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

Part 3:

Method using freeze-dried bacteria

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

ISO 11348-3:2007
Partie 3: Méthode utilisant des bactéries lyophilisées

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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 11348-3 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 11348-3:1998), 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 give 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 liquid-dried bacteria (ISO 11348-2) and freshly prepared bacteria (ISO 11348-1), a procedure with freeze-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.

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

Part 3: Method using freeze-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 this part of ISO 11348 be carried out by suitably trained staff.

1 Scope

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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 freeze-dried bacteria.

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This method is applicable to:

- waste water;
- aqueous extracts and leachates;
- fresh water (surface and ground water);
- sea and brackish water;
- eluates of sediment (freshwater, brackish 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 and optionally 5 min, taking into account a correction factor (f_{kr}), 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 freeze-dried reagents which can be stored in a freezer at $-18\text{ }^{\circ}\text{C}$ to $-20\text{ }^{\circ}\text{C}$. 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, e.g. $c(\text{NaOH}) = 1\text{ mol/l}$.

5.4 Hydrochloric acid, e.g. $c(\text{HCl}) = 1 \text{ mol/l}$.

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

5.5 Solution for freeze-dried bacteria.

20 g	Sodium chloride (NaCl)
2,035 g	Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6 \text{ H}_2\text{O}$)
0,30 g	Potassium chloride (KCl)

Dissolve in water and make up to 1 l with water. The solution may be stored in portions in a freezer 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:

19,34 mg/l	Zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7 \text{ H}_2\text{O}$)
6,8 mg/l	3,5-Dichlorophenol ($\text{C}_6\text{H}_4\text{OCl}_2$) (purity > 99 %)
105,8 mg/l	Potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$)

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 $\text{K}_2\text{Cr}_2\text{O}_7$ (titrisol) for the preparation of the stock solutions of the reference substances.

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6 Apparatus

6.1 Freezer, for the storage of preserved bacteria.

6.2 Incubator or refrigerator, to maintain the stock suspension (8.2) and, optionally, the “solution for freeze-dried bacteria” (5.5) (variant B) at a temperature of $4^\circ\text{C} \pm 3^\circ\text{C}$.

6.3 Thermostatically controlled thermo-block, to maintain the test samples at a temperature of $15^\circ\text{C} \pm 1^\circ\text{C}$. Within one test, the temperature deviation should be at most $\pm 0,3^\circ\text{C}$.

6.4 Luminometer, measuring cell maintained at $15^\circ\text{C} \pm 1^\circ\text{C}$, equipped with suitable test tubes.

6.5 Test tubes, made of a chemically inert material, appropriate for the selected luminometer and having a capacity which facilitates the taking of a reading over the largest possible surface area and able to fit into the thermo-block (6.3).

6.6 pH-meter.

6.7 Chronometer.

6.8 Piston pipettes or plastic syringes, 10 μl , 500 μl and 1 000 μl .

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

6.10 Conductometer.

6.11 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 $7,0 \pm 0,2$ or to the upper ($8,5 \pm 0,2$) and lower limits ($6,0 \pm 0,2$). 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

8.1 Initial preparations

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 to each stock suspension vial reconstituted.

Prepare the samples according to 7.2.

Prepare, in a first set of test tubes (6.5), 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 $15\text{ °C} \pm 1\text{ °C}$.

Chose test conditions which safeguard that the maximum temperature deviation in the thermo-block within one test is at most $\pm 0,3\text{ °C}$.

8.2 Preparation of stock suspension

Remove the vial of the freeze-dried culture (contents sufficient for up to 100 measurements) from the freezer immediately before reconstitution in water. For the reconstitution, cool 1 ml of distilled water in a glass test tube to $4\text{ °C} \pm 3\text{ °C}$.

Pour this volume of cooled water all at once into the lyophilized bacteria in the vial, thereby minimizing cell damage during the rehydration process.

It is important that water be added quickly, to allow the bacteria to come into contact with the water at once, thus avoiding clumping and loss of activity. Therefore, do not use a pipette. The exact volume of water is not critical.

The reconstituted luminescent bacteria suspension (cell concentration about 10^8 cells per millilitre) serves as a stock suspension; store it at $4\text{ °C} \pm 3\text{ °C}$. The stock suspension may be used for testing purposes, as long as the validity criteria stated in Clause 11 are met. Refrozen, rehydrated bacteria may be used for preliminary tests only.

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8.3 Preparation of test suspensions

8.3.1 Initial step

After a waiting time of at least 10 min, prepare the test suspensions from the stock suspension into a second corresponding set of test tubes (6.5), maintained at $15\text{ °C} \pm 1\text{ °C}$ by one of the two variants.

8.3.2 Variant A

Prepare the test suspensions directly in the test tubes.

Add 10 µl aliquots of the stock suspension to 500 µl of the solution (5.5), contained in test tubes maintained at $15\text{ °C} \pm 1\text{ °C}$, at the same time intervals (5 s to 20 s) as for later intensity measurements. Shake the mixtures by hand.

8.3.3 Variant B

Prepare the test suspensions outside the test tubes in a conical flask (volume e.g. 250 ml).

Add 1 volume of the stock suspension to 50 volumes of the solution (5.5) maintained at $4\text{ °C} \pm 3\text{ °C}$ and mix the resultant suspension thoroughly.

Pipette 500 µl of test suspension into the test tubes, maintained at $15\text{ °C} \pm 1\text{ °C}$ in the incubator, at the same time intervals (5 s to 20 s) as used for later intensity measurements.