INTERNATIONAL STANDARD

ISO 13641-2

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

Part 2: Test for low biomass concentrations

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

Partie 2: Essai à de faibles concentrations de biomasse

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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 13641-2 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 2:
Test for low biomass concentrations

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 muds, sediments and other anaerobic environments with low biomass concentration. The growth rate of anaerobic bacteria is much lower, compared with that of aerobic microorganisms. For this reason, the test periods in anaerobic methods are longer than in those with aerobic bacteria. The conditions of this test (for example amount of inoculum and substrate in the test bottles) were adopted to a defined test period over several days. The inoculum can be collected from anaerobic sediments or from large, or laboratory scale, anaerobic digesters.

This method is applicable to materials, 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].

Information obtained by this method can be helpful prior to anaerobic biodegradability testing with low inoculum mass concentrations and for estimations of the potential effects of chemicals and wastewater to anaerobic processes in habitats characterized by a relatively low anaerobic biomass, for example natural sediments and soils.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 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 mixtures of diluted digesting sludge or other sources of anaerobes, and a degradable substrate are incubated alone and simultaneously with a range of mass concentrations of the test material in sealed bottles for a defined incubation time at 35 °C. The amount of biogas (methane and carbon dioxide) produced is measured by the increase in pressure in the bottles before and after addition of acid to the release carbon dioxide from carbonates. The percentage inhibition of biogas production by 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 the plots of percentage inhibition against the logarithm of mass concentration of the test material.

It is possible to use this technique for special investigations, for example with sediments from anaerobic sites in nature. In this case, the incubation temperature in the test bottles can be that of the natural sediments. Anaerobic sediments can contain a high amount of inorganic matter and cell numbers and hence the bacterial activity can be very low in such cases, so that the incubation period needs to be extended.

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.

4.1.3 Phosphoric acid (H_3PO_4), 85 % by mass in water.

4.2 Media

4.2.1 Inoculum.

Collect active digesting anaerobic sludge from a full-scale, or a laboratory, digester or anaerobic sediment from a suitable natural source. Record the source and type of inoculum in the test report. Bottles for collection should be equipped with gas-tight seals and be made of high-density polyethylene or a similar material, which can expand. Glass is not recommended since the bottle may explode. Fill the sample bottles up to 1 cm from the top, seal them tightly and place them in insulated containers (5.1) to minimize temperature shock, until being transferred to an incubator (5.10) maintained at the desired test temperature. 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 inoculum within a few hours of collection, otherwise store at the test temperature (6.1) under a headspace of nitrogen for up to 3 days when little loss of activity will normally occur.

Immediately prior to use, mix the inoculum by gentle stirring and pass it through a mesh sieve (5.2) into a suitable bottle (5.4) through the headspace of which a stream of nitrogen (4.1.2) is passed. Set aside a sample for determination of the mass concentration of total dry solids (see for example ISO 11923). The mass concentration of digester sludge is usually between 20 g/l and 40 g/l total dry solids but anaerobic sediment will be more variable. Thus some sludges may require diluting using dilution water (4.1.1) and some sediments will require concentrating by centrifugation. Use a final mass concentration of 0,20 g/l $\pm$ 0,05 g/l of total dry solids.

Check the pH value of the inoculum and adjust if necessary to 7 $\pm$ 0,5. During centrifuging or dilution of the inoculum make sure that no oxygen penetrates into the suspension. Use, for example, for centrifugation

closed bottles and overlay the liquid with nitrogen gas. The use of a glove box (5.9), filled with nitrogen gas (4.1.2), is recommended for all preparation steps.

4.2.2 Test medium, prepared from 10-fold concentrated test medium (4.2.2.1) with a trace element solution (4.2.2.2).

Use freshly supplied sodium sulfide nonahydrate [4.2.2.1 h)] or wash and dry it before use, to ensure that it has sufficient reducing capacity. If the test is performed without using a glove box (5.9), the mass concentration of sodium sulfide in the stock solution should be increased to 2 g/l. Sodium sulfide may also be added from an appropriate anaerobic stock solution through the septum of the closed test bottles, as this procedure will decrease the risk of oxidation, to obtain a final mass concentration of 0,2 g/l. Alternatively titanium(III)citrate [4.2.2.1 h)] may be used. Add it through the septum of closed test bottles to obtain a final concentration of 0,8 mmol/l to 1,0 mmol/l. Titanium(III)citrate is a highly effective and is a low-toxicity reducing agent, which is prepared as follows. Dissolve 2,94 g of trisodium citrate dihydrate in 50 ml of oxygen-free dilution water (which results in a 200 mmol/l solution) and add 5 ml of a titanium(III)chloride solution (15 g/100 ml dilution water). Neutralize to pH 7 $\pm$ 0,5 with sodium carbonate and dispense to an appropriate serum bottle under a stream of nitrogen. The concentration of titanium(III)citrate in this stock solution is 164 mmol/l.

Use the test medium immediately or store at 4 °C for no longer than 1 day.

4.2.2.1 Tenfold concentrated test medium, prepared with the following:

a)	anhydrous potassium dihydrogenphosphate (KH_2PO_4)	2,7 g
b)	disodium hydrogenphosphate (Na_2HPO_4)	4,44 g (or 11,2 g dodecahydrate)
c)	ammonium chloride NH_4Cl	5,3 g
d)	calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	0,75 g
e)	magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	1,0 g
f)	iron(II)chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$)	0,2 g
g)	resazurin (redox indicator)	0,01 g
h)	sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$) or titanium(III)citrate	1,0 g final concentration 0,8 mmol/l to 1,0 mmol/l
i)	trace element solution (see 4.2.2.2)	10,0 ml
j)	yeast extract	50 g
Dissolve in dilution water (4.1.1) and make up to:		1 000 ml

4.2.2.2 Trace element solution, prepared with the following:

a)	manganese(II)chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)	0,5 g
b)	ortho-boric acid (H_3BO_3)	0,05 g
c)	zinc chloride ($ZnCl_2$)	0,05 g
d)	copper(II)chloride ($CuCl_2$)	0,03 g (or dihydrate: 0,035 g)

e)	sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$)	0,01 g
f)	cobalt(II)chloride hexahydrate ($CoCl_2 \cdot 6H_2O$)	1,0 g
g)	nickel(II)chloride hexaydrate ($NiCl_2 \cdot 6H_2O$)	0,1 g
h)	disodium selenite (Na_2SeO_3)	0,05 g
Dissolve in dilution water (4.1.1) and make up to:		1 000 ml

5 Apparatus

5.1 Insulated containers, for transport of inoculum.

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

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

5.4 Reservoir for digesting sludge or other inoculum, 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.

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

5.6 Pressure-resistant gas-tight closed glass test bottles, with an appropriate nominal size.

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.

5.7 Microsyringes, for the gas-tight connection of the pressure transducer to the headspace in the bottles; also for adding insoluble liquid test materials or acid to the bottles and to release biogas.

5.8 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.

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

5.10 Spark-free incubator, preferably equipped with a **shaking device,** and capable of maintaining the temperature to within $\pm$ 1 °C at 35 °C or other required temperature.

6 Test environment and interferences

6.1 Test environment

Carry out the test by incubating the sealed test bottles at a constant temperature of usually 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, if for example anaerobic sediment is used as inoculum, the test may be conducted at a temperature similar to the natural environment within a comparable range (for example 20 °C ± 1 °C).

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 7.4).

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 (6.1). Sometimes the pressure falls very slowly once the septa have been pierced with the syringe needle.

6.2.5 Remaining substrates in the inoculum

The anaerobic biogas production and the sensitivity of the inoculum are influenced by substrates that 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 can be 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 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 (5.9) 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 inoculum corresponds to the incubation temperature (6.1) during the operation and while the bottles are sealed.

7.1.2 Preliminary test

If the activity of the inoculum (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 0,1 g/l, 0,2 g/l and 0,4 g/l plus 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. Measure biogas in regular intervals. From the results of biogas produced the most suitable conditions for the main test can be deduced which allow the yield of sufficient biogas for measurements and hence the optimal sensitivity without the fear of explosions. Using results from the preliminary test, select the frequency at which pressure measurements should be made, the test duration and the need of acidification at the end of the test.

7.2 Test materials and controls

7.2.1 Test materials

7.2.1.1 Test compound solutions

Prepare separate stock solutions for each water-soluble test compound in oxygen-free dilution water (4.1.1) to contain, for example, 10 g/l of test material. 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 (5.6) and evaporate the solvent before the addition of the inoculum. Liquid water-insoluble test materials may be injected directly into inoculated serum bottles using microsyringes (5.7). For other treatments, see for example ISO 10634, but be aware that any surfactants used to produce emulsions can be inhibitory to anaerobic biogas production.

Add test compounds to the bottles (5.6) 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 (5.6) 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 (5.6) 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 and use oxygen-free dilution water (4.1.1).

7.2.2 Reference compound and controls

Prepare an aqueous 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, adapt the validity criteria.

Set up at least triplicate bottles (5.6), containing inoculum (4.2.1) and substrate only, to act as controls.

Set up further replicate bottles (5.6) containing inoculum (4.2.1) and test medium 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 113 mg/l. This mass concentration of 3,5-dichlorophenol will 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 (5.6) for pH measurement containing inoculum (4.2.1), inorganic medium and substrate. Add the test material (7.2.1) to 2 bottles at the highest mass concentration being tested and the deoxygenated dilution water to the remaining 2 bottles.

7.3 Addition of the inoculum and the test medium

Stir an appropriate volume of sieved digested sludge or other inoculum (4.2.1), previously adjusted to a mass concentration of 0,4 g/l up to 2 g/l of total dry solids in a 5-l bottle (5.4) while passing a stream of nitrogen gas (4.1.2) through the headspace. Flush the test bottles containing aqueous solutions or evaporated solvent solutions of the test compound (7.2.1.1), with a stream of nitrogen gas for about 2 min to remove air. Add deoxygenated dilution water (4.1.1) to bottles containing the insoluble test materials (7.2.1) so as to obtain the same total liquid volume in the bottles.

Dispense a suitable amount of a well-stirred prepared inoculum (4.2.1) with the desired mass concentration of total dry solids into the test bottle followed by test medium (4.2.2), dilution water (4.1.1) and stock solution of the test material (7.2.1), while keeping the bottles under a flow of nitrogen. When adding the sieved inoculum, ensure that it is very well stirred and dose the exact amount of inoculum needed. If a pipette is used, fill it in one step to the exact volume. If the required mark is passed, reject the sample and refill it. Omitting a part of the sample will selectively release solids and result in an unrepresentative sample. Preferably use fixed-volume dosing equipment, or a measuring cylinder.

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

Ensure that the headspace volume is within the range indicated by the preliminary test and that all bottles (controls, test and reference substances) contain the same volume of liquid; where necessary, add de-oxygenated dilution water to make up the volume. Choose, for example, the ratio of gas 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 — Examples of the test set-up for test batches

Reaction mixture	Example 1	Example 2
Mass concentration of prepared inoculum (4.2.1)	0,42 g/l	2,1 g/l
Added volume of inoculum (4.2.1)	45 ml	9 ml
End mass concentration of inoculum in test bottles	0,20 g/l	0,20 g/l
Added amount of test medium (4.2.2)	9 ml	9 ml
Added amount of dilution water (4.1.1)	36 ml	72 ml
Mass concentration of yeast extract in test bottles [4.2.2.1 j)]	9,7 g/l	9,7 g/l
Test compound stock solution (7.2.1)	3 ml	3 ml
Total liquid volume	93 ml	93 ml

7.4 Incubation of bottles and pressure measurement

Transfer the closed bottles to the thermostatically controlled incubator preferably equipped with a shaking device (5.10) and incubate at the desired temperature. If the bottles are incubated without a shaking device, shake 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 of the bottles is, however, not appropriate in all cases, for example if insoluble substances may adhere to the bottom of the bottles.

After about 1 h, equalize the pressure in the bottles to atmospheric pressure by inserting the syringe needle, attached to the pressure meter, through the seal of each bottle in turn. Insert the needle preferably at an angle of about 45° to prevent gas leaking from the bottles. Open the valve until the pressure reaches zero, then close the valve.

The bottles usually should be incubated for about 7 days. Use the experience of the preliminary test (7.1.2) to decide on the test duration. For example, if sediment is used as inoculum and low incubation temperatures are used for the test, it may be necessary to have to prolong the test until sufficient biogas can be produced. If sufficient biogas is produced in less than 7 days, the test would have to be shortened respectively.

Determine the total amount of evolved biogas by regular pressure measurements during the incubation period. Choose the intervals of measurement in such a way that sufficient data points are available to plot a curve of cumulative biogas production. Use the results from the preliminary test (7.1.2) to decide on the frequency of measurement. Alternatively, if for example the biogas production of the inoculum is well known, pressure may be measured only at the end of the test.

For measurement insert the syringe needle, which is connected to the pressure transducer (5.8), through the septum of the bottle. Allow the pressure reading to stabilize and record it. Then open the valve and ventilate. When the meter reads zero close the valve. Dry the needle well before inserting into the next bottle and measure in the same way. If the pressure reading is negative, do not open the valve. Moisture sometimes accumulates in the syringe needle and tubing. This is indicated by a small negative pressure reading. In this case, remove the needle, shake the tubing, dry it with a tissue, then fit a new needle.

Use the results from the preliminary test (7.1.2) to decide whether acidification at the end of the test is useful. If only one measurement is to be made or at the last measurement add 0,5 ml of phosphoric acid (4.1.3) using a microsyringe (5.7) to each bottle. Shake the bottles for at least 30 min. After this time, the carbon dioxide produced will have escaped from the liquid to the gaseous phase.

Alternatively measure the pressure before and after the addition of acid, to distinguish between biogas containing mainly carbon dioxide, which is in the liquid, and biogas containing mainly methane, which is in the headspace. This information may be useful to control the quality of the inoculum. When the rate of carbon dioxide production is much higher than that of methane production, the sensitivity of the fermenting bacteria may be altered. This may also indicate that the methanogenic bacteria are preferentially affected by the test material. Such a shift should not occur when the inoculum has few residual substrates and when the preparation of the test has taken place strictly under anaerobic conditions for example in a glove box.

7.5 pH measurement

Measure and record the pH in bottles set up for this purpose (7.2.2) at the beginning and the end of the test. In addition, the pH may be measured at the end of the test in all those bottles to which acid has not been added.

If the pH value is outside the range of 6,2 to 7,5 at the end of the test, the result is invalid and the test should be repeated (see Clause 9).

8 Calculation

Sum, and average, the pressures recorded at each interval for each set of replicate bottles and calculate the mean cumulative gross biogas pressure. 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 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) (4.2.1).

Calculate either the percentage inhibition achieved by the single mass concentrations of the reference compound (7.2.2) 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 sometimes 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 closest to linearity over a larger range.

Convert the mean pressure of the biogas produced in the control into the volume of biogas by reference to the pressure meter calibration curve (Annex A) and from this calculate the yield of biogas expressed as the volume produced at the selected time (for example 48 h) from 100 ml diluted inoculum at 0,2 g/l to 0,5 g/l of total dry solids.

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

9 Validity criteria

Values from an inter-laboratory trial showed that the reference substance 3,5-dichlorophenol caused 50 % inhibition of activity of anaerobic bacteria in a mass concentration range of 60 mg/l to 200 mg/l (mean 113 mg/l).

The test is valid when an inhibition of greater than 20 % is obtained in the control containing 110 mg/l of 3,5-dichlorophenol, more than 20 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 13641-2;

b) name, specification, mass concentration and addition mode of the test material;

c) source of the inoculum (for example actively digesting anaerobic sludge or anaerobic sediment);

d) test temperature and range;

e) name of the reference substance and result of the inhibition (for example EC_{50});

f) volume of liquid and headspace in the test bottles;

g) main characteristics of pressure measurement and type of pressure meter;

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

i) test results, especially EC_{50} (expressed as mg/l and mg/g dry matter) and the inhibition curve.

Annex A
(informative)

Calibration of the pressure meter

The meter should be calibrated at regular intervals. Pressure readings may be related to gas volumes by means of a standard curve produced by injecting known volumes of biogas at 35 °C ± 2 °C or at the used test temperature 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 ± 2 °C into five serum bottles. Seal the bottles and place in a water bath at 35 °C ± 2 °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 ± 2 °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 gas 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 gas production.

Annex B
(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), which 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 even may be determined in limit tests with two to four dilutions. For the determination of the toxic effect of material in a single test, the evaluation of EC values is preferred.

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