Short-term Ecotoxicological Method Guidelines for Effluent and Receiving Water Assessment (Draft)

Technical Report
E83

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R&D Technical Report E83

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Publishing Organisation:
Environment Agency
Rio House
Waterside Drive
Aztec West
Almondsbury
Bristol BS32 4UD

Tel: 01454 624400 Fax: 01454 624409

ISBN: 1 85705 115 7

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This report is the result of work jointly funded by the Environment Agency and the Scotland and Northern Ireland Forum for Environmental Research (SNIFFER).

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Statement of use
This report provides standard operating procedures, associated analytical control and sampling procedures for selected short-term exposure ecotoxicological methods. These are for use in the Agency's environmental protection areas such as effluent control and environmental monitoring and are specifically developed for implementation within the Direct Toxicity Assessment effluent control initiative. These guidelines are currently in draft and will be reviewed following the DTA effluent control demonstration programme.

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EXECUTIVE SUMMARY

The Environment Agency, Scotland & Northern Ireland Forum for Environmental Research (SNIFFER) and manufacturing and water industries are working collaboratively to develop procedures and methods to control and monitor complex waste materials using biological effects tests. Most research effort to date has focused on controlling and monitoring point source discharges to water. As part of this programme the Agency is identifying and developing ecotoxicological methods which are considered appropriate for effluent control. Initially effort has been directed towards selecting well-developed short-term exposure tests which will be used to target the worst-case, most toxic, discharges.

The selection of appropriate short-term exposure tests began by evaluating available tests against a number of selection criteria relevant to the application of effluent and receiving water assessment. The development of criteria and test method selection was conducted as part of a previous project (Project 494) and is described in R&D Note 389 "Identification of screening, lethal and sublethal toxicity tests for assessing effluent toxicity". Standard guidelines were required for the selected tests which were appropriate for application to whole effluent assessment and receiving water monitoring. The need for standard guidelines and internal and external quality systems for successful implementation of regulatory ecotoxicity testing (see project 493 R&D Technical Report P166) has been highlighted by the USEPA in their whole effluent toxicity testing programme (WET).

This report is the product of the selection of appropriate methods and the production of test method guidance. Guidance is given on algal growth inhibition tests, the *Daphnia magna* immobilisation test, the oyster embryo larval development test and juvenile fish lethality tests. The tests are based on national and international guidelines developed for pure chemical or environmental sample testing. The test guidelines and associated guidance on culture or maintenance methods differentiate critical steps which must be followed from those where a procedure is recommended but other approaches are allowed. They are accompanied by a glossary of terms and an appendix giving a list of suppliers of test organisms and equipment. The guidance given in the report has been prepared on the basis of comments received on a previous version during a consultation exercise (Environment Agency 1997) and the output from a workshop on the DTA Methods Guidelines organised by the DTA Methods Working Group. The Workshop was held at Sundridge Park, Bromley on the 22-23 July 1997 and was attended by representatives of the regulators, industry, consultancies, testing houses and academia (DTA Demonstration Programme 1998). In the revised guidelines considerable emphasis has been placed on the culture or maintenance of test organisms since the DTA Methods Guidelines Workshop emphasised the importance of conducting test procedures with 'healthy' organisms.

The DTA Methods Guidelines in the report are to be used initially in the DTA Demonstration Programme (Project 094) to screen and characterise effluent toxicity and to assess receiving water column toxicity. The guidelines, which are currently in draft format, will be modified following the DTA Demonstration Programme to incorporate any comments or improvements recommended by testing laboratories that have been using the guidelines during the project. They will then be transferred into the appropriate technical guidance format. In the future the Agency National Centre for Ecotoxicology & Hazardous Substances will be producing testing guidelines for screening methods and methods to measure long-term sub-lethal effects for

effluent and receiving waters. Guidelines for sediment and *in-situ* testing of receiving waters will also be produced.

KEY WORDS

Direct Toxicity Assessment (DTA), effluents, receiving waters, aquatic, ecotoxicology, test guidelines, Short-term exposure.

1. INTRODUCTION

The use of ecotoxicological methods to provide data for deriving and monitoring toxicity-based limits and for assessing receiving water column toxicity has to be carried out using standardised procedures to ensure the quality and integrity of the data generated in the course of the study (Gawadi 1990). A project was, therefore, initiated with the aim of producing Direct Toxicity Assessment (DTA) Methods Guidelines to meet the requirements of the Environment Agency and organisations within SNIFFER.

The DTA Methods Guidelines are to be used initially in the DTA Demonstration Programme to screen and characterise effluent toxicity and to assess receiving water column toxicity. The guidance given in the current document has been prepared on the basis of comments received on a previous version during a consultation exercise (Environment Agency 1997) and the output from a workshop on the DTA Methods Guidelines organised by the DTA Methods Working Group. The Workshop was held at Sundridge Park, Bromley on the 22-23 July 1997 and was attended by representatives of the regulators, industry, consultancies, testing houses and academia (DTA Demonstration Programme 1998).

In the revised guidelines considerable emphasis has been placed on the culture or maintenance of test organisms since the DTA Methods Guidelines Workshop emphasised the importance of conducting test procedures with 'healthy' organisms. Guidance is given on algal growth inhibition tests, the *Daphnia magna* immobilisation test, the oyster embryo larval development test and juvenile fish lethality tests.

The test guidelines and associated guidance on culture or maintenance methods differentiate critical steps which must be followed from those where a procedure is recommended but other approaches are allowed. They are accompanied by a glossary of terms and an appendix giving a list of suppliers of test organisms and equipment.

The guidelines given in the manual will be updated in due course following consideration of the results of the Demonstration Programme.

1.1 References

DTA Demonstration Programme (1998) Direct Toxicity Assessment (DTA) Demonstration Programme: Proceedings of the DTA Methods Workshop: Method Guidelines and Register of Approved Laboratories for Effluents and Receiving Water Assessment. 22-23 July 1997, Sundridge Park, Bromley, pp 45.

Gawadi, N. (1990) Standard operating procedures. In: *Good laboratory and clinical practices. Techniques for the quality assurance professional* (Eds P.A. Carson and N.J. Dent), Heinemann Newnes, pp 67-83.

Environment Agency (1997) The application of toxicity-based criteria for the regulatory control of wastewater discharges - Response Compendium, pp 52.

2. COLLECTION, TRANSPORT, STORAGE AND TREATMENT OF AQUEOUS ENVIRONMENTAL SAMPLES

2.1 Introduction

It is vital that aqueous environmental samples (such as effluents, leachates and receiving waters) taken for toxicity testing are considered representative and that the procedures adopted for the collection, storage and preparation of samples ensure that the toxicity of the sample obtained at source does not change markedly before a test is conducted.

This section describes general procedures for the collection, storage, preparation and disposal of environmental samples for toxicity tests. Procedures specific to a test method are given in the relevant sections of this volume of the DTA Methods Guidelines. All test reports should detail the collection, storage and preparation procedures adopted for the test substance being assessed.

2.2 Collection of Environmental Samples

2.2.1 Sampling point

The sampling of effluents or leachates for toxicity screening, discharge characterisation or monitoring against a toxicity-based limit should be carried out at the designated end of pipe points currently used for sampling for chemical analysis[1]. The sampling of receiving waters should be carried out at locations that are appropriate to the purposes of the study and representative of fully mixed conditions.

2.2.2 Sampling method

In the DTA Demonstration Programme spot samples of effluents, leachates or receiving waters are to be taken (DTA Demonstration Programme 1998). Environmental samples shall be collected in accordance with existing regulatory procedures in the National Sampling Procedures Manual (Environment Agency 1997) for obtaining samples for chemical analysis where these are appropriate. If these procedures are not considered acceptable, guidelines drawn up by the Standing Committee of Analysts (HMSO 1993) or the general procedures specified in ISO document 5667-2 (ISO 1993) should be followed.

[1] However, in some instances it may be necessary to identify other locations which are consistent with the objectives of the study. For example, when assessing toxicity at sewage treatment works samples may need to be collected from points within the plant.

2.2.3 Sample containers

Environmental samples should be collected in containers made of materials certified by manufacturers as being inert. The containers should be either new or cleaned thoroughly and rinsed at least three times with the sample to be collected. For volumes of up to 25 litres, samples should be normally be collected in one or a series of amber glass screw top bottles[2]. If a series of bottles is used the samples must be pooled before testing commences to ensure homogeneity of the samples. The minimum sample volume collected should be 1 litre. If volumes larger than 25 litres are required appropriate vessels such as 25 litre HPDE or unlacquered stainless steel drums should be used. Containers must be filled completely to minimize any air space into which volatile components of the sample could diffuse.

2.2.4 Volume of sample required

The volume of effluent, leachate or receiving waters which needs to be collected depends on the testing system used and on the number of concentrations tested. Three types of testing regime can be used:

1. Static, in which the test solutions are prepared manually by adding the environmental sample and the dilution water together at the beginning of the test;

2. Semi-static, which are similar to static tests, but with renewal of the test solutions on a predetermined schedule, for example every 24 h. The renewal can be carried out using fresh aliquots of the original sample or freshly collected samples;

3. Flow-through, which uses a continuous supply of test substance and dilution water, if required. Flow-through systems may be more resource intensive than static or semi-static systems, require complex delivery systems and large volumes of the environmental sample and dilution water.

Table 2.1 shows the volumes of environmental samples needed to conduct specific toxicity tests during effluent characterisation with a defined concentration range. The algal growth inhibition tests, *Daphnia* immobilization tests, oyster embryo-larval development tests and marine copepod lethality tests use static exposure regimes and all require comparatively small sample volumes (<2 litres) for a test with 10 exposure concentrations.

[2] If the discharge contains appreciable levels of metals and these as thought to be responsible for toxicity then polyethylene bottles should be used instead of amber glass bottles.

Table 2.1 Volumes of effluents, leachates or receiving waters required to conduct each type of test during effluent characterisation with example concentration ranges

Type of test	Test procedure	Number and range of environmental samples concentrations	Test solution volume	Minimum sample volume required	
				Per day	During test
Algal growth inhibition	Static	10 (0.1,0.22,0.46,1.0,2.2,4.6,10,22,46,100)	1 litre	-	1868 ml
Daphnia immobilization	Static	10 (0.1,0.22,0.46,1.0,2.2,4.6,10,22,46,100)	500 ml	-	934 ml
Oyster embryo larval development	Static	10 (0.1,0.22,0.46,1.0,2.2,4.6,10,22,46,100)	100 ml	-	187 ml
Fish lethality	Semi-static	10 (0.1,0.22,0.46,1.0,2.2,4.6,10,22,46,100)	10 litres	18.7 litres	74.8 litres

The DTA Methods Guidelines advocate the use of semi-static (or if appropriate static) juvenile fish lethality tests which may necessitate the collection of 75 l of sample for tests with the concentration range given in Table 2.2.

2.2.5 Monitoring of physico-chemical parameters in test samples

Measurements of the basic physico-chemical properties of the discharge given in Table 2.2 should be made both in the field at the time samples are collected and on receipt at the laboratory. Samples must be labelled with the name and location of the discharge, the date and time the sample was taken, the duration of sampling, the initials of the sampler and the number of the chain of custody record form.

Table 2.2 Parameters to be measured in the field at the time samples are collected and on receipt at the laboratory

Physico-chemical parameters to be measured at the different locations	
In the field at the sampling location	On receipt at the testing laboratory
pH	pH
Temperature	Temperature
Dissolved oxygen	Dissolved oxygen
Conductivity or salinity	Conductivity or salinity
Colour	Total hardness (if appropriate)
Whether the discharge is an emulsion	Suspended solids
Description of the sample	Ammonia

Guidance on appropriate ways of measuring these parameters and the way in which the data should be expressed is given in relevant Standing Committee of Analysts (SCA) Methods for the Examination of Waters and Associated Materials (see Table 2.3). This information shall be recorded on the Chain of Custody Record (see Appendix 2A).

Table 2.3 Relevant Standing Committee of Analysts (SCA) Methods for the Examination of Waters and Associated Materials for use in measuring physico-chemical parameters

Parameter	Report number	Year of publication
Conductivity, pH	14	1978
Dissolved oxygen	16	1979
Total hardness, calcium hardness and magnesium hardness	43	1981a
Colour and turbidity	103	1981c
Suspended, settleable and total dissolved solids	105	1980b
Colour	119	1988a
pH	120	1988b

2.3 Transport and storage

Samples should be transported to the testing facility within 24 hours and testing should commence as soon as possible after collection. Testing must be started within 48 h of the end of sampling. It is recommended that initial characterisation studies on an effluent or leachate address the issue of sample stability and temporal changes in the toxicity of collected samples. These factors have relevance both for the process of toxicity testing and for the assessment of the environmental impact of the discharge. If the rate of loss of toxicity from a sample is extremely high, it should be recognised that this will influence environmental impact and in such instances the calculation of available dilution may need to take into account the possibility that the loss of chemical toxicity may mitigate effects more rapidly than physical dilution processes.

Samples must be kept in the dark during transport and the sample temperature should not deviate markedly (± 2 °C) from that at the time of collection. The temperature history of the samples from collection to arrival at the testing facility should be recorded using disposable temperature recorders (see Appendix A).

Samples should be accompanied by triplicate blanks (for example, reference water samples) to allow cross contamination in transit to be identified. Containers of effluent, leachate or receiving water samples must also be accompanied by a sample custody record sheet (see Appendix 2A).

Samples of effluents, leachates or receiving waters requiring rapid testing should be adjusted to the required temperature for the relevant toxicity test(s) immediately on receipt at the test facility. If the sample is not to be tested immediately, it shall be stored in darkness at 2 to 8 °C.

2.4 COSHH assessment

Before any testing is carried out a COSHH assessment shall be prepared for the sample(s) by the discharger. Samplers must have access to these data prior to sampling and the COSHH assessments must be part of the Chain of Custody/Duty of Care documentation.

2.5 Preparation of samples for toxicity tests and bioassays

The extent to which environmental samples are treated prior to testing depends on the objectives of the study and should be the subject of discussions between the regulator and the discharger. There are two approaches:

1. testing of samples unadjusted to gain information on the total biological effects including the influence of physico-chemical parameters such as pH, dissolved oxygen, suspended solids and turbidity, hardness or salinity and colour. This approach could mean that in certain instances it will not be possible to carry out certain methods because physico-chemical parameters will fall outside the limits specified for the procedures (see Table 2.4);

Table 2.4 Threshold criteria for different physico-chemical parameters in test solutions in various test procedures

Test procedure	Threshold criteria for different physico-chemical parameters in test solutions					
	pH	Dissolved oxygen	Hardness (mg $CaCO_3$ l^{-1})	Salinity (‰)	Suspended solids (mg l^{-1})	Colour
Algal growth inhibition		NA	NA	≥27-36‰	<2[1]	No value, can affect growth
D. magna immobilisation	7.4-8.5	≥60% ASV in lowest concentration causing 100% immobilisation	140-250	-	<20	No value, can affect ability to observe organisms
OEL	7.8-8.5		-	≥22-36‰	<20	No value, can affect ability to count organisms
Marine copepod lethality	7.8-8.5	≥60% ASV in lowest concentration causing 100% lethality	-	≥18-36‰ for *Acartia* and ≥20-36‰ for *Tisbe*	<20	No value, can affect ability to observe organisms
Fish lethality	6.0-8.5	≥60% ASV in lowest concentration causing 100% lethality	10-250	≥27-36‰	<25	NA

NA - Not applicable
[1] - US EPA recommend filtering samples for algal growth inhibition tests through 0.45 μm membrane filters (US EPA 1994)

2. adjusting either the sample or specific test solutions so that all physico-chemical parameters specified for a particular method are met (see Table 2.4). Modification of the sample or test solutions will remove the influence of these parameters and will reflect residual chemical toxicity.

In selecting an approach there is an issue of how representative a test methodology might be of conditions in the environment and whether the sample was being modified to meet test requirements with a subsequent loss of environmental realism. The influence of physico-chemical parameters will typically be more pronounced for effluents and leachates than receiving waters where dilution may have occurred. Furthermore, problems of sample or test solution treatment for physico-chemical parameters will generally only become important if toxicity occurs at higher effluent concentrations. For samples where toxicity is evident at lower effluent concentrations, dilution with reference water will often mean that physico-chemical parameters in the test solutions meet the test method criteria.

In the DTA Demonstration Programme it is recommended that in the first instance samples should be tested without treatment and that measurements are made of all the key physico-chemical parameters (DTA Demonstration Programme 1998). Table 2.3 provides guidance on Standing Committee of Analysts (SCA) methods for physico-chemical parameters and the way in which the data should be expressed.

If one or more physico-chemical parameters are deemed to cause a given response (fully or partially) then these may need to be modified in subsequent tests (see Figure 2.1). Where treatment is necessary this should, wherever possible, be restricted to required test solutions rather than the whole sample. However, in certain instances it may be necessary to modify the whole sample and test both adjusted and unadjusted samples.

The following sections provide guidance on how to modify physico-chemical parameters in test solutions or samples to satisfy test method criteria.

pH

The pH of acidic test solutions (or samples) should be modified with 1M sodium hydroxide solution and that of alkaline test solutions (or samples) with 1M hydrochloric acid. Analytical grade reagents should be used in the preparation of the solutions. Certain test solutions or samples (for example effluent or leachate samples with highly buffered pH) may require the use of stronger acid or base solutions. Aliquots of test solutions (or samples) receiving pH-adjustment should be allowed to equilibrate after each incremental addition of acid or base (Abernethy and Westlake 1989). The amount of time required will depend on the buffering capacity of the test sample or solution. For test solutions of effluents or leachates, a period of 5 to 10 minutes should be sufficient. Test solutions which have been pH corrected should only be used when the pH has stabilised.

The greater buffering capacity of seawater compared to freshwater means that sample pH will generally have to be more extreme to affect the pH of exposure concentrations in marine tests compared to freshwater tests.

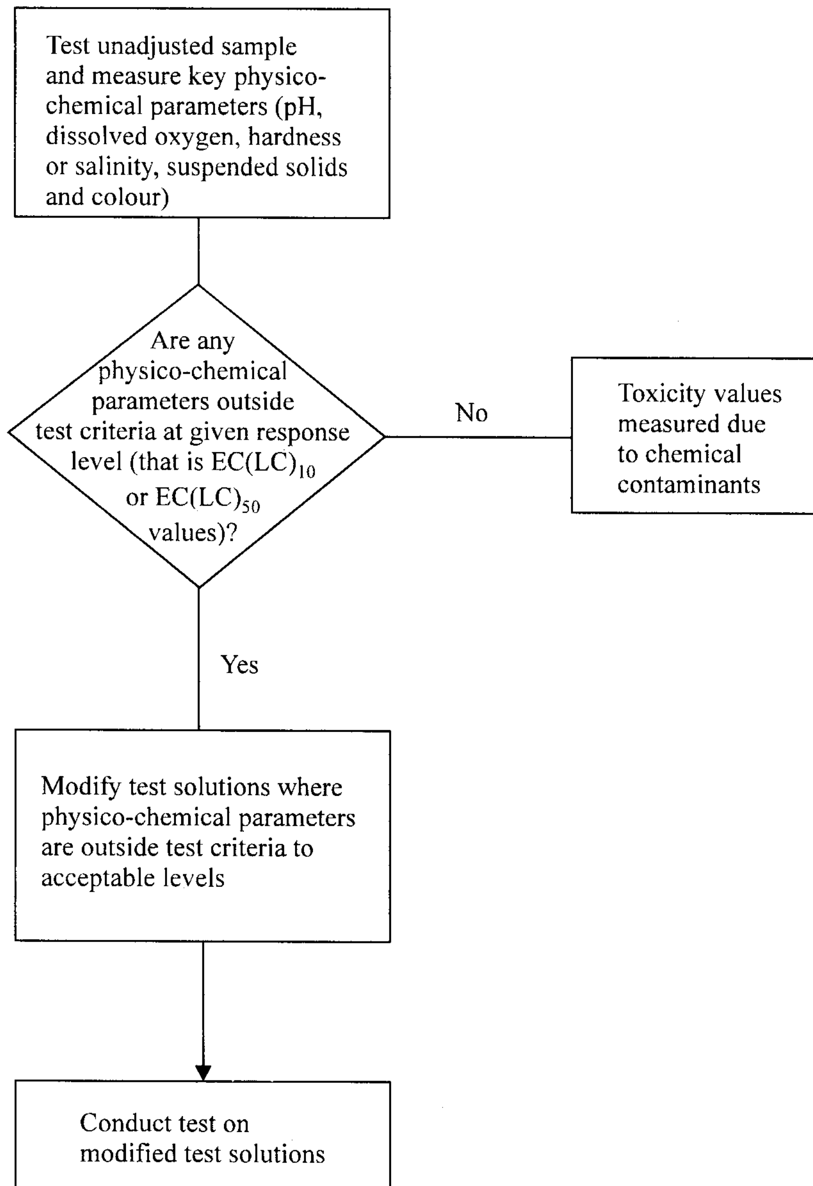

Figure 2.1 Flowchart describing the testing strategy for effluents and leachates

Dissolved oxygen

If the measured dissolved oxygen concentration in any of the test solutions is <60% or >100% of air saturation, these solutions should be pre-aerated. To achieve this, oil-free compressed air should be dispensed through a clean silica-glass air diffuser or disposable glass pipette. Any pre-aeration of test solutions or samples should be at a rate within the range of 25 to 50 ml min^{-1} l^{-1}. The duration of pre-aeration should be restricted to a period not exceeding 30 minutes. Any pre-aeration of test solutions or samples should be discontinued following this period and the test initiated, regardless of whether 40 to 100% saturation was achieved. Test solutions with sufficient oxygen (≥60% air saturation) should not be pre-aerated.

Hardness

The hardness of the test solutions of effluents or leachates (or undiluted effluent, leachate or receiving water samples) should be adjusted to within 20% of that of the test organisms culture or holding water using an appropriate hard or soft uncontaminated water.

Salinity

The salinity of test solutions of effluents or leachates (or undiluted effluent, leachate or receiving water samples) should be adjusted if they are below the salinity thresholds for the test species being used (see Table 2.4). The initial salinity of test solutions (or environmental samples) should be measured using a salinometer or another appropriate device and recorded. The adjustment should be carried out by the addition of sea salt (for example, Sigma Chemical Co.) to achieve the threshold salinity criteria. A sea salt control should be used in addition to the normal control. Salinity controls should be used if the effluent sample is tested at salinities <27 ppt. The salinity adjusted test solutions or environmental samples should be left at least 2 h to equilibrate prior to testing.

Suspended solids

Suspended solids should be removed in most instances by allowing the test solutions (or samples) to settle until there has been a noticeable reduction in the suspended solids. If there has been no apparent clearing of the sample after 2-4 h, an alternative method should be used such as:

1. filtering through a 0.45 µm cellulose acetate or cellulose nitrate filter using a vacuum filtration unit attached to a Buchner flask;

2. centrifuging samples at 5000-10 000 g for 15-60 minutes using a suitable centrifuge. Centrifuging samples at low speed (3000-5000 g) for longer periods (60 mins) can be used as an alternative to a short high speed spin (10 000 g for 15 mins). Samples should ideally be centrifuged in a cooled centrifuge to avoid any effects of temperature on the test substance.

Filtration and centrifugation may have different effects on the chemistry of test solutions (or samples) and the same procedure should be used when testing a series of samples from a location.

Coloured samples

Colour correction methodologies for algal growth tests are being considered by EU and ISO working groups (Comber *et al.* 1995, ISO 1998) and are described in the relevant sections of this document.

Emulsions

Test solutions should be shaken or stirred to produce a homogenous test solution. Further information can be obtained from Whitehouse and Mallet (1993) and ECETOC (1996).

2.6 Disposal of samples

Disposal of wastes shall be in accordance with the appropriate regulations such as the Environment Protection (Duty of Care) Regulations (1997) and the Special Waste Regulations (1996 as amended).

A COSHH assessment will have been carried out on the environmental sample before use and this should be used to determine the most suitable disposal route, taking into account the quantities involved.

- Substances with a high or extreme COSHH hazard rating will require disposal by a specialist contractor, via an appropriate member of staff.

- If the COSHH hazard rating for a substance is low, then disposal to an on-site sewage treatment works or sewer may be acceptable. However, if large quantities of waste material require disposal, special arrangements may be needed. In the case of disposal to the STW, it is not only important that hazardous chemicals are not released into a receiving water, but also that the STW is not overloaded either in terms of volume or organic loading rate. Where discharge volumes are high there may be a requirement for individual laboratories to obtain discharge consents or even undertake some form of primary treatment prior to discharge.

- Substances with a medium COSHH hazard rating will generally require disposal by a specialist contractor, unless the quantities involved are very small. However, if in doubt, the advice of an appropriate member of staff should be sought.

2.7 References

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APPENDIX 2A SAMPLE CUSTODY RECORD

CHAIN OF CUSTODY RECORD (FOR THE SAMPLING, TRANSPORT AND RECEIPT OF SAMPLES)

A) Sample Details (to be completed by sampler or following analysis at laboratory*)

Sample Details: Site Code or NGR: Place: Sample of:

Date taken: Time taken: Sample No: Receipt No (if applicable)

Sample pH: Sample temperature (°C): Sample dissolved oxygen level:

Sample hardness (mg $CaCO_3$ l^{-1})*: Sample conductivity (µS cm^{-1}) or salinity (‰): Sample suspended solids (mg l^{-1}):

Sample colour: Is sample an emulsion (Y/N):

Date and time sample deposited at depot*/handed to / /199 at hrs Mins

By (print name): Signature: * delete as appropriate

b) Transportation Details (to be completed by Courier)

Person or Depot Collected from	Date	Time		Name of Courier	Signature	Employed by	Deposited at/ handed to
		Hrs	Mins				

c) To be completed at laboratory

Sample received at (laboratory): Date: Time: Hrs Mins

Sample received by (print name): Signature:

Were seals intact? Y/N Lab number allocated: * Delete as appropriate

3. TESTING STRATEGY

The type of method(s) used to test environmental samples (such as effluents, leachates and receiving waters) and the experimental design adopted (for example number of exposure concentrations, interval between test concentrations and test duration) will depend on the objective of the study, which should be clearly defined at the outset. Guidance is given on the experimental design of toxicity tests for discharge characterisation and monitoring of toxicity-based limits and for assessing receiving water column toxicity.

3.1 Effluent and leachate testing

3.1.1 Discharge characterisation

The test design used for discharge characterisation has to consider the variability of the effluent or leachate and the practicalities of testing these unstable test substances. It is important to recognise that the constraints which apply to effluent or leachate testing are not the same as those applying to pure chemicals testing. For pure chemicals the test substances is typically consistent in character and the test concentrations used to estimate toxicity values can be progressively narrowed by an iterative process of preliminary and definitive testing. In contrast, for a given sample of an effluent or leachate, potential changes in toxicity over time mean that for tests using algae, invertebrates and fish there is only the opportunity to carry out a single definitive test. Consequently, the test design used has to be capable of providing sufficient data to derive the toxicity value(s) of interest, whilst avoiding all or nothing responses between consecutive concentrations where ever possible.

If tests are conducted to produce toxicity data to characterise discharges, 10-11 concentrations with ≤2.2 x difference between concentrations should be used in the first two tests on the discharge. The concentrations should span the range from 0.1 to 98-100% v/v test substance, for example 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 22.0, 46.0 and 98-100%. An interval of ≤2.2-fold between concentrations is necessary to allow precise toxicity (EC_{50} or LC_{50} values, EC_{10} or LC_{10} values and NOEC and LOEC) values to be calculated. If the discharge is highly acidic or alkaline it may be necessary to carry out tests on uncontrolled and pH corrected samples.

If the effluent or leachate demonstrates consistent toxicity then subsequent tests may use a modified design with 5-6 concentrations around the endpoint(s) of interest which will allow precise toxicity values to be calculated. If the discharge is of variable toxicity it may be necessary to continue with the larger number of concentrations to ensure precise toxicity values can be determined in each test.

3.1.2 Monitoring against toxicity limits

If tests are conducted to monitor a sample against a toxicity-based limit then a single concentration test may be used. In the test, responses of test organisms at the single effluent or leachate concentration (toxicity limit) are compared with those of test organisms in an appropriate control.

3.2 Assessing receiving water column toxicity

Tests (bioassays) on receiving water samples should initially be carried out using a control and the undiluted sample. In the bioassay, the responses of test organisms in the sample are compared with those in an appropriate control(s). Additional testing using a full concentration range test can be carried out if this is deemed appropriate.

4. ALGAL GROWTH INHIBITION TEST GUIDELINE

4.1 Introduction

This section of the DTA Methods Guidelines describes the procedures for the culturing of freshwater and marine algae and for conducting toxicity tests using such species to measure the effects of effluents, leachates and receiving waters on growth (and potentially mortality). The document has been compiled with reference to existing internationally recognised standard procedures (OECD 1984; ISO 1989, 1995; EC 1990; Environment Canada 1992).

Critical steps in the culturing and test procedure which **must** be followed are identified in bold type whereas instructions given in normal type are recommended and alternatives can be used.

4.2 Procedure for culturing freshwater and marine algae

4.2.1 Introduction

The purpose of culturing freshwater or marine algae using the following procedure is to provide algal cultures for toxicity tests and bioassays.

Suitable methods **must** be used to ensure that the presence of bacteria in algal cultures is minimised. Axenic cultures are preferred and unialgal cultures are essential. All operations should be carried out under sterile conditions to avoid contamination with bacteria and other algae.

4.2.2 Test species

The test species acceptable for use in toxicity tests of effluents, leachates and receiving waters are given in Table 4.1. These species have been selected on the basis of practical criteria, such as:

- fast growth rate;
- convenience for culturing and testing;
- relative sensitivity.

The species listed in Table 4.1 should be obtained from a recognised supplier (see Appendix A).

Table 4.1 Algal species recommended for growth inhibition toxicity tests and bioassays

Recommended species	CCAP number	Recommended test temperature (°C)	Freshwater/ seawater
Raphidocelis subcapitata (formerly *Selenastrum capricornutum*)	278/4	23 ± 1	Freshwater
Skeletonema costatum (Greville) Cleve	1077/3	20 ± 1	Seawater

Raphidocelis subcapitata is a ubiquitous non-motile, unicellular, crescent-shaped (40 to 60 μm^3) green alga (Chlorophyceae). This alga can be easily cultured in the laboratory and is readily available from reliable suppliers. Its uniform morphology makes it ideal for enumeration with an electronic particle counter. Clumping seldom occurs in *Raphidocelis* because it is free of complex structures and does not form chains. Growth is sufficiently rapid to accurately measure cell yield after 72 h, and the species is moderately sensitive to toxic substances (Lewis 1995).

Skeletonema costatum is very abundant in coastal waters and is generally considered to be sensitive to toxicants (Walsh 1988, Nyholm and Kusk 1990). *Skeletonema* is easily cultured in natural seawater, but is more difficult to maintain in synthetic seawater (Kusk 1989). There can be problems in estimating the density of *Skeletonema* cells from particle counts as cell size may vary greatly (Bonin *et al.* 1986) and this species forms chains of variable length, diameter and cell number. Therefore, the procedure adopted for measuring cell density (directly or indirectly) **must** take account of the number of cells in the chains in each test concentration (see Section 4.3.7).

4.2.3 Preparation of nutrient media

The appropriate medium to culture the algae **must** be prepared as described in the following sections. Care **must** be taken to ensure there is no contamination of the media. Information on the preparation of the algal culture medium **must** be recorded on an Algal Culture Medium Preparation Data Sheet (for example, see Table 4A.1).

Nutrient medium for freshwater algal toxicity tests and bioassays

The preparation of a nutrient medium for freshwater algal toxicity tests and bioassays initially involves setting up a series of four stock solutions using analytical grade reagents according to Table 4.2. The water used in the preparation of the nutrient medium **must** be distilled or deionized or be of an equivalent quality, with a conductivity less than 5 $\mu S\ cm^{-1}$. Special care **must** be taken to avoid contamination of the water by inorganic or organic substances during preparation and storage. Copper apparatus **must** not be used.

Table 4.2 Stock solution used in the preparation of the nutrient medium recommended for use in toxicity tests and bioassays with freshwater algae[1]

Nutrient	Concentration in stock solution	Final concentration in test solution
Stock solution 1 : Macro-nutrients		
NH_4Cl	1.5 g l^{-1}	15 mg l^{-1}
$MgCl_2.6H_20$	1.2 g l^{-1}	12 mg l^{-1}
$CaCl_2.2H_20$	1.8 g l^{-1}	18 mg l^{-1}
$MgSO_4.7H_20$	1.5 g l^{-1}	15 mg l^{-1}
KH_2PO_4	0.16 g l^{-1}	1.6 mg l^{-1}
Stock solution 2 : Fe-EDTA		
$FeCl_3.6H_20$	80 mg l^{-1}	80 μg l^{-1}
$Na_2EDTA.2H_20$	100 mg l^{-1}	100 μg l^{-1}
Stock solution 3 : Trace elements		
H_3BO_3	185 mg l^{-1}	185 μg l^{-1}
$MnCl_2.4H_2O$	415 mg l^{-1}	415 μg l^{-1}
$ZnCl_2$	3 mg l^{-1}	3 μg l^{-1}
$CoCl_2.6H_20$	1.5 mg l^{-1}	1.5 μg l^{-1}
$CuCl_2.2H_20$	0.01 mg l^{-1}	0.01 μg l^{-1}
$NaMoO_4.2H_20$	7 mg l^{-1}	7 μg l^{-1}
Stock solution 4 : $NaHCO_3$		
$NaHCO_3$	50 g l^{-1}	50 mg l^{-1}

The stock solutions are sterilised by membrane filtration (mean pore diameter 0.2 μm) or by autoclaving (115-121 °C for 15 minutes) and stored in the dark at 2-6 °C. Stock solution 4 **must** be sterilised only by membrane filtration as autoclaving would lead to decomposition of the $NaHCO_3$ to Na_2CO_3 and CO_2. These stock solutions are diluted to achieve the final nutrient concentrations in the test solutions.

One litre aliquots of the medium are prepared by adding 10 ml of stock solution 1 and one ml of stock solutions 2, 3, and 4 to a 1-litre volumetric flask and making up to the mark with distilled, deionized or another appropriate water.

[1] For all solutions, care must be taken to avoid precipitation of any component on mixing. If this occurs and the precipitation does not redissolve on dilution a new solution should be made up without the precipitating compound which is then added only after dilution to 950 ml. The whole is then shaken and no precipitate should occur. It can then be made up to 1 litre.

The nutrient medium should be freshly prepared before each test and should be equilibrated by either leaving the media overnight in contact with air, or by bubbling air filtered through a membrane filter (for example Microflow filters) into the solution for at least 30 minutes.

The pH of the medium after equilibration with air should be 8.25 ± 0.45. If necessary, adjust to the required pH using either 1M hydrochloric acid or 1M sodium hydroxide solution.

Nutrient medium for marine algal toxicity tests and bioassays

The preparation of the low EDTA ISO nutrient medium is described. This medium is required to avoid potential problems of deriving erroneously low toxicity values for metalliferous environmental samples due to the chelation of metals by the higher EDTA levels in the standard ISO medium.

Stock solutions are prepared using analytical grade reagents according to Table 4.3. The nutrient medium should be prepared using a reference (natural or reconstituted) seawater. Natural seawater **must** be collected from a `clean' coastal or offshore site and transported to the laboratory in clean inert (polyethylene or polypropylene) containers. If the seawater is to be stored, this should be in sealed black containers. The water **must** be filtered through a 0.2 µm cellulose acetate or cellulose nitrate filter before use. A synthetic seawater, such as Tropic Marin or Instant Ocean (see Appendix A) could be used but this increases the preparation time and cost of tests.

Table 4.3 Stock solutions required for the preparation of ISO nutrient medium[1]

Nutrient	Concentration in stock solution		Final concentration in test solution	
Stock solution 1 - Trace elements				
Na_2 EDTA.$2H_2O$	6.67	mg l^{-1}	90	µg l^{-1}
$FeCl_3.6H_2O$	5.3	mg l^{-1}	16.5	µg Fe l^{-1}
$MnCl_2.4H_2O$	144	mg l^{-1}	605	µg Mn l^{-1}
$ZnSO_4.7H_2O$	4.4	mg l^{-1}	15	µg Zn l^{-1}
$CoCl_2.6H_2O$	0.404	mg l^{-1}	1.5	µg Co l^{-1}
$CuSO_4.5H_2O$	0.157	mg l^{-1}	0.6	µg Cu l^{-1}
H_3BO_3	1140	mg l^{-1}	17 100	µg l^{-1}
Stock solution 2 - Vitamins				
Thiamin hydrochloride	50	mg l^{-1}	25	µg l^{-1}
Biotin	0.01	mg l^{-1}	0.005	µg l^{-1}
B_{12}	0.1	mg l^{-1}	0.05	µg l^{-1}
Stock solution 3				
Na NO_3	50	g l^{-1}	50	mg l^{-1}
K_3 PO_4	3	g l^{-1}	3	mg l^{-1}
Na_2 $SiO_3.5H_2O$	14.9	g l^{-1}	14.9	mg l^{-1}

Stock solutions 1 and 3 are sterilised by autoclaving (115-121 °C for 15 minutes) and stock solution 2 is sterilised by membrane filtration (mean pore diameter 0.2 µm). All stock solutions are stored in the dark at 2-6 °C. The pH of the medium after equilibration with air should be 8.0 ± 0.2.

4.2.4 Preparation of an algal stock culture

On receipt of an algal slope from a supplier, relevant information should be recorded on an Algal Inoculum Receipt and Preparation Data Sheet (for example, see Table 4A.2). The "starter" culture of *Raphidocelis*, depending on its source, may be on an agar slope, in liquid culture or suspended in a gel matrix. The "starter" culture must be aseptically transferred to and resuspended in a defined growth medium to maintain a stock culture of algae as a source for the toxicity tests. The "starter" algae can be stored in the dark at 4 °C and will remain viable for up to 6 months.

Liquid stock cultures

Prepare the appropriate nutrient medium for the test species as described in Section 4.2.3. Place the filter-sterilised medium into sterile Erlenmeyer flasks with sterile stoppers. The sterile liquid growth medium can be stored in the dark at 4 °C for up to 6 months. The volume of growth medium will be determined by the total quantity of algal cells required for a toxicity test. A volume-to-flask ratio of 20% for the growth medium is recommended to avoid growth inhibition due to carbon dioxide limitation. For example: 25 ml medium in a 125 ml flask; 50 ml medium in a 250 ml flask; 100 ml medium in a 500 ml flask.

Aseptically transfer either 1 ml of the "starter" algal culture using a disposable sterile pipette or one colony using a sterile loop to the liquid growth medium in the Erlenmeyer flask. Incubate the algal stock cultures at the appropriate temperature (23 ± 1 °C for *R. subcapitata* and 20 ± 1 °C for *S. costatum*) under continuous "cool white" fluorescent light with an intensity of 6000-10 000 at the surface of the flask (light quantal flux should approximate 60 to 120 $\mu E/(m^2.s)$). The flask with the algae should be placed on a continuous shaker at 100 rpm or shaken manually twice daily. The algal culture may take 7 to 14 days to reach the exponential growth phase.

The culture should be renewed on a weekly basis (between 4 and 7 days post-inoculation) to ensure a regular supply of exponentially growing algal cells. This can be accomplished easily by aseptically transferring 1.0 ml of a stock algal culture to a flask containing fresh liquid growth medium. Purity of the stock culture should be verified at each transfer by examining a subsample under a microscope for contamination by micro-organisms and by transferring 1 ml of algal stock culture to Petri dishes containing solid bacterial nutrient medium (for example, Standard Plate Count Agar), and incubating at 37.5 °C for 48 h. This procedure should reveal the presence of contaminating bacteria that cannot be detected microscopically, even at high magnifications.

Solid stock cultures

To ensure culture purity, periodically (for example, once every two months) streak plate algal cells from a liquid culture onto sterile solid growth medium. The solid growth medium can then be used to isolate colonies of algae to generate pure liquid stock algal cultures.

To prepare the solid growth medium, make up the appropriate liquid growth medium described in Section 4.2.3. Add 1% agar and heat to dissolve. Sterilize by autoclaving at 98 kPa (1.1 kg cm^2) and 121 °C for 30 min or 10 min l^{-1}, whichever is longer. Aseptically pour into Petri plates, cover, and leave to cool. Petri plates with solid growth medium can be stored upside down, in the dark, and at 4 °C, for up to 3 months.

Under aseptic conditions, and using streak-plate procedures, transfer algal cells from a liquid culture onto sterile solid growth medium. Incubate the plates upside down until colonies are visible (approximately 2 weeks). Store at 4 °C in the dark for future use. A fresh liquid stock algal culture should be started each month from an algal colony isolated from the solid growth medium. Cells will remain viable for up to three months if the colonized Petri plates are stored in the dark at 4 °C.

4.2.5 Quality of test organisms

Routine microscopic examination of the stock algal culture **must** be carried out to evaluate culture health in terms of cell morphology and colour, clumping and contamination of the culture by micro-organisms. Performance and culture health is evaluated by routinely measuring the rate of growth in stock cultures.

4.2.6 Pre-cultures

The pre-culture is intended to provide an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test and used during the exponentially growth phase of the algae, which is normally after an incubation period of three days. Algal cell density in the inoculum **must** be measured and recorded on an Algal Pre-Culture Preparation Data Sheet (for example, see Table 4A.3). The inoculum **must** also be examined microscopically and discarded if this reveals that algal cultures contain deformed or abnormal cells.

4.3 Guidelines for toxicity tests on effluents and leachates measuring effects on growth in freshwater and marine algae

4.3.1 Introduction

Algae exhibit several responses to toxicants (Figure 4.1) and while growth inhibition is the usual response of algae exposed to toxicants, stimulation and mortality can occur, particularly in tests with effluents (Claesson 1984).

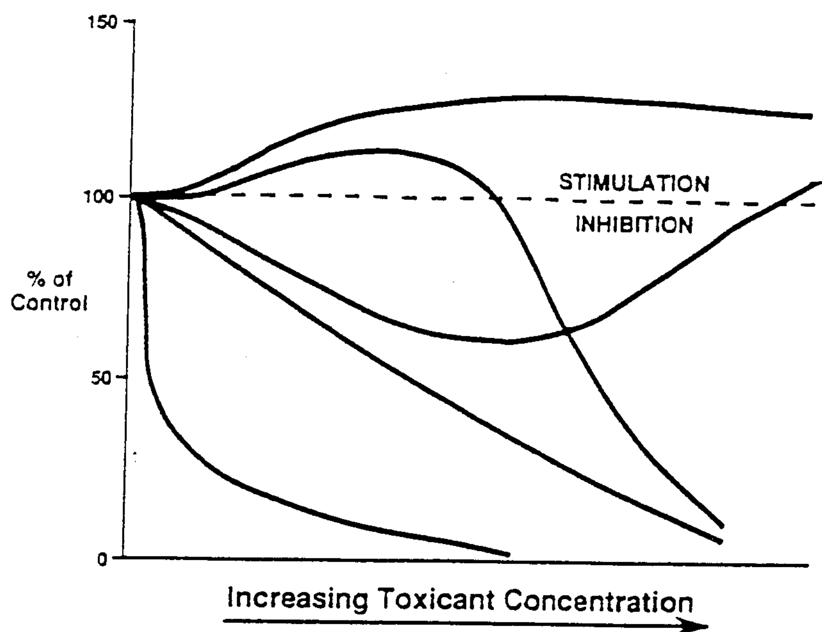

Figure 4.1 Representative response patterns of algae during exposure to toxicants in laboratory toxicity tests

Algae grow rapidly and can, therefore, recover quickly if not killed by a short-term exposure during transitory episodic events. Therefore, algistatic and algicidal effects may be as environmentally meaningful in some cases as the usually reported calculations such as the EC_{50} value which represents a partial reduction in growth rate and biomass (Payne and Hall 1979, Hughes *et al.* 1988). Consequently, it may be necessary in certain instances to establish the effects of environmental samples on both algal growth and mortality.

4.3.2 Scope of the procedure

Applications

This procedure describes a toxicity test (see glossary of terms) for the determination of the effects to unicellular freshwater and marine algae of treated and untreated industrial and sewage effluents and leachates (after either settlement, centrifugation or filtration if necessary) and receiving waters.

The experimental design adopted (for example number of exposure concentrations, interval between test concentrations and test duration) will depend on the objective of the study, which **must** be clearly defined at the outset.

Limitations

The results of toxicity tests with both freshwater and marine species can be affected by the pH, suspended solids content, colour and presence of chelating and absorptive materials in the samples. The testing of freshwater discharges to marine waters may require the use of salinity correction procedures (see Section 4.3.8).

4.3.3 Principle

In the toxicity test procedure (see Section 4.3.9), exponentially growing unialgal cultures are exposed to the environmental sample (effluent, leachate or receiving water) diluted with nutrient medium to a range of concentrations for a period of 72 h. The inhibition of growth (as the change in biomass or growth rate - see glossary of terms) over several generations, in relation to a control culture, is determined in a static system under defined conditions. The different test concentrations in an appropriate range may, under otherwise identical test conditions, exert effects on algal growth. These will extend from no inhibition of growth at lower test concentrations to complete inhibition or stimulation of growth (and possibly mortality) at higher test concentrations (Figure 4.2)[2].

The data shall be used to determine, where possible:

- the median inhibitory concentration, that is the concentration that inhibits algal growth by 50% after 72 h relative to the controls. This is referred to as the 72 h-EC_{50};
- the concentration that inhibits algal growth by 10% after 72 h relative to the controls. This is referred to as the 72 h-EC_{10};
- the highest no-observed effect concentration after 72 h (that is the NOEC);
- the lowest observed effect concentration after 72 h (that is the LOEC);
- the concentration that kills 10% of the initial algal inoculum after 72 h relative to the controls (that is the 72 h-LC_{10}).

[2] For effluents, leachates and receiving waters, algal tests may be measuring both the toxicant-induced inhibition of algal growth (and mortality) and also nutrient-induced stimulation of algal growth. The data derived as a result of these opposing effects may be difficult to interpret, particularly for samples of moderate to low toxicity (EC_{50}) between 10 and 100% v/v) where the nutrients in the samples are not well diluted. Stimulation is considered an important effect in several of the published test methods but few offer guidelines for its calculation and interpretation. The test method of US EPA (1994) describes the calculation of the SC_{20} concentration, which was first derived by Walsh *et al.* (1980). The SC_{20} value represents the concentration that increases growth 20% above that of the control population. It can be determined by graphical interpolation and by the moving-average method (see Section 4.3.10).

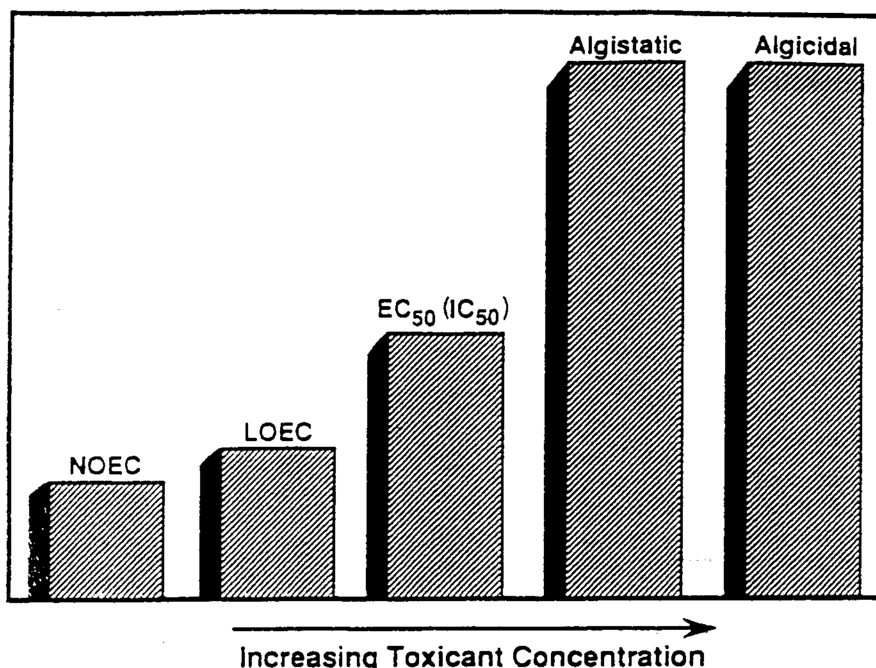

Figure 4.2 Relationship of various end points reported from phytotoxicity tests

4.3.4 Hazard

Safety procedures, such as fume hoods, eye protection and gloves, **must** be used which are appropriate to the COSHH assessment (for the sample) provided by the discharger.

4.3.5 Test facility

The test facility **must** be able to maintain the temperature of test solutions at 23 ± 1 °C for freshwater algal growth tests and 20 ± 1 °C for marine algal growth tests. This can be achieved using a temperature controlled room or cabinet such as an orbital incubator. Continuous uniform illumination **must** be in the spectral range 400-700 nm.

A light intensity in the range 60 to 120 $\mu E\ m^{-2}\ s^{-1}$ (3.5 to 7.0 x 10^{19} photons $m^{-2}\ s^{-1}$), when measured in the range 400 to 700 nm with an appropriate measuring instrument, **must** be available to the test flasks. For light measuring instruments calibrated in lux, an equivalent range of 6000 to 10 000 lux is acceptable.

The light intensity can be obtained using four to seven 30 W fluorescent lamps of the universal white type (colour temperature of approximately 4300 K), at a distance of 0.35-0.60 m from the algal culture.

If algae in control cultures have achieved the recommended growth rates, it can be assumed that the conditions for growth, including light intensity, have been adequate.

4.3.6 Reagents and materials

Test organisms

Toxicity tests **must** be carried out using:

Raphidocelis subcapitata for testing effluents or leachates discharged to freshwater.

Skeletonema costatum for testing effluents or leachates discharged to saline waters.

Source of test organisms

The strains recommended **must** be obtained from a recognized supplier (see Appendix A). The cultures **must** be of a specified strain and unialgal and, ideally, free from bacteria.

Control/dilution media

In toxicity tests, the water used for the controls and the dilution of test solutions **must** be the freshwater or seawater nutrient medium used to culture the algal test species in the facility (see Section 4.2.3).

4.3.7 Apparatus

The following apparatus (see Appendix A) is used:

- a room or cabinet in which a temperature range of 20-25 °C can be maintained at ± 1 °C, and there is continuous uniform illumination in the spectral range 400 to 700 nm.

- apparatus for measuring algal cell density, such as a particle counter (for example a Coulter counter), or microscope with counting chamber. For *Skeletonema* an assessment **must** be made of the number of cells in the chains in each treatment concentration.

  NOTE: Growth of the algal cultures can be determined by an indirect method such as a spectrophotometer, turbidimeter, fluorimeter or flow cytometry, which is sufficiently sensitive and has been calibrated with cell density. The apparatus used should be capable of measuring accurately cell densities as low as 10^3 (± 100) cells ml^{-1};

- culture flasks with air permeable stoppers (for example 250 ml conical flasks are suitable when the volume of the test solution is 100 ml). All test flasks should be identical with regard to material and dimensions;

- apparatus for membrane filtration using cellulose filters of mean pore diameter 0.2 µm;

- an autoclave, capable of operating between 115 - 121 °C;
- equipment for measuring pH, dissolved oxygen and temperature;
- apparatus for measuring light intensity.

4.3.8 Treatment and preparation of samples

The DTA Methods Working Group has recommended that initially effluent or leachate samples collected in the Demonstration Programme are tested unadjusted with measurements being made of all key physico-chemical parameters (see Section 2). If it is apparent that any physico-chemical parameter or parameters are partially or fully responsible for measured responses, then subsequent tests may need to be carried out following modification of the parameter or parameters in test solutions or samples.

General procedures for the collection, transport, storage and treatment of effluents, leachates and receiving waters have been described in Section 2. However, there are specific test requirements for dealing with salinity, suspended solids and colour.

Salinity

Freshwater effluent or leachate samples can normally only be tested with marine species at concentrations ≤20% v/v, otherwise the salinity of the test solutions will fall below the 27‰ salinity tolerance threshold for the algae. Testing at higher concentrations can be achieved by altering the salinity of the solutions by the addition of analytical quality sea salt (for example, Sigma Chemical Co.). If the salinity of any test solutions (or the sample) has to be adjusted with sea salt, an additional control should be run using sea salt at the salinity of the reference seawater (see Section 2).

Suspended solids

High background particle numbers may disturb the growth measurements when using a particle counter or a spectrophotometer. For this reason a background test substance concentration series without algae will have to be included as a background correction of the measurements. Usually quite high particle densities (that is at the same density level as the inoculum) are acceptable at the start of the test as their influence on the subsequent measurements will be progressively less due to the algal growth. If removal of suspended solids from the test solutions or sample is required this should be achieved by filtration or centrifugation (see Section 2).

Coloured samples

Light is an essential energy source for algal growth, and variation in light intensity may, therefore, influence the growth rate if the light intensity is the growth limiting factor. The change in growth rate caused by variation in the light intensity depends on whether the light intensity with which an algal culture is incubated is at the saturation intensity level or not. Above the saturation level, a change in light intensity will not change the growth rate. Below

the saturation value, there is approximately a linear relationship between the light intensity and the growth rate (if no other nutrient is limiting the growth). The saturation light intensity is different for each of the algal species recommended by the guideline, and is not exactly known. It is assumed, however, that the recommended light intensity range is below the saturation value.

Coloured and turbid (aqueous) samples and coloured substances and materials may, therefore, influence the algal growth negatively by shading or by filtering out a specific wavelength required by the algal cultures without having direct toxic effects in the same concentration range as the shading occurs. With a continuous shaken test system as described in the guideline (see Section 4.3.9), practical experience has demonstrated that significant shading effects are mainly observed with nearly opaque coloured solutions or turbid suspensions (continuous shaking assures that all algal cells will be exposed to the full light intensity for a part of the testing period).

In order to distinguish quantitatively between shading effects and inhibition effects, one of the following additional control tests may be carried out:

- increasing the light intensity in the incubator to a level that assures a saturation light intensity at the highest concentration of the coloured and/or turbid test medium in combination with a reduction of the thickness of the test medium layer (Comber *et al.* 1995);

- simulating the shading effect by using the test media without algae as a filter for algal cultures.

Both options may lead to unavoidable deviations in test conditions compared with the standard conditions.

In the first option, the saturation light intensity will have to be determined separately for each test species. The light intensity may exceed the range prescribed in the guideline and may cause an increase in the pH value of the control medium. The control test is further carried out according to the guideline. In the second option (often the more compatible with the guideline), the manipulations with the test design may lead to a lower light intensity and, therefore, to a relatively lower control growth rate. However, interlaboratory ring tests have demonstrated that, as long as the control growth rate is above the validity limit given in the guideline, the EC_{50} values will not change significantly with changes in the control growth rate. If a spectrophotometric method is used for cell density determination, a control series of the test substance without algae should be included.

Other factors

Algal tests are also subject to the interference of chelating and adsorptive materials. Since tests require the enrichment of the diluent medium, any material which removes or sequesters nutrients will create the appearance of toxicity by inhibiting growth potential. Also, where there are flocculants present, the protocol can be invalidated by the alteration of the distribution of the cells within the test vessel. The sampling procedures are only valid if the cells are randomly distributed in the medium.

4.3.9 Test procedures

Provision of exponentially growing unialgal cultures

1. Before a toxicity test or bioassay is carried out, prepare a culture of the algae in the exponential (logarithmic) growth phase for inoculation into the test flasks.

2. Inoculate the toxicity test with algae from an exponentially growing pre-culture, which has been set up 3 days before the start of the procedure (see Section 4.2.5).

3. Maintain the pre-culture under the same conditions as those in the test.

4. Measure the cell density in the pre-culture before use to determine if it is sufficient to be used as an inoculum for the test and, if this is the case, to calculate the required inoculum volume.

5. Check the test cultures, and the inoculum in particular, for possible contamination and abnormalities by microscopic examination before use in a toxicity test or bioassay (see Section 4.2.4). This information **must** be recorded in the test report (see Appendix 2B). The inoculum **must** be discarded if it is found to contain deformed or abnormal cells or is found to have excessive bacterial contamination.

Preparation of test concentration

1. Select an appropriate concentration series, **with the ratio between exposure concentrations not exceeding 2.2.** Where possible the range selected should be sufficient to give 0 and 100% inhibition of growth (and potentially mortality) and at least two intermediate degrees of growth inhibition between 0 and 100%. These results permit the calculation of the toxicity (72 h-EC_{50}, 72 h-EC_{10}, 72 h-NOEC, 72 h-LOEC and, if required, 72 h-LC_{10}) values with greater precision.

   For effluents or leachates discharged to freshwaters and marine waters an appropriate initial concentration range would be 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 22.0, 46.0 and 99% v/v effluent. The preparation of the higher concentrations (>20% v/v effluent) in tests with marine algae may require salinity correction (see Appendix B).

2. Prepare the test concentration range by adding the appropriate volumes of effluent or leachate to a series of volumetric flasks. Then add fixed volumes of each nutrient stock

(see Section 4.2.3) to each flask and dilute the solutions to the mark with reference freshwater or seawater. A control is needed for each test series which contains none of the test substance and has a volume equal to that of each exposure concentration. Record the information on the preparation of the toxicity test concentration range in the test report (see Appendix 4B).

An effluent concentration range of 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 22.0, 46.0 and 98.7% v/v effluent for a freshwater algal test would be prepared in 1 litre volumetric flasks as follows:

Nominal conc. (% effluent)	Volume of effluent (ml)	Volume of nutrient stocks (ml)* [from Table 4.1]	Volume of reference freshwater (ml)
0 (Control)	0	13	987
0.1	1.0	13	986
0.22	2.2	13	984.8
0.46	4.6	13	982.4
1.0	10	13	977
2.2	22	13	965
4.6	46	13	941
10.0	100	13	887
22.0	220	13	767
46.0	460	13	527
98.7	987	13	0

* 16.5 ml of nutrients would be added for marine algal tests using ISO medium (see Table 4.3)

Procedure for the toxicity test

The procedures given for the initiation, monitoring and termination of the toxicity test **must** be followed.

Initiation of the test

1. Once each test concentration in the range has been prepared in 1-litre stock flasks, measure the background particle concentration in each flask to allow for correction of the initial cell densities in the flasks and record these values in the test report (see Appendix 4B).

2. Transfer the required volume of test solution from each stock flask to a test vessel which has been equilibrated to the test temperature (23 ± 1 °C for freshwater tests and 20 ± 1 °C for marine tests). For example, if 250 ml flasks are used, 100 ml of test solution should be transferred. These uninoculated flasks are used to provide background particle counts for each test concentration at the end of the test. These flasks are maintained under the same conditions as the inoculated flasks for the duration of the test[3].

3. Once the cell density in the initial inoculum is known, add a sufficient volume of the inoculum to each stock flask to achieve an appropriate initial cell density[4] at the start of the test (see Table 4.4).

Table 4.4 Initial cell densities for freshwater and marine algal growth inhibition tests

Species	Medium	Initial cell density (cells ml^{-1})
Raphidocelis subcapitata	Freshwater	10^4 (± 10^3)
Skeletonema costatum	Marine	2-3 x 10^3 (± 250)

4. Use a sterile graduated pipette to add the inocula to the stock flasks.

5. Transfer a minimum of three replicate aliquots of each test concentration and control(s) to temperature equilibrated flasks. All flasks (inoculated and uninoculated) are then plugged with sterile cotton wool and placed under constant illumination at the experimental temperature (see Section 4.3.5).

6. Measure the initial cell density in each test concentration using the solution remaining in the stock flask and the data recorded in the test report (see Appendix 4B). Measure the pH of a sample of the test solutions at each concentration and control.

7. Shake the test vessels on an orbital shaker at 100-130 rpm, or if this is not possible, stirring or aerating using filtered air in order to maintain algae in suspension.

[3] The usefulness of this approach is dependent on the method used to estimate cell density, and is most appropriate for electronic particle counting (see Section 4.3.8).

[4] It is preferable to use as low an initial cell density as is technically possible to measure (see Table 4.4), thereby:

- minimizing the masking effect that sorption of test material onto algae has on toxicity;
- prolonging the time period where exponential growth and constant pH can be maintained.

Variations in pH during the test can have a significant influence on results and, therefore, limits of ±1.5 units for freshwater algal tests and ±1.0 units for marine algal tests have been specified for control solutions. However, variations in pH will increase with increasing growth rate. It should be possible to restrict pH drift to about 0.3 units in tests with the marine algal species because of the high buffering capacity of seawater. However, this may not always be possible in tests on environmental samples with the freshwater algal species.

Monitoring during the toxicity test

Measure directly or, if this is not possible, indirectly the cell density in each test vessel, including the controls and uninoculated flasks, at least every 24 h. These measurements should be made on small volumes (for example, 5 ml) removed from the test solutions with a sterile pipette and not replaced. The data should be recorded on an Algal Growth Inhibition Toxicity Test Data Sheet (for example, see Table 4B.3).

Termination of the toxicity test

Tests with freshwater and marine algae shall last for 72 h. Measure the pH of a sample of each test concentration and the controls at the end of the test using a 10-20 ml sample from the flask and record on a Water Quality Monitoring Data Sheet (for example, see Table 4B.4). This should be taken before counting and should not be returned to the flask. Measure the temperature of test vessels after final samples for cell density measurements have been taken.

4.3.10 Processing of the results

Validity of the test results

The results from algal growth inhibition toxicity tests should be considered valid if the control cell density has increased by a factor of more than 16 in 72 h, which corresponds to a growth rate of 0.9 day^{-1}. Growth rates of 1.5 to 1.9 day^{-1} can be achieved under normal experimental conditions;

Ideally, the control pH should not have varied by more than 1.5 units during a freshwater algal test or 1.0 units during a marine algal test[5].

Data from tests on effluents or leachates for discharge characterisation should only be accepted if the results of the reference toxicity test meet quality control criteria.

[5] It is possible that high growth rates in the control vessels will result in changes in pH greater than those specified.

Estimation of toxicity test endpoints

The EC_{50}, EC_{10}, NOEC and LOEC (and LC_{10} values if appropriate) are determined using an appropriate validated computer-based statistical procedure.

Estimation of the EC_{50} and EC_{10} values

Treatment of the results

Measurements of cell density, or other parameters correlated with cell density, are tabulated according to the environmental sample concentration and the time of measurement. A growth curve for each test concentration and control, as the logarithm of mean cell density (after correction for background counts) against time is then plotted. If growth in the controls is not exponential throughout the test period, then only the data points from the first 48 h **must** be used.

The assessment of the inhibition of growth in the test is based on either a comparison of area under the growth curve or a comparison of growth rates[6].

Comparison of areas under the growth curve (biomass integral)

Calculate the area, A, under the double linear growth curve for each test flask from the equation:

$$A = \frac{N_1 - N_o}{2} t_1 + \frac{N_1 + N_2 - 2N_o}{2}(t_2 - t_1) + \ldots + \frac{N_{n-1} + N_n - 2N_o}{2}(t_n - t_{n-1})$$

where:

t_1 is the time of the first measurement after the beginning of the test;

t_n is the time of the nth measurement (days) after the beginning of the test;

N_o is the nominal or measured initial cell density (after correction for background particles);

N_1 is the measured cell density (after correction for background particles) at time t_1;

N_n is the measured cell density (after correction for background particles) at time t_n.

[6] The two approaches will usually give different results. The biomass integral approach is most appropriate for biomass-based measurements such as dry weight, fluorescence or absorbance, while the growth rate approach is more broadly applicable and is certainly more appropriate for cell counts.

Calculate mean values of A for each test concentration and control. From these calculate the percentage inhibition for each test concentration, from the equation:

$$I_{A(i)} = \frac{A_c - A_i}{A_c} \times 100$$

where:

$I_{A(i)}$ is the percentage inhibition (area) for test concentration i;

A_i is the mean area for test concentration i;

A_c is the mean area for the control.

Comparison of growth rates

Calculate the growth rate, u, for each test flask from the equation:

$$u = \frac{\ln N_n - \ln N_o}{t_n}$$

where:

t_n is the time of the final measurement (days) after the beginning of the test;

N_o is the nominal or measured initial cell density (after correction for background particles);

N_n is the measured final cell density (after correction for background particles).

Alternatively, determine the growth rate from the slope of the regression line of the logarithm of cell density against time.

Calculate mean values of u for each test concentration and control. From these values calculate the percentage inhibition for each test concentration from the equation:

$$I_{u(i)} = \frac{u_c - u_i}{u_c} \times 100$$

where:

$I_{u(i)}$ is the percentage inhibition (growth rate) for test concentration i;

u_i is the mean growth rate for test concentration i;

u_c is the mean growth rate for the control.

Estimation of the EC_{50} and EC_{10} (and LC_{10} values if required)

Tabulate the values of $I_{A(i)}$ or $I_{u(i)}$ against the corresponding test concentrations and calculate the EC_{50} (and EC_{10}) value and 95% confidence intervals using an appropriate statistical procedure.

The concentration-response curve may be fitted directly by non-linear regression using an appropriate mathematical function describing a sigmoid curve or by weighted linear regression after a linearizing transformation of the data (Nyholm *et al.* 1990). Suitable functions (and associated transformations) include the probit, logit and Weibull functions (see Figure 4.3) and where the test concentration series includes concentrations at which inhibition of growth is between 0 and 100% the EC_{50} (and EC_{10}) value estimated by the different methods will be similar (Christensen and Nyholm 1984). Statistical methods (and computer programmes) developed for toxicity tests with quantal responses (such as mortality) obtained with a limited number of test organisms **must not** be used, as improper weighting of the data points may result.

The concentration-response curve is not always strictly monotonic, but may reveal an initial (slight) growth stimulation at low concentrations of test material. In calculating the EC_{50} (and EC_{10}) only the monotonic part of the curve **must** be used and a note on the concentration range where stimulation was observed is made in the test report.

Table 4.5 shows an example data which has been used to show the determination of the 72 h-EC_{50} value and 95% confidence limits for the inhibition of growth (using growth rate) by an effluent using different statistical procedures.

Table 4.6 shows the EC_{50} and EC_{10} values and 95% confidence limits estimated from the data in Table 4.5 using different statistical procedures. The results show that the EC_{50} and EC_{10} values estimated by probit, logit and Weibull functions were similar, although the confidence limits varied.

From interpolation of the graph of cumulative inhibition of growth rate against effluent concentration (log scale) shown in Figure 4.4 for the data in Table 4.5 the 72 h-ErC_{50} = 3.7% v/v effluent. Using the sample data given in Table 4.5 and Figure 4.4 the estimated 72 h-ErC_{10} value would be 0.8% v/v effluent. The estimated 72 h-LC_{10} for the data given in Table 4.5 would be 20% v/v effluent. The values obtained graphically confirm those obtained using computer-based software (see Table 4.6).

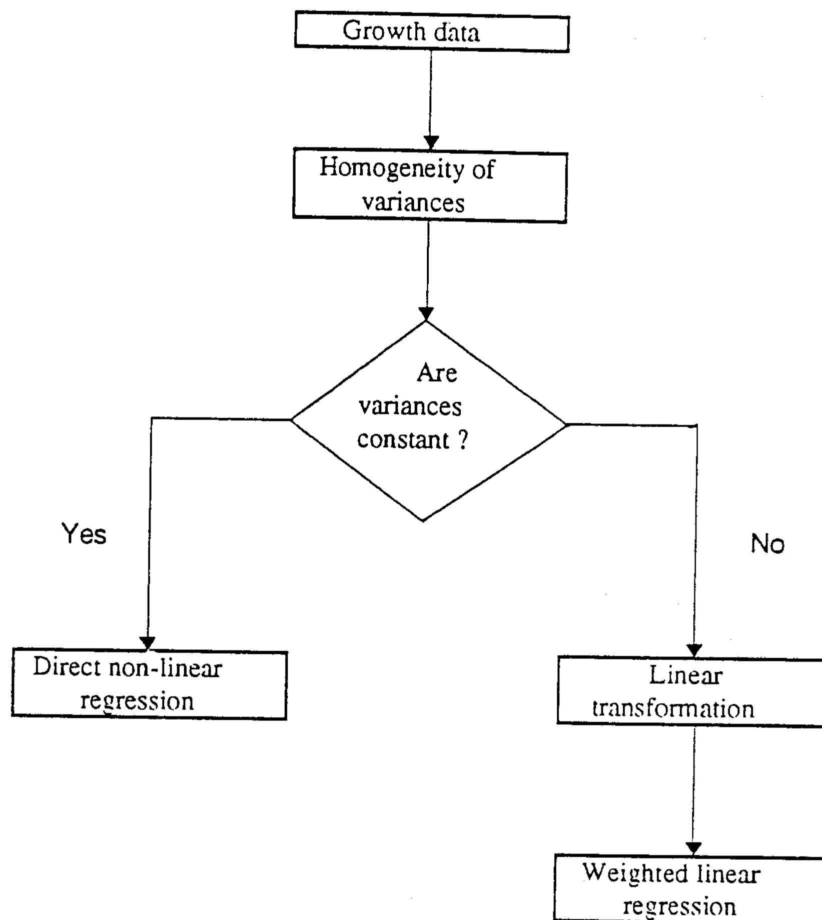

Figure 4.3 Flowchart for the estimation of the IC_{50} for full concentration range algal growth inhibition tests

Table 4.5 Example results of the effects of an effluent on the growth of algae after 72 h exposure

Treatment conc. (%)	Replicate	Initial cell density	Final cell density	Growth rate (day)	Mean growth rate	Inhibition of growth rate (%)
Control	1	10 000	2 450 000	1.834		
Control	2	10 000	2 360 000	1.821		
Control	3	10 000	2 400 000	1.827	1.827	-
0.100	1	10 000	2 390 000	1.825		
0.100	2	10 000	2 350 000	1.820		
0.100	3	10 000	2 300 000	1.819	1.819	0.44
0.220	1	10 000	2 150 000	1.790		
0.220	2	10 000	2 060 000	1.776		
0.220	3	10 000	2 100 000	1.783	1.783	2.43
0.460	1	10 000	1 870 000	1.744		
0.460	2	10 000	1 850 000	1.740		
0.460	3	10 000	1 950 000	1.758	1.747	4.38
1.000	1	10 000	1 035 000	1.547		
1.000	2	10 000	1 115 000	1.571		
1.000	3	10 000	1 165 000	1.586	1.568	14.19
2.200	1	10 000	385 000	1.217		
2.200	2	10 000	345 000	1.180		
2.200	3	10 000	395 000	1.225	1.208	33.92
4.600	1	10 000	91 500	0.738		
4.600	2	10 000	88 500	0.727		
4.600	3	10 000	95 500	0.752	0.739	59.56
10.000	1	10 000	34 000	0.408		
10.000	2	10 000	32 000	0.388		
10.000	3	10 000	28 000	0.343	0.380	79.23
22.000	1	10 000	9 500	-0.017		
22.000	2	10 000	8 500	-0.054		
22.000	3	10 000	9 000	-0.035	-0.036	101.94
46.000	1	10 000	0			
46.000	2	10 000	0			
46.000	3	10 000	0			
100.000	1	10 000	0			
100.000	2	10 000	0			
100.000	3	10 000	0			

Table 4.6 Summary of EC_{50} and EC_{10} values (and 95% confidence limits) for the data in Table 4.5 by different statistical procedures

Statistical procedure	EC_{50}		EC_{10}	
	Value	95% Confidence limits	Value	95% Confidence limits
Probit	3.6	2.0-7.1	0.76	0.05-1.5
Logit	3.6	2.7-5.0	0.73	0.25-1.2
Weibull	3.9	2.4-6.1	0.60	0.07-1.3

Estimation of the NOEC and LOEC

The NOEC and LOEC values are determined using hypothesis testing (see Figure 4.5). Initially use Shapiro-Wilk's or D'Agostino D test to test the normality of the data.

If the data do not meet the assumption of normality and there are four or more replicates of each test concentration, then the non-parametric Wilcoxon Rank Sum Test with Bonferroni Adjustment or Steels Many-One Rank Test should be used to analyse the data depending on whether there are equal numbers of replicates in each treatment.

If the data meet the assumption of normality, the Bartletts test for equality of variances is used to test the homogeneity of variance assumption. If the data meet the homogeneity of variance assumption then Analysis of Variance (ANOVA) followed by Dunnetts test, Williams' Multiple Comparison test or T-tests with Bonferroni Adjustment are used to analyse the data depending on whether there are equal numbers of replicates in each treatment. Failure of the homogeneity of variance assumption leads to the use of Wilcoxon Rank Sum Test with Bonferroni Adjustment or Steels Many-One Rank test depending on whether there are equal numbers of replicates in each treatment.

Further information on these statistical procedures can be obtained from Sokal and Rohlf (1981), Zar (1984) and US EPA (1994). In the example given in Table 4.4 the 72 h NOEC and LOEC values calculated using ANOVA and Dunnetts test were 0.1 and 0.22% v/v effluent respectively.

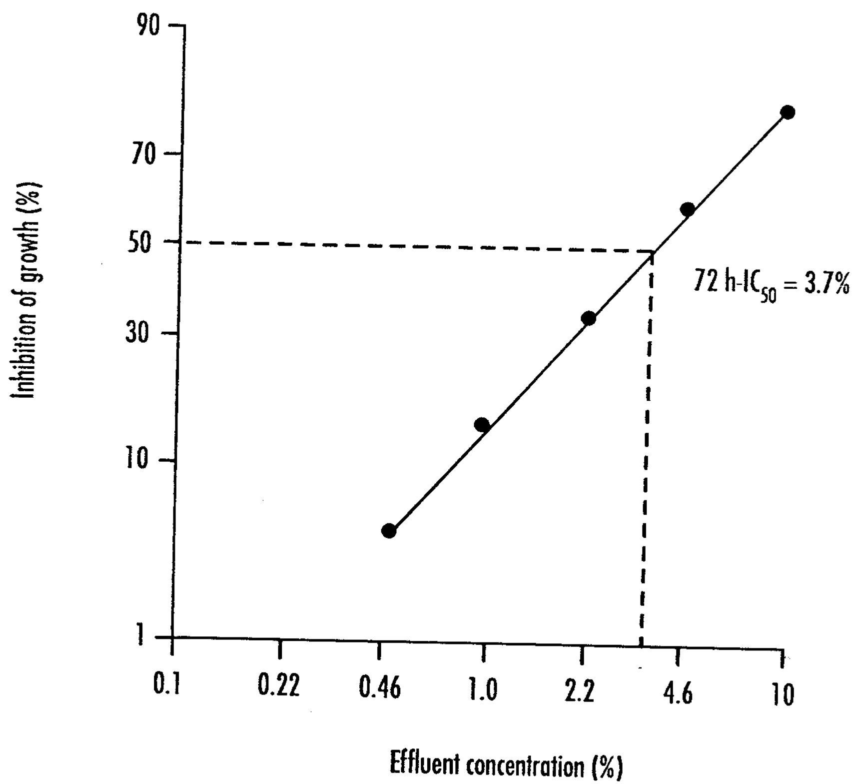

Figure 4.4 Graph of cumulative inhibition of growth rate against concentration (log scale) for an algal test

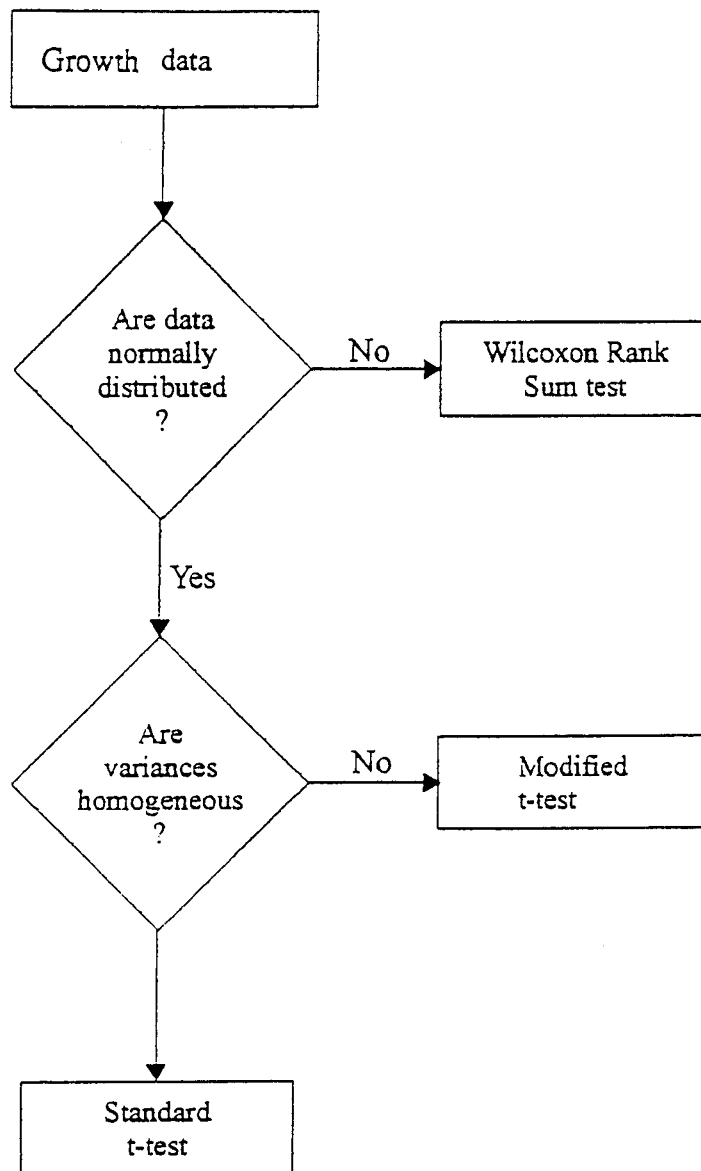

Figure 4.5 Flowchart for the estimation of NOEC and LOEC values for full concentration range algal growth inhibition tests

4.4 Guidelines for single concentration tests on effluents and leachates and bioassays on receiving waters using inhibition of growth in freshwater and marine algae

4.4.1 Monitoring against toxicity limits

Introduction

Toxicity tests with freshwater or marine algae for monitoring against toxicity limits should be carried out with a single concentration test comprising a single effluent or leachate concentration (toxicity limit) and an appropriate control(s).

Test procedure

For tests with freshwater species, the control water may be collected from upstream or adjacent to the point of collection of the effluent or leachate. For marine species the control water may be from a reference site. If water from a `clean' site is used as the control, further controls shall be prepared using the nutrient media in which the algae were cultured and documented in the test report (see Appendix 4B).

Single concentration tests should be initiated in the same way as full concentration range toxicity tests (see Section 4.3.9) with algal inocula added to the four replicates of the controls and the effluent or leachate sample to achieve the initial cell density for a species specified in Table 4.4. Cell density should be measured directly or indirectly after exposure periods including 24, 48 and 72 h and recorded on an Algal Growth Inhibition Toxicity Test Data Sheet (see Table 4B.3). Water quality monitoring should be carried out in the same way as described for the toxicity test (see Section 4.3.9) and recorded on a Water Quality Monitoring Data Sheet (see Table 4B.4).

Processing of results

Assessment of how the responses in the single effluent or leachate treatment compare to those in the control is accomplished using hypothesis testing (see Figure 4.6). The null hypothesis tested is that the responses in the treatment are not significantly different from those in the control. Initially use Shapiro-Wilk's or D'Agostino D test to test the normality of the data.

If the data do not meet the assumption of normality then the nonparametric Wilcoxon Rank Sum Test should be used to analyse the data. If the data meet the assumption of normality, the F test for equality of variances is used to test the homogeneity of variance assumption. If the data meet the homogeneity of variance assumption then the standard (homoscedastic) t test should be used to analyse the data. Failure of the homogeneity of variance assumption leads to the use of a modified (heteroscedastic) t test, where the pooled variance estimate is adjusted for unequal variance, and the degrees of freedom for the test are adjusted.

Further information on these statistical procedures can be obtained from Sokal and Rohlf (1981), Zar (1984) and US EPA (1994).

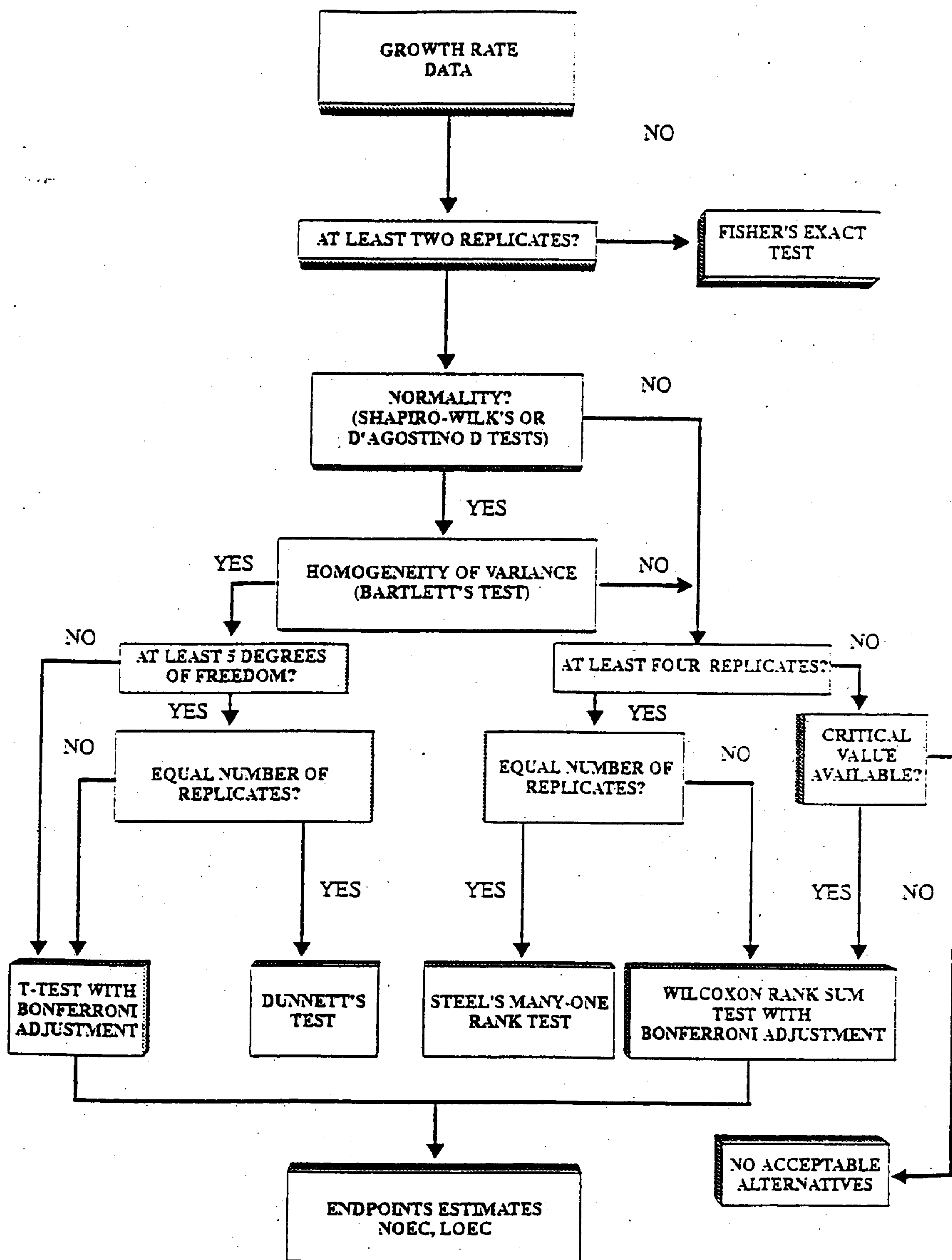

Figure 4.6 Flowchart for analysis of single concentration test data from algal growth inhibition tests

Table 4.7 shows example data sets for a single concentration test using four replicates of control and 0.22% effluent solutions. In Scenario 1 the variances are equal ($F = 5.87$, $p = 0.18$) and the standard (homoscedastic) t test indicates a significant difference between responses in the two groups ($t = 6.88$, $p < 0.05$). In Scenario 2 the variances are unequal ($F = 172$, $p = 0.015$) and the modified (heteroscedastic) t test indicates no significant difference between responses in the two groups ($t = 1.69$, $p > 0.05$).

Table 4.7 Example data set for a single concentration test and the results of statistical analysis

Effluent concentration (%)	Growth rate in replicates (day^{-1})	Method of statistical analysis	Result of statistical analysis
Scenario 1			
0 (Control)	1.834, 1.821, 1.827, 1.825	Standard t-test	Significant difference ($p<0.05$)
0.22	1.785, 1.76, 1.79, 1.776		
Scenario 2			
0 (Control)	1.834, 1.821, 1.827, 1.825	Modified t-test	NS
0.22	1.84, 1.73, 1.81, 1.685		

NS - no significant difference between control and treatment groups

4.4.2 Assessing receiving water column toxicity

The assessment of the toxicity of receiving water column samples should be carried out using the bioassay procedure given in Section 4.4.1 for an undiluted (100%) sample and the appropriate nutrient medium as a control.

4.5 Guidelines for toxicity tests on reference toxicants using freshwater or marine algae

4.5.1 Introduction

Algal growth inhibition tests which are carried out to provide data for discharge characterisation, should be accompanied by tests with the reference substance zinc.

4.5.2 Test procedure

Reference toxicant tests should be conducted according to the procedure given in Section 4.3.9 except that nutrients are added to test solutions in the nutrient media rather than directly as for environmental samples.

4.5.3 Preparation of 1000 mg l^{-1} zinc stock solution

1. Weigh out 4397 mg of zinc sulphate ($ZnSO_4.7H_2O$) in a weighing boat.
2. Add the zinc sulphate to a 1-litre volumetric flask and dilute to just below the mark with distilled water.
3. Add 1 ml of 1M Analar HCl to stabilize the stock solution.
4. Dilute to the mark with distilled water.

4.5.4 Preparation of test concentrations

For the reference toxicant zinc the concentration range given below is used in the first study to assess the sensitivity of test organisms in a facility when no previous data is available.

Nominal zinc conc. (mg l^{-1})	Volume of nutrient media (ml)*	Volume of zinc stock (ml)
0 (Control)	1000	0.0
0.1	1000	0.1
0.32	1000	0.32
1.0	999	1.0
3.2	996.8	3.2
10.0	990	10.0
32.0	968	32.0

* - See Section 4.2.3 for preparation of nutrient media

The above volumes relate to a zinc stock concentration of 1000 mg l^{-1}, which should be prepared according to the procedure given in Section 4.5.3.

The test concentration ranges of zinc for subsequent tests can be modified based on the initial results to allow the derivation of more precise LOEC and EC_{50} values.

4.5.5 Test procedure

Reference toxicant tests should be initiated in the same way as full concentration range toxicity tests (see Section 4.3.9). Cell density should be measured in each test vessel, including the control and uninoculated flasks, at least every 24 h. The data should be recorded on an Algal Growth Inhibition Toxicity Test Data Sheet (see Table 4B.3). Water quality monitoring should be carried out in the same way as described for the toxicity test and recorded on a Water Quality Monitoring Data Sheet (see Table 4B.4).

Samples of the zinc test solutions should be taken at the beginning of the test from the stock vessel for each test concentration and at the end of the test from the vessels themselves and analysed using an appropriate procedure (for example, SCA 1980, 1981, 1988).

4.5.6 Processing of results

The 72 h-LOEC and 72 h-EC_{50} values should be calculated using the procedures described in Section 4.3.10. The estimation of toxicity values should be based on measured exposure concentrations.

4.6 Test report

The test report **must** include the following information:

(a) information about the test organism such as scientific name and source, and culture conditions;

(b) the nutrient media used and major chemical characteristics such as pH and temperature;

(c) the methods of preparation of the samples including for effluents, leachates and receiving waters the manner and duration of storage of the samples and, if necessary, the procedures used to adjust physico-chemical parameters were modified, for example, whether pH was adjusted or suspended solids were treated (such as settlement, centrifugation or filtration of samples) or colour correction was applied;

(d) test details including the date of the test, test duration, inoculated algal density and light intensity and quality;

(e) the method of measuring algal density;

(f) tables showing algal growth at each control and test concentration at each time of measurement for full concentration range tests, single concentration tests and reference toxicant tests;

(g) an indication that criteria determining the validity of the test have been satisfied;

(h) for tests on effluents and leachates for discharge characterisation an indication that the responses of algal cultures in the reference toxicant test meet laboratory internal quality control criteria;

(i) the derivation of the 72 h-EC_{50} and 72 h-EC_{10} values the 95% confidence limits and the method of calculation. The 72 h NOEC and LOEC values (and if required the 72 h-LC_{10} values, where calculable, shall also be reported.

(j) any operating details not specified in this procedure and any incidents which may have affected the results.

4.7 Checklist

A checklist summarizing the test conditions and procedures for algal growth inhibition toxicity tests is given in Table 4.8.

Table 4.8 Checklist of test conditions and procedures for algal growth inhibition toxicity tests

Test procedure

Organisms	Exponentially growing unialgal cultures of freshwater or marine algae
Type	Static, 72 hours duration
Control/dilution water	Freshwater or seawater nutrient media
Temperature	23 ± 1 °C for freshwater tests, 20 ± 1 °C for marine tests
Aeration/suspension	This is achieved by either stirring the suspension or aeration. It is essential to maintain the algae in suspension during the test
Lighting	Continuous fluorescent light of 6000-10 000 lux at a distance of 0.35-0.60 m from the test vessels
Feeding	Essential nutrients supplied in test medium
Observations	Cell density (directly or indirectly) measured at specified times
Measurements	Test solution pH at the beginning and end of the test
Endpoints	72 h-EC_{50} and 72 h-EC_{10} values (± 95% confidence limits), 72 h-NOEC and LOEC values (and if required 72 h-LC_{10} values)
Reference toxicant	Zinc (as zinc sulphate) determined at the time of testing or monthly if testing is carried out infrequently
Test validity	Control growth rate ≥0.9 day^{-1} (increase in cell density by factor of more than 16) and, ideally, control pH not varied by more than 1.5 pH unit in freshwater algal tests or 1.0 pH unit in marine algal tests.

Test solutions

Effluents, leachates and receiving waters

Transport and storage	Transport at temperature not markedly different from that at time of collection. The test **must** begin within 48 h after the time of sampling has been completed. If the sample is not to be tested immediately on receipt then it should be stored at 5 ± 3 °C.
Control/dilution water	Freshwater or seawater nutrient media*1

*1 If an upstream receiving water is used as the control/dilution water, a reference freshwater or seawater control should also be run.

4.8 References

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APPENDIX 4A DATASHEETS USED IN THE CULTURING OF ALGAE (EXAMPLE)

Table 4A.1 Algal Culture Medium Preparation Data Sheet (Example)

Culture medium batch number:

Date prepared:

Operator:

Volume of medium:

Diluent:

Source of diluent:

Nutrients used:

Nutrient stock name	Nutrient stock number	Nutrient stock batch	Volume of nutrient stock (ml)
	1		
	2		
	3		
	4		

Filtered: (Y/N) µm pore-size membrane filters

Autoclaved: (Y/N) Temperature (°C): Duration (minutes):

Storage:

Expiry date: (1 month after preparation):

Water quality of culture medium:

Temperature: ____________________
pH: ____________________
Salinity: ____________________

Table 4A.2 Algal Inoculum Receipt and Preparation Data Sheet (Example)

Algal stock number:

Source of algae:

Species of algae:

Date delivered:

Algae supplied as: Slope: ____________ Liquid culture: ____________

Volume of algal stock used as inoculum:

Volume of culture medium used for inoculum preparation:

Batch number of culture medium used:

Date inoculum prepared: Operator:

Inoculum number:

Inoculum incubation location:

Inoculum incubated from: to:

Inoculum used for preparation of a slope: (Y/N)

Table 4A.3 Algal Pre-culture Preparation Data Sheet (Example)

Algal stock number:

Algal species:

Source of algae:

Date delivered:

Replicate	Background count for 100 ml of filtered dilution media	Background + x ml of algal stock	Operator	Date
1				
2				
3				
Mean				

Density of algal stock

Required algal density of pre-culture:

Volume of culture medium used:

Culture medium batch number:

Pre-culture number:

Pre-culture incubated: Location:
Temperature (°C):
Lighting regime:

Aeration (Y/N): Agitation (Y/N):

APPENDIX 4B TEST REPORT FOR AN ALGAL GROWTH INHIBITION TOXICITY TEST (EXAMPLE)

Table 4B.1 Preparation of algal culture

Data on test species

Origin of test species:

Holding conditions: Temperature (°C)
Light intensity

Data on test substance

Test substance:

Source: Hazard:

Date collected or prepared:

Date received:

Storage conditions: Temperature (°C)

Length of time before start of test (h)

Algal test culture

Date and time algal pre-culture started:

Date and time density of algal pre-culture measured:

Method used to measure algal cell density:

Evidence of contamination: Y/N

	Replicate measurements (counts ml^{-1})			Mean
	1	2	3	
Cell density of nutrient media				
Cell density of algal test culture				
Corrected cell density of algal test culture				

Volume of inoculum needed in each test vessel:

Table 4B.2 Preparation of toxicity test concentration range

Test substance:

Type of test:

Start of test: End of test:

Duration of test:

No. of test concentrations:

No. of replicates per concentration:

No. of controls: Dilution media only

Solvent controls

Concentration range

Stock solution concentration:

Type of control and dilution media:

Volume of test solution required (ml):

Nominal test substance concentration	Volume of nutrient media (ml)	Volume of test substance (ml)

Table 4B.3 Algal Growth Inhibition Toxicity Test Data Sheet

Test species:

Test substance:

Measurement method:

Type of test: Preliminary/Definitive

Date test started:

Date	Time	Exposure period (h)	Exposure conc.	Algal cell density in each replicate(cells ml^{-1})			Background particle counts (cells ml^{-1})	Corrected algal cell density in each replicate (cells ml^{-1})			Initials
				1	2	3		1	2	3	

Table 4B.4 Water Quality Monitoring Data Sheet - Toxicity test

Date	Time	Vessel No.	Nominal conc	Temp (°C)	pH	DO (%ASV)	Total hardness (mg $CaCO_3$ l^{-1})	Salinity (‰) or Conductivity (µS cm^{-1})	Initials

Table 4B.5 Expression of results of toxicity tests

Exposure period (h):

Exposure conc.	Corrected algal cell density (cells ml^{-1})				Growth (day^{-1})				Inhibition of growth (%)			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean

Method used: IC_{50} =

IC_{10} =

LC_{50} =

Method used: NOEC =

LOEC =

Concentrations used in the calculations: Nominal/measured

Operating details not specified in the standard operating procedure and any incidents which may have affected the results:

5. JUVENILE *DAPHNIA MAGNA* IMMOBILIZATION TEST GUIDELINE

5.1 Introduction

This section of the DTA Methods Guidelines describes the procedures for the culturing of the freshwater cladoceran *Daphnia magna* (Straus) and for conducting toxicity tests using such species to measure the effects of effluents, leachates and receiving waters on immobilization. The document has been compiled with reference to existing internationally recognised standard procedures (ASTM 1984, 1988; ISO 1989; EC 1990; Environment Canada 1990a,b; OECD 1984, 1997).

Critical steps in the culturing and test procedures which **must** be followed are identified in bold type whereas instructions given in normal type are recommended and alternatives can be used.

5.2 Culturing of test organisms

5.2.1 Introduction

The purpose of culturing *Daphnia magna* using the following procedure is to provide "healthy" juvenile daphnids for toxicity tests and bioassays. Table 5.1 summarises information given in existing guidelines for the culturing of *Daphnia magna*.

5.2.2 Test species

The species used in the test is *Daphnia magna* Straus and the clone should have been identified by genotyping. Research has shown that the reproductive performance of Clone A (which originated from IRCHA in France) consistently meets the health criteria described in Section 5.2 when cultured under the conditions described in this guideline (OECD 1997). However, other clones are acceptable provided that the *Daphnia* culture is shown to meet the quality criteria for the cultures and validity criteria for a test.

At the start of the test, the animals **must** be less than 24 hours old and **must** not be first or second brood progeny. Neither must the juveniles be from the seventh or eighth broods. They **must** be derived from a healthy stock and the stock animals **must** be maintained in culture conditions (light, temperature, medium, feeding and animals per unit volume) similar to those to be used in the test. If the *Daphnia* culture medium to be used in the test is different from that used for routine *Daphnia* culture, a pre-test acclimation period of normally about 3 weeks **must** be included to avoid stressing the parent animals.

Table 5.1 Summary of information given in existing guidelines for the appropriate culturing conditions for *Daphnia magna*

Parameter	Guideline			
	ASTM (1984)	Environment Canada (1990a,b)	Greene *et al.* (1988)	Poirier *et al.* (1988)
Culture water				
- Source	Receiving water	Uncontaminated ground or surface water, dechlorinated drinking water or reconstituted water	Reconstituted water	Dechlorinated drinking water or other
- Temperature (°C)	20 ± 4	20 ± 2	22 ± 4	20 ± 1
- Dissolved oxygen	>40% ASV	60-100% ASV	≥6 mg l^{-1}	No information given
- Hardness (mg $CaCO_3$ l^{-1})	No information given	140-250	80-100	120-250
Light	16 h light: 8 h dark. Light intensity ≤800 lux at surface, wide spectrum fluorescent source	16 ± 1 h light: 8 ± 1 h dark. Light intensity 400-800 lux at water surface, preferable "cool white" fluorescent source	16 h light: 8 h dark. Light intensity 540-1080 lux at water surface	16 h light: 8 h dark. Light intensity ≤800 lux at water surface, "cool white" fluorescent source
Feeding - type of food	Natural or artificial foods	Algal cultures with or without supplements (such as yeast, cerophyll™ and trout chow)	Trout chow, alfalfa, yeast and algae	Mixed algal culture

5.2.3 Culture water

Daphnia magna **must** be cultured in an appropriate natural or low EDTA reconstituted water which is capable of meeting the criteria for the reproduction of *D. magna* given in Section 5.2.5[1]. The hardness of the culture water **must** be between 140 and 250 mg $CaCO_3$ l^{-1}.

Natural waters for culturing daphnids can be an uncontaminated supply of groundwater or dechlorinated drinking water. If drinking water is to be used, the dechlorination procedure must ensure that the total residual chlorine (TRC) level in cultures is ≤0.002 mg l^{-1} (CCREM 1987). This TRC concentration can be achieved by passing the water over activated carbon filters followed subsequently by either ultraviolet radiation (Armstrong and Scott 1974) or vigorous aeration for 24 h. The addition of thiosulphate or other chemicals to water to remove total residual chlorine is not recommended as these chemical(s) could sequester trace metals essential for the health of the organisms.

The type of culture water has an important influence on culture health and fully defined media such as Elendt M4 and M7 are two such media which are known to be suitable for the long-term culture of *Daphnia magna*.

Both media contain the chelating agent EDTA and research has shown that the 'apparent toxicity' of cadmium is generally lower when the reproduction test is performed in M4 and M7 media rather than in a medium containing no EDTA (OECD 1997). Elendt M4 and M7, although suitable for culture, are not, therefore, recommended for testing environmental samples containing heavy metals, and other media containing known chelating agents **must** also be avoided.

When testing environmental samples (effluents, leachate or receiving waters) which may contain heavy metals, it is important to recognise that the properties of the test medium (such as hardness, chelating capacity) may have a bearing on the toxicity of the sample.

Consequently, for testing of environmental samples, the reconstituted media used **must** not contain EDTA (that is ASTM reconstituted hard freshwater or ISO reconstituted hard water) (see Appendix 5A) but should be supplemented with seaweed extract. Although the seaweed extract exerts a mild chelating action due to the organic components in the added extract, it has been found to be necessary for the long-term culture of *Daphnia magna*.

If reconstituted media are used which include undefined additives, these additives **must** be specified clearly and information **must** be provided in the test report on composition, particularly with regard to carbon content as this may contribute to the diet provided. It is recommended that the total organic carbon (TOC) and/or chemical demand (COD) of the stock preparation of the organic additive be determined and an estimate of the resulting

[1] The neonates of *D. magna* are larger and easier to observe in the test solutions. However, this species is found naturally only in hard (>150 mg l^{-1}) water (Pennak 1978) and the use of *D. magna* in soft water solutions may lead to mortality caused by osmotic stress (Greene *et al.* 1988). Sublethal stress from low hardness might affect resistance to the substance being tested. If the samples to be tested are of low hardness then it will be necessary to establish a *Daphnia* culture which is acclimated to the hardness levels pertaining in the test samples.

contribution to the TOC/COD in the test medium made. It is further recommended that TOC levels in the medium (that is before addition of the algae) be below 2 mg l^{-1}.

Deficiency in vitamin B_{12} or the trace element selenium can result in poor health of daphnids, and these should be routinely added to culture water, at least if reconstituted water is used. Selenium should be added at 2 µg Se l^{-1} using sodium selenate (Na_2SeO_4). Insufficient waterborne selenium may cause deterioration of the cuticle of daphnids, shorter life, and failure of progeny to mature and reproduce, according to a citation in Cowgill (1989) of work done by Keating and Dagbuson (1984). Vitamin B_{12} should be added to artificial culture water at 2 µg l^{-1} as cyanocobalamin. Stock solutions of vitamin B_{12} are unstable and should not be stored for more than two weeks. Deprivation of this vitamin may cause delayed reproduction, infrequent moulting, and reproductive failure or progeny (Cowgill 1989, citing work of Keating 1985).

Water to be used as culture medium **must** be aerated vigorously just before use to ensure the dissolved oxygen content is at least 90% of the air saturated value. The pH of the culture medium **must** be within the range 7.4 to 8.5.

Monitoring and assessment of variables such as residual chlorine, pH, hardness, alkalinity, total organic carbon, conductivity, suspended solids, dissolved oxygen, temperature, ammonia, nitrogen, nitrite, residual chlorine and total organic chlorine, metals and total organophosphorus pesticides, should be performed as frequently as necessary to document water quality (see Table 5.2).

Table 5.2 Required characteristics of acceptable freshwaters for culturing *Daphnia magna* (after OECD 1997)

Substance	Concentrations
Particulate matter	<20 mg l^{-1}
Total organic carbon	<2 mg l^{-1}
Unionised ammonia	<1 µg l^{-1}
Residual chlorine	<10 µg l^{-1}
Total organic chlorine	<25 ng l^{-1}
Total organophosphorus pesticides	<50 ng l^{-1}

For example, a holding water which is known to be relatively constant in quality should be monitored every 3 months. If water quality has been demonstrated to be constant over at least 1 year measurements can be less frequent and intervals extended (for example every six months).

5.2.4 Lighting

Light intensity should be within the range of 400 to 800 lux at the water surface, and ideally should be skewed towards the blue end of the spectrum (colour rendering index ≥90) (Buikema 1973, ASTM 1984, Poirier *et al.* 1988). Cool white fluorescent lights are suitable,

although other light sources (for example, full-spectrum fluorescent) may be used if the quality criteria in Section 5.2.5 are to be met. A photoperiod of 16 ± 1 h light and 8 ± 1 h dark should be used.

5.2.5 Quality criteria for the cultures

The juvenile daphnids used in toxicity tests need to be healthy with a documented history. This can be achieved by monitoring criteria such as time to first brood, juvenile productivity, ephippial production, the presence of dead or aborted eggs on the base of the culture vessels and the mortality of adults and juveniles.

Juveniles should produce their first brood within 12 days. Juvenile production in cultures that are greater than 10 days old should be continuous, that is juveniles should be produced from all broods. Females should produce an average of ≥15 neonates per brood.

Ephippia (black 'kidney shaped' eggs) are produced as a result of sexual reproduction and indicate that male *Daphnia* are, or have been, present in the culture. Ephippia may be present on the base of the container or in the brood pouch of an adult daphnid. If ephippia are allowed to hatch, genetic contamination of the clone could result. Therefore, if ephippia are found in the culture, dispose of the contents of the culture vessel. The cultures **must** also be examined for dead or diseased *Daphnia,* which **must** also be removed when observed.

Cultures **must** be discarded when any ephippia are produced or if there is >25% mortality in the culture during the week before the test. Cultures should also be discarded if productivity appears low (for example, if it falls below 15 juveniles per female per brood).

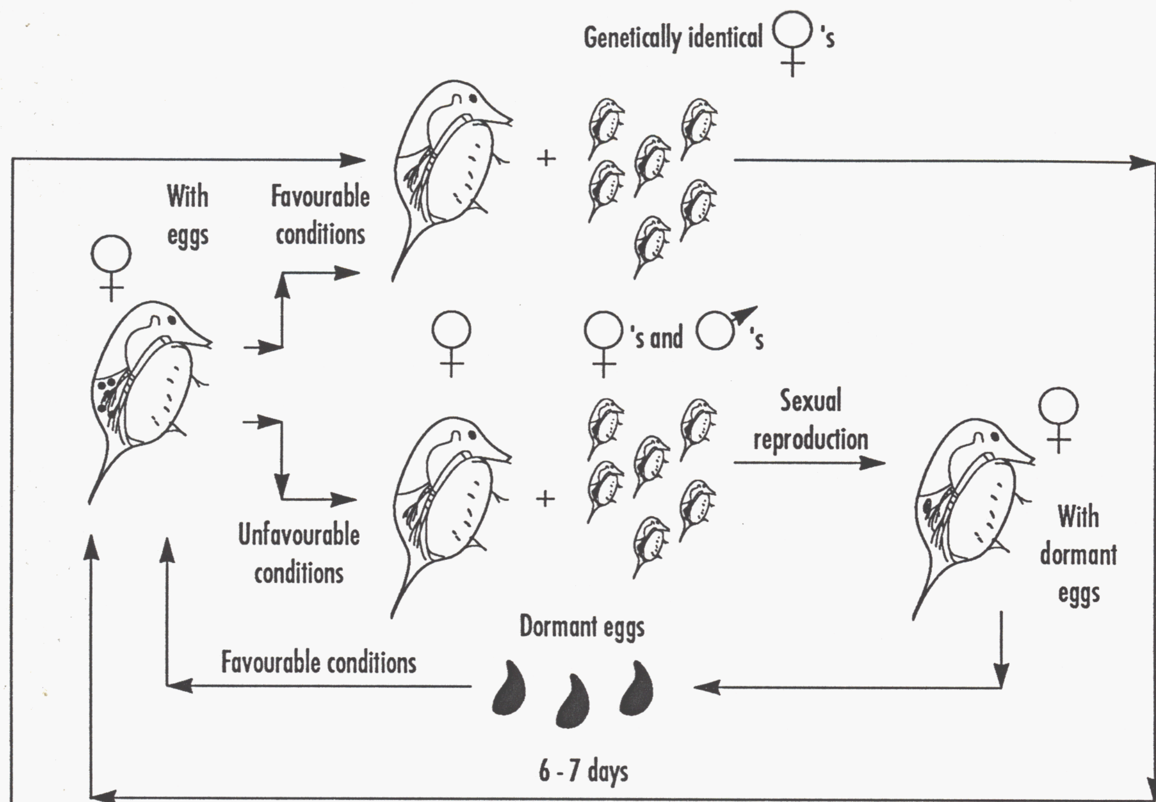
All observations on the juvenile production, the production of ephippia and the presence of dead daphnids **must** be recorded on a *Daphnia* Culture Data Sheet (for example, see Table 5B.1).

5.2.6 Culturing of stock *Daphnia*

Daphnia **must** be cultured in appropriate vessels under static conditions at 20 °C ± 2 °C. A number of successful culturing methods are in common use (see Table 5.1). The following recommendations and requirements are designed to provide a greater degree of standardisation and quality control in yielding daphnids for aquatic toxicity tests.

Culture vessels and accessories contacting the organisms and culture media **must** be made of non-toxic materials (such as glass, Nalgene™). Glass aquaria or beakers are recommended for mass cultures as they permit easy observation of the daphnids.

Materials such as copper, brass, galvanised metal, lead and natural rubber **must not** come in contact with culture vessels or media, nor with test samples, test vessels, dilution water, or test solutions. Each culture vessel should be covered to exclude dust and minimise evaporation. The number of cultures maintained is optional, but five represents a compromise between devoting excessive effort to culturing and ensuring that there will be sufficient organisms available to conduct toxicity tests when required (Environment Canada 1990a). Each of the five cultures should produce about 300 juveniles per brood if reproduction of the females is synchronized (Figure 5.1).



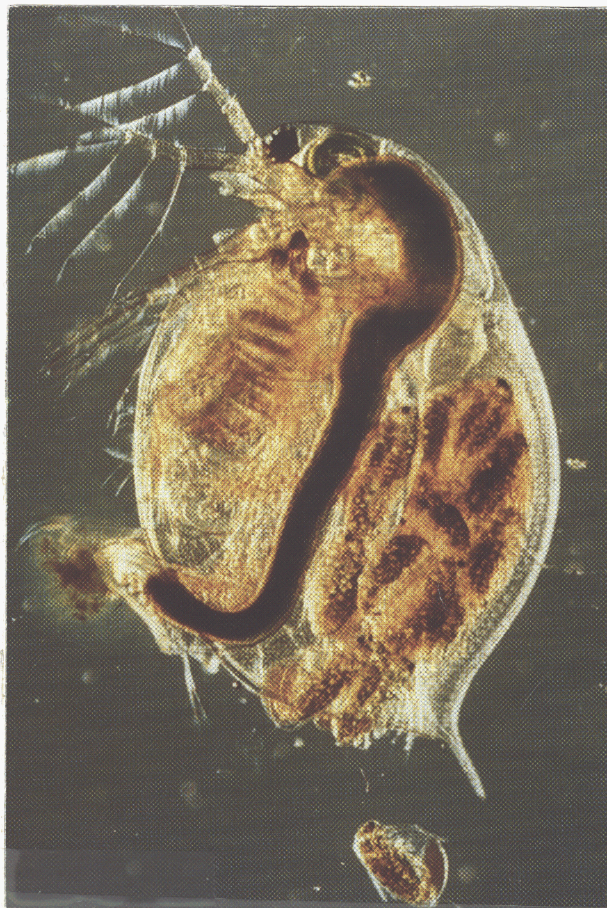


Figure 5.1 Life cycle of *Daphnia magna*

5.2.7 Feeding of organisms

Introduction

Feeding is required during culturing of daphnids. The food used **must** be sufficient and suitable to maintain the test organisms in a nutritional state that will support normal metabolic activity and achieve the quality criteria specified in Section 5.2.5. The following guidance is designed to avoid known problems.

A number of proven diets, feeding rations, and schedules for culturing daphnids are described in detail in the literature (see Environment Canada 1990a).

During culture it is recommended that the parent animals be fed living algal cells of one or more of the following: *Chlorella* sp, *Raphidocelis subcapitata* and *Scenedesmus subspicatus*. *Daphnia* should be fed once daily, however, for practical reasons feeding may be restricted to Monday through Friday with additional food being given on Friday. The supplied diet **must** be based on the amount of organic carbon (C) provided to each parent animal. Research (Sims *et al.* 1993) has shown that, for *Daphnia magna*, ration levels of between 0.1 and 0.2 mg C *Daphnia* day^{-1} are sufficient for achieving the required number of offspring to meet the quality criteria specified in Section 5.2.5.

If surrogate measures, such as algal cell number or light absorbance, are to be used to feed the required ration level (that is for convenience since measurement of carbon content is time consuming), **each laboratory must produce its own nomograph relating the surrogate measure to carbon content of the algal culture.** Nomographs should be checked at least annually and more frequently if algal culture conditions have changed. Light absorbance at 440 nm has been found to be a better surrogate for carbon content than cell number (Sims 1993).

For nomograph production, algae should be separated from the growth medium by centrifugation followed by resuspension in distilled water. Measure the surrogate parameter and TOC concentration[2] in each sample in triplicate. Distilled water blanks should be analysed and the TOC concentration deducted from that of the algal sample TOC concentration. Nomographs should be linear over the required range of carbon concentrations. Examples from OECD (1997) are shown in Figures 5.2-5.4.

Supplements such as a suspension of baking yeast or seaweed extract may be used in addition to the algae provided that their contribution (as TOC) to the quantity of food fed to the *Daphnia* is taken into consideration.

[2] TOC should be measured by high temperature oxidation rather than by UV or persulphate methods. (For advice see: The Instrumental Determination of Total Organic Carbon, Total Oxygen Demand and Related Determinands 1979, HMSO 1980; 49 High Holborn, London WC1V 6HB.)

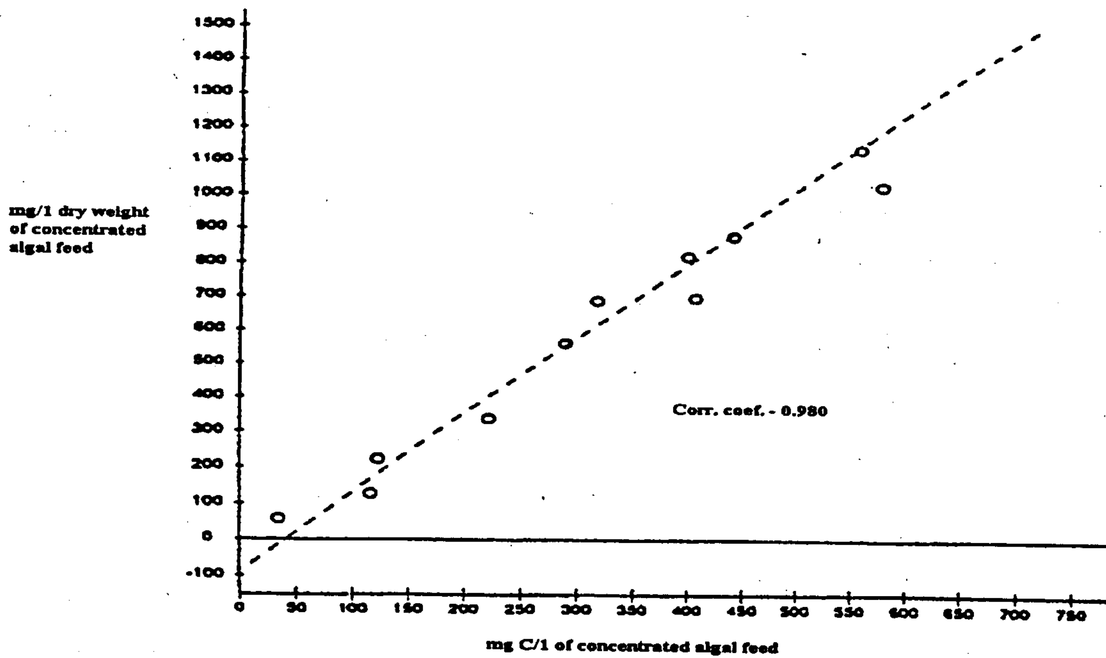


Figure 5.2 Example regression of dry weight of concentrated algal feed (mg l^{-1}) against total organic carbon of concentrated algal feed (mg C l^{-1}) for *Chlorella vulgaris* var. *viridis* (CCAP 211/12). Data from concentrated suspensions of semi continuous batch cultured cells, resuspended in distilled water

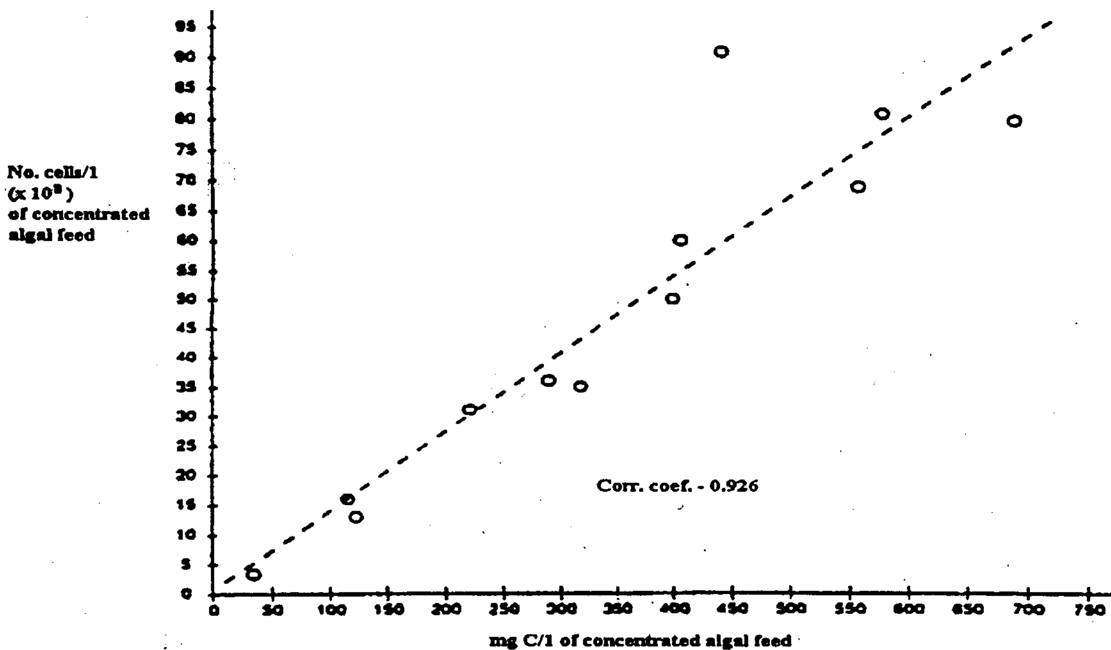


Figure 5.3 Example regression of cell number of concentrated algal feed against total organic carbon of concentrated algal feed (mg C l^{-1}) for *Chlorella vulgaris* var. *viridis* (CCAP 211/12). Data from concentrated suspensions of semi continuous batch cultured cells, resuspended in distilled water

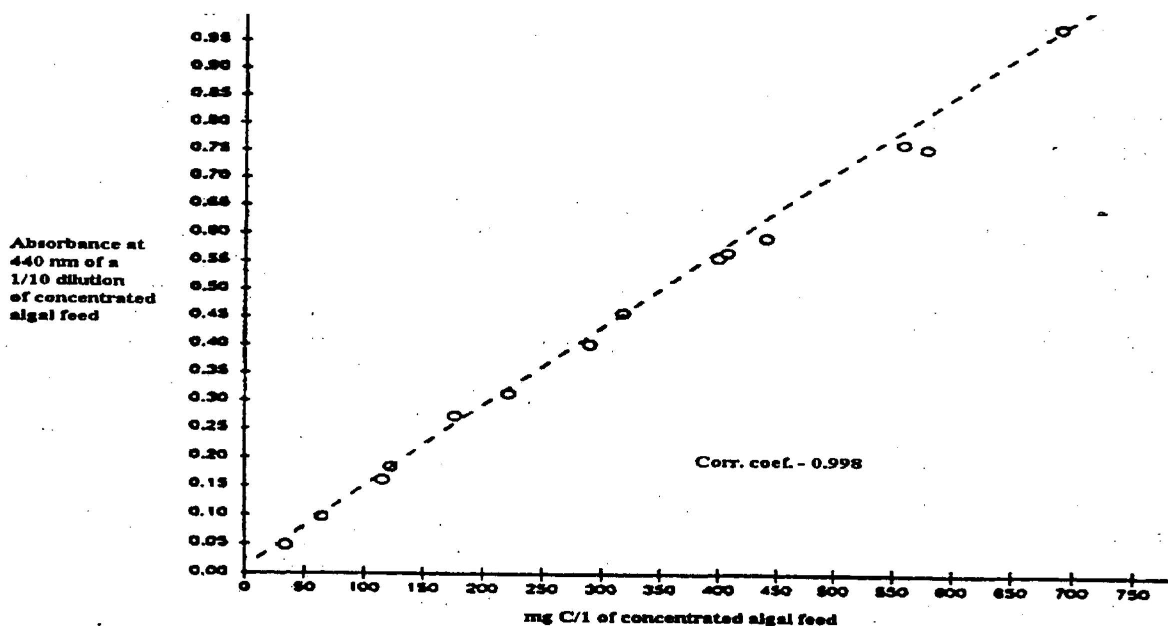


Figure 5.4 Example regression of absorbance (at 440 nm) of concentrated algal feed against total organic carbon of concentrated algal feed (mg C l^{-1}) for *Chlorella vulgaris* var. *viridis* (CCAP 211/12). Data from concentrated suspensions of semi continuous batch cultured cells, resuspended in distilled water

Preparation and addition of algal concentrates

Details of algal culture are given in Section 4 of the manual. Fresh algal suspensions should be prepared each week by centrifuging aliquots of the algal culture (for example, 5000 g for 20 minutes) or filtration. The pellet of cells or filtrate should then be re-suspended in a small volume of distilled water. Appropriate volumes of the concentrated suspension should then be added to each *Daphnia* culture to ensure each organism achieves a ration of 0.1-0.2 mg C *Daphnia* day^{-1}.

Preparation and addition of yeast supplement[3]

A 100 mg l^{-1} suspension of baking yeast can be prepared at intervals of 7 days by adding 50 mg of dried baking yeast to 500 ml of distilled water. The yeast supplement should be added to the cultures at a rate of 0.6 ml per 1500 ml of culture.

[3] Note that feeding rate is expressed on a vol to vol basis not as a quantity per animal as for algae.

Preparation and addition of seaweed extract[3]

A 1% dilution of seaweed extract can be used as a supplement and should be added to cultures vessels at a rate of 2 ml l^{-1}.

Storage of algal concentrates and yeast or seaweed suspensions

When not in use, the algal concentrates and yeast or seaweed extract suspensions should be stored in sealed glass containers in a refrigerator at 2-6 °C under low levels of illumination. Algal concentrates should not be used if they are more than one week old. Yeast suspensions should also not be used if more than seven days old.

5.2.8 Renewal of culture water and thinning of cultures

The water in the cultures **must** be renewed regularly and the temperature, pH and dissolved oxygen of the new medium should be recorded on the relevant *Daphnia* Culture Data Sheet (see Table 5B.1). The renewal of culture medium should be integrated with the thinning out of cultures.

In multiple cultures of ≥3 l (with ≤20 adults per vessel) the water in each culture vessel **must** be almost completely replaced, at least weekly (US EPA 1982, Greene *et al.* 1988). For smaller vessels, more frequent replacement of culture media will be required. If the medium is not replaced at least weekly, and if the population density is not reduced to an acceptable level (≤20 per l), waste products will accumulate which could cause a population crash or the production of males and/or ephippia[4] (Greene *et al.* 1988). Greene *et al.* (1988) provide details of a siphoning technique to replace 90% of the culture medium. The population of daphnids **must** be thinned to a maximum of twenty animals per litre. Lower numbers may produce more satisfactory results, and a rearing density of only four adult *D. magna* per litre has been recommended elsewhere, to avoid production of ephippia (Cowgill 1989). The establishment and maintenance of at least five culture vessels for each test species is desirable.

Adult daphnids are transferred between vessels using a wide bore pipette or a glass dropping tube with an internal diameter of 5 mm. The daphnids should remain immersed at all times and be expelled from the tube beneath the surface of the new medium and not onto the surface. *Daphnia* **must** only be transferred if the temperature difference between the old and new media is less than 2 °C. *Daphnia* **must never** be transferred between different cultures to avoid cross contamination of disease, etc. and isolate problems in single culture vessels. Once the transfer is complete the pH and dissolved oxygen of the old medium should be measured and recorded on a *Daphnia* Culture Data Sheet (see Table 5B.1), along with the number of

[4] This procedure of thinning down reduces the occurrence of males in the culture and hence the formation of ephippia. By the fifth day, it is possible to discriminate between the two sexes as females are usually much larger. If there is some uncertainty about the sex of the individual daphnid, then a double check can be performed by examining the antennule (the appendage in the mouth) under the microscope. Females have a small antennule; males have a greatly enlarged antennule. Periodic examination of broods for the presence of males is desirable, before waiting for the presence of ephippia to indicate that the culture is unhealthy.

juveniles produced since the previous media renewal. The old medium and the spare *Daphnia* should then be discarded.

5.2.9 Establishing a new culture

New *Daphnia* cultures should be established using an appropriate number of juvenile animals obtained from isolated gravid adults in a single existing culture. All of the juveniles used for a new culture **must** be less than 24 h old. The origin of the juveniles used to set up a new culture must be clearly identified on a *Daphnia* Culture Establishment Data Sheet (for example, see Table 5B.2), together with the number of the new culture. This should be sequential from the last culture. The isolation of adults, their subsequent feeding and the removal of the juvenile *Daphnia* should be recorded on a *Daphnia* Isolation Data Sheet (for example, see Table 5B.3). One form should be used for each isolation vessel.

It is recommended that new cultures are established from the third, fourth and fifth broods (as well as using neonates from these broods for toxicity testing). A staggered culture system is, therefore, set up ensuring continuity in neonate supply.

The juveniles shall be placed in a volume of culture medium which is appropriate to the number of organisms. An appropriate sized pipette or glass tube should be used to transfer the juveniles. The temperature of this medium **must** be within 2 °C of that in the adult isolation vessels. The measured values for the temperature, pH and dissolved oxygen of a sample of the new medium are recorded on a *Daphnia* Culture Establishment Data Sheet (see Table 5B.2). New cultures should be labelled with the culture number, the date established and the date of the next renewal.

The new culture **must** be fed each day with algae and supplements, as described in Section 5.2.7. The dates when gravid adults are first observed and the first brood of juveniles are produced should be recorded on a Daphnia Culture Establishment Data Sheet (see Table 5B.2).

5.3 Guideline for toxicity tests on effluents and leachates using the juvenile *Daphnia magna* (Straus) immobilization test

5.3.1 Introduction

Information given in internationally recognised test guidelines has been used to define the procedures for testing the toxicity of:

- effluents and leachates in full concentration range tests;
- effluents and leachates in single concentration tests and receiving waters in bioassays;
- a reference toxicant in full concentration range tests.

5.3.2 Scope of the procedure

Applications

This procedure describes a toxicity test (see glossary of terms) for the determination of the acute toxicity to juvenile *Daphnia magna* of freshwater treated and untreated industrial and sewage effluents and leachates (after either settlement, centrifugation or filtration if necessary) and receiving waters.

The experimental design adopted (for example number of exposure concentrations, interval between test concentrations and test duration) will depend on the objective of the study, which **must** be clearly defined at the outset.

Limitations

The results of toxicity tests can be affected by the pH, dissolved oxygen levels, suspended solids content and colour of samples and whether these are emulsions (see Section 5.3.8).

5.3.3 Principle

In the toxicity test procedure (see Section 5.3.9), groups of juvenile *Daphnia* are exposed to the environmental sample (effluent, leachate or receiving water) diluted with reference freshwater (see glossary of terms) to a range of concentrations for a period of 24 h or 48 h[5]. The different test concentrations in an appropriate range may, under otherwise identical test conditions, exert toxic effects on the swimming activity (and survival) of *Daphnia.* These will extend from an absence of effects at lower test concentrations to immobilization of all daphnids at higher test concentrations. The data should be used to determine:

- the median effective concentration, that is the concentration that immobilises 50% of the exposed *Daphnia* after 24 h or 48 h. The derived values are referred to as the 24 h-EC_{50} or the 48 h-EC_{50};
- the concentration that immobilises 10% of the exposed *Daphnia* after 24 h or 48 h (that is the 24 h - EC_{10} or the 48 h - EC_{10});
- the highest no-observed effect concentration after 24 h or 48 h (that is the NOEC);
- the lowest observed effect concentration after 24 h or 48 h (that is the LOEC).

In the context of these procedures, immobilization describes juvenile daphnids which do not swim within 15 seconds after gentle agitation of the test container, even if there is still movement of the antennae.

[5] Measurements of immobilisation must also be made after shorter exposure periods to allow the data to be analysed using the time to effect procedure (see Section 5.3.9).

5.3.4 Hazard

Safety procedures, such as fume hoods, eye protection and gloves, **must** be used which are appropriate to the COSHH assessment (for the sample) provided by the discharger.

5.3.5 Test facility

The test facility **must** be able to maintain the temperature of test solutions at 20 ± 2 °C. This can be achieved using a temperature controlled room or cabinet. Organisms should be maintained under "cool white" fluorescent light of 400-800 lux at the surface in a 16 ± 1 h light: 8 ± 1 h dark regime.

5.3.6 Reagents and materials

Test organisms

Juvenile *Daphnia magna* of a specific clonal type **must** be used for all the toxicity tests conducted in a facility. The clonal type used in a toxicity test should be recorded in the test report. At the start of the test, the juvenile daphnids **must** be less than 24 h old. The juveniles used to start the test must not be first or second brood progeny and **must** be derived from females 2-5 weeks old to avoid both young and senescent females.

Control/dilution water

In toxicity tests, the water used for the controls and the dilution of test solutions **must** be a reference freshwater (such as uncontaminated groundwater, dechlorinated tapwater or reconstituted water) used to culture the *Daphnia* in the facility.

The hardness of the reference freshwater **must** be between 140-250 mg $CaCO_3$ l^{-1}, within the range ± 20% of the water used for culturing the test organisms and must exclude chelators (see Section 5.2). Any greater differences in hardness between the culture water and the reference freshwater could lead to erroneous test results due to osmotic stress imposed upon the organisms.

Samples of the water used should be taken periodically and analysed to ensure no extraneous substances are present (see Section 5.2.3).

5.3.7 Apparatus

The following apparatus (see Appendix A) is used:

- glass or non-toxic inert clear plastic (Nalgene or polyethylene) containers of an appropriate volume, for example bottles, beakers or crystallizing dishes
- equipment for measuring pH, dissolved oxygen and temperature;
- equipment for the determination of water hardness;
- equipment for measuring light levels.

5.3.8 Treatment and preparation of samples

The DTA Methods Working Group has recommended that initially, effluent or leachate samples collected in the Demonstration Programme are tested unadjusted with measurements being made of all the key physico-chemical parameters (see Section 2). If it is apparent that any physico-chemical parameter or parameters are partially or fully responsible for measured responses, then subsequent tests may need to be carried out following modification of the parameter or parameters in test solutions or samples.

General procedures for the collection, transport, storage and treatment of effluents, leachates and receiving waters have been given in Section 2. However, there are specific test requirements for dealing with suspended solids, colour and oily substances and volatiles.

Suspended solids

For effluent samples with appreciable solids content, it is desirable to measure total suspended and settleable solids upon receipt, as these may influence the results of the toxicity test. In some cases, a high concentration of suspended solids may pose additional problems in the toxicity test, however, they are an integral component of the sample and should be retained. Suspended solids may impair visual observation of the test organisms and in some cases particles, by adhering to body appendages, may physically interfere with normal swimming movement. Removal of particulates may not be an option, however, some of these problems may be ameliorated by allowing time for the suspended solids to settle (to the base of the test vessel) before adding animals to the test solution.

Colour

Highly coloured solutions (or those with high levels of suspended solids) may impair visual observation of the *Daphnia* in the test vessels. Observation may be improved if each vessel is temporarily illuminated from the side or from below by placing it on a light box or other source. If this does not suffice, observations may be restricted to the termination of the test (48 h). At end of test, the solutions can be carefully poured into shallow dishes to aid observation or they can be poured gently through fine-mesh netting and the contents resuspended in dilution water for viewing.

Oily substances and volatiles

The presence of oily substances, or substances in excess of their water solubility, may cause flotation of *Daphnia* in the test solutions - by adhering to the carapace and other body appendages, the effective density of the animal in the test solution is lowered. In these circumstances, the interpretation of test data is very difficult as mortality may be influenced (positively or negatively) by the flotation. If flotation is observed in test concentrations there are practical options to employ that will enable a comparison of the toxicity of the effluent with and without the influence of flotation. By performing the experiment in sealed vessels (glass vessels with a septum cap are particularly suitable) the extrusion of all air and air bubbles will eliminate flotation. Before sealed vessels are employed, appropriate checks should be made to

ensure that survival of *Daphnia* in the dilution water control can be achieved. The loading of animals per vessel employed in the acute method should not exhaust the available dissolved oxygen during the test period.

The potential loss of volatile components from effluent samples is usually ignored in testing, however, if required, sealed systems could also be employed for testing of samples known to contain volatile components.

5.3.9 Test procedures

Provision of juvenile daphnids

The provision of <24 h old juvenile daphnids for toxicity tests can be achieved by a number of procedures including:

- isolating gravid adult *Daphnia magna* from the main culture 24 h before the start of the test. These gravid females should then be transferred to suitable vessels containing 200-500 ml of the reference freshwater to be used in the test and an algal inoculum as food. The stocking density of adult *Daphnia* in the vessels should be 10 or less. The time the females are isolated in a given vessel shall be recorded on a *Daphnia* Isolation Data Sheet (see Table 5B.3) along with the number of juveniles produced. Juveniles produced from the different isolation vessels should be combined in a common vessel prior to use in the tests. Adult *Daphnia* should be returned to the appropriate culture vessel after juveniles have been isolated.

- separating juveniles from a parent culture on the day preceding the test. A separation made in the late afternoon provides sufficient time for the operator to prepare and initiate the test before the juveniles are too old (that is 24 h). Separation may be carried out by carefully pouring the culture contents through a series of nylon meshes, the largest of which, should retain the adults only. Care should be taken not to pour the culture contents through the meshes too fast as eggs and embryos may inadvertently be 'flushed' from the brood chamber.

There has been some debate on whether the juveniles for testing, during the separation process, should not come into contact with air. Separation procedures that eliminate exposure to air may be less stressful to the juveniles, minimise the chances of damaging individuals during the separation process and avoid air entrapment. Note, however, that the juveniles do not appear to suffer any ill effects from very short exposures to air provided that they are rapidly transferred from the meshes to dilution water.

In a typical scenario, juveniles are obtained on the morning prior to test start. These cultures may not have been fed since the previous day, therefore, the *Daphnia* may not have received food for many hours. On these occasions, rather than retain juveniles for testing for long periods without food, it may be beneficial to separate the juveniles and provide them with a small quantity of algal food prior to testing to provide "healthy" organisms for the test.

Preparation of test concentrations

1. Select an appropriate concentration series **with the ratio between exposure concentrations not exceeding 2.2.** Where possible the range selected should be sufficient to give 0 and 100% immobilization and at least two intermediate degrees of immobilization between 0 and 100%. These results permit the calculation of the toxicity (24 h-EC_{50} or 48 h-EC_{50} values, 24 h-EC_{10} or 48 h-EC_{10} values and 24 h or 48 h NOEC and LOEC) values with greater precision. For effluents or leachates an appropriate initial concentration range would be 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 22.0, 46.0 and 100% v/v effluent.

For each control(s) and each effluent or leachate concentration a minimum of 20 animals, divided into four groups of five or two groups of 10, shall be used. The density of *Daphnia* per container **must** not exceed a maximum of five daphnids per 50 ml of solution (that is at least 10 ml of solution is required per animal).

2. Prepare the concentration range on the day of the test by diluting appropriate amounts of effluent or leachate with reference freshwater to provide at least 300 ml volumes of each concentration in volumetric glassware. Record the information on the preparation of the toxicity test concentration range in the test report (see Appendix 5C). In each test series, a control is needed which contains none of the test substance and has a volume equal to that of each exposure concentration.

The test vessels used should be of sufficient volume (for example 100 ml) to ensure a lack of oxygen does not cause a problem during the test. Crystallizing dishes (for example 100 ml volume) are preferred for effluents and leachates, which may deplete oxygen from solutions. The dishes permit diffusion of oxygen into the test solutions, but should be covered by watch glasses to limit evaporation and entry of dust into the solutions.

An effluent concentration range of 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 22.0, 46.0 and 100% v/v effluent using 500 ml test volumes would be prepared as follows:

Nominal conc. (% effluent)	Volume of reference freshwater (ml)	Volume of effluent (ml)
0 (Control)	500	0.0
0.1	499.5	0.5
0.22	498.9	1.1
0.46	497.7	2.3
1.0	495	5.0
2.2	489	11
4.6	477	23
10.0	450	50
22.0	390	110
46.0	270	230
100.0	0	500

Temperature, pH, dissolved oxygen and conductivity should be measured on all the test solutions remaining in the volumetric glassware after aliquots have been added to test vessels and recorded on a Water Quality Monitoring Data Sheet (for example, see Table 5C.4). Total hardness should be measured on the control and highest exposure concentration. The organisms **must** only be transferred if the temperature difference between the isolation vessels and the test vessels is less than 2 °C.

Procedure for the toxicity test

The procedures given for the initiation, monitoring and termination of the toxicity test **must** be followed.

Initiation of the toxicity test

Add *Daphnia* to each of the test containers so that there are a minimum of 20 animals per concentration in four groups of 5 or two groups of 10. The allocation of *Daphnia* to test vessels should be randomized to minimise systematic variability. Random number tables are included for use (see Appendix B).

Juvenile daphnids **must** not be fed during the course of tests[6].

It is important that the separation procedures (to provide the juveniles for testing) are performed carefully. Prior to transfer of juveniles to the test vessels, any damaged individuals or those that appear pale or exhibit weak swimming movements **must** be discarded. Juveniles should, ideally, be transferred with a glass pipette cut off and fire polished to provide a 5 mm opening. Some Guidelines recommend the addition of *Daphnia* beneath the water column, however, this will require a large stock of pipettes and there is also an increased chance of accidental cross contamination with test solution if sufficient care is not taken. As an alternative, allowing the *Daphnia* to enter the test solution within a drop of water delivered just above the surface from the tip of the pipette, has proved successful.

Although the glass pipette used for transfer must be of sufficient diameter to avoid damage to the juveniles, with practice, it is relatively easy to capture and transfer the requisite number of *Daphnia* per beaker in a very small volume of water. This process is facilitated if the juveniles are captured from a high density (that is several *Daphnia* may be captured in a single drop of water). If the quantity of *Daphnia* obtained for testing is relatively small, concentrate them in a relatively small water volume or explore their behavioural response to light as a means of

[6] The inclusion of food in the test medium may:

- either increase or decrease the toxic effects of the test substance due to adsorption onto food particles;
- alter the dissolved oxygen content by increasing the biochemical oxygen demand;
- alter the physiology of the instars and change the uptake and metabolism of the test substance;
- introduce additional variability into the test.

concentrating them in a small area. Care **must** be taken to minimize transfer of 'culture water' to test solutions and this should be restricted to ≤2 ml per test vessel to avoid markedly diluting test concentrations.

If *Daphnia* are floating in the control solution, there may be contamination of the test glassware or possibly entrapment of air as a result of a fault in the separation procedure (if the juveniles are exposed to air). Flotation, once it has occurred, may be difficult to remove although the severity of the symptoms may vary depending on the degree of contamination. The occasional 'mild' floater may be resuspended in the water column by allowing a drop of test water (delivered by a pasteur pipette) to fall on the animal thereby carrying it into the water column. If symptoms are severe, the test should be discarded and checks made to establish the likely causes of such contamination[7].

Monitoring of the toxicity test

Determine the number of mobile and immobile *Daphnia* in each test container after 24 h and 48 h and record the data on a Juvenile *Daphnia* Immobilization Toxicity Test Data Sheet (for example, see Table 5C.3). Observations **must** also be made at earlier intervals after the start of the test so that the test data can be analysed using the time to effect procedure (see Appendix B). It is recommended that measurements are made 1.5 h, 3 h and 6 h after the start of the test but this may need to be modified based on the results of initial tests. Animals which are not able to swim in the 15 seconds following agitation of the test container are considered to be immobile, even if there is still movement of the antennae. Any anomalies in the behaviour of the *Daphnia* (such as lethargy, circling or floating) should be noted and recorded on the data sheets. Observations can be made at other exposure times if these are considered necessary.

Termination of the toxicity test

Immediately after counting the immobilized *Daphnia* at the end of the test, measure the temperature, pH and DO in the controls and test concentrations and record the data on a Water Quality Monitoring Data Sheet (see Table 5C.4).

[7] Contamination may be difficult to trace and remedial action usually involves a thorough cleaning of all glassware and related items used in the test. Repeated flotation in control conditions should trigger a review of the separation procedures employed in a laboratory.

5.3.10 Processing of results

Validity of the results

The results from toxicity tests with *Daphnia magna* should be considered valid if the following conditions are satisfied:

(a) in the control(s) not more than a mean of 10% of the daphnids in the replicates have been immobilized or trapped at the surface of the water;

(b) for tests on unadjusted samples the dissolved oxygen concentration at the end of the test (see Section 5.3.9) in the control vessels is greater than or equal to 60% (ASV). If dissolved oxygen level is found to contribute to measured responses, and this parameter is subsequently modified in test solutions or samples, the DO level in the lowest test concentration causing 100% immobilization **must** be greater than or equal to 60% (ASV).

Data from tests on effluents or leachates for discharge characterisation should only be accepted if the results of the reference toxicity test met quality control criteria (see Appendix C).

Estimation of toxicity test endpoints

The EC_{50}, EC_{10}, NOEC and LOEC values are determined using an appropriate validated computer-based statistical package.

Estimation of the EC_{50} and EC_{10} values

At the end of the exposure period (24 h or 48 h), calculate the mean percentage immobilization of animals in each of the test concentrations relative to the total number of animals used for that concentration, and determine the 24 h-EC_{50} or 48 h-EC_{50} (and 24 h-EC_{10} and 48 h-EC_{10}) values by an appropriate statistical method (see Figure 5.5). Confidence limits ($p=0.95$) for the calculated EC_{50} (and EC_{10}) value should be determined using these standard procedures and quoted in the test report (see Appendix 5B).

Table 5.3 shows an example data set which has been used for the determination of the 48 h-EC_{50} (and 48 h-EC_{10}) value for the immobilization of juvenile *Daphnia* by an effluent using different statistical procedures.

Table 5.3 Example results of the immobilisation of *Daphnia magna* after exposure to an effluent for 48 h

		Scenario 1		Scenario 2		Scenario 3	
Effluent concentration (%)	Number of *Daphnia* exposed	Cumulative no. dead	Lethality (%)	Cumulative no. dead	Lethality (%)	Cumulative no. dead	Lethality (%)
Control	20	0	0	0	0	0	0
0.1	20	0	0	0	0	0	0
0.22	20	0	0	0	0	0	0
0.46	20	2	10	0	0	0	0
1.0	20	4	20	0	0	0	0
2.2	20	8	40	0	0	0	0
4.6	20	11	55	9	45	20	100
10	20	15	75	20	100	20	100
22	20	19	95	20	100	20	100
46	20	20	100	20	100	20	100
100	20	20	100	20	100	20	100

The data in Table 5.3 should be used by laboratories to check that in house statistical procedures are providing comparable results to those given in Table 5.4.

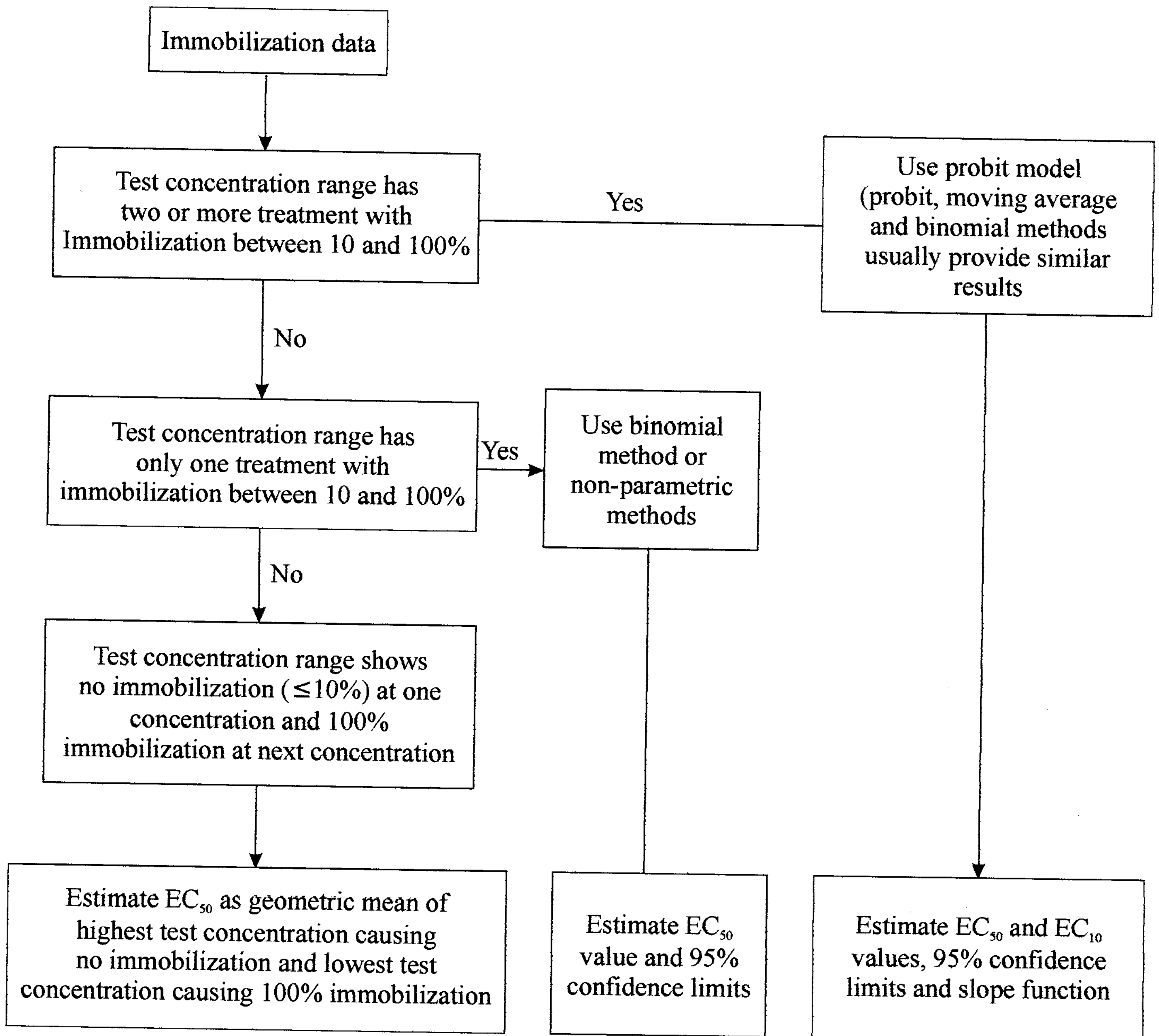


Figure 5.5 Flowchart for the estimation of the EC_{50} for full concentration range *D. magna* immobilization toxicity tests

In analysing data from *Daphnia magna* immobilization tests the following points should be considered:

1. If the results include concentrations at which there are 0-10 and 100% immobilization and also two concentrations at which the percentage immobilization is between 0-10 and 100%, and the data are smooth and regular, then probit, moving average and binomial methods should provide similar estimates of the EC_{50} value. Probit analysis should be used to estimate EC_{50} and EC_{10} values, 95% confidence limits and the slope, providing the probability is not less than 0.05.

2. If the results do not include two concentrations at which immobilization is between 0-10 and 100%, the probit and moving average methods cannot be used. The binomial method can be used to provide a best estimate of the EC_{50} and EC_{10} values with wide confidence limits. Non-parametric methods such as the Spearman-Karber or Trimmed Spearman Karber methods may allow the determination of an EC_{50}.

3. Where the data obtained are inadequate for calculating the EC_{50} by any of the standard methods or estimating the value graphically, identify the highest concentration causing no immobilization and the lowest concentration causing 100% immobilization. An approximation of the EC_{50} can be made from the geometric mean of these two concentrations. In this case, the ratio of the higher to the lower concentration should not exceed 2.2, otherwise any EC_{50} calculated will be less statistically sound.

4. In all instances, the EC_{50} derived from any of the above methods should be compared with a graphical plot on logarithmic-probability (log-probit) paper of percent immobilization for the various test concentrations. Any major disparity between the graphical estimation of the EC_{50} and that derived from the computer-based statistical programmes should be resolved by rechecking the statistical programmes.

Table 5.4 summaries the EC_{50} values derived for the different data sets in Table 5.3 using different statistical procedures. For the data in Scenario 1 the probit, moving average and binomial methods produce similar results although the confidence limits are greater for the value derived using the binomial method than those using the probit or moving average methods. Where there are less than two intermediate effect concentrations (Scenarios 2 and 3) the EC_{50} values derived are less statistically sound.

The 48 h EC_{10} value estimated by the Tox Calc software (Tide Pool Scientific Software) is 0.64% with 95% confidence limits of 0.35-0.98%.

From interpolation of the graph of cumulative immobilization of *Daphnia* (probability scale) against effluent concentration (log scale) shown in Figure 5.6 for Scenario 1 the 48 h-EC_{50} = 3.25% v/v effluent and the 48 h-EC_{10} = 0.46% v/v effluent. The values obtained graphically confirm those obtained using computer-based software (see Table 5.4).

Table 5.4 Summary of EC_{50} values (and 95% confidence limits) for the data in Table 5.3 estimated by different statistical procedures

Scenario	Statistical procedure	EC_{50} (%)		Slope
		Value	Confidence limits	
1	Probit (Tox Calc)	3.3	2.3-4.6[1]	1.8
	Probit (Stephan 1982[2])	3.3	2.8-3.8	1.8
	Moving average (Stephan 1982[2])	3.2	2.7-3.7	-
	Binomial (Stephan 1982[2])	3.6	1-10	-
2	Probit	Not valid approach		-
	Moving average	Not valid approach		-
	Binomial (Stephan 1982[2])	4.8	2.2-10	-
	Spearman-Karber (Tox Calc)	4.8	4.1-5.7	-
3	Geometric mean	3.2	-	-

[1] Fiducial limits
[2] Computer based statistical package based on Stephan (1977)

Estimation of the NOEC and LOEC

The NOEC and LOEC values are determined using hypothesis testing. If there are no replicates of each test concentration, as in the *Daphnia magna* immobilization test, the NOEC and LOEC values should be calculated using Fisher's Exact Test.

Further information on these statistical procedures can be obtained from Sokal and Rohlf (1981), Zar (1984) and US EPA (1993). In the example given in Table 5.3 the 48 h NOEC and LOEC values calculated using Fisher's Exact Test were 1.0 and 2.2% v/v effluent respectively.

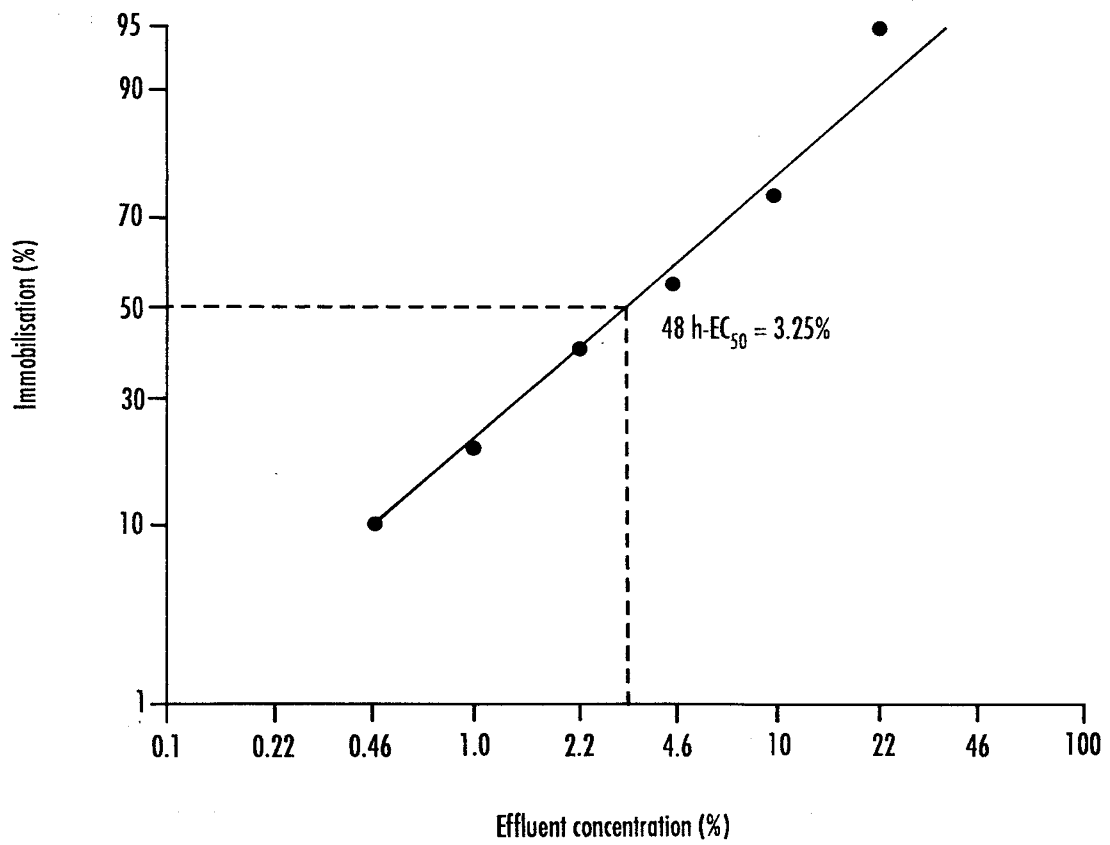


Figure 5.6 Graph of cumulative immobilization (probability scale) against effluent concentration (log scale)

5.4 Guideline for single concentration toxicity tests on effluents and leachates and bioassays on receiving waters using the juvenile *D. magna* immobilization test

5.4.1 Monitoring against toxicity limits

Introduction

Toxicity tests with *D. magna* for monitoring against toxicity limits should be carried out with a single concentration test comprising a single effluent or leachate concentration (toxicity limit) and an appropriate control(s).

Test procedure

The control water may be obtained from a 'clean' site. If water from a 'clean' site is used as the control, further controls should be prepared using the reference freshwater in which the daphnids were maintained. All relevant information should be documented in the test report (see Appendix 5C).

Single concentration tests should be initiated in the same way as full concentration range toxicity test (see Section 5.3.9) with duplicates of each control and test sample. Groups of 20 daphnids are then added to these solutions. A replicate could be prepared as four vessels containing 5 organisms or two vessels containing 10 organisms. The density of *Daphnia* per container **must** not exceed a maximum of five daphnids per 50 ml of solution (that is at least 10 ml of solution is required per animal). Immobilization should be monitored after 24 h and 48 h and recorded on a Juvenile *Daphnia* Immobilization Toxicity Test Data Sheet (see Table 5C.3). Observations can be made at other exposure times if these are considered necessary. Water quality monitoring should be carried out in the same way as described for the toxicity test (see Section 5.3.9) and the data recorded on a Water Quality Monitoring Data Sheet (see Table 5C.4).

Processing of results

Assessment of how the responses in the single effluent or leachate treatment compare to those in the control is accomplished using hypothesis testing (see Figure 5.7). The null hypothesis tested is that the responses in the treatment are not significantly different from those in the control.

Initially the proportion of organisms surviving in the control and the single treatment concentration are transformed using an appropriate procedure such as the arc sine square root transformation. The arc sine square root transformation is commonly used on proportional data to stabilise the variance and satisfy the normality and homogeneity of variance requirements. Shapiro-Wilk's or D'Agostino D test should be used to test the normality assumption.

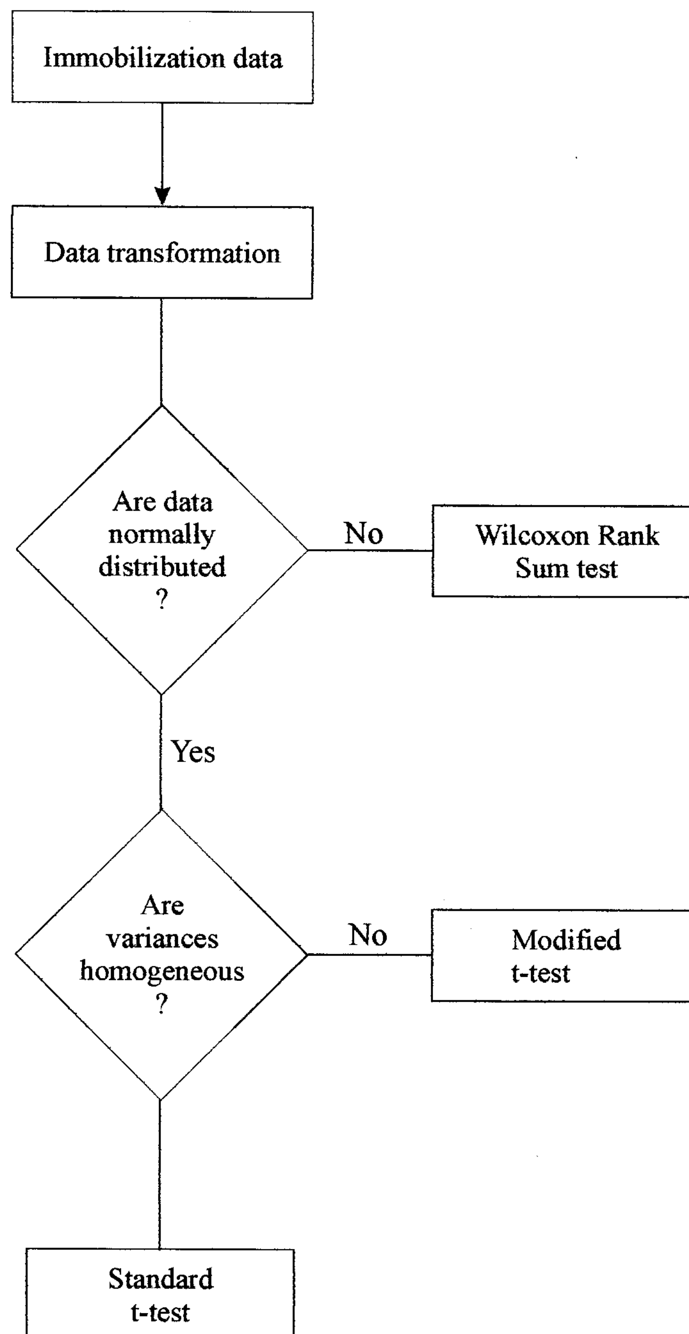


Figure 5.7 Flowchart for the analysis of single concentration test data from the *D. magna* immobilization test

If the data do not meet the assumption of normality then the non-parametric Wilcoxon Rank Sum Test should be used to analyse the data. If the data meet the assumption of normality, the F test for equality of variances is used to test the homogeneity of variance assumption. If the data meet the homogeneity of variance assumption then the standard (homoscedastic) t test should be used to analyse the data. Failure of the homogeneity of variance assumption leads to the use of a modified (heteroscedastic) t test, where the pooled variance estimate is adjusted for unequal variance, and the degrees of freedom for the test are adjusted. Further information on these statistical procedures can be obtained from Sokal and Rohlf (1981), Zar (1984) and US EPA (1993).

Table 5.5 shows example data sets for a single concentration test using duplicate control and 0.46% effluent solutions. In Scenario 1 the equality of variances cannot be confirmed and a modified (heteroscedastic) t test indicates a significant difference between responses in the two groups ($t = 6.52$, p 0.05). In Scenario 2 the variances are equal ($F = 2.23$, $p = 0.73$) and the standard (homoscedastic) t test indicates no significant difference between responses in the two groups ($t = 2.80$, $p > 0.05$).

5.4.2 Assessing receiving water column toxicity

The assessment of the toxicity of receiving water column samples should be carried out using the bioassay procedure given in Section 5.4.1 for an undiluted (100%) sample and an appropriate control(s). Receiving water samples may not meet the physico-chemical parameters required to support *Daphnia* survival. In these circumstances, the sample may have to be modified using the procedure given in Section 2 to satisfy the threshold criteria indicated in Table 5.6.

5.5 Guidelines for toxicity tests on reference toxicants using the juvenile *D. magna* immobilization test

5.5.1 Background

Acute *Daphnia* immobilization tests which are carried out to provide data for discharge characterisation or monitoring against a toxicity limit, should be accompanied by tests with the reference substance zinc (see Appendix C). If a facility only carries out toxicity tests infrequently then the sensitivity of the cultures to reference toxicants should be assessed monthly.

5.5.2 Test procedure

Reference toxicant tests should be conducted according to the procedure given in Section 5.3.9.

Table 5.5 Example data set for a single concentration test and the results of statistical analysis

Effluent concentration (%)	Replicate	No. of *Daphnia* exposed	Cumulative number of dead	Lethality (%)	Method of statistical analysis	Result of statistical analysis
Scenario 1						
0 (Control)	1	20	0	0	Modified t-test	Significant difference ($p < 0.05$)
	2	20	0	0		
0.46	1	20	2	10		
	2	20	3	15		
Scenario 2						
0 (Control)	1	20	0	0	Standard t-test	NS
	2	20	1	5		
0.46	1	20	2	10		
	2	20	3	15		

NS - no significant difference between control and treatment groups

5.5.3 Preparation of 1000 mg l^{-1} zinc stock solution

1. Weigh out 4397 mg of zinc sulphate ($ZnSO_4.7H_2O$) in a weighing boat.
2. Add the zinc sulphate to a 1-litre volumetric flask and dilute to just below the mark with distilled water.
3. Add 1 ml of 1M Analar HCl to stabilize the stock solution.
4. Dilute to the mark with distilled water.

5.5.4 Preparation of the test concentrations

For the reference toxicant zinc the concentration range given below should be used in the first study to assess the sensitivity of the test organisms in a facility when no previous data is available.

Nominal zinc conc. (mg l^{-1})	Volume of reference freshwater (ml)	Volume of zinc stock (ml)
0 (Control)	1000	0.0
0.1	1000	0.1
0.32	1000	0.32
1.0	999	1.0
3.2	996.8	3.2
10.0	990	10.0

The above volumes relate to a zinc stock concentration of 1000 mg l^{-1}, which should be prepared according to the procedure given in Section 5.5.3.

The test concentration ranges of zinc for subsequent tests can be modified based on the initial results to allow the derivation of more precise LOEC and EC_{50} values.

5.5.5 Test procedure

Reference toxicant tests should be initiated in the same way as full concentration range toxicity test (see Section 5.3.9). The test vessels used must be of sufficient volume to ensure that the density of *Daphnia* per container does not exceed a maximum of five daphnids per 50 ml of solution (that is at least 10 ml of solution is required per animal). Immobilization should be monitored after 24 h and 48 h and recorded on a Juvenile *Daphnia* Immobilization Toxicity Test Data Sheet (see Table 5C.3). Water quality monitoring should be carried out in the same way as described for the toxicity test (see Section 5.3.9) and the data recorded on a Water Quality Monitoring Data Sheet (see Table 5C.4).

Samples of the zinc test solutions should be taken at the beginning of the test from the stock vessel for each test concentration and at the end of the test from the vessels themselves and analysed using an appropriate procedure (for example, SCA 1980, 1981, 1988).

5.5.6 Processing of results

The 48 h LOEC and EC_{50} values should be calculated using the procedures described in Section 5.3.10. The estimation of toxicity values should be based on measured exposure concentrations.

5.6 Test report

The test report (see Appendix 5C) **must** include the following information:

(a) information about the test organism such as the scientific name, clonal type, source, any pre-treatment, the culture method (including type and amount of food and feeding frequency) and the age of daphnids used in the test;

(b) the source of the reference freshwater for toxicity tests and the major chemical characteristics of the water such as temperature, pH and hardness;

(c) the methods of preparation of test samples including for effluents, leachates and receiving waters, the manner and duration of storage of the samples and, if necessary, the conditions by which physico-chemical parameters were modified, for example, whether pH was adjusted or suspended solids were treated (either settlement, centrifugation or filtration);

(d) tables showing the cumulative immobilization at each control and test concentration at the end of the exposure period (that is 24 h or 48 h) for full concentration response tests, single concentration tests and reference toxicant tests;

(e) an indication that criteria determining the validity of the test (that is a control immobilization of $<10\%$ and a dissolved oxygen level of $>60\%$ ASV in the control for unmodified samples and in the lowest concentration causing 100% immobilization for modified samples) have been achieved;

(f) for tests on effluents and leachates for discharge characterisation or monitoring toxicity-based limits, an indication that the responses of juvenile daphnids in the reference toxicant test(s) met quality control criteria;

(g) the derivation of the 24 h-EC_{50} or the 48 h-EC_{50}, the 95% confidence limits and the method of calculation. The 24 h-EC_{10} or the 48 h-EC_{10}, values (and 95% confidence limits) and the NOEC and LOEC values after 24 h or 48 h are also reported;

(h) any abnormal behaviour of the *Daphnia magna* under the test conditions;

(i) any operating details not specified in this procedure and any incidents which may have affected the results.

If information is not available for any reason this shall be documented in the test report.

5.7 Checklist

A checklist summarizing the test conditions and procedures for juvenile daphnid immobilization toxicity tests is given in Table 5.6.

Table 5.6 Checklist of test conditions and procedures for acute juvenile *Daphnia magna* immobilization toxicity tests

Test procedure

Organisms	Neonates of *Daphnia magna,* ≥10 daphnids per concentration, loading density ≤ one daphnid per 10 ml
Type	Static, 24 or 48 hours duration
Control/dilution water	Reference freshwater
Temperature	20 ± 2 °C
Physico-chemical parameters	If the total biological effect of a sample is being measured, then the sample is tested unadjusted and key physico-chemical parameters (such as temperature, pH, dissolved oxygen, salinity and suspended solids) are measured. If it is apparent that any physico-chemical parameters are partially or fully responsible for measured responses, then the test solutions or sample have to be modified using the procedures given in Section 2 to satisfy the threshold criteria given below:

Physico-chemical parameter	Threshold criteria
pH	7.4-8.5 in all test vessels
Dissolved oxygen	≥60% ASV in all test vessels
Hardness	140-250 mg $CaCO_3$ l^{-1} in all test vessels
Suspended solids	<20 mg l^{-1} in all test vessels

Lighting	"Cool white" fluorescent light of ≤400-800 lux at the surface. 16 ± 1 h light : 8 ± 1 h dark regime
Feeding	No feeding during the test
Observations	Immobilization and atypical behaviour (lethargy, circling, floating) observed at specified times

Table 5.6 continued

Measurements	Test solution pH and DO at the beginning and end of the test, conductivity and hardness at the start as a minimum
Endpoints	24 h-EC_{50} and/or 48 h-EC_{50} (+ 95% confidence limits) 24 h-EC_{10} and/or 48 h-EC_{10} (+ 95% confidence limits), 24 h and/or 48 h NOEC and LOEC values
Reference toxicant	Zinc (as zinc sulphate) determined at the time of testing or monthly if testing is carried out infrequently
Test validity	Mean control immobilization ≤10% and for unadjusted samples DO in controls ≥60% ASV or for modified samples DO in lowest concentration causing 100% immobilization ≥60% ASV
Test samples	
Effluents, leachates and receiving waters	
Transport and storage	Transport at a temperature not markedly different from that measured at the time of collection. The test **must** begin within 48 h after the time of sampling has been completed. If the sample is not to be tested immediately on receipt then it should be stored at 5 ± 3 °C.
Control/dilution water	Reference freshwater[*1]

*1 If an upstream receiving water is used as the control/dilution water, a reference freshwater control should also be run.

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APPENDIX 5A PREPARATION OF RECONSTITUTED FRESHWATER

Table 5A.1 gives the types and quantities of analytical grade chemicals to distilled or deionised water with a conductivity of <10 µS cm^{-1} to prepare ASTM reconstituted hard water.

Table 5A.1 Preparation of ASTM reconstituted hard water

Water type	Reagent added (mg l^{-1})				Final water quality	
	$NaHCO_3$	$CaCl_2.2H_2O$	$MgSO_4$	KCl	Hardness[1]	pH[2]
Hard	192.0	120.0	120.0	8.0	160 - 180	7.6 - 8.0

1 - Expressed in mg $CaCO_3$ l^{-1}
2 - Approximate pH after aerating for 24 h

Table 5A.2 describes the types and quantities of analytical grade chemicals to be added to distilled or deionised water having a maximum conductivity of 10 µS cm^{-1} to prepare a hard reconstituted water (250 mg $CaCO_3$ l^{-1}) for freshwater toxicity tests (ISO 1989). Prepare the water by mixing 250 ml of each solution in a volumetric flask and make up to a total volume of 10 litres with distilled or deionized water. The water should be stored in a clean container made of an inert material (see Section 2).

The dilution water should be aerated until the dissolved oxygen concentration has reached saturation and the pH has stabilised. If necessary, adjust the pH to 7.8 ± 0.2 by adding 1M sodium hydroxide (NaOH) solution or 1M hydrochloric acid (HCl) solution. The water prepared in this way should not be aerated further before use.

Table 5A.2 Preparation of ISO reconstituted hard water

Solutions	Formula	Stock solution concentration (g in 1 litre volumetric flask)
Calcium chloride dihydrate	$CaCl_2.2H_2O$	11.76
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	4.93
Potassium chloride	KCl	0.23
Sodium hydrogen carbonate	$NaHCO_3$	2.59

APPENDIX 5B DATASHEETS USED WHEN CULTURING *DAPHNIA MAGNA* (EXAMPLE)

Table 5B.1 ***Daphnia*** **Culture Data Sheet (Example)**

Year: Period:

Sheet number of sheets Culture number:

Date ______________________

Feeding

Algae - Volume (ml) ______________________

- Batch number ______________________

Yeast - Volume (ml) ______________________

- Batch number ______________________

Initials ______________________

Ephippia/dead *Daphnia*

Number ephippia removed ______________________

Cumulative number ______________________

Number dead removed ______________________

Cumulative number ______________________

Initials ______________________

Isolation of gravid ***Daphnia*** **for juvenile production**

Number removed from culture for tests ______________________

Isolation vessel number ______________________

Initials ______________________

Return of adult *Daphnia*

Number returned ______________________

Initials ______________________

Table 5B.2 ***Daphnia*** **Culture Establishment Data Sheet (Example)**

Source of juveniles

Isolation vessel number:

Main *Daphnia* culture number (from 5B.1): New culture number:

Juveniles isolated at on :

Number of juveniles isolated: Number immobilized/dead juveniles:

Approximate age of juveniles:

Water Quality

Temperature	- Water containing juveniles	°C
	- New culture water	°C
pH	- New culture water	
DO	- New culture water	% ASV
Date	Time	Initials

Transfer of juveniles

Time juveniles transferred:

Initials

Observations

	Date	Time	Initials	Age of culture
Gravid *Daphnia* observed				
Juvenile *Daphnia* observed				
Volume of culture increased to 1.5 litres				

Culture discarded on at by

Total number of ephippia produced:

Table 5B.3 *Daphnia* Isolation Data Sheet (Example)

Year: Period:

Main culture number: Isolation vessel number:

Isolation of gravid *Daphnia*

Date	Time	Temperature (°C)		Volume of medium in isolation vessel	Number of *Daphnia*	Initials
		Culture vessel	Isolation vessel			

Isolation of juveniles

Date ____________________

Isolation vessel temp (°C) ____________________

Holding vessel temp (°C)* ____________________

Number of juveniles ____________________

Juveniles used ____________________

Time ____________________

Initials ____________________

* It is not necessary to record these data if the juveniles are not required for a test

Return of *Daphnia* to main culture

Date ____________________

Number dead removed ____________________

Number gravid animals returned to culture ____________________

Table 5B.3 Continued (Example)

Feeding of isolated *Daphnia*

Date ______________________________

Time ______________________________

Algae (ml) ______________________________

Batch number ______________________________

Yeast (ml) ______________________________

Batch number ______________________________

Initials ______________________________

APPENDIX 5C TEST REPORT FOR A JUVENILE *DAPHNIA* IMMOBILIZATION TOXICITY TEST (EXAMPLE)

Table 5C.1 Provision of juvenile daphnids

Data on test species

Origin of test species:

Clonal type:

Holding conditions: Temperature (°C)

pH

Total hardness (mg $CaCO_3$ l^{-1})

Feeding regime

Data on test substance

Test substance:

Source: Hazard:

Date collected or prepared:

Date received:

Storage conditions: Temperature (°C)

Length of time before start of test (h)

Collection of juveniles

Time collection of juveniles started:

Time collection of juveniles ended:

Age of *Daphnia* at start of test (h):

Table 5C.2 Preparation of toxicity test concentration range

Test substance:

Type of test:

Start of test: End of test:

Duration of test:

No. of test concentrations:

No. of controls:

No. of *Daphnia* per concentration:

No. of replicates per concentration:

Concentration range

Stock solution concentration:

Dilution media:

Volume of test solution required (ml):

Nominal test substance concentration	Volume of reference freshwater (ml)	Volume of test substance (ml)

Table 5C.3 Juvenile *Daphnia* Immobilization Toxicity Test Data Sheet

Test substance:

Date and time *Daphnia* added:

Exposure conc.	Replicate	Time after start of the test					
		h		h		h	
		No. of mobile *Daphnia*	No. of immobile *Daphnia*	No. of mobile *Daphnia*	No. of immobile *Daphnia*	No. of mobile *Daphnia*	No. of immobile *Daphnia*

Table 5C.4 Water Quality Monitoring Data Sheet - Toxicity Test

Date	Time	Vessel No.	Nominal conc	Temp (°C)	pH	DO (%ASV)	Total hardness (mg $CaCO_3$ l^{-1})	Initials

Table 5C.5 Expression of results of a toxicity test

Exposure period	Exposure conc.	Cumulative No. of immobile Daphnia in each replicate				Total No. of immobile *Daphnia*	Total No. of *Daphnia* exposed	Immobile *Daphnia* (%)
		1	2	3	4			

Calculation of the EC_{50} and EC_{10} values

Exposure period (h):

Method used: EC_{50} = EC_{10} =

Concentrations used in the calculation of the EC_{50}: Nominal/Measured

Calculation of the NOEC and LOEC values

Exposure period (h):

Method used: NOEC = LOEC =

Abnormal behaviour of the *Daphnia* during the test

Operating details not specified in the standard operating procedure and any incidents which may have affected the result:

6. OYSTER EMBRYO-LARVAL DEVELOPMENT TEST GUIDELINE

6.1 Introduction

This section of the DTA Methods Guidelines describes the procedures for the holding of adult oysters and for conducting toxicity test using oyster embryos to measure the effects of effluents, leachates and receiving waters on larval development. The document has been compiled with reference to an existing internationally recognised standard procedure (ICES 1991).

Critical steps in the holding of oysters and the conduct of the test procedure which **must** be followed are identified in bold type whereas instructions given in normal type are recommended and alternatives can be used.

6.2 Holding of test organisms

6.2.1 Introduction

The purpose of holding adult oysters (where necessary) using the following procedure is to provide conditioned organisms from which viable sperm and eggs are obtained to produce embryos for toxicity tests and bioassays.

6.2.2 Test species

The species to be used in toxicity tests and bioassays is the Pacific oyster (*Crassostrea gigas*). Embryos for the toxicity tests and bioassays are produced from the sperm and eggs of conditioned adult male and female oysters of individual wet weight greater than 45 g. The oysters **must** be obtained from a recognised commercial shellfish hatchery (see Appendix A) and should be delivered to the toxicity laboratory by courier within 24 h of dispatch.

Structure of the gonad

The gonad is the largest organ in a ripe oyster and typically represents 30% of the total wet weight of soft tissue (see Figure 6.1). The gonad, covered by the mantle, is normally in total a layer 5 to 8 mm thick which envelopes the digestive gland. Oysters in a poor reproductive condition have very thin gonads, in which only the digestive gland is visible, and these should be discarded.

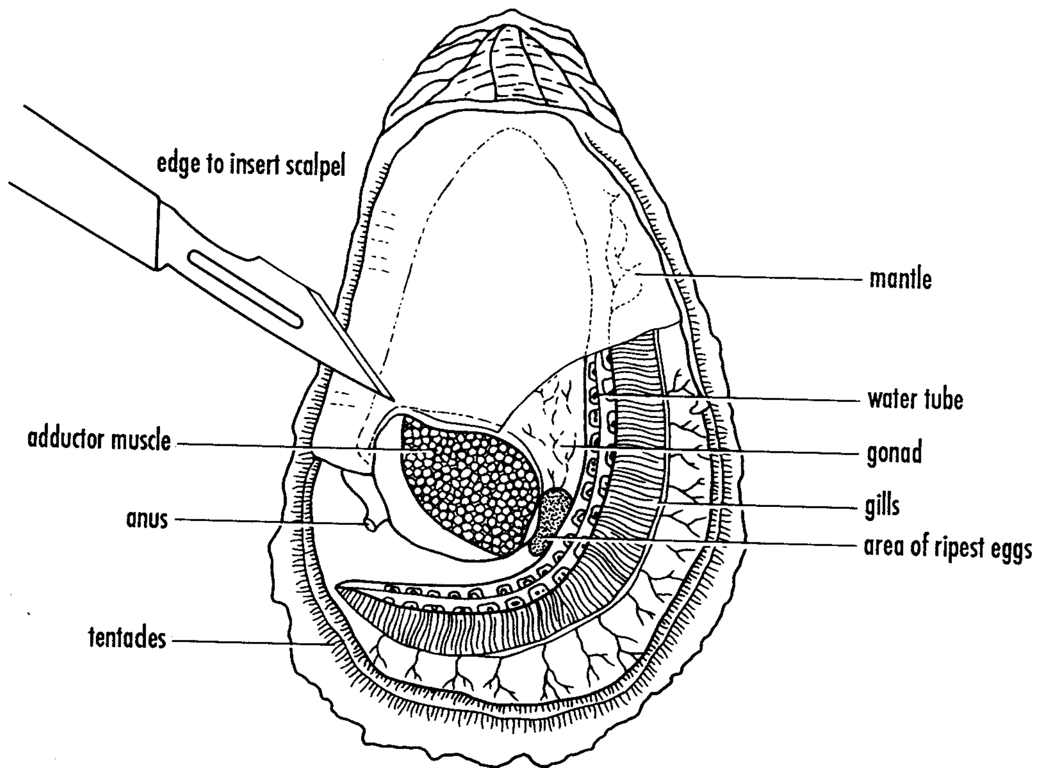


Figure 6.1 The anatomy of the Pacific oyster, *Crassostrea gigas*

Assessment of egg maturity

Eggs in the mature ovary of *Crassostrea gigas* are pear shaped and compressed with the long axis varying from 55-75 µm and the width at the broadest part measuring 35-55 µm (Galtsoff 1963). The oblong shape is retained some time after discharge into seawater, but gradually the eggs become globular and denser. The nucleus appears as a large transparent area surrounded by densely packed granules. The rounded eggs can vary from 45-62 µm in diameter (Loosanoff and Davis 1963). Immature eggs tend to be very irregular in size and shape and are often clumped together. Eggs that are undergoing resorption are characterized by a shrunken egg within the vitelline membrane.

Assessment of embryos

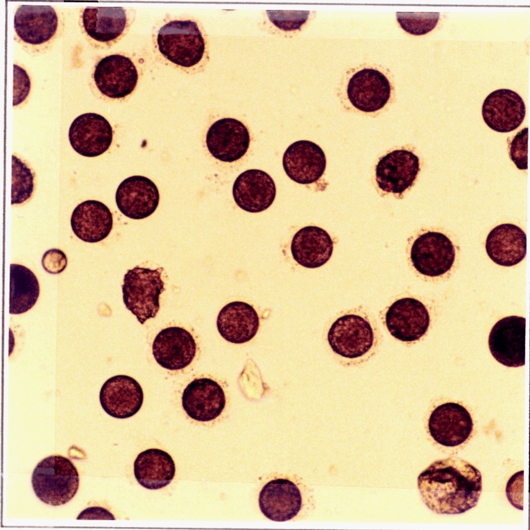
The pattern of development for normal embryos is given below and is shown in Figure 6.2:

Stage	Cell shape	Number of cells
1st division	Bilaterally symmetrical	2
2nd division	'Catspaw' shaped	4
3rd division	Becoming more spherical	8
4th division	" " "	16
5th division	Blackberry like	32

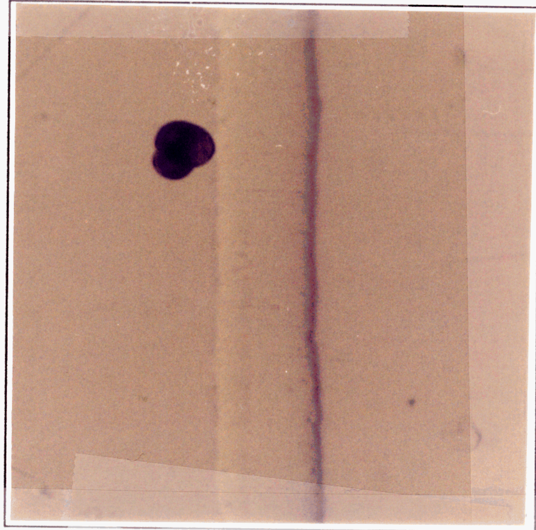
An ideal batch of normal embryos will have:

- >95% normally fertilized eggs;
- >60% of embryos at the same stage of development. Two hours after fertilization, the majority of eggs in normal batches of embryos will be at the 16 cell stage, with some unfertilized eggs and some 32 cell stage embryos;
- A uniform shape consisting of dark, granular and tightly packed cells.

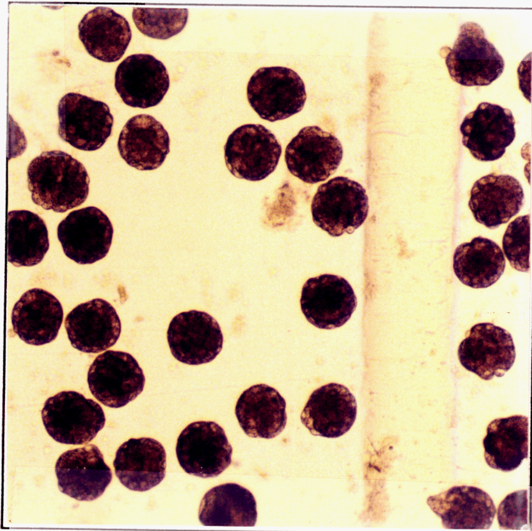
Abnormal embryos, which become apparent between the 3rd and 5th divisions and consist of loosely packed cells appearing almost separate, should be discarded. Abnormal embryos which have undergone rapid division consist of extended or oblong cells as opposed to normal rounded cells.



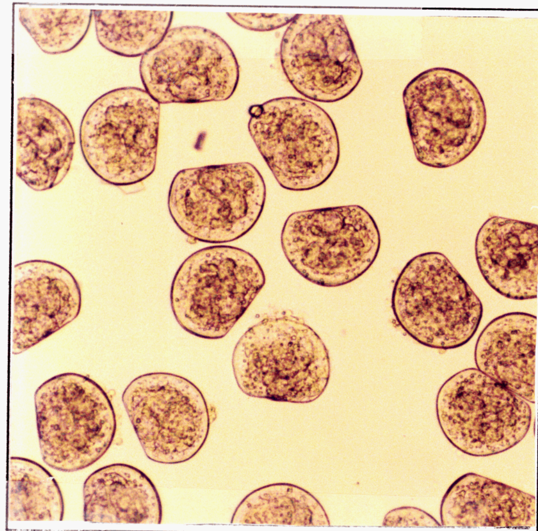
a) Eggs (Time 0h)



b) Embryos (30min Post fertilisation)



c) Embryos (2h Post fertilisation)



d) D shaped larvae (24h Post fertilisation)

Figure 6.2 Embryology of *Crassostrea gigas*

6.2.3 Conditioning of oysters

Conditioned oysters are normally used within a few hours of receipt and require no maintenance. However, if required, the oysters can be held for several days if they are kept in air under damp and refrigerated (5 ± 3 °C) conditions. The damp conditions can be achieved by wrapping the oyster in paper moistened with seawater and placing them in a closed box.

If the oysters supplied by the shellfish hatchery have not been conditioned then it will be necessary to carry this out in-house. However, since the process is time consuming it should only be adopted if it is considered to be the most cost-effective option. If conditioning is required guidance should be sought from a recognised shellfish hatchery (see Appendix A).

6.3 Guideline for toxicity tests on effluents and leachates using the Pacific Oyster (*Crassostrea gigas*) embryo-larval development test

6.3.1 Introduction

Information given in the internationally recognised test guideline (ICES 1991) has been used to define the procedures for testing the toxicity of:

- effluents and leachates in full concentration range tests;
- effluents and leachates in single concentration tests and receiving waters in bioassays;
- a reference toxicant in full concentration range tests.

6.3.2 Scope of the procedure

Applications

This procedure describes a toxicity test (see glossary of terms) for the determination of the acute toxicity to oyster embryos of treated and untreated industrial and sewage effluents and leachates (after either settlement, filtration or centrifugation if necessary) and receiving waters.

The experimental design adopted (for example number of exposure concentrations and interval between test concentrations) will depend on the objective of the study, which **must** be clearly defined at the outset.

Limitations

The results of toxicity tests can be affected by the pH, dissolved oxygen levels and suspended solids content of samples. The testing of freshwater discharges to marine waters may require the use of salinity correction procedures (see Section 6.3.8).

6.3.3 Principle

In the toxicity test procedure (see Section 6.3.9), groups of oyster embryos are exposed to the environmental sample (effluent, leachate or receiving water) diluted with reference seawater (see glossary of terms) to a range of concentrations for a period of 24 h. The different test concentrations in an appropriate test range, under otherwise identical test conditions, may exert toxic effects on the normal development of embryos into `D' shaped larvae[1]. These will extend from an absence of effects at lower test concentrations to lethality and a lack of development in all the embryos at the higher test concentrations.

The data shall be used to determine:

- the median effective concentration, that is the concentration that results in only 50% of the exposed oyster embryos developing normally to D larvae after 24 h. This median effective concentration is referred to as the 24 h-EC_{50};
- the concentration that results in only 10% of the exposed oyster embryos developing normally to D larvae after 24 h (that is the 24 h-EC_{10});
- the highest no-observed effect concentration after 24 h (that is the NOEC);
- the lowest observed effect concentration after 24 h (that is the LOEC).

6.3.4 Hazard

Safety procedures, such as fume hoods, eye protection and gloves, **must** be used which are appropriate to the COSHH assessment (for the sample) provided by the discharger.

6.3.5 Test facility

The test facility **must** be able to maintain the temperature of test solutions at 24 °C ± 2 °C. This can be achieved using a temperature controlled room or an incubator.

6.3.6 Reagents and materials

Test organisms

The test is carried out using embryos of the Pacific oyster (*Crassostrea gigas*) (see Section 6.2.2).

[1] In the context of these procedures, normal development describes the transformation of naked embryos, over a 24 h exposure period to larvae having a protective `D' shaped shell, where the paired hinged shells are visible. Although the exposure time is short, it encompasses a period of intense cellular activity, in which the impairment of a number of critical physiological and biochemical processes may result in poor growth and development. `Abnormal development' is characterised by embryos which die at an early stage or larvae which are developing but fail to reach the D stage.

Control/dilution water

In toxicity tests, the water used for the controls and the dilution of test solutions **must** be a reference seawater (see glossary of terms). A suitable reference seawater can be prepared by adjusting distilled or deionised water to 34 ± 2‰ with analytical quality sea salts (for example, Sigma Chemical Co.). However, the reconstituted water **must** be aged before use.

6.3.7 Apparatus

The following apparatus (see Appendix A) is used:

- test containers (vials) stoppered or screw capped made of non-toxic inert material (such as glass or polystyrene) and capable of holding 30 ml of test solution;
- a constant temperature room or incubator to maintain test solutions at 24 ± 2 °C;
- a microscope (inverted or binocular) providing magnification of 20-100x and a suitable gridded counting chamber such as a 1 ml Sedgewick-Rafter cell;
- equipment for measuring pH, dissolved oxygen and temperature;
- a salinity or conductivity meter or refractometer capable of measuring salinity in small volume samples;
- tally counters for recording egg and larval numbers;
- a large flat bladed scalpel or oyster knife for opening oysters.

6.3.8 Treatment and preparation of samples

The DTA Methods Working Group has recommended that initially, effluent or leachate samples collected in the Demonstration Programme are tested unadjusted with measurements being made of all the key physico-chemical parameters (see Section 2). If it is apparent that any physico-chemical parameter or parameters are partially or fully responsible for measured responses, then subsequent tests may need to be carried out following modification of the parameter or parameters in test solutions or samples.

General procedures for the collection, transport, storage and treatment of samples for testing have been given in Section 2. However, there are specific test requirements for dealing with salinity and suspended solids.

Salinity

Freshwater environmental samples can only be normally tested at concentrations ≤33% v/v, otherwise the salinity of the test solutions will fall below the 22‰ salinity tolerance threshold for oyster embryos. Testing at higher concentrations can be achieved by altering the salinity of the sample by the addition of analytical quality sea salt (for example, Sigma Chemical Co.). If the salinity of the test samples has to be adjusted with sea salt an additional control **must** be

run using a solution of sea salt in distilled or deionised water at the salinity of the reference seawater.

Suspended solids

For effluent samples with appreciable solids content, it is desirable to measure total suspended and settleable solids upon receipt, as these may influence the results of the toxicity test. In some cases, a high concentration of suspended solids may pose additional problems in the toxicity test, however, they are an integral component of the sample and should be retained. Suspended solids may impair visual observation of the test organisms. Removal of particulates may not be an option, however, some of these problems may be ameliorated by allowing time for the suspended solids to settle in the sample before preparing the test solutions.

6.3.9 Test procedure

A number of procedures for the preparation of oyster embryos have been shown to consistently result in >80% normal D larvae in test controls.

It is only necessary to strip sufficient organisms to obtain viable batches of sperm and eggs, which may mean only one male and female oysters are needed.

Preparation of oyster embryos

Obtaining gametes

Oysters are usually supplied by the hatchery conditioned and often ready sexed. Male and female gametes are obtained by stripping the gonads or by naturally spawning the adults.

Stripping the gametes does not appear to affect the quality or variability of the embryos (Allen *et al.* 1988). Irrespective of the approach used to strip the gametes it is imperative that gamete collection is synchronized. An appropriate procedure is described below:

1. Open the mature male and female conditioned oysters by cutting the adductor muscle with a standard oyster knife. The knife should be inserted in the flat edge of the oyster and held level when cutting to avoid damaging the gonads.

2. Rinse the body cavity of each oyster thoroughly with reference seawater to remove debris.

3. Prior to collecting sperm from the males a small sample from each organism should be obtained and placed on a slide with a few drops of reference seawater. After 15-30 minutes the activity of the sperm is assessed under the microscope and suitable males are selected for use.

4. Collect the gametes by one of two alternative methods, both of which have been shown to consistently result in >80% normal D larvae in test controls:

   (a) Insertion of a Pasteur pipette into the gonads

   Insert a clean Pasteur pipette into the gonad to a depth of 1-2 mm and draw the eggs or sperm collected from the area indicated in Figure 6.1. Care should be taken to avoid puncturing the gut. Transfer the gametes to separate volumes of reference seawater at 24 ± 2 °C.

   (b) Incision of gonads

   Ensure the body of the oyster has been thoroughly washed with a reference seawater to remove all debris and signs of body fluids, as contamination of gametes can lead to reduced fertilisation. The gonad should be gently incised (see Figure 6.1) with a sharp scalpel, angling the blade upwards to avoid puncturing the gut. Collect the gametes in a suitable beaker by pipetting reference seawater (at 24 ± 2 °C) over the surface of the gonad.

Alternatively natural spawning methods (such as temperature shock) can be used to obtain male and female gametes.

Identification and preparation of gametes

1. Identify the sex of the suspensions of gametes prior to use if this is unknown. In water, sperm are identified by their milky appearance and eggs by their granular appearance (see Figure 6.2). Identification and motility of the sperm should be confirmed by microscopic examination at 100x magnification.

2. Filter the egg suspensions from each female through 90-100 µm plastic mesh to remove tissue debris and collected in clean glass beakers. The filtered egg suspensions from each female can then either be held individually or mixed in a clean glass beaker. Filtration of the egg (and sperm) solutions is specified since the presence of other tissue material can interfere with the development of the embryos. A sub-sample (1-5 ml) of the individual suspensions or mixed suspension should be removed and examined microscopically using an appropriate chamber, such as a Sedgewick-Rafter cell or a Coulter counter, to assess egg densities and quality[2]. The density of the stock egg suspension should then be modified with an appropriate volume of reference seawater to achieve an egg density of 1000-4000 eggs per ml and, ideally 3000 (± 300) eggs per ml.

3. Filter the sperm solutions from the males through a 60 µm mesh to remove tissue debris and collect the filtrates in clean glass beakers. The filtered sperm suspensions should then be mixed in a clean glass beaker.

[2] Only use eggs which appear normal (see Section 6.2.2)

Fertilisation of the eggs

1. Fertilise the egg suspension within 30 minutes of obtaining the eggs and sperm by mixing the gametes at a ratio of 2-3 ml of sperm to 1-litre of the egg suspension. The embryo suspension can be prepared in a number of ways which will result in >80% normal D larvae in test control. These include:

   - fertilising the egg suspension from each female with the pooled sperm suspension and subsequently pooling the viable and healthy embryo suspensions;

   - fertilising the mixed egg suspension with the pooled sperm suspension.

   The approach adopted should not affect the outcome of the test provided the eggs used are normal (see Section 6.2.2). However, results may be affected if abnormal eggs from one or more females are included in a mixed egg suspension.

2. After mixing, leave the eggs for 2 h at 24 ± 2 °C in the dark without aeration. During this time the eggs should undergo the early stages of cleavage and typically reach the 16-32 cell stage (see Figure 6.2). After approximately 2 h the embryos should be assessed microscopically (at 20-40 x magnification) using an appropriate counting chamber, such as a Sedgewick-Rafter cell, to determine whether cell cleavage is occurring.

If cleavage has started but the embryos have not reached the 16-32 cell stage, the suspension should be left for up to an additional 2 h to allow this level of development to be reached. If it has not been reached after this time the test should be restarted and other oysters stripped for gametes.

An embryo density of approximately 50 per ml is required in each 30 ml test solution, therefore, 1500 embryos are required per test vessel. An inoculum size of 0.37-1.5 ml, and ideally 0.5 ml, should be used at each test concentration to avoid affecting the salinity of the test solutions. The embryo suspension used, therefore, needs to contain 1000-4000 embryos per ml, and ideally 3000 (± 300) embryos per ml.

Procedure for the toxicity test

1. Select an appropriate concentration series **with the ratio between exposure concentrations not exceeding 2.2**. Where possible the range selected should include concentrations at which there is no effect on larval development, relative to control, and complete cessation of development (100% inhibition), along with those causing intermediate effects on larval development. This permits the calculation of the 24 h-EC_{50}, 24 h-EC_{10} and 24 h NOEC and LOEC values with greater precision. For effluents or leachates an appropriate initial concentration range would be 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 22,0, 46.0 and 100% v/v effluent. The preparation of the higher concentrations (>20% v/v effluent) in tests with oyster embryos may require salinity correction.

2. Prepare the concentration range on the day of the test by diluting appropriate amounts of the effluent or leachate with reference seawater to provide at least 200 ml volumes of

each concentration in volumetric glassware[3]. Record the information on the preparation of the toxicity test concentration range in the test report. In each test series a control is needed which contains none of the effluent or leachate and has a volume equal to that of each exposure concentration. The test vessels used **must** be made of non-toxic materials (such as glass, polystyrene, polyethylene) with a total volume of 35-60 ml and capable of holding 30 ml of test solution with a head space.

At least three replicate vessels containing 30 ml of test solution should be used for each test concentration, along with six replicates of the control. Two additional vessels each containing 30 ml of test solution are needed at each test concentration to measure water quality parameters (see next Section).

An effluent concentration range of 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10 and 20% v/v effluent using 200 ml test volumes would be prepared as follows:

Nominal conc. (% effluent)	Volume of reference seawater (ml)	Volume of effluent (ml)
0 (Control)	200	0.0
0.1	199.8	0.2
0.22	199.5	0.44
0.46	199.1	0.92
1.0	198.0	2.0
2.2	195.6	4.4
4.6	190.8	9.2
10.0	180	20
22.0	156	44
46.0*	108	92
100*	0	200

* = Salinity correction of these test concentrations by the addition of analytical quality sea salt may be required.

Initiation of the toxicity test

1. After the test concentration range has been prepared, add an appropriate volume of the embryo suspension to each of the three replicate 30 ml test solutions using an automatic pipette, so that the final density of embryos is around 50 per ml. The embryo suspension **must** be vigorously mixed between the addition of inocula to different test concentrations to ensure the embryos remain in suspension.

[3] Test solutions can be prepared directly in the test vessels if this is deemed more appropriate.

2. Check the resultant density of eggs in the test solutions by preserving three vials of reference seawater immediately after inoculation (AQC Egg Count Check). The counts **must** be recorded on an Oyster Embryo-Larval Toxicity Test Data Sheet (for example, see Table 6A.3).

3. Incubate the test solutions at 24 °C (± 2 °C) for 24 (± 2) h under static conditions without light.

Monitoring of the toxicity test

Measurements of pH, dissolved oxygen, temperature and salinity shall be made in separate (uninoculated) vials of test solutions at the start (and end) of the test. Data should be recorded on a Water Quality Monitoring Data Sheet (for example, see Table 6A.4).

Terminating the toxicity test

1. Terminate the test after 24 (± 2) h. If the test vessels are to be assessed later preserve the larvae by adding 0.5 ml of 20% (v/v) buffered formaldehyde solution to each test vessel in a fume cupboard wearing appropriate protective clothing. The formalin solution is made up with buffer containing sodium tetraborate (20 g l^{-1}). Buffered formaldehyde can be prepared by adding 250 ml of pH 9.0 borate buffer to 250 ml of formalin solution (40% v/v formaldehyde) in a 500 ml screw top glass bottle.

2. Identify and count the normal D larvae microscopically using a suitable counting chamber[4]. When sub-samples are taken for counting care **must** be taken to ensure that they are representative of the sample as a whole. The counts of normal (and abnormal larvae) are recorded on an Oyster Embryo-Larval Toxicity Test Data Sheet (see Table 6A.3). Normal larvae possess a completely formed bivalve shell, which may be irregular (ASTM 1980).

Measure pH, DO, temperature and salinity in the uninoculated test vessels of each test concentration at the end of the test. Data should be recorded on a Water Quality Monitoring Data Sheet (see Table 6A.4).

6.3.10 Processing of results

Validity of the results

In the original method of Woelke (1972) it was recommended that control abnormalities should not exceed five per cent. However, recorded abnormalities at the end of the exposure period did not include mortalities at the early stage of development, or non-fertilized eggs. The

[4] Abnormal D larvae can also be counted if this is considered appropriate. Larvae which fail to reach D-stage, although they may be normal trocophores or other early larval stages, are recorded as abnormal (see Section 6.2).

ICES (1991) method states that control `abnormalities' of up to 20% are common, and that up to 40% is acceptable. Therefore, a test should be rejected if abnormalities in the development of control embryos are greater than 40%.

Data from tests on effluents or leachates for discharge characterisation should only be accepted if the results of the reference toxicity test met quality control criteria.

Estimation of toxicity test endpoints

The EC_{50}, EC_{10}, NOEC and LOEC values are determined using an appropriate validated computer-based statistical package.

The determination of endpoints such as the EC_{50}, EC_{10}, NOEC and LOEC for an oyster embryo-larval toxicity test is based on the level of abnormality of embryos in the different test concentrations. In the ICES (1991) method the number of normal `D' shaped larvae per ml in each replicate are counted. The number of abnormal embryos per ml is calculated to be 100 minus the number of normal larvae per ml.

However, this approach assumes that 50 embryos per ml (100 per 2 ml of solution) were added at the start of the test and requires that the embryo suspension is homogenous whilst the aliquots are dispensed and that these volumes are pipetted accurately.

Therefore, the AQC Egg Count Check (ECC) **must** be used to indicate whether the addition of correct density of embryos has been achieved.

Data handling

Calculate Percentage Normal Development (PND) for each replicate test solution as the number of normal D-shaped larvae in each replicate of a test concentration relative to the mean number of embryos in the Egg Count Check such that:

$$\text{PND} = \frac{\text{Number of normal D} - \text{shaped larvae}}{\text{Mean number of embryos in Egg Count Check}} \times 100$$

Calculate Percentage Abnormal Development (PAD) for each replicate of a test concentration as:

PAD = 100 minus PND

Calculate the mean PAD values for each exposure concentration from the PAD values for each replicate.

Calculate the Percent Net Response (PNR) for each test concentration which is adjusted for control abnormality using the equation:

$$PNR = \frac{PAD_t - PAD_c}{100 - PAD_c} \times 100$$

where: PAD_t = Mean percentage abnormality in a test concentration
PAD_c = Mean percentage abnormality in controls

Estimation of the EC_{50} and EC_{10} values

The 24 h-EC_{50} (and 24 h-EC_{10}) value should be determined by an appropriate statistical method (see Figure 6.3). Confidence limits (p=0.95) for the calculated EC_{50} value should be determined using these standard methods and should be quoted in the test report (see Appendix 6A).

Table 6.1 shows an example data set which has been used to determine the 24 h-EC_{50} (and 24 h-EC_{10}) value for the inhibition of development of oyster embryos by an effluent using different statistical procedures.

In analysing data from oyster embryo-larval development tests the following points should be considered:

1. If the results include concentrations at which there are ≥60% and 0% normal D larvae and two concentrations at which the percentage of normal D larvae is between 0 and ≥60%, the results from probit moving average and binomial methods should provide similar estimates of the EC_{50} value. Probit analysis should be used to estimate EC_{50} and EC_{10} values, 95% confidence limits and the slope, providing the probability is not less than 0.05.

2. If the results do not include two concentrations at which the percentage of normal D larvae is between 0 and 60%, the probit and moving average methods cannot be used. The binomial method can be used to provide a best estimate of the EC_{50} values with wide confidence limits. Non-parametric methods such as the Spearman Karber or Trimmed Spearman Karber methods may allow the determination of an EC_{50}.

3. Where the data obtained are inadequate for calculating an EC_{50}, identify the highest concentration causing no effect on larval development and the lowest concentration causing 100% inhibition of larval development. An approximation of the EC_{50} can then be made from the geometric mean of these two concentrations. In this case the ratio of the higher to the lower concentration should not exceed 2.2, otherwise any EC_{50} calculated will be less statistically sound.

4. In all instances, the EC_{50} derived from any of the above methods should be compared with a graphical plot on logarithmic-probability scales of percent abnormal larvae for the various test concentrations. Any major disparity between the graphical estimation of the EC_{50} and that derived from the statistical programmes should be resolved by rechecking the statistical programmes.

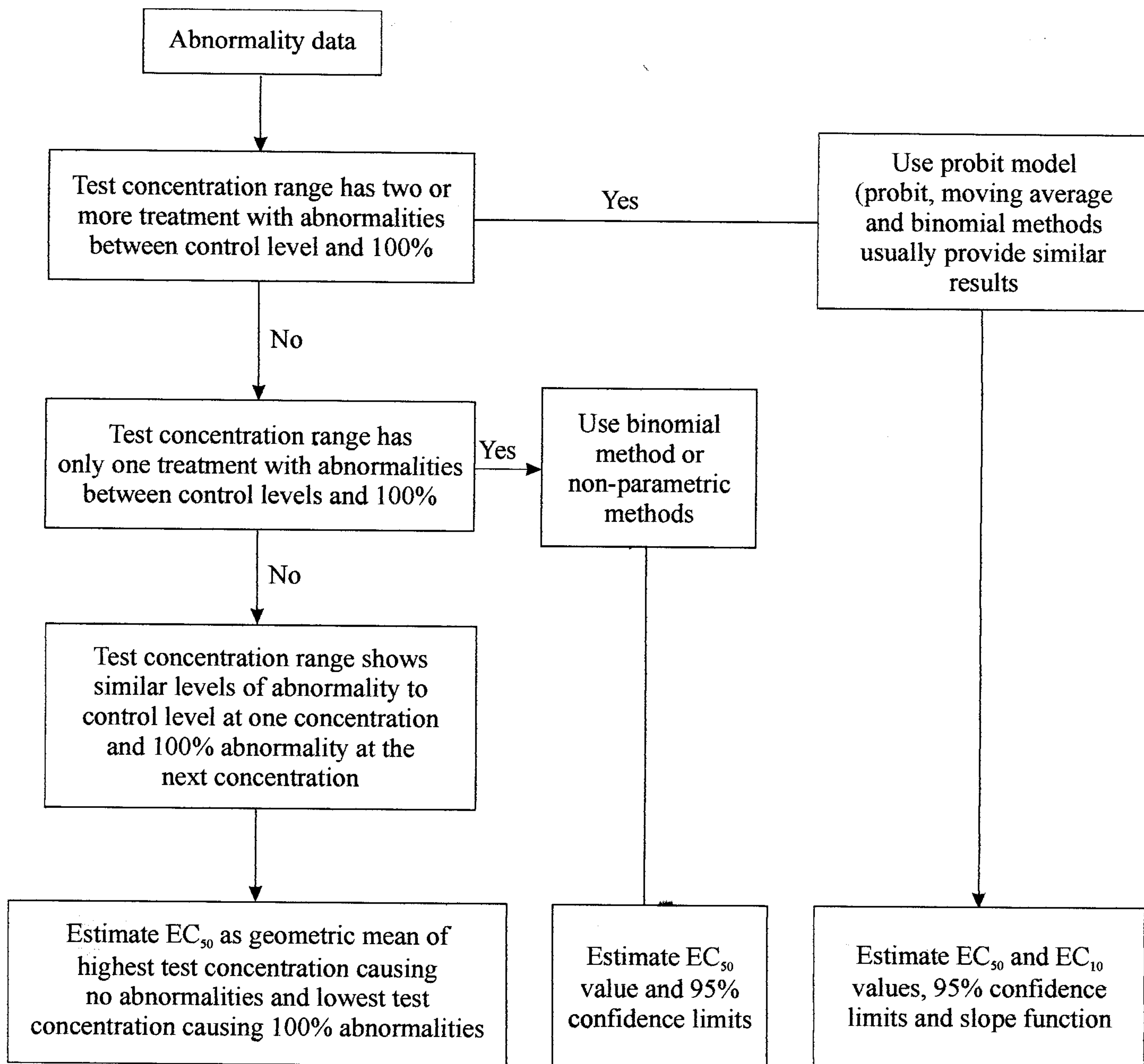


Figure 6.3 Flowchart for the estimation of the EC_{50} for full concentration range oyster embryo-larval development tests

Table 6.1 Example results of the inhibition of development of oyster embryos by an effluent after 24 h exposure

Effluent conc. (%)	Percent normal D larvae in each test vessel						Percent abnormal D larvae in each test vessel						Mean percent abnormality	PNR
	1	2	3	4	5	6	1	2	3	4	5	6		
Scenario 1														
0	86	92	90	96	89	90	14	8	10	4	11	10	10	-
0.1	94	86	84	88	-	-	6	14	16	12	-	-	12	2.2
0.22	82	88	85	85	-	-	18	12	15	15	-	-	15	5.6
0.46	66	64	62	60	-	-	34	36	38	40	-	-	37	30.0
1.0	30	26	29	23	-	-	70	74	71	77	-	-	73	70.0
2.2	0	0	0	0	-	-	100	100	100	100	-	-	100	100
4.6	0	0	0	0	-	-	100	100	100	100	-	-	100	100
10.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
22.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
46.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
100.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
Scenario 2														
0	86	92	90	96	89	90	14	8	10	4	11	10	10	-
0.1	94	86	84	88	-	-	6	14	16	12	-	-	12	2.2
0.22	82	88	85	85	-	-	18	12	15	15	-	-	15	5.6
0.46	60	59	55	58	-	-	40	41	45	42	-	-	42	35.6
1.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
2.2	0	0	0	0	-	-	100	100	100	100	-	-	100	100
4.6	0	0	0	0	-	-	100	100	100	100	-	-	100	100
10.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
22.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
46.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
100.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
Scenario 3														
0	86	92	90	96	89	90	14	8	10	4	11	10	10	-
0.1	94	86	84	88	-	-	6	14	16	12	-	-	12	2.2
0.22	82	88	85	85	-	-	18	12	15	15	-	-	15	5.6
0.46	8	85	86	89	-	-	14	15	14	11	-	-	14	4.4
1.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
2.2	0	0	0	0	-	-	100	100	100	100	-	-	100	100
4.6	0	0	0	0	-	-	100	100	100	100	-	-	100	100
10.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
22.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
46.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
100.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100

The data in Table 6.1 should be used by laboratories to check that in house statistical procedures are providing comparable results to those given in Table 6.2

Table 6.2 summarises the EC_{50} values derived for the different datasets in Table 6.1 using different statistical procedures. For the data in Scenario 1 the probit moving average and binomial methods produce similar results although the confidence limits are greater for the values derived using the binomial rather than the probit or moving average methods. Where there are less than two intermediate effect concentrations (Scenarios 2 and 3) the EC_{50} values derived are less statistically sound.

Table 6.2 Summary of EC_{50} values (and 95% confidence limits) for the data in Table 6.1 estimated by different statistical procedures

Scenario	Statistical procedure	EC_{50} (%)		Slope
		Value	Confidence limits	
1	Probit (Tox Calc)	0.67	0.58-0.76[1]	4.0
	Probit (Stephan 1982[2])	0.63	0.57-0.70	3.4
	Moving average (Stephan 1982[2])	0.59	0.53-0.67	-
	Binomial (Stephan 1982[2])	0.68	0.46-1.0	-
2	Probit	Not valid approach		-
	Moving average	Not valid approach		-
	Binomial (Stephan 1982[2])	0.52	-	-
	Spearman Karber (Tox Calc)	0.50	0.46-0.55	-
3	Geometric mean	0.68		

[1] Fiducial limits
[2] Computer based statistical package based on Stephan (1977)

The 24 h EC_{10} value estimated by the Tox Calc software (Tide Pool Scientific Software) is 0.32% with 95% confidence limits of 0.24-0.4%.

From interpolation of the graph of cumulative inhibition of embryo development (probability scale) against effluent concentration (log scale) shown in Figure 6.4 for Scenario 1 the 24 h-EC_{50} = 0.68% v/v effluent and the 24 h-EC_{10} = 0.27% v/v effluent. The values obtained graphically confirm those obtained using computer-based software (see Table 6.2).

Estimation of the NOEC and LOEC

The NOEC and LOEC values are determined using hypothesis testing (see Figure 6.5). Initially the proportion of organisms surviving in the control and treatments are transformed using an appropriate procedure such as the arc sine square root transformation.

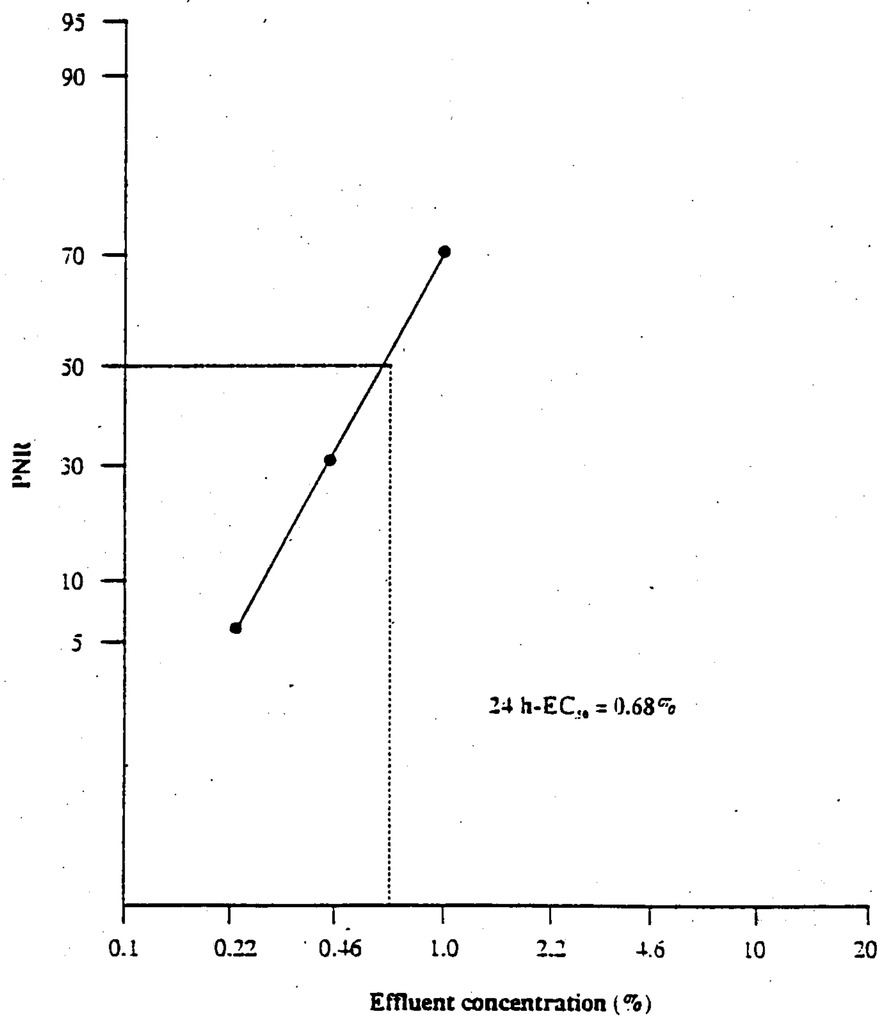


Figure 6.4 Graph of cumulative inhibition of development of D larvae (probability scale) against effluent concentration (log scale)

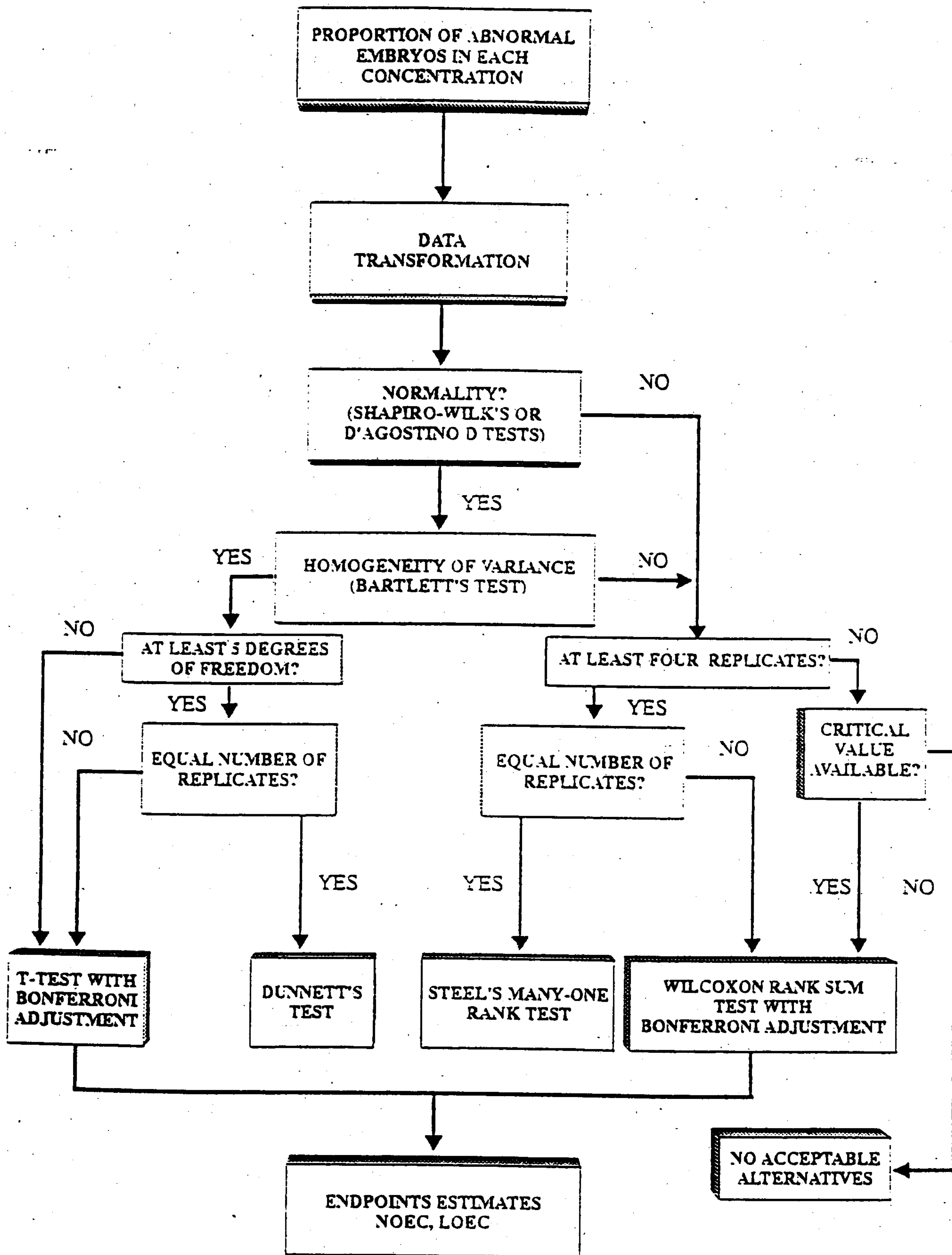


Figure 6.5 Flowchart for the estimation of NOEC and LOEC values in full concentration range oyster embryo-larval development toxicity tests

The arc sine square root transformation is commonly used on proportional data to stabilise the variance and satisfy the normality and homogeneity of variance requirements. Shapiro-Wilk's or D'Agostino D test should be used to test the normality assumption. If the data do not meet the assumption of normality, then the nonparametric Wilcoxon Rank Sum Test with Bonferroni Adjustment or Steels Many-One Rank Test should be used to analyse the data depending on whether there are equal numbers of replicates in each treatment.

If the data meet the assumption of normality, the Bartletts test for equality of variances is used to test the homogeneity of variance assumption. If the data meet the homogeneity of variance assumption then Analysis of Variance (ANOVA) followed by Dunnetts test, Williams' Multiple Comparison test or T-tests with Bonferroni Adjustment are used to analyse the data depending on whether there are equal numbers of replicates in each treatment. Failure of the homogeneity of variance assumption leads to the use of Wilcoxon Rank Sum Test with Bonferroni Adjustment or Steels Many-One Rank test depending on whether there are equal numbers of replicates in each treatment.

Further information on these statistical procedures can be obtained from Sokal and Rohlf (1981), Zar (1984) and US EPA (1993). In the example given in Table 6.1 the 24 h NOEC and LOEC values calculated using ANOVA and T-tests with Bonferroni Adjustment were 0.1 and 0.22% v/v effluent respectively.

6.4 Guidelines for single concentration toxicity tests on effluents and leachates and bioassays on receiving waters using oyster embryos

6.4.1 Monitoring against toxicity limits

Introduction

Toxicity tests with oyster embryos for monitoring against toxicity limits should be carried out with a single concentration test comprising a single effluent or leachate concentration (toxicity limit) and an appropriate control(s).

Test procedure

If water from a clean site adjacent to the discharge point is used as the control, further controls shall be prepared using the reference seawater. All relevant information shall be documented in the test report (see Appendix 6A).

Single concentration tests should be initiated in the same way as full concentration range toxicity tests (see Section 6.3.9) with at least eight replicates of each control and four replicates of each sample. Counts of normal larvae should be made on solutions preserved after 24 h exposure and recorded on an Oyster Embryo-Larval Development Toxicity Test Data Sheet (see Table 6A.3). Water quality monitoring should be carried out in the same way as described for the toxicity test (see Section 6.3.9) and recorded on a Water Quality Monitoring Data Sheet (see Table 6A.4).

Processing of results

Assessment of how the responses in the single effluent or leachate treatment compare to those in the control is accomplished using hypothesis testing (see Figure 6.6). The hypothesis tested is that the responses in the treatment are not significantly different from those in the control.

Initially the proportion of organisms surviving in the control and the single treatment concentration are transformed using an appropriate procedure such as the arc sine square root transformation. The arc sine square root transformation is commonly used on proportional data to stabilise the variance and satisfy the normality and homogeneity of variance requirements. Shapiro-Wilk's or D'Agostino D test should be used to test the normality assumption.

If the data do not meet the assumption of normality, then the non-parametric Wilcoxon Rank Sum Test should be used to analyse the data. If the data meet the assumption of normality, the F test for equality of variances is used to test the homogeneity of variance assumption. If the data meet the homogeneity of variance assumption then the standard (homoscedastic) t test should be used to analyse the data. Failure of the homogeneity of variance assumption leads to the use of a modified (heteroscedastic) t test, where the pooled variance estimate is adjusted for unequal variance, and the degrees of freedom for the test are adjusted. Further information on these statistical procedures can be obtained from Sokal and Rohlf (1981), Zar (1984) and US EPA (1993).

Table 6.3 shows example data sets for a single concentration test using duplicate control and 0.22% effluent solutions. In Scenario 1 the variances are equal ($F = 1.28$, $p = 0.70$) and the standard (homoscedastic) t test indicates a significant difference between responses in the two groups ($t = 2.04$, $p < 0.05$). In Scenario 2 the variances are unequal ($F = 26.59$, $p = 0.007$) and the modified (heteroscedastic) t test indicates no significant difference between responses in the two groups ($t = 0.40$, $p > 0.05$).

Table 6.3 Example dataset for a single concentration test and the results of statistical analysis

Effluent concentration (%)	Percent abnormalities in replicates	Method of statistical analysis	Result of statistical analysis
Scenario 1			
0 (control)	11, 8, 10, 9, 11, 15, 12, 11	Standard t-test	Significant difference ($p < 0.05$)
0.22	16, 12, 18, 12		
Scenario 2			
0 (control)	11, 8, 10, 9, 11, 15, 12, 11	Modified t-test	NS
0.22	4, 29, 24, 5		

NS - no significant difference between control and treatment groups

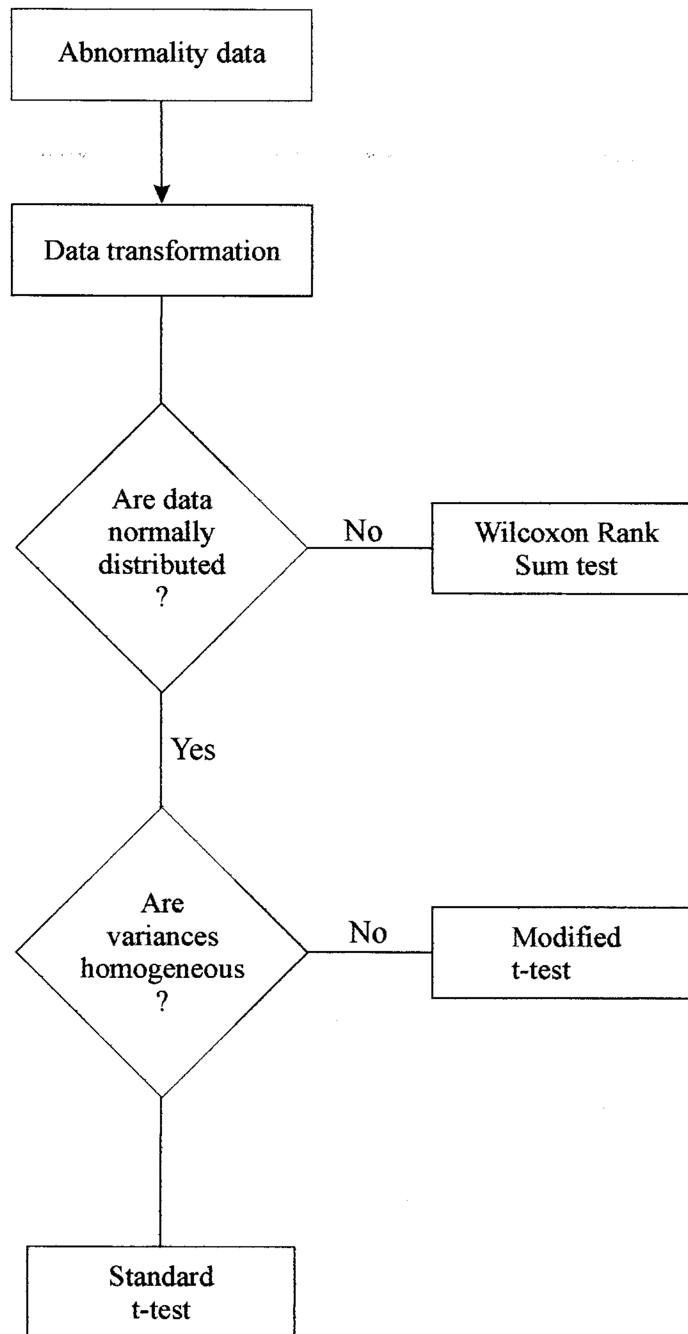


Figure 6.6 Flowchart for the analysis of single concentration test data from oyster embryo-larval development toxicity tests

6.4.2 Assessing receiving water column toxicity

The assessment of the toxicity of receiving water column samples should be carried out using the bioassay procedure given in Section 6.4.1 for an undiluted (100%) sample and an appropriate control(s).

Receiving water samples may not meet the physico-chemical parameters required to support oyster embryo development. In these circumstances, the sample may have to be modified using the procedures given in Section 2 to satisfy the threshold criteria indicated in Table 6.4.

6.5 Guidelines for toxicity tests on reference toxicants using the oyster embryo-larval development test

6.5.1 Introduction

Oyster embryo-larval development tests which are carried out to provide data for discharge characterisation or monitoring against a toxicity limit, should be accompanied by tests with the reference substance zinc. Tributyltin is often used as a reference toxicant, but the confirmation of exposure concentrations in the tests is complicated by the complexity of the analytical procedures.

6.5.2 Test procedure

Reference toxicant tests should be conducted according to the procedure given in Section 6.3.9.

6.5.3 Preparation of zinc stock solutions

Preparation of 1000 mg l^{-1} zinc stock solution

(a) Weigh out 4397 mg of zinc sulphate ($ZnSO_4.7H_2O$) in a weighing boat.

(b) Add the zinc sulphate to a 1-litre volumetric flask and dilute to just below the mark with distilled water.

(c) Add 1 ml of 1M Analar HCl to stabilize the stock solution.

(d) Dilute to the mark with distilled water.

Preparation of 50 mg l^{-1} zinc working solution

The working solution should be prepared on the day of the test by diluting 25 ml of the 1000 mg l^{-1} stock solution with reference seawater in a 500 ml volumetric flask.

6.5.4 Preparation of the test concentrations

For the reference toxicant zinc the concentration range given below is used in the initial study to assess the sensitivity of the test organisms in a facility.

Nominal zinc conc. (mg l^{-1})	Volume of reference seawater (ml)	Volume of zinc stock (ml)
0 (Control)	250	0.0
0.032	250	0.16
0.1	249.5	0.5
0.32	248.4	1.6
1.0	245	5
3.2	234	16

The above volumes relate to a zinc stock concentration of 50 mg l^{-1}, which should be prepared according to the procedure given in Section 6.5.3.

The test concentration ranges of zinc for subsequent tests can be modified based on the first results to allow the derivation of a more precise LOEC and EC_{50} values when no previous data is available.

6.5.5 Test procedure

Reference toxicant tests should be initiated in the same way as full concentration range toxicity tests (see Section 6.3.9). Counts of normal larvae should be made on solutions preserved after 24 h exposure and recorded on an Oyster Embryo-Larval Development Toxicity Test Data Sheet (see Table 6A.3). Water quality monitoring should be carried out in the same way as described for the toxicity test (see Section 6.3.9) and recorded on a Water Quality Monitoring Data Sheet (see Table 6A.4).

Samples of the zinc test solutions should be taken at the beginning of the test from the stock vessel for each test concentration and at the end of the test from the vessels themselves and analysed using an appropriate procedure (for example, SCA 1980, 1981, 1988).

6.5.6 Processing of results

The LOEC and EC_{50} values should be calculated using the procedures described in Section 6.3.10. The estimation of toxicity values should be based on measured exposure concentrations.

6.6 Test report

The test reports **must** include the following information:

(a) information about the test organism such as scientific name and source, holding conditions, any pre-treatment and, if appropriate, the conditioning method used (including type and amount of food and feeding frequency);

(b) the source of the reference seawater and the major physical and chemical characteristics of the water such as temperature, pH and salinity or conductivity;

(c) the methods of preparation of the samples including for effluents, leachates and receiving waters the manner and duration of storage of the samples and, if necessary, the conditions by which physico-chemical parameters were modified, for example, whether pH was adjusted or suspended solids were treated (settlement, centrifugation or filtration of samples);

(d) tables showing the numbers of normal and abnormal larvae at each control and test concentration at the end of the 24 h exposure period for full concentration-response tests, limit tests and reference toxicant tests;

(e) an indication that criteria determining the validity of the test (that is abnormalities in the development of control embryos of less than 40%) have been satisfied;

(f) for tests on effluents or leachates for discharge characterisation or to monitor toxicity-based limits, an indication that the responses of oyster embryos in the reference toxicant test met the quality control criteria;

(g) the derivation of the 24 h-EC_{50}, the 95% confidence limits and the method of calculation. The 24 h-EC_{10} (and 95% confidence limits), the NOEC and LOEC values after 24 h are also reported;

(h) any operating details not specified in this procedure and any incidents which may have affected the results.

6.7 Checklist

A checklist of test conditions and procedures for acute oyster embryo-larval development toxicity tests is given in Table 6.4.

Table 6.4 Checklist of test conditions and procedures for oyster embryo-larval development toxicity tests

---

Test procedure

Organisms — Embryos of the Pacific oyster (*Crassostrea gigas*), 50 per ml

Type — Static, 24 h duration

Control/dilution water — Reference seawater

Temperature — 24 ± 2 °C

Aeration — No aeration during test

Physico-chemical parameters — If the total biological effect of a sample is being measured, then the sample is tested unadjusted and key physico-chemical parameters (such as temperature, pH, dissolved oxygen, salinity and suspended solids) are measured. If it is apparent that any physico-chemical parameters are partially or fully responsible for measured responses, then the test solutions or sample have to be modified using the procedures given in Section 2 to satisfy the threshold criteria given below:

Physico-chemical parameter	Threshold criteria
pH	7.8-8.5 in all test vessels
Dissolved oxygen	-
Salinity	≥22‰
Suspended solids	<20 mg l^{-1} in all test vessels

Lighting — Exposure to contaminants is in the dark

Feeding — No feeding during the test

Observations — Number of normal larvae in each concentration after 24 h exposure

Measurements — No water quality measurements normally conducted

Endpoints — 24 h EC_{50} and 24 h-EC_{10} values (± 95% confidence limits), 24 h NOEC and LOEC values

Reference toxicant — Zinc (as zinc sulphate) determined at the time of the test

Test validity — Valid if mean normal D larvae in the controls is ≥60%

Table 6.4 continued

Test samples	
Effluents, leachates and receiving waters	
Transport and storage	Transport at a temperature not markedly different from that measured at the time of collection. The test **must** begin within 48 hours of the time of sampling. If the sample is not to be tested immediately on receipt then it should be stored at 5 ± 3 °C.
Control/dilution water	Reference seawater*1

*1 If a receiving water is used as the control/dilution water, a reference seawater control should also be run

6.8 References

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SCA (1980) Atomic Absorption Spectrophotometry 1979 Version: An Essay Review. Methods for the Examination of Waters and Associated Materials No. 20, Standing Committee of Analysts, London.

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APPENDIX 6A TEST REPORT FOR AN OYSTER EMBRYO-LARVAL DEVELOPMENT TOXICITY TEST (EXAMPLE)

Table 6A.1 Preparation of oyster embryos

Data on test species

Test species:

Origin of test species:

Oysters purchased from a supplier

Date received: Number of oysters:

Size range:

Conditioning method:

Data on test substance

Test substance:

Source: Hazard:

Date collected:

Date received:

Storage conditions: Temperature (°C)
Length of time before start of test (h)

Stripping of oysters

Number of females opened: Number of males opened:

Number of females used: Number of males used:

Wet weights (g) Wet weights (g)

Condition of oysters: Condition of oysters:

Source of water:

Water temperature (°C):

Fertilisation of gametes

Time of addition of sperm: Volume of sperm added:

Incubation temperature:

Time between addition of sperm and test inoculation:

Table 6A.2 Preparation of toxicity test concentration range

Test substance:

Type of test:

Start of test: End of test:

Duration of test:

No. of test concentrations:

No. of replicates per concentration:

No. of controls: Dilution media only

Solvent controls

Concentration range

Stock solution concentration:

Dilution media:

Volume of test solution required (ml):

Nominal test substance concentration	Volume of reference seawater (ml)	Volume of test substance (ml)

Egg suspension concentration (numbers per ml):

Volume of egg suspension added to test containers:

Table 6A.3 Oyster Embryo-Larval Development Toxicity Test Data Sheet

Test substance: Test date:

Method of preservation: Date of preservation:

Counting dates: Initials of counter:

Test solution	Larval counts	Replicates						Comments
		1	2	3	4	5	6	
Egg Count Check								
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							

Table 6A.3 continued

Test solution	Larval counts	Replicates						Comments
		1	2	3	4	5	6	
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							
	Normal D's							
	Abnormal D's							
	Eggs							
	TOTAL							

Table 6A.4 Water Quality Monitoring Data Sheet - Toxicity Test

Date	Time	Vessel No.	Nominal conc	Temp (°C)	pH	DO (%ASV)	Salinity (‰) or Conductivity ($\mu S\ cm^{-1}$)	Initials

Table 6A.5 Expression of the results of a toxicity test

Exposure conc.	Percentage normal D larvae in each replicate						Percentage abnormal D larvae in each replicate						Mean percent abnormality
	1	2	3	4	5	6	1	2	3	4	5	6	

Calculation of the 24 h-EC_{50} and 24 h-EC_{10} values

Method used: 24 h-EC_{50} = 24 h-EC_{10} =

Concentrations used in the calculation of the 24 h-EC_{50}: Nominal/Measured

Calculation of the NOEC and LOEC values

Method used: NOEC = LOEC =

Operating details not specified in the standard operating procedure and any incidents which may have affected the results:

7. JUVENILE FISH LETHALITY TEST GUIDELINE

7.1 Introduction

This section of the DTA Methods Guidelines describes the procedures for the holding of freshwater (rainbow trout) and marine (turbot) fish and for conducting toxicity tests using such species to measure the effects of effluents, leachates and receiving waters on lethality (and behavioural responses). The document has been compiled with reference to existing internationally recognised standard procedures (ASTM 1988, EC 1990; Environment Canada 1990 a,b; OECD 1992).

Critical steps in the holding and test procedures which must be followed are identified in bold type whereas instructions given in normal type are recommended and alternatives can be used.

Although concerns have been raised in the United Kingdom regarding the use of fish for assessing the toxicity of environmental samples (effluents, leachates and receiving waters) the DTA Methods Working Group recommended the inclusion of a fish test in the battery of trophic level tests to be used for effluent characterisation. The appropriateness of the test would then be reviewed in the light of the results generated in the Demonstration Programme.

Tests with juvenile fish **must** only be conducted at designated Home Office establishments under the conditions of a Home Office Project Licence. The conditions of the licence will ensure fish are treated humanely and in accordance with the Animals (Scientific Procedures) Act (HMSO 1986).

7.2 Holding of freshwater and marine fish for toxicity tests and bioassays

7.2.1 Introduction

The purpose of holding juvenile freshwater or marine fish using the following procedure is to provide "healthy" organisms for toxicity tests and bioassays. Tables 7.1 and 7.2 summarise information given in internationally recognised test guidelines for the holding of the freshwater species *Oncorhynchus mykiss* (rainbow trout) or the marine species *Scophthalmus maximus* (turbot).

Table 7.1 Summary of information given in internationally recognised test guidelines for the appropriate holding conditions for rainbow trout

Parameter	Guideline			
	ASTM (1988)	EC (1990)	Environment Canada (1990a,b)	OECD (1992)
Minimum holding period	At least 14 days	At least 12 days	At least 14 days	At least 12 days
Holding water				
- Source	Good quality natural water (preferred) Reconstituted water[1] (option) Dechlorinated drinking water[2] (option)	Good quality natural water Reconstituted water Dechlorinated drinking water	Uncontaminated groundwater or surface water (preferred) Dechlorinated drinking water[2] (preferred) Reconstituted water[1] (option)	Good quality natural water (preferred) Reconstituted water (preferred) Dechlorinated drinking water (option)
- Temperature (°C)	12	12-17	15 ± 2	13 - 17
- Dissolved oxygen (%ASV)	60-100	At least 80	80-100	At least 80
- pH	7.3-7.5	6.0-8.5	6.0-8.5 (7.5-8.0 desirable)	6.0-8.5
- Hardness (mg Ca CO_3 l^{-1})	40-48	10-250	No information given	10-250
- Light	16h light: 8h dark with 30 min dawn/dusk	12-16h light: 8 - 12 h dark	16 ± 1h light: 8 ± 1 h dark with 15-30 min dawn/dusk[3]. Light intensity 100-500 lux at water surface, full spectrum	12-16h light: 8-12h dark
Holding system	Flow through with at least 2 volume additions per day	Recirculation or flow-through depending on species	Flow-through with at least 1 litre per minute of new water for every kg of fish held (preferred) Recirculating[4] (option)	No information given

Parameter	Guideline			
	ASTM (1988)	EC (1990)	Environment Canada (1990a,b)	OECD (1992)
Feeding - rate	Daily until 48h before the start of the test	Daily or 3 times a week until 24 h before the start of the test	Once or more times daily, normally with a daily ration approximately 1-5% of wet body weight, depending on fish size and age and water temperature and manufacturers recommendations	Daily or 3 times a week until 24h before the start of the test
- type of food	No information given	No information given	A recognised standard commercial pelleted fish food appropriate for fish size and age	No information given
Stocking density	No information given	Appropriate to the system (recirculating or flow-through) and the species	10g of fish per litre of water for flow-through	No information given
Minimum period before transfer to test water (d)	At least 2 days before the start of the test	7 days before the start of the test	-	7 days before the start of the test

Notes:

1. The potentially large volumes of water needed to hold fish mean the use of reconstituted water may not be a cost-effective procedure compared to the use of natural holding waters.
2. If dechlorinated water is to be used for holding fish and as a control/dilution water, effective dechlorination must rid the water to which fish are exposed of any harmful concentration of chlorine (see Section 7.2.3).
3. A "dawn/dusk" transition period is recommended since abrupt changes in intensity startle and stress fish. Automated dimmer control systems are available for dimming and brightening the intensity of fluorescent lights, although they are costly. Alternatively, a secondary incandescent light source, regulated by time clock and automated rheostat, may be used to provide the transition period.

Notes to Table 7.1 continued

4. If necessary (for example, if fish are being acclimated to reconstituted water, receiving water or some other water source that is restricted in amount), water-volume requirements for fish acclimation may be decreased substantially by recirculating the flow to the fish tank through a filter suitable for removing metabolic wastes. If a recirculation system is used, ammonia and nitrate concentrations in the acclimation tank should be monitored and kept below levels harmful to fish health.

Table 7.2 Summary of information given in internationally recognised test guidelines for the appropriate holding conditions for turbot

Parameter	Guideline	
	ASTM (1988)	PARCOM (1995)
Minimum holding period	At least 14 days	At least 12 days
Holding water		
- Source	Good quality natural sea water (preferred) Reconstituted seawater (option)	Good quality natural seawater (preferred) Reconstituted seawater (option)
- Temperature (°C)	No data	13.5-16.5
- Dissolved oxygen (%ASV)	60-100	At least 60
- pH	No information given	8.0
- Salinity (‰)	34 ± 0.5	30-36
- Light	12-16h light: 8-12h dark	No information given
Holding system	No information given	Flow-through with at least 2 volume additions per day
Feeding		
- rate	According to suppliers recommendations until 24h before the start of the test	Daily until 48h before the test
- type of food	According to suppliers recommendations	No information given
Stocking density	No information given	No information given
Minimum period before transfer to test water (d)	At least 2 days before the start of the test	4 days before the start of the test

7.2.2 Test species

The fish species acceptable for use in toxicity tests of effluents, leachates and receiving waters are given in Table 7.3. These species have been selected on the basis of practical criteria, such as:

- ready availability throughout the year;
- ease of holding;
- convenience for testing;
- relative sensitivity;
- any economic, biological or ecological factors which affect the test.

Table 7.3 Fish species recommended for lethal toxicity tests and bioassays

Recommended species	Recommended range of test temperature (°C)	Recommended total length of test fish (cm)	Freshwater/ seawater
Oncorhynchus mykiss (Teleostei, Salmonidae) (Richardson) Rainbow trout	15 ± 2	5.0 ± 1.0	Freshwater
Scophthalmus maximus (Teleostei, Bothidae) (Linnaeus) Turbot	15 ± 2	3.0 ± 1.0	Seawater

The species listed in Tables 7.1 and 7.2 are bred and cultivated in fish farms, under controlled conditions, so that the test fish will be healthy and of known parentage. Juvenile fish should be obtained from a recognized supplier (see Appendix A). The length of the largest fish **must** not be more than twice that of the smallest in the same test.

Table 7.4 provides information on the average wet weight of turbot of different sizes. These data should be used to ensure that fish will be of the correct size for tests following a given holding period in the laboratory. Information on rainbow trout should be obtained from the supplier used.

Table 7.4 Average wet weight of turbot of different lengths

Turbot[1]	
Length (cm)	Wet weight (g)
2	0.2
3	0.5
4	1.1

1 - Information supplied by Mannin Sea Farms (see Appendix A)

7.2.3 Holding water

Freshwater species

Natural waters for holding fish should be an uncontaminated supply of groundwater or dechlorinated drinking water. The holding water used **must** have a hardness between 10 and 250 mg $CaCO_3$ l^{-1}, with a pH of 6.0 to 8.5 and a dissolved oxygen content of at least 90% of the air saturation value (ASV). The holding water **must** previously have been demonstrated to consistently and reliably support good survival, health and growth of rainbow trout. Monitoring and assessment of variables such as residual chlorine, pH, hardness, alkalinity, total organic carbon, conductivity, suspended solids, dissolved oxygen, temperature, ammonia, nitrogen, nitrite, residual chlorine and total organic chlorine, metals and total organophosphorus pesticides, should be performed as frequently as necessary to document water quality (see Table 7.5).

For example, a holding water which is known to be relatively constant in quality should be monitored every three months. If water quality has been demonstrated to be constant over at least 1 year, measurements can be less frequent and intervals extended(for example every six months).

Table 7.5 Required characteristics of acceptable freshwaters for holding fish (after OECD 1997)

Substance	Concentrations
Particulate matter	<20 mg l^{-1}
Total organic carbon	<2 mg l^{-1}
Un-ionised ammonia	<1 µg l^{-1}
Residual chlorine	<10 µg l^{-1}
Total organic chlorine	<25 ng l^{-1}
Total organophosphorus pesticides	<50 ng l^{-1}

If drinking water is to be used the dechlorination procedure **must** ensure that the total residual chlorine (TRC) level is ≤ 0.002 mg l^{-1} (CCREM 1987). This TRC concentration can be achieved by passing the water over activated carbon filters followed by either subsequent ultraviolet radiation (Armstrong and Scott 1974) or vigorous aeration for 24 h after carbon filtration. The addition of thiosulphate or other chemicals to water to remove total residual chlorine is not recommended since such chemical(s) could sequester trace metals essential for the health of the organisms.

If reconstituted water is to be used as dilution control, fish **must** be acclimated to this or a water of similar hardness for at least five days immediately prior to testing.

Holding water of a specific hardness may be prepared by adjusting the laboratory supply of uncontaminated ground or drinking water. If the hardness of the available supply is too high, dilute this freshwater with distilled or deionized water. If the available natural freshwater is too soft, add the required quantity of reconstituted hard water or the appropriate ratio and amounts of salts (see Tables 7A.1 and 7A.2 in Appendix 7A).

Marine fish

Marine fish should be held in any appropriate `clean' seawater with a salinity of 34 ± 2‰.

7.2.4 Lighting

Depending on test requirements and intent, lighting during holding can be natural or provided by overhead full-spectrum fluorescent fixtures (ASTM 1995). If photoperiod control is required, the photoperiod **must** be a constant sequence of 16 ± 1 hour of light and 8 ± 1 hour of darkness. Light intensity at the water surface should be 100 to 500 lux. A 15- to 30-minute transition period is recommended if artificial lighting is provided. Fish **must** be acclimated to lighting conditions (including photoperiod and intensity) consistent with those used in the test, for a period of at least two weeks prior to testing.

7.2.5 Stocking density

A constant flow of water through the holding and acclimation tanks is necessary. To prevent a build-up of metabolic wastes, at least one litre per minute of fresh (new) water should flow into the tank for every kilogram of fish being held. Additionally, to prevent overcrowding, a tank should contain at any given moment at least one litre of water for every 10 grams of fish held (Sprague, 1973).

If a recirculating system is used (for example with reconstituted freshwater or natural artificial seawater) the stocking density may need to be modified and ammonia and nitrite levels should be measured frequently to check that they do not reach harmful levels.

7.2.6 Monitoring of fish stocks

Following a 48h settling-in period, fish should be inspected daily or **must** as a minimum be examined five days per week (Monday - Friday) for signs of disease and mortalities. The appearance and behaviour of the fish and any deaths **must** be recorded on a Fish Holding Record Data Sheet (for example, see Table 7B.1), and a Fish Acclimation Record Data Sheet if required (for example, see Table 7B.2). Dead and moribund individuals **must** be removed from the holding tanks immediately.

The following criteria **must** be used to determine whether fish can be used in toxicity tests.

Mortality	Consequence
Greater than 10% of population in seven days before toxicity tests	Rejection of entire batch
Between 5-10% of population in seven days before toxicity tests	Continue acclimation for additional seven days
Less than 5% of population in seven days before toxicity tests	Acceptance of batch

7.2.7 Feeding of test organisms

Fish **must** be fed daily, until the day before the start of the test. The recommended food types for each species are shown in Table 7.6. Fish should be fed at a rate of 2-4% body weight on each occasion. The actual pellet size and type, feed ration and frequency should be chosen depending on the fish size and age and water temperature.

Table 7.6 Recommended food type for different fish species (after OECD 1989)

Fish species	Recommended food	Freshwater/seawater
Oncorhynchus mykiss (Rainbow trout)	Proprietary pelleted trout food (BP Fry 02)	Freshwater
Scophthalmus maximus (Turbot)	High oil pellets/ freeze dried mussels	Seawater

7.2.8 Cleaning of tanks

Troughs and tanks used for holding and acclimating fish **must** be kept clean. Siphoning of excess food and faeces should be conducted once a day or as frequently as necessary to eliminate the build-up of excess food or faecal material. Tank designs that provide partial self-cleaning (for example, those with central, double standpipes) are recommended as they reduce maintenance requirements.

To minimize the occurrence of disease, tanks should be disinfected using an appropriate commercial product (see Appendix A) prior to introducing a new batch of fish. As disinfectants are toxic to fish, tanks **must** be rinsed thoroughly with water used for holding/acclimating fish, following their use.

7.2.9 Treatment of fish

Treatment of fish with chemicals for disease prevention or control should be avoided if possible. It is strongly recommended that fish stocks showing signs of disease be discarded humanely rather than treated and that the supplier be informed of the problem. If the use of chemically-treated fish cannot be avoided any treatment of the fish for parasites or infection during holding **must** be documented on a Fish Holding Record Data Sheet (see Table 7B.1). Appropriate text books such as Roberts and Shepherd (1986) and Roberts (1989) can be consulted for information on the treatment of fish but if the person responsible is unsure of a course of action a veterinarian should be consulted. Fish which have been treated **must** not be used in toxicity tests until at least 14 days have elapsed from the date of treatment. If the treatment is not successful the batch of fish **must** be disposed of humanely according to the conditions of the Home Office Licence.

7.2.10 Acclimation to test conditions

If the holding water initially used to maintain the fish is different from the toxicity test or bioassay reference water then fish **must** be transferred to acclimation tanks at least seven days before they are to be used for a test. Water quality monitoring and lethality measurements **must** be made at least three times per week during the acclimation period and recorded on a Fish Acclimation Record Data Sheet (see Table 7B.2) along with information on the feeding rate.

7.3 Guidelines for toxicity tests on effluents and leachates using juvenile freshwater and marine fish

7.3.1 Introduction

Tables 7.7 and 7.8 summarise information given in internationally recognised test guidelines for conducting toxicity tests with rainbow trout and turbot. This information has been used to define the procedures for testing the toxicity of:

- effluents and leachates in full concentration range tests;
- effluents and leachates in single concentration tests and receiving waters in bioassays;
- a reference toxicant in a full concentration range test.

7.3.2 Scope of the procedure

Applications

This procedure describes a toxicity test (see glossary of terms) for the determination of the short-term toxicity to juvenile fish of treated and untreated industrial and sewage effluents and leachates (after either settlement, centrifugation or filtration if necessary) and receiving waters.

Table 7.7 Summary of information given in internationally recognised test guidelines for measuring toxicant-induced lethality in rainbow trout

Parameter	Guideline			
	ASTM (1988)	EC (1990)	Environment Canada (1990b)	OECD (1992)
Application of test	Chemicals	Chemicals	Effluents	Chemicals
Size of organisms at start of test	0.5-5.0 g	6.0 ± 2.0 cm	0.3-5.0 g, largest fish should not be more than twice the length of the smallest in the same test	5.0 ± 1.0 cm
Number of organisms per vessel	10	At least 7	Minimum 7	At least 7
Test Water				
- Source	Soft reconstituted water (preferred)	Good quality natural water	Uncontaminated natural, or dechlorinated drinking water	Good quality natural water (preferred)
	Good quality natural water	Reconstituted water		Reconstituted water (preferred)
		Dechlorinated drinking water		Dechlorinated drinking water (option)
- Temperature	12 (within ± 1 °C for test)	12-17 (within ± 1 °C for test)	15 ±1	13-17 (within a range of 2 °C for test)
- Dissolved oxygen (% ASV)	60-100 for first 48 h 40-100 after 48 h	>60 throughout	>70 throughout	>60
- pH	7.2-7.5	6.0-8.5	6.0-8.5	6.0-8.5
- Hardness (mg $CaCO_3$ l^{-1})	40-48	10-250	No information given	10-250
- Light	16 h light: 8h dark with 30 min dawn/dusk.	12-16h light: 8-12h dark	16 ± 1h light: 8 ± 1h dark with 15-30 min dawn/dusk.	12-16h light: 8-12h dark.

Parameter	Guideline			
	ASTM (1988)	EC (1990)	Environment Canada (1990b)	OECD (1992)
			100-500 lux light intensity at water surface	
Feeding	None	None	None	None
Observation				
- parameters	Lethality, behavioural responses	Lethality, behavioural responses	Lethality, behavioural responses	Lethality, behavioural responses
- times	At least 24,48,72 and 96h (3 and 6h desirable)	2, 4, 24, 48, 72 and 96h	At least 24, 48, 72 and 96h	At least 24, 48, 72 and 96h
Water quality measurements	pH and DO every 48h, hardness at beginning and end	pH, DO and temperature daily	pH. DO and temperature daily. Conductivity at start of test	pH, DO and temperature daily
Endpoints calculated	LC_{50} (± 95% confidence levels)	LC_{50} (± 95% confidence levels)	LC_{50} (± 95% confidence levels)	LC_{50} (± 95% confidence levels)

Table 7.8 Summary of information given in internationally recognised test guidelines for measuring toxicant-induced lethality in turbot

Parameter	Guideline	
	ASTM (1988)	PARCOM (1995)
Application	Chemicals	Chemicals
Size of organisms at start of test	0.5-5.0 g	4-6 cm
Number of organisms per vessel	10	At least 7
Test water		
- Source	Good quality natural seawater Reconstituted seawater	Good quality natural seawater (preferred) Reconstituted seawater
- Temperature	No information given	13.5-16.5
- Dissolved oxygen (%ASV)	60-100 for first 48h 40-100 after 48h	>60
- pH	No information given	8.0
- Salinity (‰)	34 ± 0.5	30-36
- Light	16h light: 8h dark	12-16h light: 8-12h dark
Feeding	None	None
Observation		
- parameters	Lethality, behavioural responses	Lethality, behavioural responses
- times	At least 24, 48, 72 and 96h (3 and 6h desirable)	At least 24, 48, 72 and 96h
Water quality measurements	pH and DO every 48h Salinity at beginning and end	pH, DO, temperature and salinity daily
Endpoints calculated	LC_{50} (± 95% confidence levels)	LC_{50} (± 95% confidence levels)

The experimental design adopted (for example number of exposure concentrations, interval between test concentrations and test duration) will depend on the objective of the study, which **must** be clearly defined at the outset.

Limitations

The results of toxicity tests with both freshwater and marine species can be affected by the pH and dissolved oxygen levels of test samples. The testing of freshwater discharges to marine waters may require the use of salinity correction procedures (see Section 7.3.8).

7.3.3 Principle

In the toxicity test procedure (see Section 7.3.9), groups of juvenile fish are exposed to the environmental sample (effluent, leachate or receiving water) diluted with reference freshwater or seawater to a range of concentrations for a period of 96 h. The different test concentrations in an appropriate range may, under otherwise identical test concentrations, exert toxic effects on the survival of fish. These may extend from an absence of effects at lower test concentrations to mortality of all the fish at higher test concentrations. The data should be used to determine, where possible:

- the median lethal concentration, that is the concentration that results in the lethality of 50% of the exposed fish after 24, 48, 72 and 96 h[1]. The value derived after 24 h is referred to as the 24 h-LC_{50} and the value after 96 h is referred to as the 96 h-LC_{50};
- the concentration that results in the lethality of 10% of the exposed fish after 24, 48, 72 and 96 h. The value derived after 24 h is referred to as the 24 h-LC_{10} and the value after 96 h is referred to as the 96 h-LC_{10};
- the highest no-observed effect concentration after 24, 48, 72 and 96 h (that is the NOEC);
- the lowest observed effect concentration after 24, 48, 72 and 96 h (that is the LOEC).

In the context of these procedures, fish are considered dead when they fail to show evidence of opercular or other activity and they do not respond to gentle prodding.

7.3.4 Hazard

Safety procedures, such as fume hoods, eye protection and gloves, **must** be used which are appropriate to the COSHH assessment (for the sample) provided by the discharger.

7.3.5 Test facility

The test facility **must** be able to maintain test solutions at the required experimental temperature for the test species (see Table 7.3). This can be achieved using a temperature controlled room.

Persons involved in carrying out toxicity tests using fish **must** be licensed by the Home Office and adhere to the relevant guidelines (HMSO 1986) at all times.

[1] Measurements of lethality must also be made after shorter exposure periods to allow the data to be analysed using the time to effect procedure (see Section 7.3.9).

7.3.6 Reagents and materials

Test organisms

Prior to a toxicity test, fish **must** be acclimated, in appropriate holding conditions for at least 14 days (see Section 7.2). All fish **must** be exposed to water of the quality to be used in the test for at least seven days immediately before testing. Test fish should be derived from a healthy, single stock of similar age and length.

Control/dilution water

In toxicity tests the media used for the controls and the preparation of test solutions **must** be a reference freshwater (such as uncontaminated groundwater, dechlorinated tapwater or reconstituted water) for freshwater species or reference seawater for marine species (see Section 7.2.2).

The hardness of the reference freshwater **must** be within the range ± 20% of the water used in the fish holding tanks (see Section 7.2.3). Any greater differences in hardness between the culture water and the reference freshwater could lead to erroneous test results due to osmotic stress imposed upon the fish.

Samples of the water used should be taken periodically and analysed to ensure no extraneous substances are present (see Section 7.2.3).

7.3.7 Apparatus

The following apparatus (see Section A) is used:

- test containers of non-toxic inert material and an appropriate volume, for example 10-40-litre glass tanks;
- equipment for measuring pH, dissolved oxygen and temperature;
- a conductivity meter, salinometer or other suitable equipment for measuring conductivity (for tests with freshwater species) or salinity (for tests with marine species);
- equipment for the determination of water hardness;
- soft nets for transferring fish.

7.3.8 Treatment and preparation of samples

The DTA Methods Working Group has recommended that initially, effluent or leachate samples collected in the Demonstration Programme are tested unadjusted with measurements being made of all the key physico-chemical parameters (see Section 2). If it is apparent that

any physico-chemical parameter or parameters are partially or fully responsible for measured responses, then subsequent tests may need to be carried out following modification of the parameter or parameters in test solutions or samples.

General procedures for the collection, storage, preparation and disposal of effluents, leachates and receiving waters have been given in Section 2.

Freshwater effluent or leachate samples can normally only be tested with marine species at concentrations ≤20% v/v, otherwise the salinity of the test solutions will fall below the 27‰ salinity tolerance threshold for the fish. Testing at higher concentrations can be achieved by altering the salinity of the solutions by the addition of analytical quality sea salt (for example, Sigma Chemical Co.). If the salinity of any test solutions (or the sample) has to be adjusted with sea salt, an additional control should be run using sea salt at the salinity of the reference seawater.

7.3.9 Test procedures

Exposure regime

To examine the toxicity of effluents and leachates, use a semi-static procedure (with replacement of solutions every 24h[2]).

Preparation of test concentration

1. Select an appropriate series **with the ratio between exposure concentrations not exceeding 2.2**. Where possible the range selected should be sufficient to give 0 and 100% lethality and at least two intermediate degrees of lethality between 0 and 100%. These results permit the calculation of the time-specific toxicity (LC_{50}, LC_{10}, NOEC and LOEC) values with greater precision. For effluents or leachates discharged to freshwaters or marine waters an appropriate initial concentration range would be 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 22.0, 46.0 and 100% v/v effluent. The preparation of the higher concentrations (>20% v/v effluent) in tests with marine fish will require salinity correction.

2. Prepare the test concentration range on the day of the test by diluting appropriate amounts of the effluent or leachate sample with reference water in volumetric glassware. Record the information on the preparation of the toxicity test concentration range in a test report (see Appendix 7C). In each test series, a control is needed which contains none of the effluent or leachate and has a volume equal to that of each exposure concentration.

[2] A static procedure with no replacement of test solutions can be used providing preliminary studies indicate that toxicity will not be underestimated using the procedure due to the loss of causative agents over the timescale of the test. Flow-through tests are not considered an appropriate option for testing due to the large volumes (1000 litres) of sample needed.

The test vessels used **must** be of sufficient volume to ensure that the maximum loading rate of 1 g fish per litre of water is not exceeded.

An effluent concentration range of 0, 0.1, 0.22, 0.46, 1.0, 2.2, 4.6, 10.0, 22.0, 46.0 and 100% v/v effluent using 10 litre test volumes would be prepared as follows:

Nominal conc. (% effluent)	Volume of reference freshwater or seawater (ml)	Volume of effluent (ml)
0 (Control)	10 000	0.0
0.1	9 990	10
0.22	9 978	22
0.46	9 954	46
1.0	9 900	100
2.2	9 780	220
4.6	9 540	460
10.0	9 000	1 000
22.0*	7 800	2 200
46.0*	5 400	4 600
100.0*	0	10 000

* = For tests with marine fish salinity correction of these test concentrations by the addition of analytical quality sea salt will be required.

Procedure for the toxicity test

The procedures given for the initiation, monitoring and termination of the toxicity test **must** be followed.

Initiation of the toxicity test

1. Before the fish are added make the following water quality measurements on the test solutions:

Freshwater tests: temperature, pH, dissolved oxygen, total hardness and conductivity

Marine tests: temperature, pH, dissolved oxygen and salinity or conductivity.

Also measure the above parameters in the water from the holding or acclimation tanks for the freshwater or marine species being used. Record all these data on a Pre-Test Water Quality Monitoring Data Sheet (for example, see Table 7C.3).

The temperature difference between the holding or acclimation tanks and test tanks **must** be no more than 2 °C. For tests on unadjusted samples measuring total biological effects the difference between the holding or acclimation tanks and the control vessel must be no more than 0.5 of a pH unit and the dissolved oxygen level in the control vessel must be at least 90% ASV at the start of the test.

2. For the control(s) and each test effluent or leachate concentration use a minimum of seven and, preferably, ten fish in sufficiently large vessels that the maximum loading rate for the test will not be exceeded.

3. Minimise systematic variability by using random number tables to randomly allocate fish to test vessels (see Appendix B). Transfer fish between holding tanks and test vessels using a soft mesh net until each vessel contains the desired number of fish.

Monitoring of the toxicity test

1. Assess lethality in each test vessel after at least 24, 48, 72 and 96 h and record the data on a Juvenile Fish Lethality Toxicity Test Data Sheet (for example, see Table 7C.4). Observations **must** also be made at earlier intervals after the start of the test so that the test data can be analysed using the time to effect procedure (see Appendix B). It is recommended that measurements are made 1.5 h, 3 h and 6 h after the start of the test but this may need to be modified based on the results of initial tests. Fish are considered dead when they fail to show evidence of opercular or other activity and do not respond to subsequent gentle prodding. Remove dead fish and those showing significant stress[3] (to avoid unnecessary suffering) from the vessels at the observation times.

2. Record observations of visible abnormalities, such as loss of equilibrium and changes in swimming behaviour, respiratory function and pigmentation.

3. On each day measure the following water quality parameters in the old and new (replacement) water of the controls and test concentrations:

Freshwater tests: temperature, pH and dissolved oxygen

Marine tests: temperature, pH, dissolved oxygen and salinity or conductivity

Measure total hardness in freshwater tests in the control and the highest exposure concentration at the end of the test as a minimum. Record these data on a Water Quality Monitoring Data Sheet (see Table 7C.5).

[3] Fish showing significant stress must be humanely killed according to the conditions of the Home Office Licence.

Termination of the toxicity test

At the end of the test after the final 96 h observations and water quality measurements have been made and recorded dispose of the surviving fish humanely using a Schedule 1 method as specified under the Home Office Licence.

7.3.10 Processing of results

Validity of the results

The results from juvenile fish lethality toxicity tests should be considered valid if the following conditions are satisfied:

(a) the lethality in the control group does not exceed 10% (or one fish if less than 10 are used) at the end of the test;

(b) for tests on unadjusted samples the dissolved oxygen concentration in the control vessel(s) is maintained above 60% of the air saturation value (ASV) during the test. If dissolved oxygen level is found to contribute to measured responses, and this parameter is subsequently modified in test solutions or samples, the DO level in all test concentrations **must** be maintained above 60% (ASV) during the test.

Data from tests on effluents or leachates for discharge characterisation should only be accepted if the results of the reference toxicity test met relevant quality control criteria (see Appendix C).

In semi-static toxicity tests and bioassays, the difference between the temperature and pH of the old and new (replacement) control water **must** be no more than 2 °C and 0.5 of a pH unit.

Estimation of toxicity test endpoints

The LC_{50}, LC_{10}, NOEC and LOEC values are determined using an appropriate validated computer-based statistical procedure.

Estimation of the LC_{50} and LC_{10} values

At the end of the exposure period, calculate the percentage lethality for each test concentration relative to the total number of animals used for that concentration and determine time-specific LC_{50} (and LC_{10}) values by an appropriate statistical method (see Figure 7.1). Confidence limits ($p = 0.95$) for the calculated LC_{50} (and LC_{10}) values should be determined using these standard procedures and shall be quoted in the test report (see Appendix 7C).

Table 7.9 shows an example data set which has been used to show the determination of the 96 h-LC_{50} (and 96 h-LC_{10}) value for the mortality of juvenile fish by an effluent using different statistical procedures.

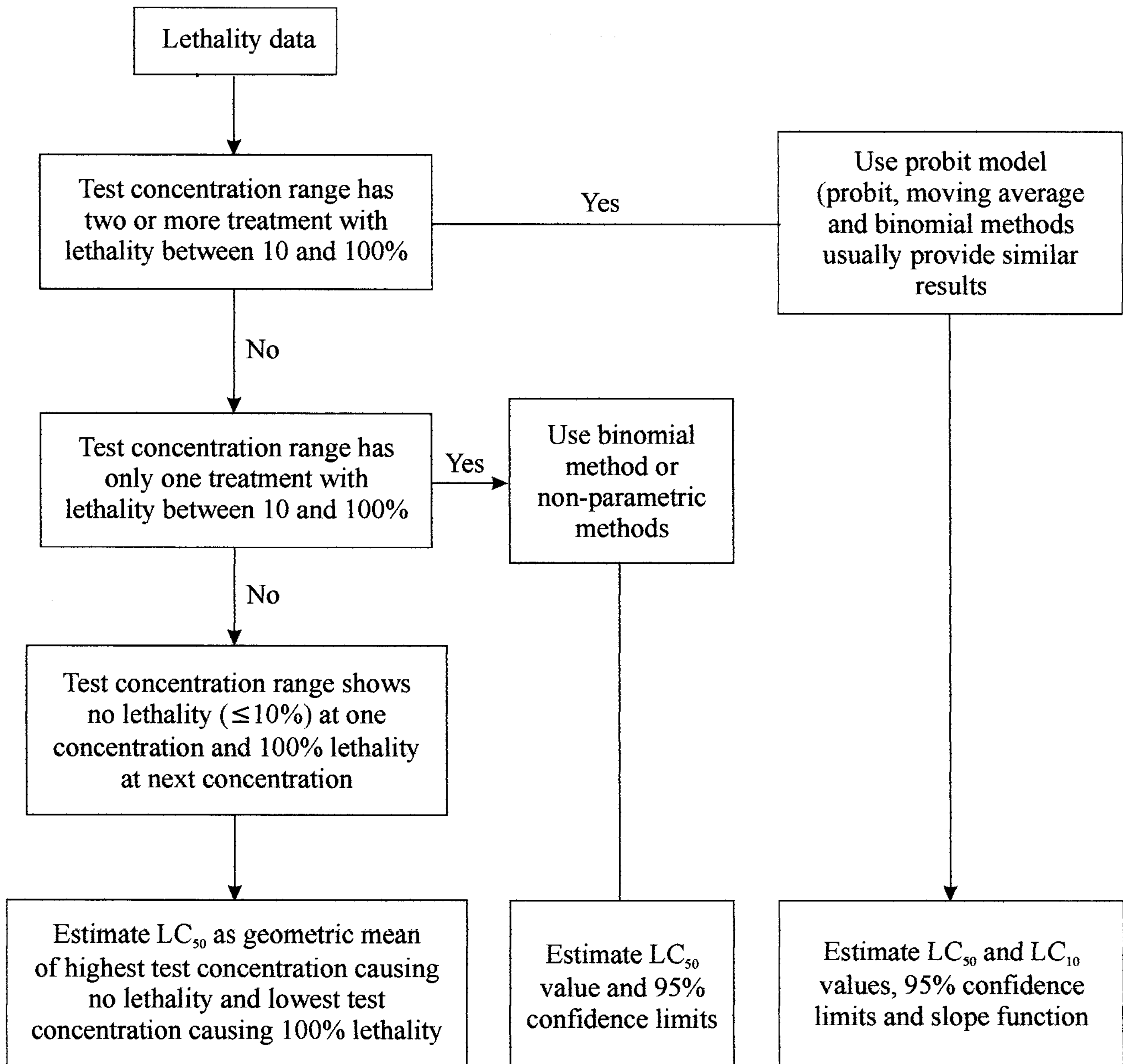

Figure 7.1 Flowchart for the estimation of the LC_{50} for full concentration range juvenile fish lethality toxicity tests

Table 7.9 Example results of the lethality of juvenile fish after exposure to an effluent for 96h

Effluent concentration(%)	Number of fish exposed	Scenario 1		Scenario 2		Scenario 3	
		Cumulative number of dead	Lethality (%)	Cumulative number of dead	Lethality (%)	Cumulative number of dead	Lethality (%)
0 (Control)	10	0	0				
0.1	10	0	0	0	0	0	0
0.22	10	0	0	0	0	0	0
0.46	10	0	0	0	0	0	0
1.0	10	0	0	0	0	0	0
2.2	10	1	10	0	0	0	0
4.6	10	4	40	4	40	0	0
10	10	6	60	10	100	10	100
22	10	9	90	10	100	10	100
46	10	10	100	10	100	10	100
100	10	10	100	10	100	10	100

The data in Table 7.9 should be used by laboratories to check that in-house statistical procedures are providing comparable results to those given in Table 7.10

In analysing data from juvenile fish lethality test the following points should be considered:

1. If the results include concentrations at which there are 0-10 and 100% lethality and also two concentrations at which the percentage lethality is between 10 and 100%, and the data are smooth and regular, then probit, moving average and binomial methods should provide similar estimates of the LC_{50} value. Probit analysis should be used to estimate LC_{50} and LC_{10} values, 95% confidence limits and the slope, provided the probability is not less than 0.05.

2. If the results do not include two concentrations at which lethality is between 10 and 100%, the probit and moving average methods cannot be used. The binomial method can be used to provide a best estimate of the LC_{50} value with wide confidence limits. Non-parametric methods such as the Spearman Karber or Trimmed Spearman Karber methods may allow the determination of an LC_{50}.

3. Where the data obtained are inadequate for calculating the LC_{50} by any of the standard methods, identify the highest concentration causing no lethality and the lowest concentration causing 100% lethality. An approximation of the LC_{50} can be made from the geometric mean of these two concentrations. In this case, the ratio of the higher to the lower concentration should not exceed 2.2, otherwise any LC_{50} calculated will be less statistically sound.

4. In all instances, the LC_{50} derived from any of the above methods should be compared with a graphical plot on logarithmic-probability scales of percent lethality for the various test concentrations. Any major disparity between the graphical estimation of the LC_{50} and that derived from the statistical programmes should be resolved by checking the statistical procedures.

Table 7.10 summarises the LC_{50} values derived for the different data sets in Table 7.9 using different statistical procedures. For the data in Scenario 1 the probit, moving average and binomial methods produce similar results although the confidence limits are greater for the value derived using the binomial method than those derived using the probit or moving average methods. Where there are less than two intermediate effect concentrations (Scenarios 2 and 3) the LC_{50} values derived are less statistically sound.

The 96 h LC_{10} value estimated by the Tox Calc software (Tide Pool Scientific Software) is 2.3% with 95% confidence limits of 0.97-3.6%.

Table 7.10 Summary of the LC_{50} values (and 95% confidence limits) for the data in Table 7.9 estimated by different statistical procedures

Scenario	Statistical procedure	LC_{50} (%)		Slope
		Value	Confidence limits	
1	Probit (Tox Calc)	6.8	4.6-10.3[1]	2.7
	Probit (Stephan 1982[2])	6.8	6.0-7.7	2.7
	Moving average (Stephan 1982[2])	6.8	5.9-7.8	-
	Binomial (Stephan 1982[2])	6.8	2.2-22	-
2	Probit	Not valid approach		-
	Moving average	Not valid approach		-
	Binomial (Stephan 1982[2])	5.1	2.2-10	-
	Spearman-Karber (Tox Calc)	5.0	4.0-6.3	-
3	Geometric mean	3.2	-	-

[1] Fiducial limits
[2] Computer based statistical package based on Stephan (1977)

From interpolation of the graph of cumulative lethality of fish (probability scale) against effluent concentration (log scale) shown in Figure 7.2 for Scenario 1 the 96 h-LC_{50} = 7.0% v/v effluent and the 96 h-LC_{10} = 2.2% v/v effluent. The values obtained graphically confirm those obtained using the computer-based software.

Estimation of the NOEC and LOEC

The NOEC and LOEC values are determined using hypothesis testing. If there are no replicates of each test concentration as in the fish lethality test then NOEC and LOEC values should be calculated using Fisher's Exact Test.

Further information on these statistical procedures can be obtained from Sokal and Rohlf (1981), Zar (1984) and US EPA (1993). In the example given in Table 7.9 the 96 h NOEC and LOEC values calculated using Fisher's Exact Test were 2.2 and 4.6% v/v effluent.

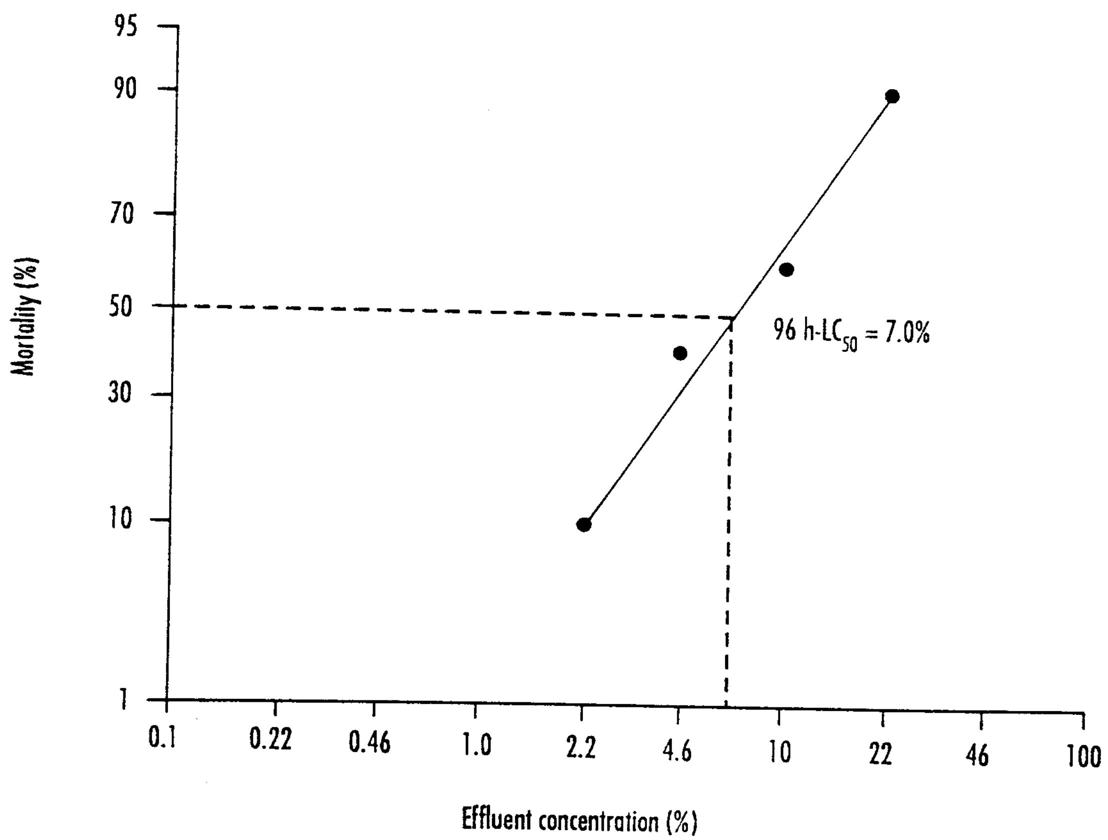

Figure 7.2 Graph of cumulative mortality (probability scale) against effluent concentration (log scale)

7.4 Guidelines for single concentration toxicity tests on effluents and leachates and bioassays on receiving waters using the juvenile fish lethality test

7.4.1 Monitoring against toxicity limits

Introduction

Toxicity tests with juvenile fish for monitoring against toxicity limits should be carried out with a single concentration test comprising a single effluent or leachate concentration (toxicity limit) and an appropriate control(s).

Test procedure

The control water may be obtained from a 'clean' site. For tests with freshwater species the water may be collected from upstream or adjacent to the point of collection of the effluent or leachate sample, whereas for marine species the control water may be from a reference site. If water from a `clean' site is used as the control, further controls should be prepared using the reference freshwater or seawater in which the fish were maintained. All relevant information should be documented in the test report (see Appendix 7C).

Single concentration tests should be initiated in the same way as full concentration range toxicity tests (see Section 7.3.9) with groups of 10 fish added randomly to the duplicates of the controls and the effluent or leachate sample. Test vessels **must** be sufficiently large that the maximum loading rate for the test will not be exceeded (see Section 7.3.9). Further replicate solutions may be tested but are not required. Lethality should be monitored after at least 24, 48, 72 and 96 h and recorded on a Juvenile Fish Lethality Toxicity Test Data Sheet in the test report (see Table 7C.4). Water quality monitoring should be carried out in the same way as described for the toxicity test and recorded on a Water Quality Monitoring Data Sheet in the test report (see Table 7C.5). In semi-static tests, measurements **must** be made on the old and new (replacement) water for each control and exposure concentration.

Processing of results

Assessment of how the responses in the single effluent or leachate treatment compare to those in the control is accomplished using hypothesis testing (see Figure 7.3). The null hypothesis tested is that the responses in the treatment are not significantly different from those in the control.

Initially the proportion of organisms surviving in the control and the single treatment concentration are transformed using an appropriate procedure such as the arc sine square root transformation. The arc sine square root transformation is commonly used on proportional data to stabilise the variance and satisfy the normality and homogeneity of variance requirements. Shapiro-Wilk's or D'Agostino D test should be used to test the normality assumption.

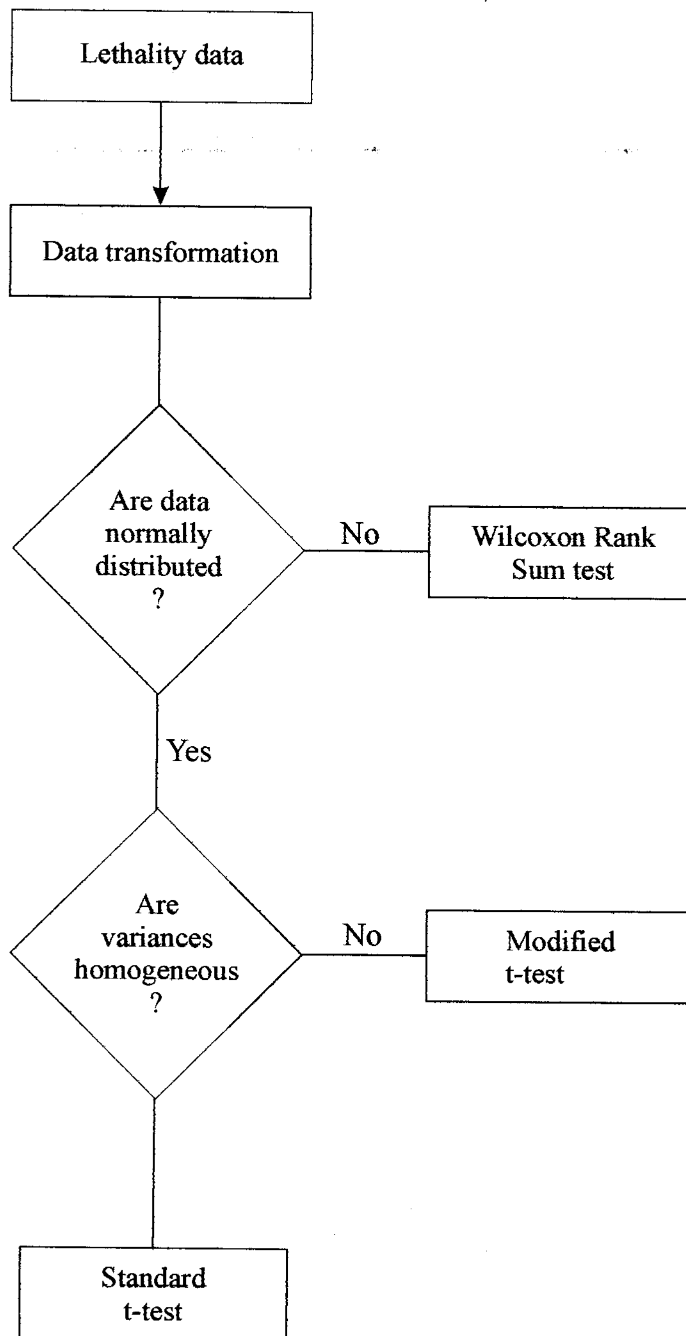

Figure 7.3 Flowchart for analysis of single concentration test data from juvenile fish lethality toxicity tests

If the data do not meet the assumption of normality, then the non-parametric Wilcoxon Rank Sum Test should be used to analyse the data providing there are at least three replicates of each treatment. If the data meet the assumption of normality, the F test for equality of variances is used to test the homogeneity of variance assumption. If the data meet the homogeneity of variance assumption then the standard (homoscedastic) t test should be used to analyse the data. Failure of the homogeneity of variance assumption leads to the use of a modified (heteroscedastic) t test, where the pooled variance estimate is adjusted for unequal variance, and the degrees of freedom for the test are adjusted. Further information on these statistical procedures can be obtained from Sokal and Rohlf (1981), Zar (1984) and US EPA (1993).

Table 7.11 shows example data sets for a single concentration test using duplicate control and 2.2% effluent solutions. In Scenario 1 the equality of variances cannot be confirmed and a modified (heteroscedastic) t test indicates no significant differences between responses in the two groups ($t = 6.26$, $p>0.05$). In Scenario 2 the variances are equal ($F = 1.97$, $p = 0.79$) and the standard (homoscedastic) t test indicates no significant differences between responses in the two groups ($t = 2.81$, $p>0.05$).

7.4.2 Assessing receiving water column toxicity

The assessment of the toxicity of receiving water column samples should be carried out using the bioassay procedure given in Section 7.4.1 for an undiluted (100%) sample and an appropriate control(s).

Receiving water samples may not meet the physico-chemical parameters required to support fish survival. In these circumstances, the sample may have to be modified using the procedures given in Section 2 to satisfy the threshold criteria indicated in Table 7.12.

7.5 Guidelines for toxicity tests on reference toxicants using juvenile fish

7.5.1 Introduction

For each batch of fish used to conduct acute lethality tests to provide data for discharge characterisation or monitoring against a toxicity limit, there should be accompanying tests with the reference substance zinc (see Appendix C).

7.5.2 Test procedure

Reference toxicant tests should be conducted according to the procedure given in Section 7.3.9.

Table 7.11 Example data set for a single concentration test and the results of statistical analysis

Effluent concentration (%)	Replicate	No. of fish exposed	Cumulative number of dead	Lethality (%)	Method of statistical analysis	Result of statistical analysis
Scenario 1						
0 (Control)	1	10	0	0	Modified t-test	NS
	2	10	0	0		
2.2	1	10	2	20		
	2	10	3	30		
Scenario 2						
0 (Control)	1	10	0	0	Standard t-test	NS
	2	10	1	10		
2.2	1	10	2	20		
	2	10	3	30		

NS - no significant difference between control and treatment groups

7.5.3 Preparation of 1000 mg l^{-1} zinc stock solution

(a) Weigh out 4397 mg of zinc sulphate ($ZnSO_4.7H_2O$) in a weighing boat

(b) Add the zinc sulphate to a 1-litre volumetric flask and dilute to just below the mark with distilled water

(c) Add 1 ml of 1M Analar HCl to stabilize the stock solution

(d) Dilute to the mark with distilled water.

7.5.4 Preparation of test concentrations

For the reference toxicant zinc the concentrations range given below should be used in the first study to assess the sensitivity of test organisms in a facility when no previous data is available.

Nominal zinc conc. (mg l^{-1})	Volume of reference freshwater or seawater (ml)	Volume of zinc stock (ml)
0 (Control)	10 000	0.0
0.1	9 999	1.0
0.32	9 996.8	3.2
1.0	9 990	10
3.2	9 968	32
10.0	9 900	100
32.0	9 680	320

The above volumes relate to a zinc stock concentration of 1000 mg l^{-1}, which should be prepared according to the procedure given in Section 7.5.3.

The test concentration ranges of zinc for subsequent tests can be modified based on the initial results to allow the derivation of more precise LOEC and LC_{50} values.

7.5.5 Test procedure

Reference toxicant tests should be initiated in the same way as full concentration range toxicity tests (see Section 7.3.9). Test vessels **must** be sufficiently large that the maximum loading rate for the test will not be exceeded (see Section 7.3.9). However, to minimize the number of fish used, only seven fish need be used in each test concentration. Lethality should be monitored after at least 24, 48, 72 and 96 h and recorded on a Juvenile Fish Lethality Toxicity Test Data Sheet (see Table 7C.4). Water quality monitoring should be carried out in the same way as described for the toxicity tests and recorded on a Water Quality Monitoring Data Sheet (see Table 7C.5). In semi-static tests, measurements **must** be made on the old and new (replacement) water for each control and exposure concentration.

Samples of the zinc test solutions should be taken at the beginning of the test from the stock vessel for each test concentration and at the end of the test from the vessels themselves and analysed using an appropriate procedure (for example, SCA 1980, 1981, 1988).

7.5.6 Processing of results

Time-specific LOEC and LC_{50} values should be calculated using the procedures described in Section 7.3.10. The estimation of toxicity values should be based measured exposure concentrations.

7.6 Test report

The test reports for toxicity tests **must** include the following information:

(a) information about the test organism, such as the scientific name, strain, supplier, any pre-treatment, size and number used in each test concentration. Holding and acclimation conditions together with lethality and feeding record for the 14-day period preceding the study should be given;

(b) the source of the reference freshwater or seawater for toxicity tests and the major chemical characteristics of the water such as temperature, pH and hardness for freshwater tests and temperature, pH and salinity or conductivity for marine tests;

(c) the methods of preparation of test samples including for effluents, leachates and receiving waters, the manner and duration of storage of the samples and, if necessary, the conditions by which physico-chemical parameters were modified, for example, whether pH was adjusted or suspended solids were treated (settlement, centrifugation or filtration of samples);

(d) test procedure used and the fish loading rate;

(e) temperature, pH and dissolved oxygen values of the test solutions at 24 h intervals for the duration of the test. In semi-static systems the temperature, pH and dissolved oxygen are measured prior to and after water renewal;

(f) tables showing cumulative lethality in each control and test concentration at the end of the exposure period for full concentration range tests, single concentration tests and reference toxicant tests;

(g) an indication that criteria determining the validity of the test (that is a control lethality of less than 10% and a dissolved oxygen concentration of >60% ASV in the control for unmodified samples and all test vessels for modified samples) have been achieved;

(h) for tests on effluents and leachates for discharge characterisation or monitoring against toxicity limits, an indication that the responses of juvenile fish in the reference toxicant test met quality control criteria;

(i) where appropriate, a graph to demonstrate the relationship between LC_{50} and test exposure period;

(j) the derivation of the time-specific LC_{50} and LC_{10} values, the 95% confidence limits and the method of calculation. Time-specific NOEC and LOEC values are also reported;

(k) any concentrations causing abnormal behaviour of the fish, and the nature of that effect under the test conditions;

(l) any operating details not specified in this procedure and any incidents which may have affected the results.

7.7 Checklist

A checklist summarizing the test conditions and procedures for juvenile fish lethality toxicity tests is given in Table 7.12.

Table 7.12 Checklist of test conditions and procedures for juvenile fish lethality toxicity tests

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Test procedure

Organisms	Juvenile fish, ≥7 fish per concentration. Loading density ≤1 g fish per litre
Type of test	96 hours duration
Exposure regime	Semi-static (or static if stability analysis indicates the approach is appropriate)
Control/dilution water	Reference freshwater or reference seawater
Temperature	15 ± 2 °C
Physico-chemical parameters	If the total biological effect of a sample is being measured, then the sample is tested unadjusted and key physico-chemical parameters (such as temperature, pH, dissolved oxygen, hardness or salinity and suspended solids) are measured. If it is apparent that any physico-chemical parameters are partially or fully responsible for measured responses then the test solutions or sample, have to be modified using the procedures given in Section 2 to satisfy the threshold criteria given below:

Physico-chemical parameter	Threshold criteria
pH	6.0-8.5
Dissolved oxygen	≥60% ASV in all test vessels
Hardness (freshwater fish tests)	10-250 mg $CaCO_3$ l^{-1} in all test vessels
Salinity (marine fish tests)	≥27-36‰ in all test vessels
Suspended solids	<25 mg l^{-1} in all test vessels

Lighting	Full-spectrum fluorescent light of 100-500 lux at the water surface, 12-16 h light : 8-12 h dark regime
Feeding	No feeding for 24 h before the start of the test or during the test
Observations	Lethality and behaviour after 24, 48, 72, 96 h (and earlier intervals - see Section 6.3.9)

Table 7.12 continued

Measurements	Test solution temperature, pH and DO daily for all tests (+ salinity or conductivity for marine tests) For freshwater tests, hardness at the start in all tests vessels and in the control and highest exposure concentration at the end as a minimum
Endpoints	Time-specific LC_{50} and LC_{10} values (± 95% confidence limits). Time-specific NOEC and LOEC values.
Reference toxicant	Zinc (as zinc sulphate) determined at the time of testing
Test validity	Mean control mortality ≤10% and for unadjusted samples DO in control vessels ≥60% ASV at all times or for modified samples DO in all test vessels ≥60% ASV at all times
Test samples	
Effluents, leachates and receiving waters	
Transport and storage	Transport at a temperature not markedly different from that measured at the time of collection. The test **must** begin within 48 h of the time of sampling. If the sample is not to be tested immediately on receipt then it should be stored at 5 ± 3 °C.
Control/dilution water	Reference freshwater or reference seawater[*1]

*1 If an upstream freshwater receiving water or seawater from a reference site is used as the control/dilution water, a reference freshwater or reference seawater control shall also be run

7.8 References

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APPENDIX 7A PREPARATION OF RECONSTITUTED FRESHWATERS

Table 7A.1 gives the types and quantities of analytical grade chemicals to be added to distilled or de-ionized water with a conductivity of $\leq 10\ \mu S\ cm^{-1}$ to prepare reconstituted water of a specific hardness, waters of intermediate hardness between the moderately hard and hard types can be prepared by adjusting the amounts of salts used.

Table 7A.1 Preparation of reconstituted water of a desired hardness (after US EPA 1985)

Water type	Reagent added ($mg\ l^{-1}$)				Final water quality	
	$NaHCO_3$	$CaCO_3$.	$MgSO_4$	KCl	Hardness[1]	pH[2]
Moderately hard	96.0	60.0	60.0	4.0	80 - 100	7.4 - 7.8
Hard	192.0	120.0	120.0	8.0	160 - 180	7.6 - 8.0

1 - Expressed in mg $CaCO_3\ l^{-1}$
2 - Approximate pH after aerating for 24 h

Table 7A.2 describes the types and quantities of analytical grade chemicals to be added to distilled or deionized water having a maximum conductivity of $10\ \mu S\ cm^{-1}$ to prepare a hard reconstituted water (250 mg $CaCO_3\ l^{-1}$) for freshwater toxicity tests (ISO 1989)

Prepare the water by mixing 250 ml of each solution in a volumetric flask and make up to a total volume of 10 litres with distilled or deionized water. The water should be stored in a clean container made of a non-toxic inert material.

The dilution water should be aerated until the dissolved oxygen concentration has reached saturation and the pH has stabilised. If necessary, adjust the pH to 7.8 ± 0.2 by adding 1M sodium hydroxide (NaOH) solution or 1M hydrochloric acid (HCl) solution. The water prepared in this way should not be aerated further before use.

Table 7A.2 Preparation of a hard reconstituted water (after ISO 1989)

Solutions	Formula	Stock solution concentration (g in 1 litre volumetric flask)
Calcium chloride dihydrate	$CaCl_2.2H_2O$	11.76
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	4.93
Potassium chloride	KCl	0.23
Sodium hydrogen carbonate	$NaHCO_3$	2.59

APPENDIX 7B DATASHEETS USED WHEN HOLDING FISH (EXAMPLE)

Table 7B.1 Fish Holding Record Data Sheet - Water quality, mortality and feeding data (Example)

Stock Number:

Species

Source:

Date received:

Number received:

Size of holding tank (litres):

Date/ Time	Temp (°C)	pH	DO (%ASV)	Total hardness (mg l^{-1})	Salinity (‰)/ conductivity (μS cm^{-1})	No of dead removed	Wt of food given (g)	Initials

Treatment for parasites or infection

Date of treatment	Nature of treatment

Table 7B.2 Fish Acclimation Record Data Sheet (Example)

Species: Source:

Location: Number present:

Volume of acclimation tank: Water used in the acclimation tank:

Food type: Amount fed on each occasion:

Date
Temp (°C)
pH
DO (% ASV)
Total hardness (mg $CaCO_3$ l^{-1})
Salinity (‰)/Conductivity (µS cm^{-1})
Aeration (Y/N)
Water flow (ml min^{-1})
Amount of food given (g)
No. of dead removed
Cumulative dead

APPENDIX 7C TEST REPORT FOR A JUVENILE FISH LETHALITY TOXICITY TEST (EXAMPLE)

Table 7C.1 Holding of test organisms

Data on test species

Test species:

Source:

Holding/Acclimation: conditions	Temperature (°C)
	pH
	Dissolved oxygen (% ASV)
	Total hardness (mg $CaCO_3$ l^{-1})
	Salinity (‰) or Conductivity (µS cm^{-1})
	Feeding regime
	Average weight of fish at start (g) (Total weight/number)

Data on test substance

Test substance:

Source: Hazard:

Date collected or prepared:

Date received:

Storage conditions:	Temperature (°C)
	Length of time before start of test (h)

Table 7C.2 Preparation of toxicity test concentration range

Test substance:

Type of test:

Type of exposure regime: Static/Semi-static

Start of test: End of test:

Duration of test:

No. of test concentrations: Renewal of test solutions (h):

No. of controls:

No. of fish per concentration:

No. of replicates per concentration:

Concentration range

Stock solution concentration:

Type of control and dilution water: Reference freshwater/Reference seawater

Source of control and dilution water:

Volume of test solutions required (l):

Nominal test substance concentration	Volume of reference water (ml)	Volume of test substance (ml)

Table 7C.3 Pre-test Water Quality Monitoring Data Sheet

Species: Source:

Water quality of holding or acclimation tank on day of test

Temperature ____________ pH: ____________

DO (%ASV): ____________

Freshwater tests

Total Hardness: ____________

Marine tests

Salinity (‰) ____________
or
Conductivity (µS cm^{-1}) ____________

Date: Initials:

Water quality of test vessels before the addition of fish

Test vessel	Temp (°C)	pH	DO (%ASV)	Total hardness (mg l^{-1})	Salinity (‰) or Conductivity (µS cm^{-1})

Date: Initials:

Table 7C.4 Juvenile Fish Lethality Toxicity Test Data Sheet

Species: Date and time fish added:

Date	Time	Exposure period	Exposure conc.	No. of fish alive	No. of dead fish removed	Cumulative no. of dead	Initials

Table 7C.5 Water Quality Monitoring Data Sheet - Toxicity Test

Date	Time	Vessel No.	Nominal conc	Temp (°C)	pH	DO (%ASV)	Total hardness (mg $CaCO_3$ l^{-1})	Salinity (‰) or Conductivity (µS cm^{-1})	Initials

For semi-static tests O = Old solutions, N = New solutions

Table 7C.6 Expression of results of a toxicity test

Exposure period	Exposure conc.	Cumulative no. of dead	Number of fish exposed	Mortality (%)

Calculation of the LC_{50} and LC_{10} values

Exposure period (h):

Method used: LC_{50} = LC_{10} =

Concentrations used in the calculation of the LC_{50} and LC_{10}: Nominal/Measured

Calculation of the NOEC and LOEC values

Exposure period (h):

Method used: NOEC = LOEC =

Concentrations used in the calculation of the NOEC and LOEC: Nominal/Measured

Abnormal behaviour of the fish during the test

Operating details not specified in the standard operating procedure and any incidents which may have affected the results:

8. GLOSSARY OF TERMS

The following definitions have been used in the methods guidelines:

Expressions

Must is used to express an absolute requirement.

Should is used to state that the specified conditions represent the preferred option and therefore be met whenever possible. If these conditions are not met then deviations from the guideline and the reasons for this action must be documented.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

General terms

Alkalinity - the acid-neutralizing (that is proton accepting) capacity of water.

Conductivity - a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentration of ions in solution, their valence and mobility and on the temperature of the solution. Conductivity in fresh waters is normally reported as millisiemens per metre ($mS\ m^{-1}$).

Hardness - the concentration of all metallic cations, except those of alkali metals, in water. In general, hardness is a measure of the concentration of calcium and magnesium ions in water and is usually expressed as mg calcium carbonate l^{-1}.

pH - the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality. Values less than 7 signify increasingly greater acidic conditions, while values greater than 7 signify increasingly greater alkaline conditions.

Salinity - the total amount of solid material (in grams) dissolved in 1 kg of aqueous solution. Salinity is usually measured directly using a salinity meter or refractometer and is reported as grams per kg ($g\ kg^{-1}$) or the approximate equivalent of that in parts per thousand (‰).

Terms for test substances

Dilution water - the water used to dilute a test substance to prepare the different concentrations used for a toxicity test.

Environmental sample - an effluent, leachate or receiving water sample.

Reference freshwater - the uncontaminated freshwater used for the controls and as dilution water in toxicity tests. This may be ground water, dechlorinated tap water or reconstituted water.

Reference seawater - the uncontaminated seawater used for the controls and as the dilution water for toxicity tests. This may be seawater collected from a 'clean' location and, ideally, filtered through a 0.2 μm filter to remove particulate matter or reconstituted water.

Reference toxicant - a standard chemical used to measure the sensitivity of the test organisms in order to establish a level of confidence in the data obtained for a toxicity test or bioassay. In most instances a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the toxicity test or bioassay is conducted and the precision of results obtained by the laboratory.

Stock solution - a concentrated aqueous solution of the test substance. Measured volumes of a stock solution are added to dilution water to prepare the required test concentrations.

Test substance - an environmental sample or pure substance such as a reference toxicant.

Toxicity terms

Acute - a short period in relation to the life span of the organism; this would be of the order of minutes for bacteria and usually ≤4 days for fish.

Arcsine transformation - the transformation of percentages or proportions to corresponding arcsine values to achieve a normal rather than a binomial distribution.

Bioassay - a procedure which evaluates the relative effects of a test substance by comparing these effects with those of a standard preparation. In the context of the guidelines bioassays are considered to be tests comparing the toxicity of undiluted effluent, leachate or receiving water samples with that of controls.

Chronic - a relatively long period of exposure, usually a significant portion of the life span of the organism such as 10% or more.

Daphnid - a freshwater crustacean commonly known as a water flea. Species of daphnids include *Daphnia magna*.

EC_{50} - the median effective concentration (that is the concentration of test substance estimated to cause a 50% effect on a test response such as immobilization). The term does not apply to a percent reduction in a rate or process in an organism or group of organisms, where the IC_{50} should be used instead.

Ephippia - egg cases that develop under the postero-dorsal part of the carapace of a female adult daphnid in response to adverse culture conditions. The eggs within have normally been fertilized indicating sexual reproduction has taken place.

Flow-through - a test procedure where the test substance is continuously replaced during the test period by the use of equipment, such as siphon dosers or electronic dosing apparatus.

Growth - an increase in algal cell number or the size or weight of invertebrates or fish as a result of proliferation of new tissue.

IC_{10} - the concentration of test substance estimated to cause 10% inhibition in a test response such as growth.

IC_{50} - the median inhibitory concentration (that is the concentration of test substance estimated to cause 50 % inhibition in a test response such as growth).

Immobilization - the inability of daphnids to swim within 15 seconds after gentle agitation of the test container, even if there is still movement of the antennae.

LC_{50} - the median lethal concentration (that is the concentration of test substance estimated to cause lethality in 50% of the test organisms).

Lethal - causing the death of organisms by direct action. Death is usually defined as the cessation of all visible signs of movement or other activity.

Loading - ratio of animal biomass to the volume of test solution in an exposure vessel.

LOEC - the lowest observed effect concentration. This is the lowest exposure concentration of a test substance which causes observed and statistically significant adverse effects compared to the controls.

NOEC - the no-observed effect concentration. This is the highest exposure concentration of a test substance which does not cause any observed and statistically significant adverse effects compared to the controls.

Semi-static - a test procedure where the test solutions are renewed in replicate test vessels at fixed intervals during the test (commonly 24 h) and the test animals transferred into the fresh solutions.

Static - a test procedure where no further replacement or replenishment of the test solutions are carried out after starting the test.

Sub-lethal - detrimental to living organisms but not resulting in death within the exposure period.

Toxicity - the inherent potential or capacity of a test substance to cause adverse effects on living organisms. The effects could be sub-lethal or lethal.

Toxicity test - a procedure which measures the toxicity produced by exposure to a series of concentrations of a test substance. In aquatic toxicity tests the effect of exposure to test substances is usually measured as either:

1. the proportion of organisms affected (quantal response) (see EC_{50} and LC_{50});
2. the degree of effect shown by the organisms (graded or non-quantal response) (see IC_{50}).

APPENDIX A LIST OF SUPPLIERS OF TEST ORGANISMS AND EQUIPMENT

A1 TEST ORGANISMS

A1.1 Algae

Scenedesmus subspicatus *Selenastrum capricornutum* *Phaeodactylum tricornutum* *Skeletonema costatum*	Culture Centre of Algae and Protozoa Institute of Freshwater Ecology The Windermere Laboratory Far Sawrey Ambleside Cumbria LA 22 0LP Tel: 015394 42468

A1.2 Invertebrates

Daphnia magna	WRc plc (IRCHA Clone 5) Henley Road Medmenham Marlow SL7 2HD Tel: 01491 571531
	DTA National Centre Laboratory 4 The Meadows Waterberry Drive Waterlooville Hampshire PO7 7XX Tel: 01903 832000
Crassostrea gigas	Guernsey Sea Farms Ltd Parc Lane Vale Guernsey Channel Islands Tel: 01481 47480

A1.3 Fish

Oncorhynchus mykiss (Rainbow trout)	Contact local Environment Agency hatchery or recognised commercial supplier in local area
Scophthalmus maximus (Turbot)	Mannin Sea Farms Castletown Derbyhaven Isle of Man Tel: 01624 824698

A2 TEST EQUIPMENT

A2.1 General equipment

Microscopes	Olympus Optical Co. (UK) Ltd 2-8 Honduras Street London EC1Y 0TX Tel: 0171 253 2772
Incubators (Cooled and Orbital), Micro-pH electrodes	BDH-Merck Ltd (Contact for local distributor) Tel: 0800 223344
	Fisher Scientific UK Bishop Meadow Road Loughborough Leicestershire LE11 ORG Tel: 01509 231166
	Jencons Scientific Ltd Cherrycourt Way Industrial Estate Stanbridge Road Leighton Buzzard LU7 8UA Tel: 01525 372010
	Philip Harris Scientific Contact Regional Office Tel: 01543 480077
	Sigma Chemical Co. Tel: 0800 447788

A2.2 Algal growth inhibition tests

Coulter Counter	Coulter Electronics Ltd Northwell Drive Luton Beds LU3 3RH Tel: 01582 491414

APPENDIX B STATISTICAL PROCEDURES

B1 TIME TO EVENT PROCEDURE

In the Demonstration Programme it is important to collect data from toxicity tests (and bioassays) in such a way that a range of statistical procedures, including non-standard methods, can be evaluated. Time to event (TTE) analysis represents a potentially useful tool for increasing the robustness of the PNEC derived following effluent characterisation. Increasing the robustness of the PNEC value for an effluent should reduce the uncertainty associated with the risk assessment process and the use of TTE analysis may offer the opportunity to consider the relative risks posed by discharges in different ways.

TTE analysis uses all the data obtained at different observation times. In the Demonstration Programme it will be necessary to collect data at additional observation times to those normally used in the *Daphnia magna* immobilization test and fish lethality tests to assess the benefits of the TTE approach. In the first instance a minimum of three additional observation times have been recommended in the relevant sections for these test methods. However, the exact timings can be adjusted based on the results obtained to ensure that the data generated is useful.

There are a series of approaches which can be used with time to event data (see table below) and, if possible, the information generated in the Demonstration Programme will be used to evaluate the usefulness of the different approaches.

Approaches to analysing time to event data

Non-parametric	Semi-parametric	Parametric
Lifetable methods Kaplin-Meier (Product limit) methods Survival curves (Lee-Desu comparison)	Cox proportional hazards model	Exponential, Weibull, Normal, Gompertz } models

B2 STATISTICAL TABLES

Table B1 Random Number Table

22808	04391	45529	53968	57136	98228	85485	13801	68194	56382
49305	36965	44849	64987	59501	35141	50159	57369	76913	75739
81934	19920	73316	69243	69605	17022	53264	83417	55193	92929
10840	13508	48120	22467	54505	70536	91206	81038	22418	34800
99555	73289	59605	37105	24621	44100	72832	12268	97089	68112
32677	45709	62337	35132	45128	96761	08745	53388	98353	46724
09401	75407	27704	11569	52842	83543	44750	03177	50511	15301
73424	31711	65519	74869	56744	40864	75315	89866	96563	75142
37075	81378	59472	71858	86903	66860	03757	32723	54273	45477
02060	37158	55244	44812	45369	78939	08048	28036	40946	03898
94719	43565	40028	79866	43137	28063	52513	66405	71511	66135
70234	48272	59621	88778	16536	36505	41724	24776	63971	01685
07972	71752	92745	86465	01845	27416	50519	48458	68460	63113
58521	64882	26993	48104	61307	73933	17214	44827	88306	78177
32580	45202	21148	09684	39411	04892	02055	75276	51831	85686
88796	30829	35009	22695	23694	11220	71006	26720	39476	60538
31525	82746	78935	82980	61236	28940	96341	13790	66247	33839
02747	35989	70387	89571	34570	17002	79223	96817	31681	15207
46651	28987	20625	61347	63981	41085	67412	29053	00724	14841
43598	14436	33521	55637	39789	26560	66404	71802	18763	80560
30596	92319	11474	64546	60030	73795	60809	24016	29166	36059
56198	64370	85771	62633	78240	05766	32419	35769	14057	80674
68266	67544	06464	84956	18431	04015	89049	15098	12018	89338
31107	28597	65102	75599	17496	87590	68848	33021	69855	54015
37555	05069	38680	87274	55152	21792	77219	48732	03377	01160
90463	27249	43845	94391	12145	36882	48906	52336	00780	74407
99189	88731	93531	52638	54989	04237	32978	59902	05463	09245
37631	74016	89072	59598	55356	27346	80856	80875	52850	36548
73829	21651	50141	76142	72303	06694	61697	76662	23745	96282
15634	89428	47090	12094	42134	62381	87236	90118	53463	46969
00571	45172	78532	63863	98597	15742	41967	11821	91389	07476
83374	10184	56384	27050	77700	13875	96607	76479	80535	17454
78666	85645	13181	08700	08289	62956	64439	39150	95690	18555
47890	88197	21368	65254	35917	54035	83028	84636	38186	50581
56238	13559	79344	83198	94642	35165	40188	21456	67024	62771
36369	32234	38129	59963	99237	72648	66504	99065	61161	16186
42934	34578	28968	74028	42164	56647	76806	61023	33099	48293
09010	15226	43474	30174	26727	39317	48508	55438	85336	40762
83897	90073	72941	85613	85569	24183	08247	15946	02957	68504
82206	01230	93252	89045	25141	91943	75531	87420	99012	80751
14175	32992	49046	41272	94040	44929	98531	27712	05106	35242
58968	88367	70927	74765	18635	85122	27722	95388	61523	91745
62601	04595	76926	11007	67631	64641	07994	04639	39314	83126
97030	71165	47032	85021	65554	66774	21560	04121	57297	85415
89074	31587	21360	41673	71192	85795	82757	52928	62586	02179
07806	81312	81215	99858	26762	28993	74951	64680	50934	32011
91540	86466	13229	76624	44092	96604	08590	89705	03424	48033
99279	27334	33804	77988	93592	90708	56780	70097	39907	51006
63224	05074	83941	25034	43516	22840	35230	66048	80754	46302
98361	97513	27529	66419	35328	19738	82366	38573	50967	72754

Table B2 The Arcsine transformation

X	0	1	2	3	4	5	6	7	8	9	X
0.000	0.00	0.57	0.81	0.99	1.15	1.28	1.40	1.52	1.62	1.72	0.000
0.001	1.81	1.90	1.99	2.07	2.14	2.22	2.29	2.36	2.43	2.50	0.001
0.002	2.56	2.63	2.69	2.75	2.81	2.87	2.92	2.98	3.03	3.09	0.002
0.003	3.14	3.19	3.24	3.29	3.34	3.39	3.44	3.49	3.53	3.58	0.003
0.004	3.63	3.67	3.72	3.76	3.80	3.85	3.89	3.93	3.97	4.01	0.004
0.005	4.05	4.10	4.14	4.17	4.21	4.25	4.29	4.33	4.37	4.41	0.005
0.006	4.44	4.48	4.52	4.55	4.59	4.62	4.66	4.70	4.73	4.76	0.006
0.007	4.80	4.83	4.87	4.90	4.93	4.97	5.00	5.03	5.07	5.10	0.007
0.008	5.13	5.16	5.20	5.23	5.26	5.29	5.32	5.35	5.38	5.41	0.008
0.009	5.44	5.47	5.50	5.53	5.56	5.59	5.62	5.65	5.68	5.71	0.009
0.01	5.74	6.02	6.29	6.55	6.80	7.03	7.27	7.49	7.71	7.92	0.01
0.02	8.13	8.33	8.53	8.72	8.91	9.10	9.28	9.46	9.63	9.80	0.02
0.03	9.97	10.14	10.30	10.47	10.63	10.78	10.94	11.09	11.24	11.39	0.03
0.04	11.54	11.68	11.83	11.97	12.11	12.25	12.38	12.52	12.66	12.79	0.04
0.05	12.92	13.05	13.18	13.31	13.44	13.56	13.69	13.81	13.94	14.06	0.05
0.06	14.18	14.30	14.42	14.54	14.65	14.77	14.89	15.00	15.12	15.23	0.06
0.07	15.34	15.45	15.56	15.68	15.79	15.89	16.00	16.11	16.22	16.32	0.07
0.08	16.43	16.54	16.64	16.74	16.85	16.95	17.05	17.15	17.26	17.36	0.08
0.09	17.46	17.56	17.66	17.76	17.85	17.95	18.05	18.15	18.24	18.34	0.09
0.10	18.43	18.53	18.63	18 72	18.81	18.91	19.00	19.09	19.19	19.28	0.10
0.11	19.37	19.46	19.55	19.64	19.73	19.82	19.91	20.00	20.09	20.18	0.11
0.12	20.27	20.36	20.44	20.53	20.62	20.70	20.79	20.88	20.96	21.05	0.12
0.13	21.13	21.22	21.30	21.39	21.47	21.56	21.64	21.72	21.81	21.89	0.13
0.14	21.97	22.06	22.14	22.22	22.30	22.38	22.46	22.54	22.63	22.71	0.14
0.15	22.79	22.87	22.95	23.03	23.11	23.18	23.26	23.34	23.42	23.50	0.15
0.16	23.58	23.66	23.73	23.81	23.89	23.97	24.04	24.12	24.20	24.27	0.16
0.17	24.35	24.43	24.50	24.58	24.65	24.73	24.80	24.88	24.95	25.03	0.17
0.18	25.10	25.18	25.25	25.33	25.40	25.47	25.55	25.62	25.70	25.77	0.18
0.19	25.84	25.91	25.99	26.06	26.13	26.21	26.28	26.35	26.42	26.49	0.19
0.20	26.57	26.64	26.71	26.78	26.85	26.92	26.99	27.06	27.13	27.20	0.20
0.21	27.27	27.35	27.42	27.49	27.56	27.62	27.69	27.76	27.83	27.90	0.21
0.22	27.97	28.04	28.11	28.18	28.25	28.32	28.39	28.45	28.52	28.59	0.22
0.23	28.66	28.73	28.79	28.86	28.93	29.00	29.06	29.13	29.20	29.27	0.23
0.24	29.33	29.40	29.47	29.53	29.60	29.67	29.73	29.80	29.87	29.93	0.24
0.25	30.00	30.07	30.13	30.20	30.26	30.33	30.40	30.46	30.53	30.59	0.25
0.26	30.66	30.72	30.79	30.85	30.92	30.98	31.05	31.11	31.18	31.24	0.26
0.27	31.31	31.37	31.44	31.50	31.56	31.63	31.69	31.76	31.82	31.88	0.27
0.28	31.95	32.01	32.08	32.14	32.20	32.27	32.33	32.39	32.46	32.52	0.28
0.29	32.58	32.65	32.71	32.77	32.83	32.90	32.96	33.02	33.09	33.15	0.29
0.30	33.21	33.27	33.34	33.40	33.46	33.52	33.58	33.65	33.71	33.77	0.30

Table B2 continued

X	0	1	2	3	4	5	6	7	8	9	X
0.31	33.83	33.90	33.96	34.02	34.08	34.14	34.20	34.27	34.33	34.39	0.31
0.32	34.45	34.51	34.57	34.63	34.70	34.76	34.82	34.88	34.94	35.00	0.32
0.33	35.06	35.12	35.18	35.24	35.30	35.37	35.43	35.49	35.55	35.61	0.33
0.34	35.67	35.73	35.79	35.85	35.91	35.97	36.03	36.09	36.15	36.21	0.34
0.35	36.27	36.33	36.39	36.45	36.51	36.57	36.63	36.69	36.75	36.81	0.35
0.36	36.87	36.93	36.99	37.05	37.11	37.17	37.23	37.29	37.35	37.41	0.36
0.37	37.46	37.52	37.58	37.64	37.70	37.76	37.82	37.88	37.94	38.00	0.37
0.38	38.06	38.12	38.17	38.23	38.29	38.35	38.41	38.47	38.53	38.59	0.38
0.39	38.65	38.70	38.76	38.82	38.88	38.94	39.00	39.06	39.11	39.17	0.39
0.40	39.23	39.29	39.35	39.41	39.47	39.52	39.58	39.64	39.70	39.76	0.40
0.41	39.82	39.87	39.93	39.99	40.05	40.11	40.16	40.22	40.28	40.34	0.41
0.42	40.40	40.45	40.51	40.57	40.63	40.69	40.74	40.80	40.86	40.92	0.42
0.43	40.98	41.03	41.09	41.15	41.21	41.27	41.32	41.38	41.44	41.50	0.43
0.44	41.55	41.61	41.67	41.73	41.78	41.84	41.90	41.96	42.02	42.07	0.44
0.45	42.13	42.19	42.25	42.30	42.36	42.42	42.48	42.53	42.59	42.65	0.45
0.46	42.71	42.76	42.82	42.88	42.94	42.99	43.05	43.11	43.17	43.22	0.46
0.47	43.28	43.34	43.39	43.45	43.51	43.57	43.62	43.68	43.74	43.80	0.47
0.48	43.85	43.91	43.97	44.03	44.08	44.14	44.20	44.26	44.31	44.37	0.48
0.49	44.43	44.48	44.54	44.60	44.66	44.71	44.77	44.83	44.89	44.94	0.49
0.50	45.00	45.06	45.11	45.17	45.23	45.29	45.34	45.40	45.46	45.52	0.50
0.51	45.57	45.63	45.69	45.74	45.80	45.86	45.92	45.97	46.03	46.09	0.51
0.52	46.15	46.20	46.26	46.32	46.38	46.43	46.49	46.55	46.61	46.66	0.52
0.53	46.72	46.78	46.83	46.89	46.95	47.01	47.06	47.12	47.18	47.24	0.53
0.54	47.29	47.35	47.41	47.47	47.52	47.58	47.64	47.70	47.75	47.81	0.54
0.55	47.87	47.93	47.98	48.04	48.10	48.16	48.22	48.27	48.33	48.39	0.55
0.56	48.45	48.50	48.56	48.62	48.68	48.73	48.79	48.85	48.91	48.97	0.56
0.57	49.02	49.08	49.14	49.20	49.26	49.31	49.37	49.43	49.49	49.55	0.57
0.58	49.60	49.66	49.72	49.78	49.84	49.89	49.95	50.01	50.07	50.13	0.58
0.59	50.18	50.24	50.30	50.36	50.42	50.48	50.53	50.59	50.65	50.71	0.59
0.60	50.77	50.83	50.89	50.94	51.00	51.06	51.12	51.18	51.24	51.30	0.60
0.61	51.35	51.41	51.47	51.53	51.59	51.65	51.71	51.77	51.83	51.88	0.61
0.62	51.94	52.00	52.06	52.12	52.18	52.24	52.30	52.36	52.42	52.48	0.62
0.63	52.54	52.59	52.65	52.71	52.77	52.83	52.89	52.95	53.01	53.07	0.63
0.64	53.13	53.19	53.25	53.31	53.37	53.43	53.49	53.55	53.61	53.67	0.64
0.65	53.73	53.79	53.85	53.91	53.97	54.03	54.09	54.15	54.21	54.27	0.65
0.66	54.33	54.39	54.45	54.51	54.57	54.63	54.70	54.76	54.82	54.88	0.66
0.67	54.94	55.00	55.06	55.12	55.18	55.24	55.30	55.37	55.43	55.49	0.67
0.68	55.55	55.61	55.67	55.73	55.80	55.86	55.92	55.98	56.04	56.10	0.68
0.69	56.17	56.23	56.29	56.35	56.42	56.48	56.54	56.60	56.66	56.73	0.69
0.70	56.79	56.85	56.91	56.98	57.04	57.10	57.17	57.23	57.29	57.35	0.70

Table B2 continued

X	0	1	2	3	4	5	6	7	8	9	X
0.71	57.42	57.48	57.54	57.61	57.67	57.73	57.80	57.86	57.92	57.99	0.71
0.72	58.05	58.12	58.18	58.24	58.31	58.37	58.44	58.50	58.56	58.63	0.72
0.73	58.69	58.76	58.82	58.89	58.95	59.02	59.08	59.15	59.21	59.28	0.73
0.74	59.34	59.41	59.47	59.54	59.60	59.67	59.74	59.80	59.87	59.93	0.74
0.75	60.00	60.07	60.13	60.20	60.27	60.33	60.40	60.47	60.53	60.60	0.75
0.76	60.67	60.73	60.80	60.87	60.94	61.00	61.07	61.14	61.21	61.27	0.76
0.77	61.34	61.41	61.48	61.55	61.61	61.68	61.75	61.82	61.89	61.96	0.77
0.78	62.03	62.10	62.17	62.24	62.31	62.38	62.44	62.51	62.58	62.65	0.78
0.79	62.73	62.80	62.87	62.94	63.01	63.08	63.15	63.22	63.29	63.36	0.79
0.80	63.43	63.51	63.58	63.65	63.72	63.79	63.87	63.94	64.01	64.09	0.80
0.81	64.16	64.23	64.30	64.38	64.45	64.53	64.60	64.67	64.75	64.82	0.81
0.82	64.90	64.97	65.05	65.12	65.20	65.27	65.35	65.42	65.50	65.57	0.82
0.83	65.65	65.73	65.80	65.88	65.96	66.03	66.11	66.19	66.27	66.34	0.83
0.84	66.42	66.50	66.58	66.66	66.74	66.82	66.89	66.97	67.05	67.13	0.84
0.85	67.21	67.29	67.37	67.46	67.54	67.62	67.70	67.78	67.86	67.94	0.85
0.86	68.03	68.11	68.19	68.28	68.36	68.44	68.53	68.61	68.70	68.78	0.86
0.87	68.87	68.95	69.04	69.12	69.21	69.30	69.38	69.47	69.56	69.64	0.87
0.88	69.73	69.82	69.91	70.00	70.09	70.18	70.27	70.36	70.45	70.54	0.88
0.89	70.63	70.72	70.81	70.91	71.00	71.09	71.19	71.28	71.37	71.47	0.89
0.90	71.57	71.66	71.76	71.85	71.95	72.05	72.15	72.24	72.34	72.44	0.90
0.91	72.54	72.64	72.74	72.85	72.95	73.05	73.15	73.26	73.36	73.46	0.91
0.92	73.57	73.68	73.78	73.89	74.00	74.11	74.21	74.32	74.44	74.55	0.92
0.93	74.66	74.77	74.88	75.00	75.11	75.23	75.35	75.46	75.58	75.70	0.93
0.94	75.82	75.94	76.06	76.19	76.31	76.44	76.56	76.69	76.82	76.95	0.94
0.95	77.08	77.21	77.34	77.48	77.62	77.75	77.89	78.03	78.17	78.32	0.95
0.96	78.46	78.61	78.76	78.91	79.06	79.22	79.37	79.53	79.70	79.86	0.96
0.97	80.03	80.20	80.37	80.54	80.72	80.90	81.09	81.28	81.47	81.67	0.97
0.98	81.87	82.08	82.29	82.51	82.73	82.97	83.20	83.45	83.71	83.98	0.98
0.990	84.26	84.29	84.32	84.35	84.38	84.41	84.44	84.47	84.50	84.53	0.990
0.991	84.56	84.59	84.62	84.65	84.68	84.71	84.74	84.77	84.80	84.84	0.991
0.992	84.87	84.90	84.93	84.97	85.00	85.03	85.07	85.10	85.13	85.17	0.992
0.993	85.20	85.24	85.27	85.30	85.34	85.38	85.41	85.45	85.48	85.52	0.993
0.994	85.56	85.59	85.63	85.67	85.71	85.75	85.79	85.83	85.86	85.90	0.994
0.995	85.95	85.99	86.03	86.07	86.11	86.15	86.20	86.24	86.28	86.33	0.995
0.996	86.37	86.42	86.47	86.51	86.56	86.61	86.66	86.71	86.76	86.81	0.996
0.997	86.86	86.91	86.97	87.02	87.08	87.13	87.19	87.25	87.31	87.37	0.997
0.998	87.44	87.50	87.57	87.64	87.71	87.78	87.86	87.93	88.01	88.10	0.998
0.999	88.19	88.28	88.38	88.48	88.60	88.72	88.85	89.01	89.19	89.43	0.999
1.000	90.00										

APPENDIX C QUALITY CONTROL CRITERIA

C1 INTRODUCTION

It is important to both regulators and dischargers that any data used to characterise discharges or generated when monitoring against toxicity-based limits is produced under a system which incorporates quality control. A quality control element is needed to ensure the tests meet acceptable performance criteria in terms of test precision and accuracy. Therefore, toxicity testing laboratories must conform with QC procedures which are to be trialed in the Register of Approved Laboratories (RAL) scheme as part of the Demonstration Programme. This section of the guidelines describes the quality control procedures which are to be used in the Demonstration Programme to provide data to assess test method precision and bias. Further details of the RAL scheme are given in 'A Register of Approved Laboratories Undertaking Toxicity Testing' (July 1997) which has been produced by the Environment Agency.

C2 ASSESSING THE PRECISION OF TEST METHODS

C2.1 Introduction

It is important to understand the level and source of variability in toxicity tests and bioassays if equitable regulatory decisions are to be made on the basis of the results obtained from these tests (Warren Hicks and Parkhurst 1992, Whitehouse *et al.* 1996a). Precision is a general term for the variability between individual measurements (including toxicity tests) and comprises *repeatability* (intra-laboratory variability) and *reproducibility* (inter-laboratory variability) (ISO 1986). A test is repeatable if similar results can be obtained from a number of tests performed by the same operator in the same laboratory. A test is reproducible if similar results can be obtained by different test operators in different laboratories. Accuracy may also be promoted by checking for evidence of bias from the expected toxicity value. The following sections describe the ways in which the repeatability and reproducibility of each test method will be assessed during the generation of data for discharge characterisation and monitoring against toxicity limits.

The acceptability of a laboratory's performance with respect to both repeatability and reproducibility will be assessed on the basis of regular toxicity tests with an approved reference toxicant. Reference toxicant tests shall be performed alongside every effluent test which is carried out for discharge characterisation or monitoring against a toxicity limit.

C2.2 Reference toxicants

It is important to select a reference toxicant for which there are analytical methods with a limit of detection significantly below the toxic concentrations and below the lowest test concentration which is likely to be used. If this can be achieved, confirmation of test concentrations by analysis is less likely to introduce uncertainties due to analytical imprecision.

To permit a valid assessment of test repeatability and reproducibility, all reference toxicant tests for a method should be performed with the same toxicant. In the ring tests of the *Daphnia magna* immobilisation and oyster embryo-larval development tests carried out as part of the SNIFFER/Environment Agency Performance Standards Project zinc (as zinc sulphate) and 3,4-dichloroaniline were used (Whitehouse *et al.* 1996b). Acceptable limits for test repeatability and reproducibility were then derived for these tests using the data from that programme of performance testing. Further ring tests are being carried out to establish acceptability criteria for other test methods.

After reviewing the data the Environment Agency has decided to carry out reference toxicant tests with zinc because it is considerably easier and cheaper to analyse than 3,4-DCA and because the measured concentrations in the ring tests were usually close to the nominal values with small levels of analytical error. Furthermore, zinc is sensitive to variations in environmental parameters which Forbes and Forbes (1994) consider to be a key criteria in selecting reference toxicants.

C2.3 Conducting reference toxicant tests

The reference toxicant tests shall be carried out using the test designs summarised in Table C1 which have been derived based on the data from the Performance Standards project. The specific procedures for conducting the reference toxicant tests are given in the relevant sections for each test method.

Table C1 Concentrations and number of replicates to be used in reference toxicant tests using zinc

Test method	Test concentrations (mg l^{-1})	No. of vessels per concentration
Daphnia magna immobilisation test	0, 0.1, 0.32, 1.0, 3.2, 10	4
Oyster embryo-larval development test	0, 0.032, 0.1, 0.32, 1.0, 3.2	3

C3 ASSESSING TEST METHOD REPEATABILITY (INTRA-LABORATORY VARIABILITY)

Estimates of repeatability for given methods are required for full concentration-response tests used to provide data to characterise discharges.

C3.1 Introduction

A two part approach to demonstrating control over the repeatability of concentration-response tests is used. This entails the construction of Shewart control charts to monitor test results against previous tests (internal quality control) and also to compare performance against externally imposed control limits (external quality control).

C3.2 Internal Quality Control

The repeatability of a method for concentration-response tests is traditionally judged by conducting a series of toxicity tests with appropriate inorganic and/or organic reference toxicants. After an acceptable number of tests have been conducted the mean and standard deviation of the test toxicity (EC_{50} or LC_{50}) values are calculated and used to derive a control chart on which the range of `normal' or `acceptable' variation is defined. On the control chart the y axis indicates the endpoint of the toxicity test (Environment Canada 1990). The US EPA requires that a minimum of five tests are conducted for each method before any control limits are established (US EPA 1994). Tests should be carried out until the limits do not change markedly with the addition of each new data point, thereby reflecting the minimum variability for that test method. A total of 15-20 tests may be necessary to obtain a representative range (Dux 1986).

The mean is calculated as the sum of the individual values (X_i) from toxicity tests divided by the number of tests (n) that is:

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

The standard deviation (S) is calculated from the equation:

$$S = \sqrt{\frac{\sum_{i=1}^{n} X^2 - \sum_{i=1}^{n} (X_i)^2 / n}{n-1}}$$

Warning limits on the control chart are defined as the values two times the standard deviation above and below the mean. For a large data set, these values represent the upper 95% confidence limits. Action limits are derived as values three times the standard deviation above and below the mean which represent the 99% confidence limits. Conventionally, if a result is outside the action limits, or two consecutive values lie outside the warning limits, the measurement process is judged to be `out of control'. A consistent trend might also suggest

that control of the measurement methods is deteriorating and that the causes of that deviation should be investigated.

The mean standard deviation data used for the control chart are also used to calculate the coefficient of variation (CV) for a test method/reference toxicant combination using the equation:

CV (%) = (Standard deviation/Mean) x 100

As part of an internal quality control process samples of reference freshwaters and seawaters should be taken and analysed quarterly to ensure no extraneous substances are present.

C3.3 External Quality Control

Whilst internal control charts are effective at stabilising the status quo, externally imposed criteria are required to control the level of variability with repeat testing. Reliance on internal control charts alone will result in different laboratories working to different acceptability thresholds and could simply reinforce bias. Therefore, the Environment Agency has defined limits for the repeatability of test methods to be used for discharge characterisation. Laboratories are encouraged to construct control charts as described for internal quality control but, in addition, external criteria for repeatability must be met.

The limits for the underlying repeatability of a test method ($\sigma^2_{repeatability}$) have been generated from the results of ring tests with the reference toxicant zinc (as zinc sulphate heptahydrate) carried out under the SNIFFER/Environment Agency Performance Standards Project (Whitehouse *et al.* 1996b). The repeat tests of a method carried out at different laboratories were analysed using Residual Maximum Likelihood (REML) analysis to estimate $\sigma^2_{repeatability}$ values for given test methods (see Table C2).

Table C2 $\sigma^2_{repeatability}$ values for a given test method using the reference toxicant zinc

Test method	$\sigma^2_{repeatability}$
Daphnia magna immobilisation test	0.0601
Oyster embryo-larval development test	0.0333

The $\sigma^2_{repeatability}$ values given in Table C2 were derived after log transforming data to obtain a normal distribution. As more reference toxicant data is gathered, the quality control criteria will be refined. These updated criteria will be made available to laboratories joining the RAL scheme. In addition, they will be accompanied by quality control criteria representing `best practice' providing a target for improvement. In the longer term, it is expected that the external quality control criteria will narrow, and, at some point, the limits representing `best practice' would be the basis on which repeatability is judged.

In the quality control assessment element of the RAL scheme, the variance estimated from the toxicity (EC_{50} and LC_{50}) values derived from repeat tests carried out within a laboratory (S^2) are compared with the expected variance ($\sigma^2_{repeatability}$) value for that method using the equation:

$$\frac{S^2}{\sigma^2_{repeatability}}(n-1) < \chi^2(n-1, \alpha)$$

If S^2 is significantly greater than $\sigma^2_{repeatability}$ for a test method (that is at the defined level of probability) then the repeatability of the test method is considered to be acceptable. If S^2 is less than $\sigma^2_{repeatability}$ then repeatability is considered not to be acceptable. Table C3 provides example zinc reference toxicant data for the OEL test and a comparison of the derived S^2 value with $\sigma^2_{repeatability}$.

Table C3 Testing the acceptability of repeatability of the OEL method (full concentration-response test)

Log transformed EC_{50} values	S^2	$\sigma^2_{repeatability}$	Test statistic χ^2	Critical value	P value
-0.509,-0.824,-0.678, -0.886,-0.420,-0.509, -0.678,-0.420,-0.292, -0.824	0.0411	0.0333	11.11	16.919	0.268

In the example S^2 is not significantly greater than $\sigma^2_{repeatability}$ and the repeatability of the test is acceptable.

C4 TEST METHOD REPRODUCIBILITY (INTER-LABORATORY VARIABILITY)

In a framework to characterise discharges, generate toxicity-based limits and monitor against these limits it is important to ensure that different laboratories deriving toxicity data show comparable results for reference toxicant tests with a given method. The accuracy of a toxicity test method is conventionally defined in terms of a `consensus mean' toxicity. This aspect of the quality control scheme compares measured toxicity values with the consensus mean. Large and especially consistent deviations outside predefined limits are taken to signify bias.

The ring tests conducted as part of the SNIFFER/Environment Agency Performance Standards Project have been used to derive acceptability criteria for test method reproducibility (Whitehouse *et al.* 1996b). This has involved applying the calculated variance, $\sigma^2_{repeatability}$, for a test method to the consensus mean incorporating a factor (1.96) to account for a 95% confidence level within which a measured toxicity (IC_{50}, EC_{50} or LC_{50}) value from a concentration-response test is expected to fall.

Table C4 shows the consensus mean and upper and lower 95% control limits for the *Daphnia magna* immobilisation and oyster embryo-larval development tests derived for the reference toxicant zinc.

Table C4 Limits of reproducibility for test methods

Test method	Consensus mean (mg Zn l^{-1})	95% control limits (mg Zn l^{-1})	
		Upper	Lower
Daphnia magna immobilisation test	1.03	3.51	0.20
Oyster embryo-larval development test	0.12	0.55	0.01

A laboratory compares the mean toxicity (EC_{50} or LC_{50}) values generated for a test with the control limits to ascertain whether it is meeting reproducibility criteria.

C5 REPORT OF QUALITY CONTROL DATA

Quality control data will be reported on the proforma datasheets examples of which are included in the relevant sections of the guidelines for a particular method.

C6 ACTIONS IN THE EVENT OF UNACCEPTABLE PERFORMANCE

The actions to be taken as a result of unacceptable performance will be described in a separate document supplied to laboratories on joining the RAL scheme.

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APPENDIX D GUIDANCE ON THE CLEANING AND STORAGE OF APPARATUS

D1 INTRODUCTION

Contaminated apparatus (whether made of glass or other inert materials) can cause serious problems for the conduct of toxicity tests and bioassays. Contamination can result from inadequate removal of contaminants during cleaning, from the use of inappropriate cleaning solutions, and from exposure of the apparatus to contaminants during storage and handling. It can lead to erroneous toxicity data being derived, which could have major implications for regulatory decisions.

This section provides guidance on the procedures which should be followed when cleaning (hand and machine washing) and storing apparatus used in toxicity tests. However, it is recognised that most ecotoxicological laboratories will have their own 'tested' glassware cleaning procedures. The types of apparatus used in these procedures includes general apparatus (such as volumetric flasks, measuring cylinders, pipettes, glass rods, conical flasks and beakers) and specific apparatus (such as test aquaria, crystallising dishes, watch glasses and sample bottles).

All apparatus previously exposed to harmful or toxic substances shall be rinsed out thoroughly before being submitted for washing up. For very lipophilic substances, such as most pesticides, oils and oil products, the apparatus should be rinsed with acetone prior to washing. Acetone may also be used to remove marker pen and residual glue from tape or labels. Excess acetone should be stored in a waste solvent bin until it can be disposed of safely. Any acetone remaining on the glassware must be allowed to evaporate before washing.

D2 SAFETY

D2.1 Acetone

Contact between acetone and the skin and eyes should be avoided by wearing a laboratory coat, gloves and safety glasses. Rinsing of glassware should be carried out in a fume cupboard since acetone fumes can be narcotic in high concentrations. Test operators should be aware of the flammable nature of acetone.

D2.2 Detergents

Commercially available detergents used to clean apparatus, such as Decon 90 or Pyroneg, should be phosphate free and rinsable. Concentrated solutions supplied by manufacturers are alkaline and skin contact should be avoided. Eye protection designated for splash protection (BS 2092) and rubber gloves should be worn at all times when handling concentrated and dilute solutions. A solution containing 2% v/v commercial detergent in tap water is recommended for hand washing apparatus.

D2.3 Acids used in hand and machine washing apparatus

Handle concentrated acids in a fume cupboard and wear rubber gloves and eye protection when handling both concentrated and dilute acids, as they attack organic material and are generally very corrosive. Limescale deposited on the inside of glassware should be removed using a dilute solution of hydrochloric acid (10% v/v acid in tap water). Nitric and sulphuric acids can be used, but these are more corrosive and more hazardous to use than hydrochloric acid.

NOTE: If any of the above solutions of dilute or concentrated acetone, detergent or acid comes in contact with skin, wash the affected area under running water immediately. Contact with eyes should be treated using either an eye wash bottle or running water. Medical attention should then be sought.

D3 BROKEN APPARATUS

Chipped or broken apparatus shall be disposed of immediately and shall not be hand washed or placed in the washing up system. However, large or specialised items of chipped or broken apparatus should be assessed for damage before they are thrown away. If the item(s) of apparatus in question is repairable it should be wrapped up in tissue and sent off for repair. Care must be exercised and thick leather gloves should be worn when handling glass during this process.

Decontamination of broken apparatus should only be carried out if the substance(s) involved is highly toxic and present in high concentrations. Decontamination should be carried out using a recognized disposal company.

D4 PROCEDURE FOR CLEANING APPARATUS IN A WASHING MACHINE

Initially, remove any labels from the apparatus by soaking in hot water or, if necessary, a solution of 2% v/v commercial detergent in tap water. After draining the items, load up the washing machine with dirty apparatus, and ensure there is cleaning material (powder or fluid) in the machine and the water supply is connected and turned on.

The cleaning action of a commercial washing machine should, ideally, comprise three cycles:

a detergent wash;

an acid wash which removes any detergent residues;

rinsing with deionised, distilled or reverse osmosis water to remove acid residues.

In hard water areas, it may be necessary to soften the water used in the washing machines. The operating instructions for the washing machine should be followed at all times.

D5 PROCEDURE FOR CLEANING APPARATUS BY HANDWASHING

D5.1 Handwashing pipettes, glass rods and tubing

These items of glassware should, ideally, be washed using a set of pipette washing tubes, but other appropriate containers can be used and the same procedure followed.

If pipette washing tubes are used, place the glassware uppermost into a basket inside the tube labelled 'detergent' and soak for at least 24 h. Transfer this basket into a second tube labelled 'tap-water' and run cold mains tap water through the tube for at least 30 minutes. A siphon action operates in this tube, whereby the tube fills with water and then empties as it refills. The pipettes in the basket are then transferred to a third tube for acid washing with a 10% v/v solution of hydrochloric acid. Nitric or sulphuric acid can be used but these are more corrosive and hazardous to use. Leave the pipettes to soak for two hours. Finally, transfer the basket to a tube containing deionised, distilled or reverse osmosis water and allow to soak for at least 30 minutes. Allow the pipettes to drain before drying.

D5.2 Handwashing all other types of apparatus

This procedure should be used for all apparatus other than pipettes, tubes and rods if a washing machine is not available and for items not suitable for machine washing (for example siphon dosers and large glass aquaria).

Initially, remove any labels by soaking the apparatus in hot water or, if necessary, a 2% v/v commercial detergent solution for at least 60 minutes. If the apparatus is particularly dirty it may need to be scrubbed. After washing the apparatus in the 2% commercial detergent solution remove it and rinse several times with tap water. Follow this by washing the apparatus with a 10% v/v solution of hydrochloric acid. Nitric or sulphuric acid can be used but these are more corrosive and more hazardous to use. Leave to soak for two hours and then thoroughly rinse the apparatus three times with deionised, distilled or reverse osmosis water. Allow the apparatus to drain before drying.

D6 PROCEDURE FOR DRYING APPARATUS

Apparatus can be dried upside down on a bench covered with tissue paper, but should, preferably, be dried in a drying cupboard. The operating instructions for the drying cupboard should be followed at all times. Ensure that the temperature of the drying cupboard does not exceed the temperature tolerance of any apparatus to be dried.

D7 STORAGE OF APPARATUS

Apparatus should be stored in clean, dry cupboards or racks in the following way:

beakers, glass aquaria, crystallising dishes etc. should be stored upside down;

fixed tanks should be covered;

volumetric flasks, bottles etc. should be stored with their tops on.