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**Prolonged Toxicity Study
with Daphnia Magna**

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ECETOC

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PROLONGED TOXICITY STUDY WITH DAPHNIA MAGNA

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1. RATIONALE

Aquatic invertebrates are the most common food chain link between plankton and fish. The chronic toxicity testing of a new chemical substance with invertebrates is therefore one of the most relevant tests for assessing more precisely the aquatic ecotoxicity of a substance.

In the EEC 6th Amendment of the 1967 Directive, a prolonged study of this type is required when (1) production volume reaches 100 tonnes per year or a total of 500 tonnes; (2) for lower tonnages when results from the base set (annex VII) suggest a risk of chronic effects.

The objective of this test is the estimation of the "No Observed Effect Concentration" (NOEC) with respect to lethality and reproduction when *Daphnia Magna* are exposed to a chemical throughout a full life-cycle for at least three consecutive broods.

2. APPLICABILITY

The test is most suitable for chemicals and compounds that are soluble or truly dispersed under the test condition. Special measures may be required to test, for example, insoluble, strongly adsorbing, readily degradable or volatile compounds. When special measures to overcome these difficulties are taken, they should be clearly mentioned together with the results. Since these results could lack environmental relevance they require careful interpretation.

3. CURRENT METHODOLOGY

Methodologies have been proposed by OECD, DIN, TNO (NEN), ASTM, and A.W. Maki - see references 1 to 5 on page 8.

Except for the paper by Maki, all the other documents are still in draft form, and therefore liable to be modified.

4. IMPORTANCE OF SOME TEST PARAMETERS

4.1. Test Design

1. OBJECTIVE

The ultimate objective of the test is to establish an NOEC for the survival and reproduction of Daphnia. The chronic toxicity testing of a new chemical substance with Daphnia must be practical and provide at the same time for each concentration a sufficient number of replicates of total Daphnia to enable a correct statistical interpretation of data.

In the 1967 Directive, a prolonged study of this type is required when (1) After a careful review of the statistics related to this type of test the following comments can be made: (Annex VII) suggest a risk of chronic effects.

i. Generally the more replicates that are used the better the precision of results and the fewer the total number of animals respect to per concentration required. Based on the limited experience with unexposed Daphnia, life-cycle a minimum of 4 replicates per concentration and a minimum of 20 parent Daphnia per concentration are considered necessary.

ii. No "optimal" test design can be specified unless the variance of daphnia juvenile production can be accurately assessed.

iii. In this type of experiment each test concentration is compared to the control. According to Dunnett (6) and Williams (7,8), for optimising the statistical interpretation the number of Daphnia in the control should be higher than that in each test concentration. When r is the number of tested concentrations (excluding the control) and n is the number of animals per test concentration, the control should contain $\sqrt{r \cdot n}$ animals.

4.2. Test Criteria

The following criteria are indicated.

4.2.1. Mandatory observations :

- i. Lethality for the parent generation (Fo) as a function of time.

ii. Gross reproduction rate : this is the total number of F_1 animals born by the end of the test divided by the number of parent animals, F_0 .

Some of the F_0 generation may die during the test, in which case the ratio F_1/F_0 is a measure of the combined effects of the test compound on lethality and reproduction. To exclude the effect on lethality, the ratio F_1/F_0 should be computed at each observation time using the mean number of the parent generation alive during the preceding stage of the test.

iii. NOEC values for all test materials should be determined as the highest measured test concentration causing no significant effect on lethality and reproduction rate.

4.2.2. Optional test observations :

- i. Any relevant observations on the growth of the parent generation and/or on the lag time before reproduction starts.
- ii. Any observable anomaly of the behaviour and appearance of F_0 and F_1 animals.

4.3. Test Duration

All methods listed above, except the OECD proposed draft, require a minimum test duration of 3 brood cycles. It appears from experience that 21 days is adequate to obtain usable results. This duration complies with the EEC proposal.

4.4. Test Concentration

The concentrations of test substances in the solutions to which the Daphniae are exposed should be kept constant so as to avoid significant variation. If possible, the concentration of the test chemical should be determined in the test solution. When no analytical verification is given it should be explained why this is not necessary or not feasible.

4.2.1. Observations to be made

- i. Lethality for the test generation (F_0)

Most tests described above are of the semi-static type where the renewal of the test solution is made every 48 hours, or on Mondays, Wednesdays, Fridays. Chemicals that are rapidly degradable or volatile, or that would easily adsorb on the glassware, might require more sophisticated techniques in order to keep the test concentrations constant. Continuous flow systems with automatic dilution of the test compound to the various test concentrations would be preferred for these chemicals.

Continuous flow systems are broadly available, but experience with these for chronic Daphnia toxicity testing is limited.

To determine the NOEC of a chemical, 5 test concentrations are usually taken within the range 24 h - LC₅₀, and 0,01 - 24 h - LC₅₀.

4.5. Water Quality

In principle synthetic water (bidistilled + adequate salts; deionised + analytical grade active carbon filtration + adequate salts) would be preferred both for culture and test, in order to improve standardisation. Synthetic media that have proved to give satisfactory results are mentioned in the Dutch NEN (5a) and German DIN (3) draft proposals (both semi-static procedures).

In some cases a good-quality water of natural origin can be used provided one can demonstrate constant quality; adequate hardness, pH and dissolved oxygen; and that it causes no more than 20% lethality in controls.

Tap water will usually not fulfil these requirements.

4.6. Temperature of the Test

Cultures and tests should be run at $20 \pm 1^{\circ} \text{C}$. This temperature should be maintained since it greatly influences the reproduction rate.

4.7. Test Animals

The most commonly used species is Daphnia Magna, whose size makes counting juveniles easy. The Daphnia must be of uniform age and be from a healthy, single-species culture in which reproduction is asexual.

The test must begin with newly hatched Daphniae (usually 24 hours maximum).

4.8. Quality of the Feed

In general the feed should meet the following criteria :

- a) good growth and minimal mortality of Daphnia Magna parent generation F_0 ;
- b) good reproduction rate of Daphnia Magna F_1 ;
- c) causes no oxygen depletion;
- d) causes no difficulties with parasitic fungi;
- e) causes no problems with trace amounts of toxic substances.

Both the quality and amount of feed are of major importance for the reproduction rate of Daphnia Magna. Current experience does not allow the definition of a standard feed which would be better than any other. The feeds described in the references seem adequate.

4.9. Lighting

The test must be conducted in a light-dark cycle adequate to prevent the production of ephippia, for which a cycle of 14 hours light and 10 hours dark has proved suitable. The light intensity should be between 2000 and 3000 lux, and the light source should be of the "cool white" type.

5. VALIDATION OF RESULTS

The results of the test can be considered as valid only when the following conditions are fulfilled :

- i. the lethality of the parent generation F_0 in the control should be equal to or less than 20%.
- ii. reproduction in the control should start on day 10 at the latest, from the beginning of the test.
- iii. The mean value for the number of young produced per surviving Daphnia in the control should be at least 60 at the end of the test.
- iv. In the source culture and in the control no ephippia should occur.

6. TREATMENT OF DATA

The determination of NOEC, either for lethality to F_0 or reproduction, implies that data from each test concentration be compared to the result obtained on the control.

The evaluation of the significance of these differences can be made with the help of statistical methods (U-test, F test); see references 6 to 12. Further experience with this test might indicate the possibility of using statistical methods applied in toxicology, which allow a determination of a theoretical No Effect Level (7,8). When the lethality of the parent Daphniae (F_0) is very high, it is meaningless to calculate reproduction rates of the surviving animals.

7. REPORTING

7.1. Ideally, the report of the results of a toxicity test should contain all the information necessary for the competent authority to judge the validity of the test and the techniques used. In addition, sufficient data should be provided to allow an independent assessment of the result which may be expressed as the No Observed Effect Concentration (NOEC) for the effect(s) being determined (i.e. survival, production of juveniles, etc...).

7.2. The following must be included in the report :

- 7.2.1. Adequate characterisation and identification of the test sample.
- 7.2.2. A description of the method of dispersing or solubilising the test material, if it is not soluble or only partially soluble.
- 7.2.3. A description of the means of dispersing in solution, and maintaining the concentration of, the test material, if it is volatile.
- 7.2.4. The Daphnia species and the strain used, its culture method, and feeding regime.
- 7.2.5. The test method used. When a standard method is used, a reference to it is sufficient. Any deviations from it should be reported, with reasons for them. When the method is not standard, a full description should be given.

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7.2.6. The specific test conditions used and their variability, particularly for dissolved oxygen, pH, temperature and water hardness.

7.2.7. Nature of the dilution water. If artificial, the formulation used should be given; if a standard formulation is used only the reference need be given. Where natural water or treated natural water is used, evidence of purity should be given.

7. REPORTING

7.1. Ideally ...

7.2.8. A statement on whether continuous flow, renewal static, or static conditions were used.

7.2.9. A table of F_0 and F_1 data at each test concentration as a function of time, including controls, and dispersant or solubiliser controls, where appropriate.

7.2.10. F_0 lethality and reproduction rate as two independent criteria. The most sensitive feature should be used to determine a No Effect Level or NOEC.

7.2.11. The statistical method used; see references 6 to 12.

7.2.12. ...

8. REFERENCES

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