
**Soil quality — Determination of potential
nitrification and inhibition of
nitrification — Rapid test by ammonium
oxidation**

*Qualité du sol — Détermination de la nitrification potentielle et inhibition
de la nitrification — Essai rapide par oxydation de l'ammonium*

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Published in Switzerland

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ISO 15685 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

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Soil quality — Determination of potential nitrification and inhibition of nitrification — Rapid test by ammonium oxidation

1 Scope

This International Standard specifies a rapid method for the determination of the potential rate of ammonium oxidation and inhibition of nitrification in soils. This method is suitable for all soils containing a population of nitrifying microorganisms. It can be used as a rapid screening test for monitoring soil quality and quality of wastes, and is suitable for testing the effects of cultivation methods, chemical substances and pollution in soils.

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 11465:1993, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

3 Terms and definitions

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

3.1 inhibitory dose ID

amount of a chemical added to soil that effectively inhibits biological activity by a stated percentage after a given time, in comparison to an untreated control

NOTE It is expressed as a percentage. For example, ID25 and ID50 indicate 25 % and 50 % inhibition of biological activity, respectively.

3.2 biosolid

organic fertiliser used in agriculture, including sewage sludge, compost, manure and industrial products

4 Principle

Ammonium oxidation, the first step in autotrophic nitrification in soil, is used to assess the potential activity of microbial nitrifying populations. Autotrophic ammonium-oxidizing bacteria are exposed to ammonium sulfate in a soil slurry buffered at pH 7,2. Chemical test substances, extracts of biosolids, as well as samples of polluted soils can be added to the slurry at various concentrations. Oxidation of the nitrite formed by nitrite-oxidizing bacteria in the slurry is inhibited by the addition of sodium chlorate. The subsequent accumulation of nitrite is measured over a 6-h incubation period, and is taken as an estimate of the potential activity of ammonium-oxidizing bacteria. As the generation time of ammonia-oxidizing bacteria is long (> 10 h), the method provides a measure of the potential activity of the nitrifying population at the time of sampling. It does not measure growth of the nitrifying population.

Nitrite can be measured quickly and accurately and hence the method is fast, accurate, reproducible and inexpensive.

Substances known to be active at $\text{pH} < 7,2$ should be added before starting the test in due time to allow substances to exhibit their effects on nitrifying bacteria.

5 Reagents

5.1 Distilled water.

5.2 Potassium dihydrogen phosphate, $c(\text{KH}_2\text{PO}_4) = 0,2 \text{ mol/l}$.

5.3 Dipotassium hydrogen phosphate, $c(\text{K}_2\text{HPO}_4) = 0,2 \text{ mol/l}$.

5.4 Sodium chlorate, $c(\text{NaClO}_3) = 0,5 \text{ mol/l}$.

5.5 Diammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$.

5.6 Sodium hydrogen carbonate, $c(\text{NaHCO}_3) = 5 \text{ mmol/l}$.

5.7 Potassium chloride, $c(\text{KCl}) = 4 \text{ mol/l}$.

5.8 Stock solution A.

Prepare stock solution A by combining the following:

KH_2PO_4 (5.2)	28 ml
K_2HPO_4 (5.3)	72 ml
Distilled water (5.1)	100 ml

5.9 Test medium.

Prepare test medium by combining the following:

Stock solution A (5.8)	10 ml
Sodium chlorate, NaClO_3 (5.4)	10 ml to 30 ml
Diammonium sulfate, $(\text{NH}_4)_2\text{SO}_4$ (5.5)	0,198 g

Dilute to 1 000 ml with distilled water (5.1).

The final medium contains 1 mmol/l of potassium phosphate buffer, 5 mmol/l to 15 mmol/l of sodium chlorate and 1,5 mmol/l of diammonium sulfate, and has a pH of approximately 7,2.

The sodium chlorate concentration selected within the range given should be sufficient for effective inhibition of biological nitrate formation, but at the same time not have negative effects on ammonium oxidation. If necessary, test the influence of sodium chlorate concentration.

Test chemicals to be added to the test medium shall be dissolved in the phosphate buffer described and added before diluting to 1 l. For test chemicals with low water-solubility, prepare solutions by mechanical dispersion or by use of vehicles such as organic solvents, emulsifiers or dispersants of low toxicity to ammonium-oxidizing bacteria. The concentration of a vehicle should not exceed 100 mg/l in the final medium.

Add extracts of biosolids, when tested, separately to the test medium before dilution to 1 l.

When a carbon source is needed, e.g. in testing of biosolids with high nitrate levels, add 10 ml of NaHCO_3 (5.6) to the test medium before diluting to 1 l.

6 Apparatus

6.1 Ordinary laboratory equipment.

6.2 Orbital shaking incubator, thermostatically controlled.

7 Sampling, storage and characterization of samples

Guidance on sampling, sample processing and storage is given in ISO 10381-6. For testing of chemicals or biosolids, or for mixing tests with polluted soils, experimental soil with ammonium-oxidizing activity of between 200 ng N/g dry mass/h to 800 ng N/g dry mass/h determined from preliminary experimentation should be chosen.

Samples of experimental soils can usually be stored in a refrigerator at approximately 4 °C for periods of up to three months prior to use (see ISO 10381-6). Some soils can only be kept in the refrigerator for up to two weeks. Storage of soil samples in a freezer (−20 °C) is not generally recommended.

It has been shown for a number of soils from temperate climates that storage at −20 °C for up to 12 months does not inhibit ammonium oxidation activity^[3]. Where such information is available for a particular soil, storage under such conditions is permissible.

Measurement of the following variables is recommended for characterization of the soil (see Bibliography):

- particle size distribution (ISO 11277);
- water content (ISO 11465);
- water-holding capacity (ISO 14238:1997, Annex A);
- pH (ISO 10390);
- effective cation-exchange capacity (ISO 11260);
- organic matter content (ISO 10694);
- total nitrogen content (ISO 11261).

8 Procedure

8.1 Testing soils

8.1.1 General

The test design should include at least three replicates of approximately 25 g of moist soil. Determine gravimetrically the water content of the soil for calculation of dry mass according to ISO 11465.

8.1.2 Initial incubation

Mix soil samples or waste materials with test medium (5.9) to form slurries. The volume of test medium should be adjusted to give a precise total liquid volume, e.g. 100 ml. Calculate the volume of medium to be added by subtracting the volume of water in the initial soil or waste sample from the desired liquid volume, e.g. 100 ml. Incubate the slurries in 250 ml flasks standing upright on an orbital shaking incubator (6.1), thermostatically controlled to 25 °C ± 2 °C. Rotation should be sufficient to keep solids suspended.

A volume of liquid greater than 100 ml is required in the slurry if the water-holding capacity of the soil is > 200 % (organic soils).

8.1.3 Sampling of soil slurry

Take samples (2 ml) of the soil slurry after 2 h and 6 h of incubation, provided that ammonium oxidation is known to be linear over this period. The soil slurry should be well shaken at sampling times to ensure that the ratio of solution/soil is constant during the test. Dispense samples into test tubes and add 2 ml of KCl (5.7) to stop the ammonium oxidation. Then centrifuge the samples at e.g. 3 000 *g* for 2 min, or filter. Filter paper should be of high filtration speed, while its chemical purity may be less than the highest grade. Determine nitrite by a suitable method of chemical analysis (see ISO 14256-2). At this stage the solutions can be stored in a refrigerator (4 °C to 8 °C) but analysis should be carried out within 24 h.

If necessary, check the linearity of the ammonium oxidation over time by sampling soil slurry a number of times during the 6 h of incubation. This is likely to be necessary if laboratories are not familiar with the soil types being used in the test. Some cases of non-linearity can be corrected by ensuring aerobic conditions or supplying a carbon source.

If the method of nitrite analysis requires larger volumes of sample solution (e.g. where activity is low and only small amounts of nitrite are formed), it may be difficult to maintain a constant solution/sample ratio over the duration of the test. In such cases, it is recommended to prepare a number of replicate soil slurries and terminate replicates at different sampling times. This procedure also allows reduced sample volumes, e.g. a total liquid volume of 10 ml with 2,5 g of moist soil.

NOTE When necessary to ensure aerobic conditions, bottles containing the soil slurries may be closed and the head space flushed with pure oxygen to provide sufficient amounts of oxygen during the test period.

8.2 Testing the effect of chemicals

Determine ammonium oxidation according to the procedure for soils with test chemicals added to the test medium (5.9) at different concentrations. Include controls with no added test chemical in the test design as reference. The number of replicates of the control should be twice the number of replicates chosen for each concentration of test chemical.

Conduct a preliminary range-finding test to select concentrations of the test chemical. In the main test, arrange at least five different concentrations in a geometric progression. The lowest concentration tested should have no effect on ammonium oxidation and the highest should inhibit by 50 % to 100 %. pH may have to be adjusted.

When a vehicle for dissolution of chemical is used, it should not exceed 100 mg/l in the test medium. Its effect on ammonium oxidation should be tested by having additional controls containing the vehicle at the highest concentration used in the test.

NOTE Volatile test substances require special arrangements which are not within the scope of this International Standard.

8.3 Testing of polluted soils

The most straightforward method is to compare the polluted soil with an unpolluted reference soil with comparable soil properties, or to make gradient studies on soil samples taken at various levels of pollution. Take a number of replicate samples from each soil.

In the absence of a suitable unpolluted soil for reference, or in cases of low potential nitrification activity, the following test procedure is recommended. Preincubate the polluted soil and an unpolluted soil of known nitrification activity at 200 ng N/g dry mass of soil/h to 800 ng N/g dry mass of soil/h at a water content of 40 % to 60 % of water-holding capacity or $-0,01$ MPa to $-0,03$ MPa suction pressure for two days at 20 °C in darkness. Mix moist amounts of the two soils comparable to 75 g of dry mass in a 1:1 ratio on a dry mass basis. Store the mixture, the polluted soil and the control soil for one day at 20 °C in darkness. Measure the potential ammonium oxidation as described in 8.1 using four replicates of the mixture and of each soil.

8.4 Testing of water extracts of biosolids

The effect on soil nitrification by extracts of biosolids, e.g. sewage sludge, can be tested. Prepare water extracts by 24 h extraction with demineralized water at a ratio of 1:10 [g biosolid (dry mass):ml water] and pH 7,5. Separate the aqueous phase from the particles by centrifugation, e.g. at 1 300 *g* for 1 h. Test the effect of the extract on soil in a concentration series, including controls with no extract. Follow the test procedure given in 8.1, adding extract of biosolid just before addition of test medium. Include extract samples without soil in the test design for subtraction of ammonium oxidation by nitrifying bacteria in the extracts.

9 Calculations

Calculate the rate of ammonium oxidation (ng NO₂-N/g dry mass of soil/h) from the difference between NO₂-N concentrations at different measuring times.

Calculate the inhibition of ammonium oxidation activity by test chemical or extract of biosolid as a change in the activity in reference soil, expressed as a percentage.

In mixtures of polluted and unpolluted soils, the polluted soil is considered toxic if the mixture shows ammonium oxidation significantly lower than 90 % of the mean activity of the two soils kept separate. Using four replicates, this is calculated using Equation (1):

$$\overline{A}_m + s_m < 0,9 \times \frac{(\overline{A}_c + \overline{A}_p)}{2} \quad (1)$$

where

\overline{A}_m is the mean ammonium oxidation activity in soil mixture;

s_m is the standard deviation of ammonium oxidation activity in soil mixture;

\overline{A}_c is the mean ammonium oxidation activity in control soil;

\overline{A}_p is the mean ammonium oxidation activity in polluted soil.

10 Test report

The following information should be reported in all cases:

- soil characteristics;
- rate of ammonium oxidation (ng NO₂-N/g dry mass of soil/h): individual values, mean values and standard deviations for each treatment and soil;
- test of reaction linearity: if reaction linearity was tested at three sampling times or more, provide a plot of nitrite-N concentration versus time;
- tests with added chemicals: graphical representation of inhibition of ammonium oxidation versus concentration of the tested chemical. Indicate inhibitory dose values ID25 and ID50 in the diagram (see Clause 3);
- test report for polluted soil in soil mixtures: qualitative classification of polluted soils as toxic or non-toxic (see Annex A);
- test report for extracts of biosolids: graphical representation of the concentration-effect relationship of added water extracts from biosolids, including estimation of inhibitory dose values ID25 and ID50.

Annex A (informative)

Results of a laboratory ring test

A laboratory ring test using four contaminated soils and a reference soil (see Table A.1) was carried out in Germany. In addition to determination of ammonium oxidation rates (see Table A.2), a toxicity test using mixtures (1:1) of the reference soil and the polluted soils was performed according to the procedure described in 8.3 (see Table A.3). Extreme values were eliminated in accordance with DIN 38402-42^[6].

Only soils with ammonium oxidation activity in the range recommended for experimental soil (Clause 7), i. e. the reference soil and soil 1, exhibited moderately low coefficients of variation in interlaboratory comparisons (Table A.2). However, a qualitative toxicity assessment using the soil mixing procedure of 8.3 showed good agreement between laboratories for all soils, including soil of low activity (Table A.3).

Table A.1 — Chemical and physical properties of the soils and their contamination

Reference soil		Soil 1	Soil 2	Soil 3	Soil 4
Textural class	Silty sand	loam	sand	sand	sand
WHC _{max} , ml/kg	290,0	511,8	347,7	241,8	268,5
pH	6,6	7,3	7,3	7,5	5,6
C _{org} , %	2,1	4,7	8,5	5,9	1,7
N, %	0,16	0,38	0,27	0,20	0,16
∑ Nitrotoluene, mg/kg	n.d.	336,3	n.d.	n.d.	n.d.
PAH, mg/kg	n.d.	77,0	60	21	2 268
Mineral oil, mg/kg	n.d.	< 50,0	43 660,1	8 798,9	< 50
As, mg/kg	n.d.	5,4	3,6	12,1	< 3
Cd, mg/kg	n.d.	6,4	29,6	9,3	< 3
Cr, mg/kg	n.d.	65,9	2 005,1	320,3	94,9
Cu, mg/kg	n.d.	104,4	10 921,3	367,8	10,0
Ni, mg/kg	n.d.	29,2	5 894,2	578,5	6,5
Pb, mg/kg	n.d.	820,8	1 082,2	547,5	10,3
Zn, mg/kg	n.d.	764,7	5 065,3	577,6	26,6
NOTE n.d. = not determined.					

Table A.2 — Ammonium oxidation rates

Soils	Reference	Soil 1	Soil 2	Soil 3	Soil 4
Mean value, µg NO ₂ -N/g h	0,54	0,35	0,08	0,01	0,01
<i>s</i>	0,09	0,14	0,12	0,02	0,01
CV, %	17,2	39,5	153,3	142,6	135,7
Number of participating laboratories	23	16	15	16	16
Number of laboratories considered	22	16	14	15	12

Table A.3 — Assessment of toxicity of contaminated soils using soil mixtures with the reference soil according to 8.3 [toxicity criteria: see Equation (1) in Clause 9]

Laboratory	Soil 1	Soil 2	Soil 3	Soil 4
6	/	/	/	/
19	—	+	+	—
23	+	+	+	+
25	—	+	+	+
26	—	+	+	+
28	—	/	+	+
30	—	+	+	—
35	—	+	+	—
41	—	+	+	+
45	/	/	/	/
46	—	+	+	+
49	—	+	+	+
51	—	+	+	+
55	—	+	+	—
57	—	+	+	+
58	—	+	+	+
60	+	+	+	+
Toxic (%)	13,3	100	100	73,3
NOTE + = toxic; — = not toxic; / = not determined.				