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**Water quality — Determination of the  
elimination and biodegradability of  
organic compounds in an aqueous  
medium — Activated sludge simulation  
test**

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*Qualité de l'eau — Détermination de l'élimination et de la  
biodégradabilité des composés organiques en milieu aqueux — Essai  
de simulation des boues activées*

ISO 11733:2004

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Published in Switzerland

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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 11733 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 11733:1995), which has been technically revised.

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# Water quality — Determination of the elimination and biodegradability of organic compounds in an aqueous medium — Activated sludge simulation test

**WARNING AND SAFETY PRECAUTIONS** — Activated sludge and sewage contain potentially pathogenic organisms, therefore appropriate precautions should be taken when handling them. Toxic test compounds and those whose properties are unknown should be handled with care.

## 1 Scope

This International Standard specifies a method for the determination of the elimination and the biodegradability of organic compounds by aerobic micro-organisms. The conditions described simulate a waste-water treatment plant. Two test systems can be used: activated sludge plants or porous pots. The tests can optionally be performed under conditions of nitrification and denitrification (Annex A) and coupling of the units (Annex B).

The method applies to organic compounds which, under the conditions of the test, are

- a) soluble in tap water at the test concentration and not expected to be transformed to insoluble metabolites if biodegradation, in addition to elimination, is determined;
- b) poorly water-soluble, but which are satisfactorily dispersible in water and allow detection with suitable analytical means (e.g. organic carbon measurements);
- c) non-volatile, or which have a negligible vapour pressure under the test conditions;
- d) not inhibitory to the test micro-organisms at the concentration chosen for the test. Inhibitory effects can be determined by using a suitable test method (e.g. ISO 8192<sup>[15]</sup> or ISO 15522<sup>[27]</sup>). Compounds inhibitory at concentrations used in this test may be tested at concentrations less than their EC<sub>20</sub> value, followed by higher practical concentrations after a period of acclimatization.

The method can also be used to measure the biodegradation and elimination of dissolved organic compounds in waste water (also called “test compound” in the method).

If more or different information is required to predict the behaviour of test compounds or waste water in a treatment plant, other degradation tests may be performed. For appropriate use of this method and for alternative biodegradation methods, see ISO/TR 15462 and for general information on biotesting, see ISO 5667-16.

## 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 10634, *Water quality — Guidance for the preparation and treatment of poorly water-soluble organic compounds for the subsequent evaluation of their biodegradability in an aqueous medium*

ISO/TR 15462, *Water quality — Selection of tests for biodegradability*

### 3 Terms and definitions

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

**3.1 accelerating removal phase**  
(activated sludge simulation test) time from the end of the lag phase until the plateau phase is reached, during which the biodegradation of a compound or organic matter increases

NOTE Accelerating removal phase is expressed in days.

**3.2 activated sludge**  
biomass and inert matter produced in the aerobic treatment of wastewater by the growth of bacteria and other micro-organisms in the presence of dissolved oxygen

**3.3 chemical oxygen demand  
COD**  
mass concentration of oxygen equivalent to the amount of a specified oxidant consumed by a chemical compound or organic matter when a water sample is treated with that oxidant under defined conditions

NOTE COD is expressed, in this case, as milligrams of oxygen consumed per milligram or per gram of test compound

**3.4 concentration of suspended solids of an activated sludge**  
amount of solids obtained by filtration or centrifugation at known conditions of a known volume of activated sludge and drying at about 105 °C to constant weight

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**3.5 degree of elimination  
biodegradation**  
(activated sludge simulation test) mean eliminated (biodegraded) amount of a chemical compound or organic matter, calculated from the measured concentrations in the inlet and the outlet of the system

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NOTE The degree of elimination (biodegradation) is determined when no further elimination can be measured and is expressed as a percentage.

**3.6 denitrification**  
reduction of nitrate and nitrite to the end product nitrogen (in the form of the gas) by the action of bacteria

**3.7 dissolved organic carbon  
DOC**  
part of the organic carbon in a sample of water which cannot be removed by specified phase separation

NOTE Phase separation may be obtained, for example, by centrifugation of the water sample at 40 000 m/s<sup>2</sup> for 15 min or by membrane-filtration using membranes with a pore size of 0,45 µm.

**3.8 lag phase**  
(activated sludge simulation test) time from the start of a test until a significant elimination (biodegradation) of a compound or organic matter can be measured (the beginning of the accelerated removal phase)

NOTE The lag phase is expressed in days.

**3.9****nitrification**

oxidation of ammonium salts by bacteria where usually the intermediate product is nitrite and the end product nitrate

**3.10****plateau phase**

(activated sludge simulation test) time from the end of the accelerating removal phase until the end of a test in which the biodegradation of a compound or organic matter is in a steady state

NOTE The plateau phase is expressed in days.

**3.11****pre-exposure**

pre-incubation of an inoculum in the presence of the test compound or organic matter, with the aim of enhancing the ability of this inoculum to biodegrade the test compound by adaptation and/or selection of the micro-organisms

**3.12****pre-conditioning**

pre-incubation of an inoculum under the conditions of the subsequent test in the absence of the test compound and other organic matter, with the aim of improving the performance of the test by acclimatization of the micro-organisms to the test conditions

**3.13****primary biodegradation**

structural change (transformation) of a chemical compound by micro-organisms resulting in the loss of a specific property

**3.14****total organic carbon  
TOC**

all the carbon present in organic matter which is dissolved and suspended in the water

**3.15****ultimate aerobic biodegradation**

breakdown of a chemical compound or organic matter by micro-organisms in the presence of oxygen to carbon dioxide, water and mineral salts of any other elements present (mineralization) and the production of new biomass

**4 Principle**

This method is designed to determine the elimination and, if possible, the primary or ultimate biodegradation of water-soluble organic compounds from water by aerobic micro-organisms in a continuously operating test system simulating the activated-sludge process. An easily biodegradable organic medium and the organic test compound are the sources of carbon and energy for the micro-organisms.

Two test units (activated sludge plants or porous pots) are run in parallel under identical conditions, normally with a mean hydraulic retention time, HRT, of 6 h (8.3.1) and a mean sludge retention time, SRT (sludge age), of 6 d to 10 d (8.3.3).

NOTE 1 HTR is the mean period of retention of waste water in the aeration vessel. It is calculated by dividing the volume of sludge, expressed in litres, by the rate of flow of waste water, expressed in litres per day.

NOTE 2 SRT is the mean period of retention of activated sludge in the aeration vessel. It is calculated by dividing the volume or weight of sludge in the aeration vessel by the volume or weight of sludge discarded per day. If a period of 8 days is chosen, remove 1/8 of the volume of the activated sludge of the aeration vessel each working day and discard it.

The test compound is added together with the organic medium, usually at a concentration equivalent to a DOC between 10 mg/l and 20 mg/l, to the influent of only one of the test units. The second unit is used as control unit to determine the degree of biodegradation of the organic medium when the analysis is based on DOC or COD.

Samples of the effluents taken at regular intervals are analyzed for DOC or COD. The difference between values in the effluent of the test and the control unit compared with the influent concentration of the test compound is used to determine the degree of elimination of the test compound. Depending on the elimination characteristics and other available information, e.g. from other tests, ultimate biodegradability can be stated.

If required, the primary biodegradation of the test compound can be determined by substance-specific analysis. Optionally, the units may be operated under denitrifying conditions (see Annex A) or be coupled (see Annex B).

## 5 Test environment

The test shall take place in diffused light or in the dark, in an enclosure which is free from vapours toxic to micro-organisms and at a controlled temperature in the range of 20 °C to 25 °C. For special purposes, it is permissible to use a test temperature in another range.

## 6 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

**6.1 Tap water**, containing less than 3 mg/l DOC.

**6.2 Deionized water**, containing less than 1 mg/l DOC.

**6.3 Organic media.** <https://standards.iteh.ai/catalog/standards/sist/1d1eed78-a3de-417c-bfcec20ad00ce008/iso-11733-2004>

**6.3.1 General.**

Synthetic sewage, domestic sewage or a mixture of both are permissible as an organic medium. Measure the DOC (e.g. ISO 8245<sup>[16]</sup>) or COD (e.g. ISO 6060<sup>[14]</sup>) concentration in each new batch of organic medium and determine the alkalinity, if required and not already known.

Experience has shown that the so-called OECD medium<sup>[29]</sup> (6.3.2) might not be suitable in some cases. Therefore, two more synthetic media which have successfully been tested in laboratories are described in this International Standard. Domestic sewage (6.3.5) may also be used. Its use is recommended, as a continuous inoculation takes place and a vastly greater number of nutrients is available to improve the biodegradation potential of the test.

**6.3.2 Synthetic sewage 1 (OECD medium)**, which gives a mean DOC concentration of about 100 mg/l and a COD of about 300 mg/l in the influent.

It is composed of the following:

— peptone	160 mg
— meat extract	110 mg
— urea	30 mg
— anhydrous potassium monohydrogenphosphate (K <sub>2</sub> HPO <sub>4</sub> )	28 mg
— sodium chloride (NaCl)	7 mg



— calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ )	4 mg
— magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ )	2 mg
— tap water (6.1)	1 l

**6.3.3 Synthetic sewage 2**, which gives a mean DOC concentration of about 150 mg/l and a COD of about 400 mg/l in the influent.

It is composed of the following:

— peptone	192 mg
— meat extract	138 mg
— glucose monohydrate	19 mg
— ammonium chloride ( $\text{NH}_4\text{Cl}$ )	23 mg
— anhydrous potassium dihydrogenphosphate ( $\text{KH}_2\text{PO}_4$ )	16 mg
— disodium hydrogenphosphate dihydrate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ )	32 mg
— sodium hydrogen carbonate ( $\text{NaHCO}_3$ )	294 mg
— sodium chloride ( $\text{NaCl}$ )	60 mg
— iron(III) chloride hexahydrate ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ )	40 mg
— tap water (6.1)	1 l

It is strongly recommended to add the iron chloride solution separately and directly to the aeration vessel to prevent precipitation, especially if a concentrated solution is sterilized (8.3.1). For example, if a stock solution of 45 g/l iron(III) chloride hexahydrate is prepared, 5 ml should be added daily to the aeration vessel.

**6.3.4 Synthetic sewage 3**, which gives a mean DOC concentration of about 180 mg/l and a COD of about 470 mg/l in the influent.

The composition is specially balanced for nutrient-removal systems as described in Annex A, but it is equally usable in the standard test system. It is composed of the following (for more information, see References [4] and [5]):

— peptone	15 mg
— meat extract	15 mg
— potato starch	50 mg
— milk powder	120 mg
— glycerol	40 mg
— sodium acetate	120 mg
— urea	75 mg
— uric acid	9 mg

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— ammonium chloride (NH <sub>4</sub> Cl)		11 mg
— magnesium hydrogen phosphate trihydrate (MgHPO <sub>4</sub> · 3H <sub>2</sub> O)		25 mg
— tripotassiumphosphate trihydrate (K <sub>3</sub> PO <sub>4</sub> · 3H <sub>2</sub> O)		20 mg
— diatomaceous earth		10 mg
— lyophilised, powdered activated sludge		50 mg;
— natural (diet) fibres		80 mg
— linear alkylbenzene sulfonate (LAS)		10 mg
— alcohol ethoxylate C <sub>12</sub> to C <sub>14</sub> EO5 or any other easily biodegradable surfactant		10 mg
— ethylene diamine tetraacetic acid tetra sodium salt (Na <sub>4</sub> -EDTA)		0,29 mg
— trace elements:		
— CaCl <sub>2</sub>	5 mg	
— NaHCO <sub>3</sub>	25 mg	
— FeSO <sub>4</sub> · 7H <sub>2</sub> O	10 mg	
— CuCl <sub>2</sub> · 2H <sub>2</sub> O	0,48 mg	
— CoCl <sub>2</sub> · 6H <sub>2</sub> O	0,05 mg	
— ZnCl <sub>2</sub>	0,18 mg	
— MnSO <sub>4</sub> · H <sub>2</sub> O	0,1 mg	
— K <sub>2</sub> MoO <sub>4</sub>	0,020 mg	
— Cr(NO <sub>3</sub> ) <sub>3</sub> · 9H <sub>2</sub> O	0,68 mg	
— NiSO <sub>4</sub> · 6H <sub>2</sub> O	0,3 mg	
— Tap water		1 l

NOTE This medium contains surfactants and therefore might not be suitable for the determination of the biodegradability of surface-active agents.

**6.3.5 Domestic sewage**, fresh, settled, largely free from coarse particles and, if necessary, neutralized to (pH 7 ± 0,5).

Preferably use sewage (8.2) from the same plant as the sludge inoculum. Sewage can be stored for several days at about 4 °C if it has been proven that the DOC or COD does not significantly decrease during storage (for example, by less than about 20 % compared to the initial concentration). In order to limit disturbances to the activated sludge system, adjust each new batch to an appropriate constant value of, for example, 100 mg/l DOC or 300 mg/l COD by dilution with tap water.

**6.3.6 Modified organic medium**, a dilution of an organic medium (6.3.2 to 6.3.5) with tap water.

EXAMPLE If synthetic sewage 1 (6.3.2) is diluted 1:1, a DOC concentration in the influent of about 50 mg/l is obtained.

Domestic sewage of low acidity or alkalinity or synthetic sewage prepared from tap water of low acidity or alkalinity can require the addition of a suitable buffer to improve the biological processes, especially nitrification. A pH of about  $7,5 \pm 0,5$  in the aeration vessel during the test may be achieved, for example, by adding a buffer solution of 1 500 mg/l potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) to the synthetic sewage 1 (6.3.2). When and how much buffer is added shall usually be decided on an individual basis, depending on the acidity or alkalinity of the organic medium and the pH values measured in the aeration vessel.

**6.4 Test compound stock solution**, a solution of a suitable concentration, e.g. 5 g/l, of the test compound in tap water (6.1) or deionized water (6.2).

Check that diluting this solution with tap water to give the required test concentration does not produce a precipitate.

Determine the DOC and TOC of the stock solution. If the difference between the DOC and TOC is  $< 20\%$ , DOC can be used as analytical parameter. If the difference between the DOC and TOC is  $> 20\%$ , check that the test compound is completely water-soluble at the desired test concentration (8.3.2). It is recommended to repeat at least the DOC measurement for each new batch of the stock solution to ensure its correct preparation. Compare the DOC of the stock solution with the theoretical value to ascertain whether the analytical recovery is good enough (normally  $> 90\%$  can be expected). Ensure, especially for dispersions, whether or not the DOC can be used as an analytical parameter. For dispersions, centrifugation of the samples is required. If primary biodegradation shall be determined, check the test-compound concentration of the stock solution measured by specific analysis with the theoretical value.

Determine the pH of the stock solution<sup>[23]</sup>. Extreme pH values indicate that the test compound may have an influence on the pH of the activated sludge in the test system. In this case, neutralize the stock solution to get a pH value of, preferably,  $7 \pm 0,5$  with small amounts of inorganic acid or base, but avoid precipitation of the test compound. In the event of precipitate formation, use another pH range which yields no precipitate.

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## 7 Apparatus

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**7.1 Test system**, consisting, for one test compound, of a test unit and a control unit.

The test unit shall be either an activated-sludge plant (a so-called Husmann apparatus) or a porous pot (see Annex C). In both cases, storage vessels sufficiently large for the influent and the effluent are needed, as well as pumps to dose the influent. One control unit can be used for several test units. In the case of coupling (see Annex B), use one control unit for each test unit.

Each activated-sludge plant consists of an aeration vessel with a capacity of about 3 l for activated sludge and a separator (secondary clarifier) which holds about 1,5 l. Vessels of different size are permissible if they are operated with comparable hydraulic loads. If it is not possible to keep the test temperature in the test room in the desired range, use, for example, water-jacketed vessels with water at a controlled temperature. Use a dosing pump or a suitable air-lift pump to recycle the activated sludge from the separator to the aeration vessel, either continuously or intermittently. The use of a dosing pump allows the recycling of settled sludge to the sewage influent and then back to the aeration vessel, so that the settled sludge does not become anaerobic. The design of the air-lift pump alone does not allow this.

The porous-pot system consists of an inner, porous cylinder with a conical bottom suspended in a slightly larger vessel of the same shape, but made of impervious material. Separation of the sludge from the treated organic medium is made by differential passage through the porous wall. The effluent collects in the annular space from where it overflows into the collecting vessel. No settlement occurs and hence there is neither sludge return nor formation of anoxic zones. The whole system may be mounted in a thermostatically controlled room or water-bath. Porous pots can sometimes block and overflow in the initial stages of the test. In such a case, replace the blocked pot with a clean pot to which the sludge from the blocked pot has been added. Clean blocked pots by soaking them in dilute sodium hypochlorite solution, then in water, followed by thorough rinsing with water.

**NOTE** As material for the cylinder, porous polyethylene with a maximum pore size of 90  $\mu\text{m}$  and a thickness of 2 mm can be used.

For aeration of the sludge in the aeration vessels of both systems, suitable techniques, e.g. sintered cubes (diffuser stones) and compressed air, are required. The air shall be cleaned, if necessary, by passing through a suitable filter and washed. Sufficient air shall pass through the system to maintain aerobic conditions and the sludge flocs in suspension at all times during the test.

Neither test system fully mimics full-scale activated sludge plants, but both systems have shown their suitability for laboratory experiments for many years.

**7.2 Analytical equipment,** use laboratory carbon analyzer to determine DOC and TOC (see e.g. ISO 8245 [16] or equipment for COD determination (see e.g. ISO 6060 [14] and, if necessary, suitable equipment for substance-specific analyses or surfactants [2], [17], [18]. Equipment to determine suspended solids [25], pH [23], oxygen concentration in water [13], temperature, if required acidity and alkalinity and, if nitrification and denitrification are being investigated, ammonium [24], nitrite and nitrate [22].

### 7.3 Filtration apparatus or centrifuge.

**7.3.1 Device for filtration,** with membrane filters of suitable porosity (nominal pore size of 0,45 µm) which adsorb organic compounds and release organic carbon to an insignificant degree.

If filters are used which release organic carbon, wash them carefully with hot water to remove leachable organic carbon. Be aware that filters treated in this way might be very fragile, therefore centrifugation is generally recommended.

**7.3.2 Centrifuge,** suitable for operating at 40 000 m/s<sup>2</sup>.

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## 8 Procedure

### 8.1 General

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The procedure is described for the activated sludge plants (Husmann apparatus). It shall be adapted for the porous-pot system.

### 8.2 Preparation of the inoculum

Inoculate the test system at the beginning of the test with either activated sludge or an inoculum containing a low concentration of micro-organisms. Keep the inoculum aerated at room temperature until it is used and use it within 24 h.

In the first case, take a sample of activated sludge from the aeration vessel of an efficiently operated biological waste-water treatment plant (e.g. from the exit end of a plug-flow type) or from a laboratory treatment plant which receives predominantly domestic sewage.

Determine the concentration of suspended solids. If necessary, concentrate the sludge by settling so that the volume added to the test system is minimal. Ensure that the starting concentration in the aeration vessel is about 2,5 g/l dry matter.

In the second case, use 2 ml/l to 10 ml/l of effluent from a domestic biological waste water treatment plant as an inoculum. The activated sludge develops and grows in the test system. To get as many different species of bacteria as possible, it might be helpful to combine inocula from various sources. When a smaller inoculum is used, it usually takes longer to achieve sufficient sludge concentrations.

### 8.3 Performance of the test

#### 8.3.1 Dosage of organic medium

Assemble the test systems (7.1) in a controlled-temperature room (Clause 5) or use water-jacketted test units.