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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Contents

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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.

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.

International Standard ISO 11348-1 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods.*

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*

Annexes A, B and C of this part of ISO 11348 are for information only.

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Introduction

Measurements according to 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 NAW WI and ISO/TC 147/SC 5 WG 1 has shown that in special cases these different techniques may give different results, especially where water samples contain 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 can 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 or cannot be revised by the user.

That is why in this International Standard, 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 (ISO 11348-1), the performance of which can be revised 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 1:
Method using freshly prepared bacteria

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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 or ground water) or salt and brackish water, especially the monitoring of changes in inhibition towards bacteria,

— pore water.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 11348. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 11348 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 5667-16:1998, *Water quality — Guidance on biotesting of samples*.

ISO 7027:—[1)], *Water quality — Determination of turbidity*.

1) To be published. (Revision of ISO 7027:1990)

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 cuvette.

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

The dilution level resulting in $<$ 20 % inhibition of light emission is determined. 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 dilutions which result in 20 % and 50 % light reduction compared to the blank (EC_{20} and EC_{50}). These values are interpolated within the dilution series.

4 Interferences

Insoluble, slightly soluble or 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.

Losses of luminescence caused by light absorption or light scattering may occur in the case of strongly coloured or turbid waters. This interference sometimes can be compensated, e.g. by using a double-chambered absorption correction cuvette (see annex A).

Since oxygen at $>$ 0,5 mg/l is required for the bioluminescence, samples with a high oxygen demand (and/or a low oxygen concentration) may cause a deficiency of oxygen and be inhibitory.

An organic contamination of the sample by readily biodegradable nutrients (e.g. urea, peptone, yeast extract, usually $\geqslant$ 100 mg/l) may cause a pollutant-independent reduction in bioluminescence.

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

5 Reagents and materials

Use chemicals of recognized analytical grade quality. Water shall be distilled or of equivalent purity.

5.1 Test bacteria

Strain of luminescent bacteria belonging to the species *Vibrio fischeri* NRRL B-11177. 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 litre with water.

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

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

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

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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 ($MgCl_2 \cdot 6H_2O$)

— 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 litre with water.

This solution can be stored in portions at −20 °C.

5.6 Reference substances

— Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)

— 3,5-Dichlorophenol ($C_6H_4OCl_2$)

— Potassium dichromate ($K_2Cr_2O_7$)

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 ($K_2HPO_4 \cdot 3H_2O$)

— 0,204 Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)

— 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 (5.3) or hydrochloric acid (5.4). Make up to 1 litre with water. Transfer 50 ml each to Erlenmeyer flasks (approx. vol. 250 ml) and sterilize in an autoclave at 121 °C for 20 min.

NOTE Caso-peptone and yeast extract offered by different suppliers can be of fluctuating quality. In case of problems (e.g. growth inhibition), purchase a product from another manufacturer.

5.8 Agar medium for stock cultures

Adjust 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

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

NOTE Damage of bacterial cells during the freezing procedure is prevented by the use of the protective medium. BSA offered by different suppliers can be of fluctuating quality. If problems occur, purchase a product from another manufacturer.

Prepare protective medium freshly before use.

6 Apparatus

6.1 Refrigerator to maintain the stock suspension at a temperature of 3 °C ± 3 °C.

6.2 Thermostatically controlled thermoblock to maintain the test samples at a temperature of 15 °C ± 1 °C. Within one test the temperature deviation shall be at most ± 0,2 °C.

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

6.4 Test tubes (vials) 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.

6.5 pH-meter.

6.6 Chronometer.

6.7 Piston pipettes for plastic syringes, nominal capacity 10 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 Incubator shaker for incubation of Erlenmeyer flasks.

6.12 Autoclave.

6.13 Incubator.

6.14 Spectral- or filterphotometer and cuvettes, optical path 1 cm.

6.15 Inoculating loop (or needle).

6.16 Conductometer.

7 Sampling and sample pretreatment

7.1 Sampling

Sampling shall be conducted in chemically inert, clean containers in accordance with ISO 5667-16. Fill the containers completely and seal them. Test the samples as soon as possible after collection. Where necessary, store samples at a temperature of 2 °C to 5 °C in the dark in glass for not longer than 48 h. For periods up to two weeks, store at −20 °C. Do not use chemicals to preserve the samples. Perform the necessary pH adjustment and salt addition just before testing.

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7.2 Sample preparation

Measure the pH of all samples. If the pH lies between 6 and 8,5 there is generally no adjustment necessary. pH-adjustment, 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 samples to 7,0 ± 0,2 by adding either hydrochloric acid (5.4) or sodium hydroxide (5.3); choose the concentration of the hydrochloric acid or the sodium hydroxide 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 brackish and saline waters, measure the salinity and calculate the amount of NaCl (if any) required to adjust the osmolarity (clause 4).

Strongly turbid samples should be allowed to sediment for 1 h or centrifuged, for example for 10 min at 5 000 *g*, or should be filtered.

8 Cultivation of luminescent bacteria

8.1 Stock culturing

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

Incubate in an incubator for 2 days to 5 days 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 maximum storage period of 2 weeks.

NOTE 1 Commercially available vials of preserved bacteria 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 should be opened approximately every 6 months.

NOTE 2 Luminescence of luminescent bacterial colonies can decrease during storage.

8.2 Preparation of precultures

Inoculate 50 ml of preculture broth (5.7) in Erlenmeyer flasks (approx. vol. 250 ml) under sterile conditions with one luminescent single colony of a stock culture aged 2 days to 5 days.

Shake for 21 h ± 1 h at 20 °C ± 1 °C at 180 r/min.

Determine the turbidity of a 1:10 dilution in sodium chloride solution (5.2), for example in formazine nephelometric units (FNU) at 578 nm in accordance with 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 preculture (8.2) to result in an estimated initially turbidity of 10 FNU.

Shake for 20 h ± 1 h at 20 °C ± 1 °C at 180 r/min.

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

NOTE Following the above conditions, the undiluted main culture will normally exhibit a turbidity of 700 FNU to 1800 FNU.

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8.4 Preparation of stock suspension

Precool sodium chloride solution (5.2) and protective medium (5.9) on ice.

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

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

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

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

Determine the turbidity of a 1:100 dilution with sodium chloride solution (5.2) photometrically.

Add precooled protective medium (5.9) more quickly up to an estimated turbidity of 2500 FNU ± 500 FNU (see note 2 in 8.1).

NOTE For the preparation of a suitable stock suspension, addition of at least 10 ml of protective medium per millilitre suspension in sodium chloride 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).

If the suspension is to be used immediately, keep it for a maximum of 4 h at 3 °C ± 3 °C before addition of the solution (5.5).

Store the stock suspension in a freezer at −20 °C; it may be used for determinations for at least one month. At −70 °C the suspension can be stored for even longer periods. Refrozen stock suspensions can be used for preliminary tests only.

The stock suspension can be used for testing purposes as long as the validity criteria (clause 12) are met.

9 Procedure

Prepare the samples according to 7.2.

Prepare the dilution series required (see annex B).

For control samples, maintain the NaCl solution (5.2) at 15 °C ± 1 °C.

Maintain the test tubes containing controls, the samples of the dilution series and the diluent (5.2) at 15 °C ± 1 °C.

If stock suspension (8.4) has been stored in a freezer, thaw it in a water bath at 20 °C ± 2 °C.

Add 0,5 ml (per 100 µl stock suspension) of solution (5.5), maintained at 15 °C ± 1 °C, and homogenize by gentle shaking of the vial. Wait about 15 min.

Pipette 500 µl of test suspension into the test tubes, maintained at 15 °C ± 1 °C in the incubator, at the same time intervals (20 s) as used for later intensity measurements.

Carry out, if possible, duplicate determinations per dilution level at a test temperature of 15 °C ± 1 °C.

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After a conditioning time of at least 15 min, determine and record the luminescence intensity I_0 of the test suspensions by means of a luminometer.

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

NOTE All samples should be measured, as differing luminescence may be expected due to possible inhomogeneities of the test suspension.

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

Immediately after the luminescence measurement of a test suspension, make up this solution to a total volume of 1 ml with samples (7.2), diluted samples (annex B) or sodium chloride solution (5.2) as necessary. Mix by hand, start the chronometer and place the cuvette back into the thermoblock at 15 °C ± 1 °C. Repeat for all the other cuvettes, leaving the same time interval between successive additions.

Determine and record the luminescence intensity in all cuvettes, including controls, again after 15 min and 30 min (I_{15}, I_{30}), optionally also after 5 min (I_5).

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.

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

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 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.

Average the f_{kt} values of the control samples.

Calculate I_{ct} using equation (2):

$$I_{ct} = I_0 \cdot \overline{f_{kt}} \quad \ldots (2)$$

where

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

I_0 [see equation (1)];

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

Calculate the inhibitory effect of a test sample using equation (3):

$$H_t = \frac{I_{ct} - I_{Tt}}{I_{ct}} \times 100 \quad \ldots (3)$$

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where

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

I_{ct} [see equation (2)];

I_{Tt} is the luminescence intensity of the test sample after the contact time of 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 deviation of the parallel determination of H_t from their respective mean for duplicates and as percentage of the mean for controls.

For evaluation of concentration-effect relationships, evaluate for each dilution level the gamma value using equation (4):

$$\Gamma_t = \frac{\overline{H}_t}{100 - \overline{H}_t} \quad \ldots (4)$$

where

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

$\overline{H}_t$ is the mean of H_t [see equation (3)].

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

10.2 Determination of EC values

Calculate the concentration-effect relationship for each exposure time using standard linear regression analysis. The concentration-effect relationship at a given exposure time often can be described by the following linear equation (5):

$$\lg c_t = b \lg \Gamma_t + \lg a \quad \ldots (5)$$

where

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

Γ_t [see equation (4)];

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 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 $\Gamma_t = 1{,}00$.

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

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11 Expression of results

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

If determined, report the LID-value (see annex B).

If determined, report the EC_{20} and EC_{50} values.

Report the type of bacterial preparation used.

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

Control experiments					
Test	**Measured values**		I_{k30}/I_0	$\overline{f_{k30}}$	**Validity test** Deviation from the mean $\overline{f_{k30}}$, in %[3]
	I_0	I_{k30}[2]			
80 %[1]	297	242	0,8148	0,8115	± 0,4
	292	236	0,8082		
50 %[1]	295	253	0,8576	0,8501	± 0,9
	305	257	0,8426		

Test experiments								
Test	**Dilution level D**	**Measured values**		I_{c30}	H_{30}	$\overline{H_{30}}$	**Validity test** Deviation from the mean, in %[4]	Γ_{30}
		I_0	I_{T30}		%	%		
80 %[1]								
1	1	300	81	243,5	66,7	65,7	± 1,0	1,919
2		297	85	241,0	64,7			
50 %[1]								
3	2	280	141	238,0	40,8	42,2	± 1,4	0,731
4		292	140	248,2	43,6			
5	3	292	193	248,2	22,3	22,95	± 0,65	0,298
6		285	185	242,3	23,6			
7	4	303	229	257,6	11,1	11,75	± 0,65	0,133
8		302	225	256,7	12,4			

1) Volume of the test suspension: 0,2 ml and 0,5 ml, respectively.

2) Final volume in cuvettes: 1 ml.

3) The deviation of the f_{k30} values in terms of percentage of the parallel determinations from their mean is a measure of the scattering of the control samples.

4) The deviation of the H_{30} values of the parallel measurements in terms of percentage from the mean is a measure of the scattering of the test samples.

The LID value in this example is LID = 4.

The EC_{20} value in this example is 31,9 %, the EC_{50} value is 58,7 %.

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12 Criteria of validity

The test is valid if

— the f_{kt} value for 30 min incubation ranges between 0,6 and 1,8;

— the parallel determinations do not deviate from their mean by more than 3 %. This holds for the control samples as well as for the test samples which determine the LID value or the EC_{20}/EC_{50} values respectively;

— the three reference substances (5.6) cause 20 % to 80 % inhibition after 30 min contact time at the following concentrations (solutions not neutralized, check separately):

6 mg/l	3,5-dichlorophenol
25 mg/l	Zn^{2+} (as zinc sulfate heptahydrate)
4 mg/l	Cr^{6+} (as potassium dichromate).

13 Precision

In a national round-robin test, carried out during summer 1991 by 22 laboratories, precision data were determined. The results are summarized annex C.

14 Test report

The test report shall refer to ISO 11348 and the modification used (ISO 11348-1, ISO 11348-2 or ISO 11348-3) and contain the following information.

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

b) pH of the original water sample;

c) date of test performance;

d) sample pretreatment, if any;

e) origin of the bacteria, batch number;

f) date of preparation of the bacteria;

g) storage temperature of the bacteria, if frozen;

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.

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Annex A
(informative)

Colour-correction method

A.1 Application

Light loss due to light 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 cuvette: double-walled cuvette, 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 ± 0,5 °C in a thermostatically controlled incubator.

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

NOTE 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 to the outer chamber of the colour-correction cuvette.

Prepare a special bacterial suspension.

NOTE With Microtox bacteria, use of 1,0 ml dilution water with 50 µl bacterial stock suspension is recommended. With Lumistox bacteria, use of 1,0 ml bacterial stock suspension is recommended.

Mix the suspension well before transferring it with a Pasteur pipette to the inner chamber of the colour-correction cuvette. 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 cuvette in the measuring chamber shall 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 sodium chloride solution.

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

NOTE The procedure can be simplified by using two identical colour-correction cuvettes. The outer chamber of the first cuvette is filled with dilution water, the outer chamber of the second cuvette 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.

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A.4 Calculations

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

Calculate B_5 with the formula:

$$B_5 = B_0 = \frac{B_0 - B_{10}}{2}$$

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

$$A_t = \frac{EC_{20,t}}{C_k} \cdot k \cdot \ln \frac{B_5}{I_5}$$

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 the formula:

$$T_t = \frac{1 - e^{-A_t}}{A_t}$$

Calculate the corrected gamma values (Γ_c) with the formulae:

$$c\Gamma_t = (5\ T_t) - 4$$

and

$$\Gamma_c = c\Gamma_t \cdot \Gamma_o$$

where

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

Γ_o is the original gamma value.

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

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

$$\Gamma_c = T_t\,(1 + \Gamma_o) - 1$$

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 ($\Gamma = 0{,}25$). The formula for calculation of the correction factor is deduced as follows:

$$c\Gamma_t = \frac{\Gamma_c}{\Gamma_o} = \frac{T_t\,(1+\Gamma_o) - 1}{\Gamma_o} = \frac{T_t\,(1+0{,}25) - 1}{0{,}25} = (5\ T_t) - 4$$

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where

C is the sample concentration;

I_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_o is the original gamma value;

Γ_c is the corrected gamma value.

A.5 Example

Colour-correction data										
C_k = 10,0 % vol. fraction		B_5 = 81			I_5 = 78			k = 3,1		
Colour-correction calculation										
C = % vol. 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_o	Γ_c	I_{15}	Γ_o	Γ_c	I_{30}	Γ_o	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 eq.:		$\ln \Gamma = 1{,}96 \times \ln C - 5{,}96$			$\ln \Gamma = 1{,}95 \times \ln C - 6{,}16$			$\ln \Gamma = 1{,}90 \times \ln C - 6{,}12$		
corrected eq.:		$\ln \Gamma = 1{,}96 \times \ln C - 6{,}30$			$\ln \Gamma = 1{,}95 \times \ln C - 6{,}56$			$\ln \Gamma = 1{,}90 \times \ln C - 6{,}53$		
original $EC_{30,t}$		10,3			11,6			12,1		
corrected $EC_{30,t}$		12,3			14,3			15,1		

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