Acute Toxicity Testing with Aquatic Organisms 1981

Methods for the Examination of Waters and Associated Materials

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Methods for the Examination of Waters and Associated Materials

This booklet contains a general introduction which should be read before any particular test is used, and also seven specific toxicity test procedures. Each of these procedures is complete in itself so as to avoid crossreferal except to Part A. This entails a small amount of repetition, but does ensure that vital points are drawn to the attention of the user.

Contents

Acknowledgement
Grateful acknowledgement is made to the Food and Agriculture Organization of the United Nations for permission to use Appendix 2 pp 19–25 of EIFAC Report 24 in section 6 of Part A of this booklet.

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Warning to Users

The analytical procedures given in this booklet should only be carried out by competent trained persons, with adequate supervision when necessary. Local Safety Regulations must be observed. Laboratory procedures should be carried out only in properly equipped laboratories. Field operations should be conducted with due regard to possible local hazards, and portable safety equipment should be carried. Care should be taken against creating hazards. Lone working, whether in the laboratory or field, should be discouraged. Reagents of adequate purity must be used, along with properly maintained apparatus and equipment of correct specification. Specifications for reagents, apparatus and equipment are given in manufacturers' catalogues and various published standards. If contamination is suspected, reagent purity should be checked before use.

There are numerous handbooks on first aid and laboratory safety. Among such publications are: 'Code of Practice for Chemical Laboratories' and 'Hazards in the Chemical Laboratory' issued by the Royal Society of Chemistry, London; 'Safety in Biological Laboratories' (Editors Hartree and Booth), Biochemical Society Special Publication No 5, The Biochemical Society, London, which includes biological hazards; and 'The Prevention of Laboratory Acquired Infection' Public Health Laboratory Service Monograph 6, HMSO, London.

Where the Committee have considered that a special unusual hazard exists, attention has been drawn to this in the text so that additional care might be taken beyond that which should be exercised at all times when carrying out analytical procedures. It cannot be too strongly emphasised that prompt first aid, decontamination, or administration of the correct antidote can save life; but that incorrect treatment can make matters worse. It is suggested that both supervisors and operators be familiar with emergency procedures before starting even a slightly hazardous operation, and that doctors consulted after any accident involving chemical contamination, ingestion, or inhalation, be made familiar with the chemical nature of the injury, as some chemical injuries require specialist treatment not normally encountered by most doctors. Similar warning should be given if a biological or radio chemical injury is suspected. Some very unusual parasites, viruses and other micro-organisms are occasionally encountered in samples and when sampling in the field. In the latter case, all equipment including footwear should be disinfected by appropriate methods if contamination is suspected.

The best safeguard is a thorough consideration of hazards and the consequent safety precautions and remedies well in advance. Without intending to give a complete checklist, points that experience has shown are often forgotten include: laboratory tidiness, stray radiation leaks (including ultra violet), use of correct protective clothing and goggles, removal of toxic fumes and wastes, containment in the event of breakage, access to taps, escape routes, and the accessibility of the correct and properly maintained first-aid, fire fighting, and rescue equipment. If in doubt, it is safer to assume that the hazard may exist and take reasonable precautions, rather than to assume that no hazard exists until proved otherwise.

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About this series

This booklet is one of a series intended to provide recommended methods for the determination of water quality. In the past, the Department of the Environment and its predecessors, in collaboration with various learned societies, has issued volumes of methods for the analysis of water and sewage culminating in 'Analysis of Raw, Potable and Waste Waters'. These volumes inevitably, took some years to prepare, so that they were often partially out of date before they appeared in print. The present series will be published as individual methods, thus allowing for the replacement or addition of methods as quickly as possible without need of waiting for the next edition. The rate of publication will also be related to the urgency of requirement for that particular method, tentative methods being issued when necessary. The aim is to provide as complete and up to date a collection of methods and reviews as is practicable, which will, as far as possible, take into account the analytical facilities available in different parts of the Kingdom, and the quality criteria of interest to those responsible for the various aspects of the water cycle. Because both needs and equipment vary widely, where necessary, a selection of methods may be recommended for a single determinand. It will be the responsibility of the users — the senior analytical chemist, biologist, bacteriologist etc to decide which of these methods to use for the determination in hand. Whilst attention of the user is drawn to any special known hazards which may occur with the use of any particular method, responsibility for proper supervision and the provision of safe working conditions must remain with the user.

The preparation of this series and its continuous revision is the responsibility of the Standing Committee of Analysts (to review Standard Methods for Quality Control of the Water Cycle). The Standing Committee of Analysts is one of the joint technical committees of the Department of the Environment and the National Water Council. It now has seven Working Groups, each responsible for one aspect of water cycle quality analysis. The are as follows:

1.0 General principles of sampling and accuracy of results
3.0 Empirical and physical methods
4.0 Metals and metalloids
5.0 General non-metallic substances
6.0 Organic impurities
7.0 Biological methods
9.0 Radiochemical methods

The actual methods etc are produced by small panels of experts in the appropriate field, under the overall supervision of the appropriate working group and the main committee. The names of those associated with this method are listed inside the back cover.

Publication of new or revised methods will be notified to the technical press, whilst a list of Methods in Print is given in the current HMSO Sectional Publication List No 5.

T A DICK
Chairman

L R PITTWELL
Secretary

30 November 1982

A Introduction to Acute Toxicity Testing with Aquatic Organisms

A1 Introduction

A1.1 Aim of this Publication

Toxicity tests with aquatic organisms have been used for many years as an aid to the management of natural waters and in the control of water pollution, and there are increasing pressures for their use in such things as the registration of new chemical products, specification of appropriate uses of agricultural chemicals, etc. The result has been a proliferation in the number and types of toxicity test available and this has led to a need for standardization of tests to meet specific objectives. In its widest sense a toxicity test is the use of living material to define the nature and degree of harmful effects produced by a single poison or by a mixture of poisons. Whilst it is possible to categorize tests on the basis of the type of living material involved or on the objective sought, it is more usual to recognise initially sub-divisions based on duration of the test (that is on the period of exposure or on the period of observation if this differs). Two main types are apparent — acute and chronic. The acute toxicity of a substance in the aquatic environment is normally expressed as the concentration of substance in the water which produces a harmful effect in 50% of a batch of the test organisms in a *short* exposure period. For longer lived organisms such as fish it is usually taken to be of about 5 days duration, or less but where appropriate it may be shorter, dependent on the lifespan of the organisms. Chronic toxicity is that resulting from much longer periods of exposure, usually at least of several weeks duration but generally a significant proportion of the lifespan of the organism. The terms "sub-acute" or "sub-chronic" are applied arbitrarily to tests falling somewhere between "acute" and "chronic" in their duration. A further categorisation of tests based on effect is common, distinguishing between lethal (in which death is the observed phenomenon) and sub-lethal (less than death), which includes effects on behaviour, growth, reproduction, biochemistry and physiology. Thus it is possible to have acute lethal tests, chronic lethal, acute sub-lethal, chronic sub-lethal, and so on.

Within each type of test there are many different methods which might be used depending on, for example, the species involved or the precision of results required, but particularly for acute tests a large part of the philosophy and fundamentals of the methodology are common to all tests. The purpose of this booklet is to set down these basic aspects as they apply to acute toxicity tests in fresh and saline waters with invertebrates and lower vertebrates. Tests involving algae, vasucular plants and micro-organisms are sufficiently different to warrant a separate treatment, as are the specialised aspects of bioassay and bioaccumulation. Similarly, the different philosophy and methodology applied to chronic testing will be described in a separate booklet.

A1.2 Topics Considered

The main topics considered in the following sections are:

(i) Establishing the conditions of the test
(ii) General procedures
(iii) Collection and analysis of results.

It is important that all experiments are carried out in properly equipped laboratories. Formal recommendations have been proposed by the Health and Safety Executive (HSE) 1982 and the Organisation for Economic Co-operation and Development (OECD) 1980 prescribing under "Good Laboratory Practice" (GLP) those conditions under which toxicity testing *must* be carried out if the results of the tests are to be considered valid and to have international acceptability. Under the Cruelty to Animals Act, 1876, specific experiments with vertebrates can be carried out only by personnel who have obtained the appropriate Home Office licence and certificate for the experiments proposed, and only in premises licensed for such experiments. This latter provision ensures that laboratories have a minimum standard of seclusion, hygiene etc, and these same requirements should be met in all laboratories where toxicity experiments are being carried out, even when invertebrates are the test organisms used. The experimental space should be free from undue disturbance and risks of contamination; appropriate washing facilities should be close at hand; stock and test animals should be well separated; and drainage facilities should take account of the disposal of contaminated waters. (See footnote).

Note: **At the time of going to press, there is considerable discussion in many places about legislation to replace or amend the Cruelty to Animals Act 1876. Users of this booklet are warned to ascertain the current state of the legislation applicable to the tests given herein before carrying out any tests, and subsequently to keep themselves informed of any changes in the legislation. To assist in this, a blank page for notes is included at the end of Part A.**

General guidelines on establishing the test system (Section A2) are concerned with the care and maintenance of test species within the laboratory area and with the specification of test conditions, inlcuding water quality. A comprehensive review of aquarium systems is given by Hawkins 1981. Detailed procedures pertinent to the different test protocols are given in the appropriate booklets but a number of procedural aspects are common to all tests and these are highlighted in Section A3. Methods for the collection and statistical treatment of results from toxicity tests are the subject of a voluminous literature but, in practice, data are analysed using only a few standard techniques and these are presented in Section A4.

A2 The Test System

A2.1 Test Species

Test organisms can be obtained by collection from populations in the wild, from commercially reared stocks, or reared in the laboratory, and appropriate methods for their capture and collection should be used in each case. For example, where animals are collected in the wild they should be obtained from a population free from overt signs of disease and from water free from pollution. The animals should then be held in isolation in the laboratory prior to testing for a period of sufficient duration to determine their satisfactory state of health. For hatchery reared fish a minimum "holding" period of one week is necessary. Newly obtained animals should not be mixed with previously quarantined stocks until proved to be healthy and during this holding time they should be observed for unusual behaviour and outbreaks of disease. Simple remedial action such as the use of formalin or antibiotics can be taken to cure disease, but if mortality is still occurring at the end of the quarantine period the animals should be rejected. The medicaments chosen to cure disease should not be used during the test period. If remedial or preventative treatment is used, the organisms should be kept for a further one week period before use. Detailed recommendations for the collection and treatment of individual species are given in the appropriate test procedure.

As soon as possible during the quarantine period the organisms should be fed, using proprietary food preparations or natural food organisms provided that these are free from poisons or disease. Feeding should be stopped prior to starting acute toxicity tests and food withheld throughout the test period. The holding tanks should be kept clean and free from faeces and uneaten food; circular tanks, through which a continuous-flow of water is maintained and discharged through a central weir tube and which are therefore largely self cleaning, make this job easier.

Before exposure to a test substance, the test-organisms should be placed in the test apparatus in dilution water of the quality to be used in the test for the period, stipulated in each protocol, to achieve some degree of acclimation. They should not be handled more than necessary and transfer should be made by use of techniques which avoid damage to the test organisms. The cut end of pipettes, for example, should be smoothed by heating and knotless nets are preferred when handling fish.

Whilst it is highly desirable to carry out tests with truly marine species of fish in sea water (SW), it is not always possible because of lack of availability and in these circumstances rainbow trout may be used. The acclimation of rainbow trout should take place in stages, eg 1 day in 50% SW, 2 days in 80% SW and finally 100% SW for at least 1 week. Smaller organisms may be capable of acclimation over longer periods with more gradual increases in salinity. The acclimated animals should be maintained under the test conditions for one week prior to testing. If mortalities occur at the end of this period the stock should be rejected.

The sizes of animals selected for use in a particular test will depend on the objectives and type of test involved but the largest in any test should not be greater than 1.5 times the length of the smallest animal. Preferably, they should be from the same age — or year — class.

Except where otherwise allowed, all test animals should be of the same species. Positive identification of all batches of test animals should be carried out and the full scientific nomenclature, including the taxonomic authority should be given in all references to the test.

A2.2 Test Apparatus

Both the physical and chemical nature of the test material and the size of test organism should be taken into consideration when designing the shape and size of the test container. All components of the test apparatus should be made of non-toxic,

non-absorptive material. Glass is recommended for this purpose because it is easy to clean and permits observation of general behaviour and of the selected effect, without causing undue disturbance to the organisms. However, a simple screen can be used to prevent animals being disturbed by the general movements made by staff in the laboratory.

The test apparatus should be cleaned thoroughly before use. To assist cleaning, solvents such as those given in Section 2.4 should be used and detergents should be avoided. Particular attention should be paid to removing traces of the previous test substance or solvent before animals are introduced. Wiping or drying with a cloth or paper towel between washings is advocated to prevent the contaminants drying onto the test vessel surface.

Test organisms can be exposed to the test chemical in "static" or "continuous flow" conditions.

The test containers must not be of such a shape and size as to restrict the ability of the organisms to move freely and should hold a volume of test solution such that the poison and dissolved oxygen concentration are not markedly reduced by the animals during the test. Generally, a suitable minimum volume for "static" tests is determined from the ratio of 0.5 litres of water per gramme of animal, renewed daily. Aeration may be provided by a gentle flow of clean air introduced throught a diffuser block, or, if foaming occurs, through a capillary tube. Care should be taken to ensure that the aeration system does not introduce unwanted materials such as oil, sulphides and high concentrations of carbon dioxide, and is not so vigorous as to disturb or damage the test animals.

For chemicals that are likely to be extensively absorbed onto the surfaces of the test apparatus thereby leading to very much lower test concentrations being present it may be prudent to 'saturate' the surface of the apparatus before testing begins. In static tests, test solutions are added some time before, and renewed immediately prior to, the introduction of the animals. In continuous flow tests the flow of test substance is started sometime before the test proper begins. In either case the test animals cannot be acclimated to the test apparatus as recommended above.

For continuous flow tests, the recommended flow rate is specified in each protocol. For fish, Sprague (1969) recommends a 90% replacement each 8–12 hours, so that the replacement flow volume within a 3–5 hour period should equal the volume of the test container. The dosing apparatus should be calibrated prior to testing by means of a dye and the flow rate should not vary by more than 10%.

There are many methods for metering, mixing and delivering test solutions and some of these have been reviewed by Marchetti 1962, Sprague 1969 and APHA 1975. The best and most reliable methods are those which work on simple principles and are "fail-safe" in their design.

Depending on the method, the following points must be considered. For continuous flow tests, pumping (at fixed rates) of both diluting water and test substance into the test vessels can be used to maintain a constant concentration throughout the test period. The same result can be achieved by a rapid intermittent introduction of diluting water and test substance and a number of gravity-fed systems have been designed (Abram, 1960; Brungs and Mount, 1967; Stark, 1967; Shurben, 1978). The majority of these have been designed for fish but in most cases scaling down is possible to achieve apparatus statisfactory for smaller organisms. It should not be assumed that the use of a continuous flow, or similar, apparatus by itself guarantees either that the planned concentrations are achieved or that the concentrations in the test aquaria are constant. Chemical analyses of the test solutions should be carried out whenever possible to determine the test concentrations.

The water temperature should be controlled throughout the test. The selected water temperature and range will depend on the objective of the test and on the species. Recommended values are given in each protocol.

A2.3 **Dilution Water**

(a) *Fresh Water*
Where possible the water should be taken directly from the potable water supply, provided that its quality is reasonably constant and suitable for the specific test. If chlorine, chloramines, pyrethroids or pyrethrum extract are

known or suspected to be present in the supply, they should be removed by filtering the water through activated charcoal or by any other suitable method. Chlorine should be removed so that its concentration is no greater than 4 $\mu g.l^{-1}$ as HOCl (EIFAC, 1973. Alabaster and Lloyd, 1980). In some cases, natural river water may be preferred. Problems which can occur with public or natural water supplies have been summarized by Hawkins 1981. A synthetic dilution water can be prepared by adding salts to deionised or otherwise appropriately treated water.

A suitable dilution water (ISO standard) can be made up as follows using analytical grade reagents:

(1) Calcium chloride solution ($CaCl_2.2H_2O$). Dissolve 11.76 g $CaCl_2.2H_2O$ in deionized water; make up to 1 litre with deionized water.

(2) Magnesium sulphate solution ($MgSO_4.7H_2O$). Dissolve 4.93 g $MgSO_4.7H_2O$ in deionized water; make up to 1 litre with deionized water.

(3) Sodium bicarbonate solution ($NaHCO_3$). Dissolve 2.59 g $NaHCO_3$ in deionized water; make up to 1 litre with deionized water.

(4) Potassium chloride solution (KCl). Dissolve 0.23 g KCl in deionized water; make up to 1 litre with deionized water.

The conductivity of the distilled or deionized water should not exceed 10 $\mu S.cm^{-1}$.
25 ml each of solutions (1) to (4) are mixed and the total volume made up to 1 litre with deionized water. The proportion of Ca–:Mg–ions is 4 : 1 and of Na–:K–ions, 10 : 1.

Aerate the dilution water until air saturation is achieved, then store it for about two days without further aeration before use. The aerated water should have a pH of 7.8 ± 0.2 and a hardness of 250 mg l^{-1} expressed as $CaCO_3$.

An alternative recipe is given by ASTM 1962. Use deionized water throughout.

Stock solution 1 — Dissolve 71 g $MgSO_4.7H_2O$, 6.5 g K_2SO_4 and 0.2 g $MnSO_4.4H_2O$ in water and make up to 1 litre.

Stock solution 2 — Dissolve 18.6 g $CaCl_2.2H_2O$ in water, and make up to 1 litre.

Stock solution 3 — Dissolve 25.0 g $NaHCO_3$, 3.0 g NH_4NO_3 and 1.1 g $K_2HPO_4.3H_2O$ in water and make up to 1 litre.

Stock solution 4 — Dissolve 32.2 g CaO in water and make up to 1 litre. Bubble CO_2 gas through mixture to make a $CaCO_3$ slurry.

Stock solution 5 — 62.6 g $Na_2SiO_3.9H_2O$ in water and make up to 1 litre.

Stock solution 6 — Dissolve 1.2 g $FeCl_3.6H_2O$ in water and make up to 1 litre.

For each 100 litres of dilution water, add 100 ml of each of solutions 1, 2 and 3. Bubble CO_2 gas into the solutions through a diffuser for 15 minutes. The pH should be 4.3. Add 100 ml of solution 4 and bubble CO_2 gas through until the solution becomes clear. The pH should be 5.1. Bubble compressed air through the solution for 25 minutes to raise the pH to 7.9. Add 100 ml each of solutions 5 and 6 and aerate for a further 60 minutes. The dilution water should have a pH of 7.9 ± 0.1. Waters of different hardness can be made up by pro-rata addition of stock solutions.

(b) *Sea Water*

Appropriate dilution waters are specified in each protocol. Sea water or a synthetic substitute is suitable for use if it is capable of supporting the test species in a healthy condition.

Sea water should be collected at points remote from sites with obvious contamination such as industrial or domestic discharges, land run-off, port facilities, etc. An acceptable sea water has a salinity within the range 30–35°/oo, although within any one test the salinity should not vary beyond ± 1°/oo. The pH of sea water should lie between 7.8 and 8.2. Suspended solids in the test water should not exceed 10 mg/l. Generally reduction to this concentration can

be achieved by allowing the solids to settle from the sea water over 48 hours and decanting off the clear water from the settled solids. Settlement in the dark has the added advantage that many species of plankton are also removed by sedimentation. Prolonged storage without removal of settled solids is to be avoided because of the potential for decomposition of the sedimented material, leading to low dissolved oxygen levels and high levels of sulphide and bacteria in the water. Aeration in the dark, of the decanted clean water, overcomes these problems. Prolonged contact of the sea water with metal surfaces during transport or storage should be avoided.

Synthetic sea water can be used for short-term tests where sea water itself is not available. There are a number of available salt mixes that can be made up with distilled or deionized water, for example "Instant Ocean", "HW Marine MIX", and there are numerous recipes for producing a suitable solution by adding reagent grade chemicals to distilled or deionized water. That reproduced below is taken from APHA 1976:-

Reagent grade chemicals are added to 890 ml glass distilled, deionized water.

Na Cl	23.50 g
Na_2SO_4	4.00
KCl	0.68
H_3BO_3	0.026
$MgCl_2.6H_2O$	10.78
$CaCl_2.2H_2O$	1.47
$NaHCO_3$	0.196
Na_4EDTA	0.0003

Each chemical must be completely dissolved before another is added. When all chemicals are dissolved, add distilled deionized water to make 1 1. The salinity should be 34°/oo + 0.5°/oo and pH 8.0 ± 0.2.

Synthetic sea water contains abnormal levels of trace metals but any potential harmful effects can be avoided by adding EDTA in only sufficient quantity to complex the metals and thereby render them unavailable to the test organisms.

A2.4 **Preparation of Experimental Solutions**

A2.4.1 *Selection of Test Concentrations*

The range of concentrations to be tested in the definitive test depends on the toxicity of the test substance. A preliminary sighting test can be used to obtain an approximate indication of the concentration range within which variable proportions of the test animals will respond.

This preliminary sighting test is carried out with the same species and stock to be used for the definitive test and in a similar dilution water. However, it is sufficient to test only three individuals in each concentration for periods of up to 24 hours is static conditions. A wide range of test solutions with successive concentrations at 10 fold intervals, and a control should be prepared. If the data obtained from this test are inadequate for establishing the range of concentrations required for the definitive test, then the preliminary procedure is repeated with either an intermediate or an extended range of concentrations.

The upper limit of the range for the definitive test is determined by the minimum concentration giving 100% response; the lower limit by the maximum concentration giving zero mortality. The particular responses occurring at intermediate concentrations will modify the concentrations selected for the definitive test.

For example:

Concentration mgl^{-1}	% response
10^4	100
10^3	100
10^2	66
10^1	0
100	0

A suitable range for the definitive test would be a geometrical series from 10 mg l^{-1} to 160 mg l^{-1}, with intermediate test concentrations of 20, 40 and 80 mg l^{-1}.

A2.4.2 *Preparation of Test Solutions*

The conditions pertaining to laboratory equipment and safety as laid down in GLP and by HSE (see A1.2) are a pre-requisite for the satisfactory preparation of test solutions but some points are worthy of emphasis.

Preparation of any stock solutions should be done well away from the experimental apparatus or holding tanks in order to reduce the risks of contamination. An additional safeguard is to always place all measuring and dispensing equipment on a clean tray which can be moved away from the experimental area.

The concentration of test solution is expressed either in terms of the amount of test material added to unit volume of water or in terms of the amount of material measured by analysis of the test solutions. In either case it is possible to describe the concentration in volumetric terms (eg ml of test material per litre of water; a number of dilutions of effluent in water) or gravimetrically (eg mg of test material per litre of water) but whenever possible the latter is to be preferred. It is advisable to avoid the use of terms such as parts per million (ppm) or parts per billion (ppb) since they have tended to be used ambiguously for both volumetric and gravimetric measurements of concentration.

For many materials particularly solids it is appropriate to prepare firstly a stock solution, using dilution water of the same quality as that to be used in the test, from which to make additions to the test aquaria. The test concentrations can be achieved in two ways. Firstly it is possible to add the required volume of stock solution direct to each aquarium, but significant volumetric errors can arise when dispensing small volumes of relatively concentrated solution. Alternatively the test concentration can be prepared by an appropriate serial dilution of the stock solution, though any errors in the accuracy of measurement are compounded at each dilution step and can be significantly large. A suitable compromise is to prepare a number of stock solutions such that only a relatively small number of serial dilutions (not more than three) are necessary to enable the final volumes to be dispensed with precision. Account should be taken of any material remaining in the measuring apparatus after adding the test substance to the test aquaria when computing nominal concentrations.

Care should be taken to ensure that test substances and stock solutions are homogeneously mixed, for example by vigorous shaking of the sample container, before any aliquot is withdrawn. The test substance should be thoroughly and quickly mixed within the test aquarium to minimize the risk of contact of the test animals with undiluted test substance.

Significant changes in aspects of the quality of the dilution water, eg salinity, hardness can occur if large volumes of effluent or stock solution are added to achieve the required test concentration (dilution). Equivalent changes in water quality should be established in a second control.

In continuous-flow tests, the range of concentrations to be tested determines the concentration of the stock solution. If the test substance is of very low solubility in water, then low concentrations of various low toxicity solvents, such as acetone, dimethyl formamide, ethanol and methanol, or the surfactant Triton X–100, may be used in preparing the stock solutions.

Where settling of emulsions or suspended solids occurs in stock or test solutions, constant mechanical stirring may be necessary. The standard methods will generally not be suitable for these substances and it will be necessary to design apparatus specific to the properties of the test compound.

A test container in which animals are exposed to dilution water only and, where applicable, the dilution water plus solvent, must be included as a control in all test procedures. The concentration of solvent should be the same as that of the highest concentration in the test flasks.

Stock solution used in the preparation of test solutions should be prepared daily, unless it is known that the material is stable, in which case sufficient stock solution may be prepared for the whole test period.

Where the toxicity of a commercial or formulated product is of concern it is important that the product itself and not just the active ingredient is tested as toxicity may be modified by the other chemicals in the mixture.

A3 Procedure

Details of the procedures pertinent to the different methods are given in the appropriate protocols but the following points are common to all procedures.

Test organisms should be selected at random from the stock tanks and distributed randomly within the test containers until the required numbers are attained in each container. Failure to randomise the animals may decrease the precision of the test since selection of active/inactive individuals can easily occur and lead to non-uniform distribution in test containers. Similarly, the concentration allocated to each test container should be randomly selected from the chosen series.

If the control organisms behave abnormally or more than 10% die during the test period the test is invalidated.

Where insufficient data are generated from a single test further tests may be carried out to include higher or lower concentrations as necessary. In these cases the new range of concentrations should include at least one dilution within the original range.

Precise definition of the effect under study is imperative. For example in acute lethal toxicity tests, care must be taken to define the criterion used to measure death. For example, "no opercular movement observed over a fixed period of time" or "no body movement" may be used. In the protocols, cessation of opercular movement for a period of 10 seconds has been selected as the criterion of death in fish. Other criteria are clearly stated in the protocols.

Frequent examination of the experimental containers during the period of the test is advocated in most protocols but this has to be considered in the light of any disturbance to the test system, particularly the animals, during the examination. Undue disturbance may hasten the onset of the effect and lead to errors in estimating the median response. This is particularly critical if the examination requires anything other than visual observation of the animals within their test containers, for example "gentle prodding" or "examination when drawn into a pipette".

In most cases it is sensible to set up all test containers at the same time and there are advantages in starting experiments early in the morning, since this provides the opportunity for observation during the first few, often critical, hours. If, however, a large proportion of the animals in one or more of the concentrations is affected overnight the experiment when repeated should be phased to begin late in the day so that the effects are observed during the following day.

In accordance with Good Laboratory Practice detailed records should be maintained throughout and should include information on the source and history of the test stock, on the water quality, on the behaviour of the test material, and on any parameters monitored throughout the test. Thus water temperature, pH and dissolved oxygen concentration should be measured at intervals throughout the test period and whenever possible chemical analyses should be carried out on test solutions to confirm that the concentration has not fallen by more than 10⅔; this is especially important for static tests. Where such confirmation is not available, the results should be expressed in terms of the initial concentration which may decrease considerably as the test progresses.

A3.1 Tests with Substances in Suspension or Sediment

Problems will arise if the substance under test is not in solution but present as a floc or sediment, or is absorbed to flocs or sediments. These tests are then not fully valid, and consideration needs to be given to the effects of smothering of the gill membranes and of ingestion by bottom feeders. In such cases a postmortem examination may be useful. Analysis of the floc or sediment should also be made to determine whether such preferential concentration is significant.

A4 Results

A4.1 General Considerations

There are numerous methods of recording and calculating the results of toxicity tests and deriving statistical data. Some of the more common methods are described below to show their interrelationships, but the recommended method is given in each test protocol.

Toxoicity is usually expressed in terms of the concentration which will produce a specified effect in a specified proportion of the population after a specified time.

The measured statistic for a population response is usually the median. For example, the concentration causing an effect on 50% of the test population after 48 hours exposure is written as 48 hr median effective concentration or 48 hr EC50. Since the time of exposure as well as the concentration is important in the evaluation of the effect of a poison, the time taken for 50% of the test population to respond can be used. The results from the latter measurement can be used to estimate the median effective time, ET50, for the test fish in a given concentration.

In tests where the measured response is death, the EC50 is equivalent to the median lethal concentration LC50; these concentrations are commonly calculated for exposure periods of 24, 48 and 96 hours. Similarly, in tests measuring the time taken for fish to die, the response is termed the median lethal time or LT50, it also has been termed the median period of survival (MPS; also known as median survival time, MST).

In lethal toxicity tests, the relationship between the LC50s at given time intervals (for example, 24, 48 and 96 hours) is important. If the concentration-response relationship (plotted on log-log graph paper) is curvilinear and becomes asymptotic to the the axis, the asymptotic concentration can be interpolated. This value has probably greater practical and theoretical significance than an LC50 at some arbitrary time. It has been previously termed the threshold or incipient median lethal concentration but the use of such terms can be misleading and should be discouraged.

It must be remembered that toxicity test results are expressed in terms of concentrations to which the fish have been exposed for the stated time interval. Acute lethal toxicity tests are sometimes carried out using static test procedures and the results expressed in terms of the initial concentration. If, by analysis, it can be shown that the concentration during the test did not fall below 90% of the initial concentration, the use of the term LC50 can be considered valid. However, if the concentration was not maintained within close limits during the test (± 10%), the use of the term LC50 is not valid. It has been proposed by Lloyd and Tooby 1979 that the term LC(I)50 should be used for those tests where the concentration decreases throughout the test period; where C(I) is the initial (nominal) concentration. Use of this nomenclature will help to identify a possible source of error so that the values would be judged in their proper context.

A4.2 **Accuracy and Precision**

One of the basic requirements of all experimental procedures is a knowledge of the precision of the method and accuracy of the results.

The precision of the method is usually expressed as confidence limits or the range of values within which, at a given level of statistical probability, the results from subsequent tests will fall, using exactly the same experimental conditions and an equally representative batch of test fish. Changes in experimental conditions or in the susceptibility of the batch of fish could give results outside the range expressed as confidence limits.

The accuracy of the results is best determined by replication of experiments under exactly the same experimental conditions so that variability of response of different representative batches of fish can be measured.

Because the resources for toxicity testing are limited, a balance has to be maintained between performing a few tests of high precision and more tests of limited precision which give some indication of accuracy. However, it is strongly recommended that within the limitations of time and facilities, every effort should be made to replicate all tests.

Calculation of the LC50 using graphical methods requires percentage responses between 0% and 100% from two or more concentrations. If only 0% and 100% responses are recorded, the interpolated LC50 is approximate. More precision, therefore, can be attained with an increase in the number of concentrations in which a partial mortality occurs; a minimum of three such concentrations is desirable.

Where, at a given time interval, no more than one concentration produces a percentage mortality between 0 and 100%, the LC50 values should be derived from the LT50 graphs. The greater the frequency of observations, and therefore the likely

number of individual responses recorded, the greater the precision of the results. It is often easier to increase the frequency of observations than to increase the number of test concentrations.

It is recommended that, wherever precision is required, the LT50 for each concentration should be determined and whenever possible the time at which each fish dies is recorded throughout the test period. The data can be presented in the form given in Table A1.

Greater precision can be achieved by increasing the number of fish used in each test container, although the use of more than 30 individuals will not decrease the confidence limits significantly. No test should be carried out with less than five fish to each container and ten is the preferred number.

A4.3 **Calculation of Results**

The complexity of data analysis will depend on the type of test and the objectives for the test. Simplified graphic methods exist for determining time-percent relationships (Litchfield, 1949) and dose-effect (Litchfield and Wilcoxon, 1949). For greater precision, probit analysis (Finney, 1971) can be used. Most of these methods have been reviewed by Sprague 1969, 1970, 1971.

Table A1. Record of cumulative percentage mortality of test fish in each concentration from an acutely lethal toxicity test.

Number of fish per concentration: 10
Test concentrations: geometric series 1.0 mgl^{-1} – 10 mgl^{-1}.

Test time in minutes	Concentration in mgl^{-1}					
	1.0	1.8	3.2	5.6	10	control
0	0	0	0	0	0	0
350					10	
540					20	
600					50	
620					70	
640					90	
690				0	100	
830				10		
850				20		
890				30		
960				40		
980			0	50		
1050			10	70		
1100				80		
1250		0		90		
1300		10	30			
1440	0	10	40	90	100	0
1500			50	100		
1650			70			
1700			80			
1950			90			
2100		20				
2400		30				
2550		40				
2700			100			
3100		50				
3700		60				
3800		70				
4500		80				
6200		90				
9800	0	100				0

A4.3.1 *Estimation of the Median Lethal Concentration (LC50)*

A4.3.1.1 *Mathematical*

The approximate mean lethal concentration can be computed from:

$$LC50 = \text{antilog}\left[\sum_{0\%}^{100\%} \frac{\log C}{n}\right]$$

where n is the number of concentrations spanning the range giving 0 to 100% effect. In all methods of computation logarithmic transformation of the test concentration data is used.

A4.3.1.2 *Graphical*

(i) In the simplest method, numbers of dead fish or percentage mortalities at a given time are plotted against their respective concentrations as in Fig A1. The approximate LC50 is them interpolated from the graph.

(ii) Alternatively, the percentage mortalities at a given time may be plotted against concentration on a logarithmic scale. An example of the estimation of the 24 hour LC50 by this method is given in Fig A2.

(iii) The values in each case can be plotted directly onto logarithmic-probability paper. An example of the estimation of the 24 hour LC50 is given in Fig A3. Note that where 10 fish are used for each concentration, the response of the first is regarded as 5%, the second 15% and so on until the tenth which is regarded as 95%. The reason for this is readily appreciated when it is realised that the LC50 should relate not to the response time of the fifth out of the total of ten, but to a response time between the fifth and sixth fish, so that the lethal concentrations for five fish are distributed on each side of the median. A straight line is fitted by eye to each set of data, giving greater weight to those values between 25% and 75% response, and the LT50 can be calculated using the Nomographic method of Litchfield 1949.

Calculation to the LC50 using these graphical methods requires partial percentage responses between 0% and 100% from two or more concentrations. Confidence intervals for the LC50 may be calculated using the Nomographic method of Litchfield and Wilcoxon 1949.

A4.3.2 *Estimation of the Median Lethal Time (LT50)*

A4.3.2.1 *Mathematical*

The approximate mean lethal time can be computed from:

$$LT50 = \text{antilog}\left[\sum_{0\%}^{100\%} \frac{\log T}{n}\right]$$

where $T_{0\text{-}100}\%$ are the individual survival times of n fish in each concentration.

A4.3.2.2 *Graphical*

(i) In the simplest case for each concentration the percentage response with time is plotted on simple linear scales and the mean response time interpolated.

(ii) More commonly the LT50 for a given concentration is obtained by separately plotting the cumulative number of fish responding in each concentration as probits against time expressed on a logarithmic scale. The values in each case can be plotted directly onto logarithmic-probability graph paper. An example of the estimation of LT50s from the data presented in Table A1 is given in Fig A4. Note that 10 fish were used for each concentration and that the response of the first fish is regarded as 5%, the second as 15% and the tenth as 95%. For an explanation see paragraph 4.3.1.2 (iii)

A straight line is fitted by eye to each set of data, giving greater weight to those values between 25% and 75% response, and the LT50 can be calculated using the Nomographic method of Litchfield 1949.

A4.3.3 *Estimation of the Median Lethal Concentration (LC50) From the Median Lethal Time (LT50)*

The logarithms of the LT50s are plotted against the logarithms of the test concentrations and a line fitted by eye through the data; arithmetic values can be plotted directly onto log-log graph paper. The LC50s for a given time period are read from the graph. An example deriving the LC50s at 24, 48 and 96 hours is given in Fig. A5.

A4.3.4 *Comparison of Results from Each Method*

Using the data presented in Table A1, and 24 hour LC50s calculated by each method are given in Table A2. Similarly using the data presented in Table A1, the LT50 values for each concentration calculated by each method are given in Table A3. The relative performance of each method is dependant on the number of data and this is taken into consideration when specifying the method in each protocol.

Table A2. Comparison of the 24 hour LC50 values calculated by each method, using data from Table A1.

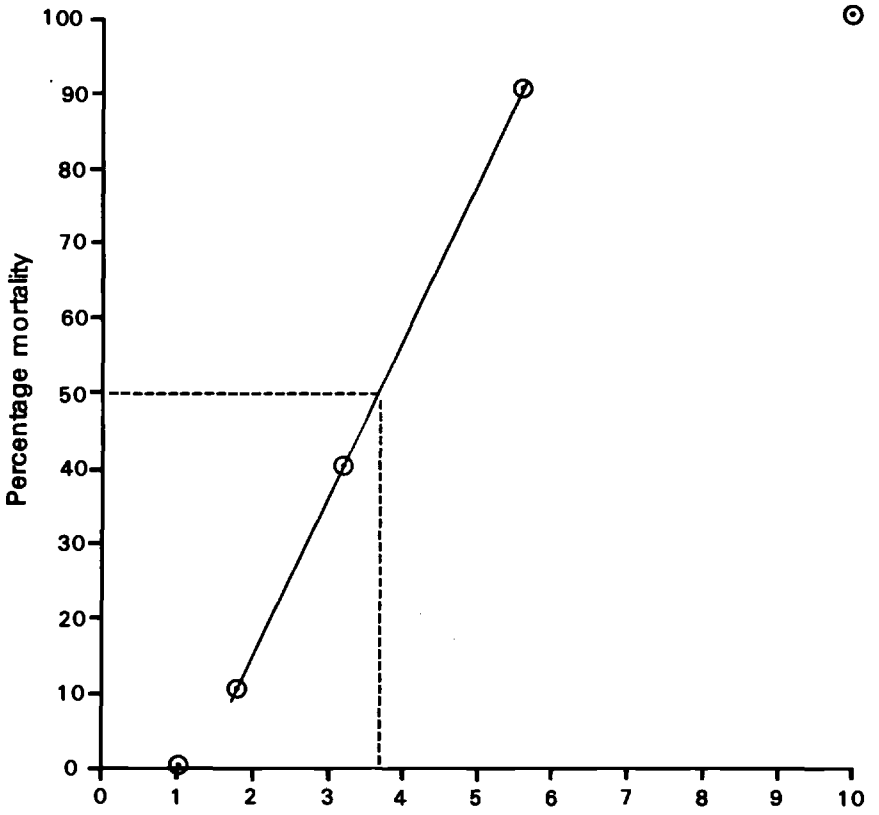
Method	24 hour LC50)mgl^{-1})
Mathematical (para 4.3.1.1)	3.2
Graphical (Fig 1)	3.6
(Fig 2)	3.1
(Fig 3)	3.8
Estimation from LT50 (Fig 5)	3.7

Table A3. Comparison of the LT50 values calculated by two methods, using data from Table A1.

	Methods	
Test Concentration (mgl^{-1})	Mathematical (para 4.3.2.1) (mins)	Graphical (Fig A4) (mins)
10	582	600
5.6	1030	1050
3.2	1574	1550
1.8	3388	3400
1.0	∞	∞
0	∞	∞

Fig.A 1 GRAPHICAL ESTIMATION OF THE 24 HOUR LC50 USING DATA FROM TABLE A1

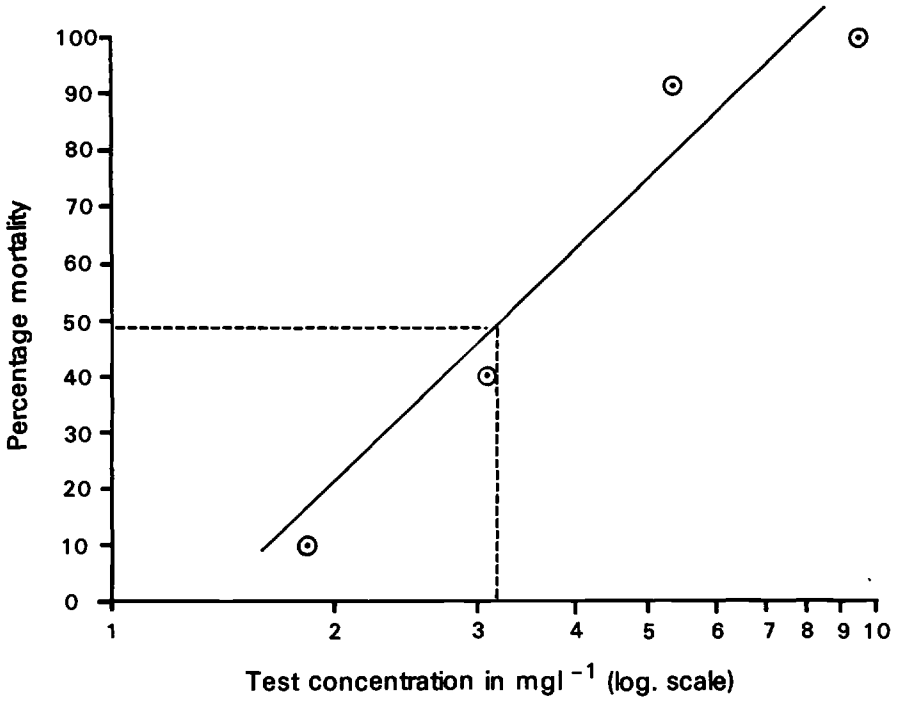
Concentration in mgl^{-1}	Percentage mortality at 24 hours
10·0	100
5·6	90
3·2	40
1·8	10
1·0	0



24 hour LC50 = 3·6mgl^{-1}

Fig.A 2 GRAPHICAL ESTIMATION OF THE 24 HOUR LC50 USING DATA FROM TABLE A1

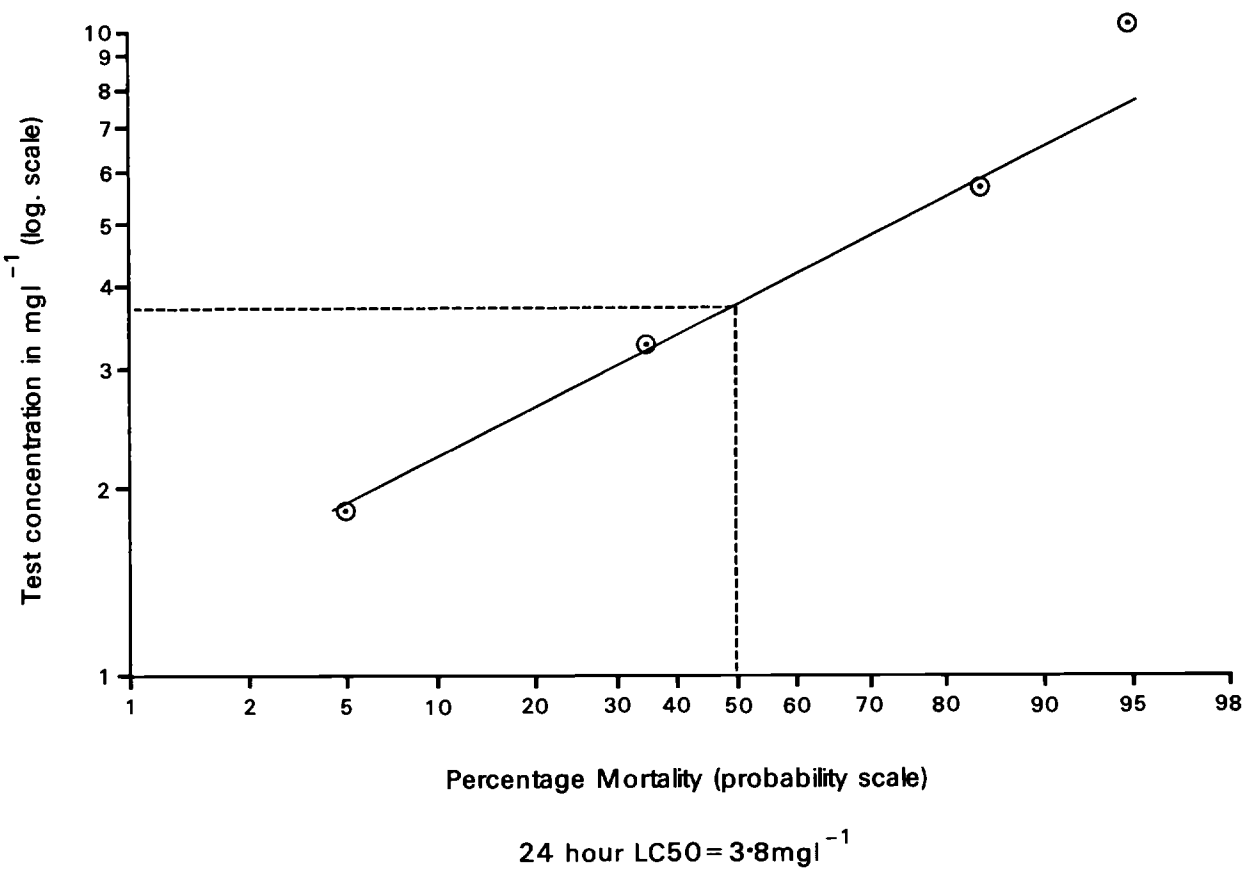
Concentration in mgl^{-1}	Percentage mortality at 24 hours
10·0	100
5·6	90
3·2	40
1·8	10
1·0	0



24 hour LC50 = 3·1 mgl $^{-1}$

Fig.A3 GRAPHICAL ESTIMATION OF THE 24 HOUR LC50 USING DATA FROM TABLE A1

Concentration in mgl^{-1}	Percentage mortality at 24 hours
10·0	100
5·6	90
3·2	40
1·8	10
1·0	0



24 hour LC50 = 3·8mgl^{-1}

Fig.A 4 GRAPHICAL ESTIMATION OF LT50 VALUES FROM THE DATA PRESENTED IN TABLE A1

concentration in mgl-1	LT50 in minutes
10·0	600
5·6	1050
3·2	1550
1·8	3400

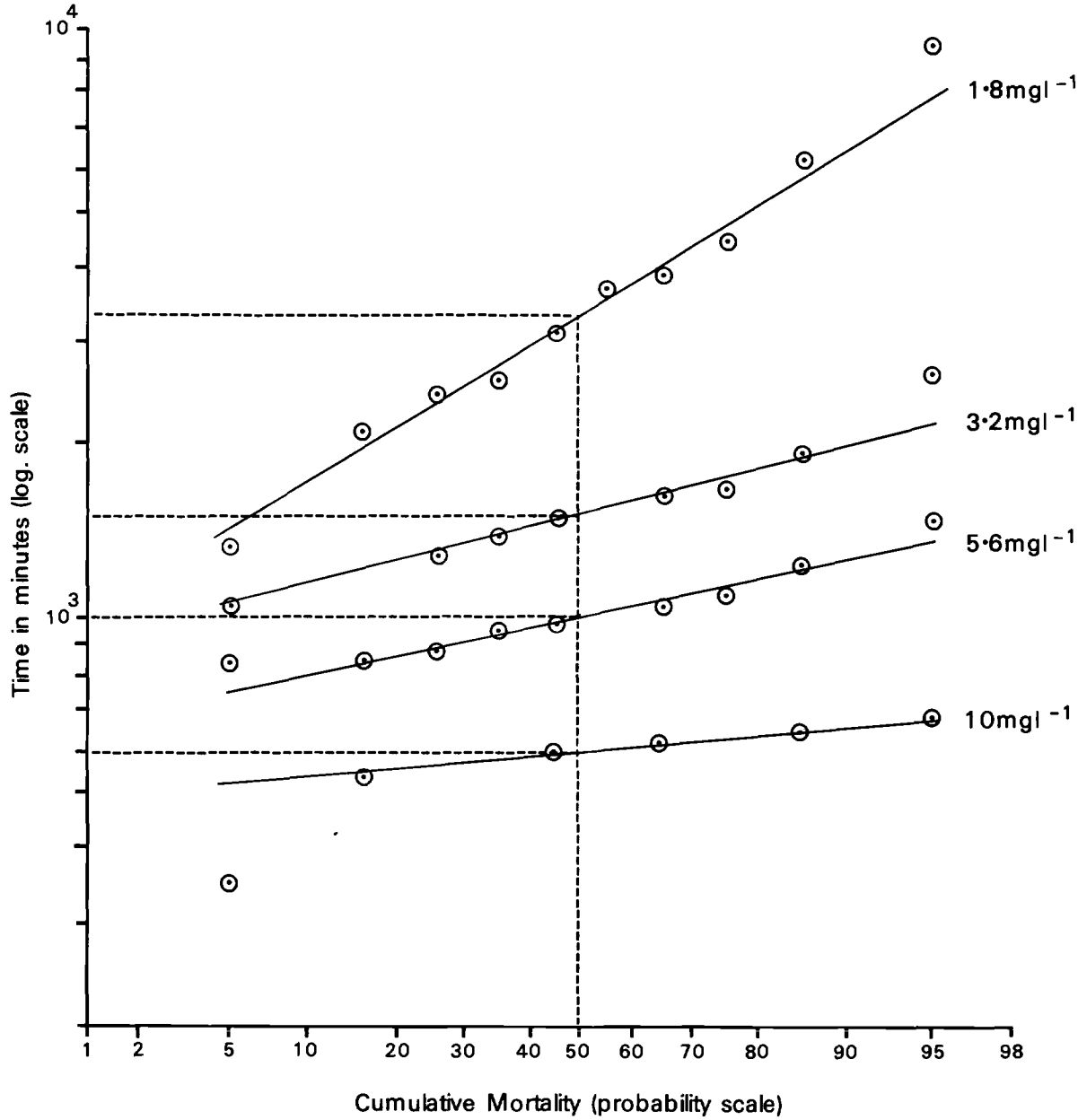
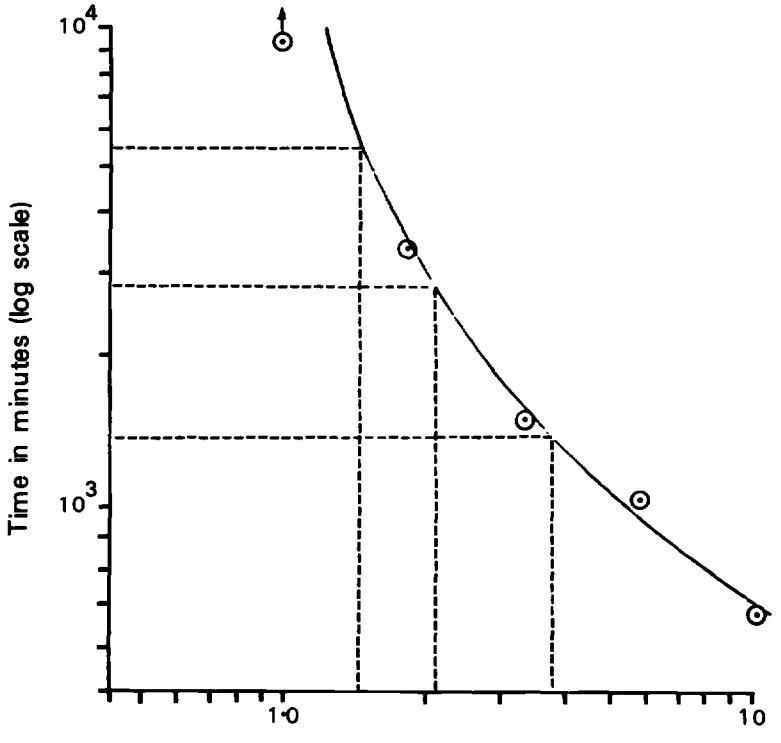


Fig.A5 GRAPHICAL ESTIMATION OF LC50 VALUES FROM LT50 DATA FROM FIG.A4

concentration in mgl-1	LT50 value in minutes
10·0	600
5·6	1050
3·2	1550
1·8	3400
1·0	>9800



24 hour LC50 = 3·7 mgl^{-1}

48 hour LC50 = 2·1 mgl^{-1}

96 hour LC50 = 1·4 mgl^{-1}

A5 Terminology used in the Fish Toxicity Testing Procedures (EIFAC 1975, Alabaster and Lloyd, 1980)

Acclimation: A physiological adaptation of fish to some selected experimental conditions, including any adverse stimulus which is involved (used by Sprague as equivalent to "tolerance" as defined here).

Acclimatization: The genetic adaptation of a fish species to some change of natural or applied environmental conditions.

Acute Toxocity: The harmful properties of a substance which are demonstrated within a short period (hours to days) of exposure. Typically associated with breakdown of tissues and physiological systems at rates which exceed rates of repair or adaptation. Commonly referred to rapidly lethal effects.

Acute toxicity test: A test made to determine the acute toxicity of a substance or waste.

Air saturation value of dissolved oxygen: The concentration of oxygen dissolved in water in equilibrium with atmospheric air at normal barometric pressure. The value varies with temperature and is, for example, 14.6 mg/l at 0°C and 8.3 mg/l at 25°C.

Asymptotic threshold concentration: That concentration of poison at which some specified proportion of a test population shows itself to be in a state of approximate homeostasis for some prolonged period of time (but not necessarily absolutely); demonstrated as the concentration at which the toxicity curve is approximately or effectively asymptotic to the time axis.

Bioassay: The use of living material to measure the concentration of a substance in water, by determining its potency in producing some specific effect. Frequently involves the use of standard reference compounds. Strictly speaking a fish toxicity test is not a bioassay.

Chronic: Of long duration; for example, a chronic toxicity is equivalent to a long term toxicity test.

Concentration-response curve: The curve obtained when, after a given period of exposure, the different percentage responses of batches of fish exposed to different concentrations of poison are plotted against those concentrations.

Constant-flow test: A toxicity test in which constant partial replacement of a test solution takes place at frequent fixed intervals, so that the flow of liquid is effectively almost continuous.

Continuous flow test: A toxicity test in which the flow of the test solution continues without interruption.

Criteria (water quality): The relation between the concentration of pollutant and its measured effect on a target organism.

Cumulative: Increasing by successive additions.

Death time: Survival time.

Delayed: Put off in time; delayed effects of a poison are those not exerted until some considerable time after first exposure to a poison or, when the period of exposure to poisoning is brief, not until after exposure has ceased.

Dose-response curve: Similar to concentration-response curve except that the dose (ie, the quantity) of poison received by the fish is known and takes the place of concentration in plotting the data.

Dynamic test: A test with constant flow or continuous flow.

EC50: Median effective concentration; the concentration of poison, or intensity of other stimulus, which produces some selected response in one half of a test population.

Effective concentration: That concentration of poisoning producing some selected/ defined effect.

ET50: Median effective time; the interval of time between initial exposure of a test population of fish to a single intensity of stimulus and the response of one half of that population.

Exposure period: The interval of time during which a fish is exposed to a stimulus.

Flow-through test: A test with constant flow or continuous flows.

Incipient: Beginning, for example, an incipient effect is an effect beginning to occur.

Incipient LC50: That level of toxicant which is lethal for 50 percent of individuals exposed for periods sufficiently long that acute lethal action has ceased (Sprague, 1969, 70, 71).

Irritation: Morbid stimulation of a fish, or of some vital function (eg, mucus secretion), without necessarily producing an inflammatory tissue response.

LC50: Median lethal concentration: the concentration of a poison lethal to one half of a test population [of fish].

LD50: Median lethal dose; the dose (ie, the quantity received by the body) which is lethal to one half of a test population [of fish]. A quantity not normally known for fish from typical toxicity tests, where LC50 is the correct term to use.

Level of no-effect: That upper concentration of a poison at which some selected effect fails to be produced, usually within some specified period of exposure. Should not be taken as indicating that the effect would not be produced with increase in the period of exposure, or that other, unsought for, harmful effects are not occurring.

Lethal: Mortal; causing death.

Lethal phase: The stage reached by a fish in a toxicity test when the progression toward death cannot be reversed.

Lethal time: Survival time.

Long-term toxicity test: A toxicity test of prolonged duration (months to years) which may include more than one generation of the test organism; effects less severe than those observed under conditions of acute poisoning, or the absence of adverse effects, are sought; chronic toxicity test.

LT50: Median lethal time; the survival time of one half of a population of fish in a given concentration of poison.

Maximum safe concentration: The maximum concentration of poison to which fish can be continuously exposed without harm (often interpreted as "without causing death").

Median period of survival: LT50; median survival time.

Mortality: Death.

Monitoring test: A toxicity test designed to be applied on a routine basis, with some implication of control, to ensure that the quality of, for example, a river water of effluent does not exceed some prescribed standard.

Ninety five percent confidence limits — see at percent.

Overturning time: The time interval between initial exposure of a fish to a poison (or other harmful stimulus) and its loss of equilibrium.

Parameter: A quantity which serves to define some item; the item itself, a numerical characterisitic of a population of items. For physical and chemical properties of water, the term "water quality characteristic" should be used.

Percentage saturation dissolved oxygen: The concentration (mg 1^{-1}) of dissolved oxygen in water at any given conditions expressed as a percentage of the air saturation value under that same condition.

Percentile: The value below which the stated percentage of a series of measurements falls (eg, the 95 percentile indicates the value for some parameter below which 95 percent of the results of a statistical population falls.)

95 percent confidence limits: The upper and lower limits of the 95 percent confidence interval; effectively the limits within which, at a probability level of 0.95, the true EC50 for the population of fish under test lies. Thus, if tests were made on a large number of separate samples of fish taken from a single large population, and the EC50 and its 95 percent confidence interval for each test sample was determined, then in about 95 tests in 100, the true value of the population EC50 would come within the sample confidence limits.

Photo period: The period fish are exposed to natural or artificial daylight which normally lies between 12 and 16 hours a day.

Poison: In the present context, any chemical in an aquatic ecosystem at such a concentration that it can, when taken into the body of a fish, impair health or cause death by its specific chemical properties. Includes all toxins.

Pollution: A change in the quality of a water which makes that water unfavourable to fish. Such impairment may occur as a result of natural phenomena, but is more usually associated with the addition, either accidental or deliberate, of wastes typically originating in the activities of man.

Probit: Unit of probability of standard deviation.

Reaction period: Manifestation time, response time.

Resistance: Ability of an animal to survive for a limited period in an environment that will eventually exert a lethal effect.

Resistance time: The finite period of time for which a fish can live beyond the incipient lethal level.

Response: The reaction of a fish (or some system thereof) to a stimulus. Both nature and time of onset of the response should be defined.

Response time: Manifestation time; reaction period, for example, of individual fish in a test batch or of a given proportion.

Safe concentration: A concentration of a chemical harmless to the greater part of a fish population even with prolonged exposure.

Screening test: A test used to detect the ability of a chemical or waste to produce some selected effect.

Short-term toxicity test: A toxicity test of short duration, usually an acute toxicity test.

Standard (water quality): The limiting concentration of a pollutant (or degree of intensity of some other adverse condition) which is permitted in a water. Standards are determined from a considered judgement of the criteria involved.

Standard test: A test in which all conditions, for example, water quality, fish species and numbers, duration, methodology, etc. conform rigidly to specified requirements.

Static test: A toxicity test in which either no replacement of test solution takes place, or in which all, or the greater part (>95%) of the test solution is replaced batchwise only after relatively prolonged intervals (eg 12– or 24 h).

Stimulus: The factor producing some response.

Sub-acute: Having effects less severe than those observed under conditions of acute poisoning and usually not demonstrated until after perhaps some weeks of exposure.

Sub-lethal: Not killing; usually applied to toxicity tests with poison concentrations which are not lethal within the period of the experiment. Both period of exposure and observation, as well as the percentage of fish referred to, should be defined.

Survival time: The time interval between initial exposure of fish to a harmful stimulus and death.

TLm: Median tolerance limit; the concentration of poison, or intensity of some harmful stimulus, lethal to one half of a test population [of fish]. ("Tolerance" here has the meaning "the intensity of stimulus required").

TL50 is equivalent ot TL_m and LC50.

Time-response curve: The curve obtained when the cumulative percentage response of a test batch of fish to a single concentration of poison is plotted against time.

Tolerance: Acquired resistance of fish to a poison or other stimulus (eg, high temperature, low dissolved oxygen) following continuous or repeated exposure.

Tolerance limit: The lowest oxygen level permitting indefinite survival of fish; the highest poison concentration permitting the indefinite survival of fish.

Toxic: Poisonous; having the properties of a poison.

Toxic substance: Poison.

Toxicant: Poison.

Toxicity: The harmful qualities of a substance.

Toxicity curve: The curve produced when, for example, the median periods of survival of test batches of fish exposed to different concentrations are expressed in a graphical form. The curve obtained when, for example, the LC50 values of a test population of fish after different periods of exposure are plotted. Resistance curve (Shepard).

Toxicity test: The use of living material to define the nature and degree of harmful effects produced by a single poison or by a mixture of poisons.

Toxin: Usually albuminous substances of high molecular weight produced by an animal or plant and which are capable of impairing health or causing death of other animals. Does not include all poisons.

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A7 Notes

B Protocol for Screening Chemicals and Products for Acutely Lethal Toxicity to Freshwater Fish

This protocol should be read in conjunction with Part A, Introduction to Acute Toxicity Testing with Aquatic Organisms.
This protocol largely conforms with 'OECD Guidelines for testing chemicals: 203 (fish)'. Differences from the OECD guidelines are noted where appropriate.

B1 Introduction

An acute lethal toxicity test provides information on the concentration of a chemical that leads to the death of a proportion of the test species under the conditions of the test, and at best this information can be used to give only a first indication of the level at which the chemical may be acutely lethal to that species in the natural environment. It is not generally possible to predict how other species would be affected, nor is the nature, degree or rate of onset of some adverse effect arising from chronic exposure to the chemical predictable from an acute test. On the other hand, acute lethal toxicity tests have a relatively long tradition and experience has shown that results from this type of test have been useful in the management and control of gross levels of water pollution. They have been extensively used in the examination of complex sewage and industrial effluents discharged to surface waters and the data obtained have been used to supplement chemical analyses in setting consents for the discharge of effluents into waters containing fish.

Three aspects are particularly important for routine acute testing, namely, the effect measured, the species used, and the exposure conditions. There are a large number of effects that can be taken as measures of intoxication, ranging from alterations at the biochemical and physiological level to changes in the behaviour of the individual or between groups of individuals. In acute tests the death of the organism is the effect most frequently used, probably because it is relatively easy and cheap to observe, it is common to all species, and it is an end-point that occurs irrespective of the nature of the toxic substance under test. The selection of species for general use is dependent

upon a wide range of factors including size, cost, availability throughout the year, ease of holding in laboratory conditions, behaviour, economic importance and the extent to which the species is used or has been used previously. In fresh water, fish have been used more than any other type of animal, probably because of their direct importance to man as food or for sport. All these considerations reduce the suitable species to a small number.

The ISO* has recommended the zebra fish (*Brachydanio rerio;* Hamilton-Buchanan) a small tropical species which breeds readily in the laboratory allowing supplies of relatively standard sized fish to be available at any time of year throughout the world. Many fish can be contained in small test vessels requiring small replacement volumes. Exposure conditions involve the duration of the experiments and maintaining the test concentrations. A test of four days duration (96 hours) can be carried out during a normal working week and this is probably the reason for its wide usage. A static procedure implies that the test solutions are not renewed during the period of the experiment or are renewed only infrequently on a batch basis. The reason for testing under conditions of constant flow is that continuous replacement of the test solution prevents significant reduction in both the test substance and in dissolved oxygen in the test solutions. There are a number of ways to achieve this objective but the method which causes the least disturbance to test organisms is to add automatically and continuously more solution at the required test concentration, allowing an equivalent volume of solution from the test chamber to run to waste. The apparatus described in this protocol and which will give the required continuous flow of test solution is chosen on the basis of simplicity and reliability. More sophisticated types of apparatus are available which incorporate fail-safe devices, an aspect of greater importance in longer term experiments. This type of apparatus is unsuitable for testing materials which may react with the flexible tubing of the pump or which contain solids which may block the metering system or settle out in the test chambers. Relatively large amounts of test substance, of stock solution, and of dilution water are required for this type of test. Where used with appropriate test materials the procedure enables much greater precision to be achieved in the extimate of toxicity especially if chemical analytical data are available for solutions. When testing formulated products, it is important to test the formulation as the toxicity of the active ingredient may be modified by the other chemicals in the mixture.

The test described in this Part will allow the acute lethal toxicity of substances to zebra fish in fresh water to be measured under conditions of constant flow.

B2 Objectives

This procedure permits determination of the concentration of the test substance which if exceeded will be acutely lethal to the test species under the test conditions.

This method is based on the draft ISO test using the zebra fish (*Brachydanio rerio*; Hamilton-Buchanan) as the test species. It will allow the acute toxicities of different substances to be compared under standardized conditions of testing and therefore the substances may be placed in broad categories of toxicity. Such results are only a guide to the implications of the appearance of these substances in the environment and cannot be used for an accurate prediction of the ecological risk involved.

Details for the maintenace and breeding of the zebra fish are given in Section B8.

B3 Test Performance Guide

B3.1 **Parameters determined**

Median lethal concentrations at 24, 48 and 96 hours.

B3.2 **Type of sample**

Soluble chemicals and products. Formulated products should be tested in preference to active ingredients (see Section A2.4.2 in the Introductory part of this Booklet).

B3.3 **Basis of method**

Measurements of mortality of fish in range of concentrations of samples with continuous replacement of test solutions.

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* Draft proposal for screening chemicals and products for acute toxicity to fish using a static, semi-state or flow-through method. ISO/TC147/SC5/WG3 — Document 7346/I, II, III, 1980/06/15 ISO/DP; also Draft BS 6068/5.

B3.4 **Test species**

Zebra fish (*Brachydanio rerio*): minimum length 2.5 cm. (OECD length 2.0 ± 1 cm).

B3.5 **Numbers of fish**

A minimum of 60 fish randomly selected into batches of 10. One batch is used as a control and the remaining 5 batches are exposed to a range of concentrations.

B3.6 **Limitations**

The method is not suitable for liquids immiscible with water or for solutions with high levels of settleable solids.

B3.7 **Other measurements**

Temperature, dissolved oxygen, pH and, where applicable, water hardness, chlorinity and concentration of test substance.

B3.8 **Time required for test**

Allow operator times of 15 h spread over 5 days for each test (excluding chemical analyses).

B4 The test System

B4.1 **Species**

Zebra fish (*Brachydanio rerio*) are used for the test. The largest fish should not be more than 1.5 times the length of the smallest and the minimum size used for the test should be 2.5 cm long.

B4.2 **Acclimation and storage**

Healthy stock fish should be held for at least a week in running water of similar quality and temperature to that of the standard dilution water used in the test. Food should be withheld for 48 hours prior to the test and throughout the test.

B4.3 **Test apparatus**

The test apparatus should be constructed mainly of glass with the internal area kept to a minimum, and with minimal use of tubing and plastic materials. Ground glass joints should be used as far as possible. It is recommended that the apparatus should comprise the following components;

B4.3.1 *Test Flask* A 1 or 2 litre spherical glass flask with three necks. The flask may be conveniently supported on small rings (Fig B1). It is recommended that the delivery adaptor should be similar to the standard Quickfit component (Cat No MF 15/38) and that the exhaust water adaptor should be protected by a line strainer screen made of unplasticized PVC*.

B4.3.2 *Water Bath* It must be capable of maintaining a temperature of 23°C (acceptable range 21–25°C) and deep enough to ensure the complete immersion of the spherical portion of the flasks.

B4.3.3 *Test solution replacement* Peristaltic pumps or other metering devices which are capable of adequately mixing the stock solution of the test substance with dilution water to give the required concentration of the test substance before addition to each flask. The apparatus should be capable of maintaining the dilutions of the stock solution within 10% of the nominal value.

B4.4 **Temperature**

The temperature of the test solutions should be 23°C and should not be allowed outside the range 21–25°C.

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* Suitable screen can be obtained from George Fischer Sales Ltd. Plastics Division London.

B4.5 **Dilution water**

Prepare the dilution water from deionized water according to the instructions given in Section A2.3 of the Introductory Booklet. The standard dilution water should have a pH value of 7.8±0.2 and a hardness of 100 mgl^{-1} expressed as calcium carbonate.

B4.6 **Preparation of experimental solutions**

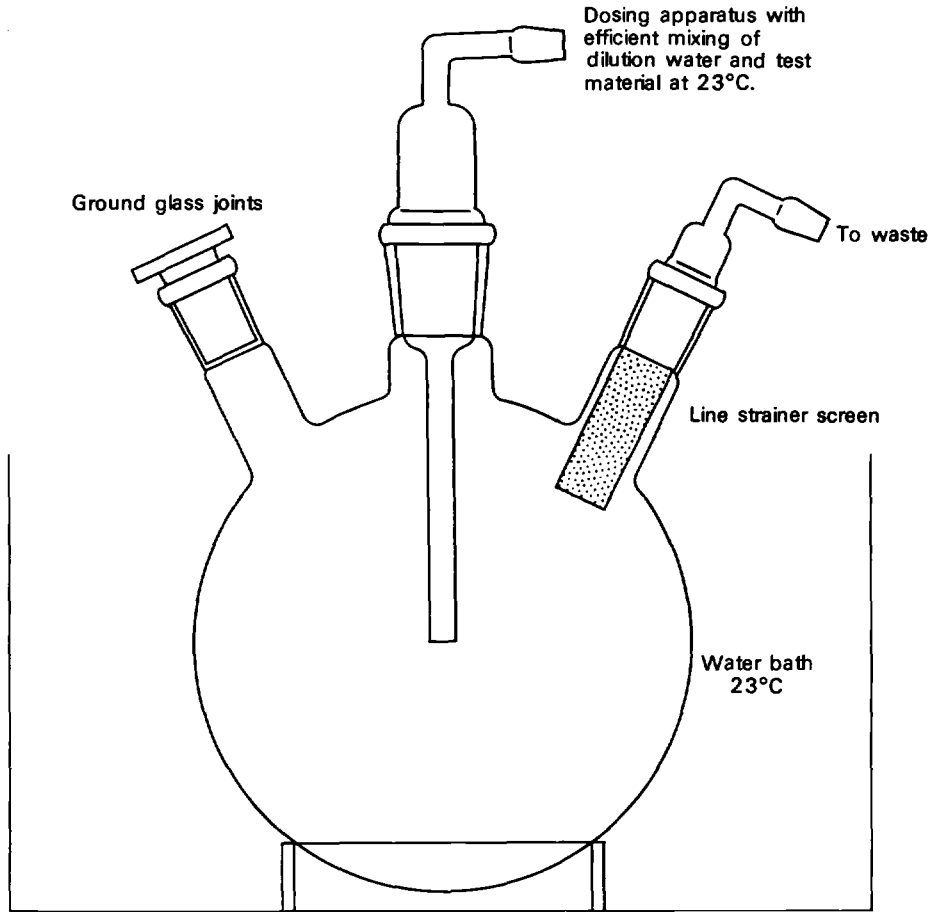
Stock solutions of chemical products should be prepared daily unless it is known that the material is stable, in which case sufficient stock solution could be prepared for the whole test.

If the test substance is marketed as a formulation both the designated active ingredient and the complete formulation should be tested.

B5 Procedure

B5.1 At least six sets of the apparatus shown in Figure B1 should be assembled to carry out tests on 5 concentrations of the test substance and a control. Completely fill all but one of the flasks with a geometric series of concentrations of the test substance.

Fig.B 1 TEST FLASK USED IN SCREENING TEST



B5.2 The concentrations should be arranged so that all the fish are killed within one day in the highest concentration and no deaths occur in the lowest concentration in the period of the test. A partial kill in at least three intermediate concentrations should be obtained within the test period. For some substances a wide range of concentrations may be required to fulfil this condition and for others a closer spacing of concentrations may be necessary. A preliminary static sighting test for establishing a suitable range of concentrations is described in Section A4.2.1 in Introductory Booklet.

B5.3 The remaining flask, the control, should be similarly filled with dilution water only, or dilution water and solvent only, and also should be included in the test series. If more than one fish die in this flask, the test becomes invalidated and should be repeated.

B5.4 When the contents of the flasks reach the temperature of 23.0°C randomly introduce fish until there are 10 in each flask. Adjust the dilution apparatus to replace each test solution at a rate of at least 25 litres per day.

B5.5 At this replacement rate, the dissolved oxygen content of incoming test solutions should be sufficient to meet the needs of the test fish without further aeration in the test flask. The rate of replacement can be reduced to 12 litres per day provided that the dissolved oxygen concentration in the effluent remains greater than 80% of the air saturation value and the concentration of chemical in the experimental solutions remains greater than 90% of the nominal concentration.

B5.6 Observe the fish in each test flask at intervals and at least 3, 6, 24, 48 and 96 hours from the start of the test. Record the number of dead fish in each flask on each occasion. Alternatively, fish can be observed at more frequent intervals and median lethal times calculated for each concentration. Any fish that are dead should be removed from the flasks. Forceps can be used for the operation. Cessation of opercular movement for a period of 10 seconds has been selected as the criterion of death.

B6 Parameters Monitored

As far as possible, the water temperature, pH and dissolved oxygen concentration of the contents of each test vessel should be monitored throughout the test. The minimum acceptable frequency of measurement is twice daily. Should the dissolved oxygen concentration of the outgoing test solution fall below 80% of the air saturation value in either the test solution or control, the test should be abandoned and repeated at a higher flow rate. It is also desirable to measure the concentration of the known test material in each vessel throughout the experimental period. The minimum acceptable frequency of measurements is once daily.

B7 Results

B7.1 **Collection of data**

The data should be collected and reported as in Figure B2 and should contain the following information:

B7.1.1 The specification of the test substance and the method of preparing the dilution water and test solution, including a detailed description of, or reference to, the dosing apparatus.

B7.1.2 All chemical, biological and physcial data pertaining to the test, including details of the acclimation conditions of the test fish.

B7.1.3 The LC50 values for 24, 48 and 96 hours stating whether they are nominal concentrations or are based on chemical analyses; for the former, the notation LC(I)50 should be used.

B7.1.4 Any unusual reactions by the fish under the test and any visible external effects produced by the test substance.

B7.2 **Calculation**

The recommended ISO test method of calculation is as follows, however for a full appreciation of the methods available refer to section 4 of the Introductory part of this Booklet.

Plot the number of deaths at a specific time against the logarithm of the concentration as in Figure B3. Interpolate from the graph the concentration which killed 5 fish under the test conditions. For the purposes of this test, the value can be regarded as the LC50 and values should be recorded for 24, 48 and 96 hours.

Fig B2 Data presentation for screening test

Acute Toxicity to *Brachydanio rerio* (Zebra Fish)

Date: Sample: Sample No: Operator:

Concentration of Stock solution:
Method of preparation of stock solution:
Type of dosing apparatus:
Size of Fish (Fork length):

Test vessel	Parameter	Time (h)				
		3 h	6 h	24 h	48 h	96 h
Flask 1 (expected initial concentration)	1. No of dead fish 2. Measured concentration 3. Temperature °C 4. pH 5. Dissolved oxygen concentration 6. Other observations					
Repeat for all test flasks						
Control Flask	1. No of dead fish 2. Measured concentration 3. Concentration of solvent if applicable 4. Temperature °C 5. pH 6. Dissolved oxygen concentration 7. Other observations					

24 h LC50 .....
48 h LC50 .....
96 h LC50 .....

B8 The maintenance and breeding of the zebra fish (Brachydanio rerio Hamilton-Buchanan)

B8.1 The Zebra Fish

The species originates from the Cormandel coast of India where it inhabits fast-flowing streams. It is a common aquarium fish, so that information about procedures for its care and culture can be found in standard reference books on tropical fish culture. Its biology has recently been reviewed by Laale 1977.

The fish rarely exceeds 4.5 cm in length. The body is cylindrical with 7–9 dark blue horizontal stripes on silver, which run into caudal and anal fins, and the back is olive green. Males are slimmer than the females and possess a golden sheen. Females are more silvery and the abdomen is distended particularly prior to spawning.

B8.2 Environmental Parameters

The fish are capable of withstanding wide ranges of temperature (15.5 to 43.3°C) and pH (6.6 to 9.2) (Axelrod, 1967). Fish may be bred, reared and maintained in dechlorinated tapwater at a temperature of about 26°C, raised to 27°C to induce spawning.

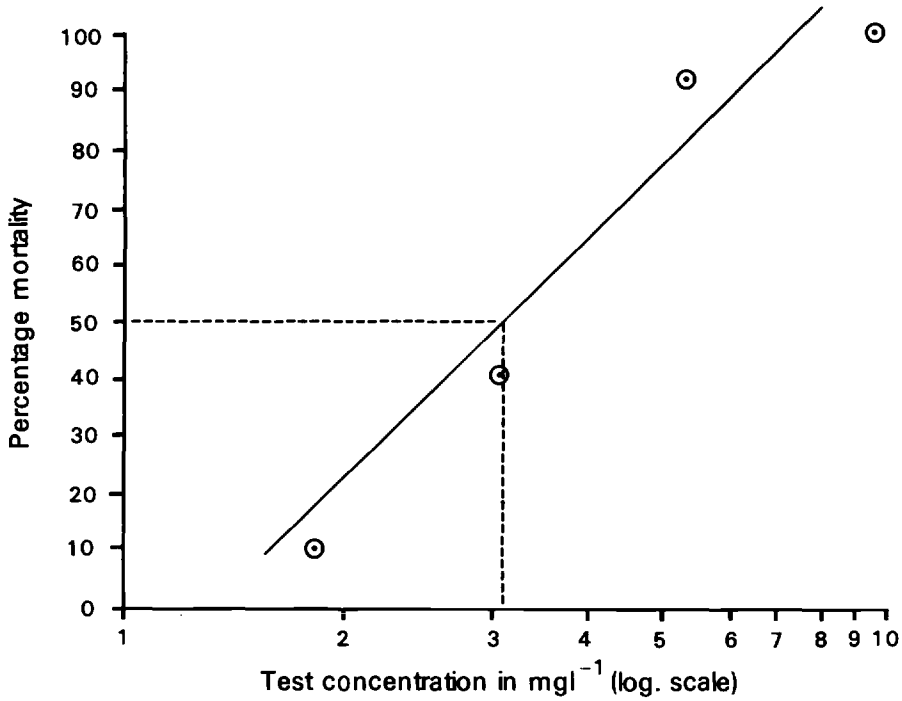
B8.3 Breeding

B8.3.1 *Materials and Methods*

The fish may be spawned in glass tanks with a capacity of about 70 litres. Since the adult fish are avid egg eaters, a method of protecting newly laid eggs and young fish is necessary. One method, used successfully, is to confine the adult fish in mesh cages in the water so that as the female lays her eggs these fall through the mesh to the bottom of the tank out of reach of the adult.

Fig.B3 GRAPHICAL ESTIMATION OF THE 24 HOUR LC50 USING THE FOLLOWING DATA

Concentration in mgl^{-1}	Percentage mortality at 24 hours
10·0	100
5·6	90
3·2	40
1·8	10
1·0	0



24 hour LC50 = 3·1 mgl^{-1}

Water filter systems should not be used in the spawning tank because of the likely damage to the eggs. The tanks should be illuminated for at least 8 hours per day.

B8.3.2 *Conditioning*

The adult fish are conditioned for a period of two weeks. The males and females are separated and fed on live food, consisting of white worms (enchytraeids), *Daphnia* and brine shrimp (*Artemia*). At the end of this period the males possess a deep golden sheen and the females are greatly distended with ova. The stocking density recommended during this period is not greater than 2 fish per litre of water.

B8.3.3 *Spawning*

An empty tank is filled with water and left for two days to condition at 27°C. A mesh cage is fitted into the tank allowing a swimming space of 1 litre volume. Six females are placed in the cage in the morning and fed with freeze-dried brine shrimp. Nine males are added to the cage in the evening and fed once more with freeze-dried brine shrimp before the lights are switched off. Spawning is induced by the first light of the morning and is completed within a period of four hours.

The eggs which are non-adhesive fall through the mesh, out of reach of the adult. When the females are exhausted of eggs, the adults are removed and the eggs left to hatch.

B8.3.4 *Fry development*

The eggs hatch in four to five days. The fry adhere to the side of the tank and remain motionless for 24–48 hours. When the fry become free-swimming they are fed on suitable proprietary fish food of small particle size. At three weeks the fry can be fed on newly hatched brine shrimp (*Artemia* sp) and then growth becomes more rapid. At one month they can be transferred to a larger tank and feed on live and proprietary food. The fish are sexually mature at three months and attain a length of 3.5 cm. Mass reproduction and the influence of breeding time intervals on egg numbers, mortality and hatching have been studied by Mertens 1973 and Niimi and Laham 1974. It should be noted that spontaneous abnormalities in the developing larvae have been observed in certain strains (Piron, 1978).

B9 References

Axelrod, H R (1967) *Breeding Aquarium Fishes. Book 1.* Tropical Fish Hobbyist Publications. Inc.

Laale, H W (1977) The biology and use of zebra fish (*Brachydanio rerio*) in fisheries research. A literature review. *J.Fish.Biol.* **10** (2): 121–173.

Merthens, J (1973) Year-round controlled mass reproduction of the Zebra fish. *Aquaculture.* **2** : 245–249.

Niimi, A J and Laham, Q N (1974) Influence of breeding time interval on egg number, mortality and hatching of the Zebra fish (*Brachydanio rerio*). *Can.J.Zoo* **52** : 515–517.

Prion, R D (1978) Spontaneous skeletal deformities in the zebra danio (*Brachydanio rerio*) bred for fish toxicity tests. *J.Fish Biol.* **13** (1) : 79–83.

C

Protocol for Measuring the Acute Lethal Toxicity of Chemical Substances, Effluents or Polluted Freshwaters to Rainbow Trout using a Static Test Procedure

This protocol should be read in conjunction with Part A, Introduction to Acute Toxicity Testing with Aquatic Organisms

This protocol largely conforms with 'OECD Guidelines for testing chemicals: 203 (fish)'. Differences from the OECD guidelines are noted where appropriate.

C1 Introduction

An acute lethal toxicity test provides information on the concentration of a chemical that leads to the death of a proportion of the test species under the conditions of the test, and at best this information can be used to give only a first indication of the level at which the chemical may be acutely lethal to that species in the natural environment. It is not generally possible to predict how other species would be affected, nor is the nature, degree or rate of onset of some adverse effect arising from chronic exposure to the chemical predictable from an acute test. On the other hand, acute lethal toxicity tests have a relatively long tradition and experience has shown that results from this type of test have been useful in the management and control of gross levels of water pollution. They have been extensively used in the examination of complex sewage and industrial effluents discharged to surface waters and the data obtained have been used to supplement chemical analyses in setting consents for the discharge of effluents into waters containing fish.

Three aspects are particularly important for routine acute testing, namely, the effect measured, the species used, and the exposure conditions. There are a large number of effects that can be taken as measures of intoxication, ranging from alterations at the biochemical and physiological level to changes in the behaviour of the individual or between groups of individuals. In acute tests the death of the organism is the effect most frequently used, probably because it is relatively easy and cheap to observe, it is common to all species, and it is an end-point that occurs irrespective of the nature of the toxic substance under test. The selection of species for general use is dependent

upon a wide range of factors including size, cost, availability throughout the year, ease of holding in laboratory conditions, behaviour, economic importance and the extent to which the species is used or has been used previously. In fresh water, fish have been used more than any other type of animal, probably because of their direct importance to man as food or for sport. All these considerations reduce the suitable species to a small number, of which the rainbow trout, *Salmo gairdneri* is pre-eminent.

Exposure conditions involve the duration of the experiments and maintaining the test concentrations. A test of four days duration (96 hours) can be carried out during a normal working week and this is probably the reason for its wide usage. A static test procedure implies that the test solutions are not renewed during the period of the experiment, or are renewed only infrequently on a batch basis and the assumption is made that the initial concentration of substance persists throughout the testing period. The limitations of this type of procedure are that the concentrations of active materials may not remain constant but may be reduced by volatilization, degradation or absorption to the test container. Further, the material may well separate from solution, either by rising to the surface or by settling out. In either case, the effect may be greater or lesser than if the test substance were present in a homogenous mixture (see Section A3.1). On the other hand, the advantages of this type of technique are that only very simple apparatus is required, only relatively small volumes of sample and dilution water are used, and it is possible to examine substances, effluents etc, that prove difficult to handle using other methods.

The simple procedure described in this Part will allow the acute lethal toxicity of substances to rainbow trout to be determined under static conditions.

C2 Objective

This procedure describes a simple method for measuring in the laboratory the acutely lethal concentrations of soluble substances, liquid effluents or polluted freshwaters to rainbow trout using a static test procedure. The main advantage compared with other test methods is that only small volumes of samples are required for the static test procedure.

C3 Test Performance Guide

C3.1 Parameters determined

(a) Median lethal time in each concentration.
(b) The median lethal concentrations at 24, 48 and 96 hours.

C3.2 Type of sample

Soluble chemicals, liquid effluents, polluted freshwater.

C3.3 Basis of method

Measurement of mortality of fish in a range of concentrations of samples. Solutions are changed daily.

C3.4 Test species

Rainbow trout (*Salmo gairdneri*); minimum weight 0.5g mean weight range 1–10g see also C4.1.
(OECD size limit 5.0 ± 1.0 cm)

C3.5 Numbers of fish

A minimum of 30 fish randomly selected into batches of 5. One batch used as a control and the remaining 5 batches are exposed to a range of concentrations.

C3.6 Limitations

The solutions are changes only daily, therefore the concentration of active material may not remain constant during the course of the test. The method is not suitable for liquids immiscible with water, for solutions with high levels of setteable solids, or for volatile or unstable compounds.

C3.7 Other measurements

Temperature, dissolved oxygen, pH and, where applicable, water hardness, chlorinity and concentration of test substance.

C3.8 **Time required for tests**

Allow operator times of 10 hours spread over 5 days for each test (excluding chemical analyses).

C3.9 **Photo period**

Not given. (The OECD require 12–16 hrs daily).

C4 The Test System

C4.1 **Species**

Rainbow trout (*Salmo gairdneri*) of average weight between 1.0 and 10 grams. The largest fish should not be more than 1.5 times the length of the smallest.

C4.2 **Acclimation and storage**

Healthy stock fish should be held for at least a week in running water of similar quality and temperature to that of the dilution water used in the test. The fish should not be fed for 48 hours prior to the test and throughout the test.

(The OECD require stock to be held for over 1–15 days in running water). If fish mortality is over 10% reject the whole batch. If fish mortality is over 5% hold for a further 7 days.

C4.3 **Test apparatus**

The test containers should be made preferably of glass although other materials may be acceptable providing there is no effect on the test solution. A minimum of 6 containers is required for each test and ideally there should be 0.5 litre of water for each grams of fish.

Each test solution should be aerated by a gentle flow of air introduced through a diffuser block or, it foaming occurs, through a capillary tube.

C4.4 **Temperature**

The temperature of the test solutions should be 15°C and should not be allowed outside the range 13–17°C. (The OECD temperature specification is 15 ± 1°C).

C4.5 **Dilution water**

The dilution water and the water used for acclimating the test fish should be the natural water (salinity < $8.5^{0}/_{00}$) receiving the effluent or of similar hardness, pH and salinity. Water hardness may affect the toxicity of chemicals to fish in which case the tests should be carried out in both soft and hard water. Ideally the soft water should have a hardness of not more than 50 mgl^{-1} expressed as calcium carbonate and that of the hard water not less than 200 mgl^{-1}. (The OECD require the hard water to the 50–250 ppm as $CaCO^{3}$).

C4.6 **Preparation of experimental solutions**

Representative samples of natural waters or effluents should be obtained. If the samples are stored for more than 3 hours or transported over long distances, they should be kept at a temperature of around 4°C.

Stock solutions of test chemical substances should be prepared daily unless it is known that the material is stable, in which case sufficient stock solutions are prepared for the whole test.

C5 Procedure

Batches of five fish, randomly selected, are exposed for 96 hours to a geometric series of concentrations of test material together with a control. The concentrations are arranged so that all the fish are killed in a few hours in the highest concentration and no deaths occur in the lowest concentration during the test period. It may be necessary to carry out the preliminary sighting test to select these concentrations (see Section A2.4.1 in Introductory part of this Booklet). As far as possible inspections should be made throughout the test period and dead fish should be removed as soon as they are observed and the mortality recorded.

Should the dissolved oxygen fall below 80% of the air-saturation value in any of the test vessels, or fish die in the control, the test should be abandoned. In static tests the dissolved oxygen concentration may be increased by increasing the rate of aeration.

The test solutions should be changed completely every 24 hours. The simplest method is to fill a spare test container with a new solution and transfer the fish. This can be done sequentially through all concentrations and control.

Cessation of opercular movement for a period of 10 seconds has been selected as the criterion of death.

C6 Parameters Monitored

During the first 6 hours of the test, water temperature, pH and dissolved oxygen should be monitored at hourly intervals to determine whether these are showing progressive changes. A reduction in dissolved oxygen concentration should be countered by increased aeration. Monitoring should continue until conditions are stable, and subsequently immediately before and after changing the test solutions and at the end of the test.

C7 Results

The recording and calculation of results are discussed in detail in Section A4 of Introductory part of this Booklet.

C7.1 Collection of data

In the test report, the following data should be presented along with the LT50 or LC50 values.

(a) average length and average weight of fish
(b) range of lengths and weights
(c) number of fish per tank
(d) Volume of test solutions
(e) period of acclimation
(f) average and range of: temperature, pH, dissolved oxygen concentration and, where applicable, water hardness, chlorinity and test chemical concentration.

C7.2 Estimation of the median lethal time (LT50)

Results are recorded as the cumulative percentage mortalities with time for each concentration. From these data, median lethal times are estimated for each concentration by separately plotting the cumulative mortality for each concentration as probits against time expressed on a logarithmic scale. A straight line is fitted by eye to each set of data so that the LT50 value for each concentration can be derived.

Confidence limits for the LT50 values may be calculated using the nomographical method of Litchfield 1949.

C7.3 Derivation of median lethal concentration at 24, 48 and 96 hours

The logarithms of the LT50 are plotted against the logarithms of the test concentrations and a line is fitted by eye through the data. The median lethal concentrations at 24, 48 and 96 hours are read from the graph.

The results of the acute toxicity tests are expressed in terms of the initial test concentration. If this concentration is not maintained at a constant level (± 10%) then the LC50 value is not valid. It has been proposed by Lloyd and Tooby 1979 that the term CL(I)50 should be used for static tests where the concentration decreases throughout the test period.

C8 References

Litchfield, J T (1949) A method for rapid graphic solution of time-percent effect curves. *Pharmacol. Exp. Ther.* **97** : 399–408.

Lloyd, R and Tooby, T E (1979) New terminology for short-term static fish bioassays : LC(I)50. *Bull. Environ. Contamin. Toxicol.* **22** (1/2): 1–3.

D Protocol for Measuring the Acute Lethal Toxicity of Chemicals, Effluents or Polluted Freshwater to Rainbow Trout using a Flow-through Procedure

This booklet should be read in conjuction with Part A, Introduction to Acute Toxicity Testing with Aquatic Organisms

This protocol largely conforms with 'OECD Guideline for testing chemicals : 203 (fish)'. Difference from the OECD guidelines are noted where appropriate.

D1 Introduction

An acute lethal toxicity test provides information on the concentration of a chemical that leads to the death of a proportion of the test species under the conditions of the test, and at best this information can be used to give only a first indication of the level at which the chemical may be acutely lethal to that species in the natural environment. It is not generally possible to predict how other species would be affected, nor is the nature, degree or rate of onset of some adverse effect arising from chronic exposure to the chemical predictable from an acute test. On the other hand, acute lethal toxicity tests have a relatively long tradition and experience has shown that results from this type of test have been useful in the management and control of gross levels of water pollution. They have been extensively used in the examination of complex sewage and industrial effluents discharged to surface waters and the data obtained have been used to supplement chemical analyses in setting consents for the discharge of effluents into waters containing fish.

Three aspects are particularly important for routine acute testing, namely, the effect measured, the species used, and the exposure conditions. There are a large number of effects that can be taken as measures of intoxication, ranging from alterations at the biochemical and physiological level to changes in the behaviour of the individual or between groups of individuals. In acute tests the death of the organism is the effect most frequently used, probably because it it relatively easy and cheap to observe, it is common to all species, and it is an end-point that occurs irrespective of the nature of the toxic substance under test. The selection of species for general use is dependent upon a wide range of factors including size, cost, availability throughout the year, ease

of holding in laboratory conditions, behaviour, economic importance and the extent to which the species is used or has been used previously. In fresh water, fish have been used more than any other type of animal, probably because of their direct importance to man as food or for sport. All these considerations reduce the suitable species to a small number, of which the rainbow trout, *Salmo gairdneri* is pre-eminent.

Exposure conditions involve the duration of the experiments and maintaining the test concentrations. A test of four days duration (96 hours) can be carried out during a normal working week and this is probably the reason for its wide usage. A static procedure implies that the test solutions are not renewed during the period of the experiment or are renewed only infrequently on a batch basis. The reason for testing under conditions of constant flow is that continuous replacement of the test solution prevents significant reduction in both the test substance and in dissolved oxygen in the test solutions. There are a number of ways to achieve this objective but the method which causes the least disturbance to test organisms is to add automatically and continuously more solution at the required test concentration, allowing an equivalent volume of solution from the test chamber to run to waste. The apparatus described in this protocol and which will give the required continuous flow of test solution is chosen on the basis of simplicity and reliability. More sophisticated types of apparatus are available which incorporate fail-safe devices, an aspect of greater importance in longer term experiments. This type of apparatus is unsuitable for testing materials which may react with the flexible tubing of the pump or which contain solids which may block the metering system or settle out in the test chambers. Relatively large amounts of test substance, of stock solution, and of dilution water are required for this type of test. Where used with appropriate test materials the procedure enables much greater precision to be achieved in the estimate of toxicity especially if chemical analytical data are available for solutions. When tests are being carried out with effluents containing high concentrations of biodegradable organic matter, difficulties may be encountered with low levels of dissolved oxygen.

The test described in this Part will allow the acute lethal toxicity of substances to rainbow trout in fresh water to be measured under conditions of constant flow.

D2 Objective

This procedure permits determination of the concentration of the test substance which if exceed will be acutely lethal to the test species under the test conditions.

A flowthrough procedure is essential for measuring the acute lethal toxicity of compounds or effluents where, under static conditions, their concentration or form does not remain constant because of physical, chemical or biological changes in the test vessels, eg volatilization, absorption on the test apparatus, photo-chemical decomposition, biological breakdown, accumulation in the test organism or reaction with excretory products from the test organism.

D3 Performance Guide

D3.1 Parameters determined

(a) Median lethal time in each concentration.
(b) The median lethal concentrations at 24, 48 and 96 hours.

D3.2 Type of sample

Soluble chemicals and also liquid effluents and polluted freshwaters if sufficient volumes are available.

D3.3 Basis of method

Measurement of mortality of fish in a range of concentrations of sample with continuous replacement of test solutions.

D3.4 Test species

Rainbow Trout (*Salmo gairdneri*);
Minimum weight 0.5g mean weight range 1–10g. See also D4.1.
(The OECD variations are the same as in method C).

D3.5 Numbers of fish

A minimum of 60 fish randomly selected into batches of 10. One batch is used as a control and the remaining 5 batches exposed to a range of concentrations.

D3.6 **Limitations**

The method is not suitable for liquids immiscible with water or for solutions with high levels of settleable solids. Very large volumes of effluent or natural waters may be required for the test.

D3.7 **Other measurements**

Temperature, dissolved oxygen, pH and, where applicable, water hardness, chlorinity and concentration of test substance.

D3.8 **Time required for test**

Allow operator times of 15 hours spread over 5 days for each test (excluding chemical analyses).

D3.9 **Photo period.**

Not given.

D4 The Test System

D4.1 **Species**

Rainbow trout (*Salmo gairdneri*) of average weight between 1.0 and 10 grams. The largest fish should not be more than 1.5 times the length of the smallest.

D4.2 **Acclimation and storage**

Healthy stocks should be held for at least a week in running water of similar quality and temperature to that of the dilution water used in the test. Food should be withheld for 48 hours prior to the test and throughout the test.

D4.3 **Test apparatus**

The apparatus is shown diagrammatically in Figure D1. It consists of 6 × 20 litre sealed spherical glass vessels with ground glass lids and stoppers. A mixing chamber is incorporated in the delivery line which discharges the test solution into the bottom of the flask.

Dosing apparatus: it is recommended that 2 series of peristaltic pumps should be used, one series maintaining a constant flow of dilution water at a nominal rate of 200 ml min^{-1}; the second series for introducing a continuous-flow of a concentrated solution of test material.

Other metering devices are acceptable provided they maintain a constant concentration of chemical in the test solution.

Ideally the concentration in each flask should be maintained within 10% of the nominal value.

D4.4 **Temperature**

The temperature of the test solution should be 15°C and should not be allowed outside the range 13–17°C.

D4.5 **Dilution water**

A local supply of potable water may be used providing the composition remains relatively constant (ie hardness ± 20%) and it is free of chlorine. Water hardness may affect the toxicity of chemicals to fish, in which case the tests should be carried out in both hard and soft water. Ideally, the total hardness of the water should not exceed 50 mgl^{-1} expressed as calcium carbonate for tests in soft water and should not be less than 200 mgl^{-1} calcium carbonate for tests in hard water.

D4.6 **Preparation of experimental solutions**

Representative samples of natural waters or effluents should be obtained. If the samples are stored for more than 3 hours or transported over long distances, they should be kept at a temperature around 4°C in a closed container made of inert material.

Stock solutions of the chemical substances should be prepared daily unless it is known that the material is stable, in which case sufficient stock solutions are prepared for the whole test.

D5 Procedure

Batches of 10 fish, randomly selected, are exposed for 96 hours to a geometric series of concentrations of test material together with a control. The concentrations are arranged so that all the fish are killed in a few hours in the highest concentration and no deaths occur in the lowest concentration during the period of the test. It may be necessary to carry out preliminary sighting tests to select these concentrations (see Section A 2.4.1 in Introductory part of this Booklet). As far as possible inspection should be made throughout the test period. Cessation of opercular movement for a period of 10 seconds has been selected as the criterion of death. Dead fish should be removed from the test solutions as soon as they are observed, and recorded. In practice, as this entails the removal of the top of the flask, it may be more convenient to do this twice a day; first of all in the morning and last of all in the evening. The dead fish should be removed by pouring the vessel contents into a beaker facilitating their removal. Faecal material can be removed at this stage.

D6 Parameters Monitored

As far as possible, the water temperature, pH and dissolved oxygen concentration of each test vessel should be monitored throughout the test. The minimum acceptable frequency of measurement is twice daily. Should the dissolved oxygen level fall below 80% of the air saturation value in either the test solution or controls, the test should abandoned and repeated at higher flow rates. It is also desirable to measure the concentration of the known test material in each vessel throughout the experimental period. The minimum acceptable frequency of measurements is once daily.

D7 Results

The recording and calculation of results are discussed in detail in Section 4 of Introductory part of this Booklet.

D7.1 Collection of data

In the test report, the following data should be presented along with the LT50 or LC50 values.

(a) average length and average weight of fish
(b) range of lengths and weights
(c) number of fish per tank
(d) volume of test solutions
(e) period of acclimation
(f) average and range of: temperature, pH, dissolved oxygen concentration and, where applicable, water hardness, chlorinity and test chemical concentration.

D7.2 Estimation of the median lethal time (LT50)

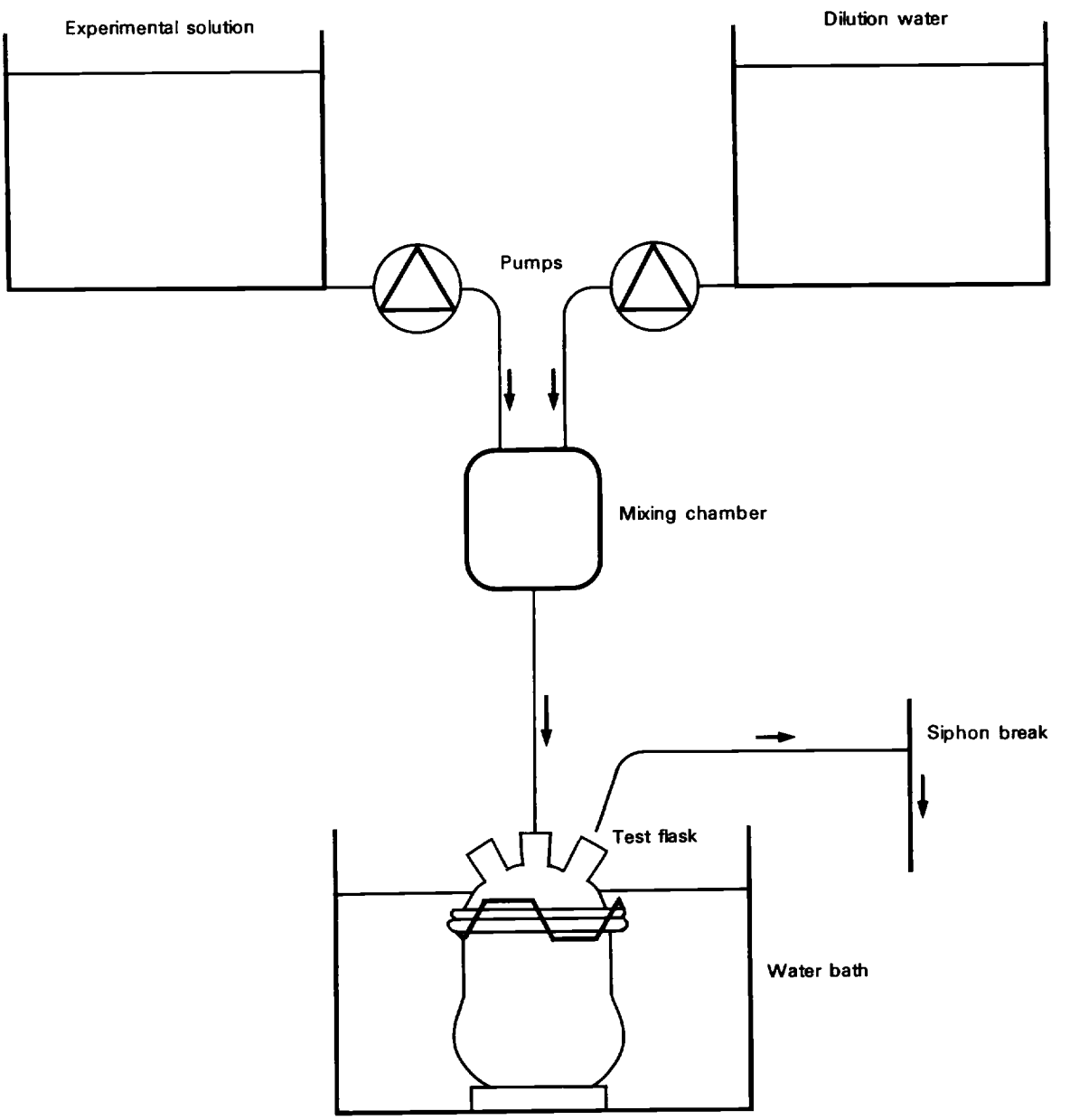
Results are recorded as the cumulative percentage mortalities with time for each concentration. From these data median lethal times are estimated for each concentration by separately plotting the cumulative mortality for each concentration as probits against time expressed on a logarithmic scale. A straight line is fitted by eye to each set of data so that the LT50 value for each concentration can be derived.

Confidence limits for the LT50 values may be calculated using the Nomographic method of Litchfield 1949 (see Sections A4.3.1.2iii and A4.3.2.2ii in the Introductory part of this Booklet).

D7.3 Derivation of the median lethal concentrations at 24, 48 and 96 h

The logarithms of the LT50 are plotted against the logarithms of the test concentration and a line is fitted by eye through the data. The median lethal concentration at 24, 28 and 96 hours are read from the graph.

Fig.D1 DIAGRAM OF A TEST FLASK AND DELIVERY SYSTEM FOR A CONTINUOUS FLOW TOXICITY TEST



E

Protocol for Measuring the Acute Toxicity of Chemicals, Effluents or Polluted Freshwater to the Crustacean, *Daphnia magna* Straus

This protocol should be read in conjunction with Part A, Introduction to Acute Toxicity Testing with Aquatic Organisms

This protocol largely conforms with 'OECD Guidelines for testing chemicals 202 (Daphnia)'. Differences from OECD guidelines are noted where appropriate.

E1 Introduction

An acute lethal toxicity test provides information on the concentration of a chemical that leads to the death of a proportion of the test species under the conditions of the test, and at best this information can be used to give only a first indication of the level at which the chemical may be acutely lethal to that species in the natural environment. It is not generally possible to predict how other species would be affected, nor is the nature, degree or rate of onset of some adverse effect arising from chronic exposure to the chemical predictable from an acute test. On the other hand, acute lethal toxicity tests have a relatively long tradition and experience has shown that results from this type of test have been useful in the management and control of gross levels of water pollution.

Three aspects are particularly important for routine acute testing, namely the effect measured, the species used, and the exposure conditions. There are a large number of effects that can be taken as measures of intoxication, ranging from alterations at the biochemical and physiological level to changes in the behaviour of the individual or between groups of individuals. In acute tests the death of the organism is the effect most frequently used, probably because it is relatively easy and cheap to observe, it is common to all species, and it is an end-point that occurs irrespective of the nature of the toxic substance under test. However, as the precise end-point of death is difficult to determine in *daphnia* tests, the accepted measured effect giving the required degree of standardization is immobilization.

Daphnia magna Straus, (Crustacea : Cladocera) have been used widely for some time for toxicity testing of chemicals and effluents. It occurs commonly in lentic freshwaters of the northern temperate zone, and considerable information is available on its biology, physiology and ecology.

Its small size, relative ease of culture and short generation time allow plentiful supplies of test organisms to be obtained throughout the year even with limited space and facilities. Under suitable conditions, females reproduce parthenogenetically (ie without sexual reproduction) producing only females, thereby minimizing genetic variability of individuals within and between tests, and potentially enabling laboratory "strains" to be established with minimum genetic drift. In addition, animals of known and limited age range can be obtained in large numbers. These practical advantages, and the frequent dominance of the daphnid family (Daphnidae) at the primary consumer trophic level in lake and pond ecosystems, make *Daphnia* a suitable representative of the freshwater crustaceans for use in laboratory toxicity tests.

The test described in this booklet will allow the acute toxicity of substances to *Daphnia magna* in freshwater to be measured.

E2 Objectives

This procedure describes a static test method for measuring in the laboratory the acute toxicity of soluble chemicals, liquid effluents or polluted freshwaters to *Daphnia magna* Straus.

This species can be bred readily in the laboratory allowing supplies of suitable animals to be available at any time of year throughout the world.

Details of the maintenance and breeding of *Daphnia* are given in Section E8.

E3 Test Performance Guide

E3.1 Parameters determined

Median effective concentration at 24 hours and 48 hours. (OECD 24 hrs only)

E3.2 Type of sample

Soluble chemicals, liquid effluents, or polluted natural freshwaters.

E3.3 Basis of method

Measurement of immobilization of *Daphnia* after 24 and 48 hours in a range of concentrations of a chemical or effluent, or in polluted natural waters, under static test conditions with no replacement of test solutions.

E3.4 Test species

Daphnia magna between 2 and 24 hours old.

E3.5 Numbers of animals

For chemicals and effluents, a minimum of 120 animals randomly divided into batches of 20, each batch consisting of 4 replicates, each of 5 animals. One batch is used as the control and the remaining batches are each exposed to one of a range of concentrations.

For natural water samples, batches of 20 animals for the control and each solution.

E3.6 Limitations

The solutions are not changed, therefore the concentration of test material may not remain constant during the course of the test.

The method is not suitable for liquids immiscible in water or for solutions with high levels of settleable solids.

Highly coloured solutions, or solutions that are opaque because of high concentrations of suspended solids, may hinder observation of the *Daphnia.*

High concentrations of solids may envelope or entrap the *Daphnia* causing difficulty in response assessment.

E3.7 **Other measurements**

Temperature, dissolved oxygen, pH and, where applicable, concentration of test chemical.

E3.8 **Time required for test**

Allow operator time of 10 hours spread over three days for each test (excluding chemical analyses).

E4 The Test System

E4.1 **Species**

Daphnia magna, between two hours and twenty four hours old at the start of the test, produced from stock cultures in the third, or later, successive parthenogenetic generation.

E4.2 **Acclimation and storage**

A procedure for maintaining stock cultures of *Daphnia* is given in Section E8. The culture temperature should be 20°C, (the same as that used in testing), and the culture water should, where possible, be of the same composition as the test dilution water.

Not more than 24 hours before starting the test, a stock culture of *Daphnia* containing reproducing adults is sieved through a square-weave nylon mesh of 1350 μm aperture, which retains only the adults. These are transferred to a separate culture vessel containing fresh dilution water and are fed a normal daily ration. No further food is added to this water and the test animals are not fed during the test.

Not less than 2 hours before starting the test, the adults are removed by a second sieving with the same mesh and may be returned to the stock cultures. The young *Daphnia* remaining, which have been produced between the sieving occasions, are the prospective test organisms. Prior to dispensing to the test vessels, they are concentrated within a sieve of 335 μm aperture square-weave nylon mesh, held within a glass vessel such that the *Daphnia* are not held out of water on the mesh at any time.

E4.3 **Test apparatus**

Glass beakers of 250 ml nominal capacity, calibrated at 200 ml, are used as test vessels.
(The OECD require volatile substances to be tested in closed vessels).

E4.4 **Temperature**

The temperature of the test solutions should be 20°C and must not be allowed outside the range 18–22°C.
(The OECD specify a temperature ± 0.5°C within range 18–22°C).

E4.5 **Dilution water**

The dilution water used in testing chemicals should be prepared from deionized or distilled water by the addition of suitable quantities of sodium, potassium, calcium and magnesium salts to give a hardness of 250 mg/l (as $CaCO_3$) and a pH of 7.8 ± 0.2. A suitable dilution water is given in Section E10. If necessary the pH is adjusted using hydrochloric acid or sodium hydroxide solutions.

Dilution water used for testing an effluent should be the natural water receiving the effluent, or the synthetic water described above modified to give a hardness and pH similar to the receiving water.

E4.6 **Preparation of experimental solutions**

Test solutions of chemicals are prepared by the addition of either weighed quantities of the chemical, or volumes of stock solutions of the chemical, to dilution water. Stock

solutions should be prepared in dilution water whenever possible. If stock solutions are prepared in distilled (or deionized) water and the maximum volume to be added to prepare a test solution does not exceed 5% v/v, add an equal volume of distilled (or deionized) water to the control solution. If other solvents are employed (see Section A2.4.2 Paragraph 7) or the addition of a stock solution in distilled (or deionized) water exceeds 5% v/v, then a separate solvent control solution should be prepared in addition to the regular control. This should contain a concentration of the solvent in dilution water equal to the maximum obtained in the preparation of a test solution. The concentration of a solvent other than distilled (or deionized) water should not exceed 0.5 ml/litre in the final test solution. (The OECD maximum solvent concentration is limited to 0.1 ml/litre).

Representative samples of natural waters or effluents should be obtained. If the samples are stored for more than 3 hours or transported over long distances, they should be kept at a temperature around 4°C.

The volume of each test solution should be greater than the volume required to fill the test vessels, to provide excess solution for initial determination of physical and chemical parameters.

Test solutions and dilution water should be at 20°C before use.

E5 Procedure

At least 5 concentrations of a chemical or effluent, and a control, should be tested. A geometric series of concentrations is used. The number and range of concentrations should be chosen such that at each observation time between 10 percent and 90 percent of the animals are immobilized in two or more of the concentrations. A preliminary sighting test should be carried out to select these concentrations. This is performed in an identical manner to the definitive test, except that a wide range of concentrations at 10-fold intervals is tested, and only 10 animals (2 replicates of 5) are used per concentration. (The highest OECD test concentration of chemical is limited to 1 g/litre).

For the definitive test, 200 ml of test solution are added to each of 4 replicate beakers, for each concentration. Five *Daphnia* are added to each beaker, using a Pasteur pipette with a tip of 1.5–2 mm bore, in such a way as to minimize the carry-over of culture water to the test solution. *Daphnia* are similarly added to each of the 4 control beakers containing untreated dilution water.

The test vessels are maintained at a temperature of 20 ± 2°C by use of a constant temperature room, cabinet or water bath. The solutions are not aerated.

After 24 hours and 48 hours the response of each *Daphnia* in each vessel is recorded. Cessation of whole body movement of the animal, over an observation period of 10 seconds, is the criterion of effect, and is termed immobilization. Gentle stirring of the test solution prior to observation can assist the assessment. The numbers of immobilized and mobile animals are recorded for each vessel.

If immobilization in the control exceeds 10%, the results of the test are considered to be invalid.

E6 Parameters Monitored

The dissolved oxygen concentration of the dilution water used for preparation of the test solutions is measured prior to the start of the test, and must be greater than 95% of the air saturation value.

The small volumes of the test solutions prevent their chemical and physical analysis during the test period. Initial measurements of pH should therefore be made using the excess test solution remaining after filling the test vessels.

For known test chemicals, it is desirable to measure each concentration of the test material, again using the excess solutions for initial determinations.

At the end of the test, the pH, dissolved oxygen concentration and, where appropriate, concentration of test material are determined for the control and each test solution immediately after the assessment of the response of the *Daphnia*. If necessary replicate solutions are bulked to provide sufficient sample.

If at the end of the test the dissolved oxygen level in the control or any test solution is less than 70% of the air saturation value the test is considered to be invalid.

The temperature of one of the control solutions should be measured, at 0, 24 and 48 hours, or the temperature of the constant temperature room, cabinet or water bath should be monitored throughout the test.

E7 Results

E7.1 Collection of data

The test report should contain the following information:

E7.1.1 The specification of the test chemical, effluent or water sample, and the method of preparation of stock solutions, test solutions and dilution water.

E7.1.2 All pH, dissolved oxygen and temperature measurements and, where appropriate, measurements of the concentration of test material.

E7.1.3 Details of the source and culture of *Daphnia*, and the age range of the animals used in the test.

E7.1.4 The number of *Daphnia* tested and the number immobilized in each concentration and in the control.

E7.1.5 The calculated 24 hour and 48 hour median effective concentrations (EC_{50} values), with 95% confidence limits, and the method of calculation.

E7.2 Estimation of EC_{50} values and 95% confidence intervals

For each concentration, at each assessment time, the numbers of immobilized *Daphnia* in the replicates are summed and expressed as a percentage.

For each assessment time, immobilization percentages between 5% and 95% are plotted as a function of concentration on a log-probability scale. A straight line is fitted by eye to each set of data, and the nomographic method of Litchfield and Wilcoxon 1949 (see Section A4.3.1.2 in the introductory part of this Booklet) is used to check the fit of the line and estimate the EC_{50} value and its 95% confidence limits.

Alternatively the EC_{50} value and its 95% confidence limits can be calculated mathematically using the probit analysis method of Finney (see Section A4.3 in the Introductory part of this Booklet) particularly if computer facilities are available.

E8 Procedures for the Culture of *Daphnia magna*

E8.1 Introduction

The mode of reproduction of *Daphnia magna* is largely dependent upon the environmental conditions and the availability of food. Under conditions of high food supply, long day length, low population density and "summer" temperatures (> 15°C), the females produce eggs which develop parthenogenetically (ie without fertilization) in the brood pouch, and are released as free swimming young which are all female. Under "winter" conditions, particularly if food supply is low or day length reduced (below ca. 10 hours), a proportion of the young produced parthenogenetically develop as males, and the females produce two eggs which require fertilization. When fertilized these "winter eggs" are enclosed, as a pair, by a dense brown/black case (the ephippium) which is subsequently released by the female. These "ephippial eggs" are resistant to adverse conditions such as drought and cold, and may remain dormant for a period of years. When hatched the young from the ephippia develop as females.

To provide offspring for use in toxicity tests the culture conditions should be such that the population reproduces solely be parthenogenesis, at a steady rate.

Various techniques have been successfully employed for the culture of *Daphnia,* differing principally with respect to the diet provided. The techniques can be broadly divided between those in which mixed and unknown populations of micro-organisms are supplied to (or cultured with) the *Daphnia*, and those in which defined species of micro-organisms (usually unicellular algae), cultured under defined conditions, are supplied (Frear and Boyd, 1967: Murphy, 1970). Methods falling into the latter category are generally preferred for supplying organisms for toxicity testing because of their more defined nature, greater consistency of reproductive mode and rate, and applicability to chronic toxicity testing procedures.

However, despite attempts to define conditions a technique successful in one laboratory often gives poor results when repeated elsewhere. For this reason the procedures described below are intended to be flexible and in some instances alternatives are suggested which may prove useful if difficulties are encountered.

E8.2 Source of *Daphnia magna*

Organisms for initiating cultures can be obtained from field populations or, usually more readily, from populations maintained by other laboratories. The identity of the species should be verified in both cases (see Brooks 1959).

E8.3 Apparatus and Conditions

E8.3.1 *Water*

The water used for culturing should be of the same quality as that intended for use as dilution water in the toxicity tests. (Details and preparation of a suitable water are given in the acute toxicity test protocol and Section E10.

E8.3.2 *Culture vessels*

Glass beakers holding 1 to 1.5 litres of culture water are recommended. By using about 6 such vessels sufficient *Daphnia* can be obtained for 2 or 3 acute tests per week. Larger vessels can be used successfully, but control of the biological and physical conditions within the cultures becomes more difficult to achieve; in particular gentle aeration (not normally required in the smaller beakers), is necessary.

E8.3.3 *Temperature and photoperiod control*

The culture vessels should be maintained at 20 ± 2°C by use of a water bath or temperature-controlled room. The cultures should be protected from direct sunlight, and artificial lighting provided, controlled to give a photoperiod of 16 hours per day. The light intensity is probably not critical. (A pair of 40 watt fluorescent tubes 1 metre from the vessels has proved satisfactory).

E8.4 Food

E8.4.1 *Composition*

Various species of unicellular algae have been used successfully as the sole source of food for *Daphnia*. *Chlorella vulgaris* (Strain 211/12, Culture Centre of Algae and Protozoa, Cambridge) is easy to culture and has given good results. The methods described below would probably be suitable for other strains of *Chlorella*.

Some laboratories have experienced low production in *Daphnia* cultures fed only a single species of alga, using methods which were successful elsewhere. Various supplements to the algal diet have proved successful in correcting these apparent deficiencies, and are described in Section E8.6.

E8.4.2 *Culture of Chlorella vulgaris*

Suitable media for the growth of *Chlorella* are described in Table E1. (p53) As far as possible, all procedures used in culturing the algae should employ normal aseptic techniques in order to exclude bacterial, algal and protozoan contaminants. If an autoclave is not available for the sterilization of apparatus and solutions, alternative methods (see Hamilton, 1973) should be used, giving particular attention to the primary and secondary cultures of the alga.

Secondary cultures of the alga (for inoculation of main cultures to be used for feeding) are maintained in 250 ml conical flasks containing 150 ml of medium, (not aerated but shaken daily) and replenished by successive subculturing at approximately weekly intervals with an inoculum of 1 ml. At 3 to 6 month intervals, or if contamination is suspected, the secondary cultures are restarted from primary cultures. Primary cultures can be maintained either as described for secondary cultures, or on agar plates or slopes (20g agar per litre of medium) subcultured in either case at 3 month intervals.

The main cultures, which provide algae for feeding, should be grown in round-bottom wide-neck glass vessels, preferably fitted with multiple entry lids to provide inlet and outlet for aeration (via cotton-wool or similar filters), an inoculation port, and harvesting tube. As a guide, three 5-litre vessels containing 3 litres of medium, with staggered inoculation dates, will provide adequate algae for the scale of *Daphnia* culture suggested earlier. Main cultures should be inoculated with approximately 2 ml of 6 to 8 days old secondary culture per litre of main culture solution.

Secondary and main cultures should be illuminated continuously (at approx 4000 Lux; 5 to 10 cm from a pair of 40 watt Warm-White fluorescent tubes has proved satisfactory). Normal laboratory temperatures and proximity to such lighting generally gives temperatures close enough to the "optimum" of 22°C. Primary cultures should be maintained under lower light intensities; normal laboratory lighting is usually sufficient.

Main cultures should be harvested after 7 days when the cell density (typically 10^7 cells/ml) is approaching maximum. The harvested algae should be concentrated approximately 10 fold, by settlement or centrifugation, to reduce the volume required for feeding, giving a cell density of ca 10^8 cells/ml. This suspension can be stored for up to 1 week in the dark at 2 to 3°C.

E8.5 Feeding Regime

Feed the *Daphnia* once daily with the concentrated algal suspension at a rate of 2 to 5 ml per litre of culture solution depending on the loading of *Daphnia*. As a guide, the feeding level can be increased provided that the *Daphnia* have filtered the water clear of suspended algae within 24 hours and no significant accumulation of algae is occurring on the bottom of the culture vessel.

E8.6 Supplements to the Algal Diet

If the rate of reproduction of the *Daphnia* is low, or gradually decreases, poor quality of, or a minor deficiency in, the algal diet is a possible cause.

Various supplements to the algal diet have been found, on occasions, to correct the problem. These have included yeast, yeast extract, beef extract and fish fry starter diet preparations. Some, or all, of these supplements may function indirectly, by encouraging a population of undefined micro-organisms to develop in the *Daphnia* cultures. Yeast cells however are ingested by *Daphnia* and probably act as a direct nutritional supplement.

Dried, "active", baker's yeast, fed as a suspension in culture water, at a daily rate of 5mg. dry yeast per litre of culture solution, has proved successful.

E8.7 Daphnia Culture Procedure

The loading of *Daphnia* should not normally exceed 50 adults, or 250 immature animals, per litre of culture, unless the feeding regime and dissolved oxygen levels are more closely monitored and controlled. If reproduction rates are low or ephippia occur, reducing the loading may resolve the problem.

The *Daphnia* culture solutions should be renewed at least twice per week, at which time the *Daphnia* should be sorted by size and excess young discarded. If reproduction rate is high, removal of young from adult cultures between the changes of culture solution may be necessary in order to stay within recommended loading limits.

Nylon, square-weave meshes, of 1350 μm, 710 μm and 335 μm aperture which retain adult, juvenile and newly released animals respectively, are used for sorting and separating young. The procedures are described in the acute test protocol Section E4.2.

E9 References

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Hamilton R D (1973) Sterilization. In : *Handbook of Physological Techniques,* J R Stein, Ed. Cambridge University Press

Murphy J S (1970) *Biol.Bull.***139**, 321–332

E10 Culture and Test Dilution Water

A suitable dilution water, based on the ISO standard, can be made up as follows using analytical grade reagents:

1. Calcium chloride solution ($CaCl_2.2H_2O$). Dissolve 11.76g $CaCl_2 2H_2O$ in deionized water; make up to 1 litre with deionized water.

2. Magnesium sulphate solution ($MgSO_4 7H_2O$). Dissolve 4.93 g $MgSO_4 7H_2O$ in deionized water; make up to 1 litre with deionized water.

3. Sodium bicarbonate solution ($NaHCO_3$). Dissolve 2.59 g $NaHCO_3$ in deionized water; make up to 1 litre with deionized water.

4. Potassium chloride solution (KC1). Dissolve 0.23 g KC1 in deionized water; make up to 1 litre with deionized water.

Distilled water may be used as an alternative to deionized water.

The conductivity of the distilled or deionized water should not exceed 10 $\mu S\ cm^{-1}$.

25 mls each of solutions (1) to (4) are mixed and the total volume made up to 1 litre with distilled or deionized water. The proportion of Ca−:Mg−ions is 4 : 1 and Na−:K−ions, 10 : 1.

Aerate the dilution water until air saturation is achieved, then store it for about two days without further aeration before use. The aerated water should have a pH of 7.8 ± 0.2 and a hardness of 250 mg 1^{-1} expressed as $CaCO_3$.

Table E1 Growth Media for *Chlorella vulgaris*

Medium A

A defined medium, after Adema (1980).

Per litre of distilled water, dissolve:

KNO_3	1.25g
$CaCl_2.2H_2O$	0.01g
$MgSO_4.7H_2O$	0.3g
Ferric citrate.$3H_2O$	0.01g
K_2HPO_4	0.29g
KH_2PO_4	0.19g
Trace-element solution	0.5 ml

Prepare the trace-element solution as follows:

H_3BO_3	2.86g
$MnCl_2.4H_2O$	1.81g
$ZnSO_4.7H_2O$	0.22g
$(NH_4)_2MoO_4$	0.02g
$CuSO_4.5H_2O$	0.079g
$Co(NO_3)_2.6H_2O$	0.49g
Distilled water to	1 litre

Medium B

A simplified medium, but with a tap water inclusion, and therefore the composition and quality are variable, (ICI plc, Brixham Laboratory).

Per litre of a 70:30 mixture of distilled: tap water (which has been autoclaved to sterilize).

add: 4.0 ml of Solution A
1.0 ml of Solution B.

Solution A:

$CaCl_2.2H_2O$	1.0 g
KNO_3	137.5 g
$MgSO_4.7H_2O$	25.0 g
KH_2PO_4	10.0 g
Distilled water to	1 litre

Solution B:

EDTA (disodium salt)	3.6 g
$FeSO_4.7H_20$	5.0 g
Distilled water to	1 litre

Autoclave Solutions A and B and add to previously autoclaved water.

F

Protocol for Measuring the Acute Lethal Toxicity of Chemicals, Effluents or Polluted Sea Water to Rainbow Trout in Sea Water using a Static Test Procedure

This protocol should be read in conjunction with Part A, Introduction to Acute Toxicity Testing with Aquatic Organisms

F1 Introduction

An acute lethal toxicity test provides information on the concentration of a chemical that leads to the death of a proportion of the test species under the conditions of the test, and at best this information can be used to give only a first indication of the level at which the chemical may be acutely lethal to that species in the natural environment. It is not generally possible to predict how other species would be affected, nor is the nature, degree or rate of onset of some adverse effect arising from chronic exposure to the chemical predictable from an acute test. On the other hand, acute lethal toxicity tests have a relatively long tradition and experience has shown that results from this type of test have been useful in the management and control of gross levels of water pollution. They have been extensively used in the examination of complex sewage and industrial effluents discharged to surface waters and the data obtained have been used to supplement chemical analyses in setting consents for the discharge of effluents into waters containing fish.

Three aspects are particularly important for routine acute testing, namely, the effect measured, the species used, and the exposure conditions. There are a large number of effects that can be taken as measures of intoxication, ranging from alterations at the biochemical and physiological level to changes in the behaviour of the individual or between groups of individuals. In acute tests the death of the organism is the effect most frequently used, probably because it is relatively easy and cheap to observe, it is common to all species, and it is an end-point that occurs irrespective of the nature of the toxic substance under test.

The availability of adequate numbers of test organisms of the required size throughout the year, together with ease of maintenance, are major factors which determine which particular species are most commonly used for toxicity testing in sea water. Seemingly

the most desirable species, because of their economic or ecological importance, have often been rejected because of scarcity of difficulties arising during experiments. For example, bivalve molluscs have been generally excluded because of their ability by and large to avoid exposure to the test solutions for an indetermined period merely by tightly closing their shells. This also gives rise to difficulties in defining whether they are dead or dying. Echinoderms are not readily available in large numbers and suitable polychaetes present problems of collection. Several species of fish have been extensively used. Plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) tend to be seasonal in size and abundance; the pogge (*Agonus cataphractus*) restricted in distribution; gobies (*Pomatoschistus minutus, P.microps*) have an intraspecific aggressive behaviour that can make them difficult to hold and use under laboratory conditions.

Whilst it is highly desirable to carry out tests with truly marine fish, it is not always possible because of lack of availability and in these circumstances rainbow trout (*Salmo gairdneri*) may be used. Rainbow trout are essentially a fresh water species and have been used extensively in toxicity tests in fresh water. However, above a minimum size (about 80 g, 20 cm body length), the species can be readily acclimated to fully saline conditions. Tests with rainbow trout acclimated to sea water will indicate the approximate level of the substance that could lead to the death of fish in sea water.

Exposure conditions involve the duration of the experiments and maintaining the test concentrations. A test of four days duration (96 hours) can be carried out during a normal working week and this is probably the reason for its wide usage. A "static" procedure implies that the test solutions are not renewed during the period of the experiment, or are renewed only infrequently on a batch basis and the assumption is made that the initial concentration of substance persists throughout the testing period. The limitations of this type of procedure are that the concentration of active materials may not remain constant but may be reduced by volatilization, degradation or absorption to the test container. Further, the material may well separate from solution, either by rising to the surface or by settling out. In either case, the effect may be greater or lesser than if the substance were present in a homogenous mixture. On the other hand, the advantages of this type of technique are that only very simple apparatus is required, only relatively small volumes of sample and dilution water are used, and it is possible to examine substances, effluents etc, that prove difficult to handle using other methods.

The simple procedure described in this booklet will allow the acute lethal toxicity of substances to rainbow trout to be determined in sea water under static conditions.

F2 Objective

This procedure describes a simple method for measuring in the laboratory the acutely lethal concentrations of soluble substances, liquid effluents or polluted sea water to rainbow trout using a static test procedure. The main advantage compared with other test methods is that only small volumes of samples and sea water are required for the static test procedure.

F3 Test Performance Guide

F3.1 **Parameters determined**

(a) Median lethal time in each concentration.
(b) The median lethal concentrations at 24, 48 and 96 hours.

F3.2 **Type of sample**

Soluble substances, liquid effluents, polluted sea-water.

F3.3 **Basis of method**

Measurement of mortality of fish in a range of concentrations of samples. Solutions are changed daily.

F3.4 **Test species**

Rainbow trout (*Salmo gairdneri*) preferably minimum weight 80g (see Section A2.1 in the Introductory Part of this Booklet).

F3.5 **Numbers of fish**

A minimum of 30 fish randomly selected into batches of 5. One batch used as a control and the remaining 5 batches are exposed to a range of concentrations.

F3.6 **Limitations**

The solutions are changed daily. The concentration of active material may not remain constant during the course of the test. The method is not suitable for liquids immiscible with sea water or for solutions with high levels of settleable solids.

F3.7 **Other measurements**

Temperature, dissolved oxygen, pH, salinity and, where applicable, concentration of test substance.

F3.8 **Time required for tests**

Allow operator times of 10 hours spread over 5 days for each test (excluding chemical analyses).

F4 The Test System

F4.1 **Species**

Rainbow trout (*Salmo gairdneri*) of minimum weight 80g (*ca* 20 cm). The largest fish should not be more than 1.5 times the length of the smallest.

F4.2 **Acclimation and storage**

The stock should be acclimated to the test dilution water over a period of three days and then held for at least a week in water of similar quality and temperature of the dilution water used in the test. The fish should not be fed for 48 hours prior to the test and throughout the test.

F4.3 **Test apparatus**

The test containers should be made preferably of glass although other materials may be acceptable providing there is no effect on the test solution. A minimum of 6 containers is required for each test. Ideally there should be 0.1 – 0.5 l of water for each grams of fish.

Each test solution should be aerated by a gentle flow of air introduced through a diffuser block or, if foaming occurs, through a capillary tube.

F4.4 **Temperature**

The temperature of the test solutions should be 15°C and should not be allowed outside the range 13–17°C.

F4.5 **Dilution water**

The dilution water used in the test should be natural or synthetic sea-water with the following characteristics

Salinity within range	30–35‰
pH	7.8 – 8.1
DO	$\not<$80%$\not>$110% air saturation value
Suspended solids	$\not>$10 mg/l

F4.6 **Preparation of experimental solutions**

Representative samples of the substance for testing should be obtained. If the samples are stored for more than 3 hours or transported over long distances, they should be kept at a temperature around 4°C.

Stock solutions of chemical substances should be prepared daily, unless it is known that the material is stable, in which case sufficient stock solutions are prepared for the whole test. Where possible stock solutions should be made up in dilution sea water. Where additions of effluents etc, to the test media are sufficiently large to significantly reduced the salinity of the test solution a control of an equivalent salinity should be prepared. Other test solutions should be adjusted to this minimum salinity by addition of distilled or suitable tap water.

F5 Procedure

Batches of five fish, randomly selected, are exposed for 96 hours to a geometric series of concentrations of test material together with a control. The concentrations are arranged so that all the fish are killed in a few hours in the highest concentration and no deaths occur in the lowest concentration during the test period. It may be necessary

to carry out the preliminary sighting test to select these concentrations (see Section A2.4.1 in the Introductory Part of this Booklet). As far as possible inspections should be made throughout the test period and dead fish should be removed as soon as they are observed and the mortality recorded. Should the dissolved oxygen fall below 80% of the air-saturation value in any tank, or fish die in the control, the test should be abandoned. In static tests the dissolved oxygen concentration may be controlled by the rate of aeration.

The test solutions should be changed completely every 24 hours. The simplest method is to prepare a spare test container with a new solution and transfer the fish. This can be done sequentially through all concentrations and control.

Cessation of opercular movement for a period of 10 seconds has been selected as the criteria of death.

F6 Parameters Monitored

During the first 6 hours of the test, water temperature, pH and dissolved oxygen should be monitored at hourly intervals to determine whether these are showing progressive changes. A reduction in dissolved oxygen concentration should be countered by increased aeration. Monitoring should continue until conditions are stable, and subsequently immediately before and after changing the test solutions and at the end of the test. Salinity measurements should also be taken at the end of the test.

F7 Results

The recording and calculation of results are discussed in detail in Section 4 of Introductory Part of this Booklet.

F7.1 Collection of data

In the test report, the following data should be presented along with the LT50 or LC50 values:

(a) average length and average weight of fish.
(b) range of length and weight.
(c) number of fish per tank.
(d) volume of test solutions.
(e) period of acclimation.
(f) average and range of: temperature, pH, dissolved oxygen concentration, salinity and, where applicable, test chemical concentration.

F7.2 Estimation of the median lethal time (LT50)

Results are recorded as the cumulative percentage mortalities with time for each concentration. From these data median lethal times are estimated for each concentration by separately plotting the cumulative mortality for each concentration as probits against time expressed on a logarithmic scale. A straight line is fitted by eye to each set of data so that the LT50 value for each concentration can be derived.

Confidence limits for the LT50 values may be calculated using the nomographical method of Litchfield (1949).

F7.3 Derivation of median lethal concentrations at 24, 48 and 96 hours

The logarithms of the LT50 are plotted against the logarithms of the test concentrations and a line is fitted by eye through the data. The median lethal concentrations at 24, 48 and 96 hours are read from the graph.

The results of the acute toxicity tests are expressed in terms of the initial test concentration. If this concentration is not maintained at a constant level (± 10%) then the LC50 value is not valid. It has been proposed by Lloyd and Tooby (1979) that the term LC(I)50 should be used for static tests where the concentration decreases throughout the test period.

F8 References

Litchfield, J T (1949) A Method for rapid graphic solution of time-percent effect curves. *Pharmacol.exp.Ther* **97**: 399–408.

Lloyd, R and Tooby, T E (1979) New terminology required for short-term static fish bioassays, LC(I)50. *Bull.Environ.Contamin.Toxicol.* **22**(1/2): 1–3.

G Protocol for Measuring the Acute Lethal Toxicity of Chemicals, Effluents or Polluted Sea Water to Brown Shrimp using a Static Test Procedure

This protocol should be read in conjunction with Part A, Introduction to Acute Toxicity Testing with Aquatic Organisms

G1 Introduction

An acute lethal toxicity test provides information on the concentration of a chemical that leads to the death of a proportion of the test species under the conditions of the test, and at best this information can be used to give only a first indication of the level at which the chemical may be acutely lethal to that species in the natural environment. It is not generally possible to predict how other species would be affected, nor is the nature, degree or rate of onset of some adverse effect arising from chronic exposure to the chemical predictable from an acute test. On the other hand, acute lethal toxicity tests have a relatively long tradition and experience has shown that results from this type of test have been useful in the management and control of gross levels of water pollution. They have been extensively used in the examination of complex sewage and industrial effluents discharged to surface waters and the data obtained have been used to supplement chemical analyses in setting consents for the discharge of effluents into waters containing fish.

Three aspects are particularly important for routine acute testing, namely, the effect measured, the species used, and the exposure conditions. There are a large number of effects that can be taken as measures of intoxication, ranging from alterations at the biochemical and physiological level to changes in the behaviour of the individual or between groups of individuals. In acute tests the death of the organism is the effect most frequently used, probably because it is relatively easy and cheap to observe, it is common to all species, and it is an end-point that occurs irrespective of the nature of the toxic substance under test.

The availability of adequate numbers of test organisms of the required size throughout the year, together with ease of maintenance, are major factors which determine which particular species are most commonly used for toxicity testing in sea water. Seemingly the most desirable species, because of their economic or ecological importance, have often been rejected because of scarcity or difficulties arising during experiments. For example, bivalve molluscs have been generally excluded because of their ability by and large to avoid exposure to the test solutions for an indetermined period merely by tightly closing their shells. This also gives rise to difficulties in defining whether they are dead or dying. Echinoderms are not readily available in large numbers and suitable polychaetes present problems of collection. Several species of fish have been widely used and several species of crustacean appear suitable, the brown shrimp (*Crangon crangon* = *C.vulgaris*) has been selected because of its extensive use.

The brown shrimp occurs abundantly around the United Kingdom coast, especially in shallow waters and estuaries over sandy and muddy substrates. In deeper waters it can occur together with the closely related and similar *Crangon aldmani.* The brown shrimp can be readily caught using a small beam trawl from a boat or push net fitted with nets of mesh size less than 10 mm. It is recommended that the duration of tow should be restricted to less than 5 minutes to avoid excessive damage to the catch. The whole catch should be carefully placed in a bin of water, sorted immediately and all shrimps transferred to clean water except for animals less than about 30 mm total length, ovigerous females and females which are conspicuously damaged (opaque areas on the abdominal muscle) or recently moulted (soft skinned) which should be discarded with the rest of the catch. **Care should be taken in sorting the catch since *Crangon* species often occur along with the lesser weaver fish, *Trachinus viperus,* which can inflict serious poisoning. As this fish can bury itself in wet sand if caught by a low tide and sting if trodden on, avoid walking barefoot on wet sand in places where it may occur.** Prior to and during transport to the laboratory, and in the laboratory, the shrimps should be kept at densities low enough to ensure that the dissolved oxygen level of the water does not fall below 80% saturation (usually aeration will be required) and the water temperature does not rise or fall by more than 3°C. The animals should be held within the laboratory for at least 6 days prior to use during which time they should be gradually acclimated to test salinity and temperature. Dead, damaged and newly moulted animals should be discarded. If more than 50% of the animals die within the first 3 days of capture, or if alternatively there is a continuing mortality greater than 5% per day, then the stock should not be used.

The animals should be fed (see Section G8) during the holding period. Feeding should be stopped 2 days prior to starting acute toxicity tests and food withheld throughout the test period. Only animals between 30 mm and 50 mm in length (from the tip of the antennal scale to the tip of the telson) should be used. Knotless nets may be used to capture the animals in the holding tanks or they can be transferred by wet hands. The use of forceps for transferring live animals should be avoided.

Setting exposure conditions involves the duration of the experiments and the maintaining of the test concentrations. A test of four days duration (96 hours) can be carried out during a normal working week and this is probably the reason for its wide usage. A "static" test procedure implies that the test solutions are not renewed during the period of the experiment, or are renewed only infrequently on a batch basis and the assumption is made that the initial concentration of substance persists throughout the testing period. The limitations of this type of procedure are that the concentrations of active materials may not remain constant but may be reduced by volatisation, degradation or absorption to the test container. Further, the material may well separate from solution, either by rising to the surface or by settling out. In either case, the effect may be greater or lesser than if the test substance were present in a homogenous mixture (see Section A3.1). On the other hand, the advantages of this type of technique are that only very simple apparatus is required, only relatively small volumes of sample and dilution water are used, and it is possible to examine substances, effluents etc, that prove difficult to handle using other methods.

The simple procedure described in this part will allow the acute lethal toxicity of substances to brown shrimp to be determined under static conditions.

G2 Objective

This procedure describes a simple method for measuring in the laboratory the acutely lethal concentrations of soluble substances, liquid effluents or polluted freshwaters to brown shrimp using a static test procedure. The main advantage compared with other test methods is that only small volumes of samples are required for the static test procedure.

G3 Test Performance Guide

G3.1 Parameters determined

(a) Median lethal time in each concentration.
(b) The median lethal concentrations at 24, 48 and 96 hours.

G3.2 Type of sample

Soluble substances, liquid effluents, polluted sea water.

G3.3 Basis of method

Measurement of mortality of shrimps in a range of concentrations of samples. Solutions are changed daily.

G3.4 Test species

Brown shrimp (*Crangon crangon*), length 30 – 50 mm (see G4.1)

G3.5 Numbers of animals

A minimum of 120 shrimp randomly selected into batches of 20. One batch used as a control and the remaining 5 batches are exposed to a range of concentrations.

G3.6 Limitations

The solutions are changed daily. The concentration of active material may not remain constant during the course of the test. The method is not suitable for liquids immiscible with sea water or for solutions with high levels of setteable solids.

G3.7 Other measurements

Temperature, dissolved oxygen, pH, salinity and, where applicable, concentration of test substance.

G3.8 Time required for tests

Allow operator times of 10 hours spread over 5 days for each test (excluding chemical analyses).

G4 The Test System

G4.1 Species

Brown shrimp (*Crangon crangon*) of average length between 30 and 50 mm. The largest shrimp should not be more than 1.5 times the length of the smallest. Ovigerous females, newly moulted ("soft") animals or any animal showing opaque musculature should not be used. Length is measured straightened, excluding antennae.

G4.2 Acclimation and storage

Healthy stock should be held for at least a week in running water of similar quality and temperature of the dilution water used in the test. The shrimps should not be fed for 48 hours prior to the test and throughout the test.

G4.3 Test apparatus

The test containers should be made preferably of glass although other materials may be acceptable providing there is no effect on the test solution. A minimum of 6 containers is required for each test and ideally there should be 0.5 litre of water for each gram of shrimp.

G4.4 Temperature

The temperature of the test solutions should be 15°C and should not be allowed outside the range 13–17°C.

G4.5 Dilution water

The dilution water used in the test should be natural or synthetic sea-water with the following characteristics:

Salinity within range	30–35‰
pH	7.8 – 8.1
Dissolved oxygen	$\not<$80% $\not>$110% air saturation value
Suspended solids	$\not>$10 mg/l

G4.6 Preparation of experimental solutions

Representative samples of natural waters of effluents should be obtained. If the samples are stored for more than 3 hours or transported over long distances, they should be kept at a temperature of around 4°C.

Stock solutions of test chemical substances should be prepared daily, unless it is known that the material is stable, in which case sufficient stock solutions are prepared for the whole test.

G5 Procedure

Batches of 20 shrimps randomly selected, are exposed for 96 hours to a geometric series of concentrations of test material together with a control. The concentrations are arranged so that all the shrimps are killed in a few hours in the highest concentration and no deaths occur in the lowest concentration during the test period. It may be necessary to carry out the preliminary sighting test to select these concentrations (see Section A2.4.1 in the Introductory part of this Booklet). As far as possible, inspections should be made throughout the test period and dead shrimps should be removed as soon as they are observed and recorded.

Shrimps can be defined as dead if they do not respond, usually by leg or pleopod movement, when gently prodded.

Should the dissolved oxygen fall below 80% of the air-saturation value in any tank, the results from this tank should be discounted. The dissolved oxygen concentration may be increased by increasing the rate of aeration.

The test solutions should be changed completely every 24 hours. The simplest method is to fill a spare container with a new solution and transfer the shrimps. This can be done sequentially through all concentrations and control.

Cannibalism can occur with *Crangon* (newly moulted animals being particularly vulnerable) and this has the effect of reducing the test population during the course of the test. There are a number of methods for making allowance for this mortality; but since it is not always clear that cannibalism has been the cause of death such techniques are not recommended. In practice the effect of cannibalism can be ignored and all mortalities regarded as due to the substance under test. This procedure results in a slight underestimation of ET50 values, and hence a slight over-estimation of toxicity. If more than 20% of the control animals die during the course of the test if should be abandoned.

Cannibalism can be reduced if the stock animals are fed (see Section G8), but the test animals should not be fed during the test.

G6 Parameters Monitored

During the first 6 hours of the test, water temperature, pH and dissolved oxygen should be monitored at hourly intervals to determine whether these are showing progressive changes. A reduction in dissolved oxygen concentration should be countered by increased aeration. Monitoring should continue until conditions are stable, and subsequently immediately before and after changing the test solutions and at the end of the test.

G7 Results

The recording and calculation of results are discussed in detail in Section A4 of the Introductory part of this Booklet.

G7.1 Collection of data

In the test report, the following data should be presented along with the LT50 or LC50 values:

(a) average length and average weight of shrimps
(b) range of length and weight
(c) number of shrimps per tank
(d) volume of test solutions
(e) period of acclimation
(f) average and range of: temperature, pH, dissolved oxygen concentration, salinity and, where applicable, test chemical concentration.

G7.2 Estimation of the median lethal time (LT50)

Results are recorded as the cumulative percentage mortalities with time in each concentration. From these data median lethal times are estimated for each

concentration by separately plotting the cumulative mortality in each concentration as probits against time expressed on a logarithmic scale. A straight line is fitted by eye to each set of data so that the LT50 value for each concentration can be derived.

Confidence limits for the LT50 values may be calculated using the nomographical method of Litchfield (1949).

G7.3 Derivation of median lethal concentrations at 24, 48 and 96 hours

The logarithms of the LT50 are plotted against the logarithms of the test concentrations and a line is fitted by eye through the data. The median lethal concentrations at 24, 48 and 96 hours are read from the graph.

The results of the acute toxicity tests are expressed in terms of the initial test concentration. If this concentration is not maintained at a constant level (± 10%) then the LC50 value is not valid. It has been proposed by Lloyd and Tooby (1979) that the term LC(I)50 should be used for static tests where the concentration decreases throughout the test period.

G8 Food suitable for shrimps

In order to reduce cannibalism and to maintain a healthy stock of test organisms it is recommended that they are fed during the acclimation and holding period.

Ingredients: 90g raw whiting flesh (pulverised)
10g frozen adult brine shrimp (pulverised)
22g gelatine (29g for temperatures above 15°C)
6ml full strength pilchard oil
22ml hot water

Preparation: Dissolve the gelatine in the hot water, add the pulverised whiting flesh and brine shrimp and stir well. Add the pilchard oil and stir vigorously to obtain an emulsion. Allow the mixuture to cool, stirring occasionally to avoid the contents setting. When the mixture is almost set pour into flat moulds approximately 1 cm deep.

The mixture will keep for about 7 days in the cool part of a refrigerator. It is removed as required and cut into approximately 1 cm cubes. It is fed at the rate of approximately 2–5% total body weight of animals present per day.

G9 References

Litchfield, J T (1949) A Method for rapid graphic solution of time-percent effect curves. *Pharmacol.exp.Ther.* **97**: 399–408.

Lloyd, R and Tooby, T E (1979) New terminology required for short-term static fish bioassays, LC(I)50. *Bull.Environ.Contamin.Toxicol.***22**.(1/2): 1–3.

H A Rapid Bioassay Method for Detecting Acutely Toxic Concentrations of Substances Poisonous to Rainbow Trout in Fresh or Saline Waters

This booklet should be read in conjuction with Part A, Introduction to Acute Toxicity Testing with Aquatic Organisms

H1 Introduction

A method is described for detecting under field conditions or in the laboratory whether contaminated fresh or saline waters are acutely toxic to rainbow trout. Compared with conventional 96 hr toxicity tests, the method is relatively quick, since it is completed within eight hours. Further advantages compared with traditional methods are that fewer fish are required, many water samples may be tested since only a small volume is used in each test and only simple equipment is necessary.

The test depends on the fact that fish placed in sealed containers lower the dissolved oxygen content of the water and this enhances the lethality of toxic substances in the water so that the fish die quickly. Thus, in waters containing toxic substances, the residual oxygen will be higher at the time the fish die than in the controls which contain water free of toxic substances (see Carter 1962). A feature of the rapid bioassay test examined over many years is that the majority of results correlate well with the results of conventional 96 hr acute lethal toxicity tests. (see Ballard and Oliff 1969, Vigers and Ellis 1976, and Vigers and Maynard 1977). Thus the rapid bioassay is very useful as a preliminary sighting test before using other rainbow trout toxicity test protocols.

The method is defined as a bioassay since it allows the potency of a solution of unknown toxic properties to be determined by measuring the degree of response to a predetermined effect.

H2 Objective

The objective of this test is to detect substances which are acutely poisonous to rainbow trout in fresh or saline waters.

Compared with other methods the results are obtained quickly and only the minimum of apparatus and volume of test solution is required. The test may be applied in both field and laboratory investigations.

H3 Test Performance Guide

H3.1 Parameters determined

The residual dissolved oxygen in test solutions and controls, upon death of all the animals.

H3.2 Type of sample

Natural waters, (fresh, estuarine or seawater), effluents or solutions prepared in the laboratory.

H3.3 Basis of method

Fish are placed in each of a number of bottles containing solutions to be tested. As the fish respire, the dissolved oxygen concentration is reduced until a point is reached when the fish die. As lowering of dissolved oxygen enhances toxicity, fish die more quickly in acutely lethal solutions. This is reflected by the presence of relatively high concentrations of oxygen after the death of the fish. Thus acutely poisonous substances are detected because the concentration of dissolved oxygen at the time of death (residual dissolved oxygen or RDO) is higher in test solutions containing toxic levels of chemicals than in controls free of toxic substances. Blanks are also run to correct for any spontaneous depletion in oxygen content due to other causes than fish respiration.

H3.4 Test species

Rainbow trout (*Salmo gairdneri*).

H3.5 Numbers of fish

At least eight fish are required for testing one sample.

H3.6 Limitation

The toxicity of all substances is not enhanced to the same degree by a fall in dissolved oxygen. Therefore some potential pollutants may not be identified so readily as others. eg respiratory poisons such as cyanide are much more readily detected than dissolved metals.

Solutions with a high oxygen demand may give anomalous results.

H3.7 Other measurements

Temperature, pH, and where applicable, water hardness, chlorinity and concentrations of pollutants.

H3.8 Time required for test

Allow operator time of 2 hours spread over a normal working day (excluding chemical analyses).

H4 The Test System

H4.1 Species

Rainbow trout of a preferred weight between 1.0 and 10 grams. The largest fish should not be greater than 1.5 times the length of the smallest.

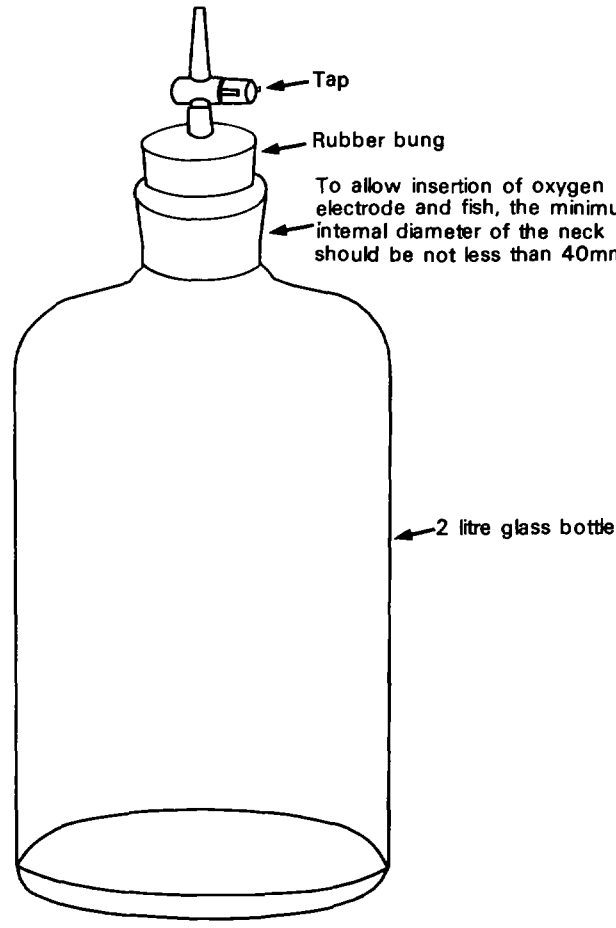
H4.2 Acclimation and storage

Healthy stock should be held for at least one week in clean running water of similar temperature to that of the test. The fish should not be fed for 48 hrs prior to the test. Experiments have shown that differences in salinity of test solutions have little or no effect on the ability of fish to remove oxygen within the period of the test.

H4.3 Test apparatus

The test containers are 2 litre glass jars fitted with rubber bungs through which a small tap has been inserted (see fig 1). A minimum of 6 containers is required for each test (2 controls 2 test solutions and 2 blanks). A meter is required for measuring pH. Preferably, a meter with a combined temperature and reference electrode should be used for the measurement of dissolved oxygen and temperature. The electrode head must be sufficiently small to pass through the neck of the containers and the oxygen demand of the instrument should be insignificant compared with oxygen levels to be measured. Alternatively the Winkler method and a mercury thermometer may be used for measuring oxygen and temperature.

Fig.H 1 RAPID BIOASSAY TEST CONTAINER



H4.4 **Temperature**

The water in the test containers should be kept at a relatively constant temperature (preferably between 13 and 17°C) throughout the test period. However, when operating in the field this is often difficult, but temperature fluctuations can be reduced by immersing the containers in the natural water body. In the laboratory the test containers should be immersed in a water bath set at 15°C.

H5 Procedure

At least four containers are filled with the test solution and a further two with water of similar quality but free of toxic substances to serve as controls. Measurements are made of the dissolved oxygen, temperature and pH of each solution. The level of dissolved oxygen must be greater than 80% of the air saturation value at the start of the test and where necessary this can be achieved by shaking the test solution in a sealed glass vessel containing air.

At least two fish are placed in each of the four or more containers, those filled with the test solution and the two controls. A weight volume ratio of 5–10 grams of fish per litre of water allows a survival time of ca 3–10 hours for the controls. The same number of fish should be added to each container. If the only fish available weigh more than 10 grams they may be used providing larger containers are employed and the 5–10g/l ratio is maintained.

All the containers are then stoppered avoiding air bubbles, the displaced solution being vented through the tap in the stopper which is then closed.

Two additional containers filled with test solution without any fish are used as blanks to test for reduction of dissolved oxygen by bacterial decomposition of organic matter. The concentrations of dissolved oxygen in these containers are measured at the start and end of the test period.

As described in H3.4 the temperature of the containers should be kept constant until the fish die (cessation of opercular movement). This is usually within 6 hours, depending upon the temperature of the solution: solutions below 10°C usually require longer periods of time. Exposed fish should be free from disturbance by the operator.

H6 Parameters Monitored

The dissolved oxygen, pH and temperature are measured when the solutions are prepared and again immediately after the test fish have died in the containers. If an oxygen meter is used, it is important to maintain an adequate movement of water over the dissolved oxygen electrode during the measurement. When using Winkler titrations to determine dissolved oxygen, water samples should be siphoned off and care taken to minimize re-aeration.

H7 Results

As soon as all the fish are dead in a container the concentration of the residual dissolved oxygen (RDO) is measured and the results recorded. Subsequently the RDO in the test containers are compared with that in the controls. Typical RDO values for controls lie in the range 1.0 – 2.0 mg/l. The greater the differential between the controls and test solutions the more acutely toxic is the solution. Similar RDO's in both the test solutions and controls indicate that death is due to asphyxiation and the solution is not acutely toxic to rainbow trout under the conditions of the test (see Table H1.).

Solutions having a high oxygen demand, as indicated by a large reduction in oxygen concentration in the blanks over the test period, will give anomalous results, and more care is required in the interpretation of the results.

Table H1 Table of typical results

All oxygen concentrations in ppm

Source/nature of sample	Control RDO	Sample RDO	Blank Oxygen Demand test	Interpretation
(1) river water	1.6, 1.8	4.8, 4.2	0.4, 0.2	***
(2) river water	1.2, 1.5	1.3, 1.4	0.2, 0.5	*
(3) river water	1.5, 1.3	1.0, 0.8	5.0, 5.6	High oxygen demand in blank invalidates results.
(4) river water	1.2, 1.4	2.2, 1.9	0.3, 0.5	**
(5) river water	1.1, 1.3	1.0, 1.4	2.3, 2.5	* The oxygen demand in the blank indicate that care is needed when interpreting this result.
Cyanide solutions (ppm)				
0.01	1.8, 1.4	1.5, 1.9		*
0.025		2.8, 2.1		**
0.05		2.7, 3.2		**
0.10		4.8, 5.1		***
0.20		7.8, 7.3		***
0.30		8.5, 7.9		***
0.50		8.7, 8.1	0.1, 0.0	***

Key:
- * No evidence of acutely toxic concentration
- ** Some evidence of acutely toxic concentration
- *** Positive evidence of acutely toxic concentration

H8 References

Ballard J A and Oliff W D (1969) A rapid method for measuring the acute toxicity of dissolved materials to marine fishes. *Water Research* 3: 313–333.

Carter L (1962) Bioassay of trade wastes. *Nature* **196**, No 4861 p. 1304.

Vigers G A and Ellis B M (1976) Assessment of the toxicity of landfill leachates by the residual oxygen bioassay. *Environmental Protection Service. Technical Report* NO EPS–AR–77 I, Halifax, Canada.

Vigers G A Maynard A W (1977) The residual oxygen bioassay: A rapid procedure to predict effluent toxicity to rainbow trout. *Water Research* **2**: 343–346.

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Any enquiries about the details of the foregoing techniques should be addressed to:

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