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

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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 document 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 of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

This second edition cancels and replaces the first edition (ISO 17244:2015), which has been technically revised.

The main changes are as follows:

— Annex C, which gives a protocol to perform the test in direct contact with sediment, has been added;

— for Japanese oyster, the revised nomenclature, *Magallana gigas*, has been used;

— the possibility to extend the test duration to 48 h has been included.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

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 bioassays (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 bioassays (see References [18] and [21]). The embryos and larvae are more sensitive to pollutants than the adults of the same species. Therefore, they represent the critical stages for the toxicity tests (see References [19] and [30]). Since 1972, it is recommended to use the Pacific oyster, *Magallana gigas*, to assess the quality of seawater (see Reference [35]). 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 bioassays.

The results of these bioassays demonstrate the necessity to determine the potential toxicity thresholds of chemicals which can 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. Potential toxicity has been defined based on teratological effects (see References [11], [27] and [26]).

This document 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 can 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 embryos and larvae of 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 bioassay, recommended by the International Council for the Exploration of the Sea (ICES),[14] has been the subject of the first European inter-calibration test performed in 1991.[31] The protocol described in this document corresponds to a modification and simplification of Reference [3].

The toxicity assessment of metals performed on *M. 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 (see Reference [4]), which compared the findings of four embryo-larval tests: *M. edulis*, *M. galloprovincialis*, *M. gigas* and *C. virginica.* Another study showed that the embryos of *M. 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 range acceptable to bivalves make them easy to use in ecotoxicity studies, particularly when assessing the quality of coastal and estuarine environments (see Reference [11]).

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WARNING — Persons using this document should be familiar with normal laboratory practice. This document 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.

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

1 Scope

This document specifies a method for assessing the effects of chemical and aqueous samples on the embryo-larval development of marine bivalves. This method 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 PSU (practical salinity unit) and 40 PSU for mussels, and

— between 25 PSU and 35 PSU for oysters.

This method in this document 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 or dilution is limited so that the aforementioned salinity ranges are respected,

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

— samples of contaminated sediment or dredged material (see Annex C).

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. 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*

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
effect concentration
EC_x
concentration at which a specific effect is detected; *x* is the percentage (10, 25, 50) of this effect, e.g. the development abnormality

EXAMPLE EC_{50} means the concentration estimated to observed 50 % of development abnormality at the end of the test compared to the control.

[SOURCE: ISO 15952:2018, 3.6, modified — "growth inhibition" has been replaced with "development abnormality" in the definition and the EXAMPLE has been replaced.]

3.2
lowest observed effect concentration
LOEC
lowest tested concentration at which the test substance or dilution (of an aqueous sample, in %, or of a sediment in g/l) is observed to have a statistically significant effect ($p < 0,05$) when compared with the control

Note 1 to entry: All test concentrations above the LOEC have a harmful effect equal to or greater than those observed at the LOEC. When these two conditions cannot be satisfied, a full explanation should be given for how the LOEC [and hence the *no observed effect concentration* (3.3)] has been selected.

[SOURCE: ISO 15952:2018, 3.8, modified — "substance or dilution (of an aqueous sample, in %, or of a sediment in g/l)" has been added to the definition.]

3.3
no observed effect concentration
NOEC
test concentration immediately below the *lowest observed effect concentration* (3.2), which, when compared with the control, has no statistically significant effect ($p > 0,05$) within a given exposure time

Note 1 to entry: The NOEC is the concentration just below the lowest observed effect concentration.

[SOURCE: ISO 15952:2018, 3.9, modified — Note 2 to entry has been deleted.]

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

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

Note 1 to entry: The normal D-shaped 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 bioassay 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-shaped larvae. This static test aims to determine the concentration level (EC_x) which results in abnormalities for *x* % of exposed larvae in 24 h (optionally 48 h) for the Japanese oyster, also named Pacific oyster (*Magallana gigas,* previously *Crassostrea gigas*), and in 48 h for the Blue and Mediterranean mussel (*Mytilus edulis* or *Mytilus galloprovincialis*). Several parameters can be assessed in 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.

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 this document.

Some regulations can extend the oyster exposure period to 48 h. In this case, the sensitivity test shall be performed in the same conditions and the test duration is mentioned clearly in the test report.

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 sampling area is subject to little or no sources of contamination and free from epizootic diseases. 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 conducting tests throughout the year.

For transportation or storage purpose, mature oyster can stay out of water up to three days as long as they are kept in cool and damp conditions.

If the bivalves are received in the laboratory within three days after sampling, it is recommended to keep them dry at 15 °C ± 2 °C until the collecting gametes or in seawater at a temperature close to their conditioning or rearing temperature (usually, 20 °C for hatchery oysters).

If the mature bivalves have to be kept for more than three days after sampling and/or dispatching, they shall be stored in water (see 5.2) at a temperature close to 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 individuals for 30 l of seawater). The water in the tanks is continuously aerated. 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.

Mussel *Mytilus edulis* and *M. galloprovincialis* basic characterization-identification should be done after organism sampling (e.g. biometry measurements or nuclear marker heterozygosity analyses).

NOTE Other species can be used, such as, *Phaeodactylum tricornutum*. Nevertheless, no information can be given regarding the suitable concentration of algae.

5.2 Reference seawater

5.2.1 General

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 artificial.

5.2.2 Natural seawater

Natural seawater shall allow a good bivalve embryo-larval development and enable at least 80 % of the normal D-shaped 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 should be checked to ensure that the water is not contaminated by any known human activity. The water should be pre-filtered with a 1 µm to 5 µm membrane. The seawater shall then be stored in the dark in controlled conditions between 2 °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 PSU to 40 PSU for mussels and 25 PSU to 35 PSU for oysters. The salinity should be close to the original location. Then, filter it through a 0,45 µm membrane. Salinity shall then be checked with a suitable probe (see 6.6).

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

5.2.3 Artificial seawater

Alternatively, artificial seawater prepared in accordance with Table 1 may be used. The composition of this seawater is similar to that suggested in Reference [36] without EDTA in order not to reduce the bio-availability of divalent metal ions, thus, resulting in a decrease in the apparent toxicity of these ions (see Reference [25]). Artificial 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 artificial seawater. Mix after each addition of salt to ensure a good dissolution.

Once ready, the artificial seawater is filtered through a 0,45 µm membrane (see 5.2.1).

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

Chemical composition	Concentration in ultrapure water g/l
NaF	0,003
$SrCl_2 \cdot 6H_2O$	0,02
H_3BO_3	0,03
KBr	0,1
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.	

Artificial seawater which only contains mineral salts can be kept for up to one year in a watertight container that is kept out of the light in a clean, dry and odourless place between 2 °C and 15 °C.

5.2.4 Hypersaline brine

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

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

Commercial sea salts may also be used to prepare 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 200×, but preferably 400×.

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 bioassay.

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. Test reference shall be performed at least twice a year but it is advisable to test it 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 EC_{50} value shall 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 of 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 in this document.

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.