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Water quality — Kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test)

Qualité de l'eau — Détermination cinétique des effets inhibiteurs des échantillons de sédiment, autres solides et des échantillons colorés sur la luminescence de Vibrio fischeri *(essai cinétique de bactéries luminescentes)*

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Foreword

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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 21338 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

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Introduction

The method specified in this International Standard is a kinetic modification of the luminescent bacteria test specified in ISO 11348. The kinetic method overcomes the problems arising from intense colour and turbidity in the samples. There is no need for sedimentation or centrifugation of turbid samples, or for the correction of colour as described in ISO 11348.

This kinetic method uses luminometers capable of dispensing luminescent bacteria to the samples and measuring the luminescent intensity over a period of time. In the method, the bacterial suspension is dispensed and mixed with the sample in the measurement chamber of the luminometer. Several suitable instruments are commercially available, but only a few of them are capable of cooling the measurement chamber to (15 ± 1) °C as specified in ISO 11348. However, if the bacterial suspension and test samples are kept at (15 ± 1) °C in a thermo-block before the measurement and during the whole incubation, the actual temperature during the contact time is (15 ± 1) °C.

The measurements specified in this International Standard can be carried out using freshly prepared bacteria, as well as freeze- or liquid-dried bacterial preparations. The various bacterial preparations can deliver different results, especially in the presence of heavy metals (see ISO 11348). The laboratories responsible for the results have the opportunity to select the most suitable bacterial preparation based on expert judgement and information about the samples to be tested.

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Water quality — Kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test)

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 trained staff.

1 Scope

This International Standard specifies the kinetic direct-contact method for determining the inhibitory effect of suspensions of sediment and other solid samples, and also for problematic turbid or coloured aqueous samples on the light emission of the marine bacterium *Vibrio fischeri* (NRRL B-11177).

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This method is applicable to:

a) sediment samples and water suspensions of sediments (fresh water, brackish, and seawater sediments);

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b) effluents (especially turbid and coloured);

c) aqueous extracts (e.g. leachates, eluates, elutriates) of soil, solid waste, and other solid material (especially turbid and coloured).

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 5667-16:1998, *Water quality — Sampling — Part 16: Guidance on biotesting of samples*

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

ISO 11348-1, *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*

ISO 11348-2, *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*

ISO 11348-3:2007, *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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3 Terms and definitions

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

3.1
contact time
duration of contact between one object or substance and another

NOTE In the test, the contact time is the time available to control or sample for contact with the test bacteria.

3.2
control sample
sample used in a laboratory in order to check or monitor the instrument or measurement performance or to monitor changes in a sample under investigation

3.3
correction factor
dimensionless multiplier to correct data for known influences affecting their values as measured

NOTE In the test, the correction factor, f_{kt}, serves to correct the initial luminescence intensity of the sample.

3.4
peak value
maximum signal recorded in response to a stimulus

NOTE In the test, the peak value is the maximum signal which is recorded immediately after all the bacteria are in contact with the sample.

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3.5
reference sample
when the effect or behaviour of a substance is known from previous tests (reference substance) and when this substance is examined within the framework of a test series as test sample, this is called the reference sample

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NOTE Adapted from ISO 5667-16:1998.

3.6
test sample
test sample is made from the sample by means of various preparatory steps specific to the sample and the test, e.g. by dissolving, homogenizing, sedimenting, filtering, neutralizing or aeration

[ISO 5667-16:1998]

4 Principle

The inhibition of light emission by cultures of *Vibrio fischeri* is measured kinetically by following the light emission of cultures from the very beginning of the assay. This is accomplished by dispensing the luminescent bacteria suspension into the sample in a cuvette or other suitable vessel (e.g. microtiter plate) already in the measuring position in the luminometer. The light emission is measured and recorded from the moment of dispensing of the bacterial suspension to the sample until the maximum value has been reached and not only at the maximum value of intensity (peak value) which usually occurs within 5 s of mixing, and after a contact time of 15 min and 30 min or optionally 5 min (Figure 1).

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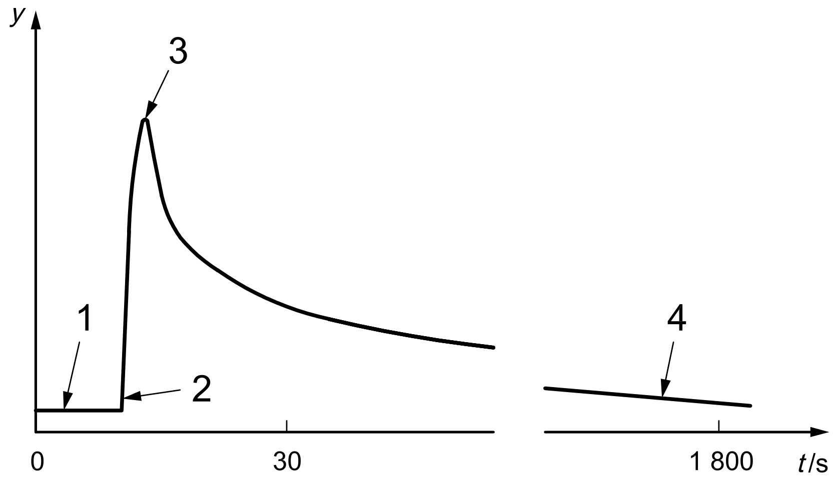

Key

t time
y relative light units
1 start measurement
2 inject bacteria
3 record peak value from 0 s to 5 s
4 mix the sample before recording signal at 30 min

Figure 1 — Principal schematic protocol for the kinetic luminescent bacteria test

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Vibrio fischeri suspension is dispensed and mixed into the sample in the measurement chamber of the luminometer.

The test criterion is the decrease of the luminescence at each endpoint compared to the peak value, taking into account a correction factor, f_{kt}, which is measured from intensity changes of control samples during the exposure time. The inhibitory effect of the sample can be determined as the lowest ineffective dilution (LID) value, or as effective concentration (EC_{20} or EC_{50}) values by means of dilution series (e.g. as described in Annex C). The LID value is the most concentrated test batch tested at which the inhibition of light emission is <20 %. For higher levels of inhibition, the dilution effect relationship can be determined graphically or by statistical analysis. The inhibition by a sample is expressed as the concentrations which results in 20 % and 50 % light reduction compared to the blank (EC_{20} and EC_{50}). This value is interpolated within the dilution series.

No extra correction procedures for colour and turbidity are needed because these factors remain the same during the whole measurement. Inhibition at different contact times and different sample concentrations yields complete kinetic toxicological data about the sample (inhibition, expressed as a percentage vs. concentration vs. time) and enables assumptions to be made about the nature of the contaminants if compared with existing data (see Annex B).

5 Interferences

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

Since oxygen is required for the bioluminescence (Reference [18]), samples with high oxygen demand (or low oxygen concentration) may cause deficiency of oxygen and be inhibitory.

Readily biodegradable nutrients in the sample may cause a pollutant-independent reduction in bioluminescence (Reference [19]).

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Samples with a pH outside the range 6,0 to 8,5 affect the luminescence of bacteria (References [18][20]). Adjust the sample when the toxic effect of pH is not of interest.

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 ISO 11348-3:2007, 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.

6 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and only distilled or demineralized water or water of equivalent purity.

6.1 Test bacteria. Use a strain of luminescent bacteria belonging to the species *Vibrio fischeri* NRRL B-11177.

The bacterial suspensions used for toxicity measurements are prepared according to the instructions in ISO 11348. Dilute the bacterial suspension before the assay from the stock suspension to the measuring concentration (example: ISO 11348-3:2007, variant B).

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6.2 Sodium chloride solution, as diluent.

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

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

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6.4 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.

6.5 Solution for freeze-dried bacteria.

— 20,0 g sodium chloride (NaCl);

— 2,035 g magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$);

— 0,30 g potassium chloride (KCl).

Dissolve in water and make up to 1 l with water. Store the solution in portions in a freezer at –18 °C to –20 °C.

6.6 Reference substances.

Prepare the following three separate reference substance solutions with sodium chloride solution (6.2) as diluent without adjustment of the pH for the use of freeze-dried bacteria:

a) 6,8 mg/l 3,5-dichlorophenol (DCP, $C_6H_4OCl_2$);

b) 19,34 mg/l zinc sulfate heptahydrate ($ZnSO_4 \cdot 7\ H_2O$);

c) 105,8 mg/l potassium dichromate ($K_2Cr_2O_7$).

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The concentrations in a) to c) are approximately twice the expected EC_{50} values for the respective reference substances in ISO 11348-3. For concentrations of reference substance solutions for freshly cultured or liquid-dried bacteria, see ISO 11348-1 or ISO 11348-2. The volumes required depend on the test set-up.

NOTE 1 It is possible to use commercially available chemical preparations with defined concentrations of $ZnSO_4$ and $K_2Cr_2O_7$ for the preparation of the solutions of the reference substances.

NOTE 2 For more information about reference substances, see Reference [22].

7 Apparatus

Usual laboratory equipment, and in particular the following.

7.1 Freezer, for the storage of preserved bacteria.

7.2 Incubator or **refrigerator**, to maintain the solution for freeze-dried bacteria (6.5) at a temperature of (4 ± 3) °C.

7.3 Thermostatically controlled thermo-block, to maintain the test samples and the *Vibrio fischeri* suspension at a temperature of (15 ± 1) °C. Within one test, the temperature deviation should be at most ±0,3 °C.

7.4 Luminometer, equipped with at least one dispenser (minimum injection volume 0,2 ml).

The instrument shall be capable of measuring and recording the luminescence continuously at least for 5 s in 0,2 s intervals or shorter. The injection and recording shall be performed simultaneously.

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7.5 Test tubes, cuvettes, test plates or any other suitable **test vessels**, made of chemically inert material, appropriate for the selected luminometer.

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7.6 pH-meter.

7.7 Chronometer.

7.8 Piston pipettes, nominal capacity 100 µl to 1 000 µl, ISO 8655-2[3].

7.9 Mixer, e.g. vortex mixer, for mixing the samples before the measurements.

7.10 Conductometer.

7.11 Oxygen probe, as specified in ISO 5814.

7.12 Sieve, nominal size of openings 2 mm, ISO 565[1].

8 Sampling and sample pre-treatment

8.1 Sampling

Collect the samples in chemically inert, clean containers as specified in ISO 5667-16. Fill the containers completely and seal them. Cool the samples on ice, or in a refrigerator or a cooling box at 2 °C to 5 °C and test them as soon as possible after collection. Where necessary, store samples at a temperature of 2 °C to 5 °C in the dark for no longer than 48 h. If the samples have to be frozen store them at a temperature of −18 °C or below in the dark for no longer than 2 months. Prepare and measure frozen samples immediately after thawing in a water bath. For long-term storage, the samples may be freeze-dried and stored in the dark at room temperature. Do not use chemicals to preserve the samples. Perform the necessary pH adjustment and salt addition just before testing.

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