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## Oyster embryo-larval bioassay (revised)

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## Contents

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<b>1</b>	<b>Abstract.....</b>	<b>1</b>
<b>2</b>	<b>Introduction .....</b>	<b>1</b>
<b>3</b>	<b>Collection, transport, storage and treatment of aqueous environmental samples .....</b>	<b>1</b>
3.1	Collection of environmental samples.....	2
3.2	Monitoring of physico-chemical parameters in test samples .....	2
3.3	Transport and storage .....	2
3.4	Preparation of samples .....	3
3.4.1	pH .....	3
3.4.2	Dissolved oxygen.....	4
3.4.3	Salinity.....	4
3.4.4	Suspended solids (optional treatment) .....	5
3.4.5	Colour.....	5
3.4.6	Other parameters .....	5
3.5	Disposal of samples .....	5
<b>4</b>	<b>Oyster (<i>Crassostrea gigas</i>) embryo-larval development test procedure.....</b>	<b>5</b>
4.1	Summary .....	5
4.1.1	Test organisms.....	6
4.1.2	Application .....	6
4.2	Oyster anatomy and embryo development .....	7
4.3	Storage and handling of oysters .....	11
<b>5</b>	<b>Guidelines for toxicity tests using a range of concentrations .....</b>	<b>11</b>
5.1	Design.....	11
5.2	Principle .....	11
5.3	Reagents and materials .....	12
5.3.1	Dilution water .....	12
5.3.2	Buffered formaldehyde solution.....	12
5.3.3	Apparatus .....	12
5.4	Test procedure.....	13
5.4.1	Obtaining oyster gametes .....	13
5.4.2	Fertilisation .....	14
5.4.3	Preparation of test dilutions .....	15
5.4.4	Initiation of the toxicity test.....	15
5.4.5	Monitoring of the toxicity test.....	16
5.4.6	Terminating the toxicity test.....	16
5.4.7	Processing of results .....	16
<b>6</b>	<b>Guidelines for single concentration toxicity tests .....</b>	<b>25</b>
6.1	Design.....	25
6.2	Test procedure.....	25

6.3	Processing of results .....	25
<b>7</b>	<b>Guidelines for reference toxicant tests using zinc.....</b>	<b>27</b>
7.1	Design.....	27
7.2	Reference toxicant preparation.....	27
7.2.1	Zinc stock solution.....	27
7.2.2	Zinc working solution.....	27
7.3	Test procedure.....	28
7.4	Processing of results .....	28
<b>8</b>	<b>References .....</b>	<b>28</b>
<b>9</b>	<b>Annex: Examples of typical record forms.....</b>	<b>29</b>
<b>10</b>	<b>Author contact information .....</b>	<b>33</b>
<b>11</b>	<b>Abbreviations .....</b>	<b>34</b>

## 1 Abstract

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The description of the oyster (*Crassostrea gigas*) embryo bioassay was initially published in the ICES TIMES series in 1991 (No.11). At the time, the bioassay was used in the United Kingdom for measuring water quality in relation to coastal waters which received anthropogenic discharges. Subsequently it was applied to measure general water quality and was taken up by the OSPAR Joint Assessment Monitoring Plan (JAMP) as a technique for measuring general biological effects in water, sediment elutriates, and pore water, and is used in several countries across the OSPAR Maritime Area.

The organism response used in the bioassay is the ability of early stage embryos to develop normally and reach the “D-shaped” larval stage (at which the paired hinged shells can be seen) within 24 hours. Although the exposure time is short, it encompasses a period of intense cellular activity during which the impairment of a number of critical physiological and biochemical processes may result in poor and abnormal growth and development.

The method described here is a complete revision of the original text, and includes a more comprehensive description of the methodology, how it can be applied for testing water quality for monitoring purposes, and for direct toxicity assessment or where a dilution series may need testing (e.g. extracts from passive samplers / sediment elutriates etc). Furthermore, there is additional information on the treatment and work up of results and analytical quality control.

**Keywords:** oyster embryo; bioassay, water quality

## 2 Introduction

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In 1991 the procedure for conducting the oyster embryo bioassay for water quality testing was published by ICES; Oyster (*Crassostrea gigas*) embryo bioassay. Techniques in Environmental Sciences No. 11. International Council for the Exploration of the Sea (Thain, ICES 1991). Subsequently, the procedure has been used widely and refined to reflect its application not only for general water quality testing but for the testing of samples for direct toxicity assessment and sample extraction – bioassay testing. It is therefore timely that the TIMES No 11 publication is revised to reflect these refinements.

The procedures described in this document follow the original protocol for water quality monitoring, but also enable direct toxicity assessments to be carried out on aqueous environmental samples using embryos of the Pacific Oyster (*Crassostrea gigas*). They take account of the previous guideline but additionally include developments based on the Environment Agency (England and Wales) project for developing test methods for effluent and receiving water assessments (Environment Agency, 2000) and more recent method developments (Leverett, Standing Committee of Analysts (SCA) 2007).

## 3 Collection, transport, storage and treatment of aqueous environmental samples

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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. 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.

### 3.1 Collection of environmental samples

Environmental samples should be collected in accordance with existing guidance given elsewhere (SCA, 1996 and ISO, 1993).

Environmental samples should be collected in containers, typically glass screw top 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 minimize any air space into which volatile components of the sample could diffuse.

### 3.2 Monitoring of physico-chemical parameters in test samples

The determination of selected physico-chemical 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 repeated on receipt at the laboratory. This enables changes (which may occur during transportation) in the physico-chemical 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 (SCA, 1979, SCA, 1986 and SCA 1998). Samples should be labelled appropriately with such details as the name and location of the site where the sample was taken and the date and time when the sample was taken. Any other relevant information, such as the name of the sampling person and chain of custody record form details (for example see Table A1) should also be recorded.

**Table 1. Parameters to be determined on site and in the laboratory.**

Sampling location / Laboratory
pH
Temperature
Dissolved oxygen
Salinity

### 3.3 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 should be allowed to reach  $24 \pm 2$  °C immediately on receipt at the laboratory. If the sample is not to be tested immediately, it should be stored in the dark at temperatures between 2–8 °C.

### 3.4 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 physico-chemical parameters such as pH, dissolved oxygen and salinity. This approach could mean that in certain instances it will not be possible to carry out the oyster embryo bioassay because the physico-chemical parameters as listed in Table 2 fall outside of the limits specified.

For Direct Toxicity Assessment (DTA) testing, modification or adjustment of the sample, or a dilution of it, is generally recommended so that all criteria for the physico-chemical parameters listed in Table 2 are met and the influence of these parameters is removed. Test results will therefore reflect the residual chemical toxicity of the discharge.

In selecting an approach there is an issue of how representative a test might be of the conditions in the environment, and whether modification or adjustment of a sample to meet test requirements causes a subsequent loss of environmental realism (due to changes in effluent character on adjustment). The influence of physico-chemical parameters will typically be more pronounced for effluents than receiving waters and problems of test dilution modification will generally only become important if toxicity occurs at higher effluent concentrations.

**Table 2. Threshold criteria for selected physico-chemical parameters for the oyster embryo-larval development test.**

pH	Dissolved oxygen (ASV)	Salinity (‰)
6.0 - 8.5	$\geq 60$	22 - 36

\* ASV is the air saturation value

Whilst no values have been specified for colour and suspended solids content, these parameters can affect the ability to physically observe the organisms.

Toxicity testing should not take place in situations where any of the threshold criteria for the physico-chemical parameters shown in Table 2 falls outside the specified range for all of the dilutions in a test. Where adjustment is required, this should, wherever possible, be restricted to 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 owing to the adjustment procedure.

#### 3.4.1 pH

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

### **3.4.2 Dissolved oxygen**

If the dissolved oxygen concentration in any of the test dilutions prior to testing is less than 60 % air saturation value (ASV) 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 gentle (for instance at a rate within the range 25–50 ml min<sup>-1</sup> l<sup>-1</sup>). 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 % ASV should not be aerated.

It is important to note that in a single-substance test, the test substance may cause high or low oxygen levels. Although this may be considered to be an ‘effect’, the test vessels may be aerated during the whole test period. This is achieved by a placing a pipette with a gentle air flow just breaking the surface of the water. The control exposure should also be aerated, but an un-aerated control should be included to assess whether there is an effect of aeration itself.

### **3.4.3 Salinity**

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

An increase in salinity is 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 (see Section 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 are then 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, provided it fulfils the requirements of the control (see Section 4.4.7.1).

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.

#### **3.4.4 Suspended solids (optional treatment)**

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 size 0.45 µm) using a vacuum filtration unit.
- (ii) Centrifuging the solution at 5000–10 000 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 (10 000 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.

#### **3.4.5 Colour**

Highly-coloured solutions may impair 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 (see Section 4.3.1) for viewing.

#### **3.4.6 Other parameters**

Test dilutions should be shaken or stirred to enhance homogeneity prior to dispensing into test vessels. Further information on other parameters which may need consideration in specific circumstances can be obtained elsewhere (ECETOC 1996, and BS EN ISO 1998) including guidance on the testing of effluents containing sparingly soluble substances (Girling *et al*, 1994).

### **3.5 Disposal of samples**

Test solutions and samples should be disposed of according to documented procedures based on the local practice of the laboratory.

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

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### **4.1 Summary**

Procedures are described, based on previously published guidance (Thain, 1991) for conducting static toxicity tests using embryos of the Pacific oyster (*Crassostrea gigas*) to assess the effects of aqueous environmental samples on embryo-larval development.

#### 4.1.1 Test organisms

Embryos are produced from the sperm and eggs of conditioned adult male and female oysters, each of individual whole wet weight greater than 45 g. Oysters can be conditioned in-house or purchased from recognised commercial sources. If oysters are purchased they should be delivered to the laboratory within 24 hours of dispatch.

#### 4.1.2 Application

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; and
- (iv) providing a general quality assessment of receiving waters (for example within monitoring programmes).
- (v) extraction-bioassay testing procedures

Two approaches to the test may be used:

- (i) A conventional procedure, based on previous guidance (Thain, 1991). 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 and more recent development of the test. This approach uses 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 but is particularly useful for the screening of effluents and TIE and/or TSE exercises in which abbreviated or 'high-throughput' versions of this procedure may be used. Such versions generally involve a reduction in the concentration range, replication, statistical analysis and quality assurance associated with the test performance and can be useful in situations where test result turnaround time and minimised costs are the primary considerations. The research which has led to the development of the miniaturised oyster-embryo test has been undertaken by the Environment Agency (UK) and addresses the issues of multi-well evaporation, gas exchange, chemical adherence to well plates, and potential loss of volatile substances (Hunt *et al*, 1998).

In both approaches;

- (i) Oyster embryos (*Crassostrea gigas*) should be exposed for  $24 \pm 2$  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 results of the toxicity test should be rejected if the percentage of abnormal larvae in the controls is greater than 40 %.

- (v) The temperature of the test dilutions should be maintained at  $24 \pm 2$  °C for the duration of the test.
- (vi) The pH of the test dilutions should be between 6.0 and 8.5 at the start of the test.
- (vii) The dissolved oxygen content in the test dilutions at the start of the test should be greater than 60 % ASV.
- (viii) The salinity of the test dilutions should be between 22–36 ‰.
- (ix) The approach taken for samples where any of the threshold criteria for the test solutions fall outside of the limits specified for the physico-chemical 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.

#### 4.2 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 (Galstoff, 1963) 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 for 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 characterized as a shrunken egg within the vitelline membrane. The development for normal embryos is given in Table 3 and is shown in Figure 2.

**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.

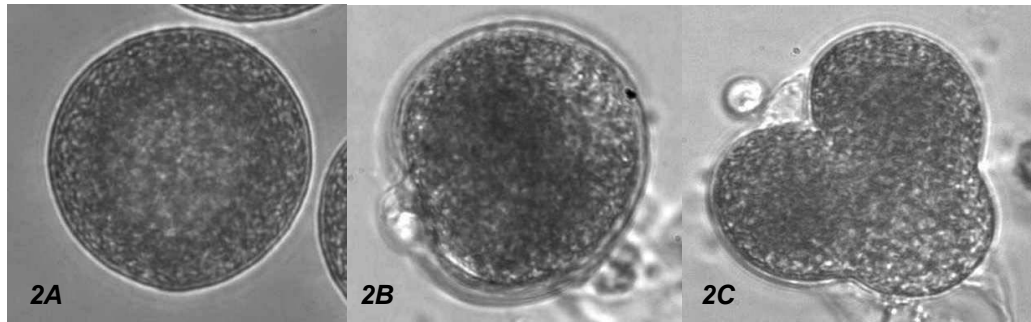


**Figure 1B.** Opened oyster with gamete tissue exposed. This is achieved by gently turning the mantle over using a flat, blunt instrument (e.g. a spatula).

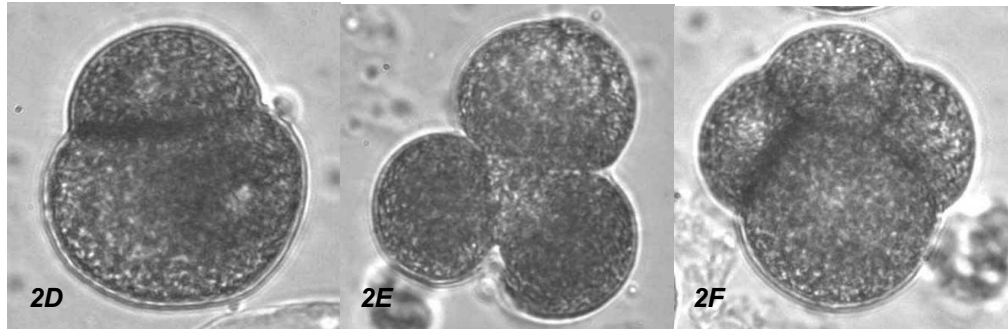


Fig 1C. Close up of oyster gamete tissue. A well-conditioned oyster will display gamete tissue which covers the entire dorsal surface of the stomach and surrounding tissue.

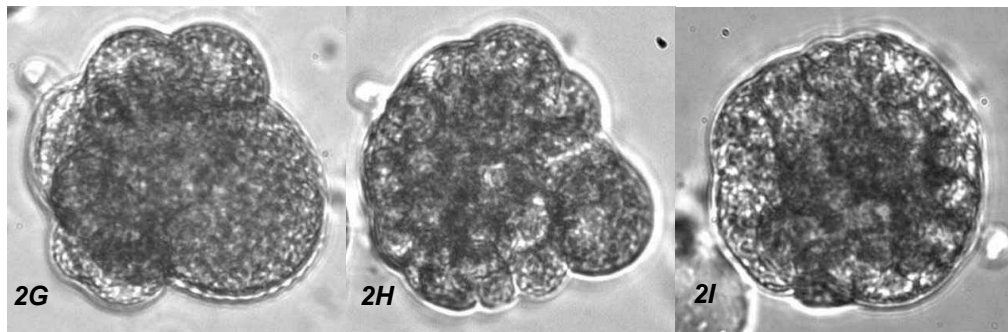
Figure 2. Embryology of *Crassostrea gigas*



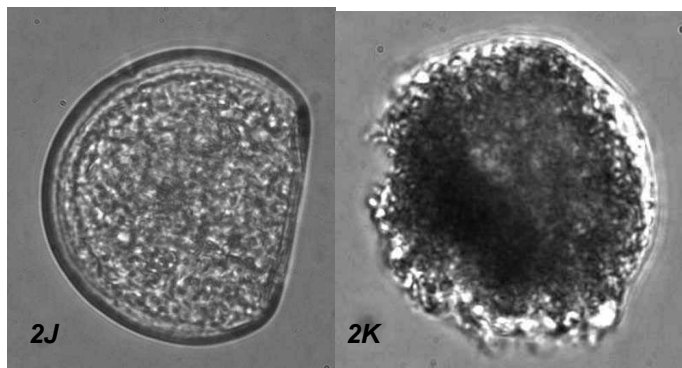
2A: Unfertilised egg (after approximately 30 minutes in seawater). 2B: Fertilised egg showing polar body. 2C: Fertilised egg undergoing 1<sup>st</sup> cell division.



2D: 2 cell embryo. 2E: 3 cell embryo. 2F: 4 cell embryo.



2G: 8 cell embryo. 2H: 16 cell embryo. 2I: 32 cell embryo.



2J: Normal 24 hour old larva. 2K: Abnormal 24 hour old larva

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:

- Greater than 95 % normally fertilised eggs;
- 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;
- 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.

### 4.3 Storage and handling of oysters

Oysters should normally be used within a few hours of being received in the laboratory and require no maintenance. However, if necessary, the oysters can be kept overnight provided they are stored in air (not submerged in water) 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.

If prolonged storage is necessary, the oysters may be placed in aerated sea water at <12°C and provided with food (suspension of algae). This way the oysters may remain in good condition for at least two or three days.

## 5 Guidelines for toxicity tests using a range of concentrations

### 5.1 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 at the outset.

### 5.2 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 solutions 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 characterized 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. percentage of normal D-shaped and abnormal larvae observed) should be used to determine:

- 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 h-EC<sub>10</sub>, 24 h-EC<sub>20</sub> and 24 h-EC<sub>50</sub> values respectively.
- The highest no-observed effect concentration after 24 hours (i.e. the 24 h-NOEC value).
- The lowest observed effect concentration after 24 hours (i.e. the 24 h-LOEC value).

### 5.3 Reagents and materials

#### 5.3.1 Dilution water

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

Artificial seawater is normally used as a substitute for natural seawater and is prepared by adjusting distilled or deionised water to a salinity of  $34 \pm 2$  ‰ with analytical grade sea salts. This should be aged by at least 24 hours prior to use and undergo continual aeration between preparation and use.

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.

#### 5.3.2 Buffered formaldehyde solution

Dissolve 5 g of sodium tetraborate in 250 ml of distilled or deionized 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.

#### 5.3.3 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 constant temperature environment to maintain test solutions at  $24 \pm 2$  °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  $\mu\text{m}$ ).

## 5.4 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 only necessary to strip sufficient adult oysters to obtain the requisite number of viable batches of sperm and eggs.

### 5.4.1 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. (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, for example see Table A2.

If stripping the gametes, the mature male and female conditioned oysters should be opened. Before opening, scrub the outside of the shell clean with a brush to remove adhering debris. The oysters can be opened by breaking the hinge and cutting the adductor muscle with a knife, 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 gametes can lead to reduced fertilization.

- 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  $24 \pm 2$  °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  $24 \pm 2$  °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  $\mu\text{m}$  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, e.g. 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 dilution water (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  $\mu\text{m}$ ) 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.

#### **5.4.2 Fertilisation**

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

- (i) Fertilizing the egg suspension from each female with the combined sperm suspension and subsequently combining individual viable and healthy embryo suspensions;
- (ii) Fertilizing 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 fertilization 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  $24 \pm 2$  °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, e.g. 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

#### 5.4.3 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 4 for the preparation of typical test solutions comprising 200 ml of test solution. Appropriate details should be recorded, for example on an oyster embryo-larval development toxicity test form (see Table A3).

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, for example on an oyster embryo-larval development toxicity test form (see Table A4).

**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

#### 5.4.4 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 50 - 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 4.4.6) 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, for example in an embryo-larval test data form (see Table A5).

The inoculated test dilutions should be incubated at  $24 \pm 2$  °C for  $24 \pm 2$  hours under static conditions without light.

#### **5.4.5 Monitoring of the toxicity test**

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, for example on a water quality monitoring data form (see Table A4).

#### **5.4.6 Terminating the toxicity test**

The test should be terminated after  $24 \pm 2$  hours. Buffered formaldehyde solution (see Section 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 is 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 is defined as those larvae that fail to reach the D-shaped stage, although they may be normal trochophores, or other early larval stages. In some cases, larvae may completely disintegrate under toxic challenge and it may not be possible to accurately determine the numbers that are abnormal. Unfertilized 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 dilutions 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 thorough mixing of the test dilution immediately 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 therefore 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.

#### **5.4.7 Processing of results**

##### **5.4.7.1 Validity of the results**

The results of the toxicity test should be rejected if the percentage of abnormal larvae in the control solutions exceeds 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.

##### **5.4.7.2 Data handling**

Endpoints such as the EC<sub>10</sub>, EC<sub>50</sub>, 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, for example see Table A6. 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 (i.e. normal D-shaped and abnormal) counts for the controls 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 total of which is by definition 100 %, i.e.  $PND + PAD = 100$ .

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

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

If counts from controls are used, then

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

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

$$PAD = 100 - PND$$

The mean  $PND_m$  and  $PAD_m$  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

$$PNR_{(TC)} = [ ( PAD_{m(TC)} - PAD_{m(C)} ) / ( 100 - PAD_{m(C)} ) ] \times 100$$

Where  $PAD_{m(TC)}$  is the mean PAD value for the test concentration and

$PAD_{m(C)}$  is the mean PAD value for the control.

Test endpoints can then be estimated using the  $PNR_{(TC)}$  values for each test concentration (see Fig. 3).

#### 5.4.7.3 Estimation of EC values

The 24 h- $EC_{10}$ ,  $EC_{20}$  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 with the test results.

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

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  $\geq 60$  %, 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.

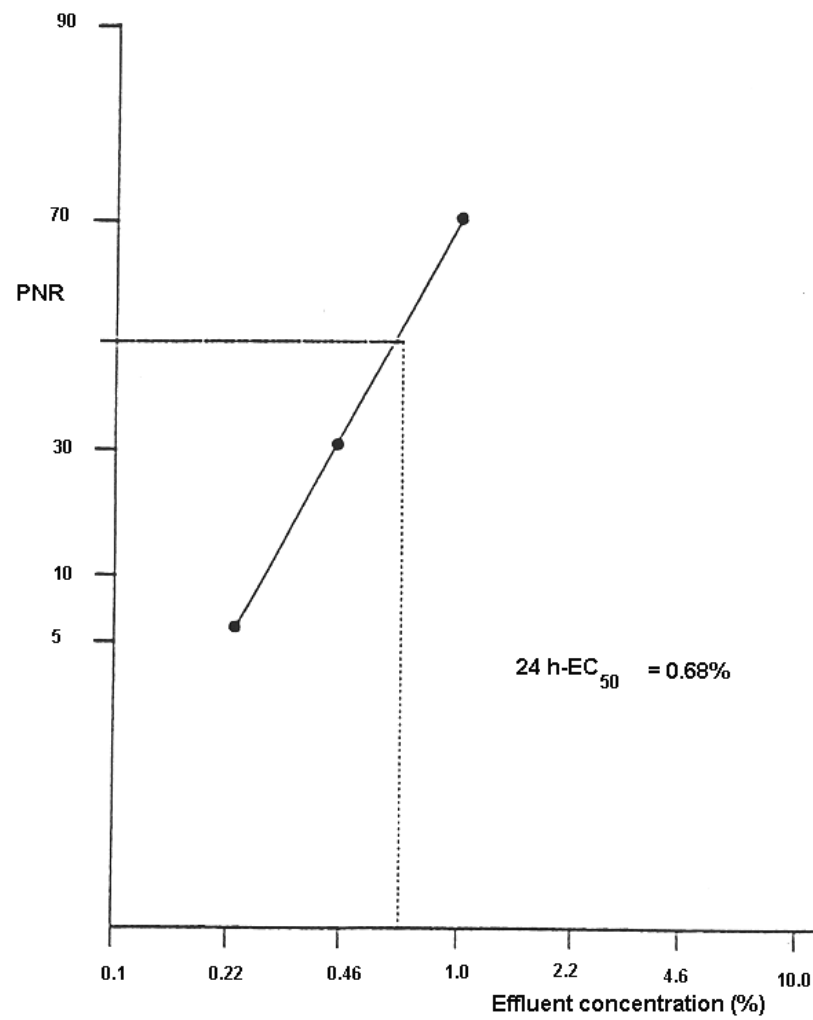


Figure 3. Graph of dose-response curve of cumulative inhibition of development of D-shaped larvae (probability scale) against effluent concentration (log scale).

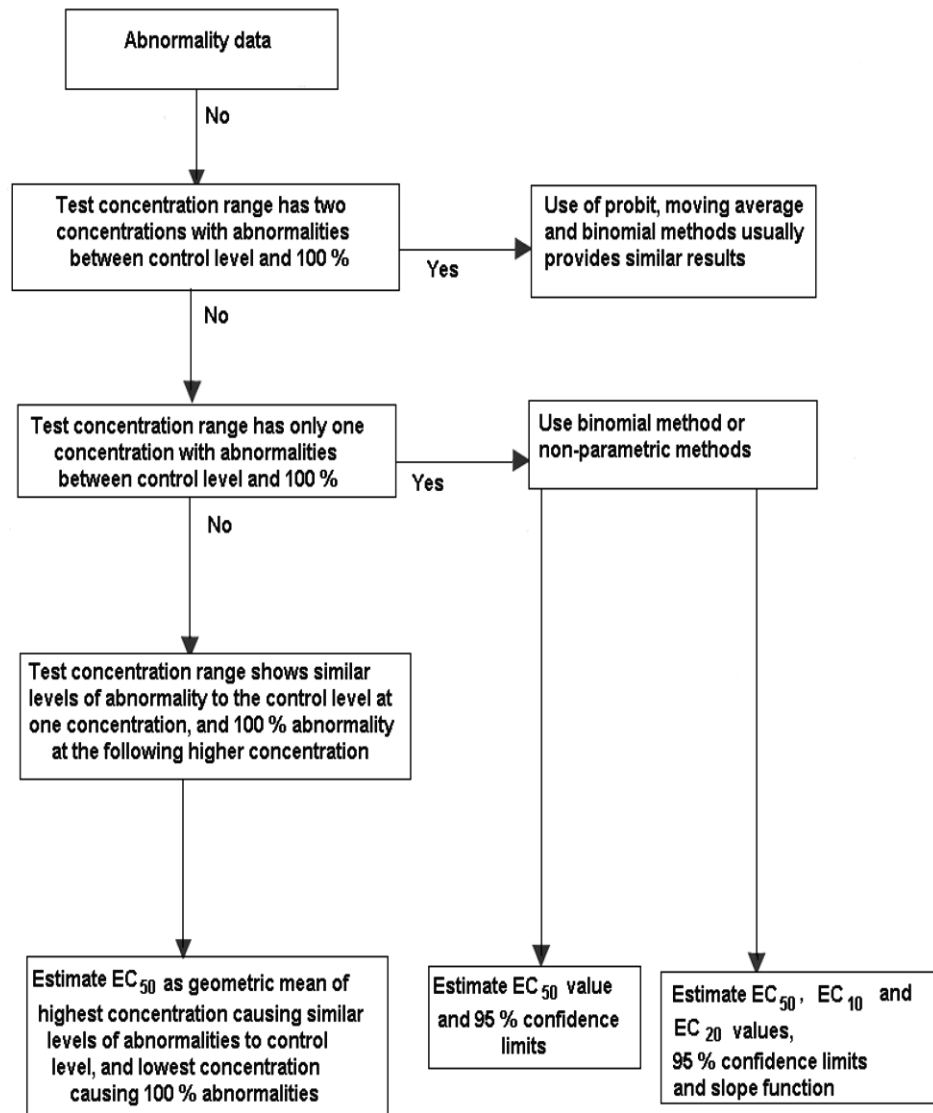


Figure 4. Flowchart for the estimation of the EC<sub>50</sub> value for full concentration range tests.



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	0	-	-	100	100	100	100	-	-	100	100
22.0	0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
46.0	0	0	0	0	0	-	-	100	100	100	100	-	-	100	100
100.0	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	2.2
0.22	82	88	85	85	-	-	18	12	15	15	-	-	15	5.6	5.6
0.46	8	85	86	89	-	-	14	15	14	11	-	-	14	4.4	4.4
1.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100	100
2.2	0	0	0	0	-	-	100	100	100	100	-	-	100	100	100
4.6	0	0	0	0	-	-	100	100	100	100	-	-	100	100	100
10.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100	100
22.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100	100
46.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100	100
100.0	0	0	0	0	-	-	100	100	100	100	-	-	100	100	100

Table 6. Summary of EC<sub>50</sub> values and 95% confidence limits for the data in Scenarios 1, 2 and 3.

Scenario	Statistical procedure	EC <sub>50</sub> value	95% percent confidence limits	Slope of dose response curve
1	Probit	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	0.50	0.46-0.55	-
3	Geometric mean	0.68		

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<sub>50</sub> values derived are less statistically sound.

From the data shown in Figure 3, the 24 h-EC<sub>10</sub> and 24 h-EC<sub>50</sub> 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 h-EC<sub>50</sub> is 0.68 % v/v effluent and the 24 h-EC<sub>10</sub> is 0.27 % v/v effluent. The values obtained graphically complement those obtained using computer-based software (see Table 6).

#### 5.4.7.4 Estimation of the NOEC and LOEC

The 24 h-NOEC and 24 h-LOEC values should be determined using hypothesis testing (see Figure 5). Initially the proportion of organisms surviving in the control solutions and test solutions are transformed using an appropriate procedure such as the arc sine square root transformation. The arc sine square root transformation is commonly used on proportional data to stabilize 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 with Bonferroni adjustment or Steel's many-one rank test should be used to analyse the data depending on whether there are equal numbers of replicates in each treatment.

If the data meet the assumption of normality, the 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 used to analyse the data depending on whether there are equal numbers of replicates in each treatment.

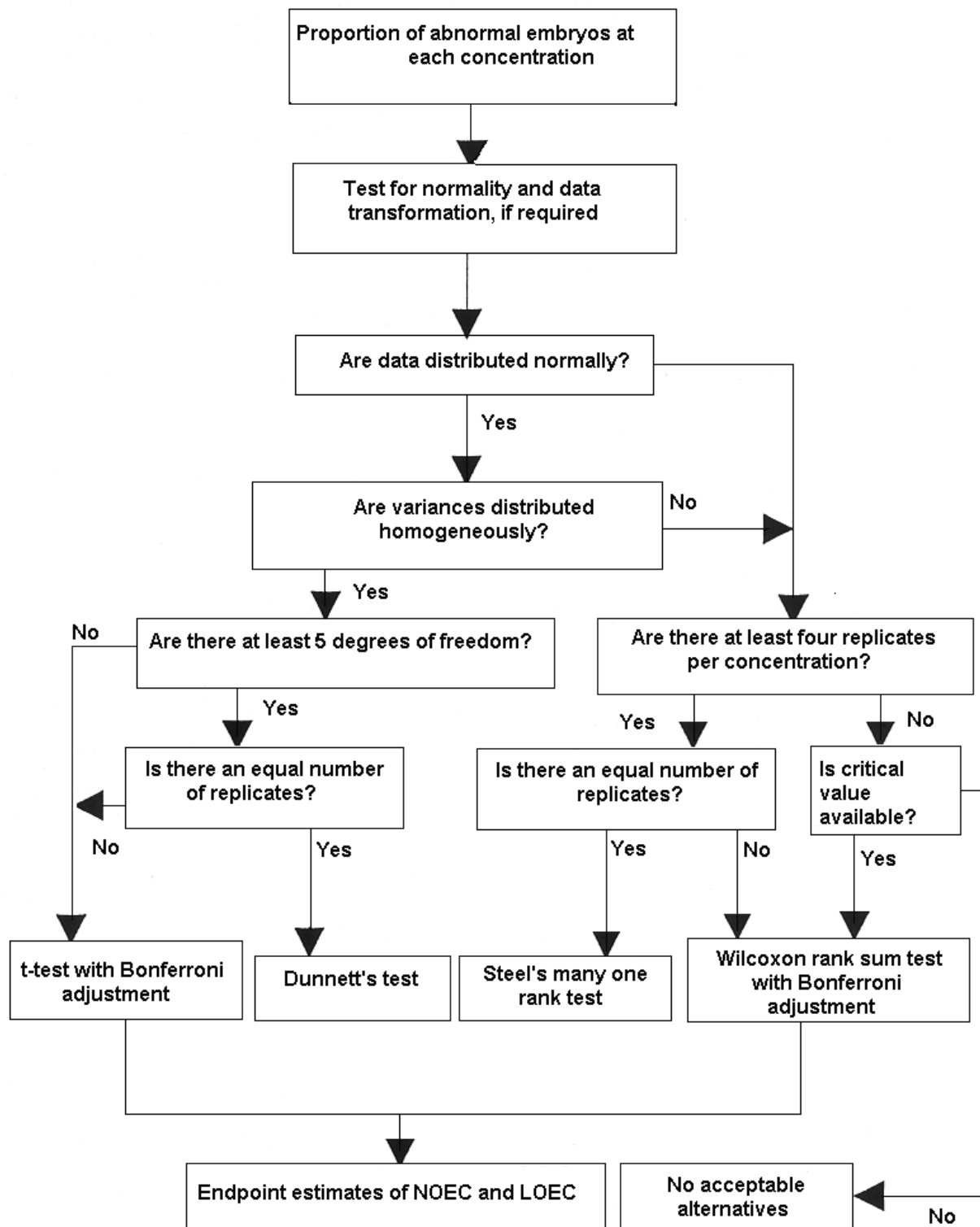


Figure 5. Flowchart for the estimation of NOEC and LOEC values for the oyster embryo-larval development toxicity test in full concentration range.

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 in each treatment. Further information on these statistical procedures can be obtained elsewhere (Stephan, 1977; Sokal, 1981;

Zar, 1984; and US EPA, 1993). In the example given in Figure 3, the 24 h-NOEC and 24 h-LOEC values calculated using ANOVA and t-tests with Bonferroni adjustment are 0.1 % and 0.22 % v/v effluent respectively.

## **6 Guidelines for single concentration toxicity tests**

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### **6.1 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 physico-chemical 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 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.

### **6.2 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, e.g. on a water quality monitoring data form (see Table A4).

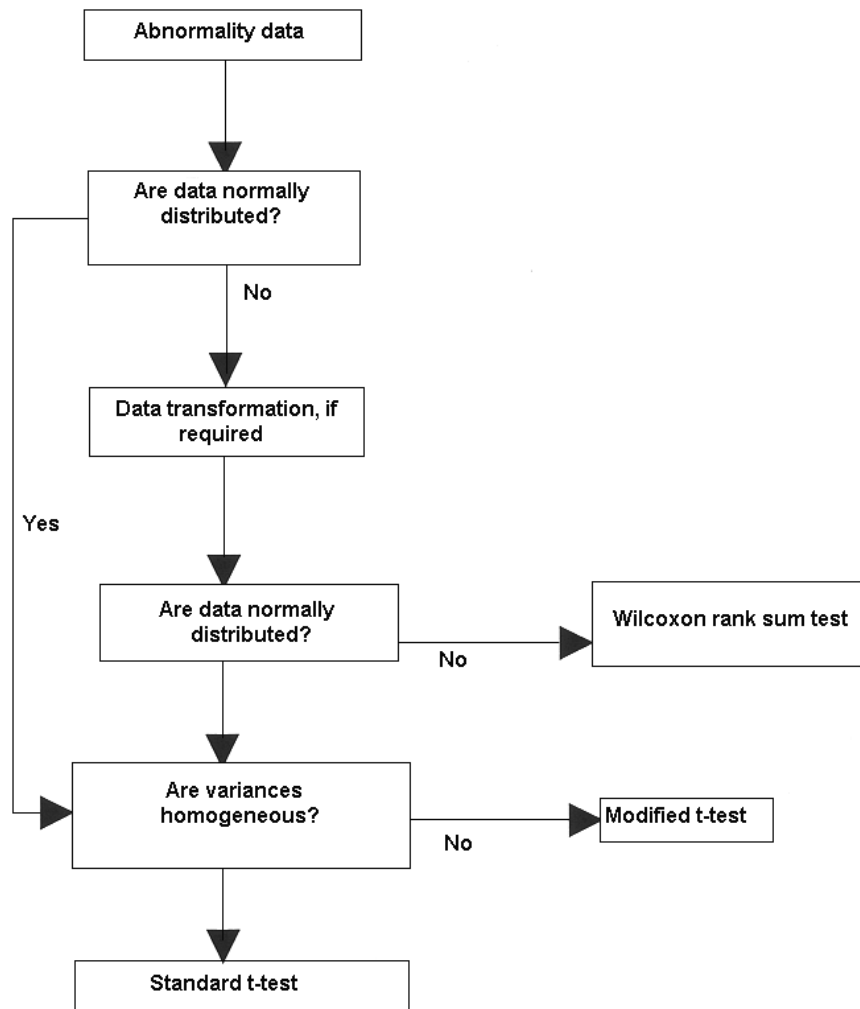
### **6.3 Processing of results**

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 (Stephan, 1977; Sokal, 1981; Zar, 1984; and US EPA, 1993).

A percentage net response (PNR) value may also be derived for the single sample concentration as described in Section 4.4.7.2.



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

Table 7 shows examples of data for a single concentration test (i.e. 0.22 % v/v effluent concentration) and control solutions. 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

## 7 Guidelines for reference toxicant tests using zinc

### 7.1 Design

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

### 7.2 Reference toxicant preparation

#### 7.2.1 Zinc stock solution

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

#### 7.2.2 Zinc working solution

A zinc working solution ( $10 \text{ mg l}^{-1}$ ) 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.

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 24 h-LOEC and 24 h-EC<sub>50</sub> values.

Table 8. Zinc concentration range

Zinc concentration ( $\text{mg l}^{-1}$ )	Volume of seawater (ml)	Volume of zinc stock 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

### 7.3 Test procedure

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

### 7.4 Processing of results

In toxicity tests the results are normally expressed as the 24 h-LOEC and 24 h-EC<sub>50</sub> values as calculated using the procedures described in Section 4.

Results for field collected water quality monitoring are usually expressed as PNR relative to the control and the design of the programme should include reference sites where water quality for embryo development is known to be good i.e. embryo development is normal. The oyster embryo test has been used for water quality monitoring in UK estuaries over many years and examples of the data can be found in the Charting Progress 2 Report, 2010 (CP2, 2010).

## 8 References

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- BS EN ISO, 1998. BS EN ISO 1998:5667-16 Water Quality – Sampling: Guidance on biotesting of samples.
- CP2, 2010. <http://chartingprogress.defra.gov.uk/feeder/CSSEG-section-3-1-hazardous-substances.pdf>
- ECETOC, 1996. Aquatic toxicity testing of sparingly soluble, volatile and unstable substances. ECETOC Monograph No. 26, 1996, pp. 1-67.
- Environment Agency, 2000. Short-term Methods Guidelines for Effluent and Receiving Water Assessment, Research and Development Technical Report E83.
- Galstoff, P.S. 1963. The American oyster, *Crassostrea gigas*, *Fishery Bulletin*, 1963, 64 pp.
- Girling, A.E., Whale, G.F., and Adema, D.M.M., 1994. A guidance supplement for determining the aquatic toxicity of poorly water soluble complex mixtures using water accommodated fractions, *Chemosphere* 29 (12), pp2645-2649.
- Hunt, J. W., Anderson, B.S., and Phillips, B.M. 1998. Recent Advances in Microscale Toxicity Testing with Marine Mollusks, in *Microscale Testing in Aquatic Toxicology, Advances, Techniques and Practice*, (Eds) P G Wells, K Lee and C Blaise, CRC Press LLC, pp. 423-436.
- ISO, 1993. ISO 1993:5667-2 Water quality - Sampling: General guidelines to sampling techniques.
- SCA, 1986. Standing Committee of Analysts, Temperature Measurement for Natural, Waste and Potable Waters and other items of interest in the Water and Sewage Disposal Industry 1986, *Methods for the Examination of Waters and Associated Materials*, ISBN 0117520179, in this series.
- SCA, 1979. Standing Committee of Analysts, Dissolved oxygen in natural and waste waters 1979, *Methods for the Examination of Waters and Associated Materials*, ISBN 011751442X, in this series.
- SCA, 1996. Standing Committee of Analysts, General Principles of Sampling Water and Associated Materials (2nd Edition) 1996: Estimation of Flow and Load (1996), *Methods for the Examination of Waters and Associated Materials*, ISBN 011752364X, in this series.



- SCA, 1998. Standing Committee of Analysts, The determination of pH in low ionic strength waters 1998, *Methods for the Examination of Waters and Associated Materials*, ISBN 0117520845, in this series.
- SCA, 2007. Standing Committee of Analysts, The Direct Toxicity Assessment of Aqueous Samples using the Oyster (*Crassostrea gigas*) Embryo-larval Development Test, Blue Book No. 209.
- Sokal, R.R. and Rohlf, F.J., 1981. Biometry, W H Freeman and Company, San Francisco, 859 pp.
- Stephan, C.E. 1977. Methods for Calculating an LC<sub>50</sub>. in Aquatic Toxicology and Hazard Evaluation, (Eds) F L Mayer and JL Hamelink, ASTM, 1977, STP 634, Philadelphia. pp. 65—84.
- Thain, J. 1991. Biological effects of contaminants: Oyster (*Crassostrea gigas*) embryo bioassay. *ICES Techniques in Environmental Sciences* No 11. International Council for the Exploration of the Sea, Copenhagen, Denmark. 12 pp.
- USEPA, 1993. Methods for measuring the acute toxicity of effluents to freshwater and marine organisms. United States Environmental Protection Agency, 1993, Report EPA/600/4-85/013, Cincinnati, OHIO.
- Zar, J.H. 1984. Biostatistical Analysis. Prentice Hall International, New Jersey, 716 pp.

9 Annex: Examples of typical record forms

Table A1. Chain of custody record form.

a) Sample details (to be completed by sampler or following analysis at laboratory*)					
Sample Details:		Site code or NGR:	Place:	Sample of:	
Date taken:		Time taken:	Sample number:	Receipt number (if applicable):	
Sample pH:		Sample temperature (°C):	Sample dissolved oxygen level:		
Sample salinity (‰):					
Date and time sample deposited at:		Is sample an emulsion (Y/N):		depot*/handed to:	
By (print name):		Signature:		* delete as appropriate	
b) Transportation details (to be completed by courier)					
Person or Collected from	Depot	Date	Time	Name of courier	Signature
				Employed by	Deposited at/handed to
c) To be completed at laboratory					
Sample received at (laboratory):		Date:		Time:	
Sample received by (print name):		Signature:			
Were seals intact? Y/N:		Laboratory code allocated:			

\* Delete as appropriate

Table A2. Oyster embryo larval development toxicity test form.

<b>Data on test species</b>	
Test species:	
Origin of test species:	
<b>Oysters purchased from a supplier</b>	
Date received:	Number of oysters:
Size range:	
Conditioning method:	
<b>Data on test substance</b>	
Test substance:	
Source:	Hazard:
Date collected:	
Date received:	
Storage conditions:	Temperature (°C)
Length of time before start of test (h)	
<b>Stripping of oysters</b>	
Number of females opened:	Number of males opened:
Number of females used:	Number of males used:
Wet weights (g):	Wet weights (g):
Condition of oysters:	Condition of oysters:
Source of water:	
Water temperature (°C):	
<b>Fertilization of gametes</b>	
Time of addition of sperm:	Volume of sperm added:
Incubation temperature:	
Time between addition of sperm and test inoculation:	

Table A3. Toxicity test concentration range

<b>Test substance:</b>		
Type of test:		
Start of test:	End of test:	
Duration of test:		
Number of test concentrations:		
Number of replicates per concentration:		
Number of controls:	Dilution water	
	Other	
Concentration range:		
Stock solution concentration:		
Dilution water:		
Volume of test solution required (ml):		
Test concentration	Volume of reference seawater (ml)	Volume of test substance (ml)
Egg suspension concentration (number per ml):		
Volume of egg suspension added to test containers:		



## 10 Author contact information

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## 11 Abbreviations

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ANOVA	analysis of variance
ASV	air saturation value
DTA	direct toxicity assessment
EC	effect concentration
LOEC	lowest observed effects concentration
NOEC	no observed effects concentration
PAD	percentage abnormal development
PND	percentage normal development
PNR	percentage net response
TIE	toxicity identification evaluation
TSE	toxicity source evaluation