Environment Agency

The direct toxicity assessment of aqueous environmental samples using the *Skeletonema costatum* marine algal growth inhibition test (2009)

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 marine *Skeletonema costatum* algal growth inhibition 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. Four documents have already been published in this series[2 - 5].

No performance data are included with this method which has been rigorously tested under Agency funded development work[6, 7]. 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.

This document replaces the earlier published version correcting the formulation for the stock solution 3 in Appendix A.

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, soils (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 these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the Agency's web-page (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. Users should ensure they are aware of the most recent version of the draft they seek.

Dr D Westwood
Secretary
July 2006

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 all regulations made under the 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, receiving waters and discharges.
ASTM	American Society for Testing and Materials.
ASV	air saturation value.
DTA	direct toxicity assessment.
EC_{10}	the concentration that results in 10 % inhibition of algal growth.
EC_{50}	the concentration that results in 50% inhibition of algal growth.
EDTA	ethylendiamine tetraacetic acid.
ISO	International Standards Organisation.
LOEC	lowest concentration where there is an observed effect compared to control dilutions.
NOEC	highest concentration where there is no-observed effect compared to control dilutions.
OECD	Organisation for Economic Co-operation and Development.
Quantal effect	An effect for which there are only two possible outcomes. In ecotoxicological terms this applies to measurements based on movement (i.e. mobile or immobile) lethality (i.e. alive or dead) or development (i.e. growth or no growth).
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 *Skeletonema costatum* marine algal growth inhibition test

1 Introduction

The procedures described in this document enable direct toxicity assessments to be carried out on aqueous environmental samples using the marine alga *Skeletonema costatum.* The procedures described are based on an Environment Agency project[6, 7] but also take into account existing guidelines and more recent method developments[8].

The marine algal growth inhibition 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[9]. 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[6, 7, 10, 11, 12].

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 the samples 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[13, 14]. 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 the sample was taken. Any other relevant information, such as the name of the sampling officer and "chain of custody" record form details should also be recorded.

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

pH
Temperature
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 this should be recorded in the test report and details given. 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 21 ± 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, salinity, colour and suspended solid content, however, this is not a requirement for regulatory effluent assessments.

For regulatory DTA testing (i.e. tests conducted on effluents), modification or adjustment of the sample, or its dilutions, should be made so that any influence from the water quality parameters determined is removed. Test results will therefore reflect the residual chemical toxicity of the discharge at the water quality ranges outlined in sections 2.4.1 - 2.4.4. 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 ranges within the area of discharge of a particular effluent, the actual measured ranges should be used.

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 lie within the ranges described.

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.

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

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. The acceptable pH range for the testing of *Skeletonema costatum* growth is 7.5 to 8.5. Test dilutions with a pH outside of this range should be adjusted accordingly.

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[15]. Test dilutions that have been pH-adjusted should only be used when the pH has stabilised.

2.4.2 Suspended solids

High levels of suspended solids may adversely affect the algae and cause inhibition of growth that is not directly attributable to the toxicity of the sample.

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 solution through a cellulose acetate or cellulose nitrate membrane filter (nominal pore size 0.45 µm) using a vacuum filtration unit.

(ii) Centrifuging the solution at 5000 - 10000 g for 15 - 60 minutes using a suitable centrifuge. Centrifuging the solution at low speeds (3000 - 5000 g) for longer periods (60 minutes) may be used as an alternative approach to short high speeds (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 solutions, or samples, and the same procedure should be used when testing a series of samples from the same location.

2.4.3 Colour

Coloured test dilutions may alter the amount of light reaching the test organisms by filtering out certain wavelengths and reducing the overall amount of light available[16]. Continuous stirring or shaking with test systems generally assists in ensuring that light can penetrate to the degree that each organism (i.e. algal cell) will be exposed for a sufficient part of the test. Consequently coloured or turbid test dilutions can be considered acceptable for testing with *Skeletonema costatum* if the absorbance of light at 440 nm is not greater than 0.5 (as measured in a 40 mm path length cell).

2.4.4 Salinity

The salinity of test dilutions should be adjusted if values fall outside the range 25 - 35 ‰. 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 the algae 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 end-point 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 end-point 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.

2.4.5 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 solutions and samples should be disposed of according to documented procedures.

3 *Skeletonema costatum* algal growth inhibition test

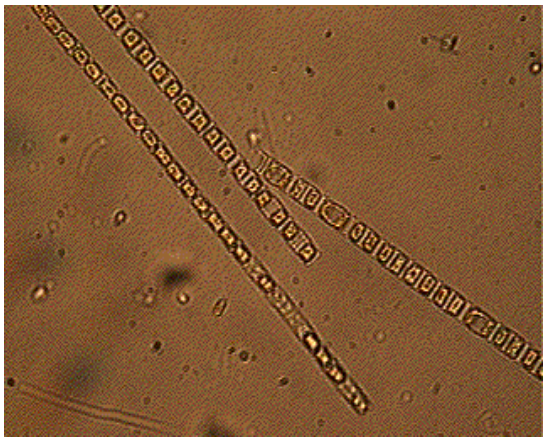
3.1 Introduction

Based on previously published guidance[6, 7, 21], procedures are described for culturing the marine alga *Skeletonema costatum*, and for conducting static toxicity tests to assess the effects of aqueous environmental samples on algal growth.

3.2 Test organism

Skeletonema costatum is a brick-shaped marine diatom (Bacillariophyta) (see Figure 1). The cells of this species form chains of varying cell numbers and length.

Figure 1 *Skeletonema costatum* cells



Algal inoculant may be derived from healthy, exponentially growing stock cultures that have been maintained under specified culture conditions.

3.3 Culturing of *Skeletonema costatum*

The following procedures should enable *Skeletonema costatum* to be laboratory cultured and used for assessing the toxicity of aqueous environmental samples.

The production and management of *Skeletonema costatum* cultures can be achieved in a number of ways without adversely affecting the quality of the algal cells produced. The following guidance enables the establishment and maintenance of an effective set of cultures, but does not preclude the use of different procedures where these have been shown to be effective in producing good algal cultures.

3.3.1 Source cultures

Source cultures (stored at approximately 15 °C under moderate illumination (approximately 1000 lux) with a 16:8 hour light:dark photo-period should remain viable for up to 10 days. After this time new cultures should be obtained. The contamination of source cultures should be avoided, for example by using sterile equipment (such as sterile pipette tips) when removing and transferring aliquots of algal cells. Any source culture, which displays signs of bacterial contamination, for example if the media becomes discoloured or gelatinous, should be discarded and replaced.

3.3.2 Nutrient medium

A nutrient medium is used to culture *Skeletonema costatum* and is prepared as described in Appendix A. Water used in the preparation of nutrient media should be sterilised seawater. The sterilisation of the seawater should be achieved by using a sterile nominal pore size 0.2 µm filter rather than by heating (for example using an autoclave) to avoid precipitation of dissolved salts.

3.3.3 Maintenance of cultures

Initially, cultures should be inoculated from the source culture by adding (under sterile conditions) 100 ml of nutrient medium (see Appendix A) and 3-5 ml of source culture to a 250 ml sterile glass conical flask. The opening of the flask should be plugged, for example with sterile, non-absorbent cotton wool. This primary culture should be incubated under continuous fluorescent illumination (between 6000 - 10000 lux) whilst being shaken or stirred at 21 ± 2 °C. After 5 - 7 days incubation, this culture should be of sufficient cell density to be used to inoculate an initial sub-culture (see below) or inoculate a pre-test culture.

A sub-culture should be prepared by transferring 5 ml of the primary culture to 100 ml of fresh nutrient medium (see Appendix A) and incubating under continuous fluorescent illumination (between 6000 - 10000 lux) whilst being shaken or stirred at 21 ± 2 °C for 5 - 7 days. From this initial sub-culture, a series of sub-cultures can subsequently be prepared in the same way, i.e. transferring 5 ml of the previously incubated sub-culture to 100 ml of fresh nutrient medium and incubating under continuous fluorescent illumination (between 6000 - 10000 lux) whilst being shaken or stirred at 21 ± 2 °C for 5 - 7 days.

In all cases, primary cultures or sub-cultures should only be used for further sub-culturing, or for inoculating a pre-test culture, if the cell density of the culture reaches a sufficient level, i.e. a light absorbance greater than or equal 0.5. This may be determined by measuring the light absorbance of the culture at 440 nm in a 40 mm path length cell.

The production of primary cultures and sub-cultures should be semi-continuous, with each primary culture being sub-cultured on no more than six occasions before a new primary culture is produced from the source culture. The continual renewing of sub-cultures prevents the accumulation of bacteria and other micro-organisms and the gradual reduction in cell numbers over successive sub-cultures owing to lower nutrient availability.

4 Guidelines for toxicity tests using a range of concentrations

Two approaches to the test may be used:

(i) A conventional approach based on previous guidance[6, 7, 21]. This approach uses glass test vessels (usually conical flasks) capable of holding 50 - 250 ml of test dilution. The assessment of algal cell density in such tests may be by direct cell counting (for example microscopic or particle analysis of the number of algal cells present) or may utilise a surrogate measure for cell density such as fluorescence or absorbance.

(ii) A contemporary approach based on a combination of previous guidance[6, 7, 21]. and more recent developments of the test[8]. This approach uses inert plastic or glass 96-well plates capable of holding up to 400 µl of test dilution (per well). The

assessment of algal growth in such tests is usually achieved using a surrogate measure for cell density such as fluorescence or absorbance.

This miniaturised approach is particularly useful for the screening of effluents and TIE and/or TSE exercises in which abbreviated or 'high-throughput' versions of the method are required. Such versions generally involve reduced 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. Recent research has led to the development of the miniaturised marine water algae growth inhibition test in the UK and addresses issues of multi-well evaporation, gas exchange, chemical adherence to well plates, and potential loss of volatile substances[8].

In both approaches,

(i) *Skeletonema costatum* should be exposed for a duration of 72 ± 4 hours.
(ii) Dilution water should comprise nutrient medium.
(iii) The temperature of the test dilutions should be 21 ± 2 °C.
(iv) The pH of the test dilutions in all of the test vessels should be between 7.5 - 8.5.
(v) The lighting regime should be continuous and comprise "cool white" fluorescent light of 6000 - 10000 lux at the surface of the test dilution.
(vi) The results from toxicity tests with *Skeletonema costatum* should only be considered valid if, in the controls, the mean algal cell density increases by a factor of more than 16.
(vii) The approach taken for samples where any of the water quality parameters for the test dilutions fall outside of the indicated ranges 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 light of the objectives of the testing programme.

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[8, 22].

4.2 Principle

In the marine algal growth inhibition test, exponentially growing uni-algal cultures of *Skeletonema costatum* should be exposed for a period of 72 ± 4 hours at 21 ± 2 °C to a range of concentrations of the environmental sample diluted with nutrient medium. The different test dilutions in an appropriate test concentration range, under otherwise identical test conditions, may exert toxic effects on the growth of *Skeletonema costatum*. The end-point values for the *Skeletonema costatum* algal growth inhibition test are based on the average estimated algal cell density for each replicated test concentration. In the context of these procedures, the inhibition of growth (relative to a control culture exposed to identical conditions) is determined under static conditions. This will extend from no inhibition of growth (at lower test concentrations) to complete inhibition of growth (at higher test concentrations).

The data (i.e. population cell density) should be used to determine:

- The effective concentrations, i.e. the concentration that results in 10% and 50% inhibition of growth after 72 hours. These effective concentrations are referred to as the 72-hour EC_{10} and 72-hour EC_{50} values respectively.

- The highest concentration where there is no-observed effect after 72 hours. This value is referred to as the no observed effect concentration (72 hour-NOEC).

- The lowest concentration where there is an observed effect after 72 hours. This value is referred to as the lowest observed effect concentration (72 hour-LOEC).

4.3 Reagents and materials

4.3.1 Dilution water

In *Skeletonema costatum* algal growth inhibition tests, dilution water used for controls and the dilution of samples should be nutrient medium (see Appendix A).

Since EDTA may chelate dissolved metals, thus making them biologically unavailable, the use of a medium containing lower than conventional amounts of EDTA (while still providing sufficient nutrient for good algal growth) reduces the likelihood of obtaining erroneously low toxicity values for environmental samples which contain significant amounts of metals.

The preparation of a 'low-EDTA' nutrient medium is described in Appendix A, however, it is critical that in tests the nutrient stock solutions are added at a rate that achieves an equivalent concentration of nutrients in all of the test dilutions. For this reason the medium should be prepared individually for each test dilution and not added as a pre-prepared medium. The medium stock solutions are therefore added to each individual test dilution at the following rates:

Stock solution 1:	15 ml per 1000 ml of test dilution.
Stock solution 2:	0.5 ml per 1000 ml of test dilution.
Stock solution 3:	1 ml per 1000 ml of test dilution.

Care should be taken to avoid contamination of the dilution water with inorganic or organic substances during preparation and storage. Copper apparatus 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 (see section 2.4.4).

Heat approximately 3 litres of natural seawater to 60 °C in an uncovered 5 litre glass beaker. Stir the contents 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 nominal pore size 1 µm glass fibre filter into a sterile glass bottle.

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

4.3.3 Apparatus

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

Test vessels (250 ml glass conical flasks or polystyrene 96-well plates).
A temperature-controlled environment to maintain test dilutions at 21 ± 2 °C.
Equipment for estimating population cell density, for example Coulter particle counter or microscope with counting chamber (for example Sedgewick-Rafter cell). Alternative equipment (for example, fluorescence plate reader or spectrophotometer) will be required if surrogate parameters are used instead of algal cell density measurements.
Equipment for providing continuous illumination at 6000 - 10000 lux.
Equipment for measuring pH.
Equipment for measuring salinity.

4.4 Test procedure

The following procedures enable marine algae to be cultured and used for conducting toxicity tests to measure the effects of environmental samples on their growth.

4.4.1 Acquisition of *Skeletonema costatum* inoculant for use in tests

Skeletonema costatum cells to be used in tests may be obtained from laboratory cultures (exhibiting exponential growth characteristics).

From laboratory sub-cultures, a pre-test culture is established by adding 5 ml of a primary culture or 5 ml of a sub-culture to 100 ml of fresh nutrient medium and incubating under continuous fluorescent illumination (between 6000 - 10000 lux) whilst being shaken or stirred at 21 ± 2 °C for 5 - 7 days.

On the day of the test, the pre-test culture should be assessed for cell density. This can be measured either directly (see section 4.4.3) or by using light absorbance as a surrogate density measurement. Pre-test cultures should only be used for subsequent tests if they attain a cell density of equal to or greater than 1×10^7 cells per ml (or an absorbance of 0.5 at 440 nm using a 40 mm path length).

4.4.2 Preparation of test dilutions

An appropriate series of concentrations should be prepared with the ratio between consecutive test concentrations not exceeding 2.2. See Table 2 for the preparation of, for example 1000 ml, of typical test dilutions. Appropriate details should be recorded. On the day of the toxicity test, the concentration range should be prepared in volumetric flasks by adding the appropriate amounts (see section 4.3.1) of nutrient stock solutions 1 - 3, and sample to each flask and making to the required volume with seawater. For each test series, a control should be prepared which only contains nutrient medium. At least four replicate test vessels should be used for tests using glass conical flasks. Tests utilising 96-well plates should include at least ten replicates of each test concentration.

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

Table 2 Preparation of test dilutions

Nominal concentration (% v/v effluent)	Volume of effluent (ml)	Volume of seawater (ml)
0 (control solution)	0	983.5
0.1	1	982.5
0.22	2.2	981.3
0.46	4.6	978.9
1.0	10	973.5
2.2	22	961.5
4.6	46	937.5
10.0	100	883.5
22.0	220	763.5
46.0	460	523.5
98.4	983.5	0

Range of concentrations is expressed as a percent of the effluent sample concentration.
The appropriate amounts (see section 4.3.1) of nutrient stock solutions also need to be added.

4.4.3 Initiation of the toxicity test

Transfer the required volume of test concentration from each flask to the test vessels. If 250 ml conical flasks are used, 100 ml of test solution should be transferred to each vessel. If 96-well plates are used, 200 µl should be transferred to each well.

Test dilutions should be inoculated with a nominal number of 2500 cells per ml. To attain this level, the amount of pre-test culture to be added to the test dilution should be determined by examining the pre-test culture microscopically. A single full grid of a 0.1 µm haemocytometer is assessed for the number of algal cells present under a magnification of 200x and the mean count converted to an estimate of the total number of cells per ml using the following calculation:

Total number of cells per ml = Mean number of cells in a haemocytometer grid x 10000

The amount of pre-test culture to add to each test vessel is then calculated from:

$$\text{Amount of pre-test culture (ml)} = \frac{2500 \times \text{Volume of test dilution (ml)}}{\text{Total number of cells per ml}}$$

To each vessel or well, add a volume of the initial inoculum, sufficient to achieve an initial cell density population of 2500 ± 250 per ml. A sterile graduated pipette should be used to transfer the inoculum volume. For tests using 250 ml conical flasks, the appropriate amount of algae should be added directly to the individual test vessel. For tests using 96-well plates, it may be beneficial to add the algae direct to the test dilution in the volumetric flask (i.e. before adding to test vessels) to prevent the need to transfer extremely small volumes of algal suspension.

The pH, salinity and absorbance (measured at 440 nm in a 40 mm path length cell) of the control solution and each concentration should be measured (on an appropriate volume of the remaining test dilution, i.e. that volume not added to the test vessels) and recorded.

If a surrogate measure is used to estimate cell density, this may be measured in each test replicate prior to commencement of the test to assist in endpoint calculation using the surrogate measure.

The neck of the flasks containing the inoculated test dilutions should then be plugged (for example using sterile cotton wool) or if 96-well plates are used, the well should be covered appropriately (for example using a plate cover or 'breathable' plastic film). The vessel or plate should then be placed under constant illumination (at 6000 - 10000 lux) and incubated at 21 ± 2 °C for 72 ± 4 hours. Care should be taken, especially if using 96-well plate systems, not to stack or otherwise cover the top of the plates, which should all receive approximately the same amount of light within the incubation chamber. During the incubation period the vessels should be gently shaken, for example at 100 - 130 rpm to maintain the algae in suspension.

4.4.4 Monitoring of the toxicity test

During the exposure period, the algal cell density (or surrogate measure) in each test vessel or well, including the controls, should be measured every 24 ± 4 hours.

Where 250 ml conical flasks have been used, these measurements should be made (and appropriate data recorded) on small aliquot volumes (for example, 5 ml) removed from the test vessel using a sterile pipette. After the determination, the aliquot removed and examined should be discarded and not returned to the test vessel.

If 96-well plate systems are used, the entire well contents should be assessed using an appropriate measuring technique. Aliquots of test dilutions should not be removed from the wells.

4.4.5 Terminating the toxicity test

The test should be terminated after 72 ± 4 hours.

The pH and salinity of the control solution and each concentration should be measured (using the remaining test dilution not added to test vessels) and recorded.

The algal cell density (or surrogate measure) of each test replicate should then be determined as described in section 4.5.2.

4.5 Processing of results

4.5.1 Validity of the results

The results from algal growth inhibition toxicity tests should only be considered valid if, in 72 hours, the algal cell density in the control vessels (as a mean) increases by a factor of more than 16, i.e. to a cell density of over 400000 per ml.

In addition, the level of variability between control replicates, as described by their coefficient of variation should be less than 20 %.

Data from tests on effluents or leachates for discharge characterisation should only be accepted if the results of the concurrent reference toxicant test (see section 6) meet the specified quality control criteria[22].

If surrogate measures (for example, fluorescence or absorbance measurements) are used to represent algal cell density, the relationship between actual algal cell density (as

determined directly) and the surrogate measure should be clearly defined and validated as a true surrogate (i.e. the general dose-response relationship observed in measurements made on algal cell numbers is demonstrated by the substituted observation). Where good quality validation data can be demonstrated, the surrogate measure may be used directly in the test end-point calculations. Where there is doubt over this relationship or evidence that it may change over time, it may be beneficial to conduct routine assessments of the relationship. These should be obtained under reference conditions and the most recent data used to convert a surrogate measurement into an estimated algal cell density.

4.5.2 Data handling

Endpoints such as the EC_{10}, EC_{50}, NOEC and LOEC values should be determined using appropriate validated computer-based statistical packages. The endpoint values for the marine water algae growth inhibition toxicity test are based on the amount of algal growth in each test concentration relative to the control.

There are a number of different mathematical techniques available for calculating the degree of growth inhibition in marine water algae tests. Traditionally, the reduction in growth rate of algal populations in test replicates relative to controls has been used as a measure of growth inhibition. This technique remains widely employed, especially in chemical-specific assessments. An alternative approach is also applied [21, 23] which uses an estimate of the biomass integral of algae present (i.e. the area under the growth curve) in each test replicate relative to control biomass to measure inhibition. The two approaches do not produce similar results and all attempts to validate them as equivalent alternatives have so far failed. In general, biomass-based endpoints tend to produce significantly lower EC_{50} values than growth rate endpoints using the same raw data.

Regulatory DTA testing is concerned primarily with protection of the environment and uses such toxicity tests to provide a broad spectrum assessment of the amount of dilution required to an effluent, in order to remove any toxic effects (i.e. the species tested is used as an indicator organism for a wide range of species which might encounter the effluent). It is unnecessary therefore to require both growth rate and 'biomass' end-points for each assessment since the most sensitive result (which should usually be 'biomass') will consistently be used in environmental risk assessments. Regulatory DTA tests require only that 'biomass'-based end-points be calculated. Growth rate end-point values need not be calculated.

The biomass intergral may be calculated for each test replicate as follows;

For each test vessel, the area, A_{tv}, under the double linear growth curve is derived :

$$A_{tv} = \frac{N_1 - N_0}{2} t_1 + \frac{N_1 + N_2 - 2N_0}{2}(t_2 - t_1) + \frac{N_2 + N_3 - 2N_0}{2}(t_3 - t_2)$$

where:
t_1 is the time (for example 24 hours) of the first cell density (or surrogate) estimate after the beginning of the test;
t_2 is the time (for example 48 hours) of the second cell density (or surrogate) estimate after the beginning of the test;
t_3 is the time (for example 72 hours) of the third cell density (or surrogate) estimate after the beginning of the test;
N_o is the initial cell density (or surrogate) estimate;

N_1 is the cell density (or surrogate) estimate at time t_1;
N_2 is the cell density (or surrogate) estimate at time t_2;
N_3 is the cell density (or surrogate) estimate at time t_3.

Once the biomass (determined from the area under the growth curve) statistics are derived for each test replicate, the inhibition of growth (i.e. reduction in biomass) relative to the control growth can be expressed. This can be achieved either by calculating the difference between the control and treatment biomass and expressing this as a proportion of the control biomass or, more simply, by expressing the treatment biomass as a proportion of the control biomass (and inferring inhibition of growth).

$I_{A(i)} = [(A_{mtv-c} – A_{tv-i}) / A_{mtv-c}]$ or

$G_{A(i)} = A_{tv-i} / A_{mtv-c}$

where:

$I_{A(i)}$ is the proportional reduction in growth for the test replicate i;
$G_{A(i)}$ is the proportional growth for the test replicate i;
A_{tv-i} is the area under the growth curve for the test replicate i;
A_{mtv-c} is the mean area under the growth curve for the control.

Both approaches should provide similar estimates of growth inhibition under appropriate statistical analysis.

4.5.3 Estimation of EC values

From the $I_{A(i)}$ or $G_{A(i)}$ values for each test replicate, the 72 hour-EC_{10} and 72 hour-EC_{50} values (and 95 % confidence intervals) should be calculated using appropriate statistical procedures.

In general, the estimation of EC_{50} values using non-linear regression (for example maximum likelihood) models for growth data can be affected by the difference in the variances of replicated test treatments. It may therefore be optimal to apply a linear interpolation method for growth data but a non-linear method is also valid if the equality of variances between treatments can be effectively demonstrated. Suitable non-linear functions (and associated transformations) include the probit, logit and Weibull functions. Where the range of test concentrations includes concentrations at which inhibition of growth is between 0 and 100 %, the EC_{50} and EC_{10} values estimated by the different methods should be similar[24]. Statistical methods (and computer programmes) developed for toxicity tests with quantal responses (such as mortality) should not be used, as improper weighting of the data may result.

The concentration-response curve for algal growth inhibition is not always strictly monotonic, (i.e. continually increasing or decreasing dose-response relationship) but may reveal an initial (slight) growth stimulation at low concentrations of toxicants under investigation. When calculating the EC_{50} and EC_{10} values, only the monotonic part of the curve should be used and a note of the concentration range where stimulation was observed should be recorded.

Table 3 shows an example data set (generated using a micro-scale approach) which has been used to show the determination of the 72 hour-EC_{50} value and 95 % confidence limits

Table 3 Example results of the effects of an effluent on the growth of algae after 72 hours exposure

Nominal concentration (% v/v effluent)	Replicate	Biomass*		Proportional growth
Control	1	3824.675		
	2	4582.125		
	3	3843.925		
	4	4622.95		
	5	4612.775		
	6	5150.15		
	7	5225.125		
	8	4207.925		
	9	3820.8		
	10	4077.14	Mean = 4396.759	1
0.01	1	3570.25		
	2	3203.875		
	3	3021.625		
	4	3429.4		
	5	4013.45		
	6	3460.5		
	7	3194.325		
	8	2883.65		
	9	3056.15		
	10	3569.25	Mean = 3340.248	0.759707
0.022	1	3844.875		
	2	2926.625		
	3	3143.375		
	4	3470.925		
	5	3513.425		
	6	3538.075		
	7	2940.625		
	8	2756.65		
	9	2915.65		
	10	3092.65	Mean =3214.288	0.731058
0.046	1	3322.575		
	2	2297.5		
	3	2840.9		
	4	3587.675		
	5	2707.9		
	6	3217.125		
	7	2704.625		
	8	2756.875		
	9	3309.525		
	10	2821.5	Mean = 2956.62	0.672454
0.1	1	2316.5		
	2	1730.375		
	3	2217.5		
	4	2419.4		
	5	2480.25		
	6	2113.5		
	7	2646.075		
	8	2241.7		
	9	2581.4		
	10	2299.275	Mean = 2304.598	0.524158
0.22	1	1323.4		
	2	1040		
	3	1226.275		
	4	1139.875		
	5	1191.25		
	6	1209.9		
	7	1076.775		
	8	1166.9		
	9	1144.625		
	10	911.025	Mean = 1143.00	0.259965
0.46	1	392		
	2	353.625		
	3	398.125		
	4	369		
	5	366.875		
	6	400.5		
	7	395.375		
	8	217.75		
	9	242.5		
	10	207.5	Mean = 334.325	0.076039

* Biomass (area under the growth curve) as determined by measuring algal cell density (indirectly using fluorescence) at the beginning of the test and at 24 hourly intervals thereafter up to 72 hours.

for the inhibition of growth by an effluent using different statistical procedures. Table 4 shows the EC_{50} value and 95 % confidence limits estimated from the data shown in Table 3 using the different statistical procedures. The results show that the EC_{50} and EC_{10} values estimated by non-linear probit, logit and Weibull functions are similar (although the 95 % confidence limits vary) but that the linear interpolation result is probably the best estimate owing to the inequality of variances across the test.

The data in Table 3 may be used to check that in-house statistical procedures provide comparable results to those given in Table 4.

Table 4 Summary of EC_{50} and EC_{10} values (and 95 % confidence limits) by different statistical procedures for the data shown in Table 5

Statistical procedure	Variance	EC_{50} value	95 % Confidence limits	EC_{10} value	95 % Confidence limits
Bartlett's test	Unequal	-	-	-	-
Control coefficient of variation	6.3 %	-	-	-	-
Linear interpolation	-	0.11	0.092 - 0.13	0.0042	0.0034 - 0.0061
Probit*	-	0.076	0.048 - 0.12	0.007	0.0013 - 0.015
Logit*	-	0.077	0.059 - 0.1	0.0065	0.0027 - 0.011
Weibull*	-	0.085	0.056 - 0.12	0.0047	0.00085 - 0.011

*maximum likelihood

4.5.4 Estimation of the NOEC and LOEC

The NOEC and LOEC values should be determined using hypothesis testing (see Figure 2). Initially, the Shapiro-Wilk's, D'Agostino or Kolmogorov D-test should be used to test the normality of the data. If the data do not meet the assumption of normality and there are four or more replicates of each test concentration, then the non-parametric Wilcoxon rank sum test with Bonferroni adjustment or Steel's many-one rank test should be used to analyse the data. This will depend on whether there are equal numbers of replicates for each test concentration (see Figure 2).

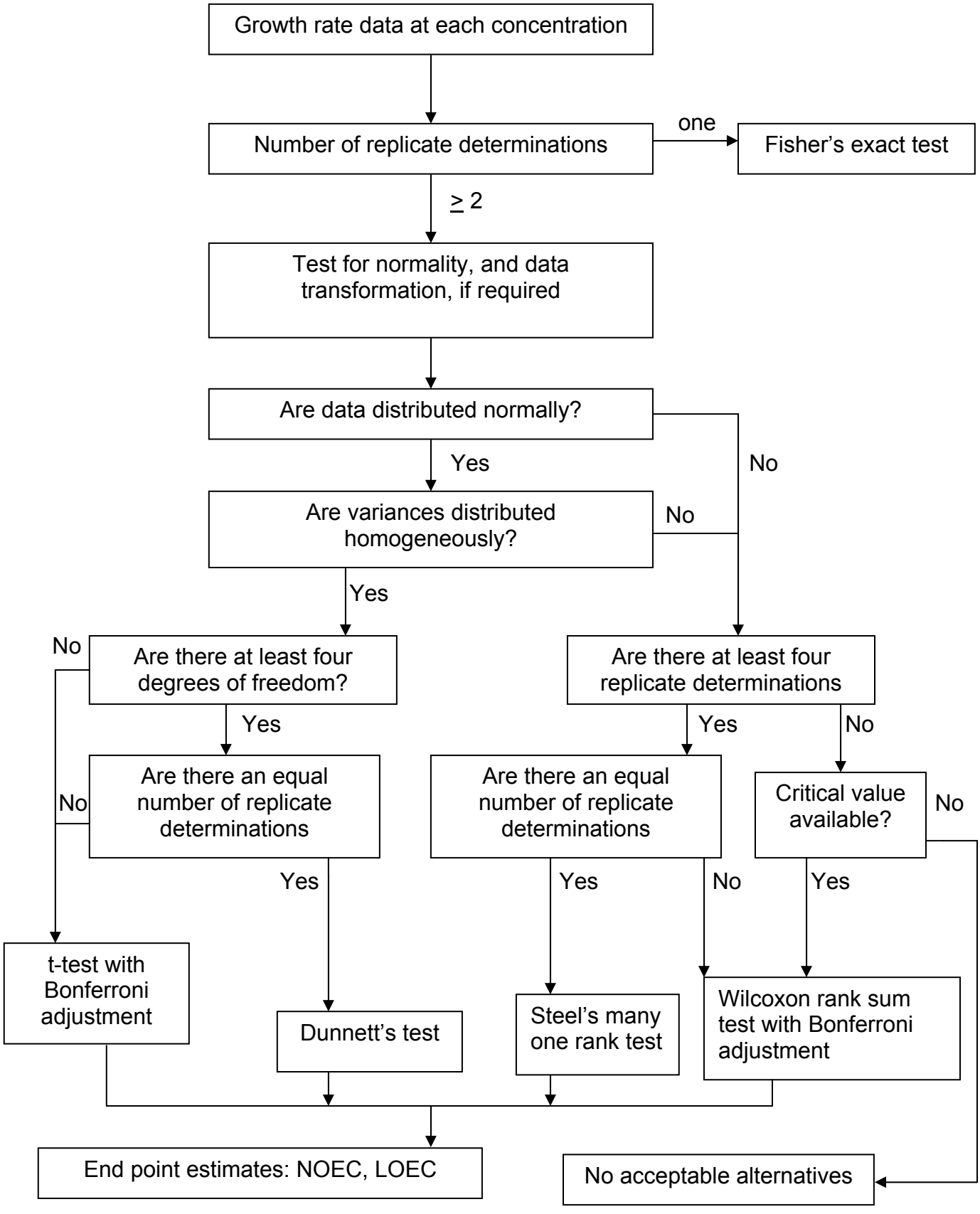
If the data meet the assumption of normality, the Bartlett' test for equality of variances should be used to test the homogeneity of variance assumption. If the data meet the homogeneity of variance assumption then analysis of variance (ANOVA) followed by Dunnett's test, Williams' multiple comparison test or t-tests with Bonferroni adjustment should be used to analyse the data depending on whether there are equal numbers of replicates for each concentration. Failure of the homogeneity of variance assumption leads to the use of Wilcoxon rank sum test with Bonferroni adjustment or Steel's many-one rank test depending on whether there are equal numbers of replicates for each test concentration.

Further information on these statistical procedures can be obtained elsewhere[23, 25, 26]. In the example shown in Table 3, the 72 hour-NOEC and 72 hour-LOEC values calculated using Steel's many-one rank test. (Table 5).

Table 5 Summary of NOEC and LOEC values for the data shown in Table 3

Statistical procedure	Variance	Distribution	NOEC	LOEC
Bartlett's test	Unequal	-	-	-
Control coefficient of variation	6.3 %	-	-	-
Kolmogorov D test	-	normal (p>0.01)	-	-
Steel's many-one rank test (1-tail, 0.05)	-	-	<0.01	0.01

Figure 2 Flowchart for the estimation of NOEC and LOEC values algal growth inhibition tests for full concentration range



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. 98.35 %) sample and appropriate controls using the procedures described in section 4.

Toxicity tests with algae 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 four replicates of each control and 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.

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 3). The hypothesis tested should be that the responses in the sample are not significantly different from those in the controls.

Initially Shapiro-Wilk's or D'Agostino D-test should be used to test the normality of the data. 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 and degrees of freedom are adjusted for unequal variance. Further information on these statistical procedures can be obtained elsewhere[23, 25, 26].

Figure 3 Flowchart for the analysis of single concentration test data from algal growth inhibition test

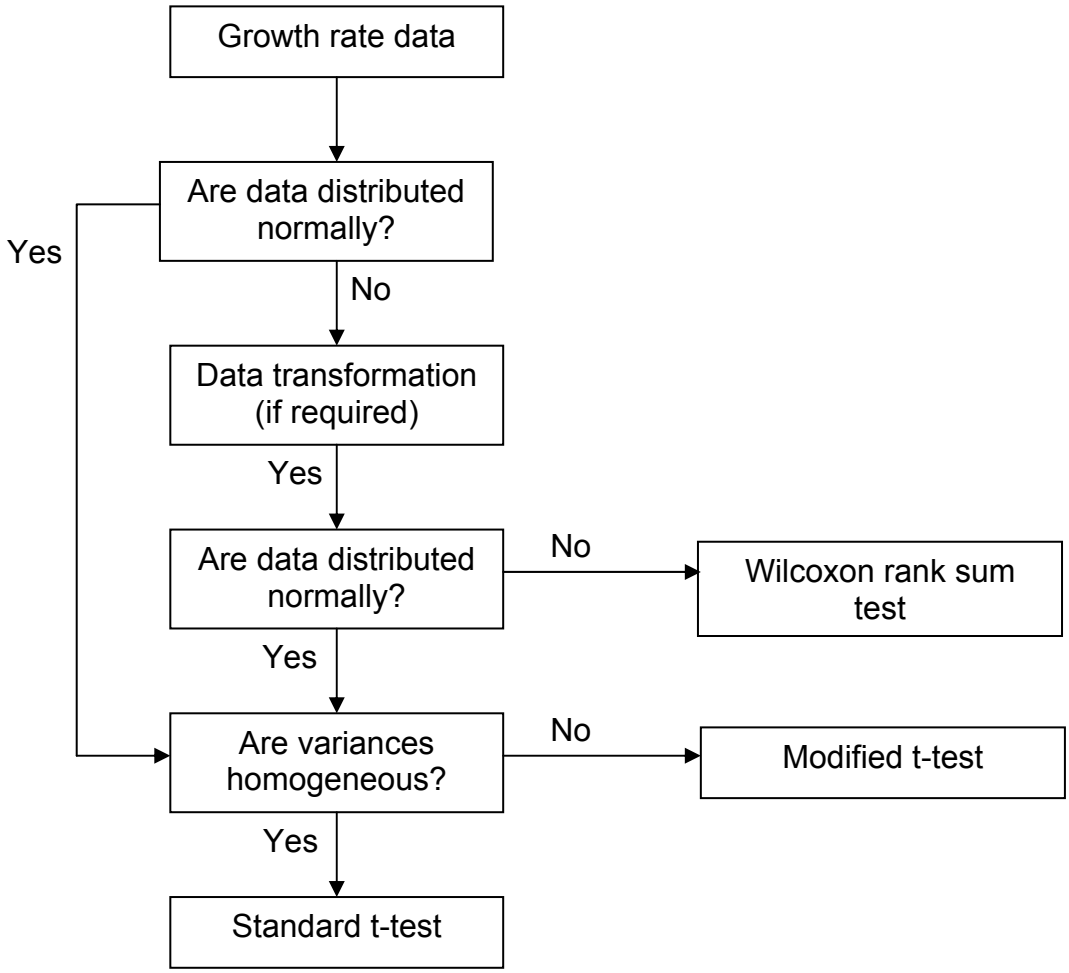


Table 6 shows example data sets for a single concentration test using the data in Table 3 (control versus 0.46 %).

Table 6 Example data set for a single concentration test and the results of statistical analysis

Statistical Procedure	Variance	Distribution	Statistical difference
F-test	Unequal	-	-
Shapiro-Wilk's test	-	normal (p>0.01)	-
Hetero-scedastic t test (2-tail, 0.05)	-	-	yes

6 Guidelines for reference toxicant tests using zinc

6.1 Design

Skeletonema costatum marine water algal growth inhibition tests which are carried out with 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 Zn l^{-1}. The range shown in Table 7 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 values of the 72 h-LOEC and 72 h-EC_{50} values.

Table 7 Zinc concentration range

Zinc concentration (mg l^{-1})	Volume of seawater (ml)	Volume of zinc stock solution 6.2.1 (ml)
0 (control solution)	196.7	0.0
0.01	196.698	0.002
0.032	196.6936	0.0064
0.1	196.68	0.02
0.32	196.636	0.064
1.0	196.5	0.2
3.2	196.06	0.64

The appropriate amounts (see section 4.3.1) of nutrient stock solutions also need to be added.

6.3 Test procedure

Reference toxicant tests should be initiated, monitored and terminated in the same way as described section 4.

6.4 Processing of results

The 72 hour-LOEC and 72 hour-EC_{50} values should be calculated using the procedures described in section 4.

7 References

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APPENDIX A Preparation of nutrient medium

Using analytical grade reagents, nutrient medium initially involves the preparation of a series of three stock solutions in sterile (autoclaved 115 - 121 °C for 15 minutes) distilled, deionised or reverse osmosis water. Following preparation, the stock solutions 1, 2 and 3 should be re-sterilised by membrane filtration (nominal pore size of 0.2 µm). All three stock solutions may be stored in the dark at 2 - 6 °C for up to 3 months.

Nutrient stock solution	Concentration in stock solution (mg l^{-1})
Stock solution 1	
$FeCl_3.6H_2O$	5.3
$MnCl_2.4H_2O$	144
$ZnSO_4.7H_2O$	4.4
$CuSO_4.5H_2O$	0.157
$CoCl_2.6H_2O$	0.404
Na_2EDTA	6.67
Stock solution 2	
Thiamin Hydrochloride	50
Biotin	0.01
Cyanobalamin	0.1
Stock solution 3	
$NaNO_3$	50000
K_3PO_4	3000
$Na_2SiO_3.5H_2O$	14900

Freshly, prepare each batch of nutrient medium by adding (under asceptic conditions) 15 ml of stock solution 1, 0.5 ml of stock solution 2, and 1 ml of stock solution 3 to 1000 ml of sterile (0.2 µm membrane filtered) seawater. The pH value of the nutrient medium should be between 7.5 - 8.5. If necessary, adjust the medium to within this pH range using 1M sodium hydroxide solution or 1M hydrochloric acid solution. The prepared nutrient medium should then be re-sterilised (nominal pore size 0.2 µm membrane filter) and may be stored at 2 - 6 °C in the dark for up to 72 hours.

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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Members assisting with this booklet

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