INTERNATIONAL STANDARD

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Water quality — Toxicity test for assessing the inhibition of nitrification of activated sludge microorganisms

Qualité de l'eau — Essai de toxicité pour l'évaluation de l'inhibition de la nitrification des micro-organismes des boues activées

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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 9509 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 9509:1989), which has been technically revised.

Introduction

Nitrification is an important process in the treatment of waste waters, since it is necessary to reduce the polluting effects of ammonium in treated discharges to receiving waters. It is further necessary to convert ammonium to nitrate in order to allow the subsequent process of denitrification (producing nitrogen gas) in the anoxic stage of the modified activated sludge process, thus considerably reducing the potential for eutrophication in the receiving waters. The nitrification process is generally performed by two separate groups of autotrophic bacterial species This International Standard describes a method for assessing the inhibition of the production of oxidized nitrogen (nitrite plus nitrate), or of the removal of ammonium, by nitrifying activated sludge.

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WARNING — Sewage and activated sludge contain potentially pathogenic organisms. Appropriate precautions are necessary when handling them.

Toxic test substances and those with unknown properties are to be handled with care.

Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted according to this standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for assessing the short-term inhibitory effect of waters, waste waters or test substances on nitrifying bacteria in activated sludge. The inhibitory effect is estimated over an exposure period of usually 3 h or up to 24 h with weakly nitrifying sludge.

The method is applicable to nitrifying activated sludge derived from domestic and synthetic sewage and also to sludges from industrial and mixed domestic and industrial waste waters.

The nitrifying activity of the sludge is verified by testing in the presence and absence of a specific inhibitor (e.g. *N*-allylthiourea; see Annex A). If the nitrification rate is within a suitable range for the test, i.e. 2 mg of nitrogen per gram of suspended solid and hour to 6,5 mg of nitrogen per gram of suspended solids and hour, the sludge may be used directly. If not, adjustments are necessary (see Clause 9).

The method is applicable to water-soluble, non-volatile chemicals, and to waste waters

Sludges from different sources respond differently to a given concentration of an inhibitor mainly due to reaction between the inhibitor and components of the sludge. This results in a partial neutralisation of the toxic effect. Also, since the test lasts only hours, any inhibitory effects may diminish or increase over a longer period, e.g. in the continuous activated sludge system (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 — Guidance on biotesting of samples*

ISO 6777, *Water quality — Determination of nitrite — Molecular absorption spectrometric method*

ISO 7150-1, *Water quality — Determination of ammonium — Part 1: Manual spectrometric method*

ISO 11733, *Water quality — Determination of the elimination and biodegradability of organic compounds in an aqueous medium — Activated sludge simulation test*

3 Terms and definitions

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

3.1
nitrification
oxidation of ammonium compounds by bacteria

NOTE Usually the intermediate product is nitrite and the end product nitrate

[ISO 6107-1:2004, 49]

3.2
test material
pure chemicals, clearly defined mixtures of chemicals, chemical products, waste waters and treated waste waters

3.3
activated sludge
accumulated biological mass (floc) produced in the treatment of waste water by the growth of bacteria and other microorganisms in the presence of dissolved oxygen

[ISO 6107-1:2004, 2]

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

[ISO 9888:1999, 3.4]

3.5
toxic range
range of concentration of a test material over which 0 % to 100 % inhibition occurs

[ISO 8192:—[1)]]

3.6
EC_{50}
effective concentration of the test material giving a calculated or interpolated inhibition of nitrification of 50 %, compared with a blank control

4 Principle

The percentage inhibition of nitrification by various concentrations of the test material is calculated by assessing the difference in concentration of oxidized nitrogen (nitrite plus nitrate) produced, or of ammonium utilized, under standard conditions by the oxidation of ammonium salts after the parallel aeration of a nitrifying sludge in the presence and absence of test material.

1) To be published. (Revision of ISO 8192:1986)

5 Reagents and materials

5.1 Deionized water, for the preparation of defined stock solutions. For washing procedures, tap water is suitable as well.

Make sure that the water is free from chemicals which may inhibit nitrification processes (e.g. Cu^{2+} ions).

5.2 Nitrifying activated sludge.

Collect a sufficient volume of a nitrifying activated sludge from a local waste water treatment plant, or from a laboratory-grown sludge (see Annex C), in which nitrification is known to be occurring. According to the purpose of the test, sludge may be collected from plants treating predominantly domestic sewage, mixed domestic industrial waste water or solely industrial waste water; the source of the sludge and the treated waste water should be reported since the results of the test often depend of the origin of the sludge used (see Reference [6]). Maintain the sludge in an aerobic condition. Since the toxicity to nitrification may change with time of storage (see Reference [1]), assessments should be made as soon as possible after collection and preferably within 24 h (see ISO 5667-16).

Instead of using activated sludge from a waste water plant, nitrifying sludge can be grown in the laboratory (see Annex C).

Although the sludge may be used as collected, it is preferable to wash the sludge to remove any inhibitors and nitrate present, before re-suspending in chlorine-free, nitrate-free tap water. This washing procedure may be carried out by centrifuging or settling and is optional. Centrifuge (e.g. 10 000 min^{-1} for 5 min) or settle the sludge and discard the supernatant liquid. Wash the residue with a volume of tap water equal to the original volume, re-centrifuge or settle and again discard the supernatant liquid. Finally, re-suspend the centrifuged or settled sludge in an appropriate volume of tap water to give the required concentration of mixed liquor suspended solids (e.g. 3 g/l) and aerate until use.

5.3 Full medium.

Dissolve 5,04 g of sodium hydrogen carbonate, $NaHCO_3$, and 2,65 g of ammonium sulfate, $(NH_4)_2SO_4$, in 1 l of water (5.1).

NOTE This medium, when diluted 1:10 (1 + 9) with water (5.1), contains 56 mg of nitrogen per litre and has a pH value of about 7,6. It allows the production of at least 25 mg/l of oxidized nitrogen without changing the pH value.

5.4 Medium for waste water samples

5.4.1 Medium A.

Dissolve 10,08 g of sodium hydrogen carbonate, $NaHCO_3$, in 1 l of water (5.1).

5.4.2 Medium B.

Dissolve 5,3 g of ammonium sulfate, $(NH_4)_2SO_4$, in 1 l of water (5.1).

5.5 Reference inhibitor.

Dissolve 1,16 g of *N*-allylthiourea (ATU) in 1 l of water (5.1).

Other inhibitors may be used as well, e.g. 2-chloro-6-(trichloromethyl)pyridine, but the concentration required and mode of addition should be investigated in advance.

5.6 Stock solution of test substance.

Prepare a stock solution or suspension of the test substance in distilled water (5.1) at a suitable concentration, e.g. 1 g/l or 10 g/l.

If necessary, adjust the pH of the stock solution to 7,6 ± 0,1.

5.7 Waste water samples.

Collect a representative sample of the waste water and store it below 4 °C for as short a period as possible (see, e.g. ISO 5667-16). The pH of the sample should be adjusted to 7,6 ± 0,1, unless the effect of the whole sample is to be determined. It is necessary to know the concentration of ammonium-N in the sample; if this is not known, determine the value.

Usually inhibition of nitrification begins to occur at concentrations above about 100 mg/l ammonium-N. When the disappearance of ammonium-N is used to measure the nitrification rate, errors increase when the initial concentration of ammonium-N is high, since, in the region of 20 mg/l N, the difference between initial and final concentrations remains low. Also, ammonium may be assimilated by heterotrophic bacteria for cell synthesis. Thus, the concentration of ammonium-N should not exceed 56 mg/l, as intended, and preferably should be the same in all vessels in a single batch of determinations. This is achieved by separating the ammonium source medium B (5.4.2), from the buffer, medium A (5.4.1), and by adding a constant volume of medium A, but differing appropriate amounts of medium B and of water.

6 Apparatus

6.1 Reaction vessels

6.1.1 Conical flasks, e.g. 200 ml or 500 ml, or

6.1.2 Measuring cylinders, 100 ml.

6.2 Pasteur pipettes, or other aeration device.

6.3 Air supply

6.3.1 Compressed air supply, humidified by passage through a wash-bottle containing water, for use with 100 ml cylinders (6.1.2).

6.3.2 Shaker, alternative to diffused air aeration for use with conical flasks (6.1.1).

6.4 Filtration apparatus.

6.5 Glass fibre filters, or **paper filters**, which neither release nor adsorb ammonium-N or oxidized-N.

6.6 Apparatus and reagents, for analytical determination of ammonium-N and/or oxidized-N in solution.

7 Procedure

7.1 Preparation

If the nitrifying activity of the sludge is not known, determine the rate according to Annex A. It is recommended that sludges be used with nitrifying rates of between 2 mg of nitrogen per gram of suspended solid and hour [mg of N per (g·h)] and 6,5 mg of nitrogen per gram of suspended solids and hour [mg of N per (g·h)] for the test period of 3 h. Sludges with activities outside this range may be brought to this range by either dilution with water (5.1) or concentration by settlement or centrifugation (see Clause 9). If this is not possible, choose a more actively nitrifying sludge from another source.

If the test samples contain no ammonium-N, add a volume of hydrogen carbonate/ammonium sulfate medium (5.3) equal to one tenth of the final reaction mixture, V_F, to each of a series of vessels (flasks 6.1.1, or cylinders, 6.1.2, respectively). Then add equal volumes (V_F/2) of washed nitrifying sludge (5.2) so that the final concentration of suspended solids will be approximately 1 500 mg/l. Finally, add a suitable volume, usually 5 ml, of test solution (5.6) and sufficient water (5.1) to make the final volume, V_F, the same in all flasks (see Annex B for an example). Ensure that sludge does not come into contact with the undiluted solution of the test substance.

Include a control vessel with sludge, medium and water but no test substance, and a reference vessel with sludge, medium, water and the reference inhibitor (V_F/100 of ATU solution (5.5), 11,6 mg/l). If required, as an extra check, take a sample of the control to measure the initial concentration of ammonium-N.

If the test sample (e.g. waste water) contains ammonium, add V_F/20 medium A (5.4.1) to each flask instead of V_F /10 full medium (5.3), then add the washed sludge (V_F/2). Finally, add sufficient volumes of medium B (5.4.2) and of water (5.1), followed by one of a range of volumes (or dilutions) of the waste water test sample, so that the final volume is V_F and the concentration of ammonium-N is 56 mg/l.

7.2 Incubation

Incubate all vessels at a constant temperature 22 °C ± 2 °C in the dark or in diffused light, for 4 h (or longer if the sludge activity is lower than 2 mg of N per (g·h) and aerate the mixtures by either bubbling humidified compressed air (6.3.1) through the measuring cylinders (6.1.2) or by shaking the conical flasks (6.1.1) at such a rate as to keep the sludge solids in suspension and the concentration of dissolved oxygen above 4 mg/l.

NOTE Strong waste waters may require extra aeration to maintain the concentration of dissolved oxygen above 4 mg/l.

At the end of incubation take a suitable volume of sample from each vessel for analysis of oxidized nitrogen (nitrate plus nitrite) (e.g. ISO 6777, ISO 7890-1) and/or ammonium (use, e.g. ISO 7150-1) concentration. Immediately, filter the samples through a glass-fibre filter or a washed paper filter (6.4, 6.5).

At the end of incubation take a suitable sample (20 ml to 25 ml) from each vessel and determine the concentration of suspended solids in the vessel. Correction in the content of the solids shall be made if the test substance contains significant amounts of suspended solids. Determine the concentration of suspended solids of the test substance and correct the concentration before calculation of the nitrification rate.

8 Calculation and expression of results

Calculate the percentage inhibition of formation of oxidized N (I_N in percent, %), as follows:

$$I_N = (\rho_c - \rho_t)/(\rho_c - \rho_b) \times 100 \quad (1)$$

where

ρ_c is the concentration of oxidized nitrogen in the control vessel, without test substances, after incubation, in milligrams per litre, mg/l;

ρ_t is the concentration of oxidized nitrogen in the vessel containing the test substance or waste water, after incubation, in milligrams per litre, mg/l;

ρ_b is the concentration of oxidized nitrogen in the vessel containing the reference inhibitor after incubation, in milligrams per litre, mg/l.

If the sample contains nitrate, e.g. a waste water from an area where tap water contains significant concentrations of nitrate, make allowance for this by subtracting from ρ_t the initial concentrations of nitrate in the reaction mixtures derived from the sample.