205

Environment Agency

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

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Methods for the Examination of Waters and Associated Materials

This booklet contains guidance on the direct toxicity assessment of aqueous environmental samples using the marine copepod *Tisbe battagliai* lethality 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. One document has already been published in this series[2] and further documents are being produced and include:

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

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[3, 4]. 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.

Contents

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.

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

‰	parts per thousand.
Aqueous environmental samples	these include effluents, leachates and receiving waters.
ASV	air saturation value.
Copepodid	*Tisbe battagliai* intermediate life stages.
DTA	direct toxicity assessment.
EDTA	ethylenediaminetetraacetic acid.
Epiphytic	A plant that grows upon or attached to another plant.
Instars	Life stages in arthropods, between each moult.
LC_{10}	the concentration that results in a lethal effect on 10% of the exposed organisms.
LC_{20}	the concentration that results in a lethal effect on 20% of the exposed organisms.
LC_{50}	the concentration that results in a lethal effect on 50% of the exposed organisms.
LOEC	lowest observed effect concentration – lowest test concentration at which there is an observed effect compared to controls.
Nauplii	*Tisbe battagliai* larval life stages.
NOEC	no observed effect concentration – the highest test concentration at which there is no observed effect compared to control dilutions.
Static tests	tests with no replacement or replenishment of the test dilutions.
USA	United States of America.

The direct toxicity assessment of aqueous environmental samples using the marine copepod *Tisbe battagliai* lethality test

1 Introduction

The procedures described in this document enable direct toxicity assessments to be carried out on aqueous environmental samples using the marine copepod *Tisbe battagliai*. The procedures described are based on an Environment Agency project[3, 4] and take into account existing guidelines[5] and more recent method developments.

The marine copepod *Tisbe battagliai* lethality 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; and
(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[6]. 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[7 - 9].

Environmental samples should be collected in containers, typically screw top glass bottles, which 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 on the sample at the location where the sample is taken (i.e. on-site determination) and on receipt 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[10 - 13]. Samples should be labelled appropriately with such details as the name

and location of the site where each of the samples was 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 record form details (for example see Table A1) should also be recorded.

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

pH
Temperature
Dissolved oxygen
Salinity

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 salinity. This approach might mean that in certain instances it will not be possible to carry out a test because the results of the parameters listed in Table 2 fall outside of the limits specified.

Table 2 Threshold criteria for selected water quality parameters for the marine copepod *Tisbe battagliai* lethality test

Parameter	Threshold criteria
pH	7.7 - 8.3
Dissolved oxygen (as a percent of the ASV) at 20 ± 2 °C	≥ 50
Salinity (‰)	20 - 36

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 DTA testing, modification or adjustment of the sample, or its dilutions, should be made so that all criteria for 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.

In selecting an approach there is an issue of how representative a test might be of the conditions in the environment, and whether modification or adjustment of a sample to meet

test requirements causes a subsequent loss of environmental realism (due to changes in effluent character on adjustment). The influence of water quality parameters will typically be more pronounced for effluents than receiving waters and problems of test dilution modification will generally only become important 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.

Test dilutions should be shaken or stirred to enhance homogeneity prior to dispensing into test vessels.

Toxicity testing should not take place in situations where any of the threshold criteria for the parameters shown in Table 2 falls outside the specified range for all of the dilutions in a test. 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.

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 solutions 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[14]. 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 50 % 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 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 ml min^{-1} l^{-1} until a dissolved oxygen concentration greater than 50 % 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 50 % of the ASV should not be aerated.

2.4.3 Salinity

The salinity of test dilutions should be adjusted if values fall outside of the range specified

in Table 2. In addition, the salinity range within a single test (i.e. between controls and test dilutions) should not exceed 3 ‰.

An increase in salinity can be achieved by the addition of hyper-saline brine solution (see section 4.3.2). An additional 'salinity adjusted' control should then be included in the test, in addition to the normal control, i.e. dilution water (4.3.1). The 'salinity adjusted' control should be prepared by adjusting a sample of the seawater used for normal controls and sample dilution to the same original salinity as the sample dilution by adding distilled or deionised water. Hyper-saline brine solution should then be added to adjust the additional control back to the required salinity range, in exactly the same way as with the sample dilution under modification. The additional control accounts for potential effects on *Tisbe battagliai* that may be caused by the addition of hyper-saline brine solution.

The results of the additional 'salinity adjusted' controls are used as a substitute for, or in addition to, dilution water control results when the endpoint values are calculated. For example, hypothesis testing can be used to investigate whether a significant statistical difference exists between the dilution water and 'salinity adjusted' controls. If no difference is shown, all the controls may be used. If a difference is found, only the 'salinity adjusted' controls should be used in endpoint value calculations.

A decrease in salinity is achieved by adding distilled or deionised water to a sample dilution. The additional dilution arising from this addition 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 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 long 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 the visual observation of *Tisbe battagliai* in the test vessels. Observation of the organisms may be improved by temporarily illuminating the vessel either from the side or below the vessel.

2.4.6 Oily substances and volatile compounds

The presence of oily substances, or substances in excess of their water solubilities, may cause *Tisbe battagliai* to float on the surface of the test solution. Oily substances may adhere to the carapace and other body appendages, and effectively change the density of the organism in the test dilution. In these circumstances, the interpretation of test data can be difficult, as the assessment of lethality may be influenced by the flotation. Tests can be carried out in sealed vessels (for example, glass vessel with a septum cap) where air has been excluded, which should inhibit flotation. Before sealed vessels are used, appropriate checks should be made to ensure the survival of *Tisbe battagliai* in the dilution water 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[15 - 17] including guidance on the testing of effluents containing sparingly soluble substances[18].

2.5 Disposal of samples

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

3 Marine copepod *Tisbe battagliai* lethality test procedure

3.1 Introduction

Based on previously guidance[3 - 5] procedures are described for culturing the marine copepod *Tisbe battagliai* and conducting static toxicity tests to assess the effects of aqueous environmental samples on their survival.

3.2 Test organism

The species used in the test is the marine copepod *Tisbe battagliai.* At the start of the test the organisms should be at the first copepodid stage and be 5 - 6 days old. Test organisms should be obtained from an age-standardised laboratory culture with a known history.

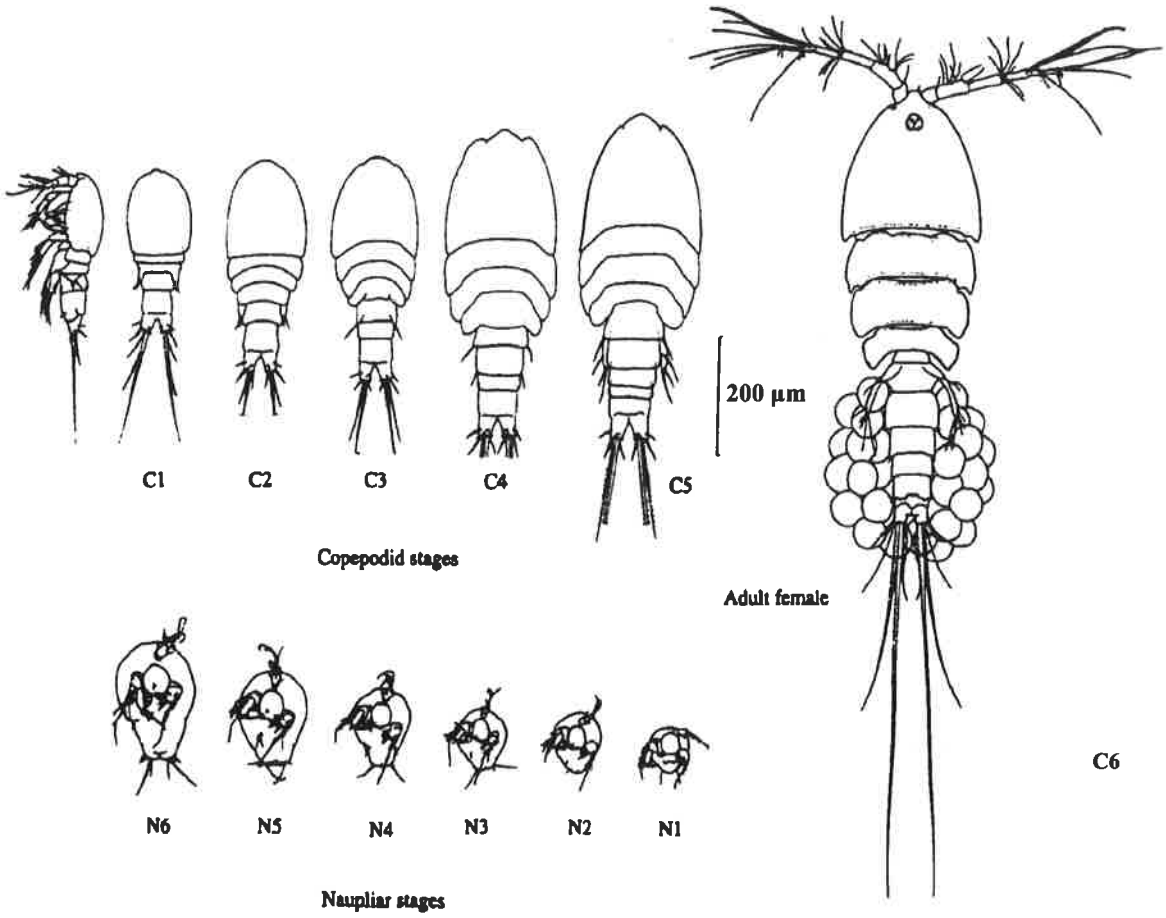
3.2.1 *Tisbe battagliai* biology and life-cycle

Tisbe battagliai (Crustacea, Copepoda, Harpacticoida) are sibling species of the holothuriae group. They are distributed throughout the shallow waters of coastal regions of Europe and the Atlantic coast of the USA, living on seaweed. They feed on epiphytic micro-algae, detritus and bacteria.

The development of *Tisbe battagliai* (see Figure 1) passes from an egg stage to six naupliar stages (N1 - N6) and five copepodid stages (C1 - C5) before reaching adulthood (C6) and sexual maturity. Reproduction is sexual and requires the transfer of a spermatophore from the male to the female of the species. Female copepods can produce multiple broods after a single copulation, releasing first-stage nauplii from a single conspicuous ovisac/brood chamber secreted from the opening of the oviduct. Under the culture conditions described within this booklet, development from first-stage nauplii to

first-stage copepodid occurs during the period 0 - 5 days and development to a sexually mature adult is usually completed within approximately 12 days. Females produce their first ovisac/brood chamber (assuming successful copulation) within about 12 - 15 days. Successive broods of nauplii are produced at intervals of 2 - 3 days. The normal life expectancy of the species is around 5 - 6 weeks. See also Figure 2.

Figure 1 Summary of the developmental stages of *Tisbe battagliai*



This figure is taken from reference 19.

Figure 2 ***Tisbe battagliai***

Figure 2A Adult female carrying ova

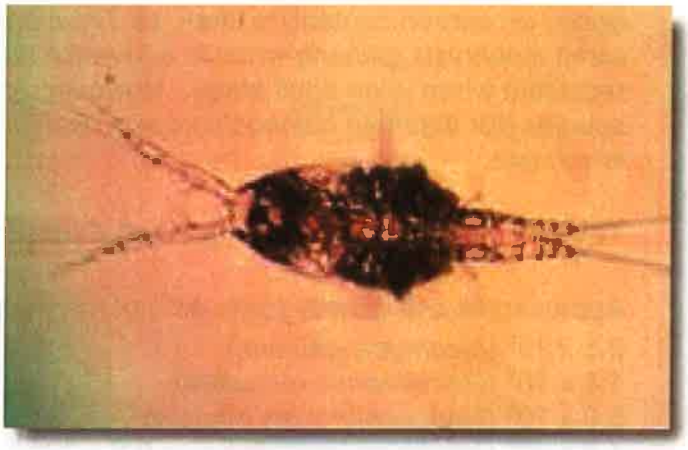


Figure 2B Second stage copepodid

3.3 Culturing of *Tisbe battagliai*

The following procedures should enable populations of *Tisbe battagliai* to be cultured in the laboratory providing organisms suitable for assessing toxicity of aqueous environmental samples.

3.3.1 Maintenance of cultures

Tisbe battagliai should be cultured in glass or polystyrene vessels capable of containing 2 - 5 litres of natural seawater that has been filtered through a 0.2 µm filter. The culture should be maintained at 20 ± 2 °C in a temperature-controlled environment, and should be protected from direct sunlight.

At the water surface of each *Tisbe battagliai* 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 light has been found suitable, although other light sources (for example, full-spectrum fluorescent light) may be used. The photo-period used in the testing should comprise 16 ± 1 hours of illumination and 8 ± 1 hours of darkness.

3.3.2 Feeding

Tisbe battagliai cultures should be fed on a diet of marine micro-algae at a rate equivalent to 3250 µg of carbon per litre of culture seawater, i.e. 3250 µg C l^{-1}. Feeding should take place at the time the seawater in each culture is renewed but more frequent feeding may be required if the population density of *Tisbe battagliai* (i.e. the number of organisms

per ml of medium) in the culture is high. As a guide, *Tisbe battagliai* cultures may need to be fed more frequently if the *Tisbe battagliai* consume most of the algal cells and no significant accumulation of algae occurs on the bottom of the culture vessel. *Tisbe battagliai* cultures that are less than one week old and contain nauplii and early copepodid stages should not normally require additional feeding.

A mixture of *Isochrysis galbana* and *Rhinomonas reticulata* cultures (in the ratio of 2:1, based on carbon content) is ideal, as *Tisbe battagliai* preferentially feed on the smaller sized *Isochrysis galbana* when in a juvenile stage, and on the larger sized *Rhinomonas reticulata* when in an adult stage. However, any combination of marine micro-algal species (for example *Nannochloropsis occulata*), fed at the prescribed rate (see Table 3), is suitable.

Table 3 Marine micro-algal cell densities to achieve 3250 µg C l^{-1} culture

Approximate cell density (cells ml^{-1}) of marine micro-algae required to provide 3250 µg C l^{-1}
2.5×10^8 (*Isochrysis galbana*)
1.8×10^8 (*Rhinomonas reticulata)*
5.0×10^8 (*Nannochloropsis oculata*)
1.5×10^8 (*Isochrysis galbana*) plus 0.6×10^8 (*Rhinomonas reticulata*)
3.0×10^8 (*Nannochloropsis oculata*) plus 0.6×10^8 (*Rhinomonas reticulata*)

Marine micro-algae should be cultured according to the guidance given in Appendix B.

3.3.3 Culture management

The seawater in each culture should be completely renewed at least once per week and replaced with natural seawater (4.3.1) that has been freshly filtered through a 0.2 µm filter. The culture volume should first be reduced by about 75 % using a slow siphoning process, ensuring the siphon intake is covered with a nylon sieve, typically of 60 µm mesh size. The remaining contents of the culture should then be carefully passed through a sequence of sieves in decreasing order of mesh size (see Table 4) to sort the *Tisbe battagliai* into age/size groups. Approximately 200 ml of the finally sieved culture water should be retained for retrospective water quality determinations.

Table 4 *Tisbe battagliai* sorting mesh sizes

Nominal mesh size (µm)	Stages retained
180	Copepodids and adults
100	All stages , except 0 - 2 day old nauplii
64	All stages

Organisms (from cultures greater than 2 weeks old) retained on the 180 µm mesh comprise the original cohort organisms and should be isolated into a suitable container (for example, a 100 ml crystallising dish) by gently back-washing the mesh with freshly filtered seawater.

The organisms retained on the 100 µm mesh comprise juvenile organisms, some of which should be discarded (in order to maintain the culture at the optimum population density) unless the culture is less than 2 weeks old, when these will represent the original cohort.

Early nauplii stages should be collected on the 64 µm mesh and these can be retained for establishing new cultures. Cultures less than 2 weeks old will be non-reproductive and are unlikely to contain any 0 - 2 day old nauplii.

Both offspring and adult stages may cling to the sides of the culture vessel after the media has been removed. A gentle stream of seawater (4.3.1) delivered from a pipette or wash bottle may be used to 'wash' these organisms onto the appropriate sieves.

The empty culture vessel should then be rinsed with hot water and the remaining seawater discarded. The rinsing process should be repeated with distilled or deionised water and the water discarded before the culture vessel is finally refilled with fresh seawater, and then food added.

The original cohort organisms should then be returned to the culture vessel. In order to maintain the culture at optimal density the culture density may be reduced, i.e. "thinned out" to approximately 100 - 150 organisms per litre of culture before returning to the culture vessel.

In order to maintain cultures in an optimal state, a new culture should be established at each seawater change by pooling the 0 - 2 day old nauplii isolated from all the individual cultures during seawater renewal and inoculating these organisms into a new culture vessel containing fresh seawater and food. The oldest culture should be discarded at this time, or in general, after 4 - 5 weeks following establishment.

The temperature, pH, dissolved oxygen and salinity of both the fresh seawater and the seawater retained from the cultures during renewal should be determined and recorded, for example on the *Tisbe battagliai* culture data form (see Table A2).

Supporting culture information, such as the time taken to produce the first brood, juvenile productivity and the mortality of adults and juveniles should also be recorded, for example on a *Tisbe battagliai* culture data form (see Table A2).

4 Guidelines for toxicity tests using a range of concentrations

The procedure described here is based on previous guidance[3 - 5]. This approach uses glass or plastic test vessels capable of holding at least 5 ml of test dilution for the determination of the toxicity of environmental samples, either as received or using a range of concentrations.

In the approach adopted

(i) The copepodids of *Tisbe battagliai* should be 5 - 6 days old at the start of the test.
(ii) The dilution water used for controls and dilution of samples should be seawater.
(iii) The test organisms should not be fed during the test.
(iv) The temperature of the test dilutions should be maintained at 20 ± 2 °C for the duration of the test.
(v) The pH of the test dilutions should be between 7.7 - 8.3 at the start of the test.
(vi) The dissolved oxygen levels in the test dilutions at the start of the test should be ≥ 50 % ASV at 20 ± 2 °C.
(vii) The salinity of the test dilutions should be between 20 - 36 ‰.
(viii) The lighting regime should comprise a photo-period of 16 ± 1 hours of light and 8 ± 1hours of darkness.

(ix) The *Tisbe battagliai* should be exposed for a duration of 48 ± 4 hours, but observations may also be made at 24 ± 2 hours.

(x) The results of the toxicity test should be rejected if the mean percentage lethality observed in the control dilutions exceeds 10 %.

(xi) The approach taken for samples where any of the threshold criteria for the test dilutions 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 the number of exposure concentrations and interval between test concentrations) will depend on the objective of the study, which should be clearly defined at the outset[1, 20].

4.2 Principle

In the *Tisbe battagliai* lethality test, *Tisbe battagliai* are exposed to aqueous environmental samples diluted with seawater to a range of concentrations for a period of 48 hours. In the context of these procedures, death (lethality) of the *Tisbe battagliai* is deemed to have occurred when no swimming or appendage movement is observed within 10 seconds of gently agitating the test container. The different test concentrations in an appropriate range may, under otherwise identical conditions, exert toxic effects on *Tisbe battagliai*. These effects will extend from the observation of no effect on the *Tisbe battagliai* at lower concentrations (0 % lethality, i.e. no deaths observed) to the observation of total lethality of all *Tisbe battagliai* at higher concentrations (100 % lethality).

The data (i.e. % lethalities observed) should be used to determine:

(i) The effective (lethal) concentrations, i.e. the concentration that results in 10 %, 20 % and 50 % of the exposed *Tisbe battagliai* being killed after 48 hours. The effective (lethal) concentrations are referred to as the 48 hour-LC_{10}, 48 hour-LC_{20} and 48 hour-LC_{50} values respectively.

(ii) The highest concentration where there is no-observed effect after 48 hours. This value is referred to as the no observed effect concentration (i.e. 48 hour-NOEC).

(iii) The lowest concentration where there is an observed effect after 48 hours. This value is referred to as the lowest observed effect concentration (i.e. 48 hour-LOEC).

4.3 Reagents and materials

4.3.1 Dilution water

In toxicity tests, the water used for the controls and the dilution of samples should be seawater.

Natural seawater should be used and should be obtained from a site known to be free of significant contamination. An assessment of the natural seawater 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 known to be toxic towards *Tisbe battagliai*. Seawater with concentrations of these parameters above those deemed 'safe' for *Tisbe battagliai* should not be used.

4.3.2 Hyper-saline brine solution

Hyper-saline brine solution should be used for the salinity adjustment of samples and sample dilutions (2.4.3).

Heat approximately 3 litres of natural seawater water (4.3.1) to 60 °C in an uncovered 5 litre glass beaker. Stir continuously and allow the aqueous contents to evaporate slowly over 24 - 48 hours but do not allow the solution to boil. The salinity of the solution should be monitored during the evaporation process (by sub-sampling and dilution). When the salinity reaches approximately 115 ‰, remove the heat source, but stir continuously while the solution cools to room temperature. When cooled, the solution should be filtered through a 1 µm glass fibre filter into a sterile glass bottle.

The resulting hyper-saline solution can be stored for up to 6 months in the dark at room temperature.

4.3.3 Apparatus

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

Test vessels (for example, multi-well plates capable of holding 5 ml of test solution) made of non-toxic inert material (such as glass or polystyrene).
A temperature environment to maintain test solutions at 20 ± 2 °C.
A low power stereo microscope with 6 - 20 x magnification.
Equipment for measuring pH, dissolved oxygen, salinity and temperature.
Wide bore (1 mm diameter) glass Pasteur pipettes for transferring *Tisbe battagliai*.

4.4 Test procedure

4.4.1 Acquisition of *Tisbe battagliai* copepodids for use in tests

Test organism acquisition should be established 5 days prior to starting the test to allow organisms that are known to be of the required age (i.e. first-stage copepodids) to be generated from mature cultures.

Prior to isolating test organisms from cultures, selected water quality parameters of the dilution water (4.3.1) should be determined and checked against the criteria given in Table 2.

An appropriate number of mature cultures (i.e. greater than 2 weeks old) should be selected and adults isolated on a 180 µm sieve. This should be carried out in the same manner as for culture seawater renewal. The sieve should then be washed to ensure that all juvenile/larval stages are not retained on the sieve. The adult organisms should then be washed into an appropriate isolation vessel (for example, a 1 litre glass beaker). The isolation vessels containing the adult *Tisbe battagliai* should then be filled to 500 ml with

dilution water (4.3.1) and the *Tisbe battagliai* fed with an appropriate volume of marine micro-algae (3.3.2). The date and time of adult isolation should be recorded, for example on a *Tisbe battagliai* culture data form (see Table A2).

Adult *Tisbe battagliai* should be removed from the isolation vessels between 20 - 24 hours after establishment by passing the contents of the vessel through a 100 µm sieve. This ensures that nauplii that are less than 24 hours old pass through the sieve. The adult *Tisbe battagliai* retained on the sieve should be returned to the appropriate culture. The date and time of adult removal from the isolation vessel should be recorded, for example on a *Tisbe battagliai* culture establishment data form (see Table A3). The nauplii remaining in the isolation vessel should then be fed with an appropriate volume of marine micro-algae (3.3.2).

On the day of the commencement of the test (i.e. 5 days after isolation vessel establishment) the contents of the isolation vessel should be passed through a 180 µm sieve followed by a 100 µm sieve. A sample of isolation water (200 ml) should be retained for water quality measurements and the remainder discarded. Any *Tisbe battagliai* retained on the 180 µm mesh should be discarded. *Tisbe battagliai* retained on the 100 µm sieve should then be washed (using as little seawater as possible) into a suitable holding vessel (for example, a 100 ml crytstallising dish). All the organisms obtained from each individual isolation vessel should be pooled together into a single holding vessel and stored at 20 ± 2 °C until required.

4.4.2 Preparation of test dilutions

An appropriate concentration range should be prepared, with the ratio between the concentrations not exceeding 2.2. See Table 5 for the preparation of typical test dilutions comprising 100 ml of test solution. Appropriate details should be recorded, for example on a *Tisbe battagliai* concentration range data form (see Table A4).

Table 5 Preparation of test solutions

Nominal concentration*	Volume of seawater (ml)	Volume of effluent (ml)
0 (control solution)	100	0.0
0.1	99.9	0.1
0.22	99.8	0.22
0.46	99.5	0.46
1.0	99.0	1.0
2.2	97.8	2.2
4.6	95.4	4.6
10.0	90.0	10.0
22.0	78.0	22.0
46.0	54.0	46.0
100.0	0	100

* % v/v

On the day the toxicity test is to commence, the concentration range should be prepared in volumetric flasks by diluting (with seawater) 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, along with six replicates of the control.

The remaining test dilution (i.e. 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, for example in a water quality monitoring data sheet (see Table A5).

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 while 30 organisms (i.e. six groups of five organisms) should be exposed in the controls. Appropriate details should be recorded, for example, see Table A6. The number of copepods in each replicate should not exceed five per 5.0 ml of test dilution (i.e. at least 1.0 ml of test dilution is required per organism).

The copepods should not be fed during the course of test as the inclusion of food in the test medium may:

(i) increase or decrease the effects of the toxicant, as a consequence of the toxicant being adsorbed onto food particles;

(ii) alter the dissolved oxygen content of the test solution by increasing the biochemical oxygen demand;

(iii) alter the physiology of the specific instars used for the test (i.e. first-stage copepodid) and change the uptake and metabolism of the toxicant;

(iv) introduce additional variability into the test.

The copepods should be transferred to the test solutions (under observation with a low power stereo microscope) using a wide bore Pasteur pipette to minimise any damage occurring to the organisms. The *Tisbe* should be checked after transfer by examining the organisms (under a microscope) against a dark background with tangential lighting. If any of the copepods are not active, or appear to have damaged antennae, they should be replaced with substitute copepods from the available pool. It is also critical that the volume of seawater transferred to the test dilution with the copepods be controlled so as not to significantly affect the test concentration. This is best achieved by initially transferring 20-30 copepods 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.

4.4.4 Monitoring water quality

Water quality measurements should be made on aliquots of test dilution (for example 200 ml) which have undergone the same exposure period under identical conditions to the test vessels but do not contain *Tisbe battagliai*.

4.4.5 Terminating the toxicity test

The number of surviving copepods in each test vessel after 48 hours should be recorded, for example on the *Tisbe battagliai* lethality toxicity test data form (see Table A7). Similar

recordings may be made at different times (for example 24 hours) but this is not usually critical for tests undertaken with environmental samples. Copepods are considered dead if they do not exhibit any swimming or appendage movement either within 10 seconds after gently prodding the organisms or following gentle agitation of the test vessel. A low power microscope should be used to aid observations. Any anomalous behaviour of the copepods should also be recorded (for example, see Table A8).

4.5 Processing of results

4.5.1 Validity of the results

The results of the toxicity test should be rejected if the percentage lethality observed in the control exposures exceeds 10 %. In addition, data from tests on effluents or leachates should only be accepted if the results of the concurrent reference toxicant test (see section 6) meet internal quality criteria[1, 20].

4.5.2 Data handling

Endpoints such as the LC_{10}, LC_{20}, LC_{50}, NOEC and LOEC values should be determined using an appropriate validated computer-based statistical package. The endpoint values for a *Tisbe battagliai* lethality toxicity test are based on the percentage of dead *Tisbe battagliai* at 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 *Tisbe battagliai* (usually 5 per replicate) and either the number which have died or the number which have survived, are entered for each test vessel and a proportional effect (taking into account the corresponding numbers for the control exposures) is calculated.

4.5.3 Estimation of LC values

The 48 hour-LC_{10}, 48 hour-LC_{20} and 48 hour-LC_{50} values (and other LC values, if necessary) should be determined using appropriate statistical procedures (see Figure 3). Confidence limits ($p = 0.95$) for the calculated LC value should be determined and quoted with the test results.

Figure 3 Flowchart for the estimation of the LC_{50} value for full concentration range tests

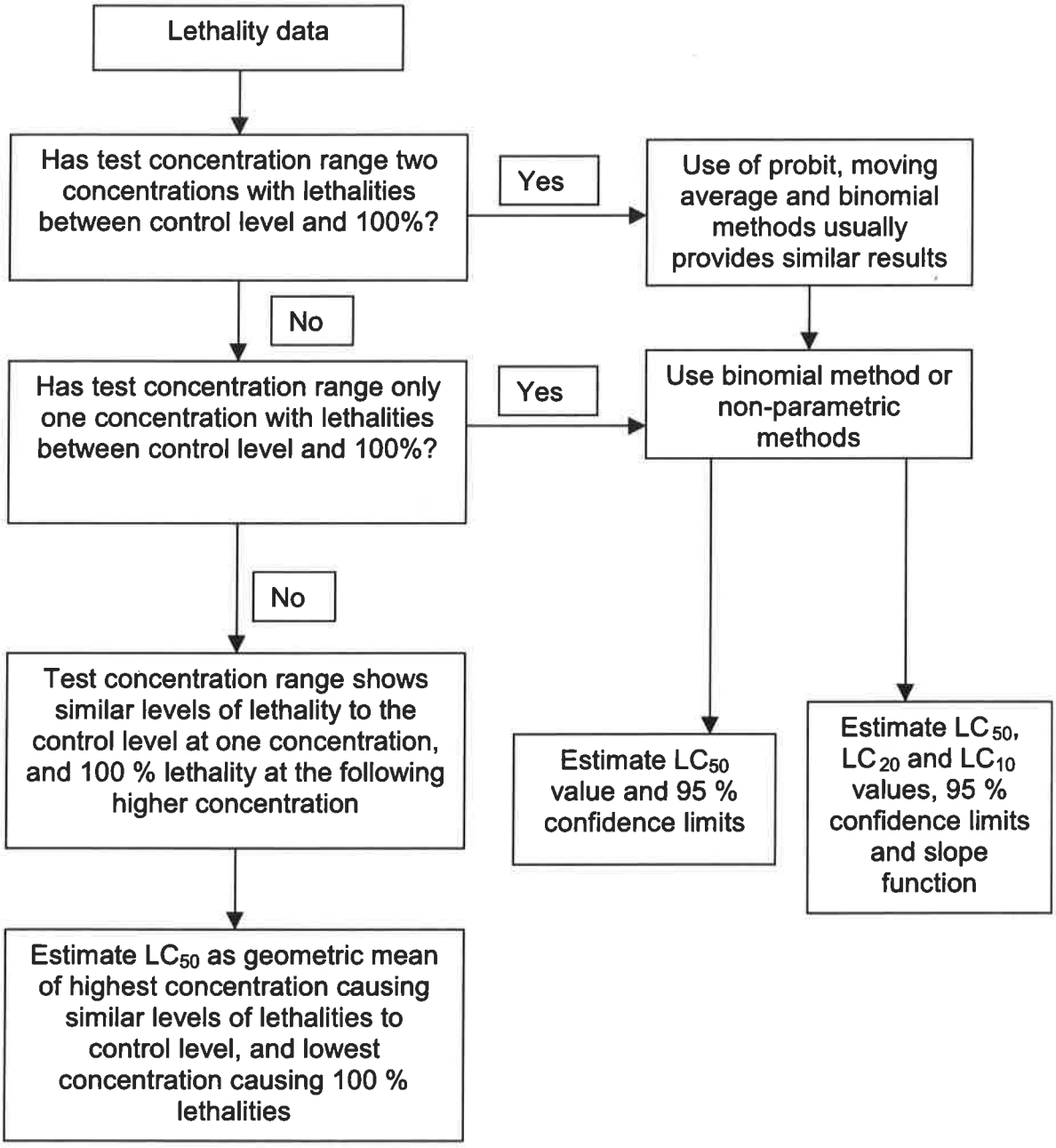


Table 6 shows examples of data produced to show the effect on *Tisbe battagliai* survival by an effluent. Table 7 summarises the LC_{50} values derived from the data in Table 6 using different statistical procedures.

Table 6 Example results of the lethality of *Tisbe battagliai* by an effluent after 48 hours exposure

		Scenario 1		Scenario 2		Scenario 3	
Effluent concentration* (%)	Number of *Tisbe battagliai* exposed	Cumulative number of dead organisms	Lethality (%)	Cumulative number of dead organisms	Lethality (%)	Cumulative number of dead organisms	Lethality (%)
Control	30	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 48 hour-LC_{50} values (and 95% confidence limits) for the data in Table 6 estimated by different statistical procedures

Scenario	Statistical procedure	48 hour-LC_{50} (%)	95 % confidence limits	Slope
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		approach not valid	
	Moving average		approach not valid	
	Binomial*	4.8	2.2 - 10	
	Spearman-Karber (Tox Calc)	4.8	4.1 - 5.7	
3	Geometric mean	3.2		

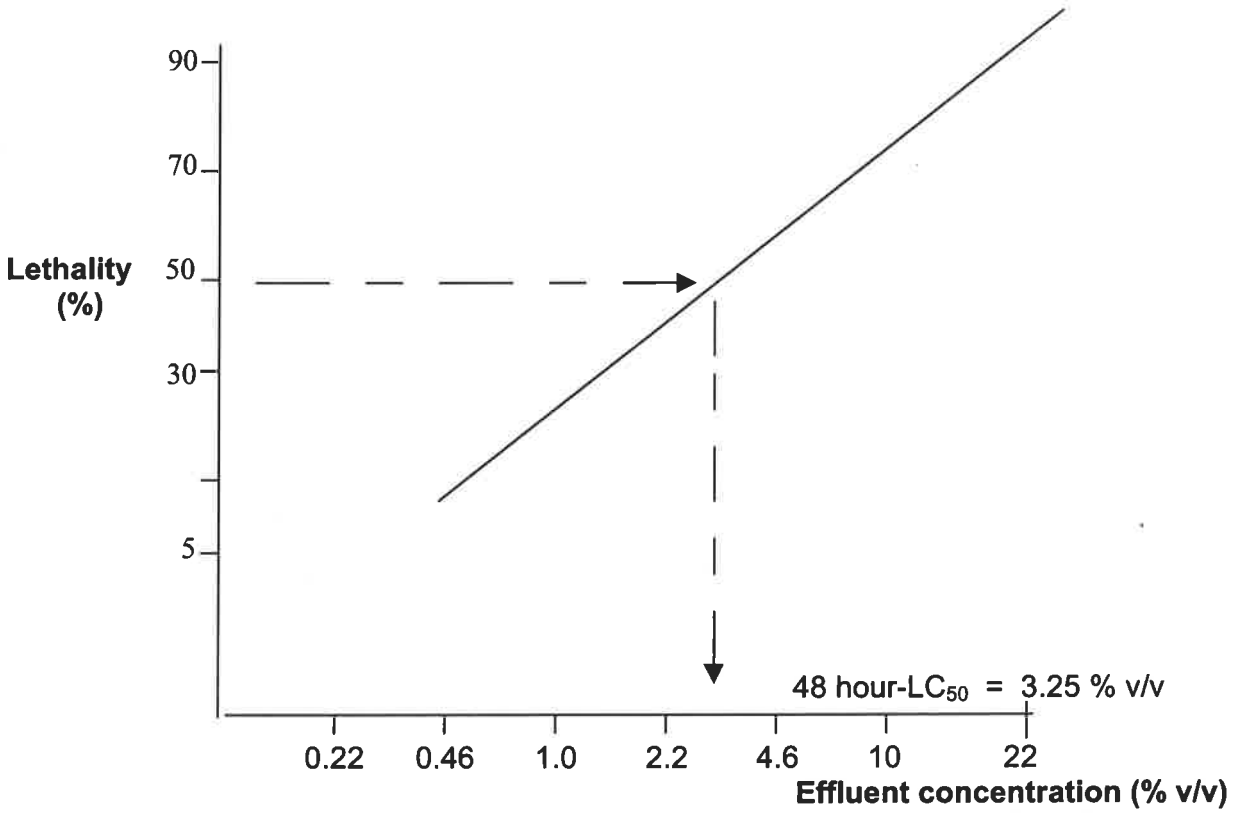
* Computer-based statistical package[21]

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 LC_{50} values derived are less statistically sound.

From the data shown in Table 6, the 48 hour-LC_{50} 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 *Tisbe battagliai* mortality (probability scale) against effluent concentration (log scale) shown in Figure 4 for Scenario 1, the 48 hour-LC_{50} is 3.25 % v/v effluent. The values obtained graphically complement those obtained using computer-based software (see Table 7).

Figure 4 Dose response curve of cumulative lethality (probability scale) against effluent concentration (log scale)



When analysing data from the *Tisbe battaglia*i lethality test the following points should be considered:

(i) 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 0 - 10 % and 100 %, then probit, moving average and binomial methods should provide similar estimates of the LC values. Probit analysis should be used to estimate LC values, 95% confidence limits and the slope of the dose response curve.

(ii) If the results do not include two concentrations at which lethality 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 LC_{50} value with wide confidence limits. The use of non-parametric methods such as the Spearman-Karber or Trimmed Spearman Karber methods may enable the determination of an LC_{50} value to be made.

(iii) Where the data obtained are inadequate for calculating an LC_{50} value, the highest concentration causing no lethality and the lowest concentration causing 100 % lethality should be identified. An approximation of the LC_{50} 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 LC_{50} calculated will be less statistically sound.

(iv) In all instances, the LC_{50} value derived from any of the above procedures should be compared with a graphical plot on logarithmic-probability (log-probit) scale of percent lethality for the various test concentrations. Any major disparity between the graphical estimation of the LC_{50} value and that derived from the statistical programmes should be resolved.

4.5.4 Estimation of the NOEC and LOEC

In the marine copepod *Tisbe battagliai* lethality test, the NOEC and LOEC values should be calculated using Fisher's exact test.

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 *Tisbe battagliai* and in these cases the sample should be adjusted using the procedures described in section 2.

Toxicity tests with *Tisbe battagliai* for monitoring or screening against defined toxicity limits may also be carried out on a single concentration of effluent or leachate sample (toxicity limit) and appropriate controls. The concentration of effluent or leachate would need to be appropriately chosen.

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) and recorded, for example on a water quality monitoring data form (see Table A5).

5.3 Processing of results

An assessment of how the responses in the single effluent or leachate concentration compare to those in the control solution 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 surviving 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[22 - 24].

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 ($t = 1.20$, $p > 0.05$).

Figure 5 Flowchart for the analysis of single concentration test data from the *Tisbe battagliai* lethality test

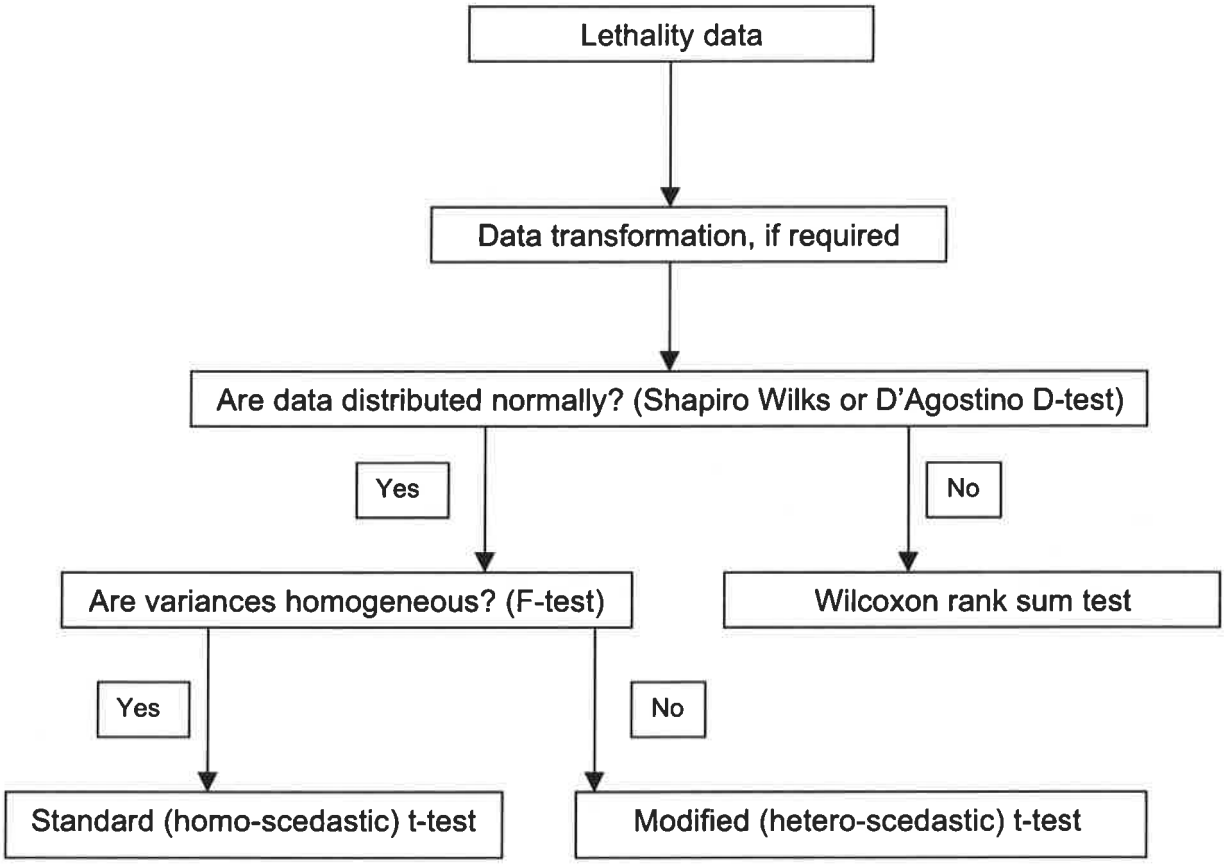


Table 8 Examples of data for a single concentration *Tisbe battagliai* lethality test and the results of statistical analysis

Effluent concentration (%)	Number of *Tisbe battagliai* exposed	Cumulative number of dead organisms	Lethality (%)	Method of statistical analysis	Result of statistical analysis
Scenario 1					
0 (Control)	30	0	0	Modified t-test	Significant difference ($p<0.05$)
0.46*	30	3	10		
Scenario 2					
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

6 Guidelines for reference toxicant tests using zinc

6.1 Design

Tisbe battagliai lethality tests which 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 ($ZnSO_4.7H_2O$) 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 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-LC_{50} 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-LC_{50} values should be calculated using the procedures described in section 4.

7 References

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22 Biometry, R R Sokal and F J Rohlf, 1981, W H Freeman and Company, San Francisco, pp859.

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APPENDIX A Examples of typical record forms

Table A1 Chain of custody record form

a) Sample details (to be completed by the sampling officer or following analysis at the laboratory*)

Sample details:
Site code or National Grid Reference:
Place:
Sample of:
Date taken:
Time taken:
Sample number:
Receipt number (if applicable):
Sample pH:
Sample temperature (°C):
Sample dissolved oxygen level (% ASV) at 20 ± 2 °C:
Sample salinity (‰):
Is sample an emulsion (Y/N):
Date and time sample deposited at:
By (print name): Signature:

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:

c) To be completed at laboratory

Sample received at (laboratory):
Date:
Time:
Sample received by (print name): Signature:
Were seals intact? Y/N Allocated laboratory code:
* Delete as appropriate

Table A2 *Tisbe battagliai* culture data form

Year: Period:

Sheet number of sheets Culture number:

Date: Time: Initials:

Renewal of water
Age of culture
Number of adults in culture
Number of juveniles in culture
Number of dead organisms
Volume of seawater replaced (litres)
Temperature (°C) - Water containing juveniles
- New culture water
pH - New culture water
Dissolved oxygen (% ASV) at 20 ± 2 °C - New culture water
Salinity (‰) - New culture water
Feeding regime
Separation of organisms
Isolation vessel number
Volume of culture water in isolation vessels
Temperature (°C) - Isolation water
pH - Isolation water
Dissolved oxygen (% ASV) at 20 ± 2 °C - Isolation water
Salinity (‰) - Isolation water
Number of adults separated
Number of nauplii recovered and number of adults retained on mesh

Table A3 *Tisbe battagliai* culture establishment data form

Source of juveniles
Isolation vessel number:
Main *Tisbe battagliai* culture number (from Table A2): New culture number:
Juveniles isolated at on :
Number of juveniles isolated: Number immobilised/dead juveniles:
Approximate age of juveniles:
Water quality
Temperature (°C) - Water containing juveniles
- New culture water
pH - New culture water
Dissolved oxygen (% ASV) - New culture water
Salinity (‰) - New culture water
Date Time Initials
Transfer of juveniles
Time juveniles transferred:
Feeding regime
Initials

Table A4 Toxicity test concentration range

Test substance:
Type of test:
Start of test: End of test:
Duration of test:
Number of test concentrations:
Number of controls:
Number of *Tisbe battagliai* per concentration:
Number of replicates per concentration:
Concentration range
Stock solution concentration:
Dilution media:
Volume of test solution required (ml):

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

Table A5 Water quality monitoring data form

Date
Time
Vessel number
Nominal concentration
Temperature (°C)
pH
Dissolved oxygen (% ASV) at 20 ± 2 °C
Salinity (‰)
Initials

Table A6 Test report for provision of copepodids

Data on test species
Origin of test species:
Holding conditions: Temperature (°C)
pH
Dissolved oxygen (% ASV) at 20 ± 2 °C
Salinity (‰)
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 (hours)

Collection of copepodids
Time collection of copepodids started:
Time collection of copepodids ended:
Age of *Tisbe battagliai* at start of test (hours):

Table A7 *Tisbe battagliai* lethality toxicity test data form

Test substance:
Date and time *Tisbe battagliai* added:
Exposure concentration: Replicate: Time after start of the test (hours):

	Number of *Tisbe battagliai* surviving	Number of dead *Tisbe battagliai*

Table A8 Expression of results

Exposure period	Exposure concentration	Cumulative number of dead *Tisbe battagliai* in each replicate	Total number of dead *Tisbe battagliai*	Total number of *Tisbe battagliai* exposed	*Tisbe battagliai* lethality (%)

Calculation of LC_{50} and LC_{10} values
Exposure period (hours):
Method used: LC_{50} LC_{10}
Concentrations used in the calculation of the LC_{50}:

Calculation of NOEC and LOEC values
Exposure period (hours):
Method used: NOEC LOEC

Abnormal behaviour of the *Tisbe battagliai* during the test

Details not specified in the procedure and any other factors that may have affected the result:

Appendix B Marine micro-algae culture as food for *Tisbe battagliai*

B1.1 Introduction

This section describes details of the procedures, materials and equipment required for the culture of marine micro-algae, as a source of food for cultures of *Tisbe battagliai.*

B1.2 Source cultures

Axenic (un-contaminated) uni-algal source cultures should be obtained from reliable sources.

Source cultures should be held at 15 ± 2 °C under moderate illumination of 1000 ± 100 lux with a photo-period of 16 hours of light and 8 hours of darkness. Under these conditions the cultures should be viable for up to 21 days after receipt.

B1.3 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 is 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. This process is then repeated.

Sub-cultures should be grown in 500 ml conical flasks containing 100 ml of growth media at 20 ± 2°C under 10000 ± 1000 lux with a photo-period of 16 hours of light and 8 hours of darkness. Flasks should be mixed intermittently every 24 - 48 hours by gentle hand-swirling.

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 lead to the culture failing to grow. Marine micro-algae are particularly susceptible to such contamination, being relatively slow growing compared to many routinely cultured freshwater species. This may allow time for bacteria to grow in sufficient quantities to compete with the algal cells for available nutrients.

Sub-cultures should be inoculated with 3.33×10^6 - 3.33×10^7 cells l^{-1} and sub-cultured at intervals of 7 - 10 days.

Mature sub-cultures (i.e. 7 - 10 days old) should be used to inoculate large scale batch cultures.

B1.4 Batch cultures

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

Batch cultures should be grown under identical conditions to sub-cultures using 2 litres of growth media in a 2.5 litre conical flask, and inoculated with 3.33×10^6 - 3.33×10^7 cells l^{-1}.

Batch cultures should be mixed and aerated using a continuous, sterile, oil-free air source and grown for up to 10 days before concentration.

Following incubation, batch cultures should be concentrated, by centrifugation, to produce algal-feeding stocks of known cell density and organic carbon content.

B1.5 Growth media

The media used to culture marine micro-algae should be natural seawater of salinity 25 - 35 ‰ filtered through a 0.2 µm filter, with additional nutrients as given in Table B1.

Nutrients should be added in the form of concentrated stock solutions (prepared using analytical grade reagents as given in Table B1) dissolved in reverse osmosis grade water. Stock solutions should be filter-sterilised and may be stored at 4 °C in the dark for up to 3 months.

Immediately before use, 1.0 ml of stock solution A and 0.05 ml of stock solution B should be added to each litre of seawater and the final solution filtered-sterilised prior to algal inoculation.

Table B1 Preparation of algal media stock solutions

Stock solutions should be prepared by dissolving the following amounts of reagents in water and making to 1000 ml.

Stock solutions	Amount
Stock solution A (Nutrients)	
sodium nitrate, $NaNO_3$	100 g
sodium di-hydrogen phosphate dihydrate, $NaH_2PO_4.2H_2O$	20 g
iron(III) chloride hexahydrate, $FeCl_3.6H_2O$	1.3 g
manganese chloride tetrahydrate, $MnCl_2.4H_2O$	0.36 g
boric acid, H_3BO_3	33.6 g
di-sodium EDTA, $Na_2EDTA.2H_2O$	45 g
stock solution C	1 ml
Stock solution B (Vitamins)	
vitamin B_1 (thiamine hydrochloride)	0.4 g
vitamin B_{12} (cyanocobalamin)	0.02 g *
Stock solution C (Trace metals) **	
zinc chloride, $ZnCl_2$	21 g
cobalt chloride hexahydrate, $CoCl_2.6H_2O$	20 g
ammonium molybdate tetrahydrate, $(NH_4)_6Mo_7O_{24}.4H_2O$	9 g
copper sulphate pentahydrate, $CuSO_4.5H_2O$	20 g

* This should be added from a concentrated solution.

** Stock solution C should be acidified by the drop-wise addition of N/10 hydrochloric acid until the solution remains clear.

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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Standing Committee of Analysts

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