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**Test Method for Acute Toxicity to
Daphnia Magna**

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TEST METHOD FOR ACUTE TOXICITY

TO DAPHNIA MAGNA

(Document EM-44bis)

APPROVED PAPER ON
TEST METHOD FOR ACUTE TOXICITY TO DAPHNIA MAGNA

1. RATIONALE

Aquatic invertebrates are the most common food link between plankton and desirable fish and shell fish. The acute toxicity testing of a new chemical to invertebrates is therefore an important element in the preliminary assessment of its impact on aquatic systems as requested at the Base Set Level of the EEC 6th Amendment.

The most widely performed and economical acute tests on invertebrates use Daphnia Magna. This convenient and representative fresh water invertebrate has a high sensitivity to chemicals, is easy to breed and culture in laboratory conditions, and has a short life-cycle.

Because of these characteristics, Daphnia Magna has been used by many researchers in chronic toxicity tests.

2. CURRENT METHODOLOGY

The most internationally recognised method is the ISO draft standard ISO/DIS 6341 for acute toxicity testing of Daphnia Magna, currently submitted to vote to become an official ISO standard (vote opened on 30 August 1979). It is a static test method.

This method aims at determining the concentration of the test chemical that inhibits the mobility of 50% of the number of Daphnia Magna after 24 hours of test exposure. This concentration is called the 24-hour EC₅₀ (instead of 24-hour LC₅₀ used when lethality is the measured effect).

An early version of ISO/DIS 6341 method was ring-tested in 1979 under the auspices of the EEC Commission. The interlaboratory reproducibility for water-soluble compound was found satisfactory in spite of some flexibility left in the method on a few parameters. No satisfactory standard methodology exists for compounds with a low water solubility (2).

Similar test methods (see Appendix) are currently being discussed in National Standardisation Associations in France, Germany and Holland.

Sometimes these offer a better definition of test parameters and are likely to show higher reproducibility than the ISO method with which, however, they are largely compatible. This higher reproducibility is not critical for the information required at the Base Set Level of the 6th Amendment. It is expected that these various test methods and the ISO/DIS 6341 standard proposal will also produce a similar acute toxicity reading on Daphnia Magna, with an acceptable interlaboratory reproducibility, well within an order of magnitude.

3. IMPORTANCE OF SOME TEST PARAMETERS

3.1. Test duration

Because of the short life-cycle of Daphnia Magna (8-10 days) a test duration of 24 hours is quite sufficient to test acute effects of chemicals. In special cases only there might be a need for 48-hour investigations, but such tests must not be conducted in test tubes where oxygen depletion could occur, but in beakers where this depletion is less likely.

With respect to the short life-cycle of Daphnia Magna, tests longer than 48 hours cannot be regarded as acute tests. Such tests would also require quite different testing procedures in order to avoid starvation of the test organism, depletion of oxygen and depletion of the test chemical concentration.

3.2. Test concentration

The objective of any EC 50 test is to determine a concentration effect, which supposes a constant concentration during the test. It will normally not be necessary to have analytical determinations. These test concentrations are thus nominal and the resulting 24 hours EC 50 is calculated from nominal concentrations. When there are reasons to believe that the concentration during the test will reduce substantially e.g. by volatility and/or biodegradation of the chemical, the test will be adapted accordingly.

3.3. Static versus continuous flow systems

Static tests are adequate for a large number of chemicals which show stable concentrations during the test exposure.

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 into various test concentrations are preferred for these chemicals.

- To our knowledge, continuous flow systems adapted for Daphnia toxicity tests, have only been experimentally examined by U.S. laboratories and are under discussion at ASTM. Currently no standard method based on a continuous flow system exists.
3. IMPORTANCE OF

3.4: Water quality

Daphnia Magna is a hard water species. For this reason water hardness limits are indicated together with pH limits in the ISO/DIS 6341 proposed standard (1). This ISO proposed standard offers, however, the flexibility to use any source of water (dechlorinated tap water, well water, reconstituted water starting from deionized or distilled water). Several ring tests have indeed shown that the standardisation of water is not critical to the test result (within an order of magnitude) provided that the water used complies with the requirements of hardness and pH and contains no toxicant. The water should, anyway, be able to sustain Daphnia Magna for long periods and allow them to reproduce normally.

It is recommended to use, as dilution water for the toxicity test, the same water quality as that used for culturing the test organisms. When a high reproducibility of the test result is required, a strict formulation of reconstituted water using deionized water and selected salts is preferable and highly recommended, instead of the flexibility described above. In this respect, the formulation of water as described in the draft of the DIN 38412 method (3) or in the draft of the Dutch NEN 6501/6503 method (4) seem quite satisfactory. Recent experiments by Müller (5) have shown the importance of defining the alkalinity (0,8 m mole/l), the Ca : Mg mole ratio (4:1), and the Na : K mole ratio (10 : 1). These parameters have an influence on the toxicity response of Daphnia Magna to K₂Cr₂O₇. Imposing a correct value on these parameters will optimize the life conditions of the test organisms and improve the reproducibility of the test method.

3.5. The use of a standard reference material

The use of a standard material gives a reasonable indication that the strain of *Daphnia* has not drifted in sensitivity. Potassium bichromate has proven to be a convenient standard in this respect in the various ring tests where it was used. It must be recognized however that its toxicity may vary if alkalinity, hardness, Ca : Mg ratio and Na : K ratio are not defined. Therefore it does not seem to make much sense to impose a range of values for $K_2Cr_2O_7$ toxicity to validate test results, as long as these water quality parameters are not rigorously fixed. The current flexibility on water sources as shown in the ISO draft is not compatible with a given narrow range of $K_2Cr_2O_7$ toxicity values.

4. CALCULATION OF THE EC₅₀

Four methods can be used to calculate the EC₅₀ :

- Litchfield, J.T. and Wilcoxon F (1949), A simplified method of evaluating dose-effects experiments, J. Pharmac. Exp. Therap. 96-99-113.
- Finney D.J. (1971) Probit Analysis, Cambridge University Press.
- Bliss C.I. (1938) The determination of the dosage-mortality curve from small numbers. Quant J. Pharm. and Pharmacol. (1938), 11-192-216.
- Thomson W.R. (1947), The use of moving-average and interpolation to estimate the medium effective dose, Bacteriological Reviews 11, 2 S. 115-145.

The graphical determination of the EC₅₀ on probit-log paper is quite acceptable. In some instances, the EC₅₀ cannot be calculated and it is often sufficient to give the maximum tested concentration with no lethal effect (EC₀) and the minimum tested concentration with 100% lethal effect (EC₁₀₀). When this range EC₀ - EC₁₀₀ is narrow this information can advantageously replace the EC₅₀.

5. RECOMMENDATION

For the type of toxicity information required in the Base Set of the EEC 6th Amendment, a very high reproducibility of this test is not essential.

Therefore it is recommended to use preferentially the ISO/DIS 6341 standard method proposal, which offers enough flexibility for easy harmonisation.

Other test protocols as listed in the Appendix should also be allowed, since they are compatible with the test conditions of ISO/DIS 6341. (1)

One must also recognize the limitation of static methods when the test compounds are volatile or rapidly degradable. In such cases, continuous flow test systems should be used in order to maintain constant the test chemical concentration throughout the time of exposure.

Tests protocols using continuous flow systems still have to be developed.

The same remark applies for the investigation of chemicals with low water solubility. For these, adequate methodology must still be developed.

References

- (1) ISO/DIS 6341
- (2) EEC ring test report by R. CABRIDENC
- (3) DIN 38412 draft method
- (4) NEN 6501/6503 draft method
- (5) The acute toxicity of $K_2Cr_2O_7$ to Daphnia Magna as a function of water quality - 22 February 1980 - by H.G. MÜLLER - BASF (internal report).

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APPENDIX

ACUTE TOXICITY TO DAPHNIA MAGNA

STATIC TESTS

| Test Method | Test Chamber | Number Daphnia per conc. | Test Duration Hours | Test Temperature °C | Test Water | Test Water Hardness (mg/L CaCO ₃) | Culture Water | Food | Use of Ref. Material |
|----------------------------------|---|--------------------------|---------------------|---------------------|-------------------------|---|-------------------------|---|---|
| ISO/DIS 6341 (static) | 10 ml test tubes or 10 to 50 ml beakers | 20 | 24 | 20° ± 2 | Optional Adjusted | 250 mg/L pH 7.8 ± 0.4 | Optional Adjusted | Optional | K ₂ Cr ₂ O ₇ |
| French AFNOR T90301 (static) | 10 ml test tubes | 20 | 24 | 20° | Synthetic reconstituted | 238 mg/L pH 8.0 ± 0.2 | Tap water | Soil micro-organisms and glucose + beef extract | K ₂ Cr ₂ O ₇ |
| German DIN 38412 (static) | 10 ml test tubes or 20 ml beakers | 20 | 24 | 20° ± 2 | Synthetic reconstituted | 250 mg/L alkalinity 0.8 m mole/l | Tap water | Green alga mainly chlorella + scenedesmus | K ₂ Cr ₂ O ₇ |
| Dutch NEN 6501 NEN 6503 (static) | 250 ml beakers | 25 | 48 | 19° ± 1 | Synthetic reconstituted | 210 mg/L pH 8.4 | Synthetic reconstituted | Chlorella Pyrenoidosa | None |

tes : 1) The Umwelthundesamt submission to the OECD Ecotoxicity Group (document B/79/CER) July, 1979, is very similar to the draft DIN 38412 method.

2) Two almost identical documents exist in the U.S. on the acute toxicity testing of aquatic species, among which is *Daphnia magna* : (1) Methods for acute toxicity tests with Fish, Macroinvertebrate and Amphibians. C.E. Stephan ; Ecological Series EPA 660/3-75009, April 1979.

(2) ASTM Draft No. 6 of Task Group E 35.21. September 9, 1977, with the same title. These documents are covering general test guidelines rather than precise protocols.