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OECD GUIDELINE FOR TESTING OF CHEMICALS

Draft revised version

Fish, Acute Toxicity Test

INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are periodically reviewed to ensure that they reflect the best available science. In the revision of this Guideline (originally adopted in 1981, updated in 1984, 1992), attention was given to possible improvements in relation to animal welfare concerns in order to minimize unnecessary testing and suffering of laboratory animals (OECD 2010), although it was concluded that the use of humane endpoints or moribundity as a surrogate for mortality cannot be recommended without further research.

2. To reflect the best available science this guideline has been updated to include responses to a series of recommendations from the OECD Fish Toxicity Testing Framework 2011, such as the possibility to use the threshold approach and other non-animal alternatives, such as QSAR-methods, as range-finders; a specification that testing the minimum concentration causing 100% and the maximum concentration causing 0% mortality are not mandatory requirements (e.g. no need to test additional concentrations just to demonstrate 0 and/or 100% mortality); guidance on the necessity of employing a water control when solvent is used; the introduction of estuarine and marine fish species, etc. Additionally, it introduces a sheet for recording clinical signs of toxicity along with guidance for individual fish identification. Collection of data on clinical signs will contribute towards the goal of identifying scientifically robust humane endpoints linked to mortality and defining moribundity for incorporation into this guideline in the future.

3. Definitions used in this Test Guideline are given in Annex 1.

PRINCIPLE OF THE TEST

4. The fish are exposed to the test chemical for a period of 96 hours. Clinical signs and mortalities are recorded and the concentrations which kill 50% of the fish (LC50) are determined, where possible

INITIAL CONSIDERATIONS

5. As this test involves treating fish with a test chemical until they die, there are significant animal welfare implications. Therefore, all other means of assessing acute toxicity in fish should be considered prior to conducting the test. These methods include the use of Quantitative Structure Activity Relationships (QSAR), read-across and importantly, fish embryos, which are regarded as non-animals in some countries.

6. Useful information about chemical-specific properties include the structural formula, molecular weight, purity, stability in water and light, pK_a and K_{ow}, water solubility (preferably in the test medium) and vapour pressure as well as results of a test for ready biodegradability (OECD TG 301 (1) or CO_2 in sealed vessels (OECD TG 310 (2)). Solubility and vapour pressure can be used to calculate Henry's law constant, which will indicate whether losses due to evaporation of the test chemical may occur. Conduct of this test without the information listed above should be carefully considered as the study design will be dependent on the physicochemical properties of the test chemical and could lead to results that are difficult to interpret or meaningless.

7. A validated analytical method for the quantification of the test chemical in test solution should be available and performance parameters reported (e.g accuracy, precision, LOD, LOQ, specificity, working range).

8. When considering testing of mixtures, difficult-to-test chemicals (e.g. unstable), or test chemicals not clearly within the applicability domain described in this Guideline, upfront consideration should be given to whether the results of such testing will yield results that are meaningful scientifically. If the Test Guideline is used for the testing of a mixture, a UVCB or a multi-constituent substance, its composition should, as far as possible, be characterized, e.g. by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties (see § 6). Recommendations about the testing of difficult substances (e.g. mixture, UVCB or multi-constituent substance) are given in Guidance Document No. 23 (3).

VALIDITY OF THE TEST

9. For a test to be valid, the following conditions should be fulfilled:

- in the control(s) (dilution water control, solvent control), the mortality should not exceed 10% (or one fish, if fewer than 10 control fish are tested) at the end of the exposure.
- the dissolved oxygen concentration should be ≥60% of the air saturation value in all test vessels throughout the exposure;
- analytical measurement of highest and lowest test concentrations and a test concentration around the LC50 at the start and the end of the exposure period should be carried out as a minimum requirement (see OECD Guidance Document No. 23, § 177, 178 (3))

DESCRIPTION OF THE METHOD

Apparatus

10. Normal laboratory equipment for the conduct of this assay, with appropriate documentation to validate that the equipment is working correctly, include:
(a) oxygen meter;
(b) pH meter;
(c) equipment for determination of hardness of water;
(d) equipment for the determination of total organic carbon concentration (TOC);
(e) equipment for the determination of chemical oxygen demand (COD);
(f) adequate apparatus for temperature control;

(g) tanks made of chemically inert material;
(h) equipment to maintain water temperature and oxygen content as appropriate;
(i) light meter.

Test Chambers

11. 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 minimized. Tubes for dosing should be made of inert material and silicone seals can be avoided by the use of e.g. monoblock glass aquaria. The dimensions of the vessels should be large enough to keep fish free of stress in the control, to maintain dissolved oxygen concentration and to comply with loading rate criteria given in § 20. Test chambers should be randomly positioned in the test area and shielded from unwanted disturbance (excessive noise, vibration, light). For some difficult-to-test chemicals (poorly water-soluble and adsorptive), pre-conditioning of the flow-through system for a suitable period with appropriate concentrations of the test chemical should be considered in order to ensure stable exposure concentrations (3) prior to the introduction of test organisms. For volatile and additional difficult-to-test chemicals, further specific measures should be taken (3).

Selection of Species

12. The selection of species depends in part on the chemical to be tested (industrial chemical, pharmaceutical, biocide or plant protection product), and on the environmental exposure that fish may receive i.e. whether a cold or warm water species, a freshwater or estuarine/marine fish should be chosen. Examples of fish species recommended for testing are given in Annex 2, Table 1. These fish are easy to rear and/or widely available throughout the year. They can be bred and cultivated either in fish farms or in the laboratory, under disease-free conditions, so that the test fish will be healthy and of known parentage. These fish are available in many parts of the world. If other species are used, the rationale for the selection of the species must be reported together with any adaptations to the test guideline's recommendations and it is suggested that the species should be selected primarily on the basis of its ready availability, ease of maintenance, and historical use in safety testing.

Age and Size of Fish

13. Fish should be juveniles (see Table 1, Annex 2, for size guidance) and originate from the same source and population to ensure genetic uniformity. The longest fish should not be more than twice the length of the shortest fish used in one test. The fish should be of the same age and have normal appearance.

Holding of Fish

14. All fish should be held in the laboratory for at least 9 days (48 hours settling-in + 7 days acclimatization) before they are used for testing. They should be held in water of adequate and sufficient quality for use in the test (see Annex 3 for relevant characteristics) for at least seven days immediately before testing and under the following conditions:

Light: 12 to 16 hours light daily;

Temperature: appropriate to the species (see Annex 2, Table 1);

Oxygen concentration: at least 60% of air saturation value;

Feeding: three times per week or daily until 24 hours before the exposure is started. Feed should be given to satiation. Surplus food and faeces should be removed as necessary to avoid accumulation of waste.

When fish are obtained from outdoor ponds (e.g. carp, bluegill) or wild populations, then they may need to be treated for ectoparasites and other diseases when first brought to the testing laboratory.

15. Following a 48-hour settling-in period, mortalities are recorded and the following criteria applied:

- mortalities >10% of population in seven days before the start of the test (not including the 48-hour settling-in period): rejection of entire batch;

- mortalities between 5 and 10% of population in seven days: acclimatization continued for seven additional days;

- mortalities of <5% of population in seven days prior to start of testing: acceptance of batch.

Fish should be free of any apparent malformations and not have been previously treated against disease within the last 14 days prior to testing.

Water

16. For freshwater fish, clean surface-, ground- or reconstituted water (4) is preferred, although if necessary, dechlorinated drinking water may also be used. For estuarine or marine species, reconstituted water is preferred to seawater and can be prepared by adding commercial sea salts (such as *Instant Ocean, Red Sea* or equivalent) to deionized or distilled water. Any water which conforms to the chemical characteristics of acceptable dilution water as listed in Annex 3 is suitable as a test water. It should be of constant quality during the period of the test. The water quality is regarded as good, if fish will survive for the duration of the culturing, acclimatization and testing without showing signs of stress (see Annex 6 for signs of stress). Total hardness and pH should be within the optimal range for the selected fish species (Annex 2, Table 1). The reagents used for the preparation of reconstituted water should be of analytical grade and the deionized or distilled water should be of conductivity ≤10 µS/cm. The dilution water is aerated prior to use for the test so that the dissolved oxygen concentration has reached saturation.

17. If natural water (surface, ground or seawater) is used, the quality parameters including conductivity and total organic carbon (TOC)[1] or chemical oxygen demand (COD) should be measured at least twice a year or whenever it is suspected that these characteristics may have changed significantly. Water quality measurements should continue until the conditions have stabilized. These measurements are required in addition to the twice per year minimum testing. Chemical

[1] High levels of TOC are an indication of high amounts of dissolved organic carbon (DOC), which potentially bind with the test chemical (organic chemicals and metal compounds that demonstrate sorption) and therefore reduce the bioavailable amount as well as the toxicity of the test chemical. DOC is operationally defined as organic molecules that pass through a filter, most often 0.45 µm.

measurements should include heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni; note that copper pipes or compositions containing copper (alloys) may cause fish to die), as well as the chemicals and maximum concentrations shown in Annex 3. Ammonia NH_3, nitrite NO_2 and nitrate NO_3^- should be regularly monitored to ensure suitable quality water and welfare. If dechlorinated tap water is used, daily chlorine analysis is desirable. As a minimum, it should be measured once prior to the test

Test Solutions

18. Test solutions of the selected concentrations can be prepared, e.g. by dilution of a stock solution. The stock solutions should preferably be prepared by simply mixing or agitating the test chemical in the dilution water by mechanical means (e.g. stirring and/or ultra-sonication). If the test chemical is not stable under test conditions and/or difficult to dissolve in water, procedures described in OECD Guidance Document No. 23 for handling of difficult substances should be followed (3). The use of solvents should be avoided, but may be required in some cases in order to produce a suitably concentrated stock solution (for recommended solvents, see OECD Guidance Document No. 23, § 69 (3)). Where a solvent is used to assist in stock solution preparation, its final concentration should be minimized as far as possible (not exceeding 100 mg/L or 0.1 mL/L) and should be the same in all test vessels. For the controls, see § 24.

19. The test should be carried out without adjustment of the pH. Where the chemical itself causes a change of the pH of the test medium outside the range of pH 6.0-8.5, it should be adjusted to lie within the specified range of pH 6.0-8.5 (OECD Guidance Document No. 23, § 139 (3)). In cases where the test item stability in water is influenced by the pH, the pH value can be adapted (within the recommended range) in order to facilitate having a constant test concentration in water.

PROCEDURE

Conditions of Exposure

20. Duration: 96 hours. If there is mortality, but the incipient LC50 is not reached within 96 hours, it is an indication of accumulative or delayed effects and the performance of chronic tests is recommended, such as TG 215, fish juvenile growth test (5), TG 210, fish early-life stage test (6) or TG 211, *Daphnia magna* reproduction test (7).

Loading: maximum loading of 0.8 g wet weight fish/L for static and semi-static testing is recommended; for flow-through systems, maximum loading of 0.5 g wet weight fish/L per 24 hours (example: in a 5 L tank with a flow rate of 5 tank volumes per 24 hours, a total of 25 L pass through in 24 hours, thus 0.5 g/L loading in 25 L correspond to 12.5 g fish/L) and not exceeding 5 g/L of solution at any time is recommended.

Light: should be within the photoperiod ranges specified for the test species (Annex 2, Table 1);

Temperature: the water temperature should not differ by more than ±2°C between test vessels or between successive days at any time during the exposure, and should be within the temperature ranges specified for the test species (Annex 2, Table 1), e.g. for zebrafish with a range of 26±2°C, the temperature selected could be 27°C and should stay between 25 and 28°C in all vessels over the exposure period;

Oxygen concentration: not less than 60% of the air saturation value. Aeration can be used provided that it does not lead to a significant loss of test chemical as verified by analytical measurements of test concentrations (see § 25).

Feeding: none.

Disturbance: disturbances, such as excessive vibration or noise, that may change the behaviour of the fish should be avoided or reduced as far as possible.

Number and Handling of Fish

21. A minimum of 7 fish, randomly distributed among treatments, must be used at each test concentration and in the control(s). 10 fish per treatment is preferred. No test tank replication is required.

Test Concentrations

22. The threshold approach should be applied whenever possible (8) to select the test concentrations for the fish test. Alternatively, predictions within the applicability domain of valid QSAR models, valid read across or grouping estimates, or fish embryos may be used as range finder, if no information on the toxicity of the test chemical is available or sufficient confidence cannot be gained (see Annex 4 for alternative range-finding procedures and *in vivo* fish confirmatory test). If the threshold approach or any other alternative to select the test concentrations as mentioned above cannot be used, a range-finder using fish is required.

23. For the definitive test with fish, at least five concentrations in a geometric series with a factor preferably not exceeding 2.2 are used, although smaller separation factors of 1.6 to 1.8 should be used whenever possible (see Annex 5). The highest test concentration should be 100 mg/L or the limit of the test chemical solubility, whichever is lowest.

Controls

24. When a solvent is used, a solvent control is required in addition to the dilution water control. However, when a low toxicity solvent recommended in OECD Guidance Document No. 23 on testing difficult substances (3) is used, i.e. acetone, ethanol, methanol, tertiary-butyl alcohol, acetonitrile, dimethyl formamide, dimethyl sulfoxide, and triethylene glycol, the dilution water control can be omitted and the test can be conducted and evaluated with a solvent control only. In the threshold approach, the dilution water and solvent control can be omitted if the test has to be repeated at a lower concentration.

Frequency of Analytical Determinations and Measurements

25. With difficult-to-test chemicals, chemical analysis of the test concentrations should be performed before initiation of the exposure to check whether target concentrations are achieved and maintained. For all chemicals, each concentration should also be analyzed individually at least twice at the beginning and termination of the exposure. Analysis of the highest and lowest test concentration and a concentration around the LC_{50} at the start and end of the exposure period is considered the **minimum requirement.** It should be ensured that the determinations reflect the

concentrations of the chemical in true solution (see Guidance Document No. 23, § 36 (3)). If samples are stored to be analyzed later, storage stability of samples should be determined.

26. During the exposure, dissolved oxygen, pH, salinity (if relevant) and temperature should be measured daily in each test vessel – temperature preferably continuously, hardness in the dilution water at the beginning of the exposure.

Observations, Humane Killing and Measurement of Fish

27. **Observations and recording:** Complex biological patterns, closely linked to exposure concentration and duration, including transient effects, can be observed in the fish acute toxicity test. The minimum number of observations required in TG203 (1992) is only once a day, a fact that limits the ability to identify such effects or patterns. Increasing the number of observations to twice a day (at least on initial day), is a relevant and sufficient way to improve the data quality of clinical signs. Observations should follow this format from test initiation (day 0), observations occurring between 3-6 h and 6-12 h of test initiation, with the third (day 1) observation occurring between 8-12 h from the last observation occurring.[2] Thereafter, only the fish tanks where clinical signs occur must be observed at least twice daily. This will increase the robustness and power of the statistical tests on clinical signs and will increase the possibility to identify response patterns,. In Annex 6, examples of clinical signs for fish, which have been collated by experts over time are described. Records should be kept of all visible abnormalities, which can be facilitated by using the description and definitions of clinical signs and the clinical signs reporting sheet in Table 1 & 2 of Annex 6. Dead fish are removed from the test chambers as soon as observed.

28. **Mortality:** Fish are considered dead (i.e. mortality) if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction.[3]

29. **Humane killing of fish:** Surviving fish are euthanized at the end of the exposure. Fish should be euthanised using appropriate lethal levels of anaesthetics followed by physical destruction of the brain (e.g. pithing or crushing the brain) (9, 10).

30. **Measurement of fish:** The individual size (wet weight, blotted dry; and total length) should be measured prior to the initiation of the exposure in at least a subsample of 10 fish from the holding tank[4] to confirm that the fish are juveniles.

LIMIT TEST

31. Using the procedures described in this Guideline, a limit test may be performed for 96 hours at 100 mg/L or at the limit of solubility in the test medium under test conditions, whichever is lower, in order to demonstrate that the LC50 is greater than this concentration. The limit test should be

[2] This will facilitate the identification of effects which may be indicative of clinical signs that precede death during the first 24 hours.

[3] It should be noted that in some jurisdictions, causing mortality in fish is not permitted by ethics or by law. In these cases, moribundity is frequently used.

[4] Measurement of a subsample at test initiation allows verification of the loading rate.

performed using at least 7 fish, with the same number in the control(s).[5] The limit test is considered valid, if the control mortality is ≤ 10%, or 1 fish if fewer than 10 control fish are used. A full study should be conducted, if > 10% dead fish are observed in the exposed fish, or more than one dead fish is observed if < 10 fish are tested. If sub-lethal effects are observed, these should be recorded (see Annex 6).

DATA AND REPORTING

Treatment and Expression of Results

32. It is recommended that results should be calculated using the measured concentrations of test chemical. However, if evidence is available to demonstrate that the concentration remains within 80-120% of the nominal concentration then the results can be based on nominal or measured values. Further guidance can be found in OECD Guidance Document No. 23, § 177, 178 (3). Data should be summarized in tabular form, showing the number of fish used, mortality and sub-lethal effects (see Annex 6) for each concentration and control(s) at each observation time. If a limit test is performed, no graphical representation of responses or statistical calculations are needed. Otherwise, the cumulative percentage mortality for each exposure period, preferably in probit or probability scale in order to produce a straight line, is plotted against concentration in logarithmic scale. The statistical methods to be used for the estimation of the LC50 depends on the number of concentrations observed with partial mortalities. When an experiment results in at least two concentrations with partial mortalities (mortality >0 and <100%), the LC50, the confidence limits (95%) and the slope of the curve should be estimated using appropriate statistical methods such as the classical maximum likelihood methods for fitting probit or logit models (11, 12 and 13). When an experiment results in only one concentration with partial mortality or none, classical maximum likelihood methods cannot be used to estimate the LC50, the slope of the concentration-response curve cannot be estimated, and a confidence interval for the LC50 may not be estimable. In such cases, estimates of the LC50 can be made using various techniques such as the Spearman-Karber method (14), the binomial method (15), the moving average method (4), or as a last resort, the graphical method (15). These non-classical methods can give precise LC50 estimates for well-designed studies (15), and are extremely useful, as up to 75% of acute fish studies yield results that cannot be analyzed using classical probit maximum likelihood techniques (16).

Test Report

33. The test report should include the following information:

Test chemical:

Mono-constituent substance:

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

[5] Binomial theory dictates that when 10 fish are used with zero mortality, there is a 99.9% confidence that the LC50 is greater than the concentration used in the limit test. With 7, 8 or 9 fish, the absence of mortality provides at least 99% confidence that the LC50 is greater than the concentration used in the limit test.

- 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:

- characterized as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Test fish:

- scientific name, strain, size (wet weight, blotted dry, and total length), supplier, any pretreatment, etc.

Test conditions:

- test procedure used (e.g. static, semi-static, flow-through; aeration; fish loading; etc.);
- water quality characteristics (pH, hardness, temperature; TOC, COD for surface, ground or seawater) and adaptations made to suit the requirements of fish species used other than those in Annex 2, Table 1;
- dissolved oxygen concentration, pH values and temperature of the test solutions at 24-hour intervals in each tank and continuous in one tank (in semi-static systems: dissolved oxygen, pH and temperature prior to and after water renewal);
- methods of preparation of stock and test solutions;
- concentrations used;
- information on concentrations of the test chemical in the test solutions;
- number of fish in each test solution.

Results:

- maximum concentration causing no mortality within the period of the exposure (but it is not a mandatory requirement to identify a maximum concentration causing 0% mortality with one of the 5 concentrations);
- cumulative mortality at each concentration at the recommended observation times;
- the LC50 values at 24, 48, 72 and 96 hours with 95% confidence limits, if possible;
- the slope of the concentration-response curve, if possible;
- graph of the concentration-mortality curve at the end of the exposure preferably on probit or probability scale versus concentration on log scale (note that the control group cannot be plotted on log scale axes). Likewise, neither 0 nor 100% mortality can be plotted on a probit scale (undefined values), and the slope cannot be meaningfully represented for experiments with less than two partial mortalities or if the 50% response is between the control and lowest test concentration. Therefore, graphs are not a requirement under such circumstances, but they might help to visualize the results;
- mortality in the control(s);
- incidence and description of visible abnormalities such as loss of equilibrium, swimming behaviour, respiratory function, pigmentation and other clinical signs including degree of the effects, if possible (according to Annex 6);
- incidents in the course of the test which might have influenced the results;

- description of the statistical methods used and treatment of data (e.g. probit analysis, logistic regression model, arithmetic or geometric mean for LC50 values, time weighted average)
- Any deviation from the Guideline and relevant explanations

LITERATURE

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(2)	OECD (2006) Ready Biodegradability, CO2 in sealed vessels, Test Guideline No. 310, Guidelines for the Testing of Chemicals, OECD, Paris.
(3)	OECD (2018) Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Series on Testing and Assessment No. 23, OECD, Paris.
(4)	ISO (1996) International Standards. Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)]. ISO 7346-3: Flow-through method. Available: [http://www.iso.org].
(5)	OECD (2000) Fish, Juvenile Growth Test, Test Guideline No. 215, Guidelines for the Testing of Chemicals, OECD, Paris.
(6)	OECD (2013) Fish, Early-life Stage Toxicity Test, Test Guideline No. 210, Guidelines for the Testing of Chemicals, OECD, Paris.
(7)	OECD (2008) *Daphnia magna* Reproduction Test, Test Guideline No. 211, Guidelines for the Testing of Chemicals, OECD, Paris.
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(15)	USEPA (2002) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. Fourth edition. US Environmental Protection Agency, Office of Water, Washington, DC. EPA-821-R-02-013. October 2002.
(16)	Rufli H, Springer TA (2011) Can we reduce the number of fish in the OECD acute fish toxicity test? *Environ Toxicol Chem* 30: 1006-1011.

ANNEX 1

DEFINITIONS

Flow-through test is a test with continued flow of test solutions through the test system during the duration of exposure.

InChI code: IUPAC International Chemical Identifier.

Incipient LC50: The curve obtained by plotting toxic concentrations to fish against time will reach a plateau value by 96h in most cases. This refers to the incipient LC50, below which 50% of exposed individuals would live indefinitely relative to the lethal effect of the toxicant.

IUPAC: International Union of Pure and Applied Chemistry.

Median Lethal Concentration (LC_{50}) is the concentration of a test chemical that is estimated to be lethal to 50% of the test organisms within the test duration.

Moribund: Approaching death, even if transferred to clean water.

Semi-static renewal test is a test with regular renewal of the test solutions after defined periods (e.g. every 24 hours).

SMILES: Simplified Molecular Input Line Entry Specification.

Static test is a test in which test solutions are not being renewed throughout the duration of the test.

Threshold Concentration (TC): The lowest EC50-value of existing and reliable algal or acute invertebrate (e.g. Daphnia) toxicity data is set as the threshold concentration, which in then used for the limit test.

Standard length (SL): 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): 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)

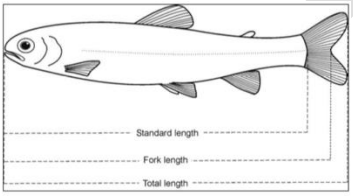

Figure 1: Description of the different lengths, used

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

377 **ANNEX 2**

378 **TABLE 1: RECOMMENDED FISH SPECIES, TOTAL LENGTHS AND TEST CONDITIONS**

Species[6]	Temperature (°C)	Salinity[7] (‰)	pH	Hardness (mg/L $CaCO_3$)	Photoperiod (hours light)	Size at first maturity[8] (cm)	Total length[9] (cm)
Freshwater:							
Danio rerio *Zebrafish*	26±2	0	6.0-8.1	>100-250	12-16	F: 2.5, M: 2.3[10]	2.0 ± 1.0
Pimephales promelas Fathead minnow	25±2	0	6.0-8.5	10-250	16	4.8[11]	2.0 ± 1.0
Cyprinus carpio Carp	22±2	0	6.0-8.5	10-250	12-16	Not relevant	3.0 ± 1.0
Oryzias latipes Japanese Medaka	25±2	0	6.0-8.5	10-250	12-16	1.6-2.5[12]	2.0 ± 1.0
Poecilia reticulata Guppy	23±2	0	6.0-8.5	10-250	12-16	F: 2.5, M :1.8[13]	2.0 ± 1.0
Lepomis macrochirus Bluegill	23±2	0	6.0-8.5	10-250	12-16	Not relevant	2.0 ± 1.0
Oncorhynchus mykiss Rainbow trout	10±2	0	6.0-8.5	10-250	12-16	Not relevant	5.0 ± 1.0
Gasterosteus aculeatus Three-spined stickleback	18±2	0-35	6.0-8.5	10-250	12-16	2.5[14]	2.0 ± 1.0

[6] If other species are used, the rationale for the selection of the species must be reported together with any adaptations to the test guideline's recommendations and it is suggested that the species is selected on the basis of their ready availability, ease of maintenance, and historical use in safety testing.

[7] For any given test this shall be performed to ± 2‰, e.g. 17±2 =15-19‰, 31±2 =29-33‰.

[8] Test fish must be juveniles. For small fish like medaka, guppy, zebrafish, stickleback and sheepshead minnow, the acceptable size in the current table of 2.0 ± 1.0 cm implies they may have reached sexual maturity and may therefore be able to lay eggs. Under such conditions, the LC50 of chemical substances which have differential toxicity between the sexes will be influenced by the sex ratio of the fish used for each test. Also, the body burden of the test chemicals might be reduced if the test fish lay eggs during the experiment. For these reasons, those species must be used when smaller than the size at first maturity.

[9] If fish of sizes other than those recommended are used, this should be reported together with developmental stage (juvenile, sub-adult, adult stage) and the rationale.

[10] Referring to standard, not total length, data from www.fishbase.org

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[13] Montag, L. F. de A., Freitas, T. M. da S., Raiol, R. D. de O., & Silva, M. V. da. (2011). Length-weight relationship and reproduction of the guppy Poecilia reticulata (Cyprinodontiformes: Poeciliidae) in urban drainage channels in the Brazilian city of Belém. Biota Neotropica, 11(3), 93–97.

[14] Wootton, R. J. (1976). The biology of the sticklebacks. London: Academic Press.

Species[6]	Temperature (°C)	Salinity[7] (‰)	pH	Hardness (mg/L $CaCO_3$)	Photoperiod (hours light)	Size at first maturity[8] (cm)	Total length[9] (cm)
Estuarine and Marine							
Cyprinodon variegatus Sheepshead minnow	20±2 and 25±2	15-35	6.0-8.5	10-250	12-16	F: 2.7, M: 3.4 [15]	2.0 ± 1.0
Menidia menidia *Atlantic* silverside ***Menidia beryllina*** Inland silverside ***Menidia peninsulae*** Tidewater silverside	25±2 25±2 22±2	15-35	6.0-8.5	10-250	12-16	Not relevant	3.0 ± 1.0
Dicentrarchus labrax European sea bass	20±2	15-35	6.0-8.5	10-250	12-16	Not relevant	6.0 ± 2.0

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[15] 10) United States Environmental Protection Agency. (2002). Method 1004.0: Sheepshead Minnow, *Cyprinodon variegatus*, Larval Survival and Growth Test. United States Environmental Protection Agency. https://doi.org/EPA-821-R-02-014

ANNEX 3

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION/TEST WATER FOR FRESHWATER, ESTUARINE AND MARINE FISH

Substance	Maximum concentration
Particulate matter	5 mg/L
Total organic carbon TOC	2 mg/L
Un-ionised ammonia NH_3	1 µg/L
Residual chlorine	10 µg/L
Total organophosphorus pesticides	50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	50 ng/L
Total organic chlorine	25 ng/L
Aluminium Al	1 µg/L
Arsenic As	1 µg/L
Chromium Cr	1 µg/L
Cobalt Co	1 µg/L
Copper Cu	1 µg/L
Iron Fe	1 µg/L
Lead Pb	1 µg/L
Nickel Ni	1 µg/L
Zinc Zn	1 µg/L
Cadmium Cd	100 ng/L
Mercury Hg	100 ng/L
Silver Ag	100 ng/L
Chemical oxygen demand COD	5 mg/L

ANNEX 4

RANGE-FINDING USING FISH EMBRYOS

When a range-finding test is required, the fish use can be reduced in some jurisdictions by using fish embryos instead of juvenile fish. For example, the 96 hours fish embryo toxicity test (1) does not fall under the scope of the European Union animal protection directive (2) because the test is terminated before the zebrafish embryos would start independent feeding (Art 1, §3) (3).

The range-finding-test with embryos is started at the *Threshold Concentration (TC)* (4), if available, or another reasonable starting concentration ≤100 mg/L based on the information available.[16] If the embryo test shows no toxicity at this concentration, it is followed by an *in vivo* fish confirmatory test performed at the concentration of the fish embryo test as a limit test, or as a full test if a concentration-effect relationship is required. If the concentration is toxic, the embryo test is repeated, stepping down from the previous test concentration until there is no toxicity, followed by the *in vivo* confirmatory limit or full test with fish as above. This procedure is shown in the flow-chart below. Testing should be ended if the mortality does not exceed 10% (or one fish, if fewer than 10 fish are tested).

Instead of the 96 hour fish embryo test, a 48 hour test might be used. However, for some chemicals like quaternary ammoniums, a 96 hour test is required to give better correlation to the fish test as embryos are protected by the selective permeability of the chorion until hatching (zebrafish hatching: after 2-3 days, fathead minnow: after 4-5 days, medaka: after 9-14 days) (6).

Limitations:

1. The fish embryo test OECD TG 236 (1) was designed for zebrafish. Although other species like medaka and fathead minnow are covered in the Background Paper (6), its application is limited as other warm water species are different with regard to hatching times; this may result in different sensitivities.
2. Care should be taken with difficult substances: if the test is performed in plastic multiwell plates, lipophilic substances may adsorb to the plastic surface.

[16] An evaluation of 694 acute algae, daphnia and fish tests revealed that fish were the most sensitive in only 15.6% of these tests whereas in 84.4%, the fish LC50 was ≥TC (5).

421 **FLOW-CHART**

1	Fish embryo test at TC (or other reasonable starting concentration ≤100 mg/L)	No toxicity Toxicity	Proceed to step 2 Repeat step 1 stepping down to lower concentration until there is no toxicity, then proceed to step 2
	↓		
2	Limit test with juvenile fish at the concentration with no toxicity or full study with juvenile fish	No toxicity Concentration -response curve	LC_{50} fish > tested concentration LC_{50} fish

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(1)	OECD (2013) Fish Embryo Acute Toxicity (FET) Test, Test Guideline No. 236, Guidelines for the Testing of Chemicals, OECD, Paris.
(2)	European Commission (2010) Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. *Official Journal of the European Union* L 276, 20.10. pp 33-79.
(3)	Belanger SE, Balon EK, Rawlings JM (2010) Saltatory ontogeny of fishes and sensitive early life stages for ecotoxicology tests. *Aquat Toxicol* 97:88-95.
(4)	OECD (2010) Short Guidance on the Threshold Approach for Acute Fish Toxicity. Series on Testing and Assessment No. 126, OECD, Paris.
(5)	Weyers A, Sokull-Klüttgen B, Baraibar-Fentanes J, Vollmer G (2000) Acute toxicity data: a comprehensive comparison of results of fish, *Daphnia* and algae tests with new substances notified in the EU. *Environ Toxicol Chem* 19:1931-1933.
(6)	Braunbeck T, Lammer E (2006) Background on Fish Embryo Toxicity Assays. UBA Contract Number 203 85 422. Prepared for German Federal Environment Agency, D-06813 Dessau.

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ANNEX 5

FACTOR BETWEEN CONCENTRATIONS

Although a factor between concentrations (separation factor) of 2.2 is permissible, when the LC50 can be estimated with sufficient confidence, separation factors of 1.6 to 1.8 are preferred for the following reasons:

1. It is not uncommon in fish tests to find none or just one concentration with a partial mortality (PM: >0 and <100% mortality). In the Industry Laboratory Database, these studies amounted to 75% (1)[17] for which classical statistical methods cannot be used.[18]
2. Little information is gained from the multiple test concentrations with no or complete mortality. In such cases, fish in 3 of 5 concentrations do not contribute to the determination of the LC50 and are, thus, wasted and suffer unnecessarily.
3. The plot of the factor between concentrations for two partial mortalities (for 13 and 87% mortality) versus the slope of the probit transformed concentration-response curve shows that separation factors of 1.2 to 2.0 would produce two partial mortalities for slopes between 7 and 30 (Fig. 1, dotted lines). To get two partial mortalities for 75% of the studies would require a ´Factor for 2PM´ of ≤1.8 for slopes up to 8.8 (75th centile) according to the U.S. EPA Oneliner Database[19] (Fig. 2a). Thus, separation factors of ≤1.8 would produce two partial mortalities if the distribution follows that of the U.S. EPA Oneliner Database (slopes ≤8.8). Even if two partial mortalities are not obtained so that mortality goes from 0 to 100% in adjacent concentrations, there is still an advantage in keeping the separation factor small, because the region of the transition from survival to mortality is more narrowly defined. The only reason to use broader spacing is to increase the probability that the test concentrations selected during study design will encompass the unknown LC50.[20]

   As a consequence, separation factors should be selected as a compromise between the need to bracket the true LC50 and the desire to minimize fish waste. A reasonable compromise appears to be using separation factors of 1.6-1.8, e.g. concentrations of 1.0, 1.8, 3.2, 5.6, 10 mg/L when using factor 1.8.

[17] Because the goal of performing a test according to OECD 203 is to estimate the 50% lethal concentration (LC50), the results of many fish acute tests performed for regulatory submission with low-toxicity chemicals must be expressed as a one-sided interval, such as LC50 >100 mg/L (37% in Industry Laboratory Database: 194 of 523 studies; 8% in U.S. EPA Oneliner Database: 326 of 4010 studies).

[18] For the use of classical probit maximum likelihood techniques, and to obtain an estimate of the slope, at least 2 partial mortalities are required.

[19] For the Industry Database, to get two partial mortalities for 75% of the studies would require a ´Factor for 2PM´ of approximately ≤1.4 for slopes up to 18.8 (75th centile), i.e. separation factors of ≤1.4 would be required to produce two partial mortalities if the distribution follows that of the Industry Database.

[20] A widespread concern is that the lower spacing factor might result in missing the LC50 leading to additional concentrations to be tested. This explains why, in practice, wider ranges are often selected. However, failures to bracket the LC50 can be minimized if suitable methods are used to select the concentrations of the definitive test. A good method to interpret the data of the rangefinder is to draw the results on probability paper on which the concentration-effect relationship forms a straight line representing all the information gathered in the rangefinder. Percentage mortalities can be deducted for each concentration. Thus, it not only provides a simple way to encompass the LC50, but also to minimize concentrations with 0 and 100% effect in which fish are wasted and suffer unnecessarily.

Figure 1. Plot of maximum ratios of adjacent test concentrations (Factor for 2PM) that would result in the expectation of two adjacent concentrations with 13 and 87% mortality if centered on the true LC50 (D-optimal dose placement). 'Factor for 2PM' is plotted as a function of concentration-response curve slopes. The 13 and 87% mortality rates correspond to one mortality in the test concentration below the LC50, and one survivor in the test concentration above the LC50. The plot shows that factors between concentrations of 1.2 to 2.0 would produce two partial mortalities for slopes between 7 and 30 (dotted lines). Concentrations for use in a study should be selected using a step size less than the 'Factor for 2PM' obtained assuming some likely slope of the concentration-response curve, thus factors <1.2 to <2.0.

PM: partial mortality (mortality in concentration >0 and <100%)

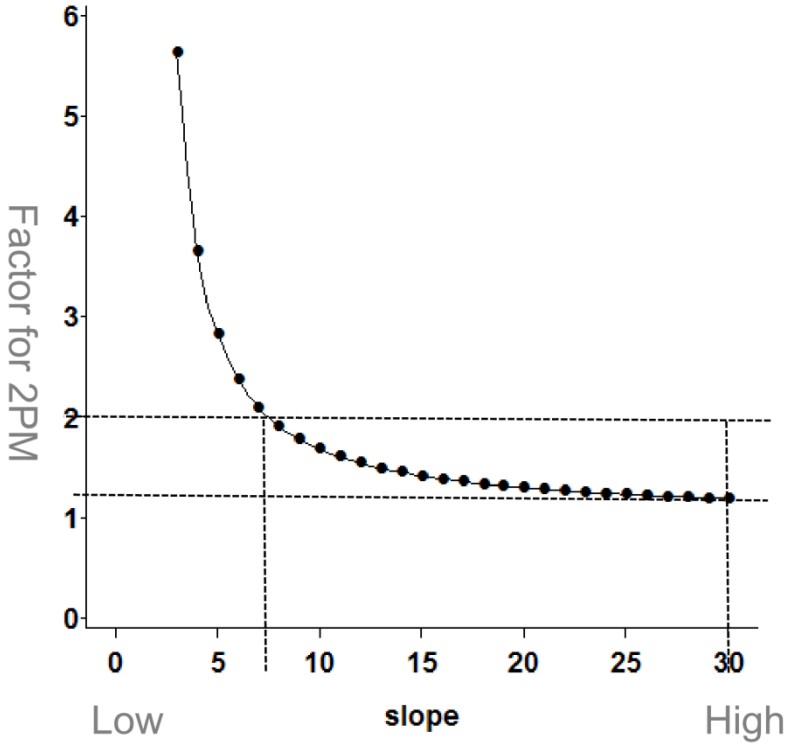
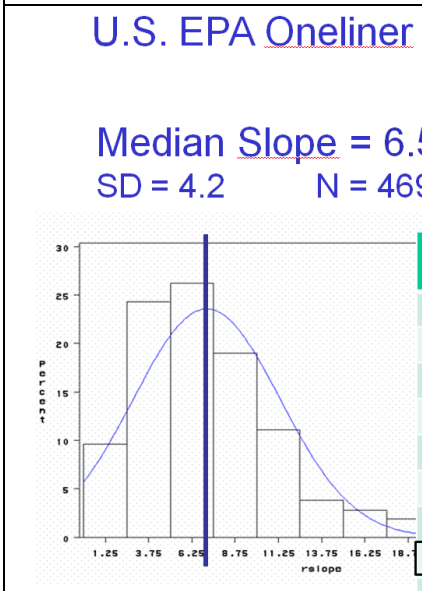


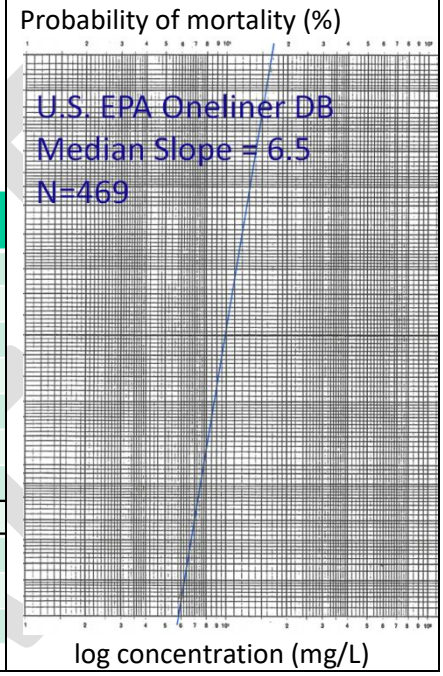
Figure 2: 2a) Distribution of the concentration-response slopes from tests performed according to regulatory test guidelines (U.S. EPA Oneliner Database). 2b) Median Slope of the Oneliner Database shown graphically on probability paper.

2a)



Quantile (%)	Estimate
100 Max	27.2
99	20.9
95	15.3
90	12.4
75	8.8
50	6.5
25	4.1
10	2.5
5	1.5
1	1.2
0	0.3

2b)



SD: standard deviation
N: total number of studies
DB: database

(1)	Rufli H, Springer TA (2011) Can we reduce the number of fish in the OECD acute fish toxicity test? *Environ Toxicol Chem* 30: 1006-1011.

ANNEX 6

CLINICAL SIGNS DESCRIPTION AND RECORDING SHEET

Introduction: It has become common practice in many laboratories to introduce early and humane endpoints in acute fish testing, as it can reduce the terminal suffering of the fish.[21] Attempts over the past decade within OECD VMG-Eco failed to define 'moribund state' in fish. This is because the original TG203 did not require the systematic collection of observations that could lead to the identification of early clinical signs predictive of moribundity or death. In addition, the limited number of observations required under TG203 were relevant to the tank rather than individual fish within the tank. At present, there is insufficient knowledge on how sub-lethal effects in fish can be used to predict death accurately. The identification of clinical signs that are predictive of moribundity and death is crucial to their effective use as experimental endpoints (3). Therefore, there is an urgent need to relate early clinical signs to moribundity and death in fish. To generate reliable data for such an analysis, systematic collection of observations on the development of clinical signs of moribundity/death over time and in the same fish, is needed. For this purpose, Annex 6 represents a tool for collecting information on clinical signs systematically and Annex 7 provides guidance on identification of individual fish.

Table 1 depicts definitions of clinical signs observed in fish toxicity studies (4, 5, 6, 7). Table 2 represents the recording sheet for those signs.22 The clinical signs are classified into three categories (minor, medium and major), according to the present state of knowledge (8, 9) although there is a spectrum of magnitude for some of these observations.23 Other sub-lethal effects should be recorded and treated in a similar way, if appropriate. However, it should always be carefully checked that any clinical sign(s) are a result of test chemical exposure only24 rather than other factors such as environmental conditions (e.g. dissolved oxygen concentration or aggression, improper handling or disease). Clear examples of chemical related clinical signs include effects on the operculum due to exposure to cationic chemicals (10) and internal haemorrhaging due to exposure to acetylcholinesterase inhibitors (11). A weight of evidence approach may aid this distinction.25 Laboratories should record the data on clinical signs in the recording sheet, including additional information available, such as physico-chemical properties (e.g. KoW), mode of action (e.g. reversible non-polar narcosis) or potential degradation (if testing in a static system). These data are then uploaded to the relevant database provided by OECD.

[21] The Directive 2010/63/EU states in Article 13 (1): "death as an endpoint of a procedure shall be avoided as far as possible and replaced by early and humane endpoints" and "procedures shall be selected which are most likely to provide satisfactory results". In the context of this guideline, this means that the implementation of humane endpoints should ensure the requirements for a determination of an LC50 are met (e.g. ensuring regulatory authority compliance).
The Canadian Council on Animal Care Guidelines (2) declares: "where feasible, the development of pre-lethal endpoints in such tests is encouraged".

[22] Examples of clinical signs:
for zebrafish: https://wiki.zfin.org/display/ZHWG/Zebrafish+Health+and+Welfare+Glossary+Home;
for salmonids: http://necropsymanual.net/en/additional-info/fpa/

[23] Note that clinical signs may be species-specific and may also be size and population specific.

[24] No effect in controls; effects and concentration-response; similar effects in range-finder; overall picture consistent with chemical toxicity.

[25] Example of weight of evidence evaluation: Consider starting time of appearance of clinical sign, development of signs over time (persistent, increasing, decreasing), number of fish affected, tanks affected (concentrations, control, holding tanks), potential origin of clinical sign (e.g. poor handling, aggression, disease, toxic effect, environmental conditions).

Table 1: Description and definitions of fish clinical signs.

Scores (0-3) to indicate scale of departure from normal, with binomial clinical signs (i.e. either absent or present) being scored as either 0 or 3.

	Clinical sign	Definition	Synonyms	0 - Normal (Absent)	1- Minor	2- Medium	3- Major (Present)
Distribution	Loss of schooling / shoaling behaviour	Individual fish show loss of aggregating & social interactions	Isolation, social isolation	Normal			Observed
	Dense schooling / shoaling behaviour	Increase in clumped association of fish	Crowding	Normal			Observed
	Vertical distribution - surface	Abnormal depth selection, close to water/air interface	On/at/near/just below surface/top	Normal			Observed
	Vertical distribution - bottom	Abnormal depth selection, close to base of tank	Lying on/ orientation to / collecting at / near / just above bottom	Normal			Observed
Equilibrium & buoyancy	Abnormal horizontal orientation	Loss of balance displaying as abnormal horizontal orientation/posture in water column	Keeling, lost righting reflex	Normal	Intermittent, partial, slight leaning, attempts to correct	Constant heavy leaning, on side	Upside down/ on back
	Abnormal vertical orientation	Head-up or head-down posture		Normal	Intermittent, partial, slight pitching, attempts to correct	Constant pitching (up or down)	Head directly up or down
	Loss of buoyancy control	Floating at surface or sinking to the bottom		Normal			Floating at surface; sinking to the bottom
Observed behaviours	Hypoactivity	Decrease in spontaneous activity	Torpid, apathy, lethargy, weak, immobility, inactivity, ceased swimming, quiescent	Normal (calm) swimming activity			No visible swimming movements
	Hyperactivity	Increase in spontaneous activity	Erratic swimming, skittering	Normal (calm) swimming activity			Rapid (erratic / irregular) movements
	Spiral swimming	Rotation on vertical or horizontal axis; erratic movements, often in bursts	Spiralling, rolling, tumbling, corkscrew swimming	None			Observed
	Hyperventilation	Increased frequency of opercular ventilatory movements	Rapid/strong respiratory rate/ function	Normal			Fast opercular movements
	Hypoventilation	Decreased frequency of opercular ventilatory movements	Reduced/laboured/weak/slow respiration/respiratory action/ventilation	Normal			Slow (and possibly shallow) opercular movements
	Irregular ventilation	Irregular opercular ventilatory movements	Sporadic / spasmodic respiration / gill movement	Regular ventilation			Erratic opercular movements
	Increased ventilation depth	Increased amplitude of opercular movements	Heavy gill movements, strong ventilation, strongly extended gills, abnormal opercular activity, operculae spread apart, mouth open	Normal			Operculae extended and/or mouth open
	Convulsions	Abnormal, involuntary and uncontrolled contraction of muscles	Seizures, twitching, muscle spasms, shaking, shuddering, vibration	None			Observed
	Coughing	Fast reflex expansion of mouth and operculae not at water surface - assumed to clear ventilatory channels	Abnormal opercular activity	None			Observed
	Gulping	Mouth (and opercular) movements at water surface, resulting in intake of water & air.	Piping	None			Observed
	Gasping	Occasional expansion of mouth and operculae not associated with ventilation; not at water surface	Yawn	None			Observed
	Surface escape / avoidance behaviours		Jumping, surfacing	None			Observed
	Bottom escape / avoidance behaviours		Diving, sounding	None			Observed
	Irritated skin behaviours	Flashing, scraping, rubbing		None			Observed
	Aggression and/or cannibalism		Aggression, direct attack, domination of choice tank locations, pick at or eat bodies of dead fish	Normal			Observed
Appearance	Tetany	Rigid body musculature	Paralysis	None		Intermittent	Permanent
	Skin colour - darkening		Changed / increased / dark(ened) colour / pigmentation / melanistic markings	Normal		Tan/brown	Black
	Skin colour - lightening		Pallor, pale; changed/weak pigmentation	Normal			Pale
	Skin colour - mottled		Discoloured	Normal			Mottled
	Oedema	Abdominal swelling due to accumulation of fluid	Distended/swollen/bloated abdomen/gut area; dropsy	Normal		Distended abdomen	Abdomen distended + scales vertical and/or fissure in abdominal wall
	Haemorrhagic areas - petechias	Pinhead sized spots due to intra-dermal or sub-mucus haemorrhage		None	<10% of skin area	10-30% of skin area	>30% of skin area
	Haemorrhagic areas - haematomas	Area of blood due to intradermal or sub-mucus haemorrhage	Small/big haematoma	None	<10% of skin area	10-30% of skin area	>30% of skin area
	Exophthalmia	Swelling within orbital socket resulting in bulging of eye out of socket	Exophthalmos, exophthalmus, popeye, protruding eyeball	Normal	1 or 2 eyes slightly extended	Unilateral - one eye fully extended	Bilateral - both eyes fully extended
	Mucus secretion	Excess mucus production	Mucus build-up (check eyes); increased secretion (mucus on skin or in water); mucus loss	None			Observed
	Faecal (anal) casts	String of faeces hanging from anus		None			Observed
Provoked behaviour -	Visual and tank knocking stimulus - over reactive	Overhead fright (startle) response to hand passing over top of tank or avoidance reaction to light beam; Fright (startle) response to tank rapped lightly	Hyperexcitability; hyperactivity after stimulus/threat	Normal			Excessive response
	Visual and tank knocking stimulus - under reactive		Not responsive to external stimulation; inactivity after stimulus/ threat	Normal		Reduced	Total loss
	Tactile stimulus - under reactive	Reduced avoidance response to touching	Not responsive to external stimulation; inactivity after stimulus/threat	Normal		Reduced	Total loss

Table 2: Clinical Signs Recording Sheet

Study & tank details																
	Day / Date															
	Observation / Time	1/			2/			3/			4/			5/		
	No. live fish in tank for scoring															
	No. moribund* removed after scoring															
	No. dead removed															
	If no abnormalities observed, record "NAO"															
	Clinical sign \ Score	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Distribution	Loss of schooling / shoaling behaviour															
	Dense schooling / shoaling behaviour															
	Vertical distribution -surface															
	Vertical distribution - bottom															
Equilibrium & buoyancy	Abnormal horizontal orientation															
	Abnormal vertical orientation															
	Loss of buoyancy control															
Observed behaviours	Hypoactivity															
	Hyperactivity															
	Spiral swimming															
	Hyperventilation															
	Hypoventilation															
	Irregular ventilation															
	Increased ventilation depth															
	Convulsions															
	Coughing															
	Gulping															
	Gasping															
	Surface escape / avoidance behaviours															
	Bottom escape / avoidance behaviours															
	Irritated skin behaviours															
	Aggression and cannibalism															
Appearance	Tetany															
	Skin colour - darkening															
	Skin colour - lightening															
	Skin colour - mottled															
	Oedema															
	Haemorrhagic areas - petechias															
	Haemorrhagic areas - haematomas															
	Exophthalmia															
	Mucus secretion															
	Faecal (anal) casts															
Provoked behaviour - responses to	Visual and tank knocking stimulus - over reactive															
	reactive															
	Tactile stimulus - under reactive															
Additional observations																

*at present there is no international agreement on the definition of moribund fish

(1)	Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L276/33
(2)	Canadian Council on Animal Care Guidelines (2005) The Care and Use of Fish in Research, Teaching and Testing. Ottawa, Canada. 87 pp. http://www.ccac.ca
(3)	Toth LA (2000) Defining the moribund condition as an experimental endpoint for animal research. ILAR J 41:72-79.
(4)	Rufli H (2012) Introduction of moribund category to OECD fish acute test and its effect on suffering and LC_{50}-values. Environ. Toxicol. Chem. 31, 2012.
(5)	Morton DB (1997) A Scheme for the Recognition and Assessment of Adverse Effects. In, Animal Alternatives, Welfare and Ethics. Eds., van Zutphen, L.F.M., Balls, M. Publ. Elsevier, Amsterdam. pp. 235-241. ISBN 0-444-82424-3.
(6)	Drummond RA, Russom CL, Geiger DL, DeFoe DL (1986) Behavioral and Morphological Changes in Fathead Minnow (Pimephales promelas) as Diagnostic Endpoints for Screening Chemicals According to Mode of Action. Aquatic Toxicology and Environmental Fate: Vol. 9, ASTM STP 921, T. M. Poston and R. Purdy, Eds., American Society for Testing and Materials, Philadelphia, pp. 415-435.
(7)	Hawkins P, Ryder K, Dennison N, Goodman G, Hetherington S, Llywelyn-Jones S, and AJ Smith (2011) Guidance on the severity classification of procedures involving fish. Poster at 8th World Congress on Alternatives and Animal Use in Montreal. http://norecopa.no/media/6975/fish-procedures.jpg
(8)	Hawkins P, Ryder K, Dennison N, Goodman G, Hetherington S, Llywelyn-Jones S, and AJ Smith (2011) Working Party Report: Guidance on the severity classification of scientific procedures involving fish: report of a Working Group appointed by the Norwegian Consensus-Platform for the Replacement, Reduction and Refinement of animal experiments (Norecopa). Laboratory Animals 45, 219–224. DOI: 10.1258/la.2011.010181
(9)	EPA-600/3-77-33 (1977) Procedures for measuring cough (gill purge) rates of fish.
(10)	Muir MM, Kosteretzoe KG, Lech JJ (1997) Localization, depuration, bioaccumulation and impairment of ion regulation associated with cationic polymer exposure in rainbow trout (Oncorhynchus mykiss). Xenobiotica 27(10), pp. 1005-1014.
(11)	McKim M, Bradbury SP, Niemi GI (1987) Fish Acute Toxicity Syndromes and Their Use in the QSAR Approach to Hazard Assessment. Environmental Health Perspectives Vol. 71, pp. 171-186

ANNEX 7

Guidance on individual fish identification techniques for TG203

1. Introduction

The main objective of this guidance is to create a robust scientific basis for replacing mortality as an endpoint by clinical signs that are predictive of death (application of humane endpoints in TG203). The ability to make observations on clinical signs and relate them to individual fish during acute toxicity testing to derive humane endpoints can only be achieved by marking (artificial or natural) or video tracking. Video tracking is completely non-invasive and involves back tracking only the behaviour of fish that demonstrated clinical signs, moribundity and/or mortality. There are numerous ways to conduct such analysis, some involving proprietary software. As long as the video analysis utilizes the same score sheet as Annex 6 for recording the clinical signs, any method is acceptable.

Various tagging and marking methods for fish exist, however, not all are applicable for TG203 as they don't meet certain key criteria as displayed below. Criteria 1-4 are essential and as such only those techniques that comply are considered. Criteria 5-9 are also very important; however, they are not incompatible with TG203. The selection of the best type of marking technique depends also on the fish species used, and since TG203 includes 13 recommended species (Annex 2, Table 1), all identification techniques that fulfil criteria 1-4 have been considered, but not necessarily criteria 5-9. These are listed in Table 1.

Criteria used to assess the suitability of fish identification techniques for TG203.

1. Individual fish identification
2. Individual fish identification while alive (not post-mortem)
3. Suitability for small size fish (≤ 5 cm)
4. Mark retention time of > 1.5 weeks (1-week acclimation followed by a 4-day test)
5. Visible and distinct under water
6. Minimal impact on welfare
7. Cost effectiveness
8. Ease of performance with minimal training
9. Rapid identification in the water

An important point to note is that the fish will need to be anaesthetised for all the procedures discussed below, unless specified.

576 Table 1. Identification techniques applicable to TG203

577

	Visibility/distinction under water	Low welfare impact	Low cost	Minimum training	Rapid identification
Mutilation clipping or punching fins or other body parts (adipose fin)	++	-	+++	++	++
Freeze branding use of cold instruments against the body of a fish	+	+	+++	++	+
Attachment tags often inserted into musculature	+++	+	+/+++	+	+++
"Tattooing" pigment injected into skin cells using high-pressure air device	++	+	++	+	++
Pigments (dyes, inks, paints) injected in small amounts into skin	++	++	+++	++	++
Visible Implant Elastomer (VIE) injected as liquid that cures into a pliable, biocompatible solid.	++	++	++	++	+++
Morphometric marking e.g. pigmentation, colouration, shape of body part	+	+++	+++	++	+

578 +: meet the criteria; -: does not meet the criteria; degrees by which the criteria have been met expressed by the
579 number of relevant symbols presented: + low, ++ medium and +++ high.

580

581 By adding up the + (advantages) for each technique it becomes clear that three techniques, namely
582 use of pigments, VIE and morphometric marking are superior to the rest. A small description for each
583 one of the three recommended techniques follows.

584

585 **2. Marking techniques recommended for TG203**

586

587 **2.1. Pigments**

588 Subcutaneous injection of pigments (dyes, inks, paint) is a valuable method for marking small fish.
589 Once injected, the colour shows through the skin and thus allows direct and rapid identification of the
590 fish.

591 In small fish, the areas for injection include:

592 a) the dorsal surface, e.g. in mollies, *Poecilia gillii* (Chapman et al., 1991), in guppy *Poecilia*
593 *reticulata* (Croft et al., 2003), in banded killifish *Fundulus diaphanous* (Hoare et al., 2000) and
594 in fathead minnow (Unger, 1983)

b) near caudal peduncle, e.g. in zebrafish (Cheung et al., 2014), in fathead minnow (Danylchuk and Tom, 2001; Unger, 1983; Vezie and Martin, 1974), in guppy (Reznick et al., 1996)
c) on several fins (except dorsal) in juvenile Atlantic salmon, *Salmo salar* (Herbinger et al., 1990) and at the base of the pectoral fins in fathead minnow (Smith, 1970).

The most common dye is alcian blue but other dyes/inks/paints have been used (e.g. dyes for tissue, acrylic paints). The advantages of the pigment injection include cost effectiveness, speed, simplicity and suitability for small fish. The use of different colours and mark position allow flexibility in individual fish marking. The choice of the colour is important as depending on the species and/or position the visibility may be impaired. In zebrafish for example, black, red and yellow are distinguishable colours while green and blue are less obvious due to similar colouration of the zebrafish body (Cheung et al., 2014). The technique seems to have no negative effects on growth, survival and behaviour (Cheung et al., 2014). However, there are minor welfare concerns as the fish still need to be anaesthetised, kept out of the water during the procedure and receive a small injection. To minimise these effects, training is recommended (Cheung et al., 2014).

2.2. Visible Implant Elastomer (VIE)

Visible Implant Elastomer is a marking system available from Northwestern Marine Technology Inc. (NMT) in 10 colours. It is a polymer that, once mixed with a curing agent and injected subcutaneously, hardens to leave a permanent, pliable, biocompatible mark. The polymer can be fluorescent (Visible Implant Fluorescent Elastomer VIFE) and is recommended over non-fluorescent elastomer for heavily pigmented species. Also, the visibility of the mark is enhanced using IV light (deep-violet beam, 405 nm). VIEs have been successfully used with many of the recommended species in TG203 (Close and Jones, 2002; Croft et al., 2004; Dewey and Zigler, 1996; Edenbrow et al., 2011; Featherstone et al., 2016; Frommen et al., 2015; Hohn and Petrie-Hanson, 2013; Im et al., 2017; Leblanc and Noakes, 2012; Wilson and Godin, 2009) and similar sized fish (Featherstone et al., 2016; Frederick, 1997; Mortensen and Hansen, 2016; Neufeld et al., 2015; Olsen and Vøllestad, 2011; Willis and Babcock, 1998).

The fish can be marked in a variety of locations including the base of the dorsal fin, abdomen, caudal fin rays/peduncle, anal and pectoral fin base. The most suitable colours reported are red, pink and green but the most visible colour to use depends on the natural pigmentation of the fish. Marking success is influenced by depth of subcutaneous tag injection, anatomical location of the tag, pigmentation of the skin at that location, and investigator's experience with the technique. VIE tags have not been shown to affect survival, growth or behaviour in either short or long-term studies. As with all tagging or marking methods, minor welfare concerns exist as the fish still need to be anaesthetised, kept out of the water during the procedure and experience skin puncture.

Given that wild caught fish such as roach and carp returned to background stress levels within 12 hours post electronic tag insertion (a technique that is more invasive than pigment or VIE application; see Lower et al, 2005), we recommend a minimum resting period for the fish post-tagging and pre-testing of 48 hours.

2.3. Natural morphological marking

Among the recommended species for TG203, only zebrafish present natural morphological marking that could be used for the identification of individuals. However, opinions seem to diverge on the presence of distinct patterns unique to individual fish, mainly due to the choice of strain. Using WIK strain zebrafish (4 fish per tank), distinguishing features such as colour/stripe pattern, body shape, and

size has allowed identification of individual fish (Paull et al., 2010). The method was slightly adapted to cope with 5 fish per tank (Reed and Jennings, 2011), so currently no positive identification of 7 fish per tank (as required in TG203) has been documented in the literature. However, since the fish do not have to be anaesthetised, taken out of the water or injected, this method is superior in terms of welfare and should be considered along with an additional method for the remaining 2 fish. Nevertheless, this method may be more difficult to apply due to logistics for recording the different fish features (individual photography, trained eye etc.) and the time required for checking previous records when the fish present clinical signs.

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