Section 2
Effects on Biotic Systems

Test Guideline No. 203
Fish, Acute Toxicity Testing

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Fish Acute Toxicity Test

INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are periodically reviewed to incorporate scientific progress, changing regulatory needs, and animal welfare considerations. The revision of this Guideline (originally adopted in 1981, updated in 1984, 1992), reflects also updates on a series of recommendations from the OECD Fish Toxicity Testing Framework 2011 (OECD, 2012), and includes:

- Alternative methods: in the interest of animal welfare and efficient use of resources, it is important to avoid/reduce the use of animals whenever possible and appropriate. Therefore, before carrying out a fish acute toxicity test according to this guideline, it should be considered whether reliable information on fish acute toxicity could be derived with alternative methods in a weight-of-evidence approach, such as the use of QSAR, read-across, fish embryos (OECD 2013), fish cell lines and others. Alternatively, the use of the threshold approach (OECD, 2010) or the limit test as described in § 30 of this guideline may be sufficient. Where testing on fish is required (i.e., alternative methods currently may not be sufficient for all jurisdictions and testing needs. Therefore, make sure the tests fulfil the regulatory requirements), alternative methods such as those listed above can be considered for range finding.
- 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 circumstances under which a water control is required when solvent is used (OECD, 2019).
- the introduction of estuarine and marine fish species in the recommended species list.
- the enhanced recording of visible abnormalities (also referred to as sublethal clinical signs) that fish may display during the exposure in order to improve our ability to predict chemical toxicity and minimise suffering of animals in the future analogously to those described in Guidance Document No. 19 on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation for mammalian studies (OECD, 2000).

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

PRINCIPLE OF THE TEST

3. The fish are exposed to the test chemical for a period of 96 hours, under either static, semi-static or flow-through conditions. Mortalities and visible abnormalities related to appearance and behaviour are recorded. Where possible, the concentrations to kill 50% of the fish (LC50) are determined.

INITIAL CONSIDERATIONS

4. Useful information about chemical-specific properties include the structural formula, molecular weight, purity, stability in water and light, acid dissociation constant (pKa), organic carbon partition coefficient (Koc) and n-octanol water partition coefficient (Kow), water solubility and vapour pressure, as well as results of a test for ready biodegradability OECD TG 301 (OECD, 1992) or OECD TG 310 (OECD, 2006a). 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 guideline 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 meaningless or difficult to interpret results. For poorly water-soluble, or other difficult to test chemicals, it should be referred to the Guidance Document No. 23 (OECD, 2019) on aquatic toxicity testing of difficult test chemicals. Where test chemicals are predicted to have no acute toxic effects at relevant test concentrations, it is recommended to consult the relevant regulatory authorities. Some regulatory authorities may prefer to omit acute toxicity tests and proceed straight to chronic toxicity testing, e.g. if steady state conditions are likely not to be reached within the duration of a short-term toxicity test. Other important information, particularly when accompanied with systematic collection of sublethal clinical signs (see Annex 4) includes mode of action (e.g. polar narcosis).

5. A validated analytical method, of known accuracy, precision, and sensitivity, for the quantification of the test chemical in the test solution should be available (OECD, 2014), where technically feasible. Performance parameters should be reported (e.g. accuracy, precision, Limit of Detection, Limit of Quantification, specificity, working range).

6. If the Test Guideline is used for the testing of a mixture, a substance of Unknown or Variable composition, Complex reaction products or Biological materials (UVCB) or a multi-constituent substance, its composition should, as far as possible, be characterised, e.g. by the chemical identity of its constituents, their quantitative occurrence and their chemical-specific properties (see § 5). Recommendations about the testing of difficult test chemicals like mixtures, UVCBs or multi-constituent substances are given in Guidance Document No. 23 (OECD, 2019). 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.

7. 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.

VALIDITY OF THE TEST

8. 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 test concentrations is compulsory (see § 24).

9. Any deviation from the validity criteria and the guideline should be reported. The reasons and consequences of the deviation(s) with regard to study outcome and validity should be included in the report. 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.

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:

- oxygen meter
- pH meter
- light meter
- adequate apparatus for temperature control
- equipment for determination of hardness of water
- equipment for determination of total organic carbon concentration (TOC) and/or chemical oxygen demand (COD)
- equipment for the determination of concentration of test chemical in test solution
- equipment to maintain water temperature and oxygen content as appropriate
- tanks made of chemically inert material

Test Vessels

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 chemicals, the use of silicone tubing in flow-through studies and use of silicone seals in contact with water should be minimised. 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 (other than from the tested chemical) and to comply with loading rate criteria given in § 20. Test vessels should be randomly positioned in the test area and shielded from unwanted disturbance (excessive noise, vibration, light). When testing difficult test chemicals, Guidance Document No. 23 (OECD, 2019) should be consulted and the study design modified appropriately. For volatile and other difficult test chemicals, further specific measures should be taken (OECD, 2019). Any silicone materials that were in contact with the test solution(s) should be preferably discarded and not reused for subsequent tests with different test chemicals.

Selection of Species

12. The selection of species depends on regulatory requirements (industrial chemical, pharmaceutical, biocide or plant protection product, etc.) and on environmental exposure scenarios (cold, temperate or warm water species, freshwater or estuarine/marine fish). Cold water fish are considered those that require holding temperatures below 20°C whilst warm water fish are typically kept in temperatures over 20°C. Temperate fish species prefer temperatures between 18-22°C. A list of recommended fish species for this test is provided in Annex 2. These fish species are readily available, are easy to maintain, and most have historical use in chemical safety testing. They can be bred and cultivated either in fish farms or in the laboratory, under disease-free conditions, providing healthy animals of known provenance for testing. If species that are not listed in Annex 2 are used, the rationale must be reported together with any adaptations to the test guideline's recommendations.

Age and Size of Fish

13. Fish should be juveniles (see Annex 2, for size guidance) and originate from the same source and population to ensure uniformity. The fish should be of the same age (if unknown it can be estimated via the size) and have normal appearance.

Holding of Fish

14. All fish should be held in the laboratory for at least 9 days before they are used for testing. The first 48 hours constitute a settling-in period. Then, fish should be acclimatised for at least 7 days (48 hours settling-in + 7 days acclimatisation = 9 days) in water similar to test water (see Annex 3 for relevant characteristics) immediately before the start of the test. Holding of fish should be under the following conditions:

- Photoperiod: appropriate to the species (see Annex 2);
- Temperature: appropriate to the species (see Annex 2);
- Oxygen concentration: at least 80% of air saturation value;
- Feeding: three times per week or daily until 24 - 48 hours before the exposure is started. Feed may be given to satiation . Surplus food and faeces should be removed as necessary to avoid accumulation of waste.

15. During the acclimatisation period, mortalities are recorded, and the following criteria are applied:

- mortalities of greater than 10% of the population in seven days: reject the entire batch;
- mortalities between 5 and 10% of the population: acclimatisation is continued for seven additional days; and if there is more than 5% mortality during the second seven day period, reject the entire batch;
- mortalities of less than 5% of population in seven days: accept the batch.

Fish should not be displaying visible signs of disease and stress and should be free of any apparent malformations and not have been previously treated against disease or parasites within the last 14 days prior to testing. When fish are obtained from outdoor ponds (e.g. carp, bluegill) or in exceptional circumstances from wild populations, they may need to be treated against disease and parasites when first brought to the testing laboratory, thus an additional 14 days of acclimatisation are required. Fish from wild populations should be avoided wherever possible.

Water (dilution water, test medium)

16. For freshwater fish, clean surface water, ground water or reconstituted water (ISO, 1996) is preferred (see Annex 2 and 3), 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 deionised 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 husbandry, acclimatisation and testing without showing signs of stress. Total hardness and pH should be within the optimal range for the selected fish species (Annex 2). The reagents used for the preparation of reconstituted water should be of analytical grade and the deionised 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. 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. Chemical analysis of the type of water used in testing should include the elements and limitations on maximum concentrations shown in Annex 3 based on at least biannual testing unless it can be demonstrated that these specifications are consistently met. If the water in the test vessels is recirculated, ammonia (NH3) should be regularly monitored to ensure suitable water quality and welfare. If natural water is used, DOC or TOC and nitrate-content (NO3) should be measured once prior to the test. If dechlorinated tap water is used, it should be demonstrated that test organism survival, growth, and reproduction are not affected and that test organisms do not show other signs of stress. Analyses of nitrate and chlorine should be performed on each batch of dilution water to demonstrate that the limits specified in Annex 3 are not exceeded.

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 Guidance Document No. 23 should be followed (OECD, 2019). The use of solvents should be avoided and only used as a last resort in order to produce a suitably concentrated stock solution. Where a solvent cannot be avoided, Guidance Document No. 23 (OECD, 2019) should be consulted. The final concentration of the solvent used should be minimised as far as possible (not exceeding 100 mg/L or 0.1 mL/L) and should be the same in all test vessels, excluding the dilution water control (OECD, 2019). For the controls, see § 23.

19. The test should be carried out without adjustment of the pH. For ionisable chemicals however, the definitive test should be conducted at a stable pH consistent with the more toxic form of the test chemical (dependent on the chemical), as described in Guidance Document No. 23 (OECD, 2019), as long as the pH does not exceed the range of pH of 6.0-8.5 (Annex 2). Where the chemical itself causes a change of the pH of the test medium outside the range of pH 6.0-8.5, the stock solution should be adjusted to lie within the specified range of pH 6.0-8.5 (OECD, 2019). In case the test is conducted with adjustment of the pH, a parallel test without adjustment is not required, except if requested by specific regulations. HCl and NaOH are preferred for pH adjustment.

PROCEDURE

Conditions of Exposure

20. Duration: 96 hours.

- Loading: for freshwater fish, maximum loading of 0.8 g wet weight fish/L for static and semi-static renewal testing is recommended. For flow-through systems, the recommended maximum loading is 0.5 g wet weight fish/L per 24 hours (example: in a 10 L tank with a flow rate of 5 tank volumes per 24 hours, a total of 50 L pass through the tank in 24 hours. With 25 g fish, this corresponds to 25 g in 50 L in 24 hours equivalent to 0.5 g/L in 24 hours). A loading 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) and with an intensity of 10-20 µE/m2/s, 540-1000 lux, or 50-100 ft-c (ambient laboratory levels).
- 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), e.g. for zebrafish with a range of 21-25°C, the temperature selected could be 24°C and should not vary more than ± 1°C between test vessels and between successive days while staying in the recommended range of 21–25°C.
- 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 must be used at each test concentration and in the control(s). The fish should be randomly distributed among treatments. No test tank replication is required.

Test Concentrations

22. When selecting the range of test concentrations, all sources of information should be considered, such as predictions within the applicability domain of valid QSAR models, valid read-across or grouping estimates and data from other tests, e.g. using fish embryos or fish cell lines. In case such data are not available or sufficient confidence cannot be gained, a range-finding test using fish, preferable with the same species (1), should be considered. In this case, use of the Threshold Concentration (OECD, 2010) derived from algae and daphnia studies (Annex 1) may guide setting the concentration range. Note that it is not a mandatory requirement to identify a maximum concentration causing 0% mortality nor a minimum concentration causing 100% mortality.

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; smaller separation factors of 1.6 to 1.8 should be used whenever possible (Rufli and Springer, 2011).

Controls

24. When a solvent is used, a solvent control is required in addition to the dilution water control. However, the dilution water control can be omitted, and the test conducted and evaluated with a solvent control only, provided it is appropriate when considering the needs for these data and the requirements of the relevant regulatory authorities. Low toxicity solvents only (i.e. acetone, ethanol, methanol, tertiary-butyl alcohol, acetonitrile, dimethyl formamide, dimethyl sulfoxide, and triethylene glycol) as recommended in Guidance Document No. 23 (OECD, 2019) should be used whilst solvents of unknown toxicity should not be used. It should be noted that in spite of the low toxicity for fish, dimethyl formamide and dimethyl sulfoxide should be avoided where possible on human health and safety grounds.

Frequency of Analytical Determinations and Measurements

25. For all test systems, analysis of the highest and lowest test concentration (or lowest quantifiable concentration, as recommended in Guidance Document No. 23 (OECD, 2019) and a concentration around the expected LC50 is considered the minimum requirement. However, measurement of each individual concentration is preferred. Furthermore, it should be ensured that the determinations reflect the concentrations of the dissolved test chemical (Guidance Document No. 23 (OECD 2019). If chemicals are not stable, ideally, analytical determination should be done immediately on fresh samples. Alternatively, determination of storage stability of the analyte in the samples is deemed useful to differentiate between potential instability under test conditions from potential instability under storage conditions. For static tests, chemical analysis of the test concentrations should be performed at the start and the end of the exposure period. For semi-static renewal tests, test concentrations should be measured at least twice over one exposure period (before and after renewal of test solutions). For flow-through tests, chemical analysis of the test concentrations should be performed before initiation of the exposure to check whether target concentrations are achieved and maintained. The necessary frequency of sampling during exposure should be decided upon based on the stability of the test chemical in the stock solution(s) and how often the stock solutions are renewed, such that the stability of test chemical exposure can be documented. There must be evidence that the concentration of the chemical being tested has been satisfactorily maintained, and preferably it should be at least 80% of the nominal concentration throughout the test. If the concentrations are expected to decline by more than 20%, then all test concentrations should be measured, and more frequent analyses are recommended, e.g. additionally at 48 hours.

26. During the exposure, dissolved oxygen, pH, salinity (if relevant) and temperature should be measured daily in each test vessel – temperature preferably continuously, hardness (unless it has demonstrated stability over time) and TOC at the beginning of the exposure in the dilution water. In semi-static systems, dissolved oxygen, pH, salinity (if relevant) and temperature should be measured prior to and after water renewal.

OBSERVATIONS, HUMANE KILLING AND MEASUREMENT OF FISH

27. **Observations and recording**: To the extent feasibly possible, a minimum of 2 observations should be conducted within the first 24 hours of the study with preferably at least 3 hours between observations. For example, fish could be inspected at 2 ± 0.5 h, 5 ± 1 h and 24 ± 2 h after the start of the exposure (day 0-1). On days 2-4 of the test, all vessels with living fish should be inspected twice per day (preferably early morning and late afternoon to best cover the 24-hour periods). Mortalities and visible abnormalities in regard to equilibrium (e.g. loss of balance, head up or down, floating at surface or sinking), appearance (weak or dark pigmentation, exophthalmia), ventilatory behaviour (e.g. hyper, hypo or irregular

ventilation, coughing) and swimming behaviour (hyper or hypo activity, immobility, convulsions, near surface or bottom, dense or loss of schooling) are recorded. If possible, additional clinical signs may be reported, as listed in Annex 4, Tables 1 and 2.

28. **Mortality:** Fish are considered dead if there is no visible movement (e.g. gill movements) and if touching of the caudal peduncle produces no reaction. Mortalities are recorded, and dead fish are removed as soon as they are observed.

29. **Humane killing of fish:** Surviving fish of the treatment groups are euthanised at the end of the exposure, whereas euthanasia of surviving control fish is not required, but they should not be used in another test. For the method of euthanising the fish, please refer to the respective national or (EU) guidance (e.g. Directive 2010/63/EU) (European Commission, 2010).

30. **Sample cryopreservation:** Plasma and excess tissues or whole organism may be preserved from surviving fish at 96 hrs for possible additional investigations such as omics. Recommended procedures for sample preservation for omics are available in OECD Guidance document No. 409 (OECD, 2025). Care should be taken when considering additional analyses so that they do not compromise the standard parameters.

31. **Measurement of fish**: The individual size (wet weight and total length) should be measured prior to the initiation of the exposure in at least a subsample of 10 fish from the designated holding tank. These fish will not be used in the test. If these fish are measured more than one week before the start of the test, then the fish from the control need to be measured at the end of the exposure to confirm the required fish length. Wet weight can be determined e.g. by placing live fish in a pre-weighed vessel containing culture water and recording the total weight. The total length can be documented e.g. by photo-imaging. Annex 2, shows the size requirements for recommended fish species.

LIMIT TEST

32. 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, or at the threshold concentration as defined in Annex 1, whichever is the lowest, in order to demonstrate that the LC50 is greater than this concentration. The limit test should be performed using at least 7 fish, with the same number in the control(s). If visible abnormalities are observed, these should be recorded (see Annex 4 for a comprehensive list of sublethal clinical signs that may be recorded on a voluntary basis in addition to the observations mentioned in paragraphs 27-31). The limit test is considered valid, if the control mortality is ≤10%, or 1 fish if fewer than 10 control fish are used.

DATA AND REPORTING

Treatment and Expression of Results

33. It is recommended that results should be calculated using the measured concentrations of the test chemical. If the deviation from the nominal concentrations is smaller than 20%, results may also be based on the nominal concentrations. It should be noted that it is often useful to have both measured and nominal effect concentrations quoted, see Guidance Document No. 23 (OECD, 2019). Data should be summarised in tabular form, showing the number of fish used, mortality and sublethal effects for each concentration and control(s) at each observation time. The reporting of clinical signs as listed in paragraph 27 is mandatory whilst reporting signs as listed in Annex 4, Tables 1 and 2) is voluntary. 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.

34. The statistical methods to be used for the estimation of the LC50 depend on the number of concentrations observed with partial mortalities (mortality >0 and <100%). When an experiment results in at least two concentrations with partial mortalities, 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 (ISO, 2006; OECD, 2006b and Finney, 1978). When an experiment results in only one concentration with partial mortality or no concentration with partial mortality, 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 (Stephan, 1977), the binomial method (USEPA, 2002), the moving average method (ISO, 1996), or as a last resort, the graphical method (USEPA, 2002). These non-classical methods can give precise LC50 estimates and are useful to evaluate acute fish studies yielding results that cannot be analysed using classical probit maximum likelihood techniques.

TEST REPORT

35. The test report should include the following information:

Test chemical:

- Mono-constituent substance:

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

o 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.

- Multi-constituent substance, UVCBs and mixtures:

o characterised as far as possible by their own chemical identifiers (see above) and/or the ones from the constituents, their relevant physicochemical properties and/or the ones of the constituents and quantitative occurrence of the constituents.

Test fish:

- scientific name, strain (where relevant), size (wet weight and total length), supplier, any pre-treatment, etc.

Test conditions:

- test procedure used (e.g. static, semi-static renewal, flow-through; frequency of renewal; aeration; fish loading; etc.);
- water quality characteristics (pH, hardness, TOC and/or COD for surface, ground or reconstituted water) and adaptations made to suit the requirements of fish species used other than those in Annex 2;
- dissolved oxygen concentration, pH values, temperature of the test solutions at 24-hour intervals in each tank and temperature continuous in one tank (in semi-static renewal systems: dissolved oxygen, pH, salinity (if relevant) and temperature prior to and after water renewal);
- methods of preparation of stock and test solutions;
- test solution appearance and any methods used to determine dissolved concentration (e.g., centrifugation or filtering);
- concentrations used;
- measured concentrations of the test chemical in the test solutions;
- number of fish in each test vessel.
- Samples cryopreserved (if applicable)

Results:

- cumulative mortality at each concentration at the recommended observation times;
- mortality in the control(s);
- the LC50 values at 24, 48, 72 and 96 hours with 95% confidence limits, if possible;
- the slope of the concentration-response curve after 96 hours exposure, if possible;
- graph of the concentration-mortality curve at the end of the exposure, if possible ;

- incidence and description of visible abnormalities observed during exposure as listed in paragraph 27; additional clinical signs are listed in Annex 4, Tables 1 and 2 can be recorded on a voluntary basis;
- 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, consequences and relevant explanations.

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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.

IUPAC: International Union of Pure and Applied Chemistry.

Median Lethal Concentration (LC50) is the concentration of a test chemical that is estimated to be lethal to 50% of the test organisms within the test duration.

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 (OECD, 2010).

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).

UVCB: Substances of Unknown or Variable composition, Complex reaction products or Biological materials.

ANNEX 2: TABLE 1: RECOMMENDED FISH SPECIES, TOTAL LENGTHS AND TEST CONDITIONS

Species[1]	Temperature[2] (°C)	Salinity[3] (‰)	pH	Hardness (mg/L $CaCO_3$)	Photoperiod (hours light)	Recommended length range[4] (cm)
Danio rerio *Zebrafish*	21-25	<0.2	6.0-8.5	40- 250, preferably <180	12-16	1-2
Pimephales promelas Fathead minnow	21-25	<0.2	6.0-8.5	40-250, preferably <180	12-16	1-3
Cyprinus carpio Carp	20-24	<0.2	6.0-8.5	40-250, preferably <180	12-16	2-4
Oryzias latipes Japanese Medaka	23-27	<0.2	6.0-8.5	40-250, preferably <180	12-16	1-2
Poecilia reticulata Guppy	21-25	<0.2	6.0-8.5	40-250, preferably <180	12-16	1-2
Lepomis macrochirus Bluegill	21-25	<0.2.	6.0-8.5	40-250,	12-16	1-3

[1] 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. It is suggested that the species is selected on the basis of their ready availability, ease of maintenance, and historical use in safety testing.
[2] Where culture temperature differs from the recommended range, the acclimatization period should be used to acclimatize the fish to the desired test temperature.
[3] For any given test this shall be performed to ± 2‰, e.g. 17±2 =15-19‰, 31±2 =29-33‰.
[4] Test fish must be juveniles when used in this test (before reaching sexual maturity). 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.

				preferably <180		
Oncorhynchus mykiss Rainbow trout	10-14[5]	<0.2	6.0-8.5	40-250, preferably <180	12-16	3-6
Gasterosteus aculeatus Three-spined stickleback	13-19	0-35	6.0-8.5	40-7500	12-16	1-2
Cyprinodon variegatus Sheepshead minnow	23-27	15-35	6.0-8.5	3000-7500	12-16	1-2
Dicentrarchus labrax European sea bass	18-22	15-35	6.0-8.5	3000-7500	12-16	4-8
Pagrus major Red sea bream	18-22	30-35	6.0-8.5	5000-7500	12-16	2-4

[5] A significant change between the previous version of TG203. With a range of 10-14°C, which is similar to the range given in OPPTS 850.1075, the range of 13-14°C overlaps the range given in the original TG203 of 13-17°C.

ANNEX 3: SOME CHEMICAL AND PHYSICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION/TEST WATER FOR FRESHWATER, ESTUARINE AND MARINE FISH

Parameter	Maximum concentration
Particulate matter	5 mg/L
Total organic carbon (TOC)[6]	2 mg/L
Un-ionised ammonia (NH_3)	1 µg/L
Nitrate (NO_3)	<9 mg/L[7]
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)[8]	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)[9]	5 mg/L

[6] High levels of total organic carbon (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.

[7] "A maximum level of 2 mg NO_3-N/L would be appropriate for protecting the most sensitive freshwater species"; equaling 8.85 mg NO_3/L (Camargo JA, Alonso A, Salamanca A. 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58(9):1255-67.

[8] Note that copper pipes or compositions containing copper (alloys) may cause fish to die (LC_{50} fathead minnow: 0.073 mg/L).

[9] COD or TOC should be measured.

ANNEX 4: DESCRIPTION OF SUBLETHAL CLINICAL SIGNS IN FISH

Introduction: European legislation[10] (European Commission, 2010) and Canadian guidance[11] (CCAC, 2005) encourage the application of early and humane endpoints in vertebrate testing. As a result, it has become common practice in laboratories to introduce sublethal endpoints in acute fish testing to reduce the terminal suffering of the fish.[12] However, besides the fact that the identification of clinical signs that are predictive of moribundity and death is crucial to their effective use as experimental endpoints (Toth, 2000), there is no international consensus on what sublethal clinical signs define moribundity or are predictive of death in fish as yet. To generate reliable scientific data that can allow such consensus in the future, the enhanced, systematic collection of observations on signs that lead to moribundity and death over time and preferably in the same individual fish is encouraged. Table 1 represents a tool for this purpose by displaying a comprehensive list of all clinical signs potentially relevant to chemical toxicity whilst Table 2 provides a means of recording those signs during daily observations. Where expertise exists, and the procedure has minimum impact on animal welfare, it is recommended to individually mark the fish prior to testing. This will allow the link between sublethal sign and outcome (survival or death) at individual fish level. Suitable identification techniques for small size fish include injection of pigments, the use of visible implant elastomers and morphometric marking. Alternatively, the fish can be filmed and the progression of sublethal symptoms to moribundity and lethality studied retrospectively.

The definitions of sublethal signs depicted on Table 1 have been observed in fish toxicity studies (Rufli, 2012; Morton, 1997; Drummond et al, 1986; Hawkins et al, 2011a and Hawkins et al, 2011b) or described elsewhere.[13] Clinical signs have been described in literature (Hawkins et al, 2011b and EPA, 1977), although there is a spectrum of magnitude for some of these observations that could be species, population or even length specific.

It is anticipated that a future version of the test guideline will include detailed guidance on how sublethal clinical signs can be used to identify which individual fish should be humanely killed before the end of the test. This guidance will be analogous to those described in Guidance Document No. 19 on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation for mammalian studies (Rufli, 2012).

When reporting on sublethal clinical signs, laboratories are encouraged to familiarise themselves with a more comprehensive list of sublethal signs listed in Table 1 and record those observed (see example score sheet in Table 2) during testing at the tank level or for individual fish where possible. The enhanced, systematic recording of sublethal signs along with any additional information that exists on the chemical (i.e. mode of action) can greatly facilitate and accelerate the purpose of this exercise.

In the future, a weight of evidence approach may aid the distinction between chemically related clinical signs or visible abnormalities due to other reasons [consider the time of appearance, the progression over

[10] Directive 2010/63/EU states in Article 13 (European Commission, 2010): “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”.

[11] The Canadian Council on Animal Care Guidelines (CCAC, 2005) declares: “where feasible, the development of pre-lethal endpoints in such tests is encouraged”.

[12] In the context of this guideline, this means that the implementation of sublethal endpoints should ensure the requirements for a determination of an LC_{50} are met (e.g. ensuring regulatory authority compliance).

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

time (persistent, increasing, decreasing), the number of fish affected, the vessels affected (concentrations, control, holding vessels), other potential origin (e.g. poor handling, aggression, disease, toxic effect, poor environmental conditions)]. Clear examples of chemical related clinical signs include effects on the operculum due to exposure to cationic chemicals and internal haemorrhaging due to exposure to acetylcholinesterase inhibitors (Muir et al, 1997, McKim et al, 1987a).

Laboratories should also include additional information available, such as physico-chemical properties (e.g. K_{ow}), mode of action, potential degradation (if testing in a static system) or any other useful information about chemical-specific properties. Mode of action in toxicology can be nonspecific (narcosis) or specific. Widely used chemical categories for describing mode of action include polar narcotics, non-polar narcotics, uncouplers of oxidative phosphorylation, electrophiles/pro-electrophiles, acetylcholinesterase inhibitors, irritants, central nervous system seizure agents, respiratory blockers (Russom et al, 1997 and McKim et al, 1987b

TABLE 1: Clinical signs observed in fish, compiled from publications (CCAC, 20015; Rufli, 2012; Drummond et al, 1986 and Midtlyng et al, 2011) and TG203 score sheets provided by individual laboratories. Non-shaded rows are the major categories of visible abnormality for which recording has been mandatory in TG203 since 1992. Shaded rows are optional explanatory sub-categories.

Clinical sign	Definition	Synonyms
LOSS OF EQUILIBRIUM (sub-categories below)		
Abnormal horizontal orientation	Loss of balance displaying as abnormal horizontal orientation/posture in water column	Keeling, lost righting reflex
Abnormal vertical orientation	Head-up or head-down posture	
Loss of buoyancy control	Floating at surface or sinking to the bottom	
ABNORMAL SWIMMING BEHAVIOUR (sub-categories below)		
Hypoactivity	Decrease in spontaneous activity	Torpid, apathy, lethargy, weak, immobility, inactivity, ceased swimming, quiescent
Hyperactivity	Increase in spontaneous activity	Erratic swimming, skittering
Corkscrew swimming	Rotation around long axis; erratic movements, often in bursts	Rolling, spiralling, spiral swimming, tumbling, circling movements
Convulsions	Abnormal involuntary and uncontrolled contraction of muscles	Seizures, twitching, muscle spasms, shaking, shuddering, vibration
Tetany	Rigid body musculature (intermittent or permanent)	Paralysis
Irritated skin behaviours		Flashing, scraping, rubbing
Abnormal surface distribution/behaviour	Abnormal depth selection, close to water/air interface	Jumping, surfacing; on/at/near/just below surface/top
Abnormal bottom distribution/behaviour	Abnormal depth selection, close to base of tank	Diving, sounding; Lying on/ orientation to / collecting at / near / just above bottom
Over-reactive to stimulus	Flight (startle) or avoidance response to: visual (hand passing over top of tank, light beam), tactile (touch) or vibration (tank rapped lightly) stimulus	Hyperexcitability; hyperactivity after stimulus/threat
Under-reactive to stimulus		Not responsive to external stimulation; inactivity after stimulus/ threat
Loss of schooling / shoaling behaviour	Individual fish show loss of aggregating and social interactions	Isolation, social isolation
Dense schooling / shoaling behaviour	Increase in clumped association of fish	Crowding
ABNORMAL VENTILATORY (RESPIRATORY) FUNCTION (sub-categories below)		
Hyperventilation	Increased frequency of opercular ventilatory movements, with possible open mouth and extended operculae	Rapid/strong respiratory rate/ function. Heavy gill movements, strong ventilation, strongly extended gills, abnormal opercular activity, operculae spread apart, mouth open
Hypoventilation	Decreased frequency of (and possibly shallow) opercular ventilatory movements	Reduced/laboured/weak/slow respiration/respiratory action/ventilation
Irregular ventilation	Irregular opercular ventilatory movements	Sporadic / spasmodic respiration / gill movement
Coughing	Fast reflex expansion of mouth and operculae not at water surface - assumed to clear ventilatory channels	Gasping, abnormal opercular activity, yawn
Gulping	Mouth (and opercular) movements at water surface, resulting in intake of water and air	Piping
Head shaking	Rapid lateral head movements	
ABNORMAL SKIN PIGMENTATION (sub-categories below)		
Darkened		Changed / increased / dark(ened) colour / pigmentation / melanistic markings
Lightened		Pallor, pale/changed/weak pigmentation
Mottled		Discoloured patches
OTHER VISIBLE (APPEARANCE & BEHAVIOUR) ABNORMALITIES (sub-categories below)		
Exophthalmia	Swelling within orbital socket(s) resulting in bulging of one or both eyes	Exophthalmos, exophthalmus, popeye, protruding eyeball
Oedema	Abdominal swelling due to accumulation of fluid. May cause protruding scales and/or fissure in abdominal wall	Distended/swollen/bloated abdomen/gut area; dropsy
Haemorrhage	Petechias (pinhead sized spots) and/or haematoma (area of blood) due to intradermal or sub-mucus bleeding	
Mucus secretion	Excess mucus production	Mucus build-up (pay close attention to eyes); increased secretion (mucus on skin or in water); mucus loss
Faecal (anal) casts	String of faeces hanging from anus or on tank floor	
Aggression and/or cannibalism		Aggression, direct attack, domination of choice tank locations, pick at or eat bodies of dead fish

TABLE 2: Example format for sheet to record clinical signs. Each column represents one set of observations. If no abnormalities observed, simply record "NAO". Otherwise, record the number of individual live fish observed displaying an abnormality. Grey rows represent optional explanatory sub-categories for recording observed visible abnormalities.

Study & tank details									
Test Day/ Observation	Day 0, 2-3 hours	Day 0, 5-6 hours	Day 1, morning	Day 1, afternoon	Day 2, morning	Day 2, afternoon	Day 3, morning	Day 3, afternoon	Day 4, morning
Approximate observation time from Start	2.5 h	5.5 h	24 h	30 h	48 h	54 h	72 h	78 h	96 h
Date / Time									
No. live fish in tank for scoring									
No. moribund* removed after scoring									
No. dead removed									
If no abnormalities observed, record "NAO"									
LOSS OF EQUILIBRIUM									
Abnormal horizontal orientation									
Abnormal vertical orientation									
Loss of buoyancy control									
ABNORMAL SWIMMING BEHAVIOUR									
Hypoactivity									
Hyperactivity									
Corkscrew swimming									
Convulsions									
Tetany									
Irritated skin behaviours									
Abnormal surface distribution/behaviour									
Abnormal bottom distribution/behaviour									
Over-reactive to stimulus									
Under-reactive to stimulus									
Loss of schooling / shoaling behaviour									
Dense schooling / shoaling behaviour									
ABNORMAL VENTILATORY FUNCTION									
Hyperventilation									
Hypoventilation									
Irregular ventilation									
Coughing									
Gulping									
Head shaking									
ABNORMAL SKIN PIGMENTATION									
Darkening									
Lightening									
Mottled									
OTHER VISIBLE ABNORMALITIES									
Exophthalmia									
Oedema									
Haemorrhage									
Mucus secretion									
Faecal (anal) casts									
Aggression and/or cannibalism									
Not listed above. Please describe.									

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

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