Section 2
Effects on Biotic Systems

Test Guideline No. 210
Fish, Early-life Stage Toxicity Test

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Fish, Early-life Stage Toxicity Test

INTRODUCTION

1. Tests with the early-life stages of fish are intended to define the lethal and sub lethal effects of chemicals on the stages and species tested. They yield information of value for the estimation of the chronic lethal and sub lethal effects of the chemical on other fish species.

2. This guideline is based on a proposal from the United Kingdom which was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988 and further updated in 2013 to reflect experience in using the test and recommendations from an OECD workshop on fish toxicity testing, held in September 2010 (1).

PRINCIPLE OF THE TEST

3. The early-life stages of fish are exposed to a range of concentrations of the test chemical dissolved in water. Flow-through conditions are preferred; however, if it is not possible semi static conditions are acceptable. For details the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures should be consulted (2). The test is initiated by placing fertilised eggs in test chambers and is continued for a species-specific time period that is necessary for the control fish to reach a juvenile life-stage. Lethal and sub lethal effects are assessed and compared with control values to determine the lowest observed effect concentration (LOEC) in order to determine the (i) no observed effect concentration (NOEC) and/or (ii) ECx (e.g. EC10, EC20) by using a regression model to estimate the concentration that would cause a x % change in the effect measured. Reporting of relevant effect concentrations and parameters may depend upon the regulatory framework. The test concentrations should bracket the ECx so that the ECx comes from interpolation rather than extrapolation (see Annex 1 for definitions).

4. This Test Guideline has been updated to include the opportunity (optional) to collect sample tissues for cryopreservation in view of further investigations. Decisions on whether to include the optional parameters set out in this test guideline should reflect existing knowledge for the test chemical or similar chemicals, as well as the needs of various regulatory authorities.

INFORMATION ON THE TEST CHEMICAL

5. Test chemical refers to what is being tested. The water solubility (see Guideline 105) and the vapour pressure (see Guideline 104) of the test chemical should be known and a reliable analytical method for the quantification of the chemical in the test solutions with known and reported accuracy and limit of quantification should be available. Although not necessary to conduct the test, results from an acute toxicity test (see Guideline 203 or the Fish Embryo Acute Toxicity Test), preferably v performed with the species chosen for this test, may provide useful information.

6. If the Test Guideline is used for the testing of a mixture, its composition should as far as possible be characterised, e.g., by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties (like those mentioned above). Before use of the Test Guideline for regulatory testing of a mixture, it should be considered whether it will provide acceptable results for the intended regulatory purpose.

7. Useful information includes the structural formula, purity of the substance, water solubility, stability in water and light, pKa, Pow and results of a test for ready biodegradability (e.g., Guideline 301 or Guideline 310).

VALIDITY OF THE TEST

8. For a test to be valid the following conditions apply:

- the dissolved oxygen concentration should be >60% of the air saturation value throughout the test;
- the water temperature should not differ by more than ± 1.5ºC between test chambers or between successive days at any time during the test, and should be within the temperature ranges specified for the test species (Annex 2);
- the analytical measure of the test concentrations is compulsory.
- overall survival of fertilised eggs and post-hatch success in the controls and, where relevant, in the solvent controls should be greater than or equal to the limits defined in Annex 2.

9. If a minor deviation from the validity criteria is observed, the consequences should be considered in relation to the reliability of the test data and these considerations should be included in the report. Effects on survival, hatch or growth occurring in the solvent control, when compared to the negative control, should be reported and discussed in the context of the reliability of the test data.

DESCRIPTION OF THE METHOD

Test chambers

10. Any glass, stainless steel or other chemically inert vessels can be used. As silicone is known to have a strong capacity to absorb lipophilic substances, the use of silicone tubing in flow-through studies and use of silicone seals in contact with water should be minimised by the use of e.g. monoblock glass aquaria. The dimensions of the vessels should be large enough to allow proper growth in the control, maintenance of dissolved oxygen concentration (e.g. for small fish species, a 7 L tank volume will achieve this) and compliance with the loading rate criteria given in paragraph 20. It is desirable that test chambers be randomly positioned in the test area. A randomised block design with each treatment being present in

each block is preferable to a completely randomised design. The test chambers should be shielded from unwanted disturbance. The test system should preferably be conditioned with concentrations of the test chemical for a sufficient duration to demonstrate stable exposure concentrations prior to the introduction of test organisms.

Selection of species

11. Recommended fish species are given in Table 1. This does not preclude the use of other species, but the test procedure may have to be adapted to provide suitable test conditions. The rationale for the selection of the species and the experimental method should be reported in this case.

Holding of the brood fish

12. Details on holding the brood stock under satisfactory conditions may be found in Annex 3 and the references cited (3)(4)(5).

Handling of fertilised eggs, embryos and larvae

13. Initially, fertilised eggs, embryos and larvae may be exposed within the main vessel in smaller glass or stainless steel vessels, fitted with mesh sides or ends to permit a flow of test solution through the vessel. Non turbulent flow-through in these small vessels may be induced by suspending them from an arm arranged to move the vessel up and down but always keeping the organisms submerged. Fertilised eggs of salmonid fishes can be supported on racks or meshes with apertures sufficiently large to allow larvae to drop through after hatching.

14. Where egg containers, grids or meshes have been used to hold eggs within the main test vessel, these restraints should be removed after the larvae hatch, according to the guidance in Annex 3, except that meshes should be retained to prevent the escape of the larvae. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release larvae from egg containers. The timing of this transfer varies with the species and should be documented in the report. However, a transfer may not always be necessary.

Water

15. Any water in which the test species shows suitable long-term survival and growth may be used as test water (see Annex 4). It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of test chemical), or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO4), ammonia, total residual chlorine pesticides, total organic carbon and suspended solids should be made, for example, on a bi-annual basis where a dilution water is known to be relatively constant in quality. If the water is known to be of variable quality the measurements have to be conducted more often; the frequency is dependent of how variable the quality is. Some chemical characteristics of an acceptable dilution water are listed in Annex 4.

Test solutions

16. For flow through tests, a system which continually dispenses and dilutes a stock solution of the test chemical (e.g. metering pump, proportional diluter, saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked

at intervals during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable (3). However, if the loading rate specified in paragraph 20 is respected, a lower flow rate of e.g. 2-3 test chamber volumes is possible to prevent quick removal of food.

17. Test solutions of the chosen concentrations are prepared by dilution of a stock solution. The stock solution should preferably be prepared by simply mixing or agitating the test chemical in dilution water by using mechanical means (e.g. stirring and/or ultrasonication). Saturation columns (solubility columns) or passive dosing methods (6) can be used for achieving a suitable concentrated stock solution. The use of a solvent carrier is not recommended. However, in case a solvent is necessary, a solvent control should be run in parallel, at the same solvent concentration as the chemical treatments; i. e. the solvent level should preferably be equal across all concentrations as well as the solvent control. For some diluter systems this might be technically difficult; here the solvent concentration in the solvent control should be equal to the highest solvent concentration in the treatment group. For difficult to test substances, the OECD Guidance Document No. 23 on aquatic toxicity testing of difficult substances and mixtures should be consulted (2). If a solvent is used, the choice of solvent will be determined by the chemical properties of the substance. The OECD Guidance Document

No. 23 recommends a maximum concentration of 100 µl/L. To avoid potential effect of the solvent on endpoints measured (7), it is recommended to keep solvent concentration as low as possible.

18. For a semi static test, two different renewal procedures may be followed. Either new test solutions are prepared in clean vessels and surviving eggs and larvae gently transferred into the new vessels, or the test organisms are retained in the test vessels whilst a proportion (at least two thirds) of the test solution / control volume is changed.

PROCEDURE

Conditions of Exposure

Duration

19. The test should start as soon as possible after the eggs have been fertilised and preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. The test duration will depend upon the species used. Some recommended durations are given in Annex 2.

Loading

20. The number of fertilised eggs at the start of the test should be sufficient to meet statistical requirements. They should be randomly distributed among treatments, and at least 80 eggs, divided equally between at least four replicate test chambers, should be used per concentration. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60% of the air saturation value can be maintained without aeration during the egg and larval stage. For flow through tests, a loading rate not exceeding 0.5 g/L wet weight per 24 hours and not exceeding 5 g/L of solution at any time has been recommended (3).

Light and temperature

21. The photoperiod and water temperature should be appropriate for the test species (see Annex 2).

Feeding

22. Food and feeding are critical, and it is essential that the correct food for each life-stage is supplied from an appropriate time and at a level sufficient to support normal growth. Feeding should be approximately equal across replicates unless adjusted to account for mortality. Surplus food and faeces should be removed as necessary, to avoid accumulation of waste. Detailed feeding regimes are given in Annex 3 but, as experience is gained, food and feeding regimes are continually being refined to improve survival and optimise growth. Live food provides a source of environmental enrichment and therefore should be used in place of or in addition to dry or frozen food whenever appropriate to the species and life stage.

Test concentrations

23. Normally five concentrations of the test chemical, with a mimimum of four replicates per concentration, spaced by a constant factor not exceeding 3.2 are required. If available, information on the acute testing, preferable with the same species and/or a range finding test should be considered (1) when selecting the range of test concentrations. However, all sources of information should be considered when selecting the range of test concentrations, including sources like e.g., read across, fish embryo acute toxicity test data. A limit test, or an extended limit test, with fewer than five concentrations as the definitive test may be acceptable where empirical NOECs only are to be established. Justification should be provided if fewer than five concentrations are used. Concentrations of the test chemical higher than the 96 hour LC50 or 10 mg/L, whichever is the lower, need not be tested.

Controls

24. A dilution water control and, if needed, a solvent control containing the solvent carrier only should be run in addition to the test chemical concentration series (see paragraph 17).

Frequency of Analytical Determinations and Measurements

25. Prior to initiation of the exposure period, proper function of the chemical delivery system across all replicates should be ensured (for example, by measuring test concentrations). Analytical methods required should be established, including an appropriate limit of quantification (LOQ) and sufficient knowledge on the substance stability in the test system. During the test, the concentrations of the test chemical are determined at regular intervals to characterise exposure. A minimum of five determinations is necessary. In flow-through systems, analytical measurements of the test chemical in one replicate per concentration should be made at least once a week changing systematically amongst replicates. Additional analytical determinations will often improve the quality of the test outcome. Samples may need to be filtered to remove any particulate matter (e.g. using a 0.45 µm pore size) or centrifuged to ensure that the determinations are made on the chemical in true solution. In order to reduce adsorption of the test chemical, the filters should be saturated before the use. When the measured concentrations do not remain within 80-120% of the nominal concentration, the effect concentrations should be determined and expressed relative to the arithmetic mean concentration for flow-through tests (see Annex 6 of the Test Guideline 211 for the calculation of the arithmetic mean (8)), and expressed relative to the geometric mean of the measured concentrations for semi-static tests (see Chapter 5 in the OECD Guidance Document No.

23 on aquatic toxicity testing of difficult substances and mixtures for more details for the calculation of the geometric mean (2)).

26. During the test, dissolved oxygen, pH, and temperature should be measured in all test vessels, at least weekly, and salinity and hardness, if warranted, at the beginning and end of the test. Temperature should preferably be monitored continuously in at least one test vessel.

Observations

27. Stage of embryonic development: the embryonic stage at the beginning of exposure to the test chemical should be verified as precisely as possible. This can be done using a representative sample of eggs suitably preserved and cleaned.

28. Hatching and survival: observations on hatching and survival should be made at least once daily and numbers recorded. If fungus on eggs is observed early in embryonic development (e.g., at day one or two of test), those eggs should be counted and removed. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to physically damage adjacent eggs/larvae. Signs of death vary according to species and life stage. For example:

- for fertilised eggs: particularly in the early stages, a marked loss of translucency and change in colouration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance;
- for embryos, larvae and juvenile fish: immobility and/or absence of respiratory movement and/or absence of heartbeat and/or lack of reaction to mechanical stimulus.

29. Abnormal appearance: the number of larvae or juvenile fish showing abnormality of body form should be recorded at adequate intervals depending on the duration of the test and the nature of the abnormality described. It should be noted that abnormal larvae and juvenile fish occur naturally and can be of the order of several percent in the control(s) in some species. Where deformities and associated abnormal behaviour are considered so severe that there is considerable suffering to the organism, and it has reached a point beyond which it will not recover, it may be removed from the test. Such animals should be euthanised and treated as mortalities for subsequent data analysis. Normal embryonic development has been documented for most species recommended in this Guideline (9) (10) (11) (12).

30. Abnormal behaviour: abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at adequate intervals depending on the duration of the test (e.g. once daily for warm water species). These effects, although difficult to quantify, can, when observed, aid in the interpretation of mortality data.

31. Weight: at the end of the test, all surviving fish are weighed at least on a replicate basis (reporting the number of animals in the replicate and the mean weight per animal): wet weight – (blotted dry) is preferred, however, dry weight data may also be reported (13).

32. Length: at the end of the test, individual lengths are measured. Total length is recommended, if however, caudal fin rot or fin erosion occurs, standard lengths can be used. The same method should be used for all fish in a given test. Individual length can be measured either by e.g. callipers, digital camera, or calibrated ocular micrometer. Typical minimum lengths are defined in Annex 2.

Sample cryopreservation:

33. Plasma and excess tissues may be preserved from surviving fish at the end of the test for possible additional investigations such as omics. Recommended procedures for sample preservation for omics are available in OECD Guidance document No. 409 (14).

34. Care should be taken when considering additional sample preservation so that this does not compromise the standard parameters.

DATA AND REPORTING

Treatment of results

35. It is recommended that the design of the experiment and selection of statistical test permit adequate power (80% or higher) to detect changes of biological importance in endpoints where a NOEC is to be reported. Reporting of relevant effect concentrations and parameters may depend upon the regulatory framework. If an ECx is to be reported, the design of the experiment and selection of regression model should permit estimation of ECx so that (i) the 95% confidence interval reported for ECx does not contain zero and is not overly wide, (ii) the 95% confidence interval for the predicted mean at ECx does not contain the control mean (iii) there is no significant lack-of-fit of regression model to the data. Either approach requires the identification of the percent change in each endpoint that is important to detect or estimate. The experimental design should be tailored to allow that. When the above conditions for determining the ECx are not satisfied, the NOEC approach should be used. It is not likely that the same percent change applies to all endpoints, nor is it likely that a feasible experiment can be designed that will meet these criteria for all endpoints, so it is important to focus on the endpoints, which are important for the respective experiment in designing the experiment appropriately. Statistical flow diagrams and guidance for each approach are available in Annexes 5 and 6 to guide in the treatment of data and in the choice of the most appropriate statistical test or model to use. Other statistical approaches may be used, provided they are scientifically justified.

36. It will be necessary for variations to be analysed within each set of replicates using analysis of variance or contingency table procedures and appropriate statistical analysis methods be used based on this analysis. In order to make a multiple comparison between the results at the individual concentrations and those for the controls, the step-down Jonckheere-Terpstra or Williams' test is recommended for continuous responses and a step-down Cochran-Armitage test for quantal responses that are consistent with a monotone concentration-response and with no evidence of extra-binomial variance (15). When there is evidence of extra-binomial variance, the Rao-Scott modification of the Cochran-Armitage test is recommended (16) (17) or Williams or Dunnett's (after an arcsin-square-root transform) or Jonckheere-Terpstra test applied to replicate proportions. Where the data are not consistent with a monotone concentration-response, Dunnett's or Dunn's or the Mann-Whitney method may be found useful for continuous responses and Fisher's Exact test for quantal responses (15) (18) (19). Care should be taken where applying any statistical method or model to ensure that the requirements of the method or model are satisfied (e.g. chamber to chamber variability is estimated and accounted for in the experimental design and test or model used). Data are to be evaluated for normality and Annex 5 indicates what should be done on the residuals from an ANOVA. Annex 6 discusses additional considerations for the regression approach. Transformations to meet the requirements of a statistical test should be considered. However, transformations to enable the fitting of a regression model require great care, as, for example, a 25% change in the untransformed response does not correspond to a 25% change in a transformed response.

In all analyses, the test chamber, not the individual fish, is the unit of analysis and the experimental unit and both hypothesis tests and regression should reflect that (3) (15) (20) (21).

Test report

37. The test report should include the following information:

Test chemical:

- physical appearance, water solubility, and additional relevant physicochemical properties;

- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible, e.g., by chemical identity, quantitative occurrence and relevant physicochemical properties of the constituents

Test species:

- scientific name, strain, source and method of collection of the fertilised eggs and subsequent handling.

Test conditions:

- test procedure used (e.g. semi static or flow through, loading);

- photoperiod(s);

- test design (e.g. number of test chambers and replicates, number of eggs per replicate, material and size of the test chamber (height, width, volume), water volume per test chamber);

- method of preparation of stock solutions and frequency of renewal (the solubilising agent and its concentration should be given, when used);

- method of dosing the test chemical (e.g. pumps, diluting systems)

- the recovery efficiency of the method and the nominal test concentrations, the limit of quantification, the means of the measured values and their standard deviations in the test vessels and the method by which these were attained and evidence that the measurements refer to the concentrations of the test chemical in true solution;

- dilution water characteristics: pH, hardness, temperature, dissolved oxygen concentration, residual chlorine levels (if measured), total organic carbon (if measured), suspended solids (if measured), salinity of the test medium (if measured) and any other measurements made;

- water quality within test vessels, pH, hardness, temperature and dissolved oxygen concentration;

- detailed information on feeding (e.g. type of food(s), source, amount given and frequency).

- samples cryopreserved (if applicable)

Results reported individually (or on a replicate basis) and as mean and coefficient of variation, as appropriate, for the following endpoints:

- evidence that controls met the overall survival acceptability standard of the test species (Annex 2);

- data on mortality at each stage (embryo, larval and juvenile) and cumulative mortality;

- days to hatch, numbers of larvae hatched each day, and end of hatching;

- number of healthy fish at end of test;

- data for length (specify either standard or total) and weight of surviving animals;

- incidence, description and number of morphological abnormalities, if any;

- incidence, description and number of behavioural effects, if any;

- approach for the statistical analysis (regression analysis or analysis of the variance) and treatment of data (statistical test or model used);

- no observed effect concentration for each response assessed (NOEC);

- lowest observed effect concentration (at p = 0.05) for each response assessed (LOEC);

- ECx for each response assessed, if applicable, and confidence intervals (e.g. 90% or 95%) and a graph of the fitted model used for its calculation, the slope of the concentration-response curve, the formula of the regression model, the estimated model parameters and their standard errors;

- any deviation from the Test Guideline

- Discussion of the results, including any influence of deviations from the Guideline on the outcome of the test.

TABLE 1: FISH SPECIES RECOMMENDED FOR TESTING

FRESHWATER	ESTUARINE and MARINE
Oncorhynchus mykiss Rainbow trout ***Pimephales promelas*** Fathead minnow ***Danio rerio*** Zebrafish ***Oryzias latipes*** Japanese ricefish or Medaka	***Cyprinodon variegatus*** Sheepshead minnow ***Menidia sp.*** Silverside

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ANNEX 1: DEFINITIONS

Fork length (FL): refers to the length from the tip of the snout to the end of the middle caudal fin rays and is used in fishes in which it is difficult to tell where the vertebral column ends

(www.fishbase.org)

Standard length (SL): refers to the length of a fish measured from the tip of the snout to the posterior end of the last vertebra or to the posterior end of the midlateral portion of the hypural plate. Simply put, this measurement excludes the length of the caudal fin. (www.fishbase.org)

Total length (TL): refers to the length from the tip of the snout to the tip of the longer lobe of the caudal fin, usually measured with the lobes compressed along the midline. It is a straight-line measure, not measured over the curve of the body (www.fishbase.org)

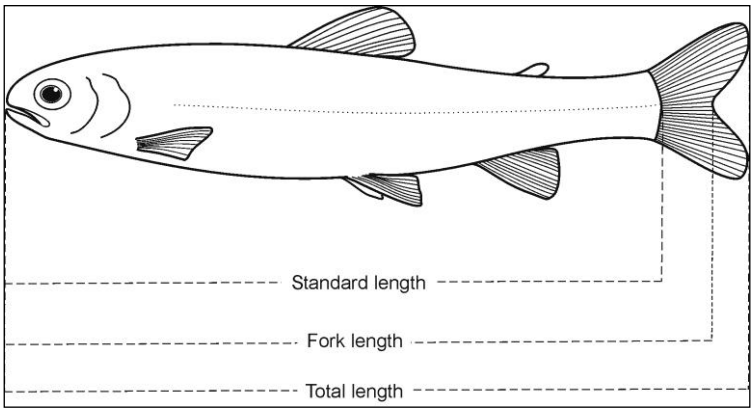

Figure 1: Description of the different lengths, used

ECx: (Effect concentration for x% effect) is the concentration that causes an x% of an effect on test organisms within a given exposure period when compared with a control. For example, an EC50 is a concentration estimated to cause an effect on a test end point in 50% of an exposed population over a defined exposure period.

Lowest observed effect concentration (LOEC) is the lowest tested concentration of a test chemical at which the chemical is observed to have a statistically significant effect (at $p < 0.05$) when compared with the control. However, all test concentrations above the LOEC should have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC (and hence the NOEC) has been selected. Annexes 5 and 6 provide guidance.

No observed effect concentration (NOEC) is the test concentration immediately below the LOEC, which when compared with the control, has no statistically significant effect ($p < 0.05$), within a stated exposure period.

UVCB: substances of unknown or variable composition, complex reaction products or biological materials

IUPAC: International Union of Pure and Applied Chemistry

SMILES: Simplified Molecular Input Line Entry Specification

ANNEX 2: TEST CONDITIONS, DURATION AND SURVIVAL CRITERIA FOR RECOMMENDED SPECIES

SPECIES	TEST CONDITIONS			RECOMMENDED DURATION OF TEST	Typical minimum mean total length of control fish at the end of the study (mm)(1)	SURVIVAL OF CONTROLS (minimum)	
	Temperature (°C)	Salinity (‰)	Photoperiod (hrs)			Hatching success	Post-hatch success
Freshwater:							
Oncorhynchus mykiss Rainbow trout	10 ± 1.5(2)		12 - 16(3)	2 weeks after controls are free-feeding (or 60 days post-hatch)	40	75%	75%
Pimephales promelas Fathead minnow	25 ± 1.5		16	32 days from start of test (or 28 days post-hatch)	18	70%	75%
Danio rerio Zebrafish	26 ± 1.5		12 - 16(4)	30 days post-hatch	11	70%	75 %
Oryzias latipes Japanese Ricefish or Medaka	25 ± 2		12 - 16(4)	30 days post-hatch	17	80%	80%
Estuarine and Marine:							
Cyprinodon variegatus Sheepshead minnow	25 ± 1.5	15-35(5)	12 - 16(4)	32 days from start of test (or 28 days post-hatch)	17	75%	80%
Menidia sp. Silverside	22 - 25	15-35(5)	13	28 days	20	80%	60%

Key:

(1) Typical minimum mean total length is not a validity criterion but deviations below the figure indicated should be carefully examined in relation to the sensitivity of the test. The minimum mean total length is derived from a selection of data available at the current time.

(2) The particular strain of rainbow trout tested may necessitate the use of other temperatures. Brood stock must be held at the same temperature as that to be used for the eggs. After receipt of eggs from a commercial breeder, a short adaptation (*e.g.* 1-2 h) to test temperature after arrival is necessary.

(3) Darkness for larvae until one week after hatching except when they are being inspected, then subdued lighting throughout test (12-16 hour photoperiod)(4).

(4) For any given test conditions, light regime should be constant.

(5) For any given test this shall be performed to ±2‰.

ANNEX 3: FEEDING AND HANDLING GUIDANCE FOR BROOD AND TEST ANIMALS OF RECOMMENDED SPECIES

SPECIES	FOOD*				POST-HATCH TRANSFER TIME	TIME TO FIRST FEEDING
	Brood fish	Newly-hatched larvae	Juveniles			
			Type	Frequency		
Freshwater:						
Oncorhynchus mykiss Rainbow trout	trout food	None [a]	trout starter BSN	2-4 feeds per day	14-16 days post-hatch or at swim-up (not essential)	19 days post hatch or at swim-up
Pimephales promelas Fathead minnow	BSN, flake food FBS	BSN	BSN48, flake food	2-3 times a day	once hatching is 90%	2 day post hatch
Danio rerio Zebrafish	BSN, flake food	Commercial larvae food, protozoa[b], protein[c]	BSN48, flake food,	BSN once daily; flake food twice daily	once hatching is 90%	2 days post hatch
Oryzias latipes Japanese Ricefish or Medaka	flake food	BSN, flake food (or protozoa or rotifers)	BSN48, flake food (or rotifers)	BSN once daily; flake food twice daily or flake food and rotifers once daily	not applicable	6-7 days post spawn
Estuarine and Marine:						
Cyprinodon variegatus Sheepshead minnow	BSN, flake food, FBS	BSN	BSN48	2-3 feeds per day	not applicable	1 day post hatch/swim-up
Menidia sp. Silverside	BSN48, flake food	BSN	BSN48	2-3 feeds per day	not applicable	1 day post hatch/swim-up

Key:
*Food should be given to satiation. Surplus food and faeces should be removed, as necessary to avoid accumulation of waste

FBS	frozen brine shrimps; adults Artemia sp
BSN	brine shrimp nauplii; newly hatched
BSN48	brine shrimp nauplii; 48 hours old
(a)	yolk-sac larvae require no food
(b)	filtered from mixed culture
(c)	granules from fermentation process

ANNEX 4: SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

Substance	Limit concentration
Particulate matter	5 mg/L
Total organic carbon	2 mg/L
Un-ionised ammonia	1 µg/L
Residual chlorine	10 µg/L
Total organophosphorous pesticides	50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	50 ng/L
Total organic chlorine	25 ng/L
Aluminium	1 µg/L
Arsenic	1 µg/L
Chromium	1 µg/L
Cobalt	1 µg/L
Copper	1 µg/L
Iron	1 µg/L
Lead	1 µg/L
Nickel	1 µg/L
Zinc	1 µg/L
Cadmium	100 ng/L
Mercury	100 ng/L
Silver	100 ng/L

ANNEX 5: STATISTICAL GUIDANCE FOR NOEC DETERMINATION

General

The replicate tank is the unit of analysis. Thus, for continuous measurements, such as size, the replicate mean or median should be calculated and these replicate values are the data for analysis. The power of the tests used should be demonstrated, preferably based on an adequate historical database for each lab. The size effect that can be detected with 75-80% power should be provided for each endpoint with the statistical test to be used.

The databases available at the time of development of this guideline establish the power possible under the recommended statistical procedures. An individual lab should demonstrate its ability to meet this power requirement either by conducting its own power analysis or by demonstrating that the Coefficient of Variation (CV) for each response does not exceed the 90th percentile of CVs used in developing the TG. Table 1 provides these CVs. If only replicate means or medians are available, then the within-replicate CV can be ignored.

Table 1: **90th Percentile CVs for selected Freshwater Species**

Species	Response	CV_Between Replicates	CV_Within Replicates
Rainbow Trout	Length	17.4	9.8
	Weight	10.1	28
Fathead Minnow	Length	16.9	13.5
	Weight	11.7	38.7
Zebrafish	Length	43.7	11.7
	Weight	11.9	32.8

For almost all statistical tests used to evaluate laboratory toxicology studies, the comparisons of interest are of treatment groups to control. For that reason, it is not appropriate to require a significant ANOVA F-test before using Dunnett's or Williams' test or a significant Kruskal-Wallis test before using the Jonckheere-Terpstra, Mann-Whitney, or Dunn test (Hochberg and Tamhane 1987, Hsu 1996, Dunnett 1955, 1964, Williams 1971, 1972, 1975, 1977, Robertson et al. 1988, Jonckheere 1954, Dunn 1964).

Dunnett's test has a built-in multiplicity adjustment and its false positive and false negative rates are adversely affected by using the F-test as a gatekeeper. Similarly, the step-down Williams and Jonckheere-Terpstra tests using a 0.05 significance level at every step preserve an overall 5% false positive rate and that rate and the power of the tests are adversely affected by using the F- or Kruskal-Wallis test as a gatekeeper. Mann-Whitney and Dunn's test have to be adjusted for multiplicity and the Bonferroni-Holm adjustment is advised.

A thorough discussion of most of the recommendations on hypothesis testing and verification of assumptions underlying these tests is given in OECD (2006), which also contains an extensive bibliography.

Treatment of Controls when a Solvent is Used

If a solvent is used, then both a dilution water control and a solvent control should be included. The two controls should be compared for each response and combined for statistical analysis if no significant difference is found between the controls. Otherwise, the solvent control should be used for NOEC determination or ECx estimation and the water control is not used. See restriction in the validity criteria (Paragraph 8)

For length, weight, proportion of egg hatch or larval mortality or abnormal larvae, and first or last day of hatch or swim-up, a T-test or Mann-Whitney test should be used to compare the dilution water- control and the solvent control at the 0.05 significance level, ignoring all treatment groups. The results of these tests should be reported.

Size Measurements (length and weight)

Individual fish length and weight values can be normally or log-normally distributed. In either case, the replicate mean values tend to be normally distributed by virtue of the Central Limit Theorem and confirmed from data from well over 100 ELS studies of three freshwater species. Alternatively, where the data or historical databases suggest a log-normal distribution for individual fish size values, the replicate mean logarithm of the individual fish values can be calculated and the data for analysis can then be the anti-logs of these replicate mean logarithms.

Data should be evaluated for consistency with a normal distribution and variance homogeneity. For this purpose, the residuals from an ANOVA model with concentration as the single explanatory class variable should be used. Visual determination from scatterplots and histograms or stem-and-leaf plots can be used. Alternatively, a formal test such as the Shapiro-Wilk or Anderson-Darling can be used. Consistency with variance homogeneity can be assessed from a visual examination of the same scatter plot or formally from Levene's test. Only parametric tests (e.g., Williams, Dunnett) need be evaluated for normality or variance homogeneity.

Attention should be paid to possible outliers and their effect on analysis. Tukey's outlier test and visual inspection of the same plots of residuals described above can be used. It should be recalled that observations are entire replicates, so omitting an outlier from analysis should be done only after careful consideration.

The statistical tests that make use of the characteristics of the experimental design and biological expectation are step-down trend tests, such as Williams and Jonckheere-Terpstra. These tests assume a monotone concentration-response and the data should be assessed for consistency with that assumption. This can be done visually from a scatter plot of the replicate means against test concentration. It will be helpful to overlay that scatter plot with a piecewise linear plot connecting the concentration means weighted by replicate sample size. Great deviation of this piecewise linear plot from monotonicity would indicate a possible need to use non-trend tests. Alternatively, formal tests can be used. A simple formal test is to compute linear and quadratic contrasts of the concentration means. If the quadratic contrast is significant and the linear contrast is not significant that is an indication of a possible problem with monotonicity which should be further evaluated from plots. Where normality or variance homogeneity may be an issue, these contrasts can be constructed from rank-order transformed data. Alternative procedures, such as Bartholomew's test for monotonicity can be used, but add complexity.

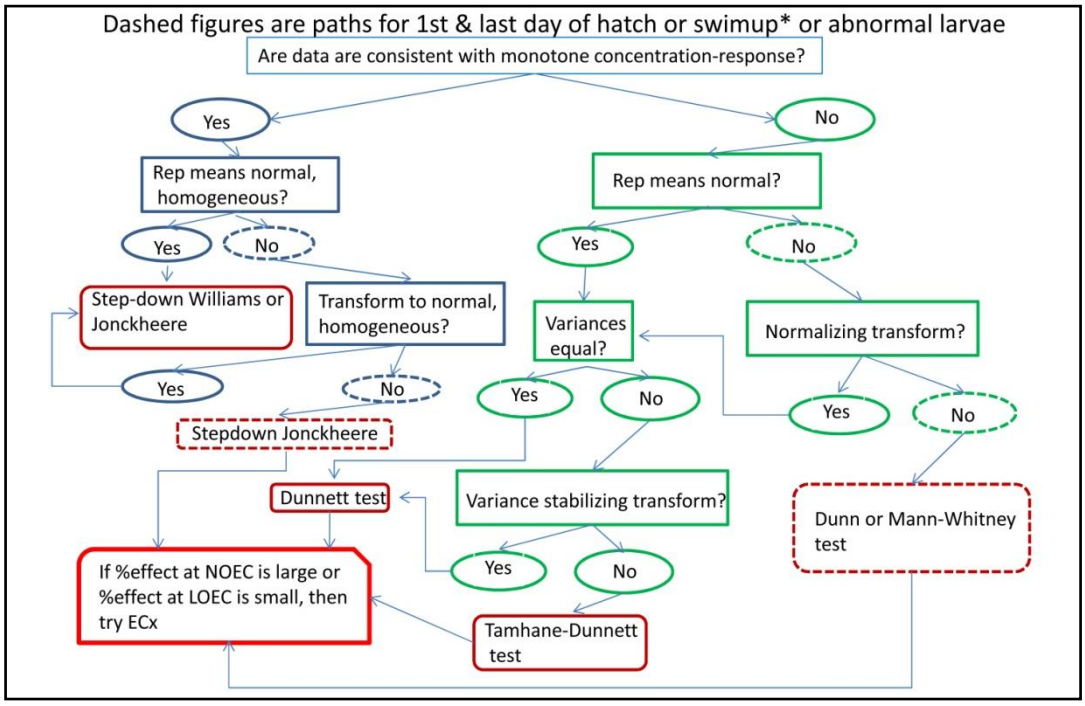

Figure 2: NOEC Flow-Chart Size Measurements (length and weight)

*These responses never satisfy assumptions for parametric analysis or models

Unless the data are not consistent with the requirements for these tests, the NOEC is determined by a step-down application of Williams' or the Jonckheere-Terpstra test. OECD (2006) provides details on these procedures. For data not consistent with the requirements for a step-down trend test, Dunnett's test or the Tamhane-Dunnett (T3) test can be used, both of which have built-in adjustments for multiplicity. These tests assume normality and, in the case of Dunnett, variance homogeneity. Where those conditions are not satisfied, Dunn's non-parametric test can be used. OECD (2006) contains details for all of these tests. Figure 2 is giving an overview, how to find the test of choice.

Egg Hatch and Larval Survival

The data are proportions of eggs that hatch or larvae that survive in individual replicates. These proportions should be assessed for extra-binomial variance, which is common but not universal for such measurements. The flowchart in figure 3 is a guidance for the test of choice; see text for detailed descriptions.

Two tests are commonly used. These are Tarone's C(α) test (Tarone, 1979) and chi-squared tests, each applied separately to every test concentration. If extra-binomial variance is found in even one test concentration, then methods that accommodate that should be used.

$$Z=(\sum_{j=1}^{m} [(x_j-n_j p')]^2/(p'(1-p'))-\sum_{j=1}^{m} n_j)/\{2\sum_{j=1}^{m} [n_j (n_j-1)]\}^{(1/2)}$$

Formula 1: Tarone's C (α) test (Tarone 1979)

where pˆ is the mean proportion for a given concentration, m is the number of replicate tanks, nj is the number of subjects in replicate j, and xj is the number of subjects in that replicate responding, e.g., not hatched or dead. This test is applied to each concentration separately. This test can be seen as an adjusted chi-squared test, but limited power simulations done by Tarone have shown it to be more powerful than a chi-squared test.

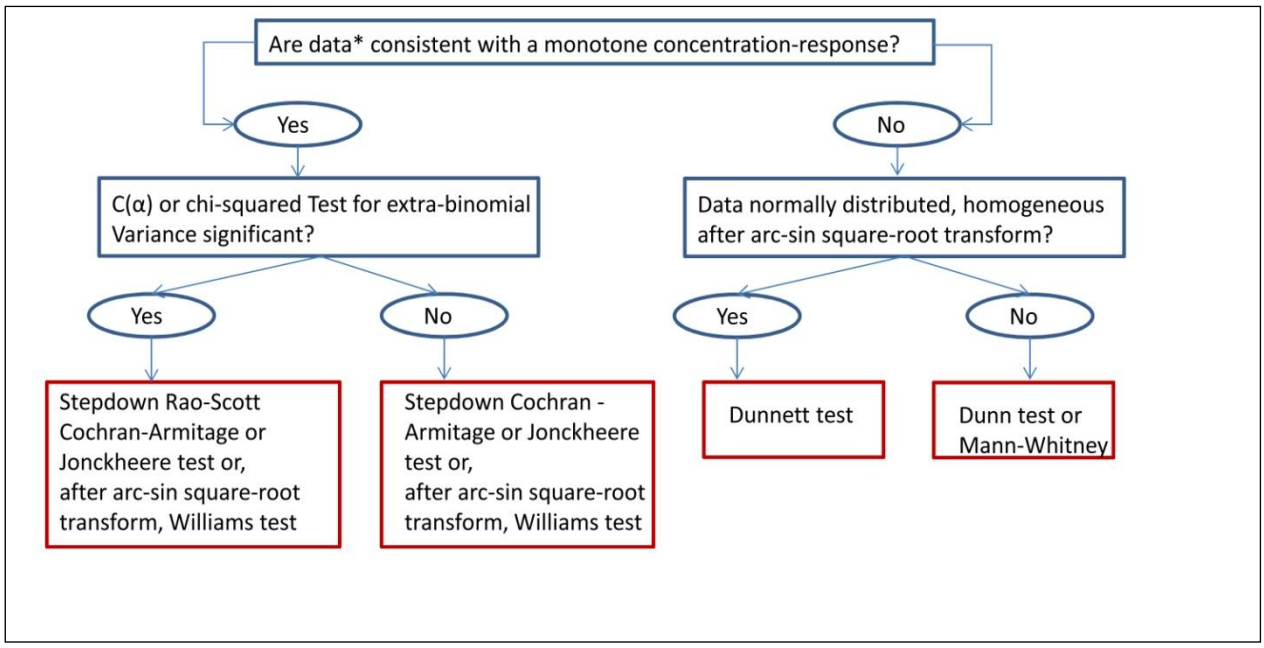

Figure 3: NOEC Flow Chart for Egg Hatch and Larval Mortality

*Data are replicate proportion

Where there is no significant evidence of extra-binomial variance, the step-down Cochran-Armitage test can be used. This test ignores replicates, so where there is such evidence, the Rao-Scott adjustment to the Cochran-Armitage test (RSCA) takes replicates, replicate sizes, and extra-binomial variance into account and is recommended. Alternative tests include the step-down Williams and Jonckheere-Terpstra tests and Dunnett's test as described for size measurements. These tests apply whether or not there is extra-binomial variance, but have somewhat lower power (Agresti 2002, Morgan 1992, Rao and Scott 1992, 1999, Fung et al. 1994, 1996).

First or Last Day of Hatch or Swim-up

The response is an integer, giving the test day on which the indicated observation is observed for a given replicate tank. The range of values is generally very limited and there are often high proportions of tied values, e.g., the same first day of hatch is observed in all control replicates and, perhaps in one or two low test concentrations. Parametric tests such as Williams and Dunnett are not appropriate for such data. Unless there is evidence on serious non-monotonicity, the step-down Jonckheere-Terpstra test is very powerful for detecting effects of the test chemical. Otherwise, Dunn's test can be used.

Larval Abnormalities

The response is the count of larvae found to be abnormal in some way. This response is frequently of low incidence and has some of the same problems as first day of hatch, as well as sometimes exhibiting erratic in concentration-response. If the data at least roughly follow a monotone concentration shape, the step-down Jonckheere-Terpstra test is powerful for detecting effects. Otherwise, Dunn's test can be used.

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ANNEX 6 : STATISTICAL GUIDANCE FOR REGRESSION ESTIMATES

General

The observations used to fit a model are replicate means (length and weight) or replicate proportions (egg hatch and larval mortality) (OECD 2006).

Weighted regression using replicate sample size as weight is generally advised. Other weighting schemes are possible, such as weighting by predicted mean response or a combination of this and replicate sample size. Weighting by reciprocal of within-concentration sample variance is not recommended (Bunke et al. 1999, Seber and Wild, 2003, Motulsky and Christopoulos 2004, Huet et al. 2003).

Any transformation of responses prior to analysis should preserve the independence of the observations and ECx and its confidence bounds should be expressed in the original units of measurement, rather than in transformed units. For example, a 20% change in the logarithm of length is not equivalent to a 20% change in length (Lyles et.al 2008, Draper and Smith 1999).

The flowchart in figure 4 gives an overview for ECx estimations. The details are described in the text below.

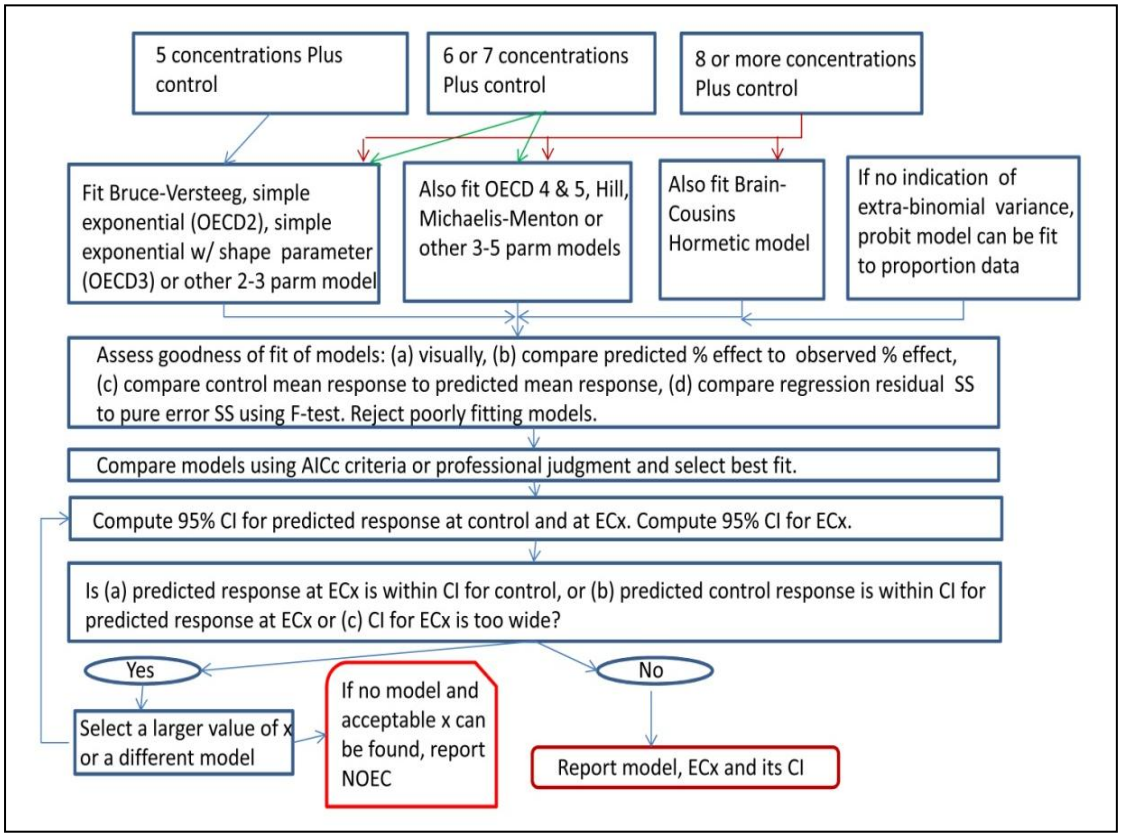

Figure 4: Flow chart for ECx Estimation of Replicate Mean Length, Weight, or Proportion of Egg Hatch or Larval Mortality, see text for more details

Considerations for Egg Hatch and Larval Mortality

For egg hatch and larval mortality, it is generally best to fit a decreasing model unless one is fitting a probit model as described below. That is, one should model the proportion of eggs that do not hatch or larvae that die. The reason for this is that ECx refers to a concentration at which there is a change equal to x% of the control mean response. If there are 5% control eggs that fail to hatch and one models failure to hatch, then EC20 refers to a concentration at which there is a change equal to 20% of the 5% control failure to hatch, and that is a change of 0.2*0.05=0.01 or 1 percentage point to 6% failure to hatch. Such a small change cannot be estimated in any meaningful way from the data available and is not biologically important. Whereas if one models the proportion of eggs that hatch, the control proportion would be 95% in this example and a 20% reduction from the control mean would be a change of 0.95*0.2=0.18, so from 95% hatch success to 77% (= 95-18) hatching success and that effects concentration can be estimated and is presumably of greater interest. This is not an issue with size measurements, though adverse effects on size generally mean a decrease in size.

Models for Size (length or weight) and Egg Hatch Success or Larval Survival.

Except for the Brain-Cousins hermetic model, all of these models are described and recommended in OECD (2006). What are called OECD 2-5, are also discussed for ecotoxicity experiments in Slob (2002). There are, of course, many other models that might be useful. Bunke, et al. (1999) lists numerous models not included here and references to other models are plentiful. Those listed below are suggested as particularly appropriate in ecotoxicity experiments and widely used.

With 5 test concentrations plus control

Bruce-Versteeg

Simple Exponential (OECD 2)

Exponential with shape parameter (OECD 3)

Simple Exponential with Lower Bound (OECD 4)

With 6 or more test concentrations plus control

Exponential with shape parameter and lower bound (OECD 5)

Michaelis-Menton

Hill

Where there is visual evidence of hormesis (unlikely with egg hatch success or larval survival, but sometimes observed in size observations)

Brain-Cousins Hormetic; Brain and Cousens (1989)

Alternative models for egg hatch failure and larval mortality

Increasing models for these responses can be fit by probit (or logistic) models if there is no evidence of extra-binomial variance and control incidence is estimated in the model fit. This is not the preferred method, as it treats the individual, not the replicate, as the unit of analysis (Morgan 1992, O'Hara Hines and Lawless 1993, Collett 2002, 2003).

Goodness of fit of a single model

- Visually compare observed and predicted percent decrease at each test concentration (Motulsky and Christopoulos 2004, Draper and Smith 1999).
- Compare regression error mean square against the pure error mean square using an F-test (Draper and Smith 1999).
- Check that every term in the model is significantly different from zero (i.e., determine whether all model terms are important), (Motulsky and Christopoulos 2004).
- Plots of residuals from regression vs. test concentration, possibly on a log(conc) scale. There should be no pattern to this plot; the points should be randomly scattered about a horizontal line at zero height.
- The data should be evaluated for normality and variance homogeneity in the same way as indicated in Annex 5.
- In addition, normality of the residuals about the regression model should be assessed using the same methods indicated in Annex 5 for the residuals from ANOVA

Compare models

- Use Akiaki's AICc criteria. Smaller AICc values denote better fits and if AICc(B)-AICc(A)≥10, the model A is almost certainly better than model B (Motulsky and Christopoulos (2004).
- Compare the two models visually by how well they meet the single model criteria above.
- The parsimony principal is advised, whereby the simplest model that fits the data reasonably well is used (Ratkowsky 1993, Lyles et.al 2008).

Quality of ECx estimate

The confidence interval (CI) for ECx should not be too wide. Statistical judgment is needed in deciding how wide the confidence interval can be and ECx still be useful. Simulations for regression models fit to egg hatching and size data show that about 75% of confidence intervals for ECx (x=10, 20 or 30) span no more than two test concentrations. This provides a general guide for what is acceptable and a practical guide for what is achievable. Numerous authors assert the need to report confidence intervals for all model parameters and that wide confidence intervals for model parameters indicate unacceptable models (Ott and Longnecker 2008, Alvord and Rossio 1993, Motulsky and Christopoulos 2004, Lyles et al 2008, Seber and Wild 2003, Bunke et al. 1999, Environment Canada 2005).

The CI for ECx (or any other model parameter) should not contain zero (Motulsky and Christopoulos 2004). This is the regression equivalent the minimum significant difference that is often cited in hypothesis testing approaches (e.g., Wang et al 2000). It also corresponds to the confidence interval for the mean responses at the LOEC not contain the control mean. One should wonder whether the parameter estimates scientifically plausible. E.g., if the confidence interval for y0 is ±20%, no EC10 estimate is plausible. If the model predicts a 20% effect at a concentration C and the maximum observed effect at C and lower concentrations is 10%, then the EC20 is not plausible (Motulsky and Christopoulos 2004, Wang et al. 2000, Environment Canada 2005).

ECx should not require extrapolation outside the range of positive concentrations (Draper and Smith 1999, OECD 2006). For example, a general guide might be for ECx to be no more than about 25% below the lowest tested concentration or above the highest tested concentration.

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