INTERNATIONAL STANDARD

ISO 10712

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Water quality — *Pseudomonas putida* growth inhibition test (*Pseudomonas* cell multiplication inhibition test)

Qualité de l'eau — Essai d'inhibition de la croissance de Pseudomonas putida *(essai d'inhibition de la multiplication des cellules de* Pseudomonas*)*

Reference number
ISO 10712:1995(E)

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 10712 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Annexes A and B of this International Standard are for information only.

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Introduction

The bacterium *Pseudomonas putida* is used as an organism representative of heterotrophic microorganisms in fresh water.

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Water quality — *Pseudomonas putida* growth inhibition test (*Pseudomonas* cell multiplication inhibition test)

1 Scope

This International Standard specifies a test method for determining the inhibitory effect of surface, ground and waste water on *Pseudomonas putida*.

This method is not suitable for highly coloured test samples, or samples containing undissolved or volatile materials or substances which react with the nutrient solution, or which undergo changes during the test (for example by precipitation, or biochemical or photochemical degradation) and may give false results and/or impair the reproducibility.

The method is also suitable for testing substances soluble in water (see annex A).

2 Normative reference

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

ISO 7027:1990, *Water quality — Determination of turbidity.*

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1 multiplication; growth: Increase in the number of cells during the test period.

3.2 concentration-effect relationship: Dependence of cell multiplication inhibition on the concentration of the test sample.

NOTE 1 The relationship is graphically represented by plotting the inhibition values along the ordinate against the sample concentrations along the abscissa.

3.3 effective concentration (EC): Concentration of the test sample giving a calculated or interpolated inhibition of cell multiplication of *Pseudomonas putida* within 16 h ± 1 h, compared to that of the control batch.

The concentrations of test samples (EC10 and EC50) are determined from the concentration-effect relationship (3.2) at which cell multiplication is inhibited by 10 % or 50 % respectively, compared to that of the control batch.

3.4 stock culture: Bacterial culture obtained from the collection strain of the laboratory and intended to provide an inoculum for the preculture in the test procedure.

3.5 preculture: Bacterial culture separately used to adapt the test bacteria to the test conditions and to produce an adequate number of exponentially multiplying bacteria as an inoculum for the test culture.

3.6 test culture: Inoculated test medium (3.9).

3.7 inoculum: Suspension of bacteria used to inoculate a nutrient solution.

3.8 nutrient solution: Aqueous solution of nutrients required for bacterial growth.

3.9 test medium: Mixture of test sample, dilution water and nutrient solution (without inoculum).

3.10 sample: The surface, ground, or waste water to be tested.

3.11 test sample: The sample, after inclusion of all preparatory steps such as homogenization, pH adjustment, filtration, centrifugation.

3.12 control: Mixture of dilution water, nutrient solution and inoculum (without test sample).

3.13 formazine nephelometric unit (FNU): Formazine turbidity units. The optical density of a bacterial cell suspension at $\lambda = 436$ nm, measured as formazine nephelometric units according to ISO 7027.

4 Principle

Determination of the inhibitory effect of the sample on *Pseudomonas putida* by measurement of cell growth under the influence of varying dilutions of the test sample, compared to the cell growth of a culture obtained under the same conditions, but without the test sample.

Determination of the cell concentration as optical density after a test period of 16 h ± 1 h.

The concentrations of the test sample at which cell multiplication is inhibited by 10 % and 50 % within 16 h ± 1 h. are the basis for assessment.

5 Reagents

Use chemicals of analytical grade and deionized water or water of equivalent purity.

5.1 Test organism

Pseudomonas putida, a gram-negative aerobic bacterium of the *Pseudomonadaceae* family; mobile rods (diameter 0,7 µm to 1,1 µm, length 2,0 µm to 4,0 µm) with polar flagellation. It occurs ubiquitously in soil and surface water. The optimal growth temperature is between 25 °C and 30 °C.

NOTE 2 The two following strains are suitable for this test:

a) MIGULA, Berlin 33/2 strain (DSM 50026)

This strain is available from the following collection:

German collection of microorganisms
Mascheroder Weg 1b
D-38124 Braunschweig
Germany

b) NCIB strain 9494

This strain is available from the following collection:

Torry Research Station
P.O. Box 31
Aberdeen, UK

Any other strain of equivalent sensitivity may be suitable.

5.2 Hydrochloric acid, $c(HCl) = 1$ mol/l.

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

More diluted or concentrated acids and alkaline solutions are permissible to adjust the pH as necessary.

5.4 Nutrient solution

Prepare the stock solutions I-IV (see 5.4.1 to 5.4.4) and then sterilize them, for example at 121 °C for 10 min.

The solutions can be stored for several weeks in the refrigerator at 2 °C to 4 °C.

5.4.1 Solution I

Dissolve the following in water and dilute to 500 ml.

— 10,0 g of sodium nitrate ($NaNO_3$)

— 2,40 g of dipotassium hydrogen phosphate (K_2HPO_4)

— 1,20 g of potassium dihydrogen phosphate (KH_2PO_4)

— 1,0 g of yeast extract

5.4.2 Solution II

Dissolve the following in water and dilute to 500 ml.

— 10,0 g of sodium nitrate ($NaNO_3$)

— 2,40 g of dipotassium hydrogen phosphate (K_2HPO_4)

— 1,20 g of potassium dihydrogen phosphate (KH_2PO_4)

5.4.3 Solution III, glucose solution

Dissolve the following in water and dilute to 500 ml.

— 40,0 g of D(+)-glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$) for biochemical and microbiological use

5.4.4 Solution IV, magnesium sulfate-iron(III) citrate solution

Dissolve the following in water and dilute to 1 000 ml.

— 4,0 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)

— 0,01 g of granulated iron(III) citrate

NOTE 3 In order to reduce the number of steps involved, solutions I and III can be combined, following sterilization, for the procedure in 5.5 and 8.1 and solutions II and III for the procedure in 8.2.

5.5 Stock culture (see table 1)

5.5.1 Nutrient medium for the stock culture (slant agar)

Dissolve 18 g of agar (high purity quality for microbiology) in water by heating.

Add 50 ml of solution I (5.4.1), 125 ml of solution III (5.4.3), 100 ml of solution IV (5.4.4) and dilute to 1 000 ml with water.

Dispense 6 ml to 10 ml portions of the nutrient medium into culture tubes while still liquid, close the tubes with plugs and sterilize for 10 min at 121 °C.

Allow the nutrient medium to gel at a slant and store at 2 °C to 4 °C.

5.5.2 Handling of stock culture

Store stock cultures of the test strain *Pseudomonas putida* in slant-agar culture tubes on the solid nutrient medium for stock cultures (5.5.1).

Start new stock cultures at intervals of one week, to preserve the test strain.

Incubate the inoculated stock cultures for 24 h at 25 °C ± 4 °C (and store at 25 °C ± 4 °C) for this purpose. Long-term recultivation of one strain may cause changes in the sensitivity of the test organisms. In this case, a new culture of the test strain has to be used.

NOTE 4 A green pigment may be produced after incubation for 24 h. This is normal and does not indicate contamination.

6 Materials and equipment

Equipment which comes into contact with the test material during preparation of the nutrient medium, or during the test period, shall consist of glass or another chemically inert material.

All glass equipment and stoppers which come into contact with the test cultures shall be sterilized before use, if they are not sterilized together with the nutrient solutions.

Table 1 — Final concentrations in the various media

Nutrients	**Stock culture** (5.5) mg/l	**Preculture** (8.1) mg/l	**Test culture** (8.2) mg/l
$NaNO_3$	1 000	500	500
K_2HPO_4	240	120	120
KH_2PO_4	120	60	60
Yeast extract	100	50	—
$C_6H_{12}O_6 \cdot H_2O$	10 000	2 000	2 000
$MgSO_4 \cdot 7H_2O$	400	200	200
Iron(III) citrate	1,0	0,5	0,5
Agar	18 000	—	—

6.1 Spectrometer or turbidimeter.

Alternatively, determine the state of growth of the culture by a different procedure, providing that this method is sufficiently sensitive and if it can be shown that the correlation with turbidimetry is acceptable.

6.2 Microscope of minimum magnification × 100.

6.3 pH-meter.

6.4 Temperature-controlled cabinet.

6.5 Culture flasks.

6.6 Autoclave.

7 Treatment of samples

Test the samples as soon as possible after collection and preparation.

If unavoidable, preserve the samples by cooling (up to two days at 2 °C to 4 °C) or freezing (up to two weeks at − 18 °C). Preserve samples only in exceptional cases, because the toxicity of the sample can change on standing.

Thoroughly shake and, if necessary, homogenize the sample before preparing the test medium.

Measure the pH of the sample.

In general, the test is carried out without adjusting the pH. If there are indications that an inhibitory effect is caused merely by an extreme pH, carry out an additional test in which the pH is adjusted to 7,4. In this case, the pH of the sample is adjusted to 7,4 ± 0,3 with hydrochloric acid (5.2) or sodium hydroxide solution (5.3); change the concentration of the water sample as little as possible by this procedure. If necessary, for example with samples contaminated with high levels of microbes, they can be sterilized by filtration, but this may change the toxicological effects of the samples.

8 Procedure

8.1 Preparation of the preculture (3.5)

8.1.1 Preparation of the preculture medium

Place 900 ml of sterilized water in a sterilized vessel, or sterilize 900 ml of water in a suitable vessel.

Add 25 ml each of solutions I and III (5.4.1 and 5.4.3) plus 50 ml of solution IV (5.4.4).

NOTE 5 The pH of the preculture medium is 7,2 ± 0,2.

Dispense the preculture medium into the culture flasks under sterile conditions (e.g. in 90 ml portions into conical flasks with nominal volumes of 250 ml).

8.1.2 Preparation of inoculum for the preculture

Prepare the inoculum for the preculture by using stock culture (5.5.2) that is up to 7 days old.

Rinse the cells from the slant agar (5.5.1) with sterile preculture medium (8.1.1).

Dilute this cell suspension with sterile preculture medium to give a calculated turbidity of 10 FNU in the preculture.

EXAMPLE

If the volume of the final preculture is to be 100 ml, the turbidity of the cell suspension has to be diluted to 100 FNU, because 10 ml of this cell suspension will be added to 90 ml of preculture medium (8.1.1).

NOTES

6 The optical density of a bacterial cell suspension is determined according to Section 3 of ISO 7027:1990, by photoelectric measurement of the attenuation of transmitted light (3.4 of ISO 7027) or by measurement of the light scattering (3.3 of ISO 7027). Only optical densities < 0,4 may be used for calibration of FNU; for optical densities > 0,4, the suspension should be diluted to an optical density between 0,1 and 0,4.

7 Instead of FNU measurements, other turbidity units may also be used. For example:

$A_{610} = 0{,}02$ (10 FNU)

$A_{610} = 0{,}2$ (100 FNU)

$A_{610} = 0{,}1$ (50 FNU)

where A_{610} is the absorbance at 610 nm.

8.1.3 Incubation and use of the preculture

Add the inoculum (8.1.2) to the preculture medium (8.1.1).

Close the culture flasks (6.5) with porous, sterile plugs.

Incubate the preculture at the same temperature as in the test (8.3) for 5 h ± 0,5 h and keep the bacteria in suspension (for example by shaking). Avoid de-

posits on the walls of the flasks (for example by shaking).

When incubation is complete, dilute the bacterial suspension with the test culture medium (see table 1) to give a specific calculated turbidity (e.g. 50 FNU).

NOTE 8 The inoculum should be taken from the preculture during the exponential growth phase. Care should be taken that the bacteria are not present in the form of chains. This can be monitored by microscopic examinations. If filaments are observed, a new culture should be prepared.

8.2 Preparation of the test cultures

Select the dilution steps (for example see table 2) and prepare the dilution series with the test sample (3.11) and deionized water (clause 5).

Set the required final volume according to the vessels used for the test culture, for example a final volume of 100 ml in 250 ml culture flasks. In the following example with a final volume of 100 ml, the test cultures thus contain the specified volumes in millilitres.

Dispense solutions II, III and IV (5.4), dilution water and the test sample into the culture flasks (6.5).

Then add the inoculum, which has been adjusted to a specific turbidity according to 8.1.2, to give a calculated initial concentration for the inoculum of 5 FNU in the test culture.

Close the flasks with sterile plugs pervious to air or aluminium caps.

The highest concentration which can be used is a test culture containing 80 % of the test sample.

If possible, each dilution step should encompass three parallel batches.

The number of parallel batches depends on the selected significance, the required confidence level and the expected variance of the individual measurements.

NOTE 9 A reduction in the number of parallel batches can be justified if additional measurements of finer concentration gradients are carried out.

If the test material is cloudy or coloured, a dilution series without inoculum has to be prepared. In this case, the inoculum is replaced by a corresponding volume of the preculture medium (8.1.1).

8.3 Incubation

Incubate the test and control cultures at a constant temperature of 23 °C ± 1 °C in the dark.

The temperature variation during the test shall not be greater than ± 1 °C.

Keep the bacteria in suspension (for example by shaking) and avoid deposits on the walls of the flasks.

Table 2 — Example of a test series

Dilution series factor, $f = 2$	**Dilution water** (clause 5) **added first**	**Test sample**	**Stock solutions** (5.4)			**Inoculum** (8.1.2) (50 FNU, $\lambda = 436$ nm)	**Final volume**
			II	III	IV		
	ml	ml	ml	ml	ml	ml	ml
Control	80	0	2,5	2,5	5	10	100
2	30	50	2,5	2,5	5	10	100
4	55	25	2,5	2,5	5	10	100
8	67,5	12,5	2,5	2,5	5	10	100
16	73,7	6,3	2,5	2,5	5	10	100
32	76,9	3,1	2,5	2,5	5	10	100
64	78,5	1,5	2,5	2,5	5	10	100