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Water quality — Test for inhibition of oxygen consumption by activated sludge for carbonaceous and ammonium oxidation

Qualité de l'eau — Essai d'inhibition de la consommation d'oxygène par des boues activées pour l'oxydation du carbone et de l'ammonium

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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 8192 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 8192:1986), which has been technically revised.

Introduction

Information generated by this method for assessing the potential toxicity of substances, mixtures and waste waters to activated sludge may be helpful in estimating the effect of a test material on mixed bacterial communities in the aquatic environment, especially in aerobic biological treatment systems. The susceptibility of oxygen uptake by different sub-populations of the bacterial communities to inhibition by chemicals and waste waters is not necessarily uniform and selective effects may profoundly influence the outcome of the test.

There are two principal groups of microorganisms contributing to the total oxygen consumption by activated sludge: heterotrophic organisms mainly responsible for the breakdown of carbon-based substrates (carbonaceous oxidation) and autotrophic nitrifying organisms causing the oxidation of ammonium to nitrate (nitrification).

This International Standard may be used to assess the toxicity of substances on total oxygen uptake (i.e. carbonaceous oxidation and nitrification combined) or, by deliberately adding a specific inhibitor of nitrification, also to assess toxicity of substances to the carbonaceous and nitrification components separately.

For the determination of the nitrification inhibition with this method, a sufficiently nitrifying activated sludge is required. Indications of nitrification may be investigated further by application of ISO 9509[4].

The user of this method should be aware that particular problems could require the specification of additional marginal conditions.

The inhibitory effect of a test material may be exerted on both components or it may be exerted predominantly on only one of them. Nitrification is the process more commonly prone to selective inhibition.

Water quality — Test for inhibition of oxygen consumption by activated sludge for carbonaceous and ammonium oxidation

WARNING — Persons using this International Standard should be familiar with normal laboratory practice. This International 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 International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for assessing the inhibitory effect of a test material on the oxygen consumption of activated sludge microorganisms.

This method is intended to represent the conditions in biological waste-water treatment plants. It gives information on inhibitory or stimulatory effects after a short exposure (usually 30 min up to 180 min or even more) of the test material on activated sludge microorganisms.

This method is applicable for testing waters, waste waters, pure chemicals and mixtures of chemicals. Concerning the chemicals, the method refers to those which are soluble under the test conditions. Special care is necessary with materials of low water solubility, high volatility and with materials abiotically consuming or producing oxygen.

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 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

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

3.1
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 oxygen

(ISO 6107-1:2004 [3], definition 2)

3.2
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 [6], definition 3.4)

3.3
oxygen consumption rate
uptake of oxygen by activated sludge microorganisms per unit volume of sludge, in unit time

NOTE This quantity is expressed in milligrams per litre per hour [mg/(l·h)].

3.4
specific oxygen consumption rate
uptake of oxygen by activated sludge microorganisms per unit mass of dry sludge (suspended solids), in unit time

NOTE This quantity is expressed in milligrams per gram per hour [mg/(g·h)].

3.5
inhibition of oxygen consumption
decrease of the oxygen consumption rate of an activated sludge plus (a) degradable substance(s) in the presence of the test material, compared with that of a similar mixture without test material

NOTE 1 This quantity is expressed as a percentage.

NOTE 2 In the absence of a substrate, some chemicals (e.g. uncouplers of phosphorylation) can increase oxygen uptake.

3.6
toxic range
range of concentration of a test material over which 0 % to 100 % inhibition occurs

3.7
EC_{50}
effective concentration of the test material giving a calculated or interpolated inhibition of oxygen consumption of 50 % compared with a blank control

3.8
nitrification
oxidation of ammonium compounds by bacteria

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

[ISO 6107-1:2004 [3], definition 49].

4 Principle

In the presence of easily biodegradable substances, activated sludge consumes oxygen at a higher rate than in their absence, depending on, among other factors, the concentration of microorganisms. Addition of a toxic concentration of a test material results in a decrease in the oxygen consumption rate. The rates are measured using an oxygen electrode. The percentage inhibition of the oxygen consumption is estimated by comparison of the rate with that of a control mixture containing no test material.

The sensitivity of the activated sludge may be checked with a suitable reference substance. The inhibition of the oxygen uptake by all sludge microorganisms, heterotrophic microorganisms and the oxidation of ammonium salts by nitrifying microorganisms may be separately expressed from measurements of the rate of uptake in the absence and presence of *N*-allylthiourea (ATU), a specific inhibitor of the oxidation of ammonium to nitrite by first-stage nitrifiers. The difference between the two oxygen values is due to nitrification and the residual value in the presence of allylthiourea is due to the heterotrophs. Any oxygen consumption due to abiotic processes may be detected by determining the rate in mixtures of the test material, synthetic medium and water, but omitting activated sludge.

Under certain (rare) circumstances, a test substance with strong reducing properties may cause measurable abiotic oxygen consumption. In such cases, abiotic controls are necessary to discriminate between oxygen uptake by the test substance and microbial respiration. Abiotic controls may be prepared either by omitting the inoculum from test mixtures, or by poisoning the inoculum with a solution of mercury(II) chloride.

5 Reagents, media and inoculum

Use only reagents of recognized analytical grade.

5.1 Water, complying with grade 1 as defined in ISO 3696, dissolved respectively distilled or de-ionized water containing less than 1 mg/l dissolved organic carbon (DOC).

5.2 Specific nitrification inhibitor, *N*-allylthiourea (ATU).

Dissolve 2,50 g of *N*-allylthiourea (ATU) in 1 000 ml of water (5.1). The addition of 2,32 ml of this stock solution to a sample of 500 ml results in a final concentration of 11,6 mg/l (10^{-4} mol/l).

5.3 Mercury(II) chloride solution

If required (see Clause 4), prepare a solution of 0,10 g of mercury(II)chloride ($HgCl_2$) in 10 ml of water (5.1).

WARNING — Stringent safety precautions and extraordinary waste disposal measures apply to the use of mercury salts in the laboratory. Routine deployment of abiotic controls poisoned with mercuric chloride is not recommended.

5.4 Antifoam agent, free from silicone.

5.5 Reference substance, stock solution.

Prepare a solution containing 1,00 g of 3,5-dichlorophenol (3,5-DCP) in 1 000 ml of water (5.1). Use warm water and/or ultrasonication to accelerate the dissolution and make the solution up to volume when it has cooled to room temperature.

Alternatively *N*-methylaniline can be used as a reference substance, especially for inhibition of nitrification processes. When using this substance, prepare a solution containing 1,00 g of *N*-methylaniline (NMA) in 1 000 ml of water (5.1).

5.6 Test medium, synthetic sewage 1 (100-fold OECD medium).

Peptone	16 g
Meat extract	11 g
Urea [$CO(NH_2)_2$]	3 g
Sodium chloride (NaCl)	0,7 g
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	0,4 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$)	0,2 g
Anhydrous potassium monohydrogenphosphate (K_2HPO_4)	2,8 g
Water (5.1)	1 l

The pH of this synthetic medium shall be 7,5 ± 0,5.

If the prepared synthetic medium is not used immediately, store it in the dark at 0 °C to 4 °C, for no longer than 1 week.

Alternatively, sterilize the synthetic medium prior to storage, or add the peptone and meat extract shortly before carrying out the test. Prior to use, ensure that the medium is mixed thoroughly and adjust the pH as necessary.

5.7 Test material, stock solution.

The test material may be a pure chemical, a mixture of chemicals, a chemical product or a waste water.

Prepare a stock solution of the test material in water (5.1) at a suitable concentration, for example 1 g/l or 10 g/l. Waste waters may be used without dilution.

For insoluble materials, a suspension or dispersion may be prepared, or the test material may be added directly to the test vessels. Take care to ensure as much homogeneity as possible. For handling insoluble materials, see, for example, ISO 10634 [7].

5.8 Inoculum

For general use, activated sludge should be taken from the exit of the aeration tank (where substrate concentrations are lowest) of a waste-water plant, treating predominantly domestic sewage, and working efficiently. Depending on the purpose of the test, any type of activated sludge, including sludge grown in the laboratory and sludge grown on industrial waste waters, may also be used at a suitable suspended-solids concentration of, for example, 2 g/l to 4 g/l. However, activated sludges from different treatment plants are likely to exhibit different characteristics and sensitivities.

6 Apparatus

General laboratory equipment, and the following (see Annex A).

6.1 Test vessels: 250 ml to 300 ml biochemical oxygen demand **(BOD) bottles** or **Erlenmeyer flasks** with stoppers are recommended (see Figure A.1). Alternatively, larger test vessels may also be used (see Figure A.2).

When using a BOD bottle for oxygen measurements, a suitable sleeve adapter may be required for sealing the oxygen electrode against the necks of the test vessels (see Figure A.1). To avoid loss of displaced liquid on insertion of the oxygen electrode, it is advisable first to insert a funnel or glass tube through the sleeve, or to use vessels with flared-out rims.

6.2 Device for measuring oxygen concentration: comprising a suitable oxygen electrode, a cell to contain the sample and a recorder (see Figure A.2).

6.3 Magnetic stirrers, covered with an inert material.

6.4 Aeration device

If necessary, pass compressed air through an appropriate filter to remove dust and oil, and through wash bottles containing water to humidify the air. Aerate the test vessels with Pasteur pipettes, or other aeration devices which do not adsorb chemicals.

6.5 pH-meter

6.6 Centrifuge, general bench-top centrifuge for sludge, capable of 10 000 m/s^2.

6.7 Apparatus for culturing nitrifying activated sludge (see Annex B).

7 Test environment

Perform the test at a temperature within the range of (22 ± 2) °C and in an atmosphere free from dust and toxic vapours.

8 Procedure

8.1 General

An overview of the test procedure is shown in Annex C.

The procedures to be applied to nitrifying sludge differ from those applied to non-nitrifying sludge. Therefore, it is advisable first to check the activated sludge for its nitrification activity (see Annex C).

The use of nitrifying sludge is only necessary when the influence of a test material on nitrification is to be determined. Nitrifying sludge is not required if only heterotrophic respiration is determined.

In order to check the nitrification activity of the sludge, apply the nitrification test (8.8) and calculate the rate of nitrification, if any, according to 9.2.

This preliminary test serves as a range-finder for the following definitive test.

See 8.9 for an outline of this preliminary test.

8.2 Elimination of foam

Difficulties can arise if foaming occurs during the incubation, to the extent that the foam, and the sludge solids carried on it, are expelled from the aeration vessels. Occasionally, foaming may simply result from the presence of the synthetic sewage, but foaming should be anticipated if the test material is, or contains, a surfactant. Loss of sludge solids from the test mixtures will result in artificially lowered respiration rates that could mistakenly be interpreted as a result of inhibition. In addition, aeration of surfactant solutions concentrates the surfactant in the foam layer; loss of foam from the test system will lower the exposure concentrations.

If foaming occurs, add a surfactant-free silicone-emulsion antifoam agent (5.4). If the problem is associated with the presence of the synthetic sewage, modify the sewage concentrate (5.6) by including an antifoam agent (5.4) at a rate of 50 µl/l. If foaming is caused by the test material, determine the quantity (generally a few drops from a Pasteur pipette) needed for abatement at the maximum test concentration, then treat all individual aeration vessels identically (including those, for example, blank controls and reference vessels, where foam is absent).

8.3 Preparation of inoculum

Where necessary, remove coarse particles by settling for a short period, for example 15 min, and decanting the upper layer of finer solids for use. Alternatively, the sludge may be homogenized by using a blender for a few seconds. Where necessary, remove coarse particles with a suitable sieve.

The sludge may be washed as follows: first centrifuge (6.6) the sludge for about 10 min at approximately 10 000 m/s^2 and discard the supernatant liquid. Re-suspend the sludge in chlorine-free tap water, remove this by re-centrifuging and then repeat, if necessary, the washing and centrifuging process. Determine the dry mass of a known volume of the sludge. Finally re-suspend the sludge in chlorine-free tap water to obtain the required activated sludge concentration, of about 3 g/l of suspended solids.

Having adjusted the concentration of suspended solids, continuously aerate the activated sludge and, where possible, use it within 24 h of collection. If this is not possible, the activated sludge may be fed for up to one additional day with synthetic medium (see 5.6) at a rate not exceeding 50 ml per litre per day, provided no significant change in its activity results and that nitrification, if initially present, is not lost. Alternatively,

changes in activity may be minimized by refrigerating the activated sludge at 4 °C for up to 4 d without feeding [13]. In all cases, the origin, the concentration, any pre-treatment and maintenance of the activated sludge shall be stated in the test report. Knowledge about possible changes occurring to sludges during storage is insufficient. Therefore, any sludge storage and/or treatment should be the same for all samples in a study under investigation.

WARNING — Laboratory-grown sludges can be less active, with a narrower spectrum of substrates than sludges from waste-water treatment plants.

8.4 Test mixtures

Incubate test mixtures under conditions of forced aeration. Start the incubation (aeration) for each preparation with the initial contact between the activated sludge inoculum and the other mixture constituents, and finalize after a specified exposure time when the rate of decline in dissolved oxygen concentration is measured.

The capacity of the equipment used to measure oxygen consumption rates determines the manner in which the incubations begin. For example, if it comprises a single probe, the measurements are made individually. In this case, prepare the various mixtures required for the test, but withhold the inoculum, adding it to each vessel of the series and starting each incubation in turn, at timed intervals of, for example, 10 min to 15 min.

Alternatively, the measuring system may comprise multiple probes that facilitate multiple, simultaneous measurements, in which case inoculum may be added at the same time to appropriate groups of vessels.

The activated sludge concentration in the test mixtures is nominally 1 500 mg/l of suspended solids. Measure the oxygen consumption after 30 min of incubation. If more information after an extended contact time is found necessary, carry out additional measurements after 180 min of incubation. Depending on the purpose of the test, the incubation time may be extended still further, e.g. up to 27 h. For a 27 h test, add the synthetic medium (5.6) after 24 h of incubation (without synthetic medium) and aerate additionally for 3 h. This shall be stated in the test report.

NOTE Usually, an incubation time of 30 min is sufficient. Longer incubation may, for example, be required for substances that are poorly soluble in water. Any extension of the incubation time will result in an increase of work.

Prepare in the test vessels (6.1) mixtures, F_T, containing dilution water (5.1), synthetic medium (5.6) and test material (5.7), to obtain different known concentrations as required. See Table D.1 of Annex D for examples of volumes of constituents. Adjust the pH to 7,5 ± 0,5, dilute with water and add the inoculum (5.8) to obtain equal final volumes. If the inhibitory effect of the pH is to be tested, do not adjust the pH.

8.5 Reference mixtures

Normally, for most cases, prepare mixtures, F_R, with a suitable reference substance (5.5) in the same way as in 8.4 (see 8.10.2).

8.6 Blank control

Carry out at least one blank control, F_B, which contains an equal volume of activated sludge and synthetic medium as the test mixture(s), but no test material. Dilute with water to the same volume as the test mixtures.

8.7 Abiotic test

If required (for example, if a test material is known or suspected to have strong reducing properties), prepare mixtures, F_A, to measure the abiotic oxygen consumption. They contain the same amount of test material, synthetic medium and water as the test mixtures, but no activated sludge. If required, add an inhibitor such as mercury chloride to prevent biological oxygen consumption, for example, 1,0 ml/l of a $HgCl_2$ solution (5.3).

8.8 Nitrification test

Prepare mixtures (F_B) as in the blank control (8.6) and additional control mixtures (F_N) but which also contain 11,6 mg/l of ATU (5.2). Aerate and incubate for 30 min (8.4) and then measure the rates of oxygen uptake (8.12) and calculate the rate of oxygen uptake due to nitrification (as indicated in 9.2).

8.9 Preliminary test

A preliminary test is useful to estimate the range of concentrations needed in a definitive test for determining the inhibition of oxygen consumption. Alternatively, the absence of inhibition of oxygen consumption in a preliminary test may demonstrate that a definitive test is unnecessary.

Carry out the test (8.10.2, 8.10.3) using at least three concentrations of test material (5.7), for example 1,0 mg/l, 10 mg/l; and 100 mg/l, a blank control (8.6) and, if necessary, an abiotic control (8.7) with the highest concentration of test material (see the example in Annex D, Table D.1).

Ideally, the lowest concentration of test material used should have no effect on the oxygen consumption.

Calculate the rates of oxygen uptake (9.1) and the rate of nitrification (9.2), if relevant; and calculate the percentages of inhibition (9.3).

NOTE Depending on the purpose of the test, it is also possible to determine the toxicity of a limit concentration, e.g. 100 mg/l, that covers all realistic scenarios for the test material in question. If no significant toxic effect occurs at this concentration, further testing at higher or lower concentrations is not necessary.

8.10 Definitive test

8.10.1 General

The inhibition of three different oxygen uptakes may be determined, namely, total, heterotrophic, and that due to nitrification. For total uptake, prepare the reaction mixtures as in 8.10.2, while for the other two, prepare mixtures as in 8.10.2 and also those in 8.10.3.

8.10.2 Inhibition of total oxygen uptake

Carry out the test using a range of concentrations deduced from the preliminary test. Use at least five concentrations in a logarithmic series and include a blank control. The abiotic control does not need to be repeated if there was no oxygen uptake in the preliminary test. However, if significant uptake occurs, include abiotic controls for each concentration of test material.

The sensitivity of the sludge may be checked using a reference substance [e.g. 3,5-dichlorophenol or *N*-methylaniline (5.5)]. Where possible, check the sensitivity for each test series.

NOTE The sensitivity of activated sludge is known to fluctuate [13], among other factors, according to the maintenance of the sludge in the laboratory during the interval between collection/preparation and use. It is therefore not permissible to rely on reference inhibitor responses obtained on other occasions, with activated sludge from the same source or the same batch.

8.10.3 Discrimination between inhibition of heterotrophic respiration and nitrification-linked oxygen uptake

The use of the specific nitrification inhibitor, ATU, enables the inhibitory effects of test substances on solely heterotrophic oxidation to be assessed directly, and, by subtracting the oxygen uptake rate in the presence of ATU from the total oxygen uptake rate (no ATU present), the effects on the rate of nitrification may be calculated (see Annex C).

Prepare two sets of reaction mixtures as in 8.10.2, but to one set add ATU to each mixture to allow a final concentration of 11,6 mg/l (5.2), which should completely inhibit nitrification. The rates of oxygen uptake of the

latter set of mixtures are a measure of heterotrophic oxidation only, and the differences between the respective individual values of the two sets give the oxygen uptake due to nitrification.

If it is not known whether the activated sludge nitrifies, prepare a blank control (F_B, 8.6) and a second mixture (denoted F_N) containing ATU at a concentration of 11,6 mg/l as in 8.8. Aerate in beakers for 30 min and then determine the rates of oxygen uptake. Oxidation of ammonia is indicated if the rate in F_B is significantly higher than that measured in F_N.

NOTE The concentration of 11,6 mg/l ATU completely inhibits nitrification over at least 180 min.

8.11 Incubation

Aerate all mixtures (8.4 to 8.9) to give as near as possible oxygen saturation, but take care not to supersaturate them. Stirring is necessary to give good mixing in the incubation vessels. Ensure that all mixtures are at approximately the same temperature (22 ± 2 °C) and that this temperature does not significantly change during the test.

The choice of 180 min is arbitrary. It is recommended, for substances that are poorly soluble in water, to allow sufficient time for effective exposure to occur. Usually the activated sludge should still be actively respiring on the synthetic sewage after this time. If not, the test should be repeated using an increased volume of concentrated medium

It is also possible to extend the incubation period up to 27 h. For more details see Reference [13].

8.12 Measurement of the rate of oxygen uptake

After 30 min from the start of the incubation of the first mixture, transfer a sample from the first aeration vessel to a measurement vessel (6.1) and immediately measure, using an oxygen probe (6.2), the rate of decrease in concentration of dissolved oxygen. Repeat the procedure with samples of each mixture, after aeration periods of 30 min and, if required, 180 min. Stirring is essential to ensure that the probe responds with minimal delay to changing oxygen concentrations, and to allow regular and reproducible oxygen measurement in the measuring vessels.

For example, place a sample in a cylindrical measuring cell of about 20 ml volume which is fitted with an oxygen electrode and a magnetic stirrer. In this case, the volume of the mixtures may be reduced to about 200 ml (from 500 ml, Table D.1). Before starting a new measurement, clean the cell with tap water. For an example of such a measuring unit, see Figure A.2 in Annex A.

Alternatively, use the sample to fill a BOD bottle fitted with a magnetic stirrer (6.3). Insert an oxygen probe with a sleeve adapter into the neck of the bottle and start the magnetic stirrer (as an example, see Figure A.1).

Measure and record continuously the concentration of dissolved oxygen for about 5 min to 10 min, or until the oxygen concentration falls below 1 mg/l. Then remove the electrode, return the mixture to the aeration vessel and continue aerating and stirring. Repeat this procedure with samples from each test vessel in turn to obtain a set of readings taken at 30 min for all test mixtures. If more information after an extended contact time is desired, repeat the procedure after 180 min from the start of incubation.

9 Calculation and expression of results

9.1 Calculation of oxygen uptake rates

Calculate the oxygen consumption rates of the test mixtures from the measured values, for example, from the linear part of the graphs of oxygen concentration versus time, ideally limiting the calculations to oxygen concentrations between 2,0 mg/l and 7,0 mg/l as higher and lower concentrations may themselves influence rates of consumption. Excursion into concentration bands below or above these values is occasionally unavoidable and necessary, for example, when respiration is heavily suppressed and consequently very slow, or if a particular activated sludge respires very quickly. This is acceptable, provided the extended sections of

the uptake graph are straight and their gradients do not change as they pass through the 2,0 mg/l or 7,0 mg/l of O_2 boundaries. Curved sections of the graph indicate that the measurement system is stabilising or the uptake rate is changing and shall not be used for the calculation of respiration rates. Express the oxygen consumption rates in milligrams per litre per hour, or milligrams per gram per hour.

The oxygen consumption rate, R, in milligrams per litre per hour, may be calculated or interpolated from the linear part of the recorded oxygen decrease graph according to Equation (1):

$$R = \left[\left(\rho_1 - \rho_2 \right) / \Delta t \right] \times 60 \quad (1)$$

where

ρ_1 is the oxygen concentration at the beginning of the selected section of the linear phase, in milligrams per litre (mg/l);

ρ_2 is the oxygen concentration at the end of the selected section of the linear phase, in milligrams per litre (mg/l);

Δt is the time interval between these two measurements, in minutes (min).

The specific respiration rate (R_S) is expressed as the amount of oxygen consumed per dry weight of sludge per hour according to Equation (2):

$$R_S = R / \rho_{SS} \quad (2)$$

where

ρ_{SS} is the concentration of suspended solids in the test mixture, in grams per litre (g/l).

A value of 20 mg/(g·h) is taken as a criterion of validity (see Clause 10).

The different indices of R, which may be combined, are explained as follows:

S specific rate

T total respiration rate

N rate due to nitrification respiration

H rate due to heterotrophic respiration

A rate due to abiotic processes

B rate based on blank assays.

9.2 Calculation of rate of nitrification

The coherence of total respiration, nitrification respiration and heterotrophic respiration is given by Equation (3):

$$R_N = R_T - R_H \quad (3)$$

where

R_N is the rate of oxygen uptake due to nitrification, in milligrams per litre and hour (mg/l·h);

R_T is the measured rate of oxygen uptake by the blank control (no ATU) (F_B), in milligrams per litre and hour (mg/l·h);

R_H is the measured rate of oxygen uptake of the blank control with added ATU (F_N), in milligrams per litre and hour (mg/l·h).

This coherence is valid for blank values (R_{NB}, R_{TB}, R_{HB}), abiotic controls (R_{NA}, R_{TA}, R_{HA}) and assays with test material (R_{NS}, R_{TS}, R_{HS}) (mg/g·h), and is calculated from Equations (4) to (6)

$$R_{NS} = R_N/\rho_{SS} \quad (4)$$

$$R_{TS} = R_T/\rho_{SS} \quad (5)$$

$$R_{HS} = R_H/\rho_{SS} \quad (6)$$

If R_N is insignificant (e.g. < 5 % of R_T in blank controls) it may be assumed that the heterotrophic oxygen uptake equals the total uptake, and that no nitrification is occurring. Hence, only one set of test vessels (8.10.2) needs to be set up for the definitive test. If R_N is significant (> 5 % of R_T in blank controls), two sets of vessels should be set up (8.10.3) to measure the inhibition of total, heterotrophic and nitrification oxygen uptakes.

In the same way, determine the oxygen uptake with different test-substance concentrations.

9.3 Calculation of percentage inhibition

The percentage inhibition, I, of *total* oxygen consumption at each concentration of test substance is given by Equation (7):

$$I = [1 - (R_T - R_{TA})/R_{TB}] \times 100\ \% \quad (7)$$

Similarly, the percentage inhibition of *heterotrophic* oxygen uptake, I_H, at each concentration is given by Equation (8):

$$I_H = [1 - (R_H - R_{HA})/R_{HB}] \times 100\ \% \quad (8)$$

Finally, the percentage inhibition of oxygen uptake due to nitrification, I_N, at each concentration is given by Equation (9):

$$I_N = [1 - (R_T - R_H)/(R_{TB} - R_{HB})] \times 100\ \% \quad (9)$$

Plot the percentage inhibition of oxygen consumption against the logarithm of the test material concentration (see an example of inhibition curves in Annex E). Inhibition curves are plotted for each aeration period, for example after 30 min and 180 min. Calculate, or interpolate from the graph, the concentration which inhibits the oxygen consumption by 50 % (EC_{50}).

If suitable data are available, the 95 % confidence limits of the EC_{50}, the slope of the curve and suitable values to mark the beginning of inhibition (for example EC_{10} or EC_{20}) and the end of the inhibition range (for example EC_{80} or EC_{90}) may be calculated or interpolated.

In view of the variability often observed in the results, it may in many cases be sufficient to express the result in order of magnitude, for example:

EC_{50} < 1 mg/l

EC_{50} 1 mg/l to 10 mg/l

EC_{50} 10 mg/l to 100 mg/l

EC_{50} > 100 mg/l.

9.4 Interpretation of results

When nitrifying sludges are used, complications may arise due to the occasionally disparate sensitivity of the nitrifying microorganisms. Thus, for example, the respiration curves may be biphasic, EC_{50} values may be significantly lower and inhibition-concentration plots may be distorted. Additionally, changes may take place in the activity of the nitrifying population of a sludge sample between a preliminary assessment and a subsequent definitive investigation giving rise to two apparently contradictory sets of results within the same test. Such interferences may be clarified by measuring the rate of oxygen uptake, in the absence and presence of a specific inhibitor of nitrification added deliberately to the test mixtures. This has several benefits for interpreting results. First, it confirms whether or not the sludge sample is nitrifying. Secondly, it quantifies the contribution of nitrification (if present) to the overall oxygen uptake, and third, it facilitates an assessment within the one test of toxicity both to heterotrophic and autotrophic oxygen.

Results with volatile materials should be interpreted with caution and are likely to underestimate any inhibitory effects because of the difficulty of maintaining the initial concentration.

Results with insoluble materials should similarly be treated with caution and cannot always be easily quantified; again, inhibitory effects may be wrongly estimated if the concentration of the compound in solution changes for whatever reason. Based on a consideration of deposition during the primary settlement stage of full-scale treatment processes, it may be more relevant to test insoluble materials for inhibition of anaerobic sludge gas production (see ISO 13641-1[10]).

The results from this test should be considered only as a guide to the likely toxicity of the test material, since sludges of different sources differ in bacterial composition and concentration. Also, laboratory tests cannot truly simulate environmental conditions. For example, no account is taken of longer-term adaptation of the activated sludge microorganisms to the test material, or of materials which may adsorb onto the sludge and build up to a toxic concentration over a longer period of time than that allowed in the test [14]. For this purpose, the incubation time may be extended even further, e.g. up to 27 h [13]. These factors may be investigated by application of appropriate simulation tests. An overview of standardized biodegradation tests is given in ISO/TR 15462[11]. For general information on biotesting see ISO 5667-16[1].

10 Validity of the results

Where possible, check the sensitivity of the activated sludge by means of a reference substance.

In an interlaboratory trial carried out in July 2004 using activated sludge from domestic sewage, the precision data (Table 1) and the valid ranges (Table 2) were obtained for the reference substances 3,5-dichlorophenol and *N*-methylaniline (only for nitrification inhibition).

Table 1 — Precision data for 3,5-dichlorophenol and *N*-methylaniline

Parameter	Mean mg/l	Standard deviation mg/l	95 % confidence interval of the mean mg/l	Number of tests performed
3,5-Dichlorophenol, 30 min incubation time				
Total respiration EC_{50}	9,8	6,5	7,1 to 12,6	24
Heterotrophic respiration EC_{50}	20,3	8,6	16,3 to 24,3	20
Nitrification respiration EC_{50}	4,6	4,7	2,3 to 6,9	19
3,5-Dichlorophenol, 180 min incubation time				
Total respiration EC_{50}	9,3	3,7	7,2 to 11,3	15
Heterotrophic respiration EC_{50}	19,3	7,7	14,1 to 24,4	11
Nitrification respiration EC_{50}	4,3	2,6	2,3 to 6,3	9
***N*-methylaniline, 30 min incubation time**				
Nitrification respiration EC_{50}	1,50	0,44	1,13 to 1,87	8
***N*-methylaniline, 180 min incubation time**				
Nitrification respiration EC_{50}	3,74	2,24	1,87 to 5,61	8

Table 2 — Proposed valid ranges for EC_{50} values

Reference substance	Test duration min	Parameter	Proposed valid range mg/l
3,5-Dichlorophenol	30	Total respiration	2 to 25
3,5-Dichlorophenol	180	Total respiration	2 to 25
3,5-Dichlorophenol	30	Heterotrophic respiration	5 to 40
3,5-Dichlorophenol	180	Heterotrophic respiration	5 to 40
3,5-Dichlorophenol	30	Nitrification respiration	0,1 to 10
3,5-Dichlorophenol	180	Nitrification respiration	0,1 to 10
N-methylaniline	30	Nitrification respiration	0,1 to 5
N-methylaniline	180	Nitrification respiration	0,1 to 5

If the EC_{50} of the reference substance does not lie in the expected range, repeat the test with activated sludge from another source.

When the total blank-control oxygen-uptake rate is less than 20 mg/g of dry weight per hour, spurious results are obtained. In the case of such low values, repeat the test with activated sludge washed before use (8.3) or use sludge from another source.

11 Test report

The test report shall include at leat the following information:

a) a reference to this International Standard (ISO 8192:2007);

b) name, specifications and properties of the test material;

c) source, concentration, pre-treatment and maintenance of the activated sludge;

d) test temperature;

e) name of the reference substance and result of the inhibition measurements with this substance (EC_{50}), stating whether total, heterotrophic and/or nitrification inhibition;

f) abiotic oxygen uptake in the physico-chemical control (if used);

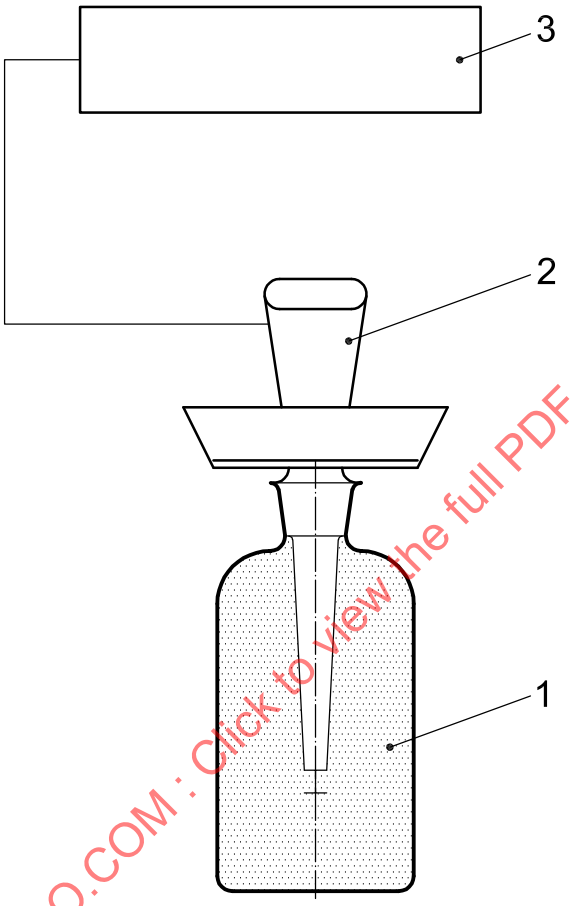
g) the test results, especially the EC_{50} and if possible other statistical data (see Clause 10), stating whether inhibition is total, heterotrophic and/or nitrification;

h) all measured data and the inhibition curve (see Clause 10 and Annex E);

i) all observations and deviations from the standard procedure which could have influenced the result.

Annex A
(informative)

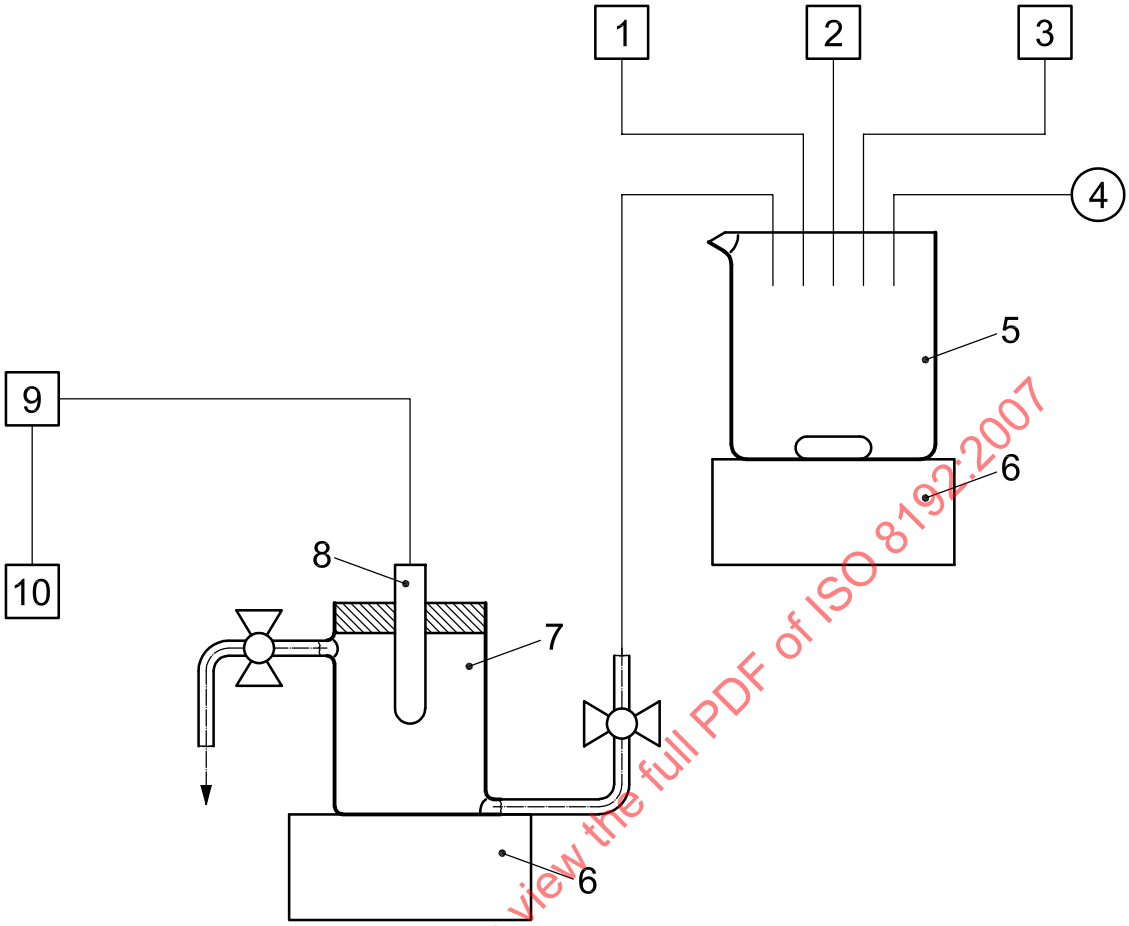
Examples of measuring units



Key

1 test vessel
2 oxygen electrode
3 oxygen-measuring instrument

Figure A.1 — Device for measuring oxygen concentration with a BOD bottle as the test vessel



Key

1 activated sludge
2 synthetic medium
3 test material
4 air
5 mixing vessel
6 magnetic stirrer
7 oxygen-measuring cell
8 oxygen electrode
9 oxygen-measuring instrument
10 recorder

Figure A.2 — Device for measuring oxygen concentration

Annex B
(informative)

Apparatus for culturing nitrifying activated sludge

B.1 General

Since nitrification should become increasingly apparent at waste-water treatment sites due to the relevant EC Directive coming into force, it should be easier to find sludges which nitrify. Failing that, this Annex describes an example of a system (Husmann unit with two secondary clarifiers) that may be used to generate nitrifying activated sludge in the laboratory, to provide a source of inoculum for the inhibition test. The Husmann unit may also be operated in a simpler, direct way with only one secondary clarifier (settlement tank).

B.2 Principle

The culturing apparatus consists of an enlarged and extended Husmann unit (see ISO 11733 [8]) comprising a single aeration basin and two secondary clarifiers connected in series. The capacity of the system shall be sufficient to provide adequate quantities of activated sludge for use in the inhibition test. Waste-water influent is dosed by means of a pump directly into the activation basin where the Waste-water and activated sludge are continuously stirred and aerated. Diffusers are used to provide fine bubble aeration. The waste-water/activated sludge mixture passes into the first clarifier where the majority of the activated sludge is separated from the treated waste water. Further separation subsequently occurs in the second clarifier. The activated sludge settles in the clarifiers and is returned to the aeration basin as return sludge, by using an air-lift or peristaltic pump.

The influent should preferably be municipal waste-water diluted with tap water, if necessary, to a dissolved organic carbon (DOC) concentration of about 50 mg/l and supplemented with mineral salts and, if necessary, a yeast extract solution. The mineral salts supplement may be necessary to prevent a lack of single nutrients. It provides NH_4Cl, ensuring a sufficient and constant ammonium concentration for the nitrifying bacteria, K_2HPO_4, necessary as a buffer to keep the pH in the optimum range and $NaHCO_3$, which is used as a carbon source by the autotrophic nitrifying bacteria, to establish sufficient nitrification activity.

B.3 Specifications

Volume of liquid in the aeration basin:	20 l;
Volume of liquid in the primary clarifier:	10 l;
Volume of liquid in the secondary clarifier:	3 l;
Sludge return rate:	approximately 99 % of the influent rate;
Operating temperature:	15 °C to 25 °C;
Lighting:	ambient laboratory conditions.