INTERNATIONAL STANDARD

ISO 11348-1

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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 1: Method using freshly prepared 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 1: Méthode utilisant des bactéries fraîchement préparées

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

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 freshly prepared bacteria.

This method is applicable to:

— waste water;

— aqueous extracts and leachates;

— fresh water (surface and ground water);

— sea and brackish water;

— eluates of sediment (fresh water, 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*

ISO 7027, *Water quality — Determination of turbidity*

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_{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 bacteria strain can be taken from commercially available freeze-dried or liquid-dried reagents or from culture collections, e.g. Deutsche Sammlung für Mikrorganismen und Zellkulturen GmbH, Mascheroder Weg 10, D-38124 Braunschweig, Germany, or NRRL, ARS Culture collection NCAUR, 1815 N, University Street, Peoria, Illinois 61604, USA. The bacterial suspensions used for toxicity measurements shall be freshly prepared from cultures.

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(NaOH) = 1 mol/l.

5.4 Hydrochloric acid, e.g. c(HCl) = 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 freshly prepared 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.

5.7 Liquid broth for pre- and main cultures.

30 g	Sodium chloride (NaCl)
6,10 g	Sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)
2,75 g	Dipotassium hydrogenphosphate trihydrate ($K_2HPO_4 \cdot 3\ H_2O$)
0,204 g	Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$)
0,500 g	Diammonium hydrogenphosphate [$(NH_4)_2HPO_4$]
3 ml	Glycerol
5,00 g	Caso-peptone
0,50 g	Yeast extract

Dissolve in water 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. Transfer 50 ml each to Erlenmeyer flasks (e.g. 250 ml) and sterilize in an autoclave at 121 °C for 20 min.

Caso-peptone and yeast extract offered by different suppliers can vary in quality. In case of problems (e.g. growth inhibition), it is recommended to purchase the product from another manufacturer.

5.8 Agar medium for stock cultures.

Adjust the liquid broth (5.7) to pH 7,0 ± 0,2.

Add 12 g of agar per litre and dissolve by gentle warming; sterilize and transfer to sterile Petri dishes.

5.9 Protective medium.

66 g	D(+)-Glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$)
4 g	Sodium chloride (NaCl)
2 g	L-Histidine
0,5 g	Bovine serum albumin, BSA

Dissolve completely in water at about 37 °C and adjust the pH to 7,0 ± 0,2 at room temperature with sodium hydroxide solution (5.3) or hydrochloric acid (5.4) as necessary. Make up to 100 ml with water.

Damage of bacterial cells during the freezing procedure is prevented by the use of the protective medium. BSA offered by different suppliers can vary in quality. If problems occur, it is recommended to purchase the product from another manufacturer.

Prepare protective medium freshly before use.

6 Apparatus

6.1 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.

6.2 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.

6.3 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 (6.1).

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 Refrigerated centrifuge.

6.10 Magnetic stirrer and **magnetic stirring bar**.

6.11 Incubated shaker, for incubation of Erlenmeyer flasks.

6.12 Autoclave.

6.13 Incubator.

6.14 Spectral- or filter-photometer and test tubes, of optical path length 1 cm.

6.15 Inoculating loop (or needle).

6.16 Conductometer.

6.17 Freezer, for the storage of solutions and suspensions.

6.18 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 $\leqslant -18$ °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 Cultivation of luminescent bacteria

8.1 Stock culturing

Transfer luminescent bacteria of strain *Vibrio fischeri* NRRL B-11177 (5.1) under sterile conditions to Petri dishes containing the agar medium for stock cultures (5.8).

Incubate in an incubator for 2 d to 3 d at 20 °C $\pm$ 1 °C.

Mark luminescent single colonies using visual observations in the dark, and store dishes in the refrigerator afterwards.

Transfer marked colonies under sterile conditions to fresh dishes after a storage period of one week to a maximum of two weeks.

Commercially available vials of preserved bacteria usually are not dispensed under sterile conditions. For cultivation of pure cultures, several single-colony passages are recommended. To prevent genetic alterations, a new vial of preserved bacteria can be opened and used approximately every 6 months.

NOTE The luminescence of luminescent bacterial colonies can decrease during storage.

8.2 Preparation of pre-cultures

Inoculate 50 ml of pre-culture broth (5.7) in Erlenmeyer flasks (e.g. 250 ml) under sterile conditions with one luminescent single colony of a stock culture aged 2 d to 3 d.

Shake for 21 h ± 1 h at 20 °C ± 1 °C with a periodicity of at least 180 $r \cdot min^{-1}$.

Determine the turbidity of a 1 in 10 dilution of the culture in sodium chloride solution (5.2), e.g. in formazine absorption units (FAU) at 578 nm, as specified in ISO 7027.

8.3 Preparation of main culture

Inoculate 50 ml of the main culture broth (5.7) in 250 ml Erlenmeyer flasks with an appropriate volume of pre-culture (8.2) to result in an estimated initial turbidity of 10 FAU.

Shake for 20 h ± 1 h at 20 °C ± 1 °C with a periodicity of at least 180 $r \cdot min^{-1}$.

Determine turbidity, in FAU, of a 1 in 10 dilution in sodium chloride solution (5.2) photometrically at 578 nm.

NOTE Following the above conditions, the undiluted main culture usually exhibits a turbidity of 700 FAU to 1 800 FAU.

8.4 Preparation of stock suspension

Pre-cool sodium chloride solution (5.2) and protective medium (5.9) on ice.

Centrifuge bacterial suspension from the main culture (8.3) at 4 °C ± 2 °C in a pre-cooled refrigerated centrifuge, for 15 min to 20 min at 6 000g ± 2 000g.

Decant supernatants and resuspend pellets in 5 ml to 10 ml, per 50 ml of main culture, of ice-cold sodium chloride solution (5.2).

Repeat centrifugation under the same conditions.

Decant supernatant and resuspend pellets in 0,5 ml, per 50 ml of main culture, of ice-cold sodium chloride solution (5.2).

Transfer the bacterial suspension to a precooled beaker (e.g. 100 ml) and place on ice.

Slowly add, under constant cooling on ice and stirring, about 4 ml of protective medium (5.9) per 50 ml of main culture.

Photometrically determine the turbidity of a 1 in 100 dilution with sodium chloride solution (5.2).

Add pre-cooled protective medium (5.9) more quickly up to an estimated turbidity of 2 500 FAU ± 500 FAU or equivalent (see Note in 8.1).