECD TG 210 – fish early life stage test</p>	<p><i>Phase II:</i> Tier B – depending on information on fate in Tier A, bioconcentration study with drug substance or its metabolites</p>
<p>Feed additives Regulation (EC) No 429/2008 (EC, 2008a) & (EFSA, 2008) Stepwise approach with initial determination (Phase I) whether a significant environmental effect of the additive is likely (based on estimated PEC). If likely, then studies are performed (Phase II)</p>	<p>Phase IIa: OECD TG 203</p>	<p>Phase IIb: OECD TG 210</p>	-
<p>CLP Regulation Regulation (EC) No 1272/2008 EC (2008b)</p>	<p>In the EU, the core classification system for aquatic environmental hazards consists of one acute (Acute 1) and three chronic (Chronic 1 – 3) hazard classification categories (EC, 2008). If adequate chronic toxicity data are not available, a combination of acute aquatic toxicity data and information on the environmental fate (degradability and bioaccumulation) is used to decide on the appropriate Chronic category. In addition, a "safety net" classification referred to as category Chronic 4 can be applied for substances which cannot be classified in any of the four categories but nevertheless raise some concerns.</p>		

Annex II. Test Guidelines covering fish toxicity and bioaccumulation

Guideline	OECD TG 236 – Fish Embryo Acute Toxicity (FET) Test	OECD TG 203 – Fish, Acute Toxicity Test	OECD TG 210 – Fish, Early-life Stage Toxicity Test (as revised in 2013)	OECD TG 212 – Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages	OECD TG 215 – Fish, Juvenile Growth Test	USEPA OCSPP 850.1500 – Fish life cycle test*	OECD TG 305 – Bioaccumulation in Fish
Species	zebrafish (<i>Danio rerio</i>); in development for fathead minnow	zebrafish (<i>Danio rerio</i>), fathead minnow (<i>Pimephales promelas</i>), Japanese medaka (<i>Oryzias latipes</i>), rainbow trout (<i>Oncorhynchus mykiss</i>) bluegill sunfish ¹ (<i>Lepomis macrochirus</i>), common carp ¹ (<i>Cyprinus carpio</i>), guppy ¹ (<i>Poecilia reticulata</i>)	rainbow trout (<i>Oncorhynchus mykiss</i>) zebrafish (<i>Danio rerio</i>), fathead minnow (<i>Pimephales promelas</i>), Japanese medaka (<i>Oryzias latipes</i>) sheepshead minnow ² (<i>Cyprinodon variegatus</i>), silverside ² (<i>Menidia</i> sp)	zebrafish (<i>Danio rerio</i>), Japanese medaka (<i>Oryzias latipes</i>), rainbow trout (<i>Oncorhynchus mykiss</i>), fathead minnow (<i>Pimephales promelas</i>), common carp (<i>Cyprinus carpio</i>), goldfish (<i>Carassius auratus</i>) bluegill (<i>Lepomis macrochirus</i>) tidewater silverside ² (<i>Menidia peninsulae</i>) herring ² (<i>Clupea harengus</i>) cod ² (<i>Gadus morhua</i>) sheepshead minnow ² (<i>Cyprinodon variegatus</i>)	rainbow trout (<i>Oncorhynchus mykiss</i>) is recommended species zebrafish (<i>Danio rerio</i>), Japanese medaka (<i>Oryzias latipes</i>)	fathead minnow (<i>Pimephales promelas</i>)	zebrafish (<i>Danio rerio</i>), fathead minnow (<i>Pimephales promelas</i>), common carp (<i>Cyprinus carpio</i>), Japanese medaka (<i>Oryzias latipes</i>), guppy (<i>Poecilia reticulata</i>), bluegill sunfish (<i>Lepomis macrochirus</i>), rainbow trout (<i>Oncorhynchus mykiss</i>); Three-spined stickleback (<i>Gasterosteus aculeatus</i>)
Life stages covered	fertilised eggs, embryos until 96h post-fertilisation	Juvenile or adult	fertilised eggs, embryo, sac-fry, larvae, juvenile fish	fertilised eggs, embryo, sac-fry	juvenile	all	adult

Guideline	OECD TG 236 – Fish Embryo Acute Toxicity (FET) Test	OECD TG 203 – Fish, Acute Toxicity Test	OECD TG 210 – Fish, Early-life Stage Toxicity Test (as revised in 2013)	OECD TG 212 – Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages	OECD TG 215 – Fish, Juvenile Growth Test	USEPA OCSPP 850.1500 – Fish life cycle test*	OECD TG 305 – Bioaccumulation in Fish
Number of animals (min)	120 (at least 20 fertilised eggs per concentration & control) ³	42 (at least 7-10 per concentrations & control)	480 (at least 80/concentration & control)	150 (at least 30 fertilised eggs per concentration & control)	Not defined; depends on the test design; at least two replicates per concentration	Start with at least 4 x 50 eggs per concentration	305 I Aqueous exposure: 36 per concentration/control (4 fish per sampling point, total of 9 sampling points) – 305 II: minimised sampling – 16 per concentration/control 305 III dietary exposure: at least 50-120 fish (treatment group), 50-110 control fish, and if needed 15 fish for lipid correction
Concentrations	At least 5	At least 5	At least 5 (or less when only NOEC needed; see limit test)	At least 5 (or less with justification)	At least 5	At least 5	At least 1 (to be justified)
Controls	Water, and if needed solvent	Water, and if needed solvent	Water, and if needed solvent	Water, and if needed solvent	Water, and if needed solvent	Water, and if needed solvent	Water, and if needed solvent
Duration	96 h	96 h	28-60 days (species dependant)	Until embryos start feeding or onset of mortality due to starvation (for zebrafish TG states 8-10 days)	28 days	At least 48 weeks	At least 42 days (28 days uptake; 14 days depuration)

Guideline	OECD TG 236 – Fish Embryo Acute Toxicity (FET) Test	OECD TG 203 – Fish, Acute Toxicity Test	OECD TG 210 – Fish, Early-life Stage Toxicity Test (as revised in 2013)	OECD TG 212 – Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages	OECD TG 215 – Fish, Juvenile Growth Test	USEPA OCSPP 850.1500 – Fish life cycle test*	OECD TG 305 – Bioaccumulation in Fish
Endpoint	Lethal effects LC ₅₀	Lethal effects LC ₅₀	<i>At all life stages:</i> hatching success, abnormal appearance, abnormal behaviour, survival/mortality <i>At the end of the test</i> wet weight, length NOEC, LOEC (or EC _x) for each observation	<i>Embryo/sac-fry stage:</i> hatching success, abnormal appearance, abnormal behaviour, survival/mortality <i>At the end of the test:</i> weight, length	Growth rate as EC _x ; NOEC, LOEC	Hatching parameters, growth, survival, abnormalities, spawning parameters	Bioconcentration factor (BCF)
Effects in control	Survival rate >90%	Max 1 control fish can die	Thresholds for hatching success, post-hatch survival (depending on the species)	Thresholds for hatching success, post-hatch survival (depending on the species)	< 10% mortality; > 50% weight gain	Not given	
Limit test	Limit test at single concentration >100 mg/L; control(s); at least 20 fertilised eggs	Limit test at single concentration >100 mg/L; control(s); 7-10 fish	Limit test or extended limit test with fewer test concentrations; when empirical NOEC needed; Concentrations of the test chemical higher than the 96 hour LC ₅₀ or 10 mg/L, whichever is the lower, need not be tested	Limit test; concentrations above LC ₅₀ derived with TG 203 or >100 mg/L not to be tested	-	-	See above; reduced sampling schemes in 305-II and 305-III

* according to US EPA User's guide for conducting life-cycle chronic toxicity tests with fathead minnows (*Pimephales promelas*)

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