OECD/OCDE **202**

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OECD GUIDELINE FOR TESTING OF CHEMICALS

Daphnia sp., Acute Immobilisation Test

INTRODUCTION

1. OECD Guidelines for the Testing of chemicals are periodically reviewed in the light of scientific progress. Guideline 202 on "*Daphnia* sp., Acute Immobilisation Test and Reproduction Test", adopted in April 1984, included two parts: Part I - the 24h EC_{50} acute immobilisation test and Part II - the reproduction test (at least 14 days). Revision of the reproduction test has resulted in the adoption and publication of Test Guideline 211 on "*Daphnia magna* Reproduction Test" in September 1998. Consequently, the new version of Guideline 202 is restricted to the acute immobilisation test.

2. This guideline describes an acute toxicity test to assess effects of chemicals towards daphnids. Existing test methods were used to the extent possible (1)(2)(3). The main differences in comparison with the earlier version are the extension of the test duration to 48 hours, the provision for more information on recommended culture and test media, and the introduction of a limit test at 100 mg/l of test substance.

PRINCIPLE OF THE TEST

3. Young daphnids, aged less than 24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilisation is recorded at 24 hours and 48 hours and compared with control values. The results are analysed in order to calculate the EC_{50} at 48h (see Annex 1 for definitions). Determination of the EC_{50} at 24h is optional.

INFORMATION ON THE TEST SUBSTANCE

4. The water solubility and the vapour pressure of the test substance should be known and a reliable analytical method for the quantification of the substance in the test solutions with reported recovery efficiency, and limit of determination should be available. Useful information includes the structural formula, purity of the substance, stability in water and light, P_{ow} and results of a test for ready biodegradability (see Guideline 301).

Note: Guidance for testing substances with physical chemical properties that make them difficult to test is provided in a separate document (4).

REFERENCE SUBSTANCES

5. A reference substance may be tested for EC_{50} as a means of assuring that the test conditions are reliable. Toxicants used in international ring-tests (1)(5) are recommended for this purpose[1]. Test(s) with a reference substance should be done preferably every month and at least twice a year.

VALIDITY OF THE TEST

6. For a test to be valid, the following performance criteria apply:

- In the control, including the control containing the solubilising agent, not more that 10 per cent of the daphnids should have been immobilised;
- The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/l in control and test vessels.

Note: For the first criterion, not more than 10 percent of the control daphnids should show immobilisation or other signs of disease or stress, for example, discoloration or unusual behaviour such as trapping at surface of water.

DESCRIPTION OF THE METHOD

Apparatus

7. Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Test vessels will normally be glass test tubes or beakers; they should be cleaned before each use using standard laboratory procedures. Test vessels should be loosely covered to reduce the loss of water due to evaporation and to avoid the entry of dust into the solutions. Volatile substances should be tested in completely filled closed vessels large enough to prevent oxygen becoming limiting or too low (see paragraphs 6 and 22).

8. In addition some or all of the following equipment will be used: oxygen-meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low volumes samples); pH-meter; adequate apparatus for temperature control; equipment for the determination of total organic carbon concentration (TOC); equipment for the determination of chemical oxygen demand (COD); equipment for the determination of hardness, etc.

Test organism

9. *Daphnia magna* Straus is the preferred test species although other suitable *Daphnia* species can be used in this test (e.g. *Daphnia pulex*). At the start of the test, the animals should be less than 24 hours old and, to reduce variability, it is strongly recommended they are not first brood progeny. They should be derived from a healthy stock (i.e. showing no signs of stress such as high mortality, presence of males and ephippia, delay in the production of the first brood, discoloured animals, etc.). All organisms used for a

[1] The results of these inter laboratory tests and a Technical Corrigendum to ISO 6341 give an EC_{50}-24 h of the potassium dichromate ($K_2Cr_2O_7$) within the range 0.6 mg/l to 2.1 mg/l

particular test should have originated from cultures established from the same stock of daphnids. The stock animals must be maintained in culture conditions (light, temperature, medium) similar to those to be used in the test. If the daphnids culture medium to be used in the test is different from that used for routine daphnids culture, it is good practice to include a pre-test acclimation period. For that, brood daphnids should be maintained in dilution water at the test temperature for at least 48 hours prior to the start of the test.

Holding and dilution water

10. Natural water (surface or ground water), reconstituted water or dechlorinated tap water are acceptable as holding and dilution water if daphnids will survive in it for the duration of the culturing, acclimation and testing without showing signs of stress. Any water which conforms to the chemical characteristics of an acceptable dilution water as listed in Annex 2 is suitable as a test water. It should be of constant quality during the period of the test. Reconstituted water can be made up by adding specific amounts of reagents of recognised analytical grade to deionised or distilled water. Examples of reconstituted water are given in (1)(6) and in Annex 3. Note that media containing known chelating agents, such as M4 and M7 media in Annex 3, should be avoided for testing substances containing metals. The pH should be in the range of 6 to 9. Hardness between 140 and 250 mg/l (as CaCO3) is recommended for *Daphnia magna*, while lower hardness may be also appropriate for other *Daphnia* species. The dilution water may be aerated prior to use for the test so that the dissolved oxygen concentration has reached saturation.

11. If natural water is used, the quality parameters should be measured at least twice a year or whenever it is suspected that these characteristics may have changed significantly (see paragraph 10 and Annex 2). Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni) should also be made. If dechlorinated tap water is used, daily chlorine analysis is desirable. If the dilution water is from a surface or ground water source, conductivity and total organic carbon (TOC) or chemical oxygen demand (COD) should be measured.

Test solutions

12. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance in the dilution water. As far as possible, the use of solvents, emulsifiers or dispersants should be avoided. However, such compounds may be required in some cases in order to produce a suitably concentrated stock solution. Guidance for suitable solvents, emulsifiers and dispersants is given in (4). In any case, the test substance in the test solutions should not exceed the limit of solubility in the dilution water.

13. The test should be carried out without the adjustment of pH. If the pH does not remain in the range 6-9, then a second test could be carried out, adjusting the pH of the stock solution to that of the dilution water before addition of the test substance. The pH adjustment should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or precipitation of the test substance is caused. HCl and NaOH are preferred.

PROCEDURE

Conditions of exposure

Test groups and controls

14. Test vessels are filled with appropriate volumes of dilution water and solutions of test substance. Ratio of air/water volume in the vessel should be identical for test and control groups. Daphnids are then placed into test vessels. At least 20 animals, preferably divided into four groups of five animals each, should be used at each test concentration and for the controls. At least 2 ml of test solution should be provided for each animal (i.e. a volume of 10 ml for five daphnids per test vessel). The test may be carried out using semi-static renewal or flow-through system when the concentration of the test substance is not stable.

15. One dilution-water control series and also, if relevant, one control series containing the solubilising agent (solvent control) at the level used in treatments must be run in addition to the treatment series.

Test concentrations

16. A range-finding test may be conducted to determine the range of concentrations for the definitive test unless information on toxicity of the test substance is available. For this purpose, the daphnids are exposed to a series of widely spaced concentrations of the test substance. Five daphnids should be exposed to each test concentration for 48 hours or less, and no replicates are necessary. The exposure period may be shortened (e.g. 24 hours or less) if data suitable for the purpose of the range-finding test can be obtained in less time.

17. At least five test concentrations should be used. They should be arranged in a geometric series with a separation factor preferably not exceeding 2.2. Justification should be provided if fewer than five concentrations are used**.** The highest concentration tested should preferably result in 100 per cent immobilisation**,** and the lowest concentration tested should preferably give no observable effect.

Incubation conditions

18. The temperature should be within the range of 18°C and 22°C, and for each single test it should be constant within ± 1°C. A 16-hour light and 8-hour dark cycle is recommended**.** Complete darkness is also acceptable, especially for test substances unstable in light.

19. The test vessels must not be aerated during the test. The test is carried out without adjustment of pH. The daphnids should not be fed during the test.

Duration

20. The test duration is 48 hours.

Observations

21. Each test vessel should be checked for immobilised daphnids at 24 and 48 hours after the beginning of the test. (see Annex 1 for definitions). In addition to immobility, any abnormal behaviour or appearance should be reported.

Analytical measurements

22. The dissolved oxygen and pH are measured at the beginning and end of the test in the control(s) and in the highest test substance concentration. The dissolved oxygen concentration in controls should be in compliance with the validity criterion (see paragraph 6). The pH should normally not vary by more than 1.5 units in any one test. The temperature is usually measured in control vessels or in ambient air and it should be recorded preferably continuously during the test or, as a minimum, at the beginning and end of the test.

23. The concentration of the test substance should be measured, as a minimum, at the highest and lowest test concentration, at the beginning and end of the test (4). It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within ± 20 per cent of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

LIMIT TEST

24. Using the procedures described in this Guideline, a limit test may be performed at 100 mg/l of test substance or up to its limit of solubility in the test medium (whichever is the lower) in order to demonstrate that the EC50 is greater than this concentration. The limit test should be performed using 20 daphnids (preferably divided into four groups of five), with the same number in the control(s). If the percentage of immobilisation exceeds 10% at the end of the test, a full study should be conducted. Any observed abnormal behaviour should be recorded.

DATA AND REPORTING

Data

25. Data should be summarised in tabular form, showing for each treatment group and control, the number of daphnids used, and immobilisation at each observation. The percentages immobilised at 24 hours and 48 hours are plotted against test concentrations. Data are analysed by appropriate statistical methods (e.g. probit analysis, etc.) to calculate the slopes of the curves and the EC_{50} with 95% confidence limits (p = 0.95) (7)(8).

26. Where the standard methods of calculating the EC_{50} are not applicable to the data obtained, the highest concentration causing no immobility and the lowest concentration producing 100 per cent immobility should be used as an approximation for the EC_{50} (this being considered the geometric mean of these two concentrations).

Test report

27. The test report must include the following:

Test substance:

- physical nature and relevant physical-chemical properties;
- chemical identification data, including purity.

Test species:

- source and species of *Daphnia*, supplier of source (if known) and the culture conditions used (including source, kind and amount of food, feeding frequency).

Test conditions:

- description of test vessels: type and volume of vessels, volume of solution, number of daphnids per test vessel, number of test vessels (replicates) per concentration;
- methods of preparation of stock and test solutions including the use of any solvent or dispersants, concentrations used;
- details of dilution water: source and water quality characteristics (pH, hardness, Ca/Mg ratio, Na/K ratio, alkalinity, conductivity, etc.); composition of reconstituted water if used;
- incubation conditions: temperature, light intensity and periodicity, dissolved oxygen, pH, etc.

Results:

- the number and percentage of daphnids that were immobilised or showed any adverse effects (including abnormal behaviour) in the controls and in each treatment group, at each observation time and a description of the nature of the effects observed;
- results and date of test performed with reference substance, if available;
- the nominal test concentrations and the result of all analyses to determine the concentration of the test substance in the test vessels; the recovery efficiency of the method and the limit of determination should also be reported;
- all physical-chemical measurements of temperature, pH and dissolved oxygen made during the test;
- the EC_{50} at 48h for immobilisation with confidence intervals and graphs of the fitted model used for their calculation, the slopes of the dose-response curves and their standard error; statistical procedures used for determination of EC_{50}; (these data items for immobilisation at 24h should also be reported when they were measured.)
- explanation for any deviation from the Test Guideline and whether the deviation affected the test results.

LITERATURE

(1) ISO 6341. (1996). Water quality - Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea) - Acute toxicity test. Third edition, 1996.

(2) EPA OPPTS 850.1010. (1996). Ecological Effects Test Guidelines - Aquatic Invertebrate Acute Toxicity Test, Freshwater Daphnids.

(3) Environment Canada. (1996) Biological test method. Acute Lethality Test Using *Daphnia* spp. EPS 1/RM/11. Environment Canada, Ottawa, Ontario, Canada.

(4) Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Environmental Health and Safety Publication. Series on Testing and Assessment. No. 23. Paris 2000.

(5) Commission of the European Communities. Study D8369. (1979). Inter-laboratory Test Programme concerning the study of the ecotoxicity of a chemical substance with respect to *Daphnia*.

(6) OECD Guidelines for the Testing of Chemicals. Guideline 211: *Daphnia magna* Reproduction Test, adopted September 1998.

(7) Stephan C.E. (1977). Methods for calculating an LC50. In Aquatic Toxicology and Hazard Evaluation (edited by F.I. Mayer and J.L. Hamelink). ASTM STP 634 - American Society for Testing and Materials. Pp65-84

(8) Finney D.J. (1978). Statistical Methods in Biological Assay. 3rd ed. London. Griffin, Weycombe, UK.

ANNEX 1

DEFINITIONS

In the context of this guideline, the following definitions are used:

EC_{50} is the concentration estimated to immobilise 50 per cent of the daphnids within a stated exposure period. If another definition is used, this must be reported, together with its reference.

Immobilisation: Those animals that are not able to swim within 15 seconds, after gentle agitation of the test vessel are considered to be immobilised (even if they can still move their antennae).

ANNEX 2

SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER

Substance	Concentration
Particulate matter	<20 mg/l
Total organic carbon	< 2 mg/l
Unionised ammonia	< 1 µg/l
Residual chlorine	<10 µg/l
Total organophosphorus pesticides	<50 ng/l
Total organochorine pesticides plus polychlorinated biphenyls	<50 ng/l
Total organic chlorine	<25 ng/l

ANNEX 3

EXAMPLES OF SUITABLE RECONSTITUTED TEST WATER

ISO Test water (1)

Stock solutions (single substance)		To prepare the reconstituted water, add the following volumes of stock solutions to 1 litre water*
Substance	**Amount added to 1 litre water***	
Calcium chloride $CaCl_2$, $2H_2O$	11.76 g	25 ml
Magnesium sulfate $MgSO_4$, $7H_2O$	4.93 g	25 ml
Sodium bicarbonate $NaHCO_3$	2.59 g	25 ml
Potassium chloride KCl	0.23 g	25 ml

* Water of suitable purity, for example deionised, distilled or reverse osmosis with conductivity preferably not exceeding 10 $\mu S.cm^{-1}$.

ANNEX 3(Cont.)

Elendt M7 and M4 medium

Acclimation to Elendt M4 and M7 medium

Some laboratories have experienced difficulty in directly transferring Daphnia to M4 and M7 media. However, some success has been achieved with gradual acclimation, i.e. moving from own medium to 30% Elendt, then to 60% Elendt and then to 100% Elendt. The acclimation periods may need to be as long as one month.

Preparation

Trace element

Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, for example deionised, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements (combined solution), i.e.:

Stock solution(s) I (single substance)	Amount added to water (mg/l)	Concentration (related to medium M4)	To prepare the combined stock solution II, add the following amount of stock solution I to water (ml/l)	
			M4	M7
H_3BO_3	57 190	20 000-fold	1.0	0.25
$MnCl_2 \bullet 4H_2O$	7 210	20 000-fold	1.0	0.25
LiCl	6 120	20 000-fold	1.0	0.25
RbCl	1 420	20 000-fold	1.0	0.25
$SrCl_2 \bullet 6H_2O$	3 040	20 000-fold	1.0	0.25
NaBr	320	20 000-fold	1.0	0.25
$Na_2MoO_4 \bullet 2H_2O$	1 230	20 000-fold	1.0	0.25
$CuCl_2 \bullet 2H_2O$	335	20 000-fold	1.0	0.25
$ZnCl_2$	260	20 000-fold	1.0	1.0
$CoCl_2 \bullet 6H_2O$	200	20 000-fold	1.0	1.0
KI	65	20 000-fold	1.0	1.0
Na_2SeO_3	43.8	20 000-fold	1.0	1.0
NH_4VO_3	11.5	20 000-fold	1.0	1.0
$Na_2EDTA \bullet 2H_2O$	5 000	2 000-fold	-	-
$FeSO_4 \bullet 7H_2O$	1991	2 000-fold	-	-
Both Na_2EDTA and $FeSO_4$ solutions are prepared singly, poured together and autoclaved immediately. This gives:				
21 Fe-EDTA solution		1 000-fold	20.0	5.0

M4 and M7 media

M4 and M7 media are prepared using stock solution II, the macro-nutrients and vitamin as follows:

	Amount added to water (mg/l)	Concentration (related to medium M4)	Amount of stock solution II added to prepare medium (ml/l)	
			M4	M7
Stock solution II (combined trace elements)		20-fold	50	50
Macro nutrient stock solutions (single substance)				
$CaCl_2 \bullet 2H_2O$	293 800	1 000-fold	1.0	1.0
$MgSO_4 \bullet 7H_2O$	246 600	2 000-fold	0.5	0.5
KCl	58 000	10 000-fold	0.1	0.1
$NaHCO_3$	64 800	1 000-fold	1.0	1.0
$Na_2SiO_3 \bullet 9H_2O$	50 000	5 000-fold	0.2	0.2
$NaNO_3$	2 740	10 000-fold	0.1	0.1
KH_2PO_4	1 430	10 000-fold	0.1	0.1
K_2HPO_4	1 840	10 000-fold	0.1	0.1
Combined Vitamin stock	-	10 000-fold	0.1	0.1
The combined vitamin stock solution is prepared by adding the 3 vitamin to 1 litre water, as shown below:				
Thiamine hydrochloride	750	10 000-fold		
Cyanocobalamine (B_{12})	10	10 000-fold		
Biotine	7.5	10 000-fold		

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added to the media shortly before use.

N.B: To avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500 – 800 ml deionised water and then fill up to 1 litre.

N.N.B: The first publication of the M4 medium can be found in Elendt, B. P. (1990). Selenium deficiency in crustacea; an ultrastructual approach to antennal damage in *Daphnia magna* Straus. Protoplasma, 154, 25-33.