INTERNATIONAL STANDARD

ISO 11348-2

Second edition
2007-12-01

Water quality — Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) —

Part 2: Method using liquid-dried bacteria

Qualité de l'eau — Détermination de l'effet inhibiteur d'échantillons d'eau sur la luminescence de Vibrio fischeri *(Essai de bactéries luminescentes) —*

Partie 2: Méthode utilisant des bactéries déshydratées

Reference number
ISO 11348-2:2007(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 11348-2 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 11348-2:1998), which has been technically revised.

ISO 11348 consists of the following parts, under the general title *Water quality — Determination of the inhibitory effect of water samples on the light emission of* Vibrio fischeri *(Luminescent bacteria test)*:

— *Part 1: Method using freshly prepared bacteria*

— *Part 2: Method using liquid-dried bacteria*

— *Part 3: Method using freeze-dried bacteria*

Introduction

The measurements specified in ISO 11348 can be carried out using freshly prepared bacteria, as well as freeze-dried or liquid-dried bacterial preparations.

Standardized work carried out by DIN Normenausschuss Wasserwesen and ISO/TC 147/SC 5/WG 1 has shown that, in special cases, these different techniques may deliver different results, especially in the presence of heavy metals.

Such varying sensitivity is caused by differences in media composition used in the preparation of freeze-dried or liquid-dried bacteria. These protective media influence the bioavailability of toxicants and/or the light emission of luminescent bacteria. This means that the origin and type of preparation need to be taken into account when interpreting the results. This may be difficult sometimes, as freeze-dried and liquid-dried bacteria may be obtained from different suppliers. This, in turn, can mean that the composition is not known in detail and therefore cannot be interpreted by the user.

For this reason, in addition to toxicity measurements with freshly prepared bacteria (ISO 11348-1) and freeze-dried bacteria (ISO 11348-3), a procedure with liquid-dried bacteria is described in this part of ISO 11348, the performance of which can be interpreted by the user in every detail.

The laboratories responsible for the results have the opportunity to select the most suitable technique based on expert judgement and information about the water sample to be tested.

Water quality — Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) —

Part 2:
Method using liquid-dried bacteria

WARNING — Persons using this part of ISO 11348 should be familiar with normal laboratory practice. This 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 to this part of ISO 11348 be carried out by suitably trained staff.

1 Scope

ISO 11348 describes three methods for determining the inhibition of the luminescence emitted by the marine bacterium *Vibrio fischeri* (NRRL B-11177). This part of ISO 11348 specifies a method using liquid-dried bacteria.

This method is applicable to:

— waste water;

— aqueous extracts and leachates;

— fresh water (surface water and ground water);

— sea water and brackish water;

— eluates of sediment (fresh water, brackish water and sea water);

— pore water;

— single substances, diluted in water.

2 Normative references

The following referenced documents are indispensable for the application 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*

ISO 5814, *Water quality — Determination of dissolved oxygen — Electrochemical probe method*

3 Principle

The inhibition of light emission by cultures of *Vibrio fischeri* is determined by means of a batch test. This is accomplished by combining specified volumes of the test sample or the diluted sample with the luminescent bacteria suspension in a test tube.

The test criterion is the luminescence, measured after a contact time of 15 min or 30 min or optionally 5 min, taking into account a correction factor (f_{kt}), which is a measure of intensity changes of control samples during the exposure time. The inhibitory effect of the water sample can be determined as LID (see Annex B) or as EC_{20}- and/or EC_{50}-values by means of a dilution series. (EC is the effective concentration.)

4 Interferences

Insoluble, slightly soluble or volatile substances or substances which react with the dilution water or the suspension, or alter their state during the test period, may affect the result or impair the reproducibility of the test results.

Losses of luminescence caused by light absorption or light scattering may occur in the case of strongly coloured or turbid waters. This interference can be compensated by a sample treatment for turbidity (7.2) or, for example, by using a double-chambered absorption correction test tube (see Annex A).

Since oxygen is required for the bioluminescence [6], samples with a high oxygen demand (and/or a low oxygen concentration) may cause a deficiency of oxygen and be inhibitory.

Readily biodegradable nutrients in the sample may cause a pollutant-independent reduction in bioluminescence [1].

Samples with a pH outside the range of pH = 6,0 and pH = 8,5 affect the luminescence of the bacteria [6], [7]. An adjustment of the sample is required when the toxic effect of pH is not wanted.

As the test organism *Vibrio fischeri* is a marine bacterium, testing salt-water samples with the standard procedure often leads to stimulation effects of bioluminescence, which may mask inhibition effects (see Annex D).

Salt concentrations in the initial sample exceeding 30 g/l NaCl, or contents of other compounds giving equal osmolarity may lead, together with the salt spiking required by the test, to hyperosmotic effects. The resulting salt concentration in the test samples should not exceed the osmolarity of a 35 g/l NaCl solution in order to avoid these effects.

5 Reagents and materials

Use chemicals of recognized analytical grade quality. Use distilled water or water of equivalent purity.

5.1 Test bacteria.

Use a strain of luminescence bacteria belonging to the species *Vibrio fischeri* NRRL B-11177. The bacterial suspensions used for toxicity measurements are prepared from commercially available liquid-dried reagents Store the liquid-dried bacteria at $\leqslant$ –18 °C and consider the recommendations of the supplier. The bacteria start glowing immediately after reconstitution and are ready to be used for the test.

5.2 Sodium chloride solution, as diluent.

Dissolve 20 g of sodium chloride (NaCl) in water and make up to 1 l with water.

5.3 Sodium hydroxide solution, c(NaOH) = e.g. 1 mol/l.

5.4 Hydrochloric acid, c(HCl) = e.g. 1 mol/l.

For the adjustment of the pH, it may be necessary to use acids or bases of lower or higher concentration.

5.5 Solution for liquid-dried bacteria.

8,0 g	D(+)-Glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$)
20,0 g	Sodium chloride (NaCl)
2,035 g	Magnesium chloride hexahydrate ($MgCl_2 \cdot 6\ H_2O$)
0,30 g	Potassium chloride (KCl)
11,9 g	*N*-(2-Hydroxyethyl)piperazine-*N*-(2-ethanesulfonic acid) (HEPES)

Dissolve in water, stir for about 30 min and adjust the pH to 7,0 ± 0,2 with sodium hydroxide solution (5.3) or hydrochloric acid (5.4). Make up to 1 l with water.

This solution may be stored in portions at –18 °C to –20 °C.

5.6 Reference substances.

Prepare the following reference-substance stock solutions with sodium chloride solution (5.2) as diluent separately, without adjustment of the pH:

219,8 mg/l	Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7\ H_2O$)
9 mg/l	3,5-Dichlorophenol ($C_6H_4OCl_2$) (purity ⩾ 99 %)
22,6 mg/l	Potassium dichromate ($K_2Cr_2O_7$)

These concentrations are approximately twice the expected EC_{50}-values for the respective reference substances in this part of ISO 11348. The volumes required depend on the test set-up.

NOTE It is possible to use commercially available chemical preparations with defined concentrations of $ZnSO_4$ and $K_2Cr_2O_7$ (titrisol) for the preparation of the stock solutions of the reference substances.

6 Apparatus

6.1 Freezer, for the storage of preserved bacteria.

6.2 Thermostatically controlled thermo-block, to maintain the test samples at a temperature of 15 °C ± 1 °C. Within one test, the temperature deviation should be at most ± 0,3 °C.

6.3 Luminometer, measuring cell maintained at 15 °C ± 1 °C, equipped with suitable test tubes.

6.4 Test tubes, made of a chemically inert material, appropriate for the selected luminometer, with a capacity which facilitates the taking of a reading over the largest possible surface area and able to fit into the thermo-block (6.2).

6.5 pH-meter.

6.6 Chronometer.

6.7 Piston pipettes or **plastic syringes**, 100 µl, 500 µl and 1 000 µl.

6.8 Piston pipettes, with variable volume, 10 ml to 200 ml and 200 µl to 5 000 µl.

6.9 Water bath, capable of maintaining a temperature of 20 °C ± 2 °C.

6.10 Water bath or thermostatically controlled thermo-block, to maintain at least 12 ml volume (e.g. reagent vessel) of the solution prepared in 5.5 at 15 °C ± 1 °C.

6.11 Conductometer.

6.12 Oxygen probe, in accordance with ISO 5814.

7 Sampling and sample pretreatment

7.1 Sampling

Collect samples in chemically inert, clean containers as specified in ISO 5667-16. Fill the containers completely and seal them. Test the samples as soon as possible after collection. Where necessary, store samples at 2 °C to 5 °C in the dark in the containers for not longer than 48 h. For periods up to two months, store at ⩽ −18 °C. Do not use chemicals to preserve the samples. Perform the necessary pH-adjustment and salt addition immediately before testing.

7.2 Sample preparation

Measure the oxygen concentration in all samples. An oxygen concentration > 3 mg/l is required for the test. If the oxygen concentration of the undiluted sample is less than 3 mg/l, use adequate methods to oxygenate the sample, e.g. aeration or stirring.

Measure the pH of all samples. If the pH is between 6,0 and 8,5, an adjustment is usually not necessary. Adjustment of the pH-value, however, may alter the nature of the sample. On the other hand, the pH of the sample and the pH of the test batch may differ because of the buffer capacity of the test medium. It may be necessary to carry out tests on both the pH-adjusted and the non-pH-adjusted samples.

If necessary, adjust the pH of the sample by adding either hydrochloric acid (5.4) or sodium hydroxide solution (5.3). Depending on the purpose of the test, the pH may be adjusted to 7,0 ± 0,2 or to the upper (8,5 ± 0,2) and lower limits (6,0 ± 0,2). Choose the concentration of the hydrochloric acid or the sodium hydroxide solution to restrict the volume added to not more than 5 % of total volume.

Add 20 g of sodium chloride per litre to the water sample or to the neutralized water sample.

For samples with high salt concentrations, measure the salinity and add the amount of salt which is necessary to adjust the osmolarity to 20 g/l NaCl.

If the sample contains between 20 g/l and 50 g/l NaCl-equivalents, add no salt. The resulting salt-concentration in the test samples shall not exceed the osmolarity of a 35 g/l sodium chloride solution.

For salt water samples, Annex D gives further information.

Strongly turbid samples should be allowed to settle for 1 h or centrifuged, for example for 10 min at 5 000g, or should be filtered. Use the supernatant or filtrate for the test.

8 Procedure

Prepare the reference samples according to 5.6. Test each batch of bacteria after delivery with all three reference substances. Test at least one of the three reference substances in parallel with each stock-suspension test tube thawed for the tests.

Prepare the samples according to 7.2.

Thaw the liquid-dried bacteria (stock suspension) in a water bath at 20 °C ± 2 °C. Refrozen stock suspensions may be used for preliminary tests only.

Prepare the test suspension from the stock suspension in two steps:

- Add 0,5 ml (per 100 µl stock suspension in the test tube) of solution (5.5), maintained at 15 °C ± 1 °C, and homogenize by gentle shaking of the test tube.

- Wait for about 15 min.

Pipette this suspension into a reagent vessel (approximately 20 ml volume) and add 11,5 ml of solution (5.5), maintained at 15 °C ± 1 °C, and homogenize by gentle shaking of the reagent vessel.

Wait for about 15 min.

Prepare, in a first set of test tubes (6.4), the sample dilution series, the reference sample (5.6) and the controls (5.2) required.

A common procedure for the preparation of the dilution series is described in Annex B. Depending on the purpose of the test and the statistical requirements concerning the test results, other dilution designs with concentrations in a geometric or a logarithmic series may be appropriate as well. Due to mixing of equal volumes of sample/diluted sample and test suspension, the highest sample concentration in the test is 50 % sample as a rule. For the testing of nearly undiluted water samples (80 % sample), an extra control batch is needed (see B.2 and Table 1).

Maintain the test tubes containing the sodium chloride solution (5.2) for controls, the reference samples (5.6), the samples (7.2) and the samples of the dilution series (Table B.1) at 15 °C ± 1 °C.

Chose test conditions which safeguard that the maximum temperature deviation in the thermo-block within one test is at most ± 0,3 °C.

For tests with equal volumes of test suspension and sample, pipette 500 µl portions of the test suspension into a second, corresponding set of test tubes (6.4), maintained at 15 °C ± 1 °C in the incubator, at the same time intervals (5 s to 20 s) as used for later intensity measurements.

Carry out two parallel determinations per dilution level at a test temperature of 15 °C ± 1 °C.

Adjust the luminometer instrument to a convenient, near-maximum setting.

Determine and record the luminescence intensity, I_0, of the test suspensions by means of a luminometer.

As the contact time for all test samples shall be equal, use a chronometer (6.6) for the measurement of the luminescence intensities at equal time intervals, seriatim. An interval of 5 s to 20 s has been found convenient.

Measure all test suspensions, as differing luminescence may be expected due to possible inhomogeneities of the test suspension.

Immediately after the initial luminescence measurement of a test suspension, make up this suspension to a total volume of 1 ml with samples (7.2), diluted samples (Annex B), reference sample (5.6) or sodium chloride solution (5.2), as appropriate. This is done by pipetting 500 µl each of samples (7.2), diluted samples (Annex B), reference sample (5.6) or sodium chloride solution (5.2), prepared in the first set of test tubes, to the test suspensions in each of the tubes in the corresponding second set of test tubes. Mix by hand, start the chronometer and place the test tubes back into the thermo-block at 15 °C ± 1 °C.

Repeat for all the other test tubes, leaving the same time interval between successive additions.

Determine and record the luminescence intensity in all test tubes of the second set of test tubes, including controls, after, optionally, 5 min (I_5) and again after 15 min and 30 min (I_{15}, I_{30}), as required, at intervals of 5 s to 20 s.

Record the instrument adjustment.