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Water quality — Determination of the toxicity of water samples on the embryo-larval development of Japanese oyster (*Crassostrea gigas*) and mussel (*Mytilus edulis* or *Mytilus galloprovincialis*)

Qualité de l'eau — Détermination de la toxicité d'échantillons aqueux sur le développement embryo-larvaire de l'huître creuse (Crassostrea gigas) *et de la moule* (Mytilus edulis *ou* Mytilus galloprovincialis)

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Contents

Page

Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT), see the following URL: Foreword — Supplementary Information.

The committee responsible for this document is ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Introduction

Traditionally, the level of pollution affecting a marine environment is shown in terms of the concentration levels of the contaminants present in the environment of interest. However, these measurements do not provide an estimation of the harmful effects on organisms and have to be complemented with the biological responses obtained through biotests (see Reference [5]).

Among the marine organisms used to assess the potential impact of chemicals or discharges into the environment, bivalve embryos and larvae are, together with sea urchins, among the organisms which are most frequently used in biotests. This has been the case since the first research undertaken by Lillies (1921) (see Reference [18]) on the sea urchin *Arbacia* and by Prytherch (1924) (see Reference [21]) on the oyster *Crassostrea virginica*. The embryos and larvae are less tolerant to pollutants than the adults of the same species. They therefore represent the critical stages for the toxicity tests (see References [19] and [30]). Since 1972, Woelke (see Reference [35]) has recommended the use of the Pacific oyster, *Crassostrea gigas*, to assess the quality of seawater. Furthermore, their worldwide distribution in coastal waters, as well as their commercial importance (see Reference [10]), make bivalves the species of choice for the undertaking of biotests.

The results of these biotests demonstrate the necessity to determine the potential toxicity thresholds of chemicals which could enter the marine environment either accidentally or chronically, as well as the "biological quality" of an environment or the potential toxicity of river water or a discharge that reach the sea. Quiniou et al. (1993, 1997) (see References [27] and [26]) and His *et. al* (1999) (see Reference [11]) defined potential toxicity on the basis of teratological effects.

This International Standard specifies a method based on the embryo-larval development of bivalves (oyster or mussel). It can be routinely used to assess development abnormalities caused by the possible presence of chemicals and mixtures in seawater. It also allows to assess the toxicity of aqueous samples like seawater, surface water, effluents (urban, agricultural, industrial effluents, etc.), aqueous extracts from sediments, and petroleum products that could be leached in the water column at the time of their resuspension or discharge and presence in the sea.

This test can be performed throughout the year with mature bivalves sampled from the natural environment during their reproduction periods or mature bivalves which come from a hatchery where they have been conditioned.

This toxicity test, recommended by the International Council for the Exploration of the Sea (ICES), (see Reference [14]), has been the subject of the first European inter-calibration test performed in 1991 (see Reference [31]). The protocol described in this International Standard corresponds to a modification and simplification of the ASTM standard method (1994) (see Reference [3]).

The toxicity assessment of metals performed on *C. gigas* and *Mytilus edulis* demonstrated that both organisms had a similar level of sensitivity (see References [19] and [15]). Two other studies performed on urban effluents showed similar findings for both species (see References [16] and [28]). These observations have been confirmed by the work carried out on mercury by Beiras and His (1994) (see Reference [4]), who compared the findings of four embryo-larval tests: *M. edulis*, *M. galloprovincialis*, *C. gigas,* and *C. virginica.* Another study showed that the embryos of *C. gigas* are more sensitive to metals and hydrocarbons than the other marine organisms which are commonly used, for example, polychaete, amphipods, fish, and crustaceans (see Reference [8]).

The sensitivity of the bivalve embryo-larval development confirms the suitability of this test to assess the toxicity of chemicals and aqueous samples. The pH, salinity, and temperature ranges acceptable to bivalves make them easy to use in ecotoxicity studies, particularly when assessing the quality of coastal and estuarine environments (see Reference [11]).

Water quality — Determination of the toxicity of water samples on the embryo-larval development of Japanese oyster (*Crassostrea gigas*) and mussel (*Mytilus edulis* or *Mytilus galloprovincialis*)

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International Standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this International Standard be carried out by suitably qualified staff.

1 Scope

This International Standard specifies a method for determining the effects of chemical and aqueous samples on the embryo-larval development of marine bivalves. It allows the determination of the concentration levels that result in an abnormality in embryo-larval development. This test is suitable for salinity ranges between 20 and 40 for mussels and between 25 and 35 for oysters. This method applies to

— chemical substances and preparations,

— marine and brackish waters,

— streams and aqueous effluents (urban, agricultural, industrial effluents, etc.) as long as the salinity is adjusted and/or dilution is limited so that the aforementioned salinity ranges are respected, and

— aqueous extracts (pore water, elutriates, eluates, and leachates) from sediments and petroleum products.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 5667-16, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

ISO 14442, *Water quality — Guidelines for algal growth inhibition tests with poorly soluble materials, volatile compounds, metals and waste water*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
EC_x
calculated concentration (of a substance) or dilution (of an aqueous sample, in %) for which an effect of x % is expected compared to the control

3.2
lowest observed effect concentration
LOEC
lowest concentration of the tested sample at which a statistically significant effect is observed

3.3
no observed effect concentration
NOEC
tested concentration just below the LOEC

[SOURCE: ISO/TS 20281:2006, 3.18]

3.4
reference seawater
natural or synthetic seawater used to induce gamete production and prepare the solutions to be tested

3.5
D-shell stage larvae
larvae stage so named due to their characteristic D-shape under microscopic examination

Note 1 to entry: The normal D larvae obtained after incubation have fully developed and symmetrical shells with a straight hinge. The larvae size is regular and around 70 µm. After fixation, the mantle nearly fills the interior of the larvae, but is totally included within the two closed shells.

4 Principle

This biotest assesses the effects of chemicals and aqueous environmental samples on the embryo-larval development of marine bivalves under static conditions.

The exposure is performed from fertilized eggs to D larvae. This static test aims to determine the concentration level (EC_x) which results in abnormalities for x % of exposed larvae in 24 h for the Japanese oyster, also named Pacific oyster (*Crassostrea gigas*), and in 48 h for the mussel (*Mytilus edulis* or *Mytilus galloprovincialis*). Several parameters can be assessed on the abnormal larvae: alteration of the shell (hinge is not straight, unequal, or incomplete valves), hypertrophy of the mantle, delayed or stopped embryonic development, and finally, death. The results are expressed as EC_x (EC_{20} or EC_{50}). The lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) can also be determined.

NOTE This method can be applied to other species of bivalves (e.g. *C. virginica*). Nevertheless, the test conditions have to be defined to reach the validity criteria of the standard.

5 Test organisms and seawater

5.1 Spawning stock or mature bivalves

The mature bivalves used for gamete production can be obtained from the natural environment during reproductive periods as long as the good quality of their sampling area has been proven. The reproduction period along the European and African coasts depends on the site. In some places, it can occur all year.

For oysters, it is also possible to use mature animals from hatcheries where they have previously undergone a conditioning cycle so that they are ready for spawning as soon as they arrive in the laboratory. This enables to perform tests throughout the year.

As soon as the bivalves are received in the laboratory, it is recommended to keep them dry until the beginning time of the experiment (for example, 15 °C) or in seawater at a temperature close to their conditioning or rearing temperature (for example, 20 °C for hatchery oysters). If the mature bivalves have to be kept for more than 48 h after sampling and/or dispatching, they shall be stored in water (see 5.2) with the same temperature as the original location and shall be provided with rich and appropriate

feeding (see Reference [9]). In this case, the spawning stock is placed in tanks (15 animals for 30 l of seawater). The water in the tanks, which is continuously aerated, is kept at a temperature of 20 °C ± 1 °C. On a daily basis, one third of the volume has to be discarded and replaced by the same volume of a single-species Prasinophycean culture (*Tetraselmis suecica*) at an average concentration of 1×10^6 cells per ml or diatom *Skeletonema costatum* at an average concentration of $2,7 \times 10^6$ cells per ml.

The pH of seawater should be between 7,0 and 8,5 for mussels and oysters.

NOTE Other species can be used, for example, *Phaeodactylum tricornutum*. Neverthelss, the appropriate concentration of algae has to be defined.

5.2 Reference seawater

This test requires good quality reference seawater. This water is used to prepare the controls and dilutions of the samples and/or chemicals to be tested. This seawater may be either natural or synthetic.

5.2.1 Natural seawater

Natural seawater shall allow a good bivalve embryo-larval development and enable at least 80 % of the normal D larvae to be free of any abnormality. The test is sensitive to high concentrations of ammonia (NH_3). Consequently, the ammonium concentration of seawater used shall not exceed 100 µmol/l (= 1,8 mg/l).

As soon as the water is collected, it is recommended that it is checked to ensure that the water is not contaminated by any known substance (discharges, human activity, etc.). The water should be pre-filtered with 1 µm membrane. The seawater shall then be stored in the dark in controlled conditions between 5 °C and 15 °C and be used within two weeks from collection. Under no circumstance shall this seawater be frozen or autoclaved.

Just before use, adjust the seawater salinity if necessary by adding ultra-pure water (for dilution) or hypersaline brine (see 5.2.3) to reach the salinity range adapted to the selected species: i.e. from 20 to 40 for mussels and 25 to 35 for oysters. Then, filter it through a 0,45 µm membrane. Salinity shall then be checked with a suitable probe (see 6.6).

Direct addition of sea salts to the sample may be a source of toxicity and should be avoided (see Reference [17]).

5.2.2 Synthetic seawater

Alternatively, synthetic seawater prepared in compliance with Table 1 may be used. The composition of this seawater is similar to that suggested by Zaroogian et al. (1969) (see Reference [36]) without EDTA in order not to reduce the bio-availability of bivalent metal ions, thus, resulting in a decrease in the apparent toxicity of these ions (see Reference [25]). Synthetic seawater is prepared by adding reagent grade chemicals to ultrapure water (distilled or demineralized water) in the order specified in Table 1. Prepare a minimum of 5 l of synthetic seawater. Mix after each addition of salt to ensure a good dissolution.

Once ready, the synthetic seawater is filtered in the same way as the natural seawater (see 5.2.1).

Table 1 — Composition of synthetic seawater for one litre of ultra-pure water

Chemical	Concentration in ultrapure water (g/l)
NaF	0,003
$SrCl_2 \cdot 6H_2O$	0,02
H_3BO_3	0,03
KBr	0,1
[a] Silicate is not needed when the water is prepared in a glass vial.	

Table 1 *(continued)*

Chemical	Concentration in ultrapure water (g/l)
KCl	0,7
$CaCl_2 \cdot 2H_2O$	1,47
Na_2SO_4	4,0
$MgCl_2 \cdot 6H_2O$	10,78
NaCl	23,5
$Na_2SiO_3 \cdot H_2O$[a]	0,2
$NaHCO_3$	0,2

[a] Silicate is not needed when the water is prepared in a glass vial.

Synthetic seawater which only contains mineral salts may be kept for up to one year in a watertight container that is kept out of the light in a clean, dry, temperate and odourless place. It may also be kept in a cold room in watertight containers.

5.2.3 Hypersaline brine (HSB)

Hypersaline brine can be made by concentrating of natural seawater by freezing or evaporation. The maximum salinity of brine prepared this way is around 100 % (USEPA method, Reference [33]).

Hypersaline brine can also be prepared following the Zaroogian's formula concentrated up to 5x maximum.

Commercial sea salts may also be used for preparing HSB, but a test with the reference substance shall be conducted to assess the absence of complexing agents.

A control test with the HSB diluted to an acceptable salinity for the embryo development has to be realized to check the lack of effect of this preparation.

6 Equipment

Usual laboratory equipment and in particular, the following.

6.1 Thermoregulated room or enclosure for the incubations.

6.2 Microscope, at least 200x, but preferably 400x.

If possible, use an inverse light microscope so that observations can be made directly in the small experiment vials (eg. microplate wells).

6.3 Culture flasks, capacity from a fraction of millilitres to several litres.

Experiments are usually performed using a volume of test solution of 50 ml. Therefore, culture flasks with the capacity of 100 ml to 200 ml are preferred. Culture flasks may be made of glass (systematically washed and sterilized) or single-use crystal polystyrene such as multi-well plates or medical sampling containers.

6.4 Cartridge or membrane based filtering device, equipped with filters and pre-filters suitable for the preparation of the test media.

6.5 Oven or autoclave, for sterilizing the equipment and glassware for the biotests.

6.6 Equipment for measuring temperature, salinity, pH, and dissolved oxygen in water.

6.7 Sieves, with mesh size of 32 µm and 100 µm for the filtering of male and female gametes, respectively.

The gametes which pass through the appropriate sieves are collected for the fertilization step.

6.8 Pipettes, single-use polyethylene transfer pipettes (1 ml to 3 ml).

6.9 Binocular magnifying glass.

6.10 Electronic particle counter (optional).

All glassware used as for isolation of the spawning stock and the collection of the gametes, as well as the pipettes, shall either be disposable or sterilized before use in the tests (e.g. placed in an oven for 2 h at 200 °C for glassware).

7 Reference substance

Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) is the recommended reference substance. This chemical is added systematically in each test series to check the sensitivity of the larvae. The test concentrations are included in the range 0 µg/l to 100 µg/l of $CuSO_4 \cdot 5H_2O$, or, 0 µg/l to 25 µg/l expressed as copper.

The value of EC_{50} should be between 4 µg/l and 16 µg/l expressed as total copper (see Annex B).

Alternately, zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) can be used as reference substance. In such case, the test concentrations should be included in the range 44 µg/l to 2 462,9 µg/l of $ZnSO_4 \cdot 7H_2O$, or, 10 µg to 560 µg expressed as zinc.

NOTE Experience gained with zinc sulfate is less than for copper sulfate. Therefore, no acceptable range can be recommended yet in this International Standard.

8 Test procedure

8.1 Collection, preparation, and preservation of aqueous samples

Collect and transport the samples in accordance with the general procedures described in ISO 5667-16.

Samples are collected in chemically inert flasks.

Undertake the toxicity test as soon as possible, ideally within 12 h after collection of the samples. If this time cannot be followed, store the samples at 4 °C and perform the test preferably within 15 d after sample collection.

8.2 Preparation of test samples

8.2.1 Chemicals

Stock solutions of test chemicals are prepared by dissolving the substances in the reference seawater (either natural or synthetic).

When the substance to be tested is poorly soluble in seawater, the stock solution may be prepared in demineralized water or according to the modifications specified in ISO 14442 and ISO 5667-16 (use of a solubilizing agent, ultrasonic dispersion, etc.).

8.2.2 Aqueous samples

Aqueous samples (seawater, river water, urban, agricultural or industrial effluents, or even aqueous extracts) are tested in their raw state and/or after filtering or centrifugation to determine the fraction

responsible for the effects observed. Furthermore, depending on the physico-chemical parameters of these aqueous samples, it may be necessary to adjust their salinity to be consistent with the recommended range for the selected species.

8.3 Selection of concentration/dilution range

For chemicals, the test solutions are prepared by diluting the stock solution in natural or synthetic seawater.

In the case of samples of low salinity water, effluents, leachates, and eluates, all dilutions shall remain within the salinity range acceptable for the species being used. For freshwater, the maximum concentration tested shall not exceed 18 % volume fraction of the initial sample. Salinity should be adjusted with hypersaline brine (see 5.2.1) if this appears to be essential.

These solutions may be prepared in advance or immediately before the test if rapid changes in the sample composition are expected.

A minimum of five dilutions shall be performed to cover a concentration range that enables the observation of a full range of effects between 0 % and 100 % of abnormal embryo-larval development. Depending on the possible effects sought, the substances may be tested alone or in mixtures. For the aqueous samples, the dilution range to be tested may be optimized in line with the test's aim: calculation of an EC_x value or determination of a no effect dilution.

The experiment should include at least three replicates per test concentration/dilution, one solvent control (if appropriate), and five to 10 reference water controls.

If the preliminary test indicates that no effect is expected under the required test conditions, a limit test can be performed to confirm the lack of effect at the highest concentration or lowest dilution of the range of the preliminary test.

If the test is performed in small vessels (i.e. multi-well plate), the number of replicates per test condition should be increased to reach the required number of larvae (300 per test condition; 500 to 1 000 for controls).

8.4 Collecting gametes

8.4.1 General

Male and female gametes are obtained by naturally spawning the adults (i.e. temperature shock) or by stripping the gonads for oysters. Before stimulating the mature bivalves to obtain the gametes, they can be placed in natural or synthetic seawater (see 5.2) so that they can recover from the stress caused by transportation and eliminate most of their faeces. Just before inducing the spawn, the bivalves are brushed and rinsed to remove the epibionts (plant or animal organisms fixed to the shell of bivalves) and sedimentary debris.

8.4.2 Thermal stimulation

Thermal stimulation is used to induce egg-laying (see Reference [3]) in seawater tanks. The temperatures used for these thermal stresses are in the interval of naturally observed spawning, namely between 14 °C and 29 °C for oysters and 15 °C and 20 °C for mussels. Every 30 min, the bivalves alternate between warm seawater tanks and cold seawater tanks (see Annex A). Spawning is generally triggered in the first 3 h with males being the first to emit their spermatozoa. This causes the females to lay their gametes a few minutes later, if the parents are very mature. In order to hasten the emission of gametes, the bivalves can be chemically stimulated by adding a few millilitres of a suspension of inactivated oocytes or spermatozoa in front of the mollusc inhaling syphon when they are widely open (which indicates that spawning is imminent).

Differentiating between male and female is simple.

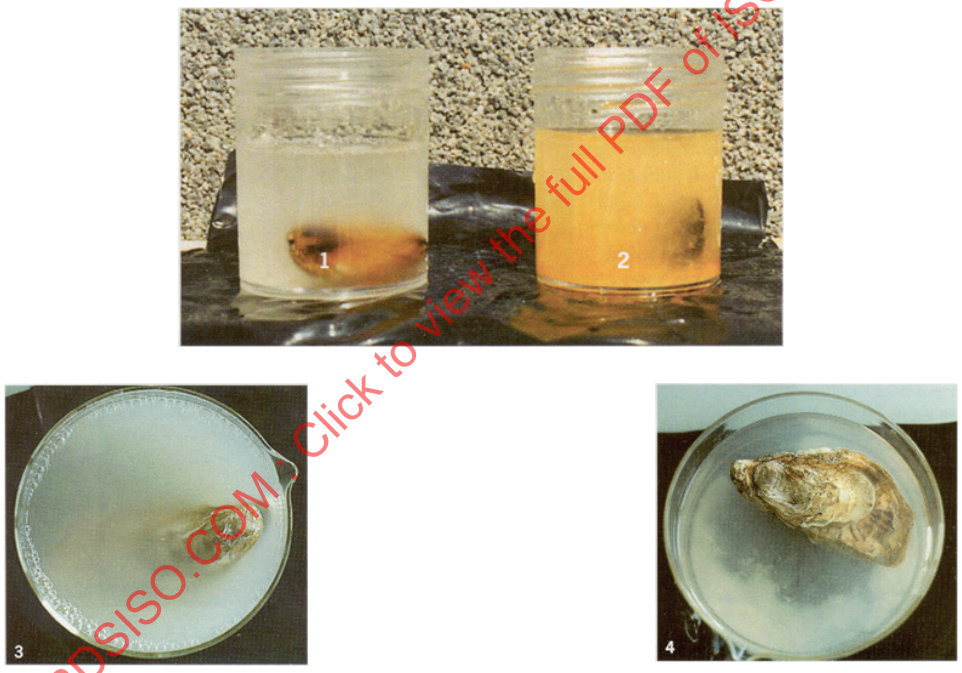
— Mussels: Spawning takes the form of a ribbon which disintegrates in water into an orange cluster for the female and a grey-white cluster for the male (see Figure 1).

— Oysters: The females expel their eggs in grey-white clouds by vigorously beating the right valve while males release sperm which turns the water milky (see Figure 1). A sample observed under a light microscope with 400x magnification, as soon as the gametes are emitted, permits to confirm the gender of the parents.

As soon as the release of the gametes is definitely in progress,

— the males are removed from the water and kept closed with an elastic band so that the spermatozoa do not lose their fertilizing ability through prolonged contact with water, and

— the females, once rinsed, are immediately placed in individual 250 ml vials filled with reference seawater. As soon as they start to release gametes, once again, they are transferred to a new seawater tank two to three times so as to only pick up the eggs of the selected spawner and eliminate those which could have been fertilized in the stimulation tanks (see Figure 1).



Key

1 male mussel
2 female mussel
3 male oyster
4 female oyster

NOTE Source: Quiniou et al. 2005, Figure 3, p.12 (Reference [25])

Figure 1 — Emission of gametes by the mussel (*Mytilus edulis*) (1, 2) and the Japanese oyster (*Crassostrea gigas*) (3, 4)

As soon as the first females have emitted their gametes, an aliquot is collected to select the female which will be used in the test. A female is retained when its oocytes, a few minutes after emission, are as uniform as possible, for example, regular, spherical (for mussels) or slightly pyriformic (for oysters) oocytes, and a uniformly granular cytoplasm. Once the female has been selected, the suspension of

oocytes is filtered through a 100 µm sieve in order to remove faeces and debris. The oocytes are then placed in a beaker containing 2 l of reference seawater and their density is determined (see 8.5).

The males are then placed in individual 250 ml vials and covered with just enough water to obtain a very dense suspension of sperm. After two or three bath changes, the sperm solution is filtered through a 32 µm sieve (the gametes pass through the sieves). A male is retained when the sperm is highly concentrated (see Figure 1) and the spermatozoa are very mobile (checked under a stereoscopic inverted light microscope).

8.4.3 Stripping the gametes

The mature male and female oysters should be opened. This can be achieved 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 might 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 min to 30 min, the activity of the sperm should be assessed using a microscope and male oysters with the most motile sperm are selected for stripping.

The gametes can be stripped by one of the methods described below.

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

— Alternatively, the gonad is 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 °C ± 2 °C) over the surface of the gonad and washing the gametes into a suitable vessel.

Care should be taken to avoid puncturing the gut as contamination of gametes can lead to reduced fertilization.

8.5 Measurement of egg density

A part of the initial egg suspension is diluted 1/100 in seawater. The egg density is evaluated by three counts of 0,1 ml of this solution using a binocular magnifying glass (see 6.9). The initial solution is then adjusted to obtain a final density in the test replicates between 20 000 and 50 000 eggs/l.

8.6 Fertilization and inoculation of fertilized eggs

Fertilization is carried out by adding a few millimetres of the dense suspension of sperm in the beaker containing the oocytes. The egg suspension should be fertilized within 60 min after obtaining the egg and the sperm suspensions.

The content of the beaker is homogenized by gently shaking the beaker (every 1 min to 2 min) in order to avoid polyspermy (see References [3], [12], [22], [24], [27], and [28]). A sample is taken from the beaker to check the fertilization rate (each oocyte shall be surrounded by six to 10 spermatozoa which can be observed on an equatorial plan). The fertilization rate should have reached 90 % to perform the inoculation.

From 15 min to 20 min post fertilization, the fertilized eggs (visualization of the polar bodies; see Figure 2), whose density has been previously measured (see 8.5), are quickly inoculated in the test solutions on the basis of 20 000 to 50 000 eggs/l.

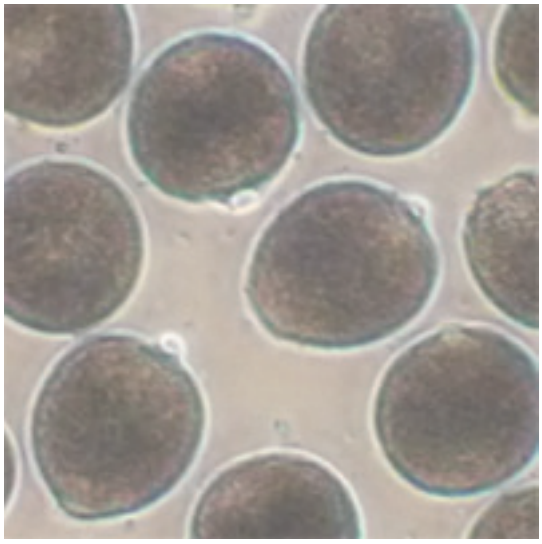


Figure 2 — Fertilized eggs showing polar bodies (M. Régis Delesmont, France)

The inoculation starts when polar bodies can be observed in order to end the inoculation in all the test replicates before the 4-cell stage.

For each experiment, a unique couple from the spawning stock is usually used. Nevertheless, it is possible to mix oocytes or spermatozoa from several organisms. Each test can be performed in duplicate, in two simultaneous or successive series, using two batches of fertilized eggs.

NOTE Experiments performed showed little differences between individuals. Therefore, it is advised to use only one couple.

8.7 Incubation

The test vials are placed in the dark without aeration and food addition for a period of 24 h ± 2 h at 24 °C ± 2 °C for the Japanese oyster and 48 h ± 2 h at 20 °C ± 2 °C for the mussel.

The dissolved oxygen content in the test concentrations/dilutions and controls should be greater than 60 % ASV at the start of the test.

NOTE Experience has shown that the tests can be performed in vials containing less than 1 ml to several litres (see 6.3). Nevertheless, experiments are usually performed using a volume of test solution of 50 ml.

8.8 Observation

After the incubation period, approximatively 10 % neutral formaldehyde, namely 20 ml/l, is added to each test vessel to fix and preserve the larvae. The observations of the larvae can then be carried out immediately or up to several weeks later if the test vials are kept hermetically sealed at the ambient temperature of the laboratory.

The observations can be carried out directly in the incubation glassware with a stereoscopic inverted light microscope fitted with a magnification of at least 200x, but preferably 400x.

If aliquots are sampled for counting, care should be observed to ensure that they are representative of the selected replicate. This can be achieved by mixing thoroughly the vessel, prior to sub-sampling, to ensure that the fixed larvae are in suspension.

8.9 Analytical measurements

Measure and record pH, oxygen concentration, and salinity at the beginning and end of the test, at least at the minimum and maximum test concentrations, as well as in the control. These analytical measurements are performed in one additional vessel for each test condition.

In case of chemical testing, the concentration of the test substance should be measured, as a minimum, at the highest and lowest test concentration, at the beginning and end of the test.

9 Expression of results

Toxicity is expressed as a percentage of abnormal or dead larvae versus the "D-Shaped" normal larvae obtained at the end of the exposure period.

The abnormal larvae are counted on 100 individuals per replicate. If multi-well plates are used, all the larvae in each well of interest shall be observed to reach the required number of larvae per test condition (300 per test concentration/dilution and 500 to 1 000 for controls).

Larvae are considered abnormal if they present at least one of the following anomalies:

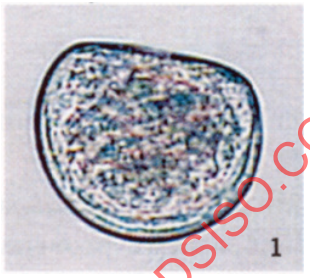
— shell abnormality: concave or convex hinge, irregular edge (a hinge is considered abnormal if it can be considered that it is not functional);

— mantle abnormality: mantle retracted between the shell's valves or hypertrophied which can go as far as expulsion (the larvae are then categorized as dead);

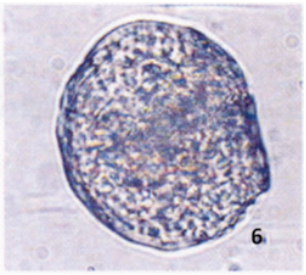
— segmentation abnormalities: blockage of the embryogenesis;

— empty shell: dead larva.

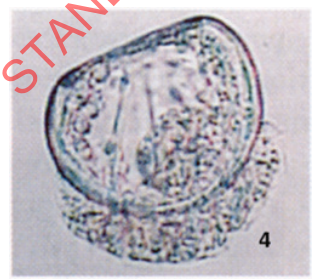
Figure 3 illustrates the normal "D" larvae and the various abnormalities that can be observed.



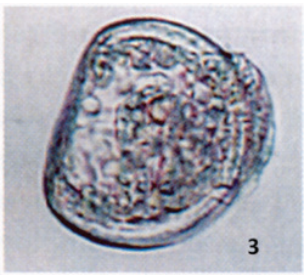
oyster normal D larvae



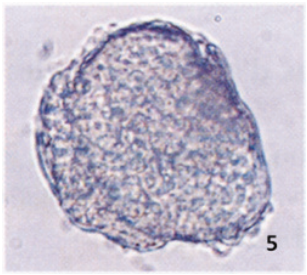
abnormal oyster larvae: shell abnormality



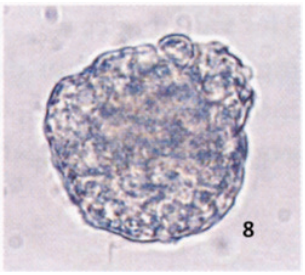
abnormal oyster larvae: hypertrophy of the mantle



abnormal oyster larvae: hypertrophy of the mantle

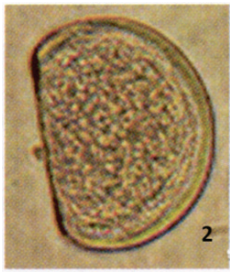


abnormal oyster larvae: hinge abnormality and hypertrophy of the mantle

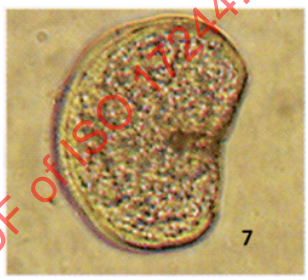


development of the oyster blocked at the embryonic stage

a) Oyster



mussel normal D larvae



abnormal mussel larvae: hinge abnormality

b) Mussels

NOTE Source: Quiniou et al. 2005, Figure 5, p.15 (Reference [25])

Figure 3 — Normal D larvae and different embryonic development abnormalities in the Japanese oyster (3a) and the mussel (3b) (60 µm to 80 µm length)

Test results may be presented as a raw percentage (RPA) and compared with the control, or transformed into a net percentage of abnormal larvae (NPA). Calculating the net percentage allows different experiments to be compared with one another when the success rates differ between the series. Although there are several options, Abbott's formula [2] allows raw data to be transformed into net percentages.

RPA = [(number of abnormal larvae) / (total number of larvae)] × 100

NPA = [(test RPA − control RPA) / (100 − control RPA)] × 100

The results can be expressed in the form of tables or graphs which show the average values, standard deviations, and confidence intervals for each concentration or dilution tested. Furthermore, the rates for the two main types of abnormality (shell and mantle abnormality, segmentation abnormality) can be given. To estimate the concentration that causes x % of effects (EC_x), a model can be adjusted to the test results. For this, the EC_x values and the parameters which characterize this model shall be estimated, if possible, with their confidence interval at 95 %. ISO/TS 20281 describes the various models applicable.

If necessary, the LOEC and NOEC are determined with suitable multiple comparison tests (for example, the Dunnett and Williams tests). The hypothesis regarding homogeneity of variance shall be verified.

The values are expressed as a mass concentration value for chemicals or as a rate of dilution (volume fraction) for aqueous samples.

It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within

20 % of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

10 Validity criteria

Consider the test valid when the average rate of normal D-shaped larvae among the controls is greater than or equal to 80 %, compared to the total number of abnormal + normal D-shell stage larvae.

11 Test report

The test report shall include the following information:

a) a reference to this International Standard, i.e. ISO 17244:2015;

b) all of the data required to identify the sample and the substance studied;

c) the method to prepare the samples;

   — for the effluents, water and aqueous extracts, the storage conditions and duration, pH and dissolved oxygen concentration of the initial sample, and if necessary, the pretreatment steps (decantation, filtration, or centrifugation);

   — for the chemicals, the preparation of stock and test solution;

d) the test species used and the origin of the mature bivalves;

e) the quality of the reference seawater used (the origin, if natural seawater, or the composition, if synthetic seawater) as well as its physico-chemical parameters;

f) the volumes of the incubation solutions;

g) the density of the fertilized eggs which are inoculated in the solutions to be tested;

h) the development abnormality observation tables for each replicate of the solutions tested;

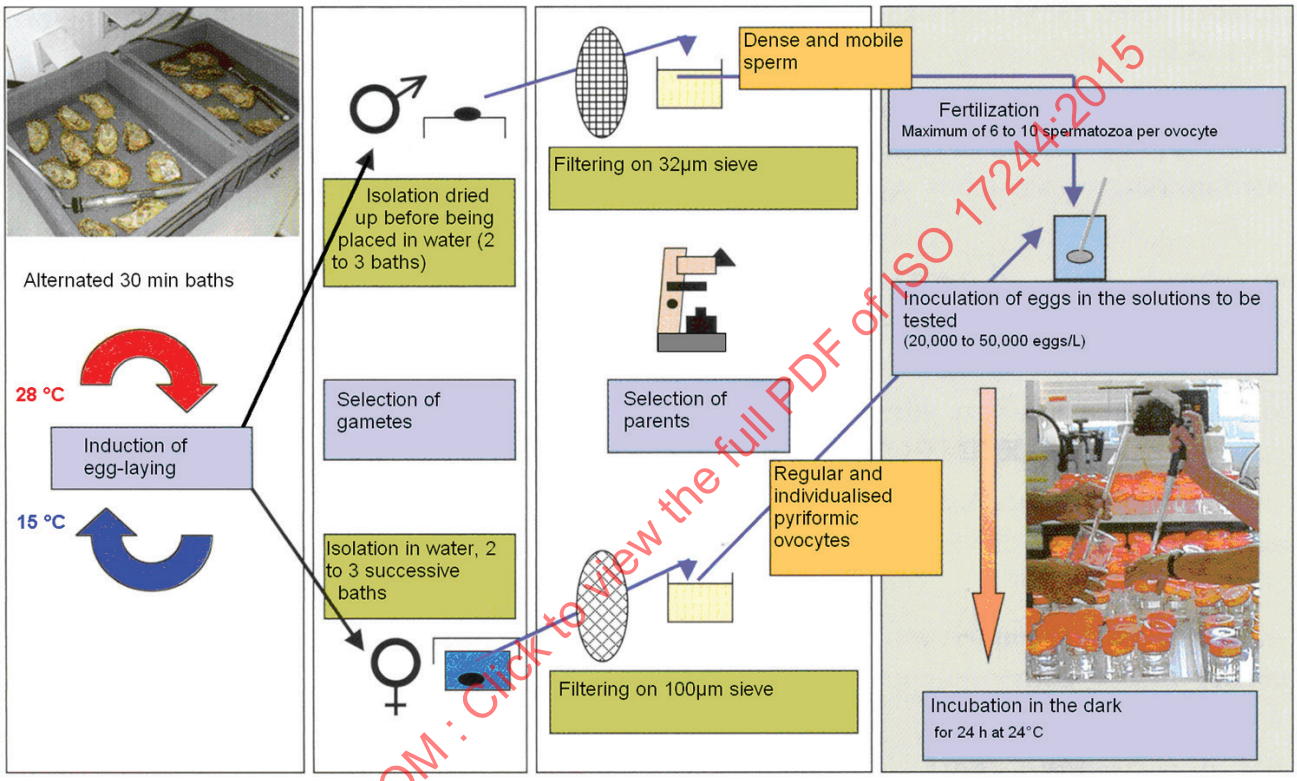
i) the curves and graphs of the effects observed;

j) the EC_x, LOEC, NOEC values, and other effects observed as well as the calculation method used to determine these values;

k) the EC_{50} for the reference substance and its confidence interval.

Annex A
(informative)

Overview of the test applied to the Japanese oyster *Crassostrea gigas*



NOTE Source: Quiniou et al. 2005, p.22 (Reference [25])

Figure A.1 — Overview of the "bivalve embryo-larval development" test applied to the Japanese oyster *Crassostrea gigas*

Annex B
(informative)

Performance data

Tables B.1 to B.3 present the values of EC_{50} obtained on the Japanese oyster and the mussel: data from two inter-calibration tests performed by five laboratories and internal values from four laboratories.

Table B.1 — Results of the two inter-calibration tests performed on the mussel and Japanese oyster in 2006 and 2007

Origin of mature bivalves	Species	Partner	Salinity	Exposure duration h	T °C	pH	Type of water	EC_{50} µg/l Cu ion	Test volume ml	Date	Average	Standard deviation	
Galicia	Mg	1	34,5	48	20	8,46	S	9,41	20	2006	9,51	0,14	Galicia mussels synthetic seawater
Galicia	Mg	1	34,5	48	20		S	9,60	20	2006			
Brittany	Me	2	30	48	20	7,5 to 8,1	N	12,08	30	2006	11,17	0,84	Brittany mussels natural seawater
Brittany	Me	5	30	48	20	7,97	N	11,02	100	2006			
Brittany	Me	5	30	48	20	7,97	N	10,41	100	2006			
Brittany	Me	5	30	48	20	8,01	S	6,89	100	2006	7,47	0,81	Brittany mussels synthetic seawater
Brittany	Me	5	30	48	20	8,01	S	8,04	100	2006			
											9,64	**1,76**	**Average value for mussels**
											11,17	0,84	Average value for mussel in natural seawater
											8,49	1,27	Average value for mussel in synthetic seawater
Barfleur	Cg	2	30	24	24	7,5 to 8,1	N	13,21	30	2007	13,54	0,46	Barfleur oysters natural seawater
Barfleur	Cg	2	30	24	24	7,5 to 8,1	N	13,86	30	2007			

Cg *Crassostea gigas*

Me *Mytilus edulis*

Mg *Mytilus galloprovincialis*

N natural seawater

S synthetic seawater

NOTE 1 EC_{50} calculated with REGTOX.

NOTE 2 Values expressed in µg of Cu/l.

Table B.1 *(continued)*

Origin of mature bivalves	Species	Partner	Salinity	Exposure duration h	T °C	pH	Type of water	EC_{50} µg/l Cu ion	Test volume ml	Date	Average	Standard deviation	
Guernsey	Cg	2	30	24	24	7,5 to 8,1	N	12,81	30	2006	11,80	1,76	Guernsey oysters natural seawater
Guernsey	Cg	2	30	24	24	7,5 to 8,1	N	13,13	30	2006			
Guernsey	Cg	2	30	24	24	7,5 to 8,1	N	9,25	30	2007			
Guernsey	Cg	2	30	24	24	7,5 to 8,1	N	12,03	30	2007			
La Tremblade	Cg	3	30	24	24	7,5 to 8,1	N	4,40	30	2006	6,59	2,62	La Tremblade oysters natural seawater
La Tremblade	Cg	3	30	24	24	7,5 to 8,1	N	4,64	30	2006			
La Tremblade	Cg	3	30	24	24	7,5 to 8,1	N	9,96	30	2007			
La Tremblade	Cg	4	30	24	24	7,5 to 8,1	N	7,37	2	2007			
Guernsey	Cg	5	33,6	24	24	7,98	N	13,59	100	2006	15,33	2,13	Guernsey oysters natural seawater
Guernsey	Cg	5	33,6	24	24	7,98	N	13,19	100	2006			
Guernsey	Cg	5	33,6	24	24	7,98	N	13,19	100	2006			
Guernsey	Cg	5	33,4	24	24	7,95	N	15,66	100	2007			
Guernsey	Cg	5	33,4	24	24	7,95	N	15,57	100	2007			
Guernsey	Cg	5	33,4	24	24	7,95	N	18,04	100	2007			
Guernsey	Cg	5	33,4	24	24	7,95	N	18,09	100	2007			

Cg *Crassostea gigas*

Me *Mytilus edulis*

Mg *Mytilus galloprovincialis*

N natural seawater

S synthetic seawater

NOTE 1 EC_{50} calculated with REGTOX.

NOTE 2 Values expressed in µg of Cu/l.

Table B.1 *(continued)*

Origin of mature bivalves	Species	Partner	Salinity	Exposure duration h	T °C	pH	Type of water	EC_{50} µg/l Cu ion	Test volume ml	Date	Average	Standard deviation	
Guernsey	Cg	5	33	24	24	7,96	S	11,62	100	2006	10,19	1,41	Guernsey oysters synthetic seawater
Guernsey	Cg	5	33	24	24	7,96	S	8,39	100	2006			
Guernsey	Cg	5	33,2	24	24	8,03	S	9,90	100	2007			
Guernsey	Cg	5	33,2	24	24	8,03	S	9,66	100	2007			
Guernsey	Cg	5	33,2	24	24	8,03	S	9,42	100	2007			
Guernsey	Cg	5	33,2	24	24	8,03	S	12,13	100	2007			
											11,70	**3,60**	**Average value for oysters**
											12,23	4,00	Average value for oysters in natural seawater
											10,19	1,41	Average value for oysters in synthetic seawater

Cg *Crassostea gigas*

Me *Mytilus edulis*

Mg *Mytilus galloprovincialis*

N natural seawater

S synthetic seawater

NOTE 1 EC_{50} calculated with REGTOX.

NOTE 2 Values expressed in µg of Cu/l.

Table B.2 — Tests prior to 2006: Internal values regarding the mussel and the Japanese oyster from four different partner laboratories

Origin of mature bivalves	Species	Partner	Salinity	Exposure duration h	T °C	pH	Type of water	EC_{50} µg/l Cu ion	Test volume ml	Internal values	Average	Standard deviation	
Galicia	Mg	1	34,5	48	20		N + S	10	20	internal	10		Galicia mussels synthetic seawater
Arcachon	Me	6	33	48	20		N	15,1	20	internal	15,1		Arcachon mussels natural seawater
Brittany	Me	5	30	48	20	7,97	N	9,14	100	internal 2006	9,03	0,16	Brittany mussels natural seawater
Brittany	Me	5	30	48	20	7,97	N	8,92	100	internal 2006			
Brittany	Me	5	30	48	20	8,01	S	7,27	100	internal 06	7,13	0,21	Brittany mussels synthetic seawater
Brittany	Me	5	30	48	20	8,01	S	6,98	100	internal 06			
Barfleur	Cg	2	30	24	24	7,5 to 8,1	N	15,76	30	internal 2005	15,76		Barfleur oysters natural seawater
Guernsey	Cg	2	30	24	24	7,5 to 8,1	N	8,37	30	18/01/ 2006	8,37		Guernsey oysters natural seawater

Cg *Crassostea gigas*

Me *Mytilus edulis*

Mg *Mytilus galloprovincialis*

N natural seawater

S synthetic seawater

NOTE 1 EC_{50} calculated with REGTOX.

NOTE 2 Values expressed in µg of Cu/l.

Table B.2 *(continued)*

Origin of mature bivalves	Species	Partner	Salinity	Exposure duration h	T °C	pH	Type of water	EC_{50} µg/l Cu ion	Test volume ml	Internal values	Average	Standard deviation	
Guernsey	Cg	5	30	24	24	7,9	S	11,47	100	internal 06	10,74	0,90	Guernsey oysters synthetic seawater
Guernsey	Cg	5	30	24	24	7,9	S	10,91	100	internal 06			
Guernsey	Cg	5	33,6	24	24	7,98	S	11,47	100	internal 06			
Guernsey	Cg	5	33,6	24	24	7,98	S	10,91	100	internal 06			
Guernsey	Cg	5	33,2	24	24	8,03	S	10,64	100	internal 07			
Guernsey	Cg	5	33,2	24	24	8,03	S	9,02	100	internal 07			
Barfleur, Brittany, Arcachon,		2 and 5	30 to 33	24	24		S and N			average of about 100 tests before 2006	8,9	3,10	Oysters with various origins and natural or synthetic seawater

Cg *Crassostea gigas*

Me *Mytilus edulis*

Mg *Mytilus galloprovincialis*

N natural seawater

S synthetic seawater

NOTE 1 EC_{50} calculated with REGTOX.

NOTE 2 Values expressed in µg of Cu/l.