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ISO 13641-1

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Water quality — Determination of inhibition of gas production of anaerobic bacteria —

Part 1: General test

Qualité de l'eau — Détermination de l'inhibition de la production de gaz des bactéries anaérobies —

Partie 1: Essai général

Reference number
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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 13641-1 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

ISO 13641 consists of the following parts, under the general title *Water quality — Determination of inhibition of gas production of anaerobic bacteria*:

— *Part 1: General test*

— *Part 2: Test for low biomass concentrations*

Water quality — Determination of inhibition of gas production of anaerobic bacteria —

Part 1: General test

WARNING — Sludge samples may contain hazardous and inflammable substances. They contain pathogens and are liable to biological action. Consequently, it is recommended that samples be handled with special care. The gases that may be produced by microbiological activity are potentially inflammable and will pressurize sealed bottles. Exploding bottles are likely to result in infectious shrapnel and/or pathogenic aerosols. Glass bottles should be avoided wherever possible. Care is necessary when sampling, transporting and utilizing the sludge and when using microsyringes and pressure-meter syringe needles. National regulations should be followed with respect to microbiological hazards associated with this method. Toxic test materials and those with unknown properties should be handled with care.

1 Scope

This part of ISO 13641 specifies a screening method for assessing the potential toxicity of substances, mixtures, surface waters, groundwaters and wastewaters, effluents, sludges or other environmental samples by determining the production of biogas (carbon dioxide and methane) from the anaerobic digestion of sewage sludge over periods of up to 3 days. The growth rate of anaerobic bacteria is much lower compared with that of aerobic microorganisms. For this reason, the test periods are longer for anaerobic methods than those used for aerobic bacteria.

This method is applicable to substances, soluble or insoluble in water, including volatile chemicals (see Reference [1] in the Bibliography).

NOTE Special care is necessary with compounds of low water-solubility, and in these cases, see for example, ISO 10634. For general information on biotesting see ISO 5667-16[2].

This method can provide information that is useful in predicting the likely effect of a test material on biogas production in anaerobic digesters. For example, only longer tests, which simulate working digesters more closely can indicate whether adaptation of the microorganisms to the test material can occur or whether compounds likely to be adsorbed onto sludge can build up to a toxic concentration over a longer period than allowed in this test.

Information obtained by this part of ISO 13641 can also be helpful in choosing suitable initial mass concentrations for anaerobic biodegradability tests (e.g. ISO 11734). However, this part of ISO 13641 using undiluted sludge is less suitable for testing dilute digesting sludge than the method described in Part 2 of ISO 13641 where the mass concentrations are a hundredfold more dilute.

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

3 Principle

Aliquots of a mixture of undiluted anaerobically digesting sludge (20 g/l to 40 g/l of total dry solids) and a degradable substrate are incubated alone and simultaneously with a range of mass concentrations of the test material in sealed bottles for 2 days to 3 days. The amount of biogas (methane plus carbon dioxide) produced is measured by the increase in pressure in the bottles. The percentage inhibition of biogas production at the various mass concentrations of the test material is calculated from the amounts produced in the respective test and control bottles. The EC_{50} and other effective mass concentrations are calculated from plots of percentage inhibition against the logarithm of mass concentration of the test material.

4 Reagents and media

4.1 Reagents

4.1.1 Dilution water, previously de-aerated and de-ionized.

Analytical controls of this water are not necessary, but make sure that the deionizing apparatus is regularly maintained. Prior to addition of the anaerobic inoculum to any solution or dilution of test material, make sure that these are oxygen-free. Therefore, either bubble nitrogen gas (4.1.2) through the dilution water or through the dilutions for 1 h before adding the inoculum, or alternatively heat the dilution water to boiling then cool it to room temperature in an oxygen-free atmosphere.

4.1.2 Nitrogen gas, of high purity with a content of less than 5 µl/l oxygen.

4.2 Media

4.2.1 Inoculum (digesting sludge).

Collect active digesting anaerobic sludge from the digester at a sewage treatment plant or, alternatively, from a laboratory digester, treating sludge from primarily domestic sewage into collection bottles (5.1). Add sludge to these bottles to about 1 cm from the top, seal them tightly and place in insulated containers (5.2) to minimize temperature shock, until being transferred to an incubator (5.12) maintained at 35 °C ± 2 °C. When opening the bottles, take care to release excess gas pressure either by periodically loosening the seal, or by fitting a three-way pressure-release valve (5.3) to the bottle cap. It is preferable to use the sludge within a few hours of collection, otherwise store the bottles at 35 °C ± 2 °C under a headspace of nitrogen for up to 3 days when little loss of activity normally occurs.

Immediately prior to use, mix the sludge by gentle stirring and pass it through a mesh sieve (5.5) into a suitable reservoir (5.6) through the headspace of which a stream of nitrogen is passed. Set aside a sample for determination of the mass concentration of total dry solids (e.g. see ISO 11923). In general, use the sludge without dilution. The solids mass concentration should be between 20 g/l and 40 g/l dry material. Check the pH value of the sludge and, if necessary, adjust it to 7 ± 0,5.

4.2.2 Substrate.

Dissolve 10 g of nutrient broth, 10 g of yeast extract and 10 g of D-glucose in deionized water (4.1.1) and dilute to 100 ml. Filter-sterilize (0,2 µm) (5.7) and use immediately or store at 4 °C for no longer than 1 day.

5 Apparatus

5.1 Inoculum collection bottles, equipped with gas-tight seals and made of high density polyethylene or a similar material, which can expand. Glass is not recommended since the bottle may explode.

5.2 Insulated containers, for transport of sludge.

5.3 Three-way pressure-release valves, capable of being fitted to the caps of inoculum collection bottles.

5.4 Centrifuge, for determination of the mass concentration of inoculum solids.

5.5 Sieve, having a mesh size of 1 mm^2.

5.6 Reservoir for digesting sludge, consisting of a glass or plastic bottle (capacity about 5 l) fitted with a stirrer and facilities for passing a stream of nitrogen gas through the headspace.

5.7 Membrane filters, with a pore size of 0,2 µm for sterilizing the substrate.

5.8 Microsyringes, for the gas-tight connection of the pressure meter to the headspace in the bottles; also for adding insoluble liquid test materials into the bottles.

5.9 Pressure-resistant gas-tight closed glass test bottles, with an appropriate nominal size (0,1 l to 1 l).

Use, for example, nominal 125-ml serum bottles with an actual total volume of 160 ml, gas-tight sealed with septa and crimped aluminium rings. Use septa preferably made of silicone or polytetrafluoroethene-coated butyl rubber capable of withstanding a pressure of about 2×10^5 Pa. Gas-tightness of the caps used, especially butyl rubber septa, should be tested in advance because several commercially available septa are not sufficiently gas-tight against methane, and some septa do not stay tight when they are pierced with a needle as required under the conditions of this test.

5.10 Precision pressure meter for measuring total biogas production (methane plus carbon dioxide).

A needle attachment is adapted to enable measurement and venting of the biogas produced. An example of a suitable instrument is a hand-held precision pressure meter connected to a suitable syringe needle; a 3-way gas-tight valve facilitates the release of excess pressure. Calibrate the meter (see Annex A) in order to allow, if necessary, the conversion of pressure measurements to gas volumes. The internal volume of the pressure meter tubing and valve should be kept as low as practically possible, so that errors introduced by neglecting the volume of the equipment are insignificant.

If a pressure meter of the described quality is used (for example capsulated with a steel membrane), no calibration is necessary in the laboratory. It should be calibrated by a licensed institute at the intervals recommended by the manufacturer. The accuracy of the calibration can be checked at the laboratory with a one-point measurement at 1×10^5 Pa against a pressure meter with a mechanical display. When this point is measured correctly, the linearity will also be unaltered. If other measurement devices are used (without certified calibration by the manufacturer), calibration is recommended over the total range at regular intervals.

5.11 Glove box (optional), with a slight positive pressure of nitrogen.

5.12 Spark-free incubator, preferably equipped with a **shaking device,** and capable of maintaining the temperature at 35 °C ± 1 °C.

6 Test environment and interferences

6.1 Test environment

Carry out the test by incubating the sealed test bottles at a constant temperature of 35 °C ± 1 °C in the absence of oxygen, initially in an atmosphere of nitrogen, in the dark or in diffused light. Use nitrogen of high purity (4.1.2). In special cases, the test may be conducted at other temperatures.

6.2 Interferences

6.2.1 Moisture in the needle of the syringe

Moisture in the needle and connecting tubing of the pressure-transducer can lead to inaccurate pressure readings (see 7.5).

6.2.2 Oxygen contamination

Anaerobic methods are subject to error from oxygen contamination. In this method, this interference is minimized by the use of strictly anaerobic handling techniques.

6.2.3 pH of the medium

Activity of anaerobic cultures is very sensitive to the pH value. Ensure that the pH of the reaction mixture is adjusted to 7 ± 0,5 and remains within the range of 6,2 to 7,5 until the end of incubation (see 7.5).

6.2.4 Quality of the caps of the bottles

Different types of septa are commercially available. Many of them do not remain gas-tight after being pierced with a needle under the test conditions. Sometimes the pressure falls very slowly once the septa have been pierced with the syringe needle.

6.2.5 Remaining substrates in the sludge

The anaerobic biogas production and the sensitivity of the sludge are influenced by substrates, which are transferred with the inoculum into the test bottles. Digested sludge from domestic anaerobic digesters often contains materials such as sand, hair or plant residues of cellulose. Sieving the sludge will remove gross insoluble matter, thus making it easier to take representative samples.

6.2.6 Volatile chemicals

Volatile chemicals are released into the headspace of the serum bottles. This can result in the loss of some of the test material from the system during venting after pressure measurements, yielding falsely high EC_{50} values. For details see Reference [1] in the Bibliography.

7 Procedure

7.1 Test set-up and preliminary test

7.1.1 Test set-up

The number of necessary replicates depends on the degree of precision required to obtain acceptable inhibition indices. If the bottle seals are sufficiently gas-tight over the duration of the test, set up just one batch preferably in triplicates of test bottles at each mass concentration required. Similarly set up one batch of bottles for the reference substance and one set for the controls.

However, if the seals of the bottles are reliable for only one or a few piercings, set up a batch preferably in triplicates of test bottles for each time interval (t) for which results are required for all mass concentrations of a test material to be tested. Similarly set up "t" batches of bottles for the reference substance and for the controls.

The use of a glove box (5.11) is recommended. At least 30 min before starting the test, let nitrogen flow into the glove box containing all necessary test equipment. If a glove box is not used, de-gas the bottles using nitrogen for air displacement. Make sure that the temperature of the sludge is within the range of 35 °C ± 1 °C during handling and sealing of the bottles.

7.1.2 Preliminary test

If the activity of the sludge (4.2.1) is unknown, it is recommended to carry out a preliminary test. Set up controls to give, for example, mass concentrations of solids of 10 g/l, 20 g/l and 40 g/l in addition to substrate but use no test material. Also, use different volumes of reaction mixture in order to have 3 or 4 different ratios of volume of headspace to volume of liquid. From the results of biogas volumes produced at various time intervals, the most suitable conditions can be deduced which allow two daily measurements yielding significant volumes of biogas and pressure release per day at optimal sensitivity without the fear of explosions.

7.2 Test materials and controls

7.2.1 Test materials

7.2.1.1 Test compound solutions

Prepare a separate stock solution for each water-soluble test compound in oxygen-free dilution water (4.1.1) to contain, for example, 10 g/l of testmaterials. Use appropriate volumes of these stock solutions to prepare the reaction mixtures containing graded mass concentrations. Alternatively, prepare a dilution series of each stock solution so that the volume added to the test bottles is the same for each required final mass concentration.

Add substances with little or no water-solubility, for example, as solutions in a volatile solvent. Prepare such a solution at an appropriate mass concentration in a suitable solvent, for example, acetone or diethyl ether (but do not use inhibitory solvents such as trichloromethane or tetrachloromethane). Add the solutions to the empty test bottles (5.9) and evaporate the solvent before the addition of the sludge. Liquid water-insoluble test materials may be injected directly into inoculated serum bottles using microsyringes (5.8). For other treatments, use ISO 10634, but be aware that any surfactants used to produce emulsions can be inhibitory to anaerobic biogas production.

Add test materials to the bottles (5.9) to give a geometric series of mass concentrations, for example, 500 mg/l, 250 mg/l, 125 mg/l, 62,5 mg/l, 31,2 mg/l and 15,6 mg/l. If the range of the toxicity is not known from similar compounds, carry out a preliminary range-finding test with mass concentrations of, for example, 1 000 mg/l, 100 mg/l and 10 mg/l so as to ascertain the appropriate range.

7.2.1.2 Waters and wastewaters

Use the original sample of waters and wastewaters as stock solution, and, if necessary, adjust the pH to $7 \pm 0{,}5$ if inhibition due to an acidic or alkaline sample is not to be determined.

Add waters and wastewaters to the bottles (5.9) to give a geometric series of final dilution steps as follows: 1:2, 1:4, 1:8, 1:16 and so forth, where these dilution ratios are expressed as volume of water or wastewater to the total end-volume.

When testing wastewater, the highest possible test mass concentration corresponds to 50 % of the wastewater sample. It results by adding the original wastewater to the test bottles (5.9) and an equal volume of the inoculum suspension. Make sure that wastewaters or other test waters are sufficiently free of oxygen. For example, bubble nitrogen gas (4.1.2) through the dilutions for at least 1 h or use oxygen-free dilution water (4.1.1).

7.2.2 Reference compound and controls

Prepare an aqueous stock solution of the reference compound, 3,5-dichlorophenol (10 g/l), by gradually adding aqueous sodium hydroxide solution (250 g/l) to the solid, while shaking, until it has dissolved. Then add de-oxygenated dilution water (4.1.1) to the required volume. Sonication can aid dissolution.

Other reference substances may be used, when the average range of the EC_{50} has been tested. In this case, add the data to Clause 9 for the validity criteria.

Set up at least triplicate sets of bottles (5.9), containing sludge (4.2.1) and substrate (4.2.2) only, to act as controls.

Set up further replicate bottles (5.9) containing sludge (4.2.1) and substrate (4.2.2). Add enough stock solution of the reference substance (3,5-dichlorophenol) to these bottles to obtain a final mass concentration of 150 mg/l. This mass concentration of 3,5-dichlorophenol should inhibit biogas production by about 50 %. Alternatively, set up a suitable range of mass concentrations with the reference substance.

In addition, set up 4 extra bottles (5.9) for pH measurement containing sludge (4.2.1), de-oxygenated dilution water and substrate. Add the test material (7.2.1) to 2 bottles at the highest mass concentration being tested and add de-oxygenated dilution water to the remaining 2 bottles.

7.3 Addition of the test materials

Add water-soluble test materials (7.2.1) to empty test bottles (5.9) as aqueous solutions (7.2.1.1). Add insoluble and poorly water-soluble test materials, for example, as solutions in a suitable volatile solvent by use of a microsyringe to empty test bottles to give replicate sets of each of five mass concentrations of the test materials. Evaporate the solvent by passing a stream of nitrogen gas (4.1.2) over the surface of the solution in the test bottles. If liquid-insoluble and poorly water-soluble test materials are not added using a solvent, add them directly after the inoculum. See Annex B for the addition of wastewater or sludges to be tested.

7.4 Addition of the inoculum and the test medium

Stir an appropriate volume of sieved digesting sludge (4.2.1) in a 5 l bottle (5.6), while passing a stream of nitrogen through the headspace. Flush test bottles (5.9), containing aqueous solutions or evaporated solvent solutions of test materials (7.2.1), with a stream of nitrogen gas for about 2 min to remove air. Dispense aliquots of the well-mixed sludge (see 4.2.1), for example 100 ml, into the test bottles using a wide-bored tipped pipette, alternatively use a measuring cylinder. Fill the pipette in one step to the exact volume of sludge required. If more is taken up, empty the pipette and start again. Then add sufficient substrate solution (4.2.2) to give a mass concentration of 2 g/l of each of the nutrient broth, yeast extract and D-glucose in the mixture, while keeping the bottles under a flow of nitrogen.

An example of the test set-up for test batches is given in Table 1.

Ensure that the headspace volume is between 10 % and 40 % of the total bottle volume and that all bottles (controls, test and reference substances) contain the same volume of liquid; where necessary, add de-oxygenated dilution water (4.1.1) to make up the volume. Choose, for example, the ratio of biogas to liquid volume from the results of the preliminary test (7.1.2).

After each bottle is dosed, remove the needle supplying the nitrogen gas and seal the bottle with the rubber stopper and aluminium cap and moisten the stopper with a few drops of deionized water to aid insertion. Mix the contents of each bottle by shaking and incubate.

Table 1 — Example of test set-up for test batches

Final mass concentration of test material in test bottles	Volume of test material stock solution (7.2.1)		Reagents and media		
	ml		Dilution water (4.1.1)	Inoculum (4.2.1)	Substrate (4.2.2)
mg/l	Stock solution a) 10 g/l	Stock solution b) 1 g/l	ml	ml	ml
0	—	0	1,0	100	2
1	—	0,1	0,9	100	2
3,3	—	0,33	0,67	100	2
10	0,1	—	0,9	100	2
33	0,33	—	0,67	100	2
100	1,0	—	0	100	2

7.5 Incubation of bottles and pressure measurement

Transfer the bottles to the thermostatically controlled incubator (5.12) maintained at 35 °C ± 1 °C. After about 1 h, equalize the pressure in the bottles to atmospheric pressure by inserting the syringe needle, attached to the pressure meter (5.10), through the seal of each bottle in turn, opening the valve until the pressure meter reads zero, and finally close the valve. Insert the needle preferably at an angle of about 45° to prevent gas leaking from the bottles.

If the bottles are incubated without a shaking facility (see 5.12), shake them manually twice each day during the total incubation period to equilibrate the system. It may be helpful to invert the bottles to prevent loss of biogas through the septum. Inversion is, however, not appropriate in cases in which insoluble substances may adhere to the bottom of the bottles.

When the bottles reached 35 °C ± 1 °C, measure and record the pH of the contents of 2 of the 4 bottles set up for the purpose and discard the contents. Continue incubating the remaining bottles in the dark. Twice a day over the following 48 h to 72 h measure and record the pressure in the bottles by inserting the needle of the pressure meter through the seal of each bottle, in turn. Allow the pressure reading to stabilize, and record it. Then open the valve for ventilation. When the pressure reads zero close the valve.

If the pressure reading is negative, do not open the valve. Moisture sometimes accumulates in the syringe needle and tubing. The presence of moisture is indicated by a small negative pressure reading. In this case remove the needle, shake the tubing, dry with a tissue and fit a new needle.

From the time of first equalizing the pressure, designated “time 0”, continue the test, usually for 48 h.

7.6 pH measurement

Measure the pH of the contents of each bottle after the final pressure measurement.

8 Calculation

Sum, and average, the pressures recorded at each interval for each set of replicate bottles and calculate the mean cumulative gross biogas pressures. Plot curves of mean cumulative biogas production (p, i.e. the pressure expressed in Pa) against time for control, test and reference bottles. Select a time on the linear part of the curve, usually 48 h, and calculate the percentage inhibition (I) for each mass concentration of test material from Equation (1):

$$I = \left(1 - \frac{p_t}{p_c}\right) \times 100 \quad (1)$$

where

I is the percentage inhibition;

p_t is the biogas pressure, expressed in pascals (Pa), produced with test material at the selected time;

p_c is the biogas pressure, expressed in pascals (Pa), produced in the control at the same time.

Plot I against the logarithm of the mass concentrations of test material. Assess the EC_{50} (mg/l) value visually or by regression analysis.

For comparative purposes it is sometimes more useful to express the mass concentration of the substance as a mass fraction (mg/g) of total dry solids. To obtain this mass fraction, divide the volumetric concentration (mg/l) by the mass concentration of dry sludge solids (g/l) (see 4.2.1).

Calculate either the percentage inhibition achieved by the single mass concentrations of the reference substance or the EC_{50}, if a sufficient number of mass concentrations have been investigated.

In aerobic bacterial inhibition tests, I is usually plotted against the logarithm of the mass concentration of the test material. In anaerobic sludge inhibition tests with some substances, the linear plot of I against the mass concentration is more suitable to show the dependency between mass concentration and inhibition than the semi-logarithmic plot. Use the type of plot that shows a curve closer to linearity over a larger range.

Convert the mean pressure of the biogas produced in the control to the volume by reference to the pressure meter calibration curve and from this calculate the yield of biogas, expressed as the volume produced in 48 h from 100 ml undiluted sludge at a solids mass fraction of 2 % to 4 % or a mass concentration of 20 g/l to 40 g/l.

In wastewater testing, the lowest ineffective dilution (LID-value), which was observed within the test, may be given as the test result. For details see Annex C.

9 Validity criteria

Values from an inter-laboratory trial (1999) showed that the reference substance 3,5-dichlorophenol caused 50 % inhibition of activity of anaerobic bacteria in a mass concentration range of 32 mg/l to 510 mg/l (mean 153 mg/l).

The volumes of biogas produced in the control bottles by the end of the test ranged from 21 ml/g dry matter to 149 ml/g dry matter (mean 72 mg/g). There was no obvious relation between the volume of biogas produced and the corresponding EC_{50} value. The final pH varied between 6,1 and 7,5.

The test is valid when an inhibition of greater than 20 % is obtained in the control containing 150 mg/l of 3,5-dichlorophenol, more than 50 ml of biogas per gram dry matter are produced in the control and the pH value is within the range 6,2 to 7,5 at the end of the test.

10 Test report

The test report shall contain the following information:

a) reference to this part of ISO 13641, i.e. ISO 13641-1;

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

c) source of the actively digesting anaerobic sludge;

d) test temperature and range;

e) reference compound and result of the inhibition (at mass concentration used or EC_{50});

f) volume of digester liquor and headspace in the test bottles;

g) mass concentrations of test material and addition mode;

h) main characteristics of biogas measurement and type of pressure meter;

i) all measured data in test, control and reference substance bottles (for example pressure p in pascals);

j) test results, especially EC_{50} (expressed as mg/g of dry matter) and the inhibition curves.

Annex A
(informative)

Calibration of the pressure meter

The meter should be calibrated at regular intervals. Pressure readings may be related to biogas volumes by means of a standard curve produced by injecting known volumes of air at 35 °C ± 1 °C into serum bottles containing a volume of water equal to that of the reaction mixture, V_R.

— Dispense V_R ml aliquots of water, kept at 35 °C ± 1 °C into five serum bottles. Seal the bottles and place in a water bath at 35 °C for 1 h to equilibrate.

— Switch on the pressure meter, allow to stabilize, and adjust to zero.

— Insert the syringe needle through the seal of one of the bottles, open the valve until the pressure meter reads zero and close the valve.

— Repeat the procedure with the remaining bottles.

— Inject 1 ml of air at 35 °C ± 1 °C into each bottle. Insert the syringe needle (on the meter) through the seal of one of the bottles and allow the pressure reading to stabilize. Record the pressure, open the valve until the pressure reads zero and then close the valve.

— Repeat the procedure for the remaining bottles.

— Repeat the total procedure above using 2 ml, 3 ml, 4 ml, 5 ml, 6 ml, 8 ml, 10 ml, 12 ml, 16 ml, 20 ml and 50 ml of air.

— Plot a calibration curve of pressure p (Pa) against air volume injected V_b (ml). The response of the instrument is linear over the range 0 Pa to 70 000 Pa, and 0 ml to 50 ml of air production.

Annex B
(informative)

Testing of liquid samples and sludges

B.1 General

Collect fresh samples of wastewater, sludges, etc. from their sources. Depending on the objective of testing, collect timed "snap" samples or 24-h samples weighted for flow. Test the samples as soon as possible, preferably on the day of collection or store adequately to minimize possible changes. Test the untreated samples. In cases where they are sufficiently acidic or alkaline to change the pH of the sludge inoculum significantly, it may be necessary to neutralize them (pH 7 $\pm$ 0,5) before testing. If this is necessary, state the neutralization conditions in the test report. Samples containing suspended matter should generally be tested without further treatment. However, if they contain gross particles, these should be removed by coarse filtration.

Modifications to the procedure may be necessary to accommodate the expected wide range of toxicity of these samples. In particular, the mass concentration of the inoculum (digesting sludge) in the final reaction mixture may be lowered, and instead of a synthetic substrate, the added substrate may be raw sludge.

B.2 Liquid samples

B.2.1 As an example, for a reaction mixture with a final volume of 100 ml, transfer volumes (1 ml to 50 ml) of the test sample to five sets of triplicate test bottles while being purged with nitrogen and add de-oxygenated dilution water to adjust the volumes to 50 ml (see Table B.1). Add 50 ml of de-oxygenated dilution water to the control bottles. Add a volume of substrate stock solution to all bottles to produce a final mass concentration of 2 g/l of each of the nutrient broth, yeast extract and D-glucose. Finally, transfer 50-ml aliquots to the digesting sludge to all bottles. Then proceed after sealing the bottles.

B.2.2 Alternatively prepare sludges (undigested) from the wastewater mixed with raw sewage sludge.

Mix a suitable volume of the test material with a raw sewage sludge taken from a treatment plant receiving predominantly domestic sewage. Ideally, the volume ratio of test material to raw sludge should reflect the situation in practice. If this is not known, a few probable ratios should be chosen.

Let the mixture settle, withdraw and discard a volume of supernatant liquor equal to that of the test material added. Use the amended raw sludge in the following way.

Table B.1 — Example of liquid test samples

Test sample ml	Deionized dilution water ml	Digesting sludge ml	Nutrient[a] stock solution ml
0 (control)	50	50	2
1	49	50	2
5	45	50	2
10	40	50	2
20	30	50	2
50	0	50	2

[a] See 4.2.2 — Nutrient broth, yeast extract and D-glucose.

B.3 Sludges

If the volume of test sludge to be discharged is known, use this knowledge to prepare reaction mixtures with raw and digesting sludges in test bottles, in the proportion(s) likely to be found in practice. Otherwise prepare mixtures of arbitrary proportions following the procedure in 7.2.1.

For example, transfer test sludge (2 ml to 20 ml) to test bottles, purged with nitrogen, adding de-oxygenated dilution water to a total volume of 20 ml. Add 20 ml of de-oxygenated dilution water to the control bottles.

Prepare a mixture of digesting sludge and the reference raw sludge (from a treatment plant receiving predominantly domestic sewage) in the ratio 3:1 and add 80 ml to all test bottles (see Table B.2). Close the bottles and proceed as in 7.4.

If the test sludges contain much degradable compound they should be adjusted to approximately the same total dry solids mass concentration as present in the raw reference sludge, i.e. by settling or centrifugation, or by dilution. The pattern of addition of these sludges would then be according to the example in Table B.3, that is the total volume of sludge plus test sludge would be the same in all bottles, unlike the situation according to Table B.2.

Table B.2 — Example of sludge samples

Test sample ml	Deionized dilution water ml	Digesting sludge[a] ml	Reference[a] raw sludge ml
0 (control)	20	60	20
2	18	60	20
5	15	60	20
10	10	60	20
15	5	60	20
20	0	60	20

[a] Alternatively, these sludges may be mixed in the ratio 3:1 (digesting:raw) just before use and dispensed in 80 ml portions.

Table B.3 — Alternative example of samples of sludge containing much degradable matter

Test sample ml	Deionized dilution water ml	Digesting sludge[a] ml	Reference[a] raw sludge ml
0 (control)	20	60	20
2	20	60	18
5	20	60	15
10	20	60	10
15	20	60	5
20	20	60	0

[a] Previously adjusted to approximately the same mass concentration of total dry solids as in the reference raw sludge.

Annex C
(informative)

Expression of results in tests with wastewater

In wastewater testing, the lowest ineffective dilution that was observed within the test (LID-value) may be given instead of, or additionally to the EC-values. The LID-value is the lowest tested dilution (highest mass concentration of the wastewater) that resulted in an inhibition below 20 % in the test. The determination of LID-values is in many cases sufficient for the purpose of control of wastewater at regular intervals. If the range of the LID-values is small, they may even be determined in limit tests with two to four dilutions. For the determination of the toxic effect of compound in a single test, the evaluation of EC values is preferred.

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