Environment Agency

The direct toxicity assessment of aqueous environmental samples using the oyster (*Crassostrea gigas*) embryo-larval development test (2007)

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 oyster embryo-larval development test, and replaces the previous version published in 2006. Using the procedures described in this booklet should enable laboratories to satisfy the requirements of the Environment Agency's Monitoring Certification Scheme (MCERTS) for laboratories undertaking direct toxicity assessment of effluents(1). However, if appropriate, laboratories should clearly demonstrate they are able to meet the MCERTS requirements. Two documents have already been published in this series(2, 3). Further documents are being produced and include:

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

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

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

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

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.
DTA	direct toxicity assessment.
EC_{10}	the concentration that results in an effect on 10 % of the exposed organisms.
EC_{20}	the concentration that results in an effect on 20 % of the exposed organisms.
EC_{50}	the concentration that results in an effect on 50 % of the exposed organisms.
Gametes	sperm or eggs, as appropriate.
LOEC	lowest observed effect concentration – lowest test concentration at which there is an observed effect compared to controls.
NOEC	no observed effect concentration – the highest test concentration at which there is no observed effect compared to control solutions.
Static test	a test with no replacement or replenishment of the test dilutions.
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 oyster (*Crassostrea gigas*) embryo-larval development test

1 Introduction

The procedures described in this document enable direct toxicity assessments to be carried out on aqueous environmental samples using embryos of the Pacific Oyster (*Crassostrea gigas*). The procedures described are based on an Environment Agency project[4, 5] and take into account existing guidelines[6] and more recent method developments.

The oyster embryo-larval development test can be used in the following roles:

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

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

Aqueous environmental samples submitted for toxicity testing should be representative of the material being sampled. Depending upon the design of the sampling programme, different approaches may need to be adopted[7]. 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[8 - 10].

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 at the location where the sample is taken (i.e. on-site determination) and on receipt of the sample at the laboratory. This enables changes (which may occur during transportation) in the water quality parameters to be assessed, and if necessary, appropriate measures taken if these changes are considered to impact on the toxicity test. The on-site determinations should be accompanied with details of a description of the sample and whether the sample contains or comprises an emulsion. Details of appropriate methodology can be found elsewhere[11 - 14]. Samples should be labelled appropriately with such details as the name and location of the site where each of the samples were taken and the date and time when each sample was taken. Any other relevant information, such as the name of the sampling officer and chain of custody details should also be recorded.

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

pH
Temperature
Dissolved oxygen
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 24 ± 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, however, this is not recommended 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 all criteria for all the parameters listed in Table 2 are met and the influence of these parameters is removed. Test results will therefore reflect the residual chemical toxicity of the discharge at the water quality ranges specified in Table 2. These ranges are generally representative of the conditions found in the receiving environments to which effluents are likely to be discharged. If these ranges are not representative of the known water quality in the area of discharge of a particular effluent, the actual measured water quality values should be substituted for those given in Table 2.

Table 2 Threshold criteria for selected water quality parameters for the oyster embryo-larval development test

Parameter	Threshold criteria
pH	6.0 - 8.5
Dissolved oxygen (as a percent of the ASV) at 24 ± 2 °C	≥ 60
Salinity (‰)	22 - 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.

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

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

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

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

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.

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

2.4.2 Dissolved oxygen

If the dissolved oxygen concentration in any of the test dilutions prior to testing is less than 60 % of the air saturation value (ASV) at 24 ± 2 °C the solution should be aerated, even though this may result in the potential loss of volatile substances from the solutions. 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 60 % of the ASV is reached. The duration of aeration should not exceed 30 minutes. Any aeration of test dilutions should be discontinued following this period and the test initiated. Test dilutions with dissolved oxygen concentrations greater than 60 % of the ASV should not be aerated.

2.4.3 Salinity

The salinity of test dilutions should be adjusted if values fall outside of the range specified in Table 2.

An increase in salinity can be achieved by the addition of analytical grade sea salts. An additional ‘salinity adjusted’ control should then be included in the test in addition to the normal control solution, 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 to the same original salinity as the sample dilution by adding distilled or deionised water. Sea salts should then be added to bring the additional control back into the required salinity range, in exactly the same way as with the sample dilution under modification. The additional control accounts for potential effects on oyster embryos that may arise when portions of the added sea salt fail to dissolve fully. This may occur if appropriate ageing of the seawater does not take place.

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 can be 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 observed 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 solution 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 solutions, 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 the oyster embryos in the test vessels. If this occurs, the solution can be poured gently through fine-mesh netting (nominal size of 100 µm) and the organisms re-suspended in dilution water (4.3.1) for viewing. In these cases, care should be taken to ensure the integrity of the oyster embryos as the process of sieving may damage the embryos.

2.4.6 Other parameters

Further information on other parameters which may need consideration in specific circumstances can be obtained elsewhere[16 - 18] including guidance on the testing of effluents containing sparingly soluble substances[19].

2.5 Disposal of samples

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

3 Oyster (*Crassostrea gigas*) embryo-larval development test procedure

3.1 Introduction

Based on previously published guidance[4 - 6], procedures are described for conducting static toxicity tests to assess the effects of aqueous environmental samples on the embryo-larval development of the Pacific oyster (*Crassostrea gigas*).

3.2 Test organism

Embryos are produced from the sperm and eggs of conditioned adult male and female oysters, each of individual wet weight greater than 45 g. The adult oysters should be in a breeding conditioned before removing the gametes for testing. Adult oysters can be brought into a breeding condition within the laboratory or purchased “ready conditioned” from recognised commercial sources. If oysters are purchased they should be delivered to the laboratory within 24 hours of despatch.

3.2.1 Oyster anatomy and embryo development

The gonad is the largest organ in a ripe oyster and typically represents 30 % of the total wet weight of soft tissue (see Figure 1). The gonad, covered by the mantle, is normally a layer 5 - 8 mm thick which surrounds the digestive gland. Oysters in a poor reproductive condition have very thin gonads, in which only the digestive gland is visible, and these should be discarded.

Eggs in the mature ovary of *Crassostrea gigas* are pear shaped and compressed[20] with the long axis varying from 55 - 75 µm and the width at the broadest part measuring 35 - 55 µm. The oblong shape is retained some time after discharge into seawater, but gradually the eggs become globular and increase in density. The nucleus appears as a large transparent area surrounded by densely packed granules. The rounded eggs can vary from 45 - 62 µm in diameter.

Immature eggs tend to be very irregular in size and shape and are often clumped together. Eggs undergoing resorption are characterised as a shrunken egg within the vitelline membrane. The development for normal embryos is shown in Figure 2 and given in Table 3.

Table 3 Development for normal oyster embryos

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

Ideally, a batch of normal embryos will have:

(i) Greater than 95 % normally fertilised eggs:

(ii) Greater than 60 % of the embryos will be at the same stage of development. Two hours after fertilisation, the majority of eggs in normal batches of embryos will be at the 16-cell stage, with some unfertilised eggs and some 32-cell stage embryos:

(iii) A uniform shape consisting of dark, granular tightly packed cells.

Abnormal embryos, which become apparent between the 3rd and 5th divisions, consist of loosely packed extended or oblong cells, appearing almost separate.

3.2.2 Storage and handling of oysters

Supplied oysters should normally be used within a few hours of being received in the laboratory and require no maintenance. Oysters that have been brought into a breeding condition within the laboratory should be used as soon as the conditioning procedure is completed. If necessary, the oysters can be stored for up to 24 hours provided they are stored in air under damp and refrigerated (6 - 10 °C) conditions. The damp conditions can be achieved by wrapping the oyster in paper that has been moistened with seawater. The wrapped oyster should then be placed in a box and kept cool. If oysters are held in this manner they should be allowed to recover before being used. Such recovery can be achieved by placing the oysters in individual beakers of aerated seawater (1 litre) for about 1 hour at 20 - 22 °C. Oysters kept under these conditions may spawn naturally and the resulting gametes can be used in the test.

Figure 1 The anatomy of the Pacific oyster, *Crassostrea gigas*

Figure 1A: Opened oyster with minor (ventral) valve removed exposing mantle. Note that the majority of gamete tissue is situated dorsally in the mantle surrounding the stomach and intestine.

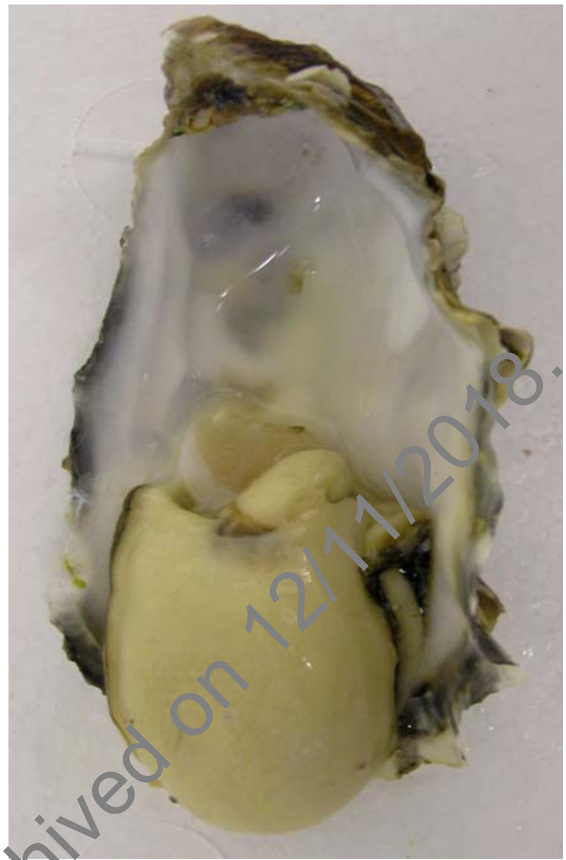


Figure 1B: Opened oyster with gamete tissue exposed. This is achieved by gently turning the mantle over using a flat, blunt instrument (for example a spatula).

Figure 1C: Close up of oyster gamete tissue. A well-conditioned oyster will display gamete tissue that covers the entire dorsal surface of the stomach and surrounding tissue.

Figure 2 Embryology of *Crassostrea gigas*

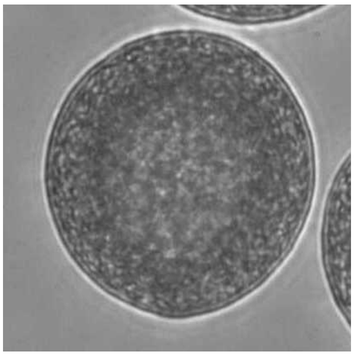


Figure 2A: Unfertilised egg (after approximately 30 minutes in sea water).

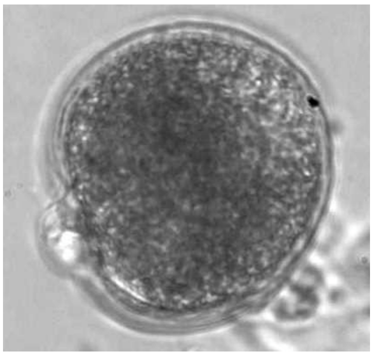


Figure 2B: Fertilised egg showing polar body.

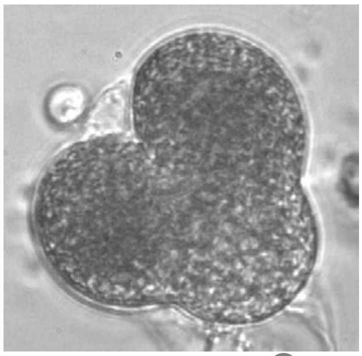


Figure 2C: Fertilised egg undergoing cell division.

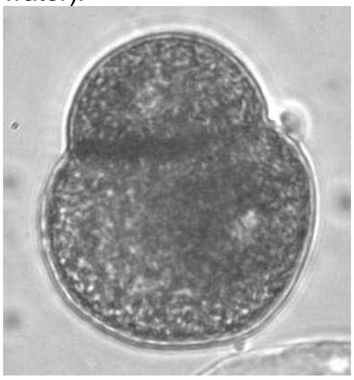


Figure 2D: 2-cell embryo.

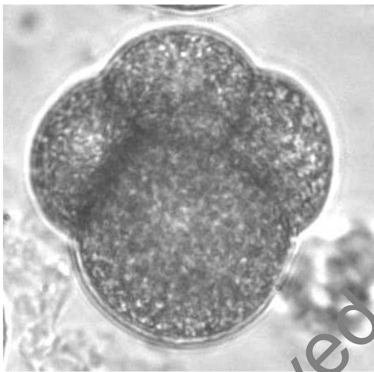


Figure 2E: 4-cell embryo.

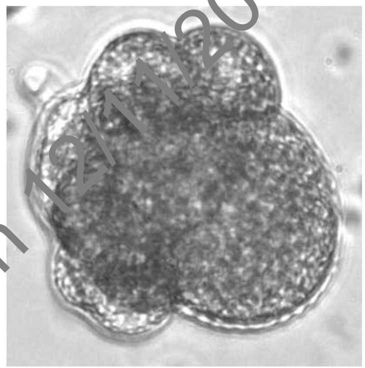


Figure 2F: 8-cell embryo.

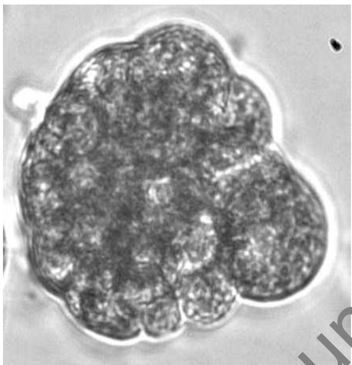


Figure 2G: 16-cell embryo.

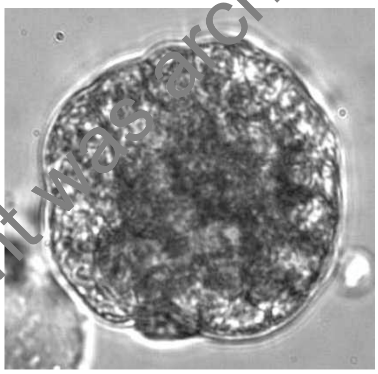


Figure 2H: 32-cell embryo.

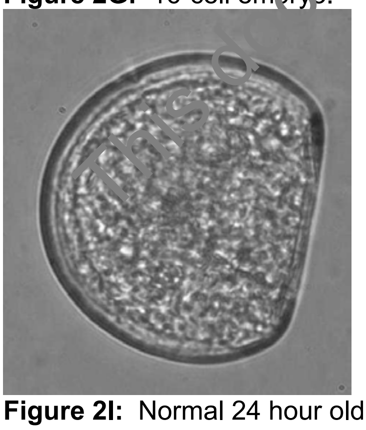


Figure 2I: Normal 24 hour old larva.

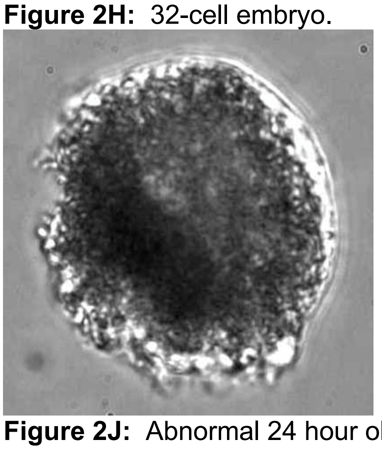


Figure 2J: Abnormal 24 hour old larva.

4 Guidelines for toxicity tests using a range of concentrations

Two approaches to the test may be used:

(i) A conventional procedure, based on previous guidance[4 - 6]. This approach uses glass test vessels capable of holding 30 ml of test solution for the determination of the toxicity of environmental samples, either as received or using a range of concentrations.

(ii) A contemporary procedure based on a combination of previous guidance [4 - 6] and more recent developments of the test. This approach uses inert plastic or glass multi-well plates capable of holding 5 ml of test solution for the determination of the toxicity of environmental samples, either as received or using a range of concentrations. This miniaturised approach can be used in place of the conventional approach and is particularly useful in the screening of effluents and TIE and/or TSE exercises in which abbreviated or 'high-throughput' versions of this procedure is required. Such versions generally involve replication, statistical analysis and quality assurance associated with the test performance and a reduction in the concentration range, and can be useful in situations where test result reporting times and minimised costs are primary considerations. Research which has led to the development of the miniaturised oyster-embryo test has been undertaken by the Environment Agency and addresses issues of multi-well evaporation, gas exchange, chemical adherence to well plates, and potential loss of volatile substances[21 - 23].

In both approaches;

(i) Oyster embryos (*Crassostrea gigas*) should be exposed for a duration of 24 ± 2 hours.
(ii) The dilution water used for controls and dilution of samples should be seawater.
(iii) The number of embryos in each test vessel at the start of the test should be approximately 100 per ml.
(iv) The temperature of the test dilutions should be maintained at 24 ± 2 ^{o}C for the duration of the test.
(v) The pH of the test dilutions should be between 6.0 to 8.5 at the start of the test.
(vi) The dissolved oxygen content in the test dilutions at the start of the test should be greater than 60 % ASV.
(vii) The salinity of the test dilutions should be between 22 - 36 ‰.
(viii) The results of the toxicity test should be rejected if the percentage of abnormal larvae in the controls is greater than 40 %.
(ix) 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 solutions 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 prior to analysis[1, 24].

4.2 Principle

In the test, groups of oyster embryos are exposed to the environmental sample diluted with seawater to a range of concentrations for a period of 24 hours. The different test dilutions in an appropriate test concentration range, under otherwise identical test conditions, may exert toxic effects on the normal development of the embryos. In the context of these procedures, normal development describes the transformation of embryos, over a 24-hour period, into "D-shaped" larvae having a protective D-shaped shell where the paired hinged shells are visible (see Figure 2). Although the exposure time is short, it encompasses a period of intense cellular activity, in which the impairment of a number of critical physiological and biochemical processes may result in poor growth and development. Abnormal development is characterised by embryos which die at an early stage or larvae which develop but which fail to reach the D-shaped stage. This will extend from an absence of abnormal effects (at lower test concentrations) to a lack of development (at higher test concentrations) in all the embryos, and possibly death (lethality).

The data (i.e. percent of normal D-shaped and abnormal larvae observed) should be used to determine:

(i) The effective concentrations, i.e. the concentration that results in 10 %, 20 % and 50 % of the exposed oyster embryos failing to develop into normal D-shaped larvae after 24 hours. The effective concentrations are referred to as the 24 hour-EC_{10}, 24 hour-EC_{20} and 24 hour-EC_{50} values respectively.

(ii) The highest no-observed effect concentration after 24 hours (i.e. the 24 hour-NOEC value).

(iii) The lowest observed effect concentration after 24 hours (i.e. the 24 hour-LOEC value).

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.

Artificial seawater may be used as a substitute for natural seawater, and is prepared by adjusting distilled or deionised water to a salinity of 34 ± 2 ‰ with analytical grade sea salts. The resulting solution should be "aged" for at least 24 hours prior to use, and should undergo vigorous continual aeration between preparation and use. If un-dissolved salt crystals are observed in the solution after ageing, it should be appropriately filtered.

Natural seawater may be used and should be obtained from a site known to be free from 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 oyster embryo development. Seawater with concentrations above those deemed ‘safe’ for oyster embryo development should not be used.

4.3.2 Buffered formaldehyde solution

Dissolve 5 g of sodium tetraborate in 250 ml of distilled or deionised water. This solution is then added to 250 ml of 40 % v/v formaldehyde solution. The combined mixture may be stored in a screw top glass bottle. This solution may be freshly prepared or stored for up to six months at 4 °C.

4.3.3 Apparatus

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

Test vessels (stoppered vials capable of holding 30 ml of test solution or 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 24 ± 2 °C.
A microscope (inverted or binocular) providing 20 - 100 times magnification and a suitable grid marked counting chamber, for example a 1 ml Sedgewick-Rafter cell.
Equipment for measuring pH, dissolved oxygen, salinity, temperature and other parameters as appropriate.
Recording equipment for counting egg and larval numbers.
A large flat-bladed scalpel or oyster knife.
Plastic meshes (60 and 100 µm).

4.4 Test procedure

The following procedures should enable oyster embryos to be prepared that result in greater than 60 % normal D-shaped larvae in test controls. It is necessary to strip only sufficient adult oysters to obtain the requisite number of viable batches of sperm and eggs.

4.4.1 Obtaining oyster gametes

Oysters are usually supplied in a conditioned state and identified as male or female. If unknown, the sex of the adult oysters should be identified prior to use. In water, sperm are identified by their milky appearance; eggs are identified by their granular appearance (see Figure 2). Male and female gametes are obtained by stripping the gonads or by naturally spawning the adults. Irrespective of the approach used to strip the gametes, it is essential that all the gametes be collected at the same time. All appropriate information should be recorded.

If stripping the gametes, the mature male and female conditioned oysters should be opened. This can be undertaken by breaking the hinge and cutting the adductor muscle, for example with a standard oyster knife. The knife should be inserted in the flat edge of the oyster and held level when cutting to avoid damaging the gonads. The body cavity of each oyster should be thoroughly rinsed with seawater to remove any debris, which may be present.

Prior to stripping sperm from a male oyster, a small sample of sperm from each male should be transferred to a slide or small vessel, together with a few drops of seawater. After 15 - 30 minutes the activity of the sperm should be assessed using a microscope at 100 times objective magnification and male oysters with the most motile sperm selected for stripping.

The gametes may be stripped by one of the methods described below. Care should be taken to avoid puncturing the gut as contamination of the gametes can lead to a lower level of fertilisation. If the gut is punctured then the particular batch of gametes should be discarded.

A clean Pasteur pipette may be inserted into the gonad to a depth of 1 - 2 mm and the eggs or sperm collected. The gametes should be transferred to separate volumes of seawater and held at 24 ± 2 °C.

Alternatively, the gonad may be gently cut into with a sharp scalpel, angling the blade upwards to avoid puncturing the gut. Gametes should be collected, by pipetting seawater (at 24 ± 2 °C) over the surface of the gonad and washing the gametes into a suitable vessel.

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

The suspension of eggs from each female oyster should be filtered through a 90 - 100 μm plastic mesh sieve, and collected in a clean glass beaker. This should remove any tissue debris. The filtered egg suspension from each female can then be stored individually (to ensure selected eggs are obtained from the ripest gonads) or mixed egg suspensions can be prepared in a single beaker from oysters that all possess ripe gonads. An aliquot, typically, 1 - 5 ml, of the individual suspensions, or mixed suspension, should then be removed and examined microscopically using an appropriate chamber, for example a Sedgewick-Rafter cell, to assess the quality and egg population densities, i.e. the number of eggs per ml of suspension. Egg population densities may also be assessed using a Coulter (or other particle) counter. The egg suspension should then be adjusted with an appropriate volume of reference seawater to achieve an egg density of approximately 3000 - 6000 eggs per ml. The egg suspension should be gently mixed every 5 minutes. Egg quality should also be assessed (see section 3.2) microscopically. Batches of eggs with high proportions of abnormal eggs (for example those that fail to 'round up' after exposure to seawater) should be discarded.

The sperm suspensions from the male oysters should be filtered through a mesh sieve (nominal size of 60 μm) and collected in a clean glass beaker. This removes any tissue debris. The filtered sperm suspensions should then be mixed in a clean glass beaker.

4.4.1.1 Fertilisation

Within 30 minutes of obtaining the egg and sperm suspensions, the egg suspension should be fertilised with the sperm suspension. The volume ratio should be 2 - 3 ml of sperm suspension to 1 litre of egg suspension. Fertilisation can be carried out in either of the ways detailed below:

(i) Fertilising the egg suspension from each female with the combined sperm suspension and subsequently combining individual viable and healthy embryo suspensions:

(ii) Fertilising the combined egg suspension with the combined sperm suspension.

The approach adopted should not affect the outcome of the test provided that the majority of eggs used are normal (see section 3.2). Results may be affected if abnormal eggs from one or more female oysters are included in a combined egg suspension, and batches of eggs with a high proportion of abnormal eggs should not be used.

After mixing the suspensions, the eggs should be checked within 15-30 minutes to ensure fertilisation is occurring. By this time, sperm should surround each egg and polar bodies should be evident. The embryo suspension should then be incubated for 2 hours at 24 ± 2 °C in the dark, without aeration. The suspension should be stirred at least every 10 - 15 minutes to prevent excessive settling of embryos and potential oxygen starvation. During this 2 hour period, the eggs should undergo the early stages of cleavage and typically reach the 16 - 32 cell stage (see Figure 2). After approximately 2 hours the embryos should be assessed microscopically (at 20 - 40 times magnification) using an appropriate counting chamber, for example a Sedgewick-Rafter cell, to determine whether cell cleavage is occurring.

The embryo suspension may be left for up to an additional 2 hours if the embryos have not yet reached the 16 - 32 cell stage to allow this level of development to be attained. If, after this time, this level of development has not been reached, the preparation should be discontinued and other oysters stripped for gametes.

4.4.2 Preparation of test dilutions

An appropriate series of concentrations of the sample should be prepared with the ratio between consecutive test concentrations not exceeding 2.2. See Table 4 for the preparation of typical test solutions comprising 200 ml of test solution. Appropriate details should be recorded.

On the day the toxicity test is to be carried out, the concentration range should be prepared in volumetric flasks by diluting (with 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 solution.

The remaining test solution (i.e. not added to the test vessels) should be used to determine the selected water quality parameters shown in Table 2. Appropriate details should be recorded.

Table 4 Preparation of test dilutions

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

* % v/v

4.4.3 Initiation of the toxicity test

An appropriate volume of the embryo suspension should be added to each of the replicated test solutions so that the final number of oyster embryos in each test vessel is approximately 100 per ml. Typically, a volume of approximately 20 µl of embryo suspension per ml of test solution is required. The embryo suspension should be vigorously mixed between each transfer to maximise homogeneity in transfers between each test vessel.

The number of inoculated embryos in each test vessel may be checked using the egg count check procedure. For this procedure, three additional control replicates should be inoculated and then immediately fixed (see section 4.4.5) i.e. they do not undergo test incubation. These embryos can then be counted to provide an estimate of the total numbers of larvae supplied to each test vessel. These numbers should then be reported. The inoculated test dilutions should be incubated at 24 ± 2 °C for 24 ± 2 hours under static conditions without light.

4.4.4 Monitoring water quality

At the beginning and end of the test period, the determination of pH, dissolved oxygen, temperature and salinity should be made on the test solutions specifically reserved for monitoring water quality. The data should be recorded.

4.4.5 Terminating the toxicity test

The test should be terminated after 24 ± 2 hours. Buffered formaldehyde solution (4.3.2) should be added to each test vessel (20 µl of buffered formaldehyde solution per ml of test dilution) to fix and preserve the larvae. This operation should be carried out in a fume cupboard.

The number of normal D-shaped larvae should be counted microscopically using a suitable counting chamber. Normality is defined as those larvae possessing a completely formed D-shaped bivalve shell, which may be irregular to some degree (see Figure 2). The number of abnormal larvae may also be counted and are defined as those larvae that fail to reach the D-shaped stage, although they may be normal trocophores, or other early larval stages. In some cases, larvae may completely disintegrate under toxic challenge and it may not be possible to determine accurately the numbers that are abnormal. Unfertilised eggs (see Figure 2) should not be included in the count, as they do not represent viable test organisms undergoing development.

When aliquots of the test solutions are taken for counting, care should be taken to ensure they are representative of the test replicate as a whole. This can be achieved by aspirating the test dilution prior to sub-sampling to ensure the majority of fixed larvae are in suspension.

Digital imaging systems have been shown to be effective in counting normal D-shaped and abnormal oyster larvae and may be used as an effective alternative to visual assessment. Such equipment should, however, be validated before being used as an alternative to conventional assessment.

4.5 Processing of results

4.5.1 Validity of the results

The results of the toxicity test should be rejected if the percentage of abnormal larvae in the control solutions exceed 40 %. 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 control criteria[24].

4.5.2 Data handling

Endpoints such as the EC_{10}, EC_{50}, NOEC and LOEC values should be determined using an appropriate validated computer-based statistical package. The endpoint values for an oyster embryo-larval development toxicity test are based on the percentage of abnormal embryos in each test concentration.

In all cases, the determination of the total numbers of larvae exposed in each test vessel is required to determine the percentage of normal D-shaped and abnormal larvae, and should be reported. This can be achieved in a number of ways such as using the counts derived from the 'egg count check' procedure, or counting both the normal D-shaped and abnormal larvae in each test vessel.

Some computer-based statistical software packages will enable the calculation of endpoint values without further data manipulation. The numbers of normal D-shaped and abnormal larvae are entered for each test vessel and a proportional effect (taking into account the corresponding larvae numbers for the control solutions) is calculated. Such direct statistical analysis can provide good results if there are sufficient total numbers of larvae present in the test vessels.

Abnormal larvae may disintegrate entirely, especially at higher test concentrations, and in these cases, it may not be possible to determine a robust estimate of the total numbers of normal D-shaped and abnormal larvae. In addition, owing to the inherent irregularity of abnormal larvae, some image analysis systems may be unable to count abnormal larvae accurately. Even when traditional visual assessments are used, it may be a more effective use of resources to count only normal D-shaped larvae. In general, the mean 'egg count check' value or mean control total value may be used to provide an estimate of total numbers in the test vessels. This is because neither of these values will be affected by potential larval disintegration. However, this means that an estimated total value is used for all test vessels (unlike the more direct procedure described above where all test vessels undergo specific counting of normal and abnormal larvae). In these situations, further manipulation of the data is required before they can be used to estimate the test endpoints. This includes calculating for each test vessel, the percentage normal development (PND) and percentage abnormal development (PAD) values.

The PND value depends on the manner by which the mean values of estimating the total numbers of larvae in each test vessel are estimated.

If the egg count check procedure is used, then

$$\text{PND} = \frac{\text{Number of normal D-shaped larvae} \times 100}{\text{Mean number of embryos from egg count check}}$$

If counts from controls are used, then

$$\text{PND} = \frac{\text{Number of normal D-shaped larvae} \times 100}{\text{Mean total number of normal D-shaped + abnormal embryos in controls}}$$

The percentage abnormal development (PAD) value is then calculated by subtracting the PND value from 100, i.e.

$$\text{PAD} = 100 - \text{PND}$$

The mean PND and PAD values for each test concentration should then be determined from the replicate values.

For each test concentration, the percentage net response (PNR) value (which is effectively the response adjusted for the control (background) levels of abnormality) should then be calculated. Hence

$$\text{PNR} = [\,(\text{PAD}_{m(TC)} - \text{PAD}_{m(C)}) / (100 - \text{PAD}_{m(C)})\,] \times 100$$

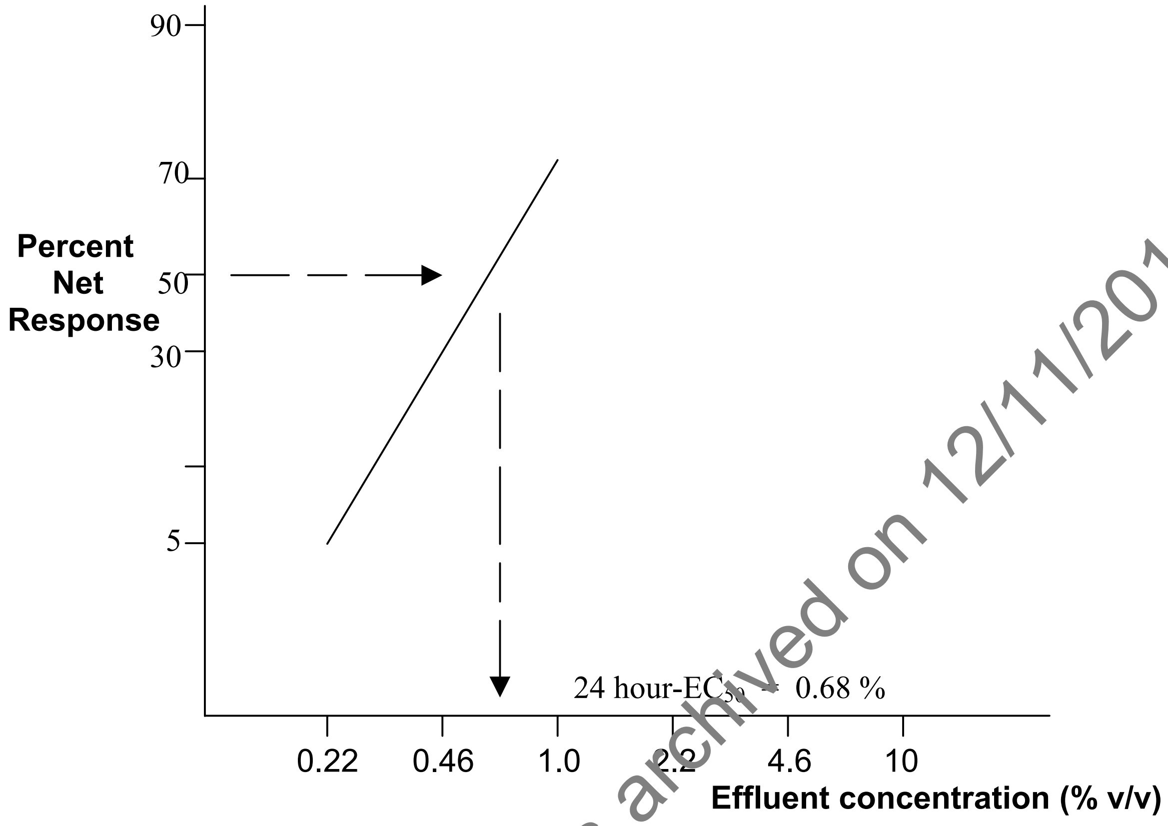
Where $\text{PAD}_{m(TC)}$ is the mean PAD value for the test concentration and
$\text{PAD}_{m(C)}$ is the mean PAD value for the control.

Test endpoints can then be estimated using the PNR values for each test concentration. See Figure 3.

4.5.3 Estimation of EC values

The 24 hour-EC_{10}, EC_{20} and EC_{50} values (and other EC values, if necessary) should be determined using appropriate statistical procedures (see Figure 4). Confidence limits (p=0.95) for the calculated EC value should be determined and quoted in with the test results.

Figure 3 Graph of dose-response curve of cumulative inhibition of development of D-shaped larvae (probability scale) against effluent concentration (log scale)



When analysing data from the oyster embryo-larval development test the following points should be considered:

(i) If the results include concentrations at which there are > 60 % normal D-shaped larvae and two concentrations at which the percentage of normal D-shaped larvae is between 0 and ≥ 60 %, the results from probit, moving average and binomial procedures should provide similar estimates of the EC_{50} value. Probit analysis should be used to estimate EC_{50} values, 95 % confidence limits and the slope of the dose-response curve.

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

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

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

Figure 4 Flowchart for the estimation of the EC_{50} value for full concentration range test

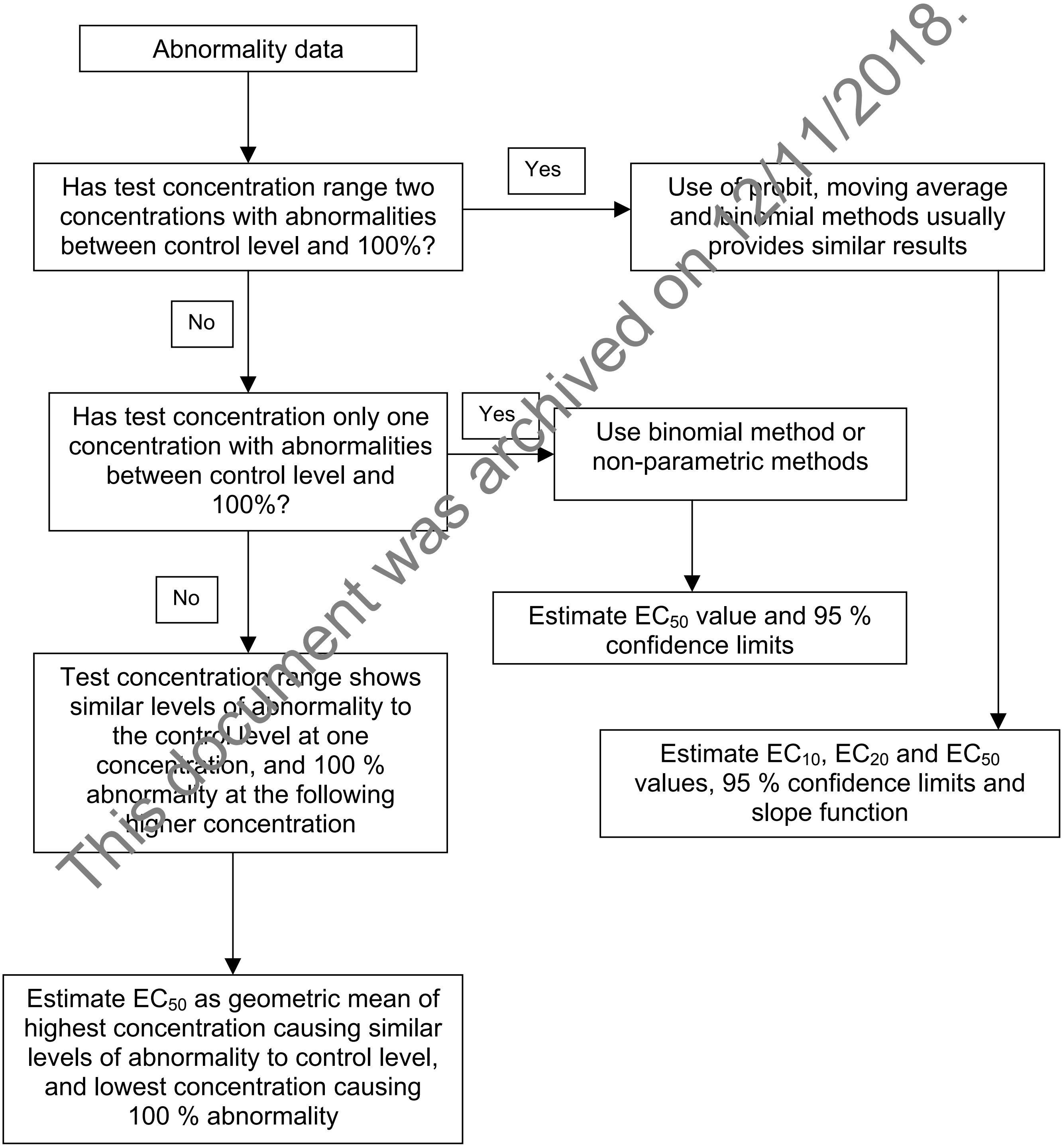


Table 5 shows examples of data produced to show the effect on the development of oyster embryos by an effluent. Table 6 summarises the EC_{50} values derived from the data in Table 5 using different statistical procedures.

Table 5 Example results of the effect on the development of oyster embryos by an effluent after 24 hours exposure

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

The data in Table 5 may be used to check that in-house statistical procedures provide comparable results to those given in Table 6.

Table 6 Summary of EC_{50} values and 95% confidence limits for the data in Table 5 estimated using different statistical procedures

Scenario	Statistical procedure	24 hour-EC_{50} value	95% percent confidence limits	Slope of dose response curve
1	Probit (Tox Calc)	0.67	0.58-0.76	4.0
	Probit*	0.63	0.57-0.70	3.4
	Moving average*	0.59	0.53-0.67	
	Binomial*	0.68	0.46-1.0	
2	Probit	No valid approach		
	Moving average	No valid approach		
	Binomial*	0.52		
	Spearman Karber (Tox Calc)	0.50	0.46-0.55	
3	Geometric mean	0.68		

* Computer based statistical package[25].

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 EC_{50} values derived are less statistically sound.

From the data shown in Table 5, the 24 hour-EC_{10} and 24 hour-EC_{50} values estimated by the Tox Calc software (Tide Pool Scientific Software) are 0.32% v/v effluent and 0.67% v/v effluent respectively with 95 % confidence limits of 0.24 - 0.4 % and 0.58 - 0.76 % respectively.

From the interpolation of the dose response curve of cumulative inhibition of embryo development (probability scale) against effluent concentration (log scale) shown in Figure 3 for Scenario 1, the 24 hour-EC_{50} is 0.68 % v/v effluent and the 24 hour-EC_{10} is 0.27 % v/v effluent. The values obtained graphically complement those obtained using computer-based software (see Table 6).

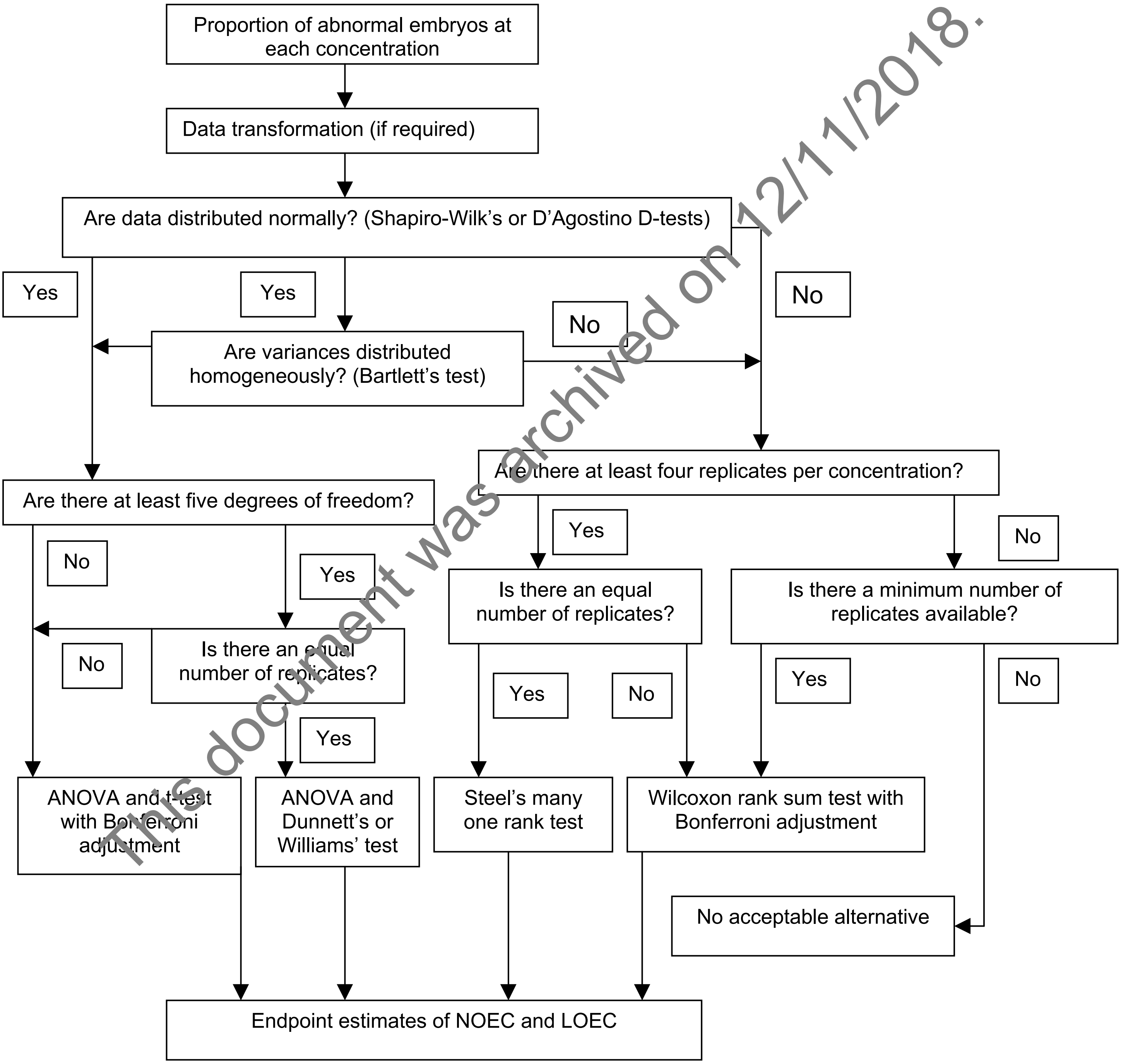
4.5.4 Estimation of the NOEC and LOEC

The 24 hour-NOEC and 24 hour-LOEC values should be determined using hypothesis testing (see Figure 5). Initially the proportion of abnormal embryos in the control and test dilutions should be tested for normality using the Shapiro-Wilk's or D'Agostino D-test. If the data are not normally distributed, they 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 the normality and homogeneity of variance requirements. Following transformation, the Shapiro-Wilk's or D'Agostino D-test should then be re-applied to test the normality assumption.

If the data meet the assumption of normality (either before or after transformation) Bartlett's 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 applied to analyse the data. This will depend on whether there are at least five degrees of freedom and equal numbers of replicates in each treatment.

Failure of the homogeneity of variance assumption leads to the use of Wilcoxon rank sum test with Bonferroni adjustment or Steel's many-one rank test depending on whether there are a minimum, equal number of replicates in each treatment. If the critical minimum number of replicates for the test (depending on the specific application of the results) has not been achieved or is not characterised, no further statistical analysis is reliable. Further information on these statistical procedures can be obtained elsewhere[26 - 28].

Figure 5 Flowchart for the estimation of NOEC and LOEC values for the oyster embryo-larval development toxicity test in 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.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 oyster embryo development and in these cases, the sample should be adjusted using the procedures described in section 2.

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

5.2 Test procedure

Single concentration tests should be initiated in the same way as full concentration range toxicity tests (see section 4) with at least eight replicates of each control and four 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.

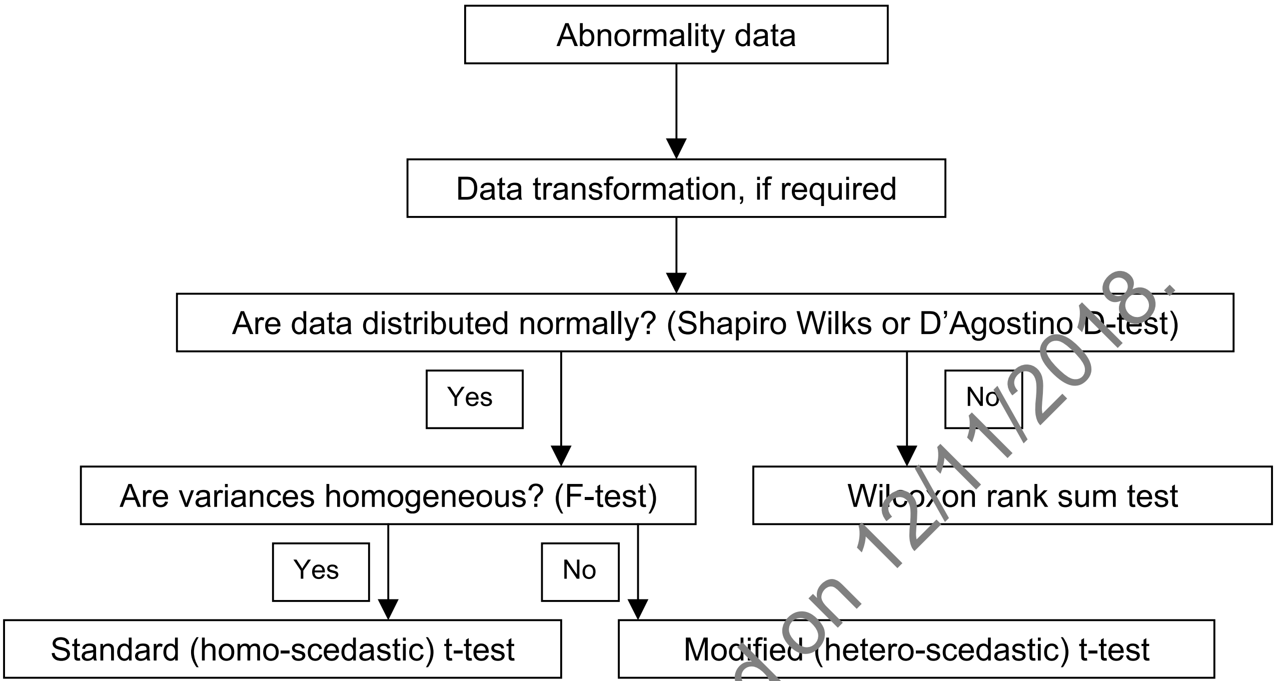
5.3 Processing of results

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 6). 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 control solution and the sample 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 the normality and homogeneity of variance requirements. Shapiro-Wilk's or D'Agostino D test should be used to test the normality assumption.

If the data do not meet the assumption of normality, then the non-parametric Wilcoxon rank sum test should be used to analyse the data. If the data meet the assumption of normality, the F-test for equality of variances 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[26 - 28].

Figure 6 Flowchart for the analysis of single concentration test data from oyster embryo-larval development toxicity tests



A percentage net response (PNR) value may also be derived for the single sample concentration as described in section 4.5.2.

Table 7 shows examples of data for a single concentration test (i.e. 0.22 % v/v effluent concentration) and control dilutions. In Scenario 1, the variances are equal ($F = 1.28$, $p = 0.70$) and the standard (homo-scedastic) t-test indicates a significant difference between the responses of the control solution and the sample effluent ($t = 2.04$, $p <0.05$). In Scenario 2, the variances are unequal ($F = 26.59$, $p = 0.007$) and the modified (hetero-scedastic) t-test indicates no significant difference between responses of the control solution and the sample effluent ($t = 0.40$, $p >0.05$).

Table 7 Examples of data for a single concentration test and the results of statistical analysis

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

NS - no significant difference between control and treatment groups

6 Guidelines for reference toxicant tests using zinc

6.1 Design

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

6.2 Reference toxicant preparation

6.2.1 Zinc stock solution

Weigh out 4.397 ± 0.002 g of zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) 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 (as Zn) of this solution is 1000 mg l^{-1}.

6.2.2 Zinc working solution

A zinc working solution (10 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 8 should be used when no previous data are available.

Based on initial results, the test concentration range of zinc for subsequent tests can be modified to enable more precise values of the 24 hour-LOEC and 24 hour-EC_{50} values to be derived.

Table 8 Zinc concentration range

Zinc concentration (mg l^{-1})	Volume of seawater (ml)	Volume of zinc working solution 6.2.2 (ml)
0 (control solution)	250	0.0
0.01	249.75	0.25
0.032	249.2	0.8
0.056	248.6	1.4
0.1	247.5	2.5
0.32	242	8
0.56	286	14

6.3 Test procedure

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

6.4 Processing of results

The 24 hour-LOEC and 24 hour-EC_{50} values should be calculated using the procedures described in section 4.

7 References

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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.

Standing Committee of Analysts
Environment Agency (National Laboratory Service)
56 Town Green Street
Rothley
Leicestershire, LE7 7NW
http://www.environment-agency.gov.uk/nls

Standing Committee of Analysts

This document is based on an Environment Agency funded project for the development of methods to assess effluent and receiving water quality with comments provided by Environment Agency ecotoxicology specialists, SCA members of Working Group 8 and the Main Committee.

CONTACTS:

ENVIRONMENT AGENCY HEAD OFFICE

Rio House, Waterside Drive, Aztec West, Almondsbury, Bristol BS32 4UD

www.environment-agency.gov.uk
www.environment-agency.wales.gov.uk

ENVIRONMENT AGENCY REGIONAL OFFICES

ANGLIAN
Kingfisher House
Goldhay Way
Orton Goldhay
Peterborough PE2 5ZR

MIDLANDS
Sapphire East
550 Streetsbrook Road
Solihull B91 1QT

NORTH EAST
Rivers House
21 Park Square South
Leeds LS1 2QG

NORTH WEST
PO Box 12
Richard Fairclough House
Knutsford Road
Warrington WA4 1HG

SOUTHERN
Guildbourne House
Chatsworth Road
Worthing
West Sussex BN11 1LD

SOUTH WEST
Manley House
Kestrel Way
Exeter EX2 7LQ

THAMES
Kings Meadow House
Kings Meadow Road
Reading RG1 8DQ

WALES
Cambria House
29 Newport Road
Cardiff CF24 0TP

ENVIRONMENT AGENCY
GENERAL ENQUIRY LINE
08708 506 506

ENVIRONMENT AGENCY
FLOODLINE
0845 988 1188

ENVIRONMENT AGENCY
EMERGENCY HOTLINE
0800 80 70 60

ENVIRONMENT AGENCY