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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 LC₅₀ 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 of 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 especially 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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