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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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Foreword

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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 (e.g. 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).

