
**Soil quality — Guidance on laboratory
testing for biodegradation of organic
chemicals in soil under anaerobic
conditions**

*Qualité du sol — Lignes directrices relatives aux essais en laboratoire pour
la biodégradation de produits chimiques organiques dans le sol sous
conditions anaérobies*



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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.

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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 International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 15473 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

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Introduction

Organic chemicals can be introduced into the soil both intentionally and accidentally, after which they can degrade as a result of biological action. For chemicals which do degrade, the rate of degradation can vary considerably, depending not only on the molecular structure of the chemical, but also on soil conditions such as temperature, water and oxygen availability which influence microbial activity. The activity of microorganisms often plays a major role in degradative processes.

ISO 11266 [3] gives general guidelines for the selection and method of tests to determine the biodegradation of organic chemicals in soils under aerobic conditions.

It is necessary to have laboratory tests available to estimate the rate and extent of biodegradation under anaerobic conditions, and to assess the capability of soil to degrade organic chemicals under these conditions.

This International Standard gives guidance for the method of tests to determine the biodegradation of organic chemicals in soils under anaerobic conditions.

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Soil quality — Guidance on laboratory testing for biodegradation of organic chemicals in soil under anaerobic conditions

1 Scope

This International Standard gives guidance on the selection and method of appropriate tests for the determination of biodegradation of organic chemicals in soil samples under anaerobic conditions.

NOTE If the method is intended for tests in the framework of the registration of chemicals, an OECD Guideline on soil degradation [20] gives useful guidance on additional test requirements.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 10381-6:1993, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory*

ISO 10390:1994, *Soil quality — Determination of pH*

ISO 10694:1995, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

ISO 11260:1994, *Soil quality — Determination of effective cation exchange capacity and base saturation level using barium chloride solution*

ISO 11261:1995, *Soil quality — Determination of total nitrogen — Modified Kjeldahl method*

ISO 11271, *Soil quality — Determination of redox potential — Field method*

ISO 11274:1998, *Soil quality — Determination of the water retention characteristic — Laboratory methods*

ISO 11277:1998, *Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation*

ISO 14239:1997, *Soil quality — Laboratory incubation systems for measuring the mineralization of organic chemicals in soil under aerobic conditions*

3 Terms and definitions

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

3.1

biodegradation

molecular degradation of an organic substance resulting from the actions of living organisms

[ISO 11266]

3.2

primary biodegradation

the degradation of a substance to an extent sufficient to remove some characteristic property of the parent molecule. In practice this will be determined by analysis as a loss of parent compound or some specific function of the parent compound

[ISO 11266]

3.3

ultimate biodegradation

breakdown of an organic compound to carbon dioxide, methane, water, mineral salts of any other elements present, and products associated with the normal anaerobic processes of microorganisms

3.4

anaerobic transformation

reaction occurring under exclusion of oxygen (reducing conditions)

NOTE Such a reaction generally occurs when the redox potential (E_h) is less than 200 mV [17].

3.5

persistence

residence time of a chemical species in a specifically defined compartment of the environment

[ISO 11266]

3.6

DT-50

disappearance time 50

time taken for the concentration of a given compound to be reduced by 50 % of its original value

[ISO 11266]

3.7

DT-90

disappearance time 90

time taken for the concentration of a given compound to be reduced by 90 % of its original value

[ISO 11266]

3.8

bound residue

non-extractable residue

chemical species in soils originating from, for example, organic molecules that are not extracted by methods which do not significantly change the chemical nature of the residue

NOTE These non-extractable residues are considered to exclude fragments recycled through metabolic pathways leading to natural products [12].

3.9

soil

upper layer of the earth's crust composed of mineral parts, organic substances, water, air and living matter

[ISO 11074-1]

3.10**test substance**

chemical substance under investigation added to the test system

3.11**saturated soil**

that part of the soil which is completely saturated by water

4 Principle

Two appropriate test methods are described:

- a) incubation of a test compound in the soil under methanogenic conditions and monitoring its biodegradation;
- b) incubation of a test compound in the soil under water-logged conditions and monitoring its biodegradation.

The latter method simulates conditions under natural anaerobic circumstances, whereas the former method makes use of chemicals to induce a low redox potential in soil, and is the method of choice to measure the potential for degradation in soil under methanogenic conditions. In the water-logged soil method, the establishment of a low redox potential takes more time than under the methanogenic test conditions.

If the water-logged ("flooded") conditions are chosen, the soil will establish conditions depending on the nature of the soil. Such conditions can be nitrate-reducing (450 mV to 200 mV, pH 7), Fe-reducing (+ 150 mV to – 100 mV, pH 7), or sulfate-reducing (– 50 mV to – 200 mV, pH 7). If "methanogenic" conditions are chosen, the redox potential will be less than – 200 mV.

The water-logging method is more appropriate for aerobic soils that may be transiently anaerobic. The methanogenic conditions are more appropriate for organic marsh (permanently flooded) surface soils, soils of landfills and sludge-amended soils.

NOTE Organic soils containing easily degradable organic matter may eventually achieve methanogenic conditions under water-logged test conditions.

After addition of the test compound to a selected soil (5.1), biodegradation is measured under anaerobic conditions by following the production of carbon dioxide, methane and other volatiles. If such volatile compounds have to be determined, the use of ^{14}C (radioactive) substances is highly recommended. The disappearance of the test compound can also be followed by substance-specific analysis.

It is also possible to use radio-labelled compounds to determine the rate of disappearance of the test compound and the formation of metabolites and bound non-extractable residues. The metabolites can be identified using appropriate analytical methods.

5 Materials**5.1 Soil****5.1.1 Selection and sampling**

If practical, soils selected for testing should come directly from the site where chemical contact is anticipated. However, if it is not possible to obtain samples owing to contamination which has already been introduced, the soil selected should have properties as close as possible to the contaminated soil.

The field history of the soil used should be considered, and recent amendments (e.g. pesticide applications) and tillage practices noted. Precise data should be provided on the sampling site, its location, its status of aeration (e.g. colour, water content, smell), the presence of plants or previous crops, the date of removal of the sample from the field, and the sampling depth.

5.1.2 Soil characteristics

A knowledge of soil characteristics is essential for full interpretation of the results of the study. It is therefore recommended that at least the following tests be performed on the selected soil:

a) physical properties:

- 1) particle size analysis in accordance with ISO 11277;
- 2) field water content by an appropriate method;
- 3) total water-holding capacity and/or water-retention characteristics in accordance with ISO 11274;

b) chemical properties:

- 1) pH of the soil in accordance with ISO 10390, or the pH in KCl or CaCl₂ solution;
- 2) organic matter content in accordance with ISO 10694;
- 3) cation exchange capacity (CEC) in accordance with ISO 11260;
- 4) nitrogen content in accordance with ISO 11261;
- 5) redox potential in accordance with ISO 11271;

c) biological properties:

It may be useful to determine the microbial biomass of soil. This should be done by an appropriate method, e.g. the substrate-induced respiration method [4]. However, if anaerobic biodegradation prevails in the collected soil, the fumigation method [5] should be used.

5.2 Test substance

Ideally, substances to be tested should be pure compounds (chemical purity > 95 % mass fraction). The influence of any carriers or formulation ingredients should also be considered.

The following data on compounds are important for the interpretation of results:

- name (IUPAC);
- structure;
- relative molecular mass;
- data on purity and the chemical nature of major impurities;
- stability in water and in organic solvents;
- solubility in water;
- vapour pressure;
- octanol/water partition coefficient;
- sorption constant;
- acid dissociation constant;

- for radio-labelled chemicals:
 - the nature and position of the label;
 - specific activity;
 - radiochemical purity.

NOTE The results of studies using radio-labelled materials depend on the position of the radio-label. Therefore the labelling positions within the molecular structure need careful consideration.

5.3 Glassware and apparatus

General laboratory equipment and glassware, in particular the following.

- 5.3.1 Round-bottomed flask** (of about 250 ml and 500 ml).
- 5.3.2 Ice bath.**
- 5.3.3 Column** containing reduced copper.
- 5.3.4 Gassing line** with syringes and gassing needles.
- 5.3.5 Glass tubes or flasks** with butyl rubber stoppers.
- 5.3.6 Pipettes** with PVC tubes of internal diameter 0,5 mm to 1 mm.
- 5.3.7 Gas-tight syringes** (10 ml, 20 ml, 50 ml and 100 ml).
- 5.3.8 Apparatus and electrodes** to measure redox potentials.

In addition, for studies with ^{14}C -labelled test materials:

- 5.3.9 Scintillation cocktails.**
- 5.3.10 Liquid scintillation counter.**
- 5.3.11 Scintillation vials.**

5.4 Reagents

All chemicals used should be of analytical grade.

- 5.4.1 Oxygen-free nitrogen, helium (pure) or argon.**
- 5.4.2 Titanium(III) chloride.**
- 5.4.3 Sodium citrate.**
- 5.4.4 Potassium dihydrogenphosphate** (KH_2PO_4).
- 5.4.5 Disodium hydrogenphosphate dihydrate** ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$).
- 5.4.6 Sodium carbonate.**

5.4.7 Titanium(III) citrate.

Prepare the titanium citrate as follows. Neutralize 5 ml of titanium(III) chloride (15 %) (5.4.2) in 50 ml of 0,2 mol/l sodium citrate (5.4.3) solution with saturated sodium carbonate (5.4.6) solution, filter if necessary, and store under oxygen-free gas.

6 Collection, handling and storage of soil

If soils in which aerobic processes prevail are collected, it is important that ISO 10381-6 be followed to ensure that viability of soil microorganisms is maximized during sampling. If soils are collected in which anaerobic biodegradation prevails, the sampling, handling and storage should minimize exposure of the soil samples to oxygen.

7 Procedure

7.1 Addition of test substance

The concentration to be used in the test depends on the experimental objectives (e.g. concentration to be expected in the field situation), and the test substance shall be applied either:

- to the soil; or
- to the aqueous phase covering the soil.

Anaerobic conditions shall be established in the test system before the test substance is applied to the water phase.

The test substance may be added in a number of ways:

- a) as an aqueous solution (depending on the solubility in water);
- b) dissolved in organic, water-miscible solvents (depending on the solubility in the solvent). The amount of solvent should be restricted to the minimum (< 1 %) necessary for the application of the compound. The possible toxicity and biodegradability of the solvent should be taken into account;
- c) as a solid, e.g. coated on quartz sand (prior to mixing with the soil).

Care should be taken to avoid adding the test substance at toxic levels. Compounds which are toxic, or have inhibitory effects on soil microorganisms at the applied concentration, interfere with the determination of biodegradability.

7.2 Incubation under methanogenic conditions

7.2.1 Preparation of oxygen-free incubation medium

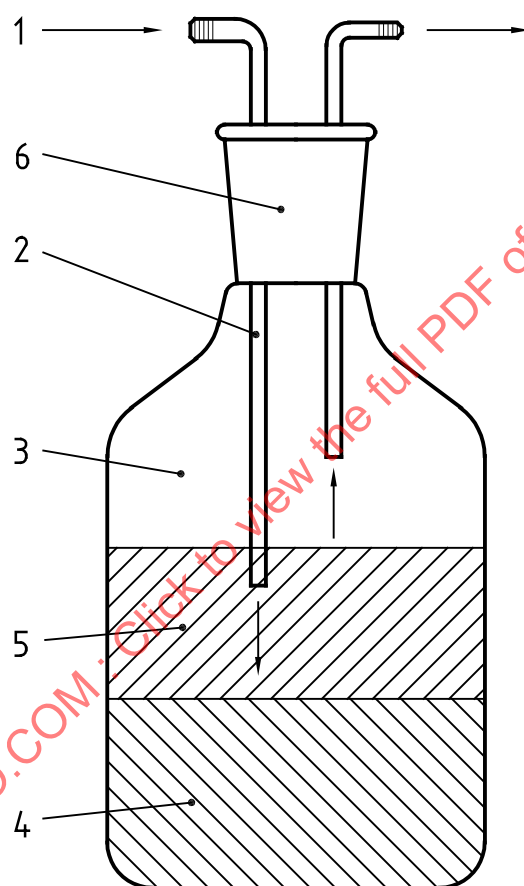
Place approximately 100 ml of water into a 250 ml or 500 ml round-bottomed flask (5.3.1), put the gassing needle (5.3.4) into the water and flush it with a small stream of O₂-free nitrogen or helium (5.4.1) which has passed a column of heated copper (350 °C) (5.3.3). Boil the water vigorously under continuous gassing with the oxygen-free gas for about 1 min. The water can then be kept anaerobic by replacing the vapour with oxygen-free nitrogen or helium as the container cools in ice. After cooling, titanium(III) citrate (5.4.7) $c = 0,8 \text{ mmol/l}$, KH₂PO₄ (5.4.4) $\rho = 0,27 \text{ g/l}$ and Na₂HPO₄ · 2H₂O (5.4.5) $\rho = 0,56 \text{ g/l}$, are added and dissolved (hereafter referred to as "oxygen-free buffer") [7].

7.2.2 Incubation system

The incubation system used should ensure that anaerobic conditions are maintained.

A rather small number of systems is available [7, 8, 9, 13, 14, 16, 18].

The system described in this International Standard consists of a glass flask (250 ml) (5.3.5) and a gassing system with syringes and gassing needles (5.3.4). See for example the system in Figure 1. When tubing is used this should be impermeable for gases (e.g. thick butyl rubber). The flask can be closed with a rubber stopper. However, any other appropriate system can be used as well, provided that it follows the guidance of this International Standard.



Key

- 1 Oxygen-free nitrogen
- 2 Glass tube or needle
- 3 Flask
- 4 Soil
- 5 Buffer
- 6 Stopper

Figure 1 — Example of an incubation system for anaerobic degradation studies in soil

If volatile substances such as $^{14}\text{CO}_2$ have to be measured, flow-through or biometer incubation systems as described in ISO 14239 should be used. In the flow-through system an inert gas should be used. The system in Figure 1 is suitable as a flow-through system.

7.2.3 Incubation

7.2.3.1 Preparation

Divide the soil into aliquots of at least 40 g (dry mass equivalent) and place them in incubation flasks (5.3.5). Transfer enough of the prepared oxygen-free buffer (but at least approx. 1 cm of liquid should be above the soil surface) into each flask containing soil, which shall be flushed continuously during preparation with an oxygen-free stream of inert gas via a gassing needle. The buffer should be transferred via pipettes fitted with a long, thin PVC tube (5.3.6; see also [7]). Immediately after removing the needle, close the flask with a butyl rubber stopper. Pierce the stopper by a needle attached to an airtight syringe to prevent excess pressure and to allow the withdrawal of gas samples for analyses, or connect to tubing as shown in Figure 1.

Generally at least two replicates per sampling point should be incubated. However, increasing the number of replicates increases the precision of the test.

Controls should be run simultaneously and should contain soil plus the amount of buffer which was used for the application of the test material in the treated samples.

Soil without any additives may be used as an additional control.

7.2.3.2 Incubation conditions

7.2.3.2.1 Maintaining anaerobicity

Anaerobic biodegradation in soils is considered to prevail if the redox potential is less than about 200 mV. For proof of anaerobicity, see 7.2.3.3.

7.2.3.2.2 Temperature

The incubation temperature should be selected according to the specific goals of the study. In general, maximum microbial activity in soil is found between 25 °C and 35 °C. For soils from temperate zones, a temperature between 10 °C and 25 °C is adequate and more representative of natural conditions.

The minimum and maximum temperatures should be measured and recorded at regular intervals throughout the incubation and should not vary by more than ± 2 °C.

7.2.3.2.3 Illumination

The test should be conducted in the dark.

7.2.3.3 Proof of anaerobicity

Check the anaerobicity occasionally during the study by measuring the redox potential in accordance with ISO 11271.

At the end of the test, measure dissolved Fe, dissolved Mn and $\text{NH}_4^+/\text{NO}_2^-/\text{NO}_3^-$ concentrations.

7.2.3.4 Test duration

There is no minimum duration recommended for a test but, as microbial activity in soil decreases during longer incubation periods, it is recommended that tests not be continued for longer than 100 days.

7.2.3.5 Sampling

Sampling should be carried out at regular intervals during the incubation period, the frequency depending on the duration of the test and the rate of biodegradation of the test substance. At least five sampling points are required to establish a degradation curve. Where materials degrade rapidly during the early stages of incubation, the

following sampling frequency is recommended: 0, 2, 4, 8, 16, 40 and 100 days after application of the test substance. For destructive sampling methods, e.g. direct analysis of the soil, it is recommended that the entire contents of an individual incubation flask be tested.

7.3 Incubation under water-logged conditions

It is common practice to cover soil with water (approx. 1 cm above the soil) to reach anaerobic conditions in the soil [19, 20].

Proceed as under 7.2.1 to 7.2.3.5, but instead of using buffer (7.2.3), use water.

This water-logged system has a much higher redox potential value than the system treated with oxygen-free buffer (7.2). Normally the water-logged system has a positive redox potential value, while the oxygen-free-buffered system has a negative value.

7.4 Degradation monitoring

7.4.1 General

The type of analyses chosen to monitor the degradation process will depend on the aims of the study and whether primary and/or ultimate biodegradation data are required.

The analyses are dependent on the chemical itself and whether a radio-labelled compound has been used.

In general the following analyses should be considered:

- a) For primary degradation (unlabelled substances):
 - loss of parent compound.
- b) For metabolism, including ultimate degradation (labelled substances):
 - determination of volatile compounds, both parent compound and metabolites;
 - determination of water/or solvent extractables;
 - determination of "bound", non-extractable residues.

For determination of extractable materials, solvents which do not alter the parent compound or its metabolites should be used. Care should be taken to follow extraction procedures which will remove as much of the extractable material as possible. The analysis of metabolites and parent compound may be performed using thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC).

7.4.2 Measurement of $^{14}\text{CO}_2$ production

$^{14}\text{CO}_2$ production can be determined using methods specified in ISO 14239.

7.4.3 Measurement of $^{14}\text{CH}_4$ production

$^{14}\text{CH}_4$ production can be determined via oxidation to $^{14}\text{CO}_2$ using copper oxide. Before carrying out this procedure, means should be provided to trap by soda lime the natural CO_2 produced in this experiment.

8 Expression of results

All data should be presented in tabular and graphic form (degradation curve).

DT-50 and DT-90 values should be calculated using, for example, the models described in [15] or other appropriate models.

Additional useful information includes the determination of volatile compounds, formation and persistence of metabolites and non-extractable residues.

NOTE If no biodegradation is observed, the likely reasons are that:

- a) the test substance is toxic;
- b) the test substance does not biodegrade;
- c) the microbial activity of the soil is zero;
- d) the test substance is not bioavailable (because of adsorption or volatilization).

9 Test report

The test report should include the following information:

- a) data on the test substance (see 5.2);
- b) data on the soils used (see 5.1);
- c) data on the test procedure, test method used, concentrations used, methods of application, data on performance of the test, proof of anaerobicity, sampling data etc. (see clause 5);
- d) data on the analytical methods used, e.g. detection limits, quality control procedures, reference substances analysed;
- e) raw data on the results of the analysis;
- f) if ^{14}C -labelled test substance is used, a mass balance (optional);
- g) evaluation and conclusions of the evaluation.