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The EEC Sixth Amendment: Prolonged Fish Toxicity Tests

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THE EEC SIXTH AMENDMENT :

PROLONGED FISH TOXICITY TESTS

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A. SUMMARY

For fish toxicity studies at Level 1, the EEC 6th Amendment requires a prolonged toxicity study for a period of at least 14 days which should include the determination of the "threshold level". This report reviews three test procedures that fulfill the Level 1 requirement. The first is simply an extended version of an acute toxicity test using death as the observed effect. The other two procedures are based on recent developments. One determines and records the presence of visible symptoms of toxicity and was developed using Brachydanio rerio. The other test determines effects on the growth rate of individually marked fish over a period of 14-28 days, for which the rainbow trout (Salmo gairdneri) is the recommended test species. A version of OECD Test Guideline 204 modified to encompass the use of the three proposed Level 1 test procedures is given, as are outline methods for the conduct of each.

The so-called "Early Life Stage test" (ELS test) and its variants were considered worthy of examination as tests suitable for fulfilling the Level 2 requirements of the 6th Amendment.

B. INTRODUCTION

The internationally agreed approach to the assessment of the potential hazard to man and the environment posed by new chemicals is to compare estimates of the environmental exposure and experimentally determined effect concentrations. By taking into account the particular circumstances of production, use and disposal, the significance of any potential hazard may be judged on the margin of safety which exists between the estimates of exposure and effect concentrations. To provide the necessary data for this purpose, testing is arranged in a series of stages in a step-sequence in which the tests tend to progress from the relatively simple to the more complex.

The EEC Directive 67/548/EEC (1979) adopts the above principle. Part of the technical dossier which has to be supplied at the different stages of notification of a new chemical will contain ecotoxicological data which should serve for "evaluating the foreseeable risks, whether immediate or delayed, which the substance may entail for ..... the environment" under normal conditions of use and disposal.

Base Set information (6th Amendment, Annex VII) must be provided when the marketed volume of a new substance exceeds one tonne per year. The required tests allow an initial evaluation of potential hazard to be made and where the conclusions are equivocal further tests may be required. In any case when the tonnage marketed reaches 10 tonnes per year or a total of 50 tonnes is produced, tests specified in Annex VIII at Level 1 may be required. If a reassessment of the potential hazard associated with the new substance remains equivocal or when the tonnage marketed reaches 1000 tonnes per year or a total of 5000 tonnes, tests specified at Annex VIII-Level 2 may be required. To be of value the tests at levels subsequent to the Base Set must provide information which allows the initial estimates of the exposure and effect concentrations to be refined or confirmed which, in turn, implies that the tests must provide additional information in order to be able to resolve outstanding questions.

Information on fish toxicity is required at the three levels but the specific requirements in the 6th Amendment are not made clear for tests beyond the Base Set. In Annexes VII and VIII the requirements are :

"Base Set :

- Acute toxicity for fish, LC_{50}...(ppm), duration of exposure determined in accordance with Annex V (C). Species selected (one or more)...

Level 1 :

- Prolonged toxicity study with fish (e.g. Oryzias, Jordanella, etc.; at least a period of 14 days; this study should also include determination of the "threshold level").

  The conditions under which this test is carried out shall be determined in accordance with the procedure described in Article 21 in the light of the methods adopted under Annex V (C) for acute toxicity tests with fish.

Level 2 :

- Prolonged toxicity study with fish (including reproduction)".

Test Guidelines (TGs) and other standard tests are established for the determination of the acute toxicity of substances to fish (e.g. OECD - TG 201,1984; EEC, 1984; ISO, 1984) but those for Level 1 (e.g. OECD - TG 204) are more open and none are available for Level 2. Testing requirements at Level 1 and level 2 have therefore been subject to different interpretation in EEC member states. An ECETOC TF was therefore established to review and assess the possible fish tests for the two levels beyond the Base Set and recommend the most appropriate methods.

Definitions of the most important terms used in this report are given in Appendix 1.

C. FISH TOXICITY TESTS AT LEVEL 1

1. Introduction

The EEC requires a prolonged toxicity study for at least a period of 14 days that should include the determination of the "threshold level". The OECD Chemicals Programme went some way towards providing the basis for a test at Level 1 (TG 204, 1984) but this makes only general observations about the use of endpoints other than lethality.

The OECD - TG 204 defines the "observed effects" as follows.

> "Lethal effects: a fish is presumed to be dead if no respiratory movement and no reaction to a slight mechanical stimulus can be detected.
>
> Effects other than lethal effects: these include all effects observed on the appearance, size and behaviour of the fish that make them clearly distinguishable from the control animals, e.g. different swimming behaviour, different reaction to external stimuli, changes in appearance of the fish, reduction or cessation of food intake, changes in length or body weight".

Within the EEC different national viewpoints exist on what constitutes an appropriate test for Level 1. These differences are centered on the type of information thought to be required from the test. Some interpreted the test as an extended LC_{50} determination when the acute (96h) test in the Base Set did not indicate an asymptotic threshold concentration for lethal effects. Others considered it should be used to gain information relating to an endpoint other than death.

To date no well described test methods have been available for the determination of effects other than lethal effects in subchronic toxicity tests at Level 1.

In view of this, ECETOC recognised the need for test methods to be available to allow the production of data on both lethality and other toxic effects in a subchronic test.

In order to provide a basis for developing test methods we defined the criteria that should be met for a Level 1 test. The test should :

i) be compatible with the requirements of the 6th Amendment;
ii) provide more reliable information on subchronic concentration dependent effects other than reproduction;
iii) where possible, detect sublethal and delayed toxic effects and adaptation phenomena after exposure periods longer than those used in acute tests (14 days or more);
iv) be scientifically justifiable and capable of producing quantitative data;
v) be done under environmentally relevant conditions;
vi) provide environmentally relevant information useful to evaluate the potential hazard of chemicals (no observable effect levels);
vii) be acceptable outside the EEC (e.g. OECD).

2. Test Methods

A number of alternative approaches may be considered :
- a test on lethality after prolonged exposure;
- a test on visible sublethal effects;
- a test on growth;
- a test covering a sensitive life stage;
- biochemical and/or histological studies.

Appropriate test methods for determining biochemical and histological effects are not yet sufficiently well developed to be considered as a suitable basis for Level 1 tests. Tests on sensitive life-stages are almost synonymous with tests on early-life stages and ECETOC is of the opinion that these are related to the reproduction cycle and hence belong to Level 2.

The first three mentioned approaches have been shown to be feasible and likely to meet some or all of the criteria for a Level 1 test.

2.1. Mortality data

The methodology is widely used and well understood and falls within the scope of OECD-TG 204 (cf. Appendix 2 - a modified OECD-TG 204, Annex 1).

On the basis of the evidence available cases where prolonged lethal effects testing is required are relatively rare. Experience has shown, that in the majority of cases with the commonly used species of fish, the 96 h LC_{50} approximates to the asymptotic threshold concentration. The latter is defined by EIFAC (1980) as the concentration of a toxicant at which 50% of the

population shows itself to be in approximate homeostasis for some prolonged period of time. In a few cases this is clearly not true, and it is possible to make a logical and scientifically justifiable case for the extension of the time period of an acute test in order to establish this asymptotic threshold concentration. A deviation of more than a factor 5 was found in only 11% of 375 tests by Sprague (1969), in 13% of guppy tests for 8 substances by Adema et al.(1981), in 3% of guppy tests with 30 substances by Hermens et al.(1982; 1984; 1985) and in 15% of zebrafish tests for 13 substances by UBA (1985).

2.2. Visible symptoms of toxicity

Visible symptoms of toxicity that may pose hazard for the survival of fish populations can be recognised at non-lethal concentrations. Paralysis, narcosis and respiratory problems are immediate threats to survival, whereas changes in escape reflex, food intake, swimming behaviour, reactions on external stimuli and appearance of the fish might affect their potential for long term survival in the natural environment. The quantitative description of these effects can be used to statistically determine significant threshold levels of subchronic effects. A test method, in annex to a modified OECD-TG 204, is given in Appendix 2 - Annex 2.

This test method depends on the observation of symptoms of toxicity including aberrant behaviour and appearance and requires training and experience on the part of the test operator. Associated with this is the difficulty of ensuring uniform standards of assessment between operators within and between laboratories. The observation of such symptoms in fish also implies a good knowledge of the behaviour, activity and appearance of the particular species.

In the Federal Republic of Germany the UBA (1985) co-ordinated a programme of testing with 25 substances of which 13 were subject to a prolonged fish toxicity test based on the observation of visible symptoms of toxicity with Zebrafish (Brachydanio rerio). Appendix 3 summarises the most important results.

The objective of the UBA test programme was to assess the applicability of the proposed test method to a broad range of chemicals with different

physico-chemical properties. The test programme was not conceived as a ring test with the aim to determine the reproducibility of the test results but, in those cases where different laboratories tested the same chemical (e.g. Lindane, Atrazine, TPBS) for the same test period, a reasonable level of reproducibility was found. When the lowest observed lethal concentrations (LOLetC) are compared with the lowest observed effect concentrations (LOEC) for 5 out 13 chemical substances (Lindane, Atrazine, PCP, TPBS and styreneoxide), indications of effects were found at sublethal concentrations. For the other 8 chemicals there was no significant difference between the lethal concentration and the LOEC.

3. Measurements of Growth

Effects on growth provide a valuable end-point because growth reflects the summation and integration of many biochemical, physiological and behavioural aspects of the organism under test. The growth from the fry stage until the onset of maturity is exponential, provided that environmental factors and food supply are non-limiting. Thus fish growth can be defined in terms of a rate which remains constant for a substantial part of the life-cycle. Moreover the environmental significance of effects on growth found in laboratory tests is obvious and can be validated by field experiments.

Crossland (1985) published a method for determining the effects of chemicals on the growth rate of rainbow trout (Salmo gairdneri). To obtain sufficient sensitivity to allow the use of the growth response as a toxicity end-point it is necessary to have a rapid marking technique which allows for the identification of individual fish throughout the experiment. Freeze branding has proved to be a suitable technique. Under these conditions the test provides a means of determining effects which are easily quantifiable and which permit a mathematical statistical evaluation of the results. Following the test method, differences in growth rate of the order of 15-25% can be detected, though the sensitivity of the method can be improved by increasing the number of fish tested at each exposure level. This test method (Appendix 2, Annex 3) was ring-tested in the UK using dichloroaniline and methyl parathion primarily to assess the feasibility of the method. The results available at the time of reporting are summarised in Appendix 4. Though no detailed analysis of the results is yet available the figures suggest that it is a satisfactory, reproducible test. Comments from participants have included a number of minor modifications that will improve the conduct of the test in practice.

This growth test, developed using rainbow trout, may be limited in the species that can be used since the fish must be of an adequate size for cold branding and in the exponential phase of growth. This implies a limit on the periods during the year when suitable stocks of the correct age will be available but, with judicious choice of hatchery and with planning, this should not present a significant practical problem to most testing laboratories. The test principles and practical techniques involved are theoretically applicable to other species and with suitable modifications the utility of the test may be proven on a broader range of species.

D. FISH TOXICITY TESTS AT LEVEL 2

The EEC Directive 67/548/EEC identifies a "prolonged toxicity study with fish (including reproduction)" as one of the key aspects of a Level 2 test. The details of the required study programme will, however, be agreed in discussions between the notifier and the competent authority and will depend to a large extent on the information already available from the Base Set and Level 1. It is important that this principle is sustained and the important criteria for Level 2 studies are that they should provide information which is environmentally relevant and which enables the margin of safety between the estimates of exposure and effect concentrations identified at Level 1 to be verified. Experiments at Level 2 must be designed to ensure that the results allow an unequivocal judgement to be made on acceptability. For this reason studies should be designed on a case by case basis and therefore only general guidance as to the tests to be carried out can be given at this Level.

Specifically in regard to the fish test requirement at Level 2 it should be noted that at the time of writing no internationally approved standard test methods or guidelines exist. The situation is further confused by the fact that neither the Directive nor associated guidance documents define what is meant by "reproduction" in this context. Several important events can be recognised in the reproductive cycle (e.g. ECETOC, 1983), in mammalian toxicological evaluation tests have been devised to determine the toxicity of a chemical to each important event and period. No similar rationalisation has been presented for fish and it is therefore unclear what type of test or tests would qualify under this Level 2 requirement.

At its broadest, this requirement could encompass any effect on any stage or activity during the complete life cycle which could be interpreted as resulting in reduced fecundity. Full life cycle tests have been done, even over more than one generation, which take account of all the reproductive stages. Our experience suggests that this type of test is time consuming and vulnerable to failure through practical difficulties and its adoption as a standard is not desirable aspecially as other options exist.

One such option that has been developed relies on the principle of ensuring that the most sensitive stages of the life cycle are exposed to the toxicant rather than the reproductive process per se. This approach originated with the work reviewed by McKim (1977) who showed that the embryo-larval stages of fish were among the most sensitive stages. In 46 (82%) of the 56 tests the endpoints derived from this test were "essentially identical" to the chronic effect levels estimated from whole life cycle tests. In the remaining 10 (18%) tests the endpoints derived from the two data sets were within a factor of 2. Since this initial work the results of well over 150 early life stage tests have been reported and reviewed for their utility (Woltering, 1984), ring tests have been performed (Hansen and Cripe, 1984) and draft standards prepared (ASTM, 1986). Several variants of the ELS test have also emerged including the Birge et al.(1976) and Balk methods (1986) and the 7-day test of Mount (1983) which variously focus on hatchability, survival, embryonic development and growth. Other fish ELS test methods are also under development in Sweden and Canada. The principles of these different ELS test methods are given in Appendix 5.

There is no doubt that those working in the field have found the tests useful in that they are sensitive, reliable and cost-effective alternatives to full life cycle tests. They have been widely used in the USA but less so in Europe where more practical experience should be acquired. They appear to predict correctly the LOEC and NOEC for whole life cycle studies in the majority of cases. According to Macek and Sleight (1977) this is likely to hold true except where some mechanism such as bioaccumulation is operating. These cases are likely to be identified by earlier tests which will reveal this potential. Considerable progress has been made towards standardisation of the ELS test (ASTM, 1986). Use in all its forms appears to find wide support from the controlling authorities in relation to meeting the requirements of the Toxic Substances Control Act (TSCA) and for the development of water and effluent quality criteria.

ECETOC is of the opinion that the ELS test is an important and useful test and that every effort should be made to develop a test guideline approved at international level. We are convinced that the ELS test and its variants are appropriate options to fulfill the Level 2 requirements.

E. CONCLUSIONS

1. No internationally agreed fish toxicity test methods are currently available for use at Levels beyond the Base Set of the 6th Amendment.

2. Three approaches are proposed for Level 1 fish toxicity tests. They are based on the observation of i) lethal effects, ii) symptoms of toxicity and iii) growth.

3. The OECD TG 204 gives sufficient information for the determination of longer term lethal effects but gives inadequate guidance for the determination of sublethal effects.

4. Two test methods have been produced for determining the sublethal effects based on the observation of growth and symptoms of toxicity. In general these fall within the scope of OECD TG 204 and the necessary minor modifications are proposed (cf. Appendix 2). These test methods have been shown to be workable in a number of laboratories.

5. The requirement of the EEC 6th Amendment for a Level 2 "prolonged toxicity study with fish (including reproduction)" is not sufficiently specific to determine the nature of the data sought.

6. The fish early life stage tests are not comprehensive reproduction tests, but they are the only tests sufficiently well validated at the present time to warrant consideration for inclusion at Level 2.

7. There is evidence from the literature that in the majority of cases NOECs derived from fish early life stage tests are similar to those obtained from whole life-cycle tests.

F. RECOMMENDATIONS

1. The OECD - TG 204 should be updated and should take account of the proposed minor modifications to the original text and the test methods described in this report.

2. ECETOC believes that the test methods described for the determination of NOECs based on the observation of lethality, growth and symptoms of toxicity are satisfactory for use at Level 1 of the EEC 6th Amendment. The methods associated with the determination of NOECs based on observation of both growth and symptoms of toxicity may require international validation.

3. Further comparison of the results from the three test methods should be made and sufficient data produced in order to determine the most satisfactory endpoint.

4. In the absence of a clear definition of the term "reproduction" in the requirements for a fish toxicity test at Level 2 of the 6th Amendment, the fish early life stage tests are considered satisfactory for use at Level 2 of the EEC 6th Amendment.

5. Further evidence should be obtained that the various fish early life stage tests provide end-points which fully encompass the chronic effects of a substance.

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APPENDICES

APPENDIX 1: DEFINITIONS

Acute Toxicity : the harmful properties of a substance which are demonstrated within a short period (hours to days) of exposure. They are typically associated with breakdown of tissues and physiological systems at rates which exceed rates of repair or adaptation.

Acute Toxicity Test : an experiment which provides information on acute toxicity over a range of concentration levels. This may include information on the lethal concentration, the organs, tissues and functions affected and the time to onset, duration and severity of effects.

Chronic Toxicity : the harmful properties of a substance which are demonstrated only after long term exposure in relation to the life of the test organism.

Chronic Toxicity Test : a toxicity test of long duration in relation to the life of the test organism which may include more than one generation.

Flow-through Test: a test in which water is renewed continuously in the test chambers, the test substance being transported with the water used to renew the test medium.

LC_{50} Value (Median Lethal Concentration) : a statistically-derived concentration which over a defined period of exposure, is expected to cause death in 50% of the animals.

LC_{50} Test : an experiment which aims at determining an LC_{50} value and in which only the mortality incidence is recorded.

LOEC (Threshold Level of Observed Effects) : the lowest test concentration at which the substance is observed to have a "statistically significant" effect on the test species.

LOLetC (Threshold Level of Lethal Effect) : the lowest concentration of the test solution at which the substance has a lethal effect.

NOEC (No Observed Effect Concentration) : the highest test concentration at which the test substance has no "statistically significant" effect on the test species.

"r" : the instantaneous growth rate is defined by the equation $W_t = W_o e^{(r)t}$ where W_o and W_t are the fish weights in grammes at time o and t.

Subchronic Toxicity Test : a toxicity test designed to investigate possible adverse effects occurring as a result of continuous or repeated exposure of several groups of experimental animals to a series of concentrations of the test chemical for part (not exceeding 10%) of their lifespan.

Toxicity : the inherent property of a substance to cause adverse biological effects at specific concentrations.

Threshold Concentration : see LOEC.

Semi-static Test is a test without flow, but with periodical batchwise renewal of the test solution.

"Statistically Significant" Effect : is an effect considered to be significant according to mathematical statistical and/or descriptive methods.

APPENDIX 2 : A MODIFIED OECD - TG 204 "FISH, SUBCHRONIC TOXICITY TEST

1. INTRODUCTORY INFORMATION

° Prerequisites

- Water solubility
- Vapour pressure

° Guidance information

- Structural formula
- Purity of the test substance
- Methods of analysis for the quantification of the substance in water
- Chemical stability in water and light
- n-Octanol/water partition coefficient
- Results of a ready biodegradability test (see Test Guidelines 301A-E)

° Qualifying statements

- Constant conditions should be maintained as far as possible throughout the test. A flow-through procedure should normally be used; if adequate, a semi-static procedure may be adopted.

- For chemicals with limited solubility under the test conditions it may not be possible to determine values called for in this Test Guideline.

- This Test Guideline is only suitable for freshwater fish species.

° Standard documents

There are no relevant international standards.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

This Test Guideline presents guidance for measurement of lethal and other observed effects in fish exposed to test substances. It should be used in place of Test Guideline 203 (Fish, Acute Toxicity Test) if a longer observation period is considered useful and appropriate and the reporting of additional information deemed necessary. Annexes to the guideline give additional details of three test methods suitable for the determination of threshold effects on lethality, visible symptoms of toxicity and growth.

° Definitions

Semi-static test is a test without flow, but with periodical batchwise renewal of the test solution.

Flow-through test in this Test Guideline is a test in which water is renewed continuously in the test chambers, the test substance being transported with the water used to renew the test medium.

LOEC (Threshold Level of Observed Effects) is the lowest test concentration at which the substance is observed to have a statistically significant effect on the test species.

Threshold level of lethal effect is the lowest concentration of the test substance in the test solution at which the substance has a lethal effect.

NOEC (No Observed Effect Concentration) is the highest tested concentration at which the test substance has no statistically significant effect on the test species.

"r" : the instantaneous growth rate is defined by the equation $W_t = W_o e^{(r)t}$ where W_o and W_t are the fish weights in grammes at time o and t.

° Reference substances

No reference substances are recommended for this test. However, if a reference substance has been tested, the results should be given.

° Principle of the test method

Threshold levels of lethal or other observed effects like visible symptoms of toxicity, measurement of weight and length and NOEC are determined at intervals during the test period, which is at least fourteen days. If necessary, the test period can be extended beyond 14 days.

° Conditions for the validity of the test

- The mortality in the controls should not exceed 10% at the end of the test.

- The dissolved oxygen concentration should be at least 60% of the air saturation value throughout the test.

- In semi-static procedures, aeration can be used, provided it does not lead to a significant loss of test substance.

- There should be evidence that the concentration of the substance being tested has been satisfactorily maintained (it should be at least 80% of the nominal concentration) over the test period. The results should be based on measured concentrations if the deviation from the nominal concentration is greater than 20%.

B. DESCRIPTION OF THE TEST PROCEDURES

° Preparations

Equipment

Normal laboratory equipment and especially the following is necessary :

- Equipment for determination of temperature, pH, oxygen concentration and hardness of water.

- Adequate apparatus for temperature control.

- Test tanks made of chemically inert material and of a suitable capacity.

- Equipment for cold-branding of fish.

Solutions of the test substance

Stock solutions of the appropriate concentrations are prepared by dissolving the appropriate amount of the test substance in the required volume of dilution water. Stock solutions of test substances of low water solubility may be prepared by mechanical dispersion or, if necessary, by use of vehicles, such as organic solvents, emulsifiers or dispersants of low toxicity to fish. The concentration of organic solvents, emulsifiers or dispersants should preferably not exceed 100 mg/l in the test solution.

Test solutions of chosen concentrations are prepared by dilution of the stock solution.

The test should be carried out without adjustment of pH. If there is evidence of marked change in the pH of the tank water after addition of the test substance, it is advised that the test be repeated, adjusting the pH of the stock solution to that of the tank water before addition of the test substance.

This 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 physical precipitation of the test substance is caused. HCl or NaOH are preferred.

° Experimental animals

Selection of species

One or more species may be used, the choice being at the discretion of the testing laboratory. However, it is recommended that the species used for this test be selected from those recommended for the Acute Toxicity Test (OECD Test Guideline 203, Table 1, p.5). The species used should be selected on the basis of such important practical criteria as, for example, their suitability for observation of the chosen effect(s), their ready availability, their ease of maintenance, their convenience for testing and any relevant economic, biological or ecological factors. The fish should be in good health and free from any apparent malformation.

The fish mentioned in Table 1 of OECD Test Guideline 203 are easy to rear and/or widely available throughout most of the year. They can be bred and cultivated either in fish farms or in the laboratory, under disease- and parasite-controlled conditions, so that the test animal will be healthy and of known parentage. These fish are available in many parts of the world.

If other species fulfilling the above criteria are used the test method should be adapted in such a way as to provide suitable test conditions.

Holding

Acclimati- : At least 12-15 days. All fish must be
sation exposed to water of the quality to be

used in the test for at least seven days before they are used. Any disturbances that may change the behaviour of the fish should be avoided.

Prophylactic treatment : Prophylactic treatments should be avoided but reported when used.

Mortality : Following a 48h settling-in period, mortalities are recorded and the following criteria applied :

- >10% of population in seven days : rejection of entire batch.
- between 5 and 10% of population : acclimatisation continued for seven days.
- <5% of population : acceptance of batch.

° Performance of the test

If a vehicle is used in the preparation of the stock solution of the test substance, it is necessary to run, in addition to the control group, a control group of fish exposed to the highest concentration of the vehicle used in the test.

In the flow-through test, the concentration of the substance in the test solution may be determined at the beginning of the test and at adequate intervals to confirm the proper functioning of the dosing equipment; in the semi-static test at the beginning, immediately prior to the first renewal of the test solution and at the termination of the test. Appropriate procedures other than analysis for giving evidence that adequate concentrations of the test substance have been maintained can also be used.

Conditions of exposure

Duration :	Normally 14 days, but can be extended if threshold levels for observed effects have not been reached.
Tanks :	Of suitable capacity in relation to the recommended loading and the purpose of the test.
Loading :	For semi-static tests maximum loading of 1.0 g fish/litre is recommended; for flow-through systems higher loading can be acceptable.
Number of animals :	At least 10 for each concentration and control.
Test concentration :	The concentration interval should be determined in relation to the concentration/response curve in the acute study. The test concentrations chosen must permit the determination of the threshold level and NOEC for the chosen effect. Normally a minimum of three concentrations are required between a concentration lower than the NOEC, depending on the mode of action of the compound, and the threshold level of lethal effects attained in the preceeding acute study. Concentration of the substance in excess of 100 mg/l need not be tested if a threshold level has not been reached up to this concentration.
Water :	Drinking water supply (dechlorinated

if necessary), good quality natural water or reconstituted water (see Annex to Test Guideline 203). Waters with a total hardness of between 50 and 250 mg of $CaCO_3$ per litre, and with a pH 6.0 to 8.5 are preferable. The reagents used for the preparation of the dilution water should be analytical grade and the deionised or distilled water should be of conductivity equal to or less than 10 μScm^{-1}.

Light : 12-16 hours photoperiod daily.

Temperature : Appropriate to the species (see Table 1, Test Guideline 203) constant within $\pm$ 2°C.

Oxygen concentration : Not less than 60% of the maximum air saturation value throughout the test.

Feeding : Preferably more than once daily, the quantity of food being kept constant and related to the initial fish weight, at least 1% body weight.

Cleaning : Inside surfaces of the test tank in the flow-through test must be cleaned if necessary and excrement removed, at least twice weekly; in the semi-static test the test tank is replaced by a clean one each time the water is changed.

Observations

- The fish are observed once a day on working days

and when effects are expected, every day. Observed effects are defined as follows :

Mortality : a fish is presumed to be dead if no respiratory movement and no reaction to a slight mechanical stimulus can be detected. The fish are inspected at least once a day for mortality. Dead fish are removed when observed and mortalities are recorded (Annex 1).

Visible symptoms of toxicity : these include all effects observed on the appearance, size and behaviour that make them clearly distinguishable from the control animals, e.g. different swimming behaviour, different reaction to external stimuli, changes in appearance of the fish, reduction or cessation of food intake (Annex 2).

Growth rate : these observations include determination of changes in weight and length and food conversion efficiency (Annex 3).

- Measurements of pH, dissolved oxygen and temperature must be carried out at least twice a week.
  As a minimum a representative sample of the test population should be weighed and measured before and after the test.

3. DATA AND REPORTING

° Interpretation of results

If it is observed that the stability or homogeneity of the test solutions cannot be maintained, care should be taken in the interpretation of the results and note made that these may not be reproducible.

° Test report

The test report should include the following information:

- Test substance : chemical identification data.
- Test organisms : scientific name, strain, size, supplier, any pretreatment, etc.
- Test conditions :
    - test procedure used (eg. semi-static or flow-through, aeration, fish loading, etc.);
    - water quality characteristics (treatment, including dechlorination, dissolved oxygen concentration, pH, hardness, temperature, any other information available);
    - dissolved oxygen concentration, pH values and temperature of the test solutions at each of the recommended observation times;
    - methods of preparation of stock and test solutions;
    - concentrations used;
    - information on the maintenance of the concentration of the test substance in the test solutions;
    - number of fish at each test concentration;

Values from the fish acute toxicity test.

Results :

- observed effects at each concentration for each observation time in tabular form;
- concentrations that produce effects can be presented graphically with respect to time;
- threshold level of observed effects;
- NOEC;
- mortality in the controls;
- incidents in the course of the test which might have influenced the results;
- any deviation from the Test Guideline.

A N N E X E S

ANNEX 1: Test Method for the Determination of Mortality in a Subchronic Fish Toxicity Test

The results of an acute toxicity test may indicate that prolonged exposure to the test substance could result in continued lethal effects. This is indicated when the LC_{50} values from an acute test are plotted onto a toxicity curve which does not become asymptotic to the time axis.

OECD TG 204 can be used as a basis for a test method to investigate the effect of prolonged exposure on mortality with particular attention to the following :

- selection of the test concentrations should be made with reference to the lethality curve from an acute toxicity test (OECD TG 203) on the test substance.

- observed effect is mortality and this should be presented in the results both graphically to demonstrate the asymptotic threshold concentration and also as the threshold of lethal effect (EIFAC, 1980).

ANNEX 2: Test Method for the Determination of Visible Symptoms of Toxicity in a Subchronic Fish Test

Fish should be observed once a day on working days and when effects are expected, every day. Visible symptoms of toxicity will depend on the fish species or the mode of action of the test substance.

Frequently observed symptoms of toxicity are :

- gasping : the fish swim immediately beneath the water surface and gasp air;

- hyper-ventilation : the frequency of the opercular movement is increased;
- convulsions: trembling for short intervals; bent back for short intervals;
- gaping : open mouth for long periods without gasping;
- irritability: increased escape reflex;
- restlessness: the fish swim agitatedly;
- narcosis : the fish lie on the bottom without moving when touched;
- tumbling: the fish swim on their sides or backs turning frequently;
- side position: the fish lie on the bottom on their sides or back but respond to touch;
- "frog eyes": the eyes are protuberant;
- distended body: the abdomen is abnormally swollen with liquid;
- shoaling : some or all fish are unable to shoal;
- reduced food intake : the fish eat less food;
- discolouration: the fish change colour.

The number of fish with each symptom should be recorded and the intensity of each symptom scored for each fish e.g. 1 to 3 (1= mild, 2 = moderate, 3 = severe).

For example :

gasping 8/2; 1/3 : means 8 fish with moderate gasping and 1 fish with severe gasping.

Concentration/effect relationships can be established for each visible symptom of toxicity that occurs.
The test report should contain :

- the observed symptoms of toxicity at each concentration for each observation time;
- threshold level of observed effects at each observation time;
- NOEC's at each observation time;
- the occurrence of increased sensitivity or adaptation.

ANNEX 3 : Test Method for the Determination of Effects on Fish Growth in a Subchronic Fish Toxicity Test.

The growth of fish from the fry stage until the onset of maturity is exponential, provided that environmental factors and the food supply are non-limiting. Thus fish growth can be defined in terms of a rate which remains constant for a substantial part of the life cycle. The rainbow trout, Salmo gairdneri (Richardson) is particularly suitable for this test but alternative species may be used with appropriate modification to the method (Crossland, 1985).

To obtain maximum sensitivity of the growth response as a toxicity endpoint it is necessary to have a simple and rapid marking technique which allows for the identification of individual fish throughout the experiment. Freeze branding has proved to be a suitable technique. A stainless steel wire number cooled in liquid nitrogen is applied to fish lightly anaesthetised with e.g. 0.05 to 0.1 g/l tricaine methane sulphonate (MS222).

Trout of approximately 4 g are acclimated to laboratory conditions for at least two weeks prior to testing. During this period they are fed a maintenance ration of 1% of their body weight per day.

During the test the fish are held in suitable vessels e.g. 40 l glass aquaria with the sides covered with black polythene to minimise disturbance, under continuous flow conditions (2-3 l/g of fish/day at 15°). The fish are starved for 24 h before starting the test and, after anaesthetising and branding, the fish are weighed (± 0.1g) and measured (± 1 mm). It is important to minimise the weight range of fish used. After recovery from the anaesthetic the fish are allocated at random, 16 fish to each aquarium, and fed 4% of wet body weight per day given in two feeds. Fourteen days later the fish are again anaesthetised, weighed and measured and the test terminated or they may be returned for a further 14 days under the same conditions.

From the weights obtained the relative growth rate (RGR) or the instantaneous growth rate (r) may be calculated respectively as :

$$RGR = \frac{W_t - W_o}{t\ (W_o)}$$

$$\text{and} \quad W_t = W_o e^{rt}$$

where W_o and W_t are weights at o and t days.

It is also possible to calculate food conversion efficiency (%) as :

$$\frac{\text{Wet weight gain of fish (g)}}{\text{Weight of ration (g)}} \times 100$$

and a condition index as :

$$\frac{\text{Wet weight of fish (g)}}{\text{Length of fish (mm)}} \times 100$$

The data for weights, lengths, condition index and growth rates may be analysed by one-way analysis of variance and the treatment means compared with that of the controls using a multiple comparison test such as Dunnett's (1955) or Williams (1972) test.

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APPENDIX 3 : UMWELTBUNDESAMT CHEMICAL PROGRAMME (1985)

Comparison of Substance Concentrations Causing Lethal and Sublethal Effects

Test species : Brachydanio rerio

(concentration mg/l)

	LC 50 48 h	LOEC 14 d	LOLetC 14 d	LC_{50}/LOE1	LOLetC/LOEC
Lindane	± 0.09	0.00008**	0.05**	1130	625
		0.011	0.075	8.2	6.8
		0.03	0.053	3	1.8
PCP	0.195	0.025**	0.20**	7.8	7.8
	0.17	0.074	0.15	2.3	2.0
Atrazine	37	0.16**	20**	230	130
	37				
	37	2.1	>19	17	8.7
		0.6	20	62	33
TPBS (Tetrapropylene benzene sulphonate)	20.8	2.3**	20**	9.2	9
	20.8	6.7	20	3.1	2.9
	23	7**		3.3	
	±20				
4-Cloro-aniline	>22/<32	3.2*	3.2*	6.9/10	1
	27			8.4	
	46			14.4	
$CdCl_2.H_2O$	0.9				
		0.2	0.2	4.5	1
$K_2Cr_2O_7$	117	80**		1.5/2	0.75
	145	60	60	1.8/2.4	1
	106			1.3/1.8	
	110-180				
	166				
	105			2.1/2.8	
2-Nitrophenol	72.5	6.3**	20**	12	3.2
Styreneoxide	17.1	0.68	5.2	25/27	7.6
	17.4	0.63**		26/28	
	7.2			11/12	
	>15/<22				
1,2,4-Trichlorobenzene	11	0.18*	0.18*	61	1
	6.6			37	
Trichloroethylene (volatile)	±240	5.1	5.1	47	1
	148			29	
	120				
	>320/<460				
Thiourea	16000	>7000	>10000	2.3	1.4
2,4,6-Trichlorophenol	±1.1	1.8	3.2	0.6	1.8

* 21 d
** 28 d
Where several values are given they are obtained from different laboratories.

APPENDIX 4 : FISH GROWTH RATE - UK RING TEST *

(Preliminary results based on calculation of the instantaneous growth rate)

Period (days)	Lowest Observed Effective Concentration (µg/l nominal)					
	HRC	ICI	MAFF	SHELL	UNILEVER	WRC
Dichloroaniline						
0 - 14	>300 (N)	220	2.6 (D)	210	210	75
0 - 28	30 (N)	220	1.1 (D)	39	210	75
Methyl parathion						
0 - 14	(NT)	400	22	170	(NT)	230
0 - 28	(NT)	400	160	170	(NT)	230

* Organisation of the ring test was funded by the UK Dept. of the Environment.

(N) Nominal concentration - all other results are based on measured exposure concentrations.

(NT) Not tested.

(D) Disease in some of the fish is thought to have been a factor in this experiment - there was a high mortality shortly after the end of the experiment which was not dose dependent.

APPENDIX 5 : THE EARLY LIFE STAGE TESTS

1. The Early Life Stage Tests

The fish early life stage can be used to determine the effect of a substance on hatching, survival, development or growth of fish exposed during the period of their early development (US EPA, 1982; ASTM 1986). The tests may be done using a variety of fish species and generally involve exposure of newly fertilised eggs to a range of test substance concentrations or effluent dilutions and a control. The duration of exposure can vary from one week up to 120 d depending on the test species and the criteria chosen to be studied. Both freshwater and marine species have been used successfully.

In the test methods described in US EPA (1982) and ASTM (1986) the eggs are tested in a flow-through system. The test is run at least in duplicate and with 30 eggs per replicate. Fish are fed during the test with a food, feeding regime and start time appropriate to the species used. Dead embryos and larvae are removed and survivors observed for abnormal development or behaviour. Growth is determined by weighing fish at the end of the test when dry weight is preferred to wet weight.

In Adema et al.(1981) and Canton et al.(1984) fish are studied in a semi-static test system, where the test medium is renewed several times per week, the frequency depending on the chemical properties of the test substance. Fish species used are Jordanella floridae, Oryzias latipes, and Brachydanio rerio. Eggs are exposed within 36 hours after fertilisation. When growth is to be measured, a test period extending three weeks after hatching is sufficient. The fish are fed with an appropriate food. This test is also described for a marine species, Pleuronectes platessa (Adema, 1980).

There are shorter variants of the early life stage test in which no food is supplied to the larvae. The tests either continue until 90% of the larvae have died (Brachydanio rerio), (Dave, 1984, 1985) or until they have resorbed the major part of their yolk sacs (Salmo gairdneri) (Van Leeuwen, 1986). Very short variants of the early life stage test are applied by Birge et al.(1976), when Pimephales promelas was used to study the toxicity of effluents, and by Balk (1986) for Brachydanio rerio. In both test systems, embryos a few hours old are exposed during 7 or 8 days to a series of test concentrations or effluent dilutions in a semi-static test. Criteria for toxic effects are the

survival of embryos, the success and rate of hatching, embryo and larval development and survival of the larvae.

A very short exposure time is also used in the so-called subchronic toxicity test or the rapid chronic test as was originally described for Pimephales promelas by Norberg and Mount (1985). In this test, newly hatched larvae, less than 24 h old are tested in a semi-static system for 7 d. The larvae are fed during the test. Survival is followed throughout the test and growth is determined as increase in dry weight.

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