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

Reference number
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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 ± 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).

For the preparation of a suitable stock suspension, addition of at least 10 ml of protective medium per millilitre suspension in sodium chloride solution is recommended. On addition of protective medium, bioluminescence is markedly decreased but reappears after addition of dilution solution.

Continue stirring for about 15 min to obtain a homogeneous mixture.

Dispense aliquots of 100 µl into suitable test tubes (6.4).

It is convenient to use the type of tube to be used in the subsequent procedure (Clause 9).

For future use, store stock suspensions in a freezer at $\leqslant -20$ °C.

NOTE The stock suspension can be used for determinations for several months. At –70 °C the suspension can be stored for even longer periods.

Refrozen stock suspensions may be used for preliminary tests only.

The stock suspensions may be used for testing purposes as long as the validity criteria (see Clause 12) are met.

9 Procedure

Prepare the reference samples according to 5.6. Test each batch of bacteria 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.

Take the tube containing the stock suspension (8.4) out of the freezer and place it in a water bath at 20 °C ± 2 °C to thaw the suspension.

Prepare the test suspension in two steps:

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

— Wait for about 15 min.

Pipette this suspension into, for example, a 20 ml reagent vessel 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 samples (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 samples (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.

10 Evaluation

10.1 Inhibitory effect on luminescent bacteria

Calculate the correction factor (f_{kt}-value) from the measured luminescence intensity using Equation (1). This factor serves to correct the initial values I_0 of all test samples before they can be used as reference values for the determination of the water-dependent decrease in luminescence.

$$f_{kt} = I_{kt}/I_0 \ (t = 5 \text{ min, } 15 \text{ min, } 30 \text{ min}) \quad (1)$$

where

f_{kt} is the correction factor for the contact time of 5 min, 15 min or 30 min;

I_{kt} is the luminescence intensity in the control sample after the contact time of 5 min, 15 min or 30 min, in relative luminescence units;

I_0 is the luminescence intensity of the control test suspension, immediately before the addition of the diluent (5.2), in relative luminescence units.

Calculate the mean correction factor $\overline{f}_{kt}$ and the deviation of the individuals from the means in percent (one significant digit):

$$\left[\left(\overline{f}_{kt} \pm f_{kti}\right)/\overline{f}_{kt}\right] \times 100 \quad (2)$$

where

f_{kti} is either of the two individual values of the correction factor and $\overline{f}_{kt}$ is the mean value.

Calculate I_{ct} using Equation (3):

$$I_{ct} = I_0 \times \overline{f}_{kt} \quad (3)$$

where

$\overline{f}_{kt}$ is the mean of f_{kt};

I_0 is the luminescence intensity of the test sample suspension, immediately before the addition of the sample (7.2) or the diluted sample (Annex B), in relative luminescence units;

I_{ct} is the corrected value of I_0 for test sample tubes immediately before the addition of the test sample.

Calculate the inhibitory effect of a test sample using Equation (4):

$$H_t = [(I_{ct} - I_t)/I_{ct}] \times 100 \quad (4)$$

where

H_t is the inhibitory effect of a test sample after the contact time of 5 min, 15 min or 30 min, in percent;

I_{ct} see Equation (3);

I_t is the luminescence intensity of the test sample after the contact time of 5 min, 15 min or 30 min, in relative luminescence units.

Calculate the mean of the inhibitory effect H_t for each dilution level, in percent.

Calculate the arithmetic difference of the parallel determinations of H_{ti} from their respective mean $\overline{H}_t$, in percent points (one significant digit):

$$\overline{H}_t(\%) - H_{ti}(\%)$$

where

H_{ti} is either of the two individual values of the inhibitory effects of a test sample and $\overline{H}_t$ is the mean value.

10.2 Determination of EC-values

Calculate the concentration-effect relationship for each exposure time using suitable standard linear or non-linear regression analysis [8].

For evaluation of concentration-effect relationships using a linear regression technique, evaluate, for each dilution level, the gamma value (ratio of light lost to the amount of light remaining at time t) using Equation (5):

$$\Gamma_t = \overline{H}_t / \left(100 - \overline{H}_t\right) \quad (5)$$

where

Γ_t is the gamma value of the test sample after the contact time of 5 min, 15 min or 30 min;

$\overline{H}_t$ is the mean of H_t, see Equation (4).

NOTE When a certain test concentration gives 0 % or 100 % inhibition of bioluminescence, the gamma value cannot be calculated. Therefore, usually only H_t-values between 10 % and 90 % are used in the calculation of the concentration-effect relationship.

The concentration-effect relationship at a given exposure time often can be described by the following linear equation:

$$\lg c_t = b \lg \Gamma_t + \lg a \quad (6)$$

where

c_t is the portion of the water sample within the test sample, in percent;

Γ_t see Equation (5);

b is the value of the slope of the described line;

$\lg a$ is the value of the intercept of the described line.

By means of standard least-squares regression statistics, calculate the EC_{20}- and EC_{50}-values with corresponding confidence limits, in which:

$c_t = EC_{20,t}$ at $\Gamma_t = 0{,}25$;

$c_t = EC_{50,t}$ at $\Gamma_t = 1{,}00$.

For non-linear regression analysis, various models are available within standard graphic or statistical software packages. They are typically based on functions of the normal distribution (i.e. Probit analysis), the logistic distribution (i.e. Logit analysis), or the Weibull distribution (i.e. Weibull analysis). Calculated inhibitory effects (H_t) can be used directly to estimate parameters of the nonlinear concentration-effect relationship, from which EC values for any level might subsequently be derived [8].

If the range of value pairs cannot be curve-fitted, the EC-values can be estimated graphically using a double logarithmic coordinate system.

11 Expression of results

Report the results in accordance with the example in Table 1.

Report the test duration (5 min, 15 min or 30 min).

If determined, report the LID_{lb}-value (see Annex B).

If determined, report the EC_{20}- and EC_{50}-values and the method for the derivation of these values.

Report the type of bacterial preparation used.

Table 1 — Example of test evaluation – Sample: effluent from a sewage treatment plant

Control experiments						
Control batch number	Dilution level D	Measured values I_0	Measured values I_{k30}	I_{k30}/I_0	$\overline{f}_{k30}$	Validity test Deviation from the mean $\overline{f}_{k30}$ in %[b]
1	1[a]	93	76	0,817 2	0,815 2	± 0,3
2		91	74	0,813 2		
3	⩾ 2[a]	92	79	0,858 7	0,850 4	± 1,0
4		95	80	0,842 1		

Test experiments								
Test batch number	Dilution level D	Measured values I_0	Measured values I_{30}	I_{c30}	H_{30} %	$\overline{H}_{30}$ %	Validity test Deviation from the mean, in % points[c]	Γ_{30}
1	1	92	25	75,0	66,7	65,53	± 1,1	1,901
2		93	27	75,8	64,4			
3	2	86	43	73,1	41,2	42,51	± 1,3	0,740
4		90	43	76,5	43,8			
5	3	91	60	77,4	22,5	22,92	± 0,5	0,297
6		89	58	75,7	23,4			
7	4	95	72	80,8	12,4	11,65	± 0,8	0,132
8		94	70	79,9	10,9			
	Reference substance							
9	3,4 mg/l DCP, or 2,2 mg/l Zn, or 18,7 mg/l Cr	91	32	77,4	58,7	57,85	± 0,9	1,372
10		93	34	79,1	57,0			

[a] See Annex B.

[b] For the control batch, the deviation from the mean $\overline{f}_{k30}$ is determined by the arithmetic difference of the parallel determinations from the mean, divided by the mean expressed in percent [Equation (2) in 10.1)].

[c] For the test batch, the deviation of the H_{30}-values (in percent) of the parallel measurements from the mean is calculated as the arithmetic difference of each H_{30}-value (in percent) from the mean $\overline{H}_{30}$ (in percent) (called percent points).

The LID_{lb}-value in this example is $LID_{lb} = 4$.

The EC_{20}-value in this example is 31,9 %; the EC_{50}-value is 58,8 % (standard least square statistics).

12 Criteria of validity

The test is valid if:

— the $\overline{f}_{kt}$-value for 15 min or 30 min incubation ranges between 0,6 and 1,3;

— the parallel determinations do not deviate from their mean by more than 3 % for the control samples;

— for the test samples which determine the LID_{lb}-value or the EC_{20}/EC_{50}-values, respectively, the deviation from their mean in “percent points” does not exceed 3 % points;

— for the batch of stock suspensions cultured according to 8.4, the three reference substances (5.6) (solutions not neutralized, check separately) cause 20 % to 80 % inhibition after 30 min contact time at the following concentrations in the final test suspension:

4,5 mg/l 3,5-dichlorophenol

25 mg/l Zn(II) (equivalent to 109,9 mg/l of zinc sulfate heptahydrate)

4 mg/l Cr(VI) (equivalent to 11,3 mg/l of potassium dichromate);

— one of these three reference substances (5.6) (solution not neutralized) tested in parallel to each stock suspension tube thawed for the actual test (Clause 9) causes 20 % to 80 % inhibition after 30 min contact time.

13 Precision

In a national interlaboratory trial, carried out during summer 1991 by 22 laboratories, precision data were determined. The results are summarized in Annex C.

14 Test report

The test report shall refer to this part of ISO 11348. The documentation should contain the following information:

a) identity of the water sample, including sampling, storage time and conditions;

b) pH and oxygen concentration, in mg/l or % saturation of the original water sample;

c) date of test performance;

d) sample pretreatment, if any, e.g. pH after adjustment;

e) origin of the bacteria, batch number;

f) date of preparation of the bacteria;

g) storage temperature of the bacteria;

h) expression of the results in accordance with Clause 11 and Table 1;

i) any deviation from this method and information on all circumstances which might affect the results;

j) test results with reference substances for the batch of bacteria and the actual test.

Annex A
(informative)

Colour-correction method

A.1 Application

Loss of light due to absorption can occur when a sample shows a visible colour in the dilution series, especially in the red to brown colour range. If there is a visible colour at the EC_{20} concentration, the following procedure is performed to check if colour correction is needed. In any case, when the test sample concentration is close to the EC_{50}-value, a colour correction should be made.

A.2 Additional apparatus

A.2.1 Colour-correction tube, double-walled tube, fitting the light meter.

A.2.2 Pasteur pipettes.

A.3 Procedure

Carry out the complete colour-correction procedure at a temperature of 15 °C ± 1 °C in a thermostatically controlled incubator.

Prepare a dilution of the test sample with diluent (5.2) with a concentration close to the $EC_{20,t}$-value (C_k). When the $EC_{20,t}$-values differ much, the C_k should be close to the lower $EC_{20,t}$.

NOTE 1 It is not necessary to choose a different C_k for each exposure time (5 min, 15 min, 30 min).

Transfer 2,0 ml of 2 % sodium chloride solution (5.2) to the chamber of the colour-correction tube.

Prepare a special bacterial suspension.

With fresh bacteria prepared according to 8.4, the use of 1,0 ml of bacterial stock suspension is recommended.

Mix the suspension well before transferring it with a Pasteur pipette to the inner chamber of the colour-correction tube. Add suspension to the same level as the solution in the outer chamber. Measure the light level (B_0) after at least 15 min, and start the chronometer.

From this moment on, the position of the colour-correction tube in the measuring chamber should remain the same for all readings.

Remove, with a pipette, the sodium chloride solution from the outer chamber and replace it by 2,0 ml of the diluted test sample (Annex B), precooled to 15 °C ± 1 °C.

Measure the light level (I_5) 5 min after the first measurement.

Remove, with a pipette, the diluted test sample from the outer chamber, and replace it by 2,0 ml of sodium chloride solution.

Measure the light level (B_{10}) 10 min after the first measurement.

NOTE 2 The procedure can be simplified by using two identical colour-correction tubes. The outer chamber of the first tube is filled with dilution water, the outer chamber of the second tube is filled with the diluted sample. After 15 min, light levels B_0 and I_0 can be measured. These values can then replace the values for B_5 and I_5 in the calculations in A.4.

A.4 Calculation

The calculations assume that the coloured sample behaves according to the Beer-Lambert law, which is usually the case.

Calculate B_5 with Equation (A.1):

$$B_5 = B_0 - \frac{B_0 - B_{10}}{2} \tag{A.1}$$

Calculate for a given exposure time (t) the absorption (A_t) of the uncorrected $EC_{20,t}$ concentration with Equation (A.2):

$$A_t = \frac{EC_{20,t}}{C_k} \times k \times \ln\frac{B_5}{I_5} \tag{A.2}$$

where

C_k is the concentration of the sample or chemical in the (colour) tested concentration;

k is an empirically derived system constant;

$\ln\frac{B_5}{I_5}$ is the absorption of the tested dilution in the colour correction cuvette.

Calculate the corresponding transmission (T_t) with Equation (A.3):

$$T_t = \frac{1 - e^{-A_t}}{A_t} \tag{A.3}$$

Calculate the corrected gamma values (Γ_c) with Equation (A.4):

$$c\Gamma_t = (5T_t) - 4 \tag{A.4}$$

and with Equation (A.5):

$$\Gamma_c = c\,\Gamma_t \times \Gamma_0 \tag{A.5}$$

where

$c\Gamma_t$ is the correction factor for gamma values at a given exposure time (t);

Γ_0 is the original gamma value.

Perform a recalculation of the test results with the corrected values.

At a given exposure time, the absorption (A_t) and the transmission (T_t) for each test concentration can be calculated, and from this the uncorrected gamma value can be calculated with Equation (A.6):

$$\Gamma_c = T_t(1 + \Gamma_0) - 1 \tag{A.6}$$

The correction factor is the same for each gamma value, assuming that the slope of the original line is correct. It is therefore sufficient to calculate the correction factor for one gamma value only. In this calculation, the gamma value corresponding to the uncorrected $EC_{20,t}$ concentration is used ($\Gamma = 0{,}25$). Equation (A.7) for calculation of the correction factor is deduced as follows:

$$c\Gamma_t = \frac{\Gamma_c}{\Gamma_0} = \frac{T_t(1+\Gamma_0)-1}{\Gamma_0} = \frac{T_t(1+0{,}25)-1}{0{,}25} = (5T_t)-4 \qquad \text{(A.7)}$$

where

T_t is measured bioluminescence value at a given exposure time (t);

$c\Gamma_t$ is the correction factor for gamma values at a given exposure time (t);

Γ_0 is the original gamma value;

Γ_c is the corrected gamma value.

A.5 Example

Table A.1 — Example

Colour-correction data										
C_k = 10,0 % volume fraction		$B_5 = 81$			$I_5 = 78$			$k = 3{,}1$		
Colour-correction calculation										
C_k = % volume fraction		5 min			15 min			30 min		
		$c\Gamma_5 = 0{,}708$			$c\Gamma_{15} = 0{,}670$			$c\Gamma_{30} = 0{,}657$		
	I_0	I_5	Γ_0	Γ_c	I_{15}	Γ_0	Γ_c	I_{30}	Γ_0	Γ_c
blank	100	90			80			70		
5,625	98	82	0,076	0,054	74	0,059	0,040	65	0,055	0,036
11,250	94	63	0,343	0,243	60	0,253	0,170	53	0,242	0,159
22,500	96	45	0,920	0,651	42	0,829	0,556	38	0,768	0,505
45,000	97	15	4,820	3,412	17	3,565	2,389	17	2,994	1,967
original equation:		$\ln \Gamma = 1{,}96 \times \ln C - 5{,}96$			$\ln \Gamma = 1{,}95 \times \ln C - 6{,}16$			$\ln \Gamma = 1{,}90 \times \ln C - 6{,}12$		
corrected equation:		$\ln \Gamma = 1{,}96 \times \ln C - 6{,}30$			$\ln \Gamma = 1{,}95 \times \ln C - 6{,}56$			$\ln \Gamma = 1{,}90 \times \ln C - 6{,}53$		
original $EC_{20,t}$		10,3			11,6			12,1		
corrected $EC_{20,t}$		12,3			14,3			15,1		

Annex B
(informative)

Dilution level D – Preparation of the dilution series

B.1 Principle

When testing waste water by means of a graduated dilution (D), the most concentrated test batch tested at which no inhibition, or only minor effects not exceeding the test-specific variability, were observed, is expressed as “Lowest Ineffective Dilution (LID)”. This dilution is expressed as the reciprocal value of the volume fraction of waste water in the test batch [e.g. if the waste water content is 1 in 4 (25 % volume fraction) the dilution level is D = 4].

In the luminescent bacteria test, usually equal volumes of test suspension and water sample or diluted sample are mixed. Therefore, the dilution levels within a dilution series are $D \geqslant 2$ (highest sample concentration in the test: 50 % sample) as a rule.

B.2 Preparation of sample

Prepare the samples according to 7.2.

Waste-water samples should be homogenized by shaking by hand or mixing with a magnetic stirrer.

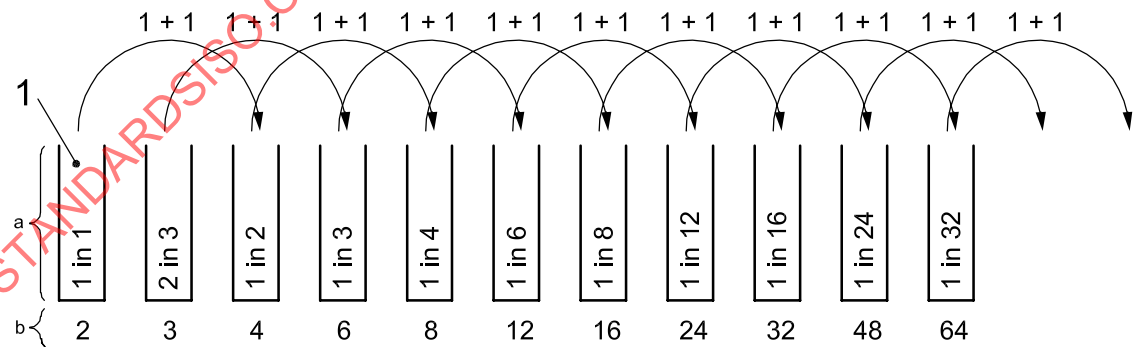
Add 20 g of sodium chloride per litre to the water sample or the pH-adjusted water sample. For waste-water samples with high salt concentrations, measure the salinity and calculate the amount of NaCl (if any) required to adjust osmolarity. The resulting salt-concentration in the test samples should not exceed the osmolarity of a 35 g/l sodium chloride solution.

Prepare the dilution series required in a first set of test tubes. A dilution series according to Table B.1 is recommended. It is prepared by means of a graduated dilution and combines two geometric series (D = 2, 4, 8, 16, etc., and D = 3, 6, 12, 24, etc.) of dilutions. For the two parallel determinations, a volume of 1 500 µl of sample or diluted sample or controls is sufficient.

Table B.1 — Preparation of dilution series

	Dilution level D	Composition of sample dilution series (total volume for parallel determinations: 1 500 µl)	
		Water sample (7.2) µl	Dilution water (5.2) µl
	For D = 1		
Control batches		0	1 500
Test batches	1	1 500	0
	For D ⩾ 2		
Control batches		0	1 500
Test batches	2	1 500	0
	3	1 000	500
	4	750	750
	6	500	1 000
	8	375	1 125
	12	250	1 250
	16	187,5	1 312,5
	24	125	1 375
	32	93,75	1 406,25
Reference batches (Concentrations see 5.6)		1 500	0

The easiest way to prepare this dilution series is to first make two stock dilutions in separate test tubes (see Figure B.1).



Key

1 sample

a Dilution of sample to make in the first set of test tubes.

b Final dilution level D after addition to test suspension.

Figure B.1 — Dilution scheme

Dilution 1 in 1, undiluted sample 3 000 µl (final dilution in test after mixing with equal volumes of test suspension will be 1 in 2).

Dilution 2 in 3, e.g. 2 000 µl of sample + 1 000 µl of solution 5.2 (final dilution in test after mixing with equal volumes of test suspension will be 1 in 3).

For the preparation of further dilutions, transfer 1 500 µl of sample/diluted sample to 1 500 µl of solution 5.2 in the test tubes, mix after each transfer.

In the luminescent bacteria test, usually 500 µl of water sample or diluted sample prepared in the first set of test tubes are mixed with 500 µl of test suspension (see Clause 9) prepared in the second set of test tubes, after measurement of initial light intensities of test suspensions. Therefore, the resulting dilution level D in the test batch for light inhibition measurements is twice the dilution of the sample or diluted sample.

If it is desired to test nearly undiluted water samples, it is possible to add 800 µl of the undiluted water sample (7.2) to 200 µl of a test suspension. The dilution of sample then is 1 in 1,25 (final sample concentration in the test: 80 % sample). The corresponding D value may be called $D = 1$. The test suspension for $D = 1$ is prepared by adding 4,5 ml of solution (5.5) to the resuspended bacteria, instead of 11,5 ml. For this value, an extra control batch is needed for the calculation of the correction factor serving to correct the initial values. The control batch is made by adding 800 µl of sodium chloride solution (5.2) to 200 µl of test suspension.

B.3 Procedure

Carry out the test according to Clause 9.

Carry out duplicate determinations for each dilution.

Determine and record the luminescence intensity in all test tubes, including controls, optionally after 15 min, and after 30 min (I_{30}).

B.4 Evaluation

Calculate the mean of the inhibitory effect $\overline{H}_{30}$ for each dilution level, in percent (see 10.1).

Calculate the deviation of the parallel determination H_{30} from their respective mean for duplicates H_{30} (%) (mean) – H_{30} (%) in percent points (one significant digit), and as a percentage of the mean for controls (correction factors, see 10.1).

B.5 Expression of results

Express the results according to Clause 11.

The lowest D-value tested at which the mean inhibitory effect $\overline{H}_{30}$ is < 20 % is called LID_{lb}.

B.6 Test report

See Clause 14.

In addition to the data required by this part of ISO 11348, the test report should include information on the appearance of waste water, adjustment of salinity and pre-treatment (settling, filtration, centrifugation, aeration).