



Environment Agency

The direct toxicity assessment of aqueous environmental samples using the juvenile *Daphnia magna* immobilisation test (2007)

Methods for the Examination of Waters and Associated Materials

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This booklet contains guidance on the direct toxicity assessment of aqueous environmental samples using the juvenile *Daphnia magna* immobilisation test. Using the procedures described in this booklet should enable laboratories to satisfy the requirements of the Environment Agency's Monitoring Certification Scheme (MCERTS) for laboratories undertaking direct toxicity assessment of effluents⁽¹⁾. However, if appropriate, laboratories should clearly demonstrate they are able to meet the MCERTS requirements. Two documents have already been published in this series^(2, 3) and further documents are being produced and include:

The direct toxicity assessment of aqueous environmental samples using the freshwater algal growth inhibition test with *Pseudokirchneriella subcapitata*

The direct toxicity assessment of aqueous environmental samples using the marine algal growth inhibition test with *Skeletonema costatum*

No performance data are included with this method which has been rigorously tested under Agency funded development work^(4, 5). However, inter- and intra-laboratory data are being collected under the MCERTS scheme. Information on the routine use of this method is welcomed to assess its full capability.

Whilst this booklet may report details of the materials actually used, this does not constitute an endorsement of these products but serves only as an illustrative example. Equivalent products are available and it should be understood that the performance characteristics of the method might differ when other materials are used. It is left to users to evaluate methods in their own laboratories.

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soil (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials"

and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

- 1 General principles of sampling and accuracy of results
- 2 Microbiological methods
- 3 Empirical and physical methods
- 4 Metals and metalloids
- 5 General non-metallic substances
- 6 Organic impurities
- 7 Biological methods
- 8 Biodegradability and inhibition methods
- 9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with this booklet are listed at the back of the booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advance notice of forthcoming publications, or obtain details of the index of methods then contact the Secretary on the Agency's internet web-page (<http://www.environment-agency.gov.uk/nls>) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
Secretary
December 2004

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and regulations made under this Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is

noted. Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

Glossary

Aqueous environmental samples	these include effluents, leachates, receiving waters and discharges.
ASTM	American Society for Testing and Materials.
ASV	air saturation value.
daphnid	Individual <i>Daphnia</i> .
DTA	direct toxicity assessment.
EC ₁₀	the concentration that results in an effect on 10 % of the exposed organisms.
EC ₂₀	the concentration that results in an effect on 20 % of the exposed organisms.
EC ₅₀	the concentration that results in an effect on 50 % of the exposed organisms.
EDTA	ethylenediamine tetraacetic acid.
gravid <i>Daphnia magna</i>	female <i>Daphnia</i> bearing developing embryos.
ISO	International Standards Organisation.
LOEC	lowest concentration where there is an observed effect compared to control dilutions.
neonate	juvenile <i>Daphnia</i> , less than 24 hours old.
NOEC	highest concentration where there is no-observed effect compared to control dilutions.
OECD	Organisation for Economic Co-operation and Development.
Static test	a test procedure where no further replacement or replenishment of the test solutions is carried out after starting the test.
TIE	toxicity identification evaluation – a procedure for identifying the toxicants responsible for the ecotoxicity of samples.
TSE	toxicity source evaluation – a procedure for identifying the origins of toxicants present in samples that comprise fractions derived from unrelated and often geographically separated processes.

The direct toxicity assessment of aqueous environmental samples using the juvenile *Daphnia magna* immobilisation test

1 Introduction

The procedures described in this document enable direct toxicity assessments to be carried out on aqueous environmental samples using the freshwater cladoceran *Daphnia magna*. The procedures described are based on an Environment Agency project^(4, 5) but also take into account existing guidelines^(6, 7) and more recent method developments.

The juvenile *Daphnia magna* immobilisation test can be used in the following roles:

- (i) effluent screening and characterisation;
- (ii) monitoring effluent toxicity against a toxicity limit;
- (iii) assessing the impact of point source discharges on receiving waters;
- (iv) providing a general quality assessment of receiving waters (for example within monitoring programmes).

2 Collection, transport, storage and treatment of aqueous environmental samples

Aqueous environmental samples submitted for toxicity testing should be representative of the material being sampled. Depending upon the design of the sampling programme, different approaches may need to be adopted⁽⁸⁾. The procedures used for the collection, storage and preparation of samples should ensure that the toxicity of the sample does not change significantly before the test is conducted. All reports should contain details of the collection, storage and preparation of samples used in the toxicity assessment.

2.1 Collection of environmental samples

Environmental samples should be collected in accordance with existing guidance given elsewhere^(9, 10, 11).

Environmental samples should be collected in containers, typically screw top glass bottles, that are inert and do not adversely affect the sample or sample toxicity. The container should be new (or thoroughly cleaned) and rinsed at least three times with the sample to be collected. If a series of bottles is used for the collection of one sample, the portions should be combined and mixed before testing begins in order to ensure the pooled sample is homogeneous. The minimum sample volume collected should be 1 litre. Containers should be filled completely to minimise any air space into which volatile components of the sample might diffuse.

2.2 Monitoring of water quality parameters in test samples

The determination of selected parameters (see Table 1) should be carried out at the location where the sample is taken (i.e. on-site determination) and on receipt of the sample at the laboratory. This enables changes (which may occur during transportation) in the water quality parameters to be assessed, and if necessary, appropriate measures taken if these changes are considered to impact on the toxicity test. The on-site determinations should be accompanied with details of a description of the sample and whether the sample contains or comprises an emulsion. Details of appropriate methodology can be found elsewhere^(12 - 15). Samples should be labelled appropriately with such details as the name

and location of the site where each of the samples were taken and the date and time when each sample was taken. Any other relevant information, such as the name of the sampling officer and chain of custody details should also be recorded.

Table 1 Water quality parameters to be determined on-site and in the laboratory

pH
 Temperature
 Dissolved oxygen
 Total hardness

2.3 Transport and storage

Samples should be transported to the laboratory within 24 hours of being taken. In addition, testing should commence within 48 hours of sampling. In situations where testing is not started within 48 hours of sampling appropriate details should be recorded in the test report. During transportation, samples should be stored in the dark at temperatures between 2 - 8 °C.

Samples requiring immediate testing on receipt at the laboratory should be allowed to equilibrate to 20 ± 2 °C. If the sample is not to be tested immediately, it should be stored in the dark at temperatures between 2 - 8 °C.

2.4 Preparation of samples

The extent to which environmental samples are treated prior to testing depends on the objectives of the study.

Samples may be tested unadjusted to gain information on the total biological effects including the influence of water quality parameters such as pH, dissolved oxygen and total hardness, however, this is not recommended for regulatory effluent assessments.

Table 2 Threshold criteria for selected water quality parameters for the *Daphnia magna* immobilisation test

Parameter	Threshold criteria
pH	7.4 - 8.5
Dissolved oxygen (as a percent of the ASV) at 20 ± 2 °C	≥ 60
Total hardness (expressed as mg l ⁻¹ CaCO ₃)	140 - 320

Whilst no values have been specified for colour and suspended solids content, these parameters can have an effect on the physical observation of the organisms.

For regulatory DTA testing (i.e. tests conducted on effluents), modification or adjustment of the sample, or its dilutions, should be made so that all criteria for all the parameters listed in Table 2 are met and the influence of these parameters is removed. Test results will therefore reflect the residual chemical toxicity of the discharge at the water quality ranges specified in Table 2. These ranges are generally representative of the conditions found in the receiving environments to which effluents are likely to be discharged. If these ranges are not representative of the known water quality in the area of discharge of a particular effluent, the actual measured water quality values should be substituted for those given in Table 2.

Sample modification is not generally recommended for tests conducted on receiving waters.

The influence of water quality parameters on the toxicity of the sample will typically be more pronounced for effluents than receiving waters, and direct modification (as outlined below) will generally only be necessary if toxicity occurs at higher effluent concentrations. For samples where toxicity is evident at lower sample concentrations, dilution will often mean that the water quality parameters in the test dilutions meet the limits specified in Table 2.

Where adjustment is required, this should, wherever possible, be restricted to the specific test dilutions rather than to the whole sample and, if possible, both adjusted and unadjusted dilutions should be tested concurrently. For any adjustment, a record of adjustment should be made which includes the extent of any resultant further dilution of samples or changes in other water quality parameters arising from the adjustment procedure.

The measurement of the toxicity of an effluent under environmentally unrealistic water quality conditions and the effect on toxicity caused by the modification of water quality parameters are not relevant to the regulatory DTA process. This process is concerned primarily with assessing the dilution at which an effluent ceases being acutely toxic under conditions likely to be encountered in the receiving environment. The results of toxicity tests undertaken with effluents at extreme water quality values require additional interpretation and should not be used in environmental hazard and risk assessments.

2.4.1 pH

The pH of test dilutions may potentially affect the speciation of substances (for example ammonia and certain heavy metals) contained in the sample and result in the observation of different toxic effects. For example, the toxicity of ammonia increases with increasing pH values, principally in the range 6.0 to 9.5. This is due to an increasing proportion of the ammonia being present in the test dilutions in the unionised (toxic) form.

The pH of acidic test dilutions, or samples, should be adjusted with 1M sodium hydroxide solution, whilst the pH of alkaline test dilutions, or samples, should be adjusted with 1M hydrochloric acid solution. Certain test dilutions, or samples, for example effluent samples with highly buffered pH capacities, may require the use of stronger acid or alkaline solutions. Aliquots of test dilutions, or samples, that are pH-adjusted should be allowed to equilibrate after each incremental addition of acid or base⁽¹⁶⁾. Test dilutions that have been pH-adjusted should only be used when the pH has stabilised.

2.4.2 Dissolved oxygen

If the dissolved oxygen concentration in any of the test dilutions prior to testing is less than 60 % of the air saturation value (ASV) at 20 ± 2 °C the dilution should be aerated, even though this may result in the potential loss of volatile substances from the solution. To achieve this, oil-free compressed air should be dispensed through a clean silica-glass air diffuser or disposable glass pipette. Any aeration of test dilutions should be at a rate within the range $25 - 50 \text{ ml min}^{-1} \text{ l}^{-1}$ until a dissolved oxygen concentration greater than 60 % of the ASV is reached. The duration of aeration should not exceed 30 minutes. Any aeration of test dilutions should be discontinued following this period and the test initiated. Test dilutions with dissolved oxygen concentrations greater than 60 % of the ASV should not be aerated.

2.4.3 Hardness

Modifying the hardness of test solutions can modify the speciation and toxicity of certain heavy metals (for example cadmium, chromium, copper and zinc). The toxicity of these metals decreases with increasing water hardness due to the changes in the proportion of the metals present in the form of toxic species.

The hardness of the test dilutions should be adjusted to be within the limits specified in Table 2 and also within the limits specified in Table 3 (see section 3.3.3). Total hardness may be increased by the addition of appropriate concentrated stock solutions. Hardness may be reduced by addition of distilled or deionised water. In both cases, the additional dilution arising from the additions should then be calculated and used to determine the concentration after adjustment.

2.4.4 Suspended solids

Suspended solids may be removed in most cases by allowing the test dilutions to settle until there is a noticeable reduction in the suspended solids content. If no apparent clearing of the sample is noticeable after 2 - 4 hours, an alternative approach should be used. These may include:

- (i) Filtering the sample through a cellulose acetate or cellulose nitrate membrane filter (nominal size 0.45 µm) using a vacuum filtration unit.
- (ii) Centrifuging the sample at 5000 - 10000 g for 15 - 60 minutes using a suitable centrifuge. Centrifuging the sample at low speeds (3000 - 5000 g) for longer periods (60 minutes) may be used as an alternative approach to high speeds for short periods (10000 g for 15 minutes). Dilutions should, ideally, be centrifuged in a cooled state to avoid adverse effects occurring due to rising temperatures during centrifugation.

Filtration and centrifugation can exhibit different effects on the chemistry of test dilutions, or samples, and the same procedure should be used when testing a series of samples from the same location.

2.4.5 Colour

Highly coloured solutions may impair visual observation of *Daphnia magna* in the test vessels. The observation of the organisms may be facilitated by temporary illumination either from the side or below the vessel.

2.4.6 Oily substances

The presence of oily substances, or any substance present as a saturated salt, may cause *Daphnia magna* to float to the surface of the test dilution. These substances may adhere to the carapace and other body appendages, and effectively change the density of the organism in the test solution. In these circumstances, the interpretation of test data can be difficult, as immobility may be influenced by the flotation. Tests can be carried out in sealed vessels (for example, a glass vessel with a septum cap) where air bubbles have been excluded, which should inhibit flotation. Before sealed vessels are used, appropriate checks should be made to ensure the survival of *Daphnia magna* in the controls is not affected. The number of organisms in each vessel used in the test should not deplete the

available dissolved oxygen during the test period.

2.4.7 Other parameters

Further information on other parameters which may need consideration in specific circumstances can be obtained elsewhere^(17 - 19) including guidance on the testing of effluents containing sparingly soluble substances⁽²⁰⁾.

2.5 Disposal of samples

Test dilutions and samples should be disposed of according to documented procedures.

3 Juvenile *Daphnia magna* immobilisation test

3.1 Introduction

Based on previously published guidance^(4, 5) procedures are described for culturing the freshwater cladoceran *Daphnia magna*, and for conducting static toxicity tests to assess the effects of aqueous environmental samples on their mobility.

3.2 Test organism

The species generally used in the test is *Daphnia magna* (Straus) clone A which should have previously been identified by genotyping. The performance of clone A has been shown to meet, consistently, the test and culture criteria outlined in this booklet.

Other species (for example *Daphnia pulex*) may also be used provided they are shown to meet the quality and validity criteria for culturing and testing. It should, however, be recognised that different species may produce different results⁽²¹⁾.

At the start of the test, the test organisms must be less than 24 hours old.

Test organisms should be derived from healthy stock cultures that should be maintained under specified culture conditions, similar to those used in the test. If the *Daphnia magna* culture medium to be used in the test is different from that used for routine *Daphnia magna* culture a pre-test acclimation period of 2 - 3 weeks should be included to avoid stressing the test organisms. Only juvenile *Daphnia magna* derived from each female's third to seventh brood progeny should be used, and should be derived from female *Daphnia magna* which are between 2 - 5 weeks old.

An alternative approach is to use juvenile *Daphnia magna* derived from dormant (i.e. cryptobiotic) eggs, and hatch these prior to the start of the test. Dormant eggs are protected by a chitinous capsule (the ephippium) and can be stored for long periods without loss of viability. When the ephippia are placed in specific environmental conditions they are triggered to develop (within 3 - 4 days) into neonates which can be used immediately for toxicity testing. The ephippia may be supplied as part of a kit and can be used as required. The process of producing ephippia may have an effect on the genetic purity of a particular *Daphnia magna* clone, and contamination might occur. Neonates obtained from ephippia may be more or less sensitive to toxicants than those obtained by more traditional procedures and may require more careful handling. The results of reference toxicant tests (for example with zinc) should be used to demonstrate that sample

tests using ephippial juveniles are likely to generate results which are comparable to those generated using juveniles produced by laboratory cultures.

3.2.1 *Daphnia magna* biology and life-cycle

Daphnia magna (Crustacea, Brachiopoda, Diplostraca, Cladocera) are small freshwater crustaceans inhabiting hard water streams, ponds and lakes in Europe and North America, at latitudes ranging from Mexico to the Arctic regions. The organisms feed on water-borne suspensions of bacteria, algae, zooplankton and detritus.

Under optimal conditions, the reproductive pattern of *Daphnia magna* (see Figure 1) is parthenogenetic (asexual) and diploid eggs hatch into females. A single female can thus produce a succession of genetically identical broods. Development is direct (i.e. no distinct life stages) and when the young leave the brood chamber beneath the carapace, the skeleton is moulted and a new batch of eggs is released into the brood chamber. Certain factors, such as temperature or decrease in food supply induce the appearance of males, and fertilised eggs are produced. The fertilised eggs are large, and only two are produced in a single clutch. The walls of the brood chamber are then transformed into a protective capsule, the ephippium. This is cast off at the next moult and usually sinks where it can withstand drying and freezing. By means of such protection, *Daphnia magna* may be dispersed for some distances and can survive certain adverse conditions.

3.3 Culturing of *Daphnia magna*

The following procedures enable *Daphnia magna* to be laboratory cultured and used for assessing toxicity of aqueous environmental samples.

The management of *Daphnia magna* cultures can be achieved in a number of different ways without detriment to the quality of juvenile animals produced for tests. The following guidance is provided to enable the establishment of an effective set of culture procedures where required, but does not preclude the use of different methods where these have been shown to be effective in producing good quality juvenile *Daphnia magna*.

3.3.1 Maintenance of cultures

Daphnia magna should be cultured in glass or polystyrene vessels capable of holding 1-2 litres of culture media. The culture should be maintained at 20 ± 2 °C in a temperature-controlled environment, and should be protected from direct sunlight.

The media used to culture *Daphnia magna* should be similar to that used for toxicity testing (i.e. for test controls and the dilution of samples).

Deficiency in vitamin B₁₂ or selenium (an essential trace element) can result in poor health of daphnids. Insufficient water-borne selenium may cause deterioration of the cuticle of daphnids, shorter life, and failure of progeny to mature and reproduce^(22, 23). Deprivation of vitamin B₁₂ may cause delayed reproduction, infrequent moulting, and reproductive failure or progeny^(22, 24). These substances should therefore be added to media used to culture *Daphnia magna*. Selenium should be added to provide a concentration of 2 µg l⁻¹ and vitamin B₁₂ should be added to provide a concentration of 2 µg l⁻¹ as cyanocobalamin. Stock solutions of vitamin B₁₂ can be unstable and should not be stored for more than two

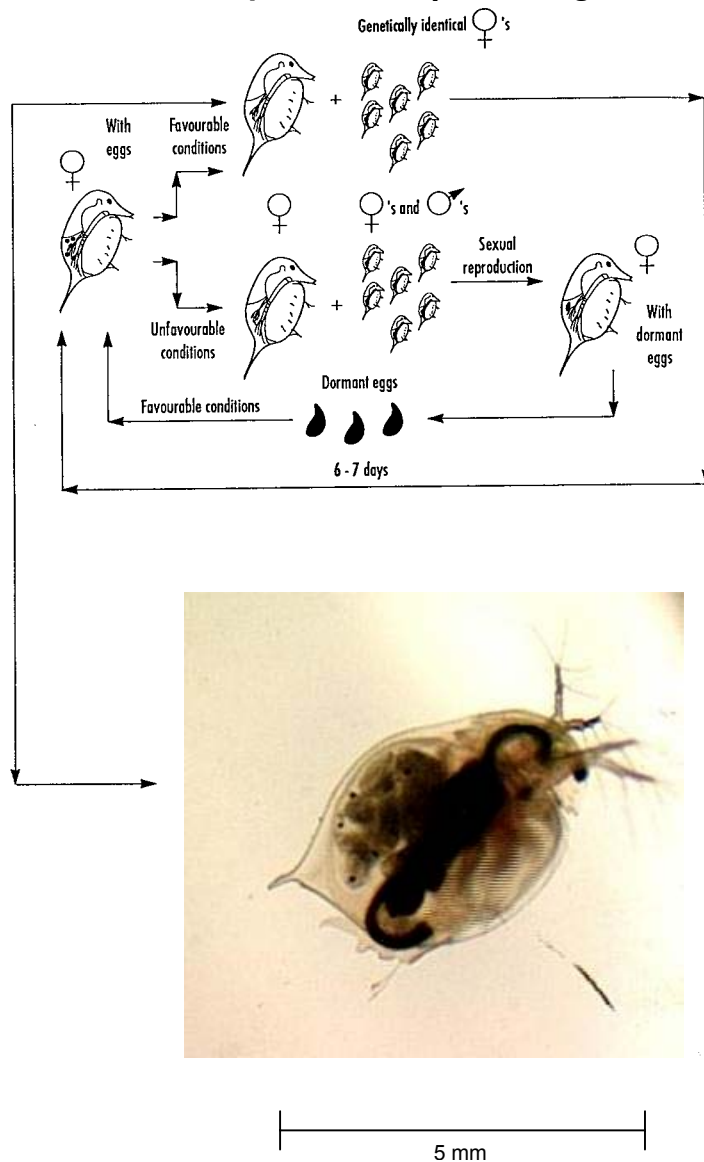
weeks unless frozen. Frozen stock solutions may be stored for up to six months but should not be used for longer than two weeks after thawing.

Water used in the preparation of culture medium should be aerated vigorously just before use to ensure the dissolved oxygen content is at least 90 % of the ASV. Other water quality parameters should comply with the criteria shown in Table 2.

At the water surface of each *Daphnia magna* culture the light intensity should be within the range of 400 - 800 lux, and, ideally, should be skewed towards the blue end of the spectrum (colour rendering index ≥ 90). Cool white fluorescent lights have been found suitable, although other light sources (for example, full-spectrum fluorescent lights) may be used. The photo-period should comprise 16 ± 1 hours of illumination and 8 ± 1 hours of darkness.

Culture information, such as the time taken to produce the first brood, juvenile productivity and the mortality of adults and juveniles should be recorded.

Figure 1 Development of *Daphnia magna*



3.3.2 Feeding

Cultures of adult *Daphnia magna* should be fed concentrated freshwater suspensions of unicellular green algae at a rate equivalent to 0.1 - 0.2 mg of carbon per adult per day. Feeding should take place on a daily basis unless over-feeding is suspected when the feeding rate should be reduced (for example, if the culture media is very green or large amounts of accumulated algae are present on the base of the culture vessel).

A uni-algal or mixed feeding regime of freshwater algal species such as *Chlorella vulgaris*, *Scenedesmus subspicatus* or *Pseudokirschneirella subcapitata* is ideal, see Appendix A. However, any combination of freshwater unicellular species found to maintain the culture at sufficient quality and fed at the prescribed rate is suitable.

Freshwater suspensions of yeast cells, seaweed extract solution or infusoria may also be fed as nutritional supplements but their contribution to the overall carbon ration should be accounted for.

3.3.3 Culture management

The media in each culture should be completely renewed at intervals not exceeding 14 days and replaced with fresh media. Prior to renewal, samples of both original and fresh media should be taken and appropriate water quality measurements performed (see Table 3) and details recorded. Significant differences in the water quality between the old and fresh media can impose considerable stresses upon *Daphnia magna* and adults should therefore only be transferred between old and fresh media if the differences in the water quality results are within the maximum differences shown in Table 3. If the differences in the specified water quality criteria are within those specified in Table 3, the adults should be carefully transferred from the original culture vessel to a new vessel containing the fresh media. This should be performed using a wide bore glass tube (of not less than 5 mm internal diameter) ensuring no juvenile *Daphnia magna* are added to the fresh media. This should ensure all the productive adults in each culture are of the same age.

Table 3 Maximum allowable differences in water quality values

Parameter	Maximum differences
Temperature	2 °C
pH	0.5 units
Total hardness	20 %

In addition to media renewal, the number of *Daphnia magna* should be reduced, i.e. thinned out, in each culture, as necessary. The accumulation of large numbers of juveniles in the culture reduces the food and dissolved oxygen available for adults and may result in the production of males or ephippia, and a subsequent decrease in juvenile productivity.

To thin out the cultures, approximately 400 ml of culture medium should be transferred from the culture vessel to a suitable holding vessel. Adult *Daphnia magna* should then be removed from the culture using a wide-bore glass tube and transferred to the holding vessel. The culture medium remaining in the culture vessel should then be poured (through a 180 µm mesh) into another vessel. All juvenile *Daphnia magna* removed from the culture should be discarded. The thinned culture medium should then be returned to the original culture vessel, and the adult *Daphnia* returned to the culture from the holding vessel.

Cultures should be discarded 4 to 5 weeks after establishment. Any culture in which ephippia, males or dead juvenile *Daphnia magna* are observed should also be discarded if and when such observations are made. Any dead adult *Daphnia magna* should be removed from the culture whilst retaining the remainder.

New cultures should be established to replace any cultures that have been discarded, or to increase the overall productivity of the culture process. The source organisms for establishing new cultures should be obtained in exactly the same manner as the procedure for attaining organisms for testing. Approximately 20 juvenile *Daphnia magna* (each of less than 24 hours old) should initially be housed in approximately 1 litre of culture medium and fed a proportion of the adult culture food ration, i.e. (1 litre / adult culture volume) x adult culture food ration. After 5 - 7 days, ten of the original source organisms should be selected and transferred to new culture media under standard adult conditions (for example, culture volume and food ration).

4 Guidelines for toxicity tests using a range of concentrations

Two approaches to the test may be used:

(i) A conventional procedure, based on previous guidance^(4, 5). This approach uses glass test vessels capable of holding 50 - 100 ml of test solution for the determination of the toxicity of environmental samples, either as received or using a range of concentrations.

(ii) A contemporary procedure based on a combination of previous guidance^(4, 5) and more recent developments of the test. This approach uses inert plastic or glass multi-well plates capable of holding 10 ml of test solution for the determination of the toxicity of environmental samples, either as received or using a range of concentrations. This miniaturised approach can be used in place of the conventional approach and is particularly useful for the screening of effluents and TIE and/or TSE exercises in which abbreviated or 'high-throughput' versions of the method is required. Such versions generally involve reduced replication, statistical analysis and quality assurance associated with the test performance and a reduction in the concentration range, and can be useful in situations where test result reporting times and minimised costs are primary considerations. Research which has led to the development of the miniaturised *Daphnia magna* immobilisation test has been undertaken by the Environment Agency and addresses the issues of multi-well evaporation, gas exchange, chemical adherence to well plates, and potential loss of volatile substances^(25 - 27).

In both approaches;

- (i) Juvenile *Daphnia magna* should be exposed for a duration of 48 ± 4 hours.
- (ii) Dilution water should comprise any suitable natural water (surface or groundwater), reconstituted freshwater or de-chlorinated drinking water.
- (iii) Daphnids should not be fed during the course of the test.
- (iv) The temperature of the test dilutions should be 20 ± 2 °C.
- (v) The pH of the test dilutions in all of the test vessels should be between 7.4 - 8.5.
- (vi) The dissolved oxygen content in the test dilutions at the start of the test should be greater than 60 % ASV at 20 ± 2 °C.
- (vii) The hardness of the test dilutions should be between 140 - 320 (expressed as $\text{mg l}^{-1} \text{CaCO}_3$).

- (viii) The lighting regime should comprise “cool white” fluorescent light of 400 - 800 lux at the surface of the test dilution with a lighting period of 16 ± 1 hours of light and 8 ± 1 hours of darkness.
- (ix) The results from toxicity tests with *Daphnia magna* should be considered valid if, in the controls, not more than 10 % of the daphnids have been immobilised or trapped at the surface of the water.
- (x) The approach taken for samples where any of the threshold criteria for the test solutions fall outside of the limits specified for the water quality parameters is described in section 2. This involves testing adjusted test dilutions and may involve testing samples that have not been adjusted to establish the extent of this issue. The approach should always be considered in the light of the objectives of the testing.

4.1 Design

The experimental design adopted (for example number of exposure concentrations and interval between test concentrations) will depend on the objective of the study, which should be clearly defined prior to analysis^(1, 28).

4.2 Principle

In the *Daphnia magna* immobilisation test, *Daphnia magna* are exposed to aqueous environmental samples diluted with freshwater to a range of concentrations for a period of 48 hours. In the context of these procedures, immobilisation 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. 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 magna*. These effects will extend from an absence of effects at lower test concentrations (0 % immobilisation) to complete immobilisation of all the daphnids at higher test concentrations (100 % immobilisation).

The data should be used to determine:

- The effective concentrations, i.e. the concentration that results in 10 %, 20 % and 50 % of the exposed *Daphnia magna* being immobilised after 48 hours. These effective concentrations are referred to as the 48 hour-EC₁₀, 48 hour-EC₂₀ and 48 hour-EC₅₀ values respectively.
- The highest concentration where there is no-observed effect after 48 hours. This value is referred to as the no observed effect concentration (48 hour-NOEC).
- The lowest concentration where there is an observed effect after 48 hours. This value is referred to as the lowest observed effect concentration (48 hour-LOEC).

4.3 Reagents and materials

4.3.1 Dilution water

In the context of this method, dilution water used for cultures, controls and the dilution of samples should be freshwater. Surface or groundwater, de-chlorinated drinking water and some reconstituted (artificial) freshwater media are all suitable for testing and culturing

Daphnia magna. The total hardness of the freshwater should be between 140 - 320 (expressed as mg l⁻¹ CaCO₃) and should exclude chelating agents such as EDTA.

Natural freshwater (including drinking water), where used, should be obtained from a source known to be free from significant contamination. An assessment of the water quality should be carried out, by monitoring parameters that are known to be toxic to aquatic organisms. These parameters include ammonia, nitrite, nitrate, common heavy metals, organophosphates and suspended solids. The results of these analyses should be compared with those concentrations deemed 'safe' for *Daphnia magna*, and if found to be above these values, the water should not be used.

Some artificial media, for example Elendt M4 and M7, contain the chelating agent EDTA, which can prevent some metals being available to the test organisms. Although suitable for some culture purposes, Elendt M4 and M7 should not be used for toxicity testing of environmental samples. Media containing other known chelating agents should also be avoided.

Artificial freshwaters, such as ASTM or ISO reconstituted hard waters may be used, see Table 4

Table 4 Preparation of artificial freshwaters

Compound	ASTM (mg l ⁻¹)	ISO (mg l ⁻¹)
Sodium hydrogen carbonate	192	64.75
Calcium chloride dihydrate	120	29.4
Magnesium sulphate heptahydrate	120	123.25
Potassium chloride	8	5.75

Individual concentrated stock solutions of each compound should be prepared in distilled or deionised water. An appropriate volume of each stock should then be added to distilled or deionised water to give the final concentration as indicated in Table 4. The final dilution water should be aerated for 24 hours before use or until the pH has stabilised.

Dilution water (both natural and artificial) should be aerated vigorously just before use to ensure the dissolved oxygen content is at least 90 % of the air saturated value (ASV) at 20 ± 2 °C. Other water quality parameters should comply with those shown in Table 2.

4.3.2 Apparatus

In addition to normal laboratory glassware and apparatus, the following equipment may be required:

Test vessels (crystallising dishes capable of holding 100 ml of test dilution or multi-well plates capable of holding 10 ml of test dilution) made of non-toxic inert material (such as glass or polystyrene).

A constant temperature environment to maintain test solutions at 20 ± 2 °C.

Equipment for measuring pH, dissolved oxygen, temperature and total hardness.

Wide bore (2 mm diameter) glass or polystyrene Pasteur pipettes for transferring juvenile *Daphnia magna*.

4.4 Test procedure

The following procedures should enable *Daphnia magna* to be cultured that result in not more than 10 % being immobilised in test controls.

4.4.1 Acquisition of juvenile *Daphnia magna* for use in tests

Juvenile *Daphnia magna* to be used in tests may be obtained from laboratory cultures or dormant eggs.

From laboratory cultures, gravid female adult *Daphnia magna* should be transferred from the main culture into isolation vessels (see section 3.3.3) on the day preceding the start of the test. The time the female *Daphnia magna* are transferred to a given vessel should be recorded. Adult *Daphnia magna* should be fed at an appropriate rate based on the differential between the isolation and culture volume, i.e. (isolation volume / culture volume) x culture food ration. On the day of the test, isolation vessels should be inspected for the presence of any juvenile *Daphnia magna*, which should be removed and counted. All the juvenile organisms from each isolation vessel should be pooled together for use in the test. Adult *Daphnia magna* can then be returned to produce more juvenile organisms. Juvenile *Daphnia magna* must be less than 24 hours old at the start of the test. Organisms older than 24 hours undergo moulting and become less sensitive to toxicants. Hence, the time that adult *Daphnia magna* are isolated from cultures and juvenile *Daphnia magna* collected should be recorded.

Alternatively, juvenile *Daphnia magna* derived from dormant eggs (ephippia) may be used. These should be incubated 3 days prior to the start of the test to optimise the hatching process in the 24 hours preceding the start of the test. Juvenile *Daphnia magna* must be less than 24 hours old at the start of the test. Organisms older than 24 hours undergo moulting and become less sensitive to toxicants. Hence, the time that ephippia are incubated and hatched, and juvenile *Daphnia magna* collected should be recorded.

4.4.2 Preparation of test dilutions

An appropriate series of concentrations should be prepared with the ratio between the concentrations not exceeding 2.2. See Table 5 for the preparation of 500 ml of typical test dilutions. Appropriate details should be recorded. On the day the toxicity test is to be carried out, the concentration range should be prepared in volumetric flasks by diluting (with dilution water) appropriate amounts of the effluent or leachate. If appropriate, test dilutions can be prepared directly in the test vessels. For each test series, a control should be prepared which contains dilution water only. At least four replicate test vessels should be used for each test concentration and control.

The remaining test dilution (i.e. that volume not added to the test vessels) should be used to determine the selected water quality parameters shown in Table 2, both at the beginning and end of the test. Appropriate details should be recorded.

Table 5 Preparation of test dilutions

Nominal concentration*	Volume of 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

* v/v

4.4.3 Initiation of the toxicity test

For each sample dilution, a minimum of 20 organisms (i.e. four groups of five organisms) should be exposed. The number of organisms in each replicate should not exceed five per 10 ml of test dilution (i.e. at least 2 ml is required per organism).

Daphnia magna should be checked prior to use by examining them against a dark background with tangential lighting. If any of the daphnids are not active, or appear to have damaged antennae, they should not be used in the test and should be discarded and replaced.

Juvenile *Daphnia magna* should be transferred to the test dilutions using a wide bore Pasteur pipette to minimise any damage occurring to the organisms. (This process may be observed using a low power stereo microscope). It is critical that the volume of culture media transferred with the organisms to the test dilution should be as small as possible so as not to significantly affect the test concentration. This is best achieved by initially transferring 20 - 30 daphnids into a 'pre-exposure' vessel (for example an unused well on the plate) containing the same concentration of test dilution as that to which they are to be exposed, immediately prior to transfer to the exposure vessels. By minimising the volume of culture water added to this vessel, and also the volume of 'pre-exposure' water added to the exposure vessels, the potential dilution effects of test organism addition can be minimised.

Juvenile daphnids should not be fed during the course of tests, as the inclusion of food in the test medium may:

- increase or decrease the effects of the toxicant under investigation due to adsorption of the toxicant onto food particles;
- affect the dissolved oxygen content by increasing the biochemical oxygen demand;
- change the physiology of the organisms used for the test and affect the uptake and metabolism of the toxicant under investigation;
- introduce additional variability into the test.

4.4.4 Monitoring water quality

Water quality measurements should be made on aliquots of test dilution which have undergone the same exposure period under identical conditions to the test vessels but which do not contain test organisms.

4.4.5 Terminating the toxicity test

The number of mobile *Daphnia magna* in each test vessel after 48 hours should be recorded. *Daphnia magna* are considered immobile if they do not move within 15 seconds following gentle agitation of the test vessel, even if there is still movement of the antennae.

4.5 Processing of results

4.5.1 Validity of the results

The results of the toxicity test should be rejected if the percentage mortality observed in the controls exceeds 10 %. In addition, data from tests should only be accepted if the results of the concurrent reference toxicant test (see section 6) meet internal quality criteria⁽²⁸⁾.

4.5.2 Data handling

Endpoints such as the EC₁₀, EC₅₀, NOEC and LOEC values should be determined using an appropriate validated computer-based statistical package. The endpoint values for a *Daphnia magna* immobility toxicity test are based on the percentage of immobile *Daphnia* in each test concentration.

Most computer-based statistical software packages will enable the calculation of endpoint values without further data manipulation. The total number of exposed *Daphnia* (5 per replicate) and either the number which were immobile, or the number which were mobile, are entered for each test vessel and a proportional effect (taking into account the corresponding effect in the controls) is calculated.

4.5.3 Estimation of EC values

The 48 hour-EC₁₀, 48 hour-EC₂₀ and 48 hour-EC₅₀ values (and other EC values, if necessary) should be determined using appropriate statistical procedures (see Figure 3). Confidence limits (p = 0.95) for the calculated EC value should be determined and recorded with the test results.

Figure 3 Flowchart for the estimation of the EC_{50} value for full concentration range test

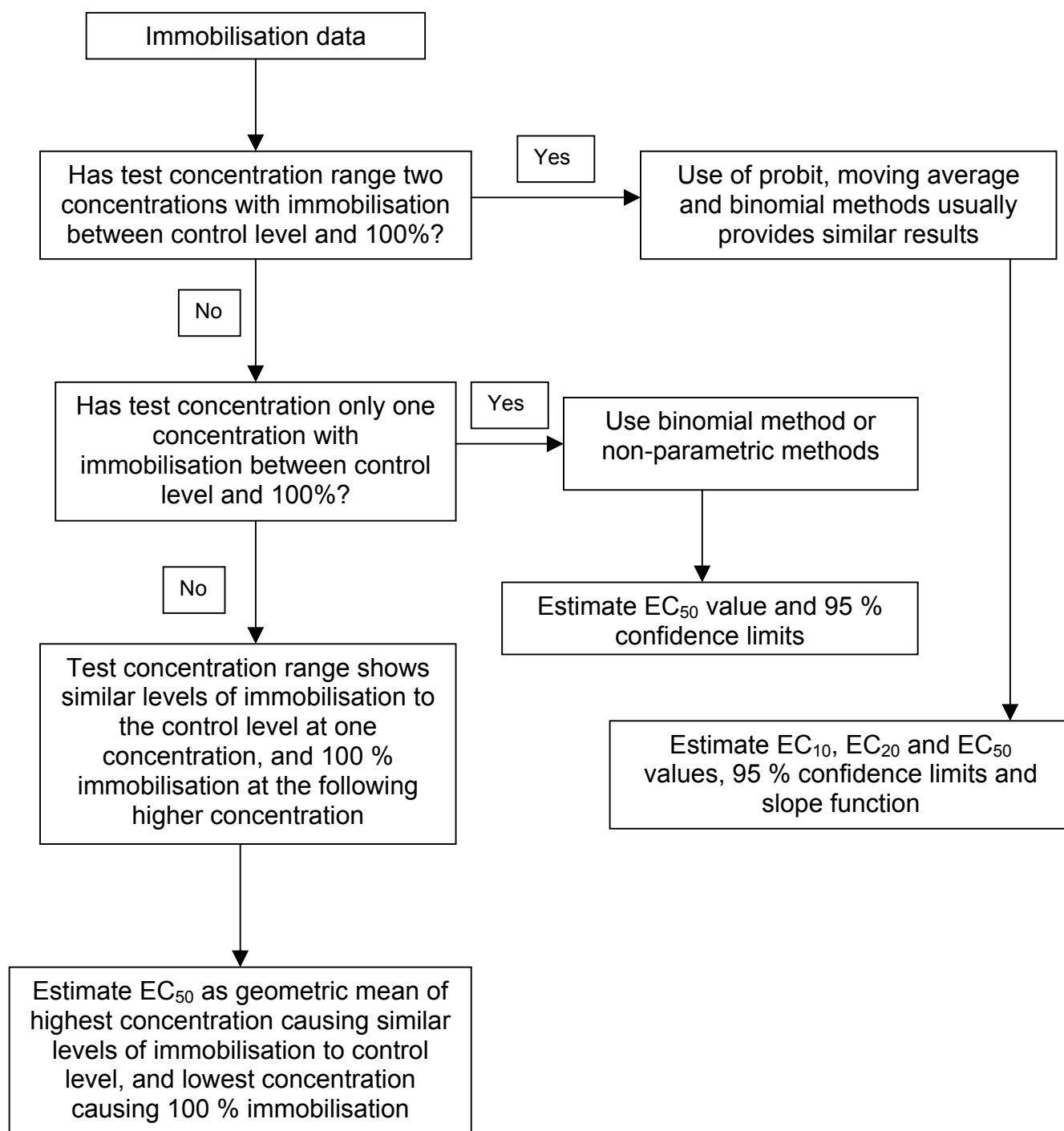


Table 6 shows examples of data produced to show the effect on *Daphnia magna* mobility by an effluent. Table 7 summarises the EC_{50} values derived from the data in Table 6 using different statistical procedures.

Table 6 Examples of results of the immobilisation of *Daphnia magna* after exposure to an effluent for 48 hours

Effluent concentration* (%)	Number of <i>Daphnia magna</i> exposed	Scenario 1		Scenario 2		Scenario 3	
		Cumulative number of immobilised <i>Daphnia magna</i>	Immobilisation (%)	Cumulative number of immobilised <i>Daphnia magna</i>	Immobilisation (%)	Cumulative number of immobilised <i>Daphnia magna</i>	Immobilisation (%)
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

* Range of concentrations expressed as a percent of the effluent sample concentration.

The data in Table 6 may be used to check that in-house statistical procedures provide comparable results to those given in Table 7.

Table 7 Summary of EC₅₀ values (and 95 % confidence limits) for the data in Table 6 estimated by different statistical procedures

Scenario	Statistical procedure	EC ₅₀ Value	Confidence limits (95 %)	Slope of dose response curve
1	Probit (Tox Calc)	3.3	2.3-4.6	1.8
	Probit*	3.3	2.8-3.8	1.8
	Moving average*	3.2	2.7-3.7	-
	Binomial*	3.6	1-10	-
2	Probit	No valid approach	-	-
	Moving average	No valid approach	-	-
	Binomial*	4.8	2.2-10	-
	Spearman-Karber (Tox Calc)	4.8	4.1-5.7	-
3	Geometric mean	3.2	-	-

* Computer based statistical package⁽²⁹⁾

For the data in Scenario 1, the probit, moving average and binomial procedures produce similar results although the confidence limits are greater for the values derived using the binomial procedure. Where there are less than two intermediate effect concentrations (Scenarios 2 and 3) the EC₅₀ values derived are less statistically sound.

From the data shown in Table 6, the 48 hour-EC₅₀ value estimated by Tox Calc software (Tide Pool Scientific Software) is 3.3 % v/v effluent with 95 % confidence limits of 2.3 - 4.6 %.

From the interpolation of the dose response curve of cumulative *Daphnia* immobility (probability scale) against effluent concentration (log scale) shown in Figure 4 for Scenario 1, the 48 hour-EC₅₀ is 3.25 % v/v effluent. The values obtained graphically complement those obtained using computer-based software (see Table 7).

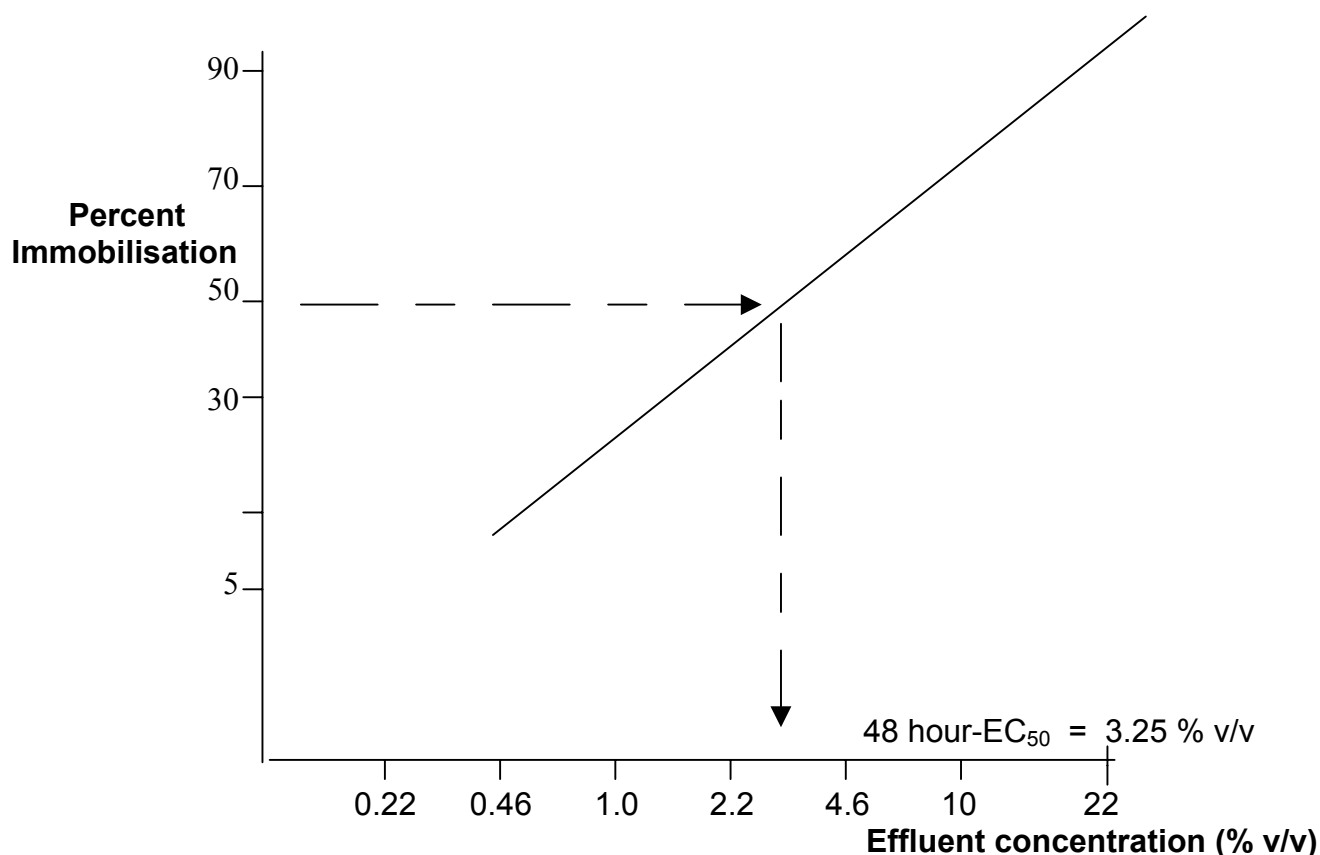
When analysing data from the *Daphnia magna* immobility test the following points should be considered:

- (i) If the results include concentrations at which there are 0 - 10 and 100 % immobility, and also two concentrations at which the percentage immobility is between 0 - 10 and 100 %, then probit, moving average and binomial methods should provide similar estimates of the EC values. Probit analysis should be used to estimate EC values, 95% confidence limits and the slope of the dose response curve.
- (ii) If the results do not include two concentrations at which immobility 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 EC₅₀ value with wide confidence limits. The use of non-parametric methods such as the Spearman-Kärber or trimmed Spearman-Kärber methods may enable the determination of an EC₅₀ value to be made.
- (iii) Where the data obtained are inadequate for calculating an EC₅₀ value, the highest concentration causing no immobility and the lowest concentration causing 100 % immobility should be identified. An approximation of the EC₅₀ value 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 EC₅₀ calculated will be less statistically sound.
- (iv) In all instances, the EC₅₀ value derived from any of the above procedures should be compared with a graphical plot on logarithmic-probability (log-probit) scales of percent immobility for the various test concentrations. Any major disparity between the graphical estimation of the EC₅₀ value and that derived from the statistical programmes should be resolved.

4.5.4 Estimation of the NOEC and LOEC

In the juvenile *Daphnia magna* immobilisation test, the NOEC and LOEC values should be calculated using Fisher's exact test.

Figure 4 Graph of cumulative immobilisation (probability scale) against effluent concentration (log scale)



5 Guidelines for single concentration toxicity tests

5.1 Design

The assessment of the toxicity of receiving waters should be carried out on an undiluted (i.e. 100 %) sample and appropriate controls using the procedures described in section 4. Receiving waters may not meet the criteria specified in Table 2 for the selected water quality parameters required to support *Daphnia magna* and in these cases the sample should be adjusted using the procedures described in section 2.

Toxicity tests with *Daphnia magna* for monitoring or screening may also be carried out on a single concentration of effluent or leachate sample and appropriate controls. The concentration of effluent or leachate used in such tests would need to be appropriately chosen with due consideration given to the objectives of the study.

5.2 Test procedure

Single concentration tests should be initiated in the same way as full concentration range toxicity tests (see section 4) with at least six replicates of each control and six replicates of the sample concentration. Water quality monitoring should be carried out in the same way as described for the full concentration range toxicity test (see section 4).

5.3 Processing of results

An assessment of how the responses in the single effluent concentration compare to those in the control dilution should be carried out using hypothesis testing (see Figure 5). The hypothesis tested should be that the responses in the sample are not significantly different from those in the controls.

Initially, the proportion of organisms remaining mobile in the controls and the single test concentration should be 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 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 should be used to test the homogeneity of variance assumption. If the data meet the homogeneity of variance assumption then the standard (homo-scedastic) t-test should be used to analyse the data. Failure of the homogeneity of variance assumption leads to the use of a modified (hetero-scedastic) 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 elsewhere^(30 - 32).

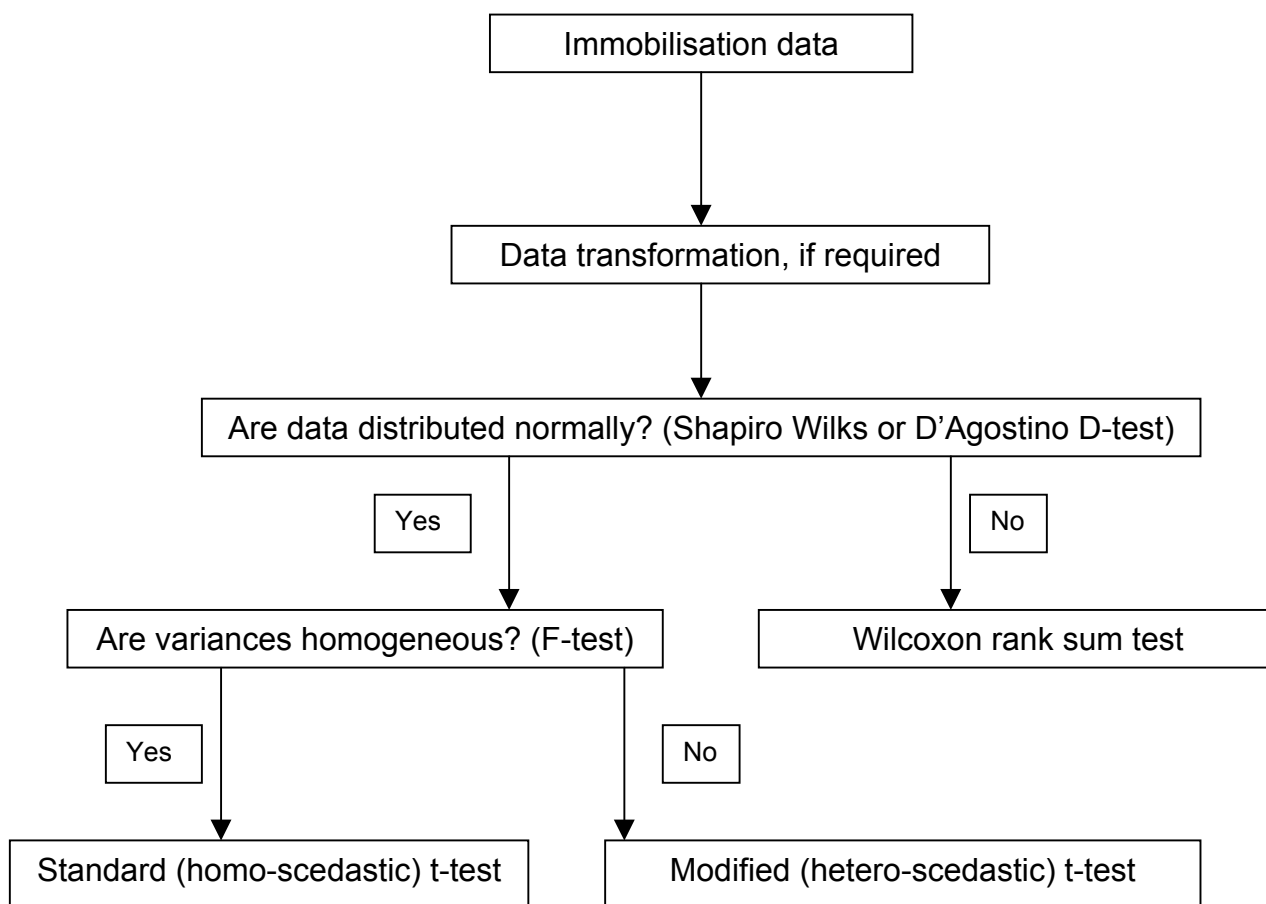
Table 8 shows example data sets for a single concentration test (i.e. 0.46 % v/v effluent) and controls. In Scenario 1, the equality of variances cannot be confirmed and a modified (hetero-scedastic) t-test indicates a significant difference between responses in the two groups ($t = 2.24$, $p < 0.05$). In Scenario 2, the variances are equal ($F = 1.80$, $p = 0.53$) and the standard (homo-scedastic) t-test indicates no significant difference between the responses in the two groups ($t = 1.20$, $p > 0.05$).

Table 8 Example of data for a single concentration *Daphnia magna* immobilisation test and the results of statistical analysis

Effluent concentration (%)	Number of <i>Daphnia magna</i> exposed	Cumulative number of immobile organisms	Immobility (%)	Method of statistical analysis	Result of statistical analysis
<i>Scenario 1</i>					
0 (Control)	30	0	0	Modified t-test	Significant difference ($p < 0.05$)
0.46*	30	3	10		
<i>Scenario 2</i>					
0 (Control)	30	1	3.33	Standard t-test	No significant difference ($p > 0.05$)
0.46*	30	3	10		

* Expressed as a percent of the effluent sample concentration

Figure 5 Flowchart for the analysis of single concentration test data from the *Daphnia magna* immobilisation test



6 Guidelines for reference toxicant tests using zinc

6.1 Design

Daphnia magna immobility tests that are carried out to provide toxicity data on environmental samples should be accompanied by tests with the reference toxicant zinc (as zinc sulphate). Reference toxicant tests should be conducted according to the procedures described in section 4.

6.2 Reference toxicant preparation

6.2.1 Zinc stock solution

Weigh out 4.397 ± 0.002 g of zinc sulphate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) into a 1-litre volumetric flask and dilute to just below the mark with distilled or deionised water. Add 1 ml of 1M hydrochloric acid solution to the flask and make to the mark with distilled or deionised water. The concentration (as Zn) of this solution is 1000 mg l^{-1} .

6.2.2 Zinc working solution

A zinc working solution (100 mg l^{-1}) should be prepared on the day the test is carried out and used to prepare an appropriate range of concentrations. The range shown in Table 9 should be used when no previous data are available.

The test concentration range of zinc for subsequent tests can be modified based on initial results to allow the derivation of more precise 48 hour-LOEC and 48 hour-EC₅₀ values.

Table 9 Zinc concentration range

Zinc concentration (mg l ⁻¹)	Volume of seawater (ml)	Volume of zinc working solution 6.2.2 (ml)
0 (control solution)	1000	0.0
0.1	999	1.0
0.32	996.8	3.2
1.0	990	10.0
3.2	968	32
10.0	900	100

6.3 Test procedure

Reference toxicant tests should be initiated in the same way as described section 4.

6.4 Processing of results

The 48 hour-LOEC and 48 hour-EC₅₀ values should be calculated using the procedures described in section 4.

7 References

- 1 Monitoring Certification Scheme (MCERTS) Performance Standard for Laboratories Undertaking Direct Toxicity Assessment of Effluents, Version 1, Environment Agency, January 2006.
- 2 Standing Committee of Analysts, The direct toxicity assessment of aqueous environmental samples using the oyster (*Crassostrea gigas*) embryo-larval development test (2006), *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
- 3 Standing Committee of Analysts, The direct toxicity assessment of aqueous environmental samples using the marine copepod *Tisbe battagliai* lethality test (2006), *Methods for the Examination of Waters and Associated Materials*, in this series, Environment Agency.
- 4 Ecotoxicity test methods for effluent and receiving water assessment - Comprehensive guidance, Environment Agency, EP236, 2001.
- 5 Ecotoxicity test methods for effluent and receiving water assessment - Supplementary advice to international guidelines, Environment Agency, EP236, 2001.
- 6 Organisation for Economic Cooperation & Development (OECD) (2004), Guideline for the testing of chemicals 202, *Daphnia* sp., Acute Immobilisation Test & Reproduction Test.
- 7 Organisation for Economic Cooperation & Development (OECD) (2002), Guideline for the testing of chemicals 201, Freshwater Alga & Cyanobacteria, Growth Inhibition Test.

- 8 Technical Guidance for the Implementation of Direct Toxicity Assessment (DTA) for Effluent Control: Addressing water quality problems in catchments where acute toxicity is an issue. Technical Report TX02B 217, UKWIR, 2000.
- 9 Programme for the Monitoring of Water Quality, Manual Volume 018, Environment Agency, 1997.
- 10 Standing Committee of Analysts, General Principles of Sampling Water and Associated Materials (2nd Edition) 1996: Estimation of Flow and Load (1996), *Methods for the Examination of Waters and Associated Materials*, ISBN 011752364X, in this series.
- 11 ISO 2006:5667-1 Water quality - Sampling - Part 1: Guidance on the design of sampling programmes and sampling techniques.
- 12 Standing Committee of Analysts, The determination of pH in low ionic strength waters 1998, *Methods for the Examination of Waters and Associated Materials*, ISBN 0117520845, in this series.
- 13 Standing Committee of Analysts, Temperature Measurement for Natural, Waste and Potable Waters and other items of interest in the Water and Sewage Disposal Industry 1986, *Methods for the Examination of Waters and Associated Materials*, ISBN 0117520179, in this series.
- 14 Standing Committee of Analysts, Total hardness, Calcium hardness, and magnesium hardness in raw and potable waters by EDTA titrimetry 1981, *Methods for the Examination of Waters and Associated Materials*, ISBN 0117520179, in this series.
- 15 Standing Committee of Analysts, Dissolved oxygen in natural and waste waters 1979, *Methods for the Examination of Waters and Associated Materials*, ISBN 011751442X, in this series.
- 16 Guidelines for pH adjustment of effluent samples for toxicity testing, S G Abernethy and G F Westlake, 1989, Report of the Ontario Ministry of the Environment, Ontario.
- 17 Aquatic toxicity testing for notification of new substances, an advisory document on dealing with "difficult" substances, P Whitehouse and M Mallet, 1993, Report No. CP 722, pp1-34, WRc plc, Medmenham.
- 18 Aquatic toxicity testing of sparingly soluble, volatile and unstable substances. ECETOC Monograph No. 26, 1996, pp1-67.
- 19 BS EN ISO 1998:5667-16 Water Quality – Sampling: Guidance on biotesting of samples.
- 20 A guidance supplement for determining the aquatic toxicity of poorly water soluble complex mixtures using water accommodated fractions, A E Girling, G F Whale and D M M Adema, *Chemosphere*, 1994, **29 (12)**, pp2645-2649.

- 21 Development and validation of Toxkit microbiotests with invertebrates, in particular crustaceans, G Personne, In: *Microscale Testing in Aquatic Toxicology: Advances Techniques and Practice* (Eds: P G Wells, K Lee and C Blaise), 1998, CRC Press, Boca Raton, Florida, pp437-450.
- 22 Nutritional Considerations in Toxicity Testing: Invertebrate Nutrition (*Daphnia*, *Ceriodaphnia*), U M Cowgill, in *Nutritional Considerations in Toxicity Testing*, (Ed) R P Lanno, Proceedings from short course presented at 10th Annual Meeting, *Soc Environ Toxicol Chem*, 1989, Toronto, Ontario (29 October 1989).
- 23 Effect of Selenium Deficiency on Cuticle Integrity in the Cladocera (Crustacea), K I Keating and B C Dagbuson, *Proc (US) Nat Acad Sci*, 1984, **81**, pp3433-3437.
- 24 The Influence of Vitamin B₁₂ Deficiency on the Reproduction of *Daphnia pulex* Leydig (Cladocera), K I Keating, *J Crust Biol*, 1985, **5**, pp130-136.
- 25 Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*. Environmental Protection Series Report EPS 1/RM/14. 1990, Environment Canada.
- 26 ISO 1989:6341 - Water Quality - Determination of inhibition of mobility of *Daphnia magna* Straus.
- 27 Assessment of the feasibility of using image analysis in the oyster embryo-larval development and *Daphnia magna* growth tests, Environment Agency, 1999.
- 28 Guidance on the use of Direct Toxicity Assessment in PPC Impact Assessments, Integrated Pollution Prevention & Control (IPPC) Guidance Note, Version 1, Environment Agency, January 2006.
- 29 Methods for Calculating an LC₅₀, C E Stephan, pp65-84, in *Aquatic Toxicology and Hazard Evaluation*, (Eds) F L Mayer and JL Hamelink, ASTM, 1977, STP 634, Philadelphia.
- 30 Biometry, R R Sokal and F J Rohlf, 1981, W H Freeman and Company, San Francisco, pp859.
- 31 Biostatistical Analysis. J H Zar, 1984, Prentice Hall International, New Jersey, pp716.
- 32 Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. United States Environmental Protection Agency, Report EPA/600/4-85/013, 1993, Cincinnati, OHIO

Appendix A Freshwater unicellular algae culture as food for *Daphnia magna*

A1.1 Introduction

This method describes procedures, materials and equipment required for the culture of freshwater algae as food for cultures of *Daphnia magna*.

A1.2 Source cultures

Axenic (uncontaminated) uni-algal source cultures should be obtained from reliable sources. *Chlorella vulgaris*, *Scenedesmus subspicatus* and *Pseudokirschneriella subcapitata* are suitable species.

Source cultures should be held at 4 ± 4 °C in the dark. Under these conditions cultures should be viable for up to 3 months after receipt.

A1.3 Laboratory cultures

A series of sub-cultures should be established from each source culture to provide a supply of algae as inocula for larger scale batch culturing. A series of sub-cultures should comprise, essentially, an exponentially growing population of algae maintained in a nutrient unlimited environment. As the population of cells in one sub-culture approaches nutrient limitation an aliquot of cells should be inoculated into fresh media, where the population should continue to grow exponentially.

Sub-cultures should be grown in 500 ml conical flasks containing 100 ml of growth media at 20 ± 2 °C under continuous illumination at 10000 ± 1000 lux. Flasks should be constantly mixed, for example on an orbital shaker or stirrer.

A single series of sub-cultures should not be maintained for greater than three months before a new series is initiated with fresh source culture. This should avoid the accumulation of bacterial contamination in the sub-culture, which may result in an overall failure of the culture.

Primary sub-cultures should be inoculated with 0.1 ml of source culture (which should be shaken to re-suspend any settled algal cells) and sub-cultured at intervals of 3 - 5 days by transferring 0.5 ml of sub-culture to fresh media.

Mature (i.e. 3 - 5 day-old) sub-cultures should be used to inoculate large scale batch cultures.

A1.4 Batch cultures

Batch cultures comprise large volume cultures that are used to grow sufficient biomass of algae as food for *Daphnia magna*.

Batch cultures should be grown under identical conditions used for sub-cultures, using 2 litres of growth media in a 2.5 litre conical flask, and inoculated with 20 ml of sub-culture. A chemostat (continuous large scale algal culture) may also be employed as a substitute for batch cultures and should be incubated under the same conditions of temperature and light as batch cultures.

Batch cultures (or chemostats) should be mixed and aerated using a continuous, sterile, oil-free air source and incubated for up to 5 days before concentration. Following incubation, batch cultures should be concentrated, by centrifugation, to produce algal stocks of known cell density and organic carbon content. Cell density can be measured directly by counting the number of cells in a given volume of algal suspension or indirectly by a surrogate method (e.g. absorbance at 440nm) for which the relationship with actual cell numbers has been characterised. The amount of algal food provided to *Daphnia* cultures is generally based on the optimal ration of organic carbon required by the animals to allow continuous parthenogenetic reproduction. The total organic carbon (TOC) present in a given number of algal cells will usually be dependent on the specific culturing conditions of the algae and should be determined on an annual basis.

A1.5 Growth media

The media used to culture freshwater algae should be Bold's Basal Medium 1 (BBM1) for *Chlorella vulgaris* and OECD algal growth medium for *Scenedesmus subspicatus* or *Pseudokirschneriella subcapitata*.

Nutrients should be added in the form of concentrated stock solutions prepared using analytical grade reagents dissolved in sterile distilled, deionised or reverse osmosis grade water.

A1.6 Preparation of BBM1 algal growth media

The preparation of BBM1 nutrient media initially involves setting up a series of fourteen stock solutions using analytical grade reagents. Sterilisation of the stocks is not required. Store the solutions at ambient temperature in the dark. Stock solutions 1 to 6 may be stored up to three months, and stock solutions 7 to 14, up to 12 months.

Table A1 Reagents used to Prepare BBM1 Medium

Stock solution	Nutrients	Concentration in stock solution (g l ⁻¹)
1	K ₂ HPO ₄	7.5
2	KH ₂ PO ₄	17.5
3	MgSO ₄ ·7H ₂ O	7.5
4	NaNO ₃	25
5	CaCl ₂ ·2H ₂ O	2.5
6	NaCl	2.5
7	C ₁₀ H ₁₂ N ₂ O ₈ Na ₄ (EDTA) ⁽¹⁾	50
8	FeSO ₄ ·7H ₂ O ⁽²⁾	4.9
9	H ₃ BO ₃	11.42
10	ZnSO ₄ ·7H ₂ O	17.64
11	MnCl ₂ ·4H ₂ O	2.92
12	CuSO ₄ ·5H ₂ O	3.144
13	Co(NO ₃) ₂ ·6H ₂ O	1
14	Na ₂ MoO ₄ ·2H ₂ O	2.384

⁽¹⁾ in alkaline solution containing 31 g of potassium hydroxide per litre.

⁽²⁾ in acid solution containing 1 ml of concentrated sulphuric acid (SG = 1.84) per litre.

Prepare batches of fresh media by adding the following amounts of the stock solutions to sterile distilled, deionised or reverse osmosis water in appropriate sterile glassware:

- stock solutions 1 to 6, add 10 ml per litre
- stock solutions 7 to 9, add 1 ml per litre
- stock solutions 10 to 14, add 0.5 ml per litre

Allow the solution to equilibrate after preparation by leaving it to stand overnight, or by continual gentle stirring for one hour. The pH of the medium after equilibration should be between 6.5 - 7.2. No adjustment of the pH should be required. If the pH of the medium is outside the range 6.5 - 7.2 after equilibration, stir for a further 30 minutes or discard and prepare a new solution.

A1.7 Preparation of OECD algal growth medium

The preparation of OECD nutrient medium initially involves setting up a series of four sterile stock solutions using analytical grade reagents. Store the solutions at 2-6 °C in the dark. Stock solutions may be stored up to three months.

Prepare batches of fresh medium by adding 15 ml per litre of stock solution 1 and 1.5 ml per litre of stock solutions 2, 3 and 4 to sterile distilled, deionised or reverse osmosis water in appropriate sterile glassware. Allow the medium to equilibrate after preparation by passing air, filtered, for example through a polytetrafluoroethylene bacterial membrane filter (nominal size 0.2 µm) into the solution for at least 30 minutes. The pH of the medium after equilibration should be between 8.0 - 8.5. If necessary, adjust the pH of the medium to within this range using 1M sodium hydroxide solution or 1M hydrochloric acid solution. Prepare OECD medium on the day required for semi-continuous algal culture.

Table A2 Reagents used to prepare OECD Medium

Stock solution	Nutrients	Concentration in stock solution (mg l ⁻¹)
1 Macro-nutrients (Autoclave for 15 minutes at 115 - 121 °C)	NH ₄ Cl	1500
	MgCl ₂ .6H ₂ O	1200
	CaCl ₂ .2H ₂ O	1800
	MgSO ₄ .7H ₂ O	1500
	KH ₂ PO ₄	160
2 Fe-EDTA (Autoclave for 15 minutes at 115 - 121 °C)	FeCl ₃ .6H ₂ O	80
	C ₁₀ H ₁₄ N ₂ O ₈ Na ₂ .2H ₂ O	100
3 Trace elements (Autoclave for 15 minutes at 115 - 121 °C)	H ₃ BO ₃	185
	MnCl ₂ .4H ₂ O	415
	ZnCl ₂	3
	CoCl ₂ .6H ₂ O	1.5
	CuCl ₂ .2H ₂ O	0.01
	NaMoO ₄ .2H ₂ O	7
4 NaHCO ₃ (Sterilise by membrane filtration (0.2 µm))	NaHCO ₃	50000

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users would like to receive advanced notice of forthcoming publications please contact the Secretary on the Agency's web-page.

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