

OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Determination of the inhibition of the activity of anaerobic bacteria – reduction of gas production from anaerobically digesting (sewage) sludge

INTRODUCTION

1. Chemicals discharged to the aquatic environment pass through both aerobic and anaerobic zones, where they may be degraded and/or can inhibit bacterial activity; in some cases they can remain in anaerobic zones undisturbed for decades or longer. In waste water treatment the first stage, primary settlement, is aerobic in the supernatant liquid and anaerobic in the subnatant sludge. This is followed in the secondary stage by an aerobic zone in the activated sludge aeration tank and an anaerobic zone in the subnatant sludge in the secondary settlement tank. Sludge from both of these stages is usually subjected to anaerobic treatment, producing methane and carbon dioxide which are normally used to produce electricity. In the wider environment, chemicals reaching sediments in bays, estuaries and the sea are likely to remain in these anaerobic zones indefinitely if they are not biodegradable. Larger proportions of some chemicals will preferably reach these zones because of their physical properties, such as low solubility in water, high adsorption to suspended solids, as well as inability to be biodegraded aerobically.

2. While it is desirable that chemicals discharged to the environment should be biodegradable under both aerobic and anaerobic conditions, it is essential that such chemicals do not inhibit the activity of microorganisms in either zone. In the UK there have been a few cases of complete inhibition of methane production caused by, for example, pentachlorophenol in industrial discharges, leading to very costly transportation of inhibited sludge from the digesters to 'safe' sites and importation of healthy digesting sludge from neighbouring installations. But there have been many cases of less severe disruption of digestion by several other chemicals, including aliphatic halohydrocarbons (dry-cleaning) and detergents, leading to significant impairment of digester efficiency.

3. Only one OECD Test Guideline, 209 (1), deals with inhibition of bacterial activity (Respiration of activated sludge), which assesses the effect of test substances on the rate of oxygen uptake in the presence of substrate. The method has been widely used to give early warning of possible harmful effects of chemicals on the aerobic treatment of wastewaters, as well as indicating non-inhibitory concentrations of test substances to be used in the various tests for biodegradability. OECD Test Guideline 311 (2) offers a limited opportunity for determining the toxicity of a test substance to gas production by anaerobic sludge, diluted to one tenth of its normal concentration of solids to allow the required precision in the assessment of percentage biodegradation. Because diluted sludge could be more sensitive to inhibitory substances, the ISO group decided to prepare a method using undiluted sludge. At least three texts were examined (from Denmark, Germany and the UK) and finally two ISO standards were prepared, one using undiluted sludge, ISO 13 641-1 (3) and the other using one hundredth diluted sludge, ISO 13 641-2 (4), to represent muds and sediments having low bacterial populations. Both methods were subjected to a ring-test (5); part 1 was confirmed as an acceptable standard but there was disagreement over part 2. The UK considered that, because a significant proportion of participants reported very little or no gas production, partly because the percentage gas space was too high (at 75 %) for optimal sensitivity, the method requires further investigation.

4. Earlier work in the UK (6)(7) described a manometric method using undiluted digesting sludge, plus raw sewage sludge as the substrate, in 500 mL flasks; the apparatus was cumbersome and the stench of the raw sludge was offensive. Later the more compact and convenient apparatus of Shelton and Tiedje (8) as developed by Battersby and Wilson (9) was successfully applied by Wilson et al. (10). Kawahara et al (11) successfully prepared more standard sludges in the laboratory for use in tests for anaerobic biodegradability and inhibition on a number of chemicals. Also, raw sludge as the substrate was replaced to carry out a test either with one hundredth diluted anaerobic sludge or with muds, sediments etc. of low bacterial activity.

5. This method can provide information that is useful in predicting the likely effect of a test substance on gas production in anaerobic digesters. However, only longer tests simulating working digesters more closely can indicate whether adaptation of the microorganisms to the test substance can occur or whether substances likely to be absorbed and adsorbed onto sludge can build up to a toxic concentration over a longer period than allowed in this test.

PRINCIPLE OF THE TEST

6. Aliquots of a mixture of anaerobically digesting sludge (20 g/L to 40 g/L total solids) and a degradable substrate solution are incubated alone and simultaneously with a range of concentrations of the test substance in sealed vessels for up to 3 days. The amount of gas (methane plus carbon dioxide) produced is measured by the increase in pressure (Pa) in the bottles. The percentage inhibition of gas production brought about by the various concentrations of the test substance is calculated from the amounts produced in the respective test and control bottles. The EC₅₀ and other effective concentrations are calculated from plots of percentage inhibition against the concentration of the test chemicals or, more usually, its logarithm.

INFORMATION ON THE TEST SUBSTANCE

7. Test substances should normally be used in the purest form readily available, since impurities in some substances, e.g. chlorophenols, can be much more toxic than the test substance itself. The use of formulated products is not routinely recommended, but for poorly soluble test substances the use of formulated material may be appropriate. Properties of the test substance which should be available include solubility in water and some organic solvents, vapour pressure, adsorption coefficient, hydrolysis and biodegradability under anaerobic conditions.

APPLICABILITY OF THE METHOD

8. The test is applicable to substances which are soluble or insoluble in water, including volatile substances. But special care is necessary with materials of low water-solubility (see ref. (12)) and of high volatility. Also, inocula from other anaerobic sites, e.g. muds, saturated soils, sediments, may be used. Anaerobic bacterial systems that have previously been exposed to toxic substances may be adapted to maintaining their activity in the presence of xenobiotic chemicals. Inocula from adapted bacterial systems may show a higher tolerance to the test substances compared to inocula obtained from non-adapted systems.

REFERENCE SUBSTANCES

9. To check the procedure, a reference substance is tested by setting up appropriate vessels in parallel as part of normal test runs; 3, 5-dichlorophenol has been shown to be a consistent inhibitor of anaerobic gas production, as well as of oxygen consumption by activated sludge and other biochemical reactions. Two other chemicals have been shown to be more inhibitory to methane production than 3, 5-dichlorophenol, namely methylene bis-thiocyanate and pentachlorophenol but results with them have not been validated. Pentachlorophenol is not recommended since it is not readily available in a pure form.

REPRODUCIBILITY OF THE RESULTS

10. In an international ring test (5) there was only fair reproducibility in EC₅₀ values between the 10 participating laboratories for 3, 5-dichlorophenol and 2-bromo-ethane sulphonic acid. (The range for the former was 32mg/L to 502 mg/L and for the latter 220-2190 mg/L.)

Number of laboratories	As mg/L			As mg/g sludge		
	mean	s.d.	cv(%)	mean	s.d.	cv(%)
10	<u>3, 5-Dichlorophenol</u>					
	153	158	103	5	4.6	92
10	<u>2-Bromo-ethane sulphonic acid</u>					
	1058	896	85	34	26	76

EC₅₀ data from ring test – undiluted sludge

11. The high coefficients of variation between laboratories to a large extent reflect differences in the sensitivity of the sludge microorganisms due to either pre-exposure or no pre-exposure to the test substance or other chemically related substances. The precision with which the EC₅₀ value based on the sludge concentration was determined was barely better than the 'volumetric' value (mg/L). The three laboratories which reported the precision of their EC₅₀ values for 3.5-dichlorophenol showed much lower coefficients of variation (22, 9, and 18 % respectively for EC₅₀ mg/g) than those of the means of all ten laboratories. The individual means for the three laboratories were 3.1, 3.2 and 2.8 mg/g, respectively. The lower, acceptable coefficients of variation within laboratories compared with the much higher coefficients between laboratory values, namely 9-22 % cf. 92 %, indicate that there are significant differences in the properties of the individual sludges.

DESCRIPTION OF THE METHOD**Apparatus**

12. Usual laboratory equipment and the following are required:

- (a) Incubator – spark-proof and controlled at 35 °C ± 2°C;

(b) Pressure-resistant glass test vessels of an appropriate nominal size¹, each fitted with a gas-tight coated septum, capable of withstanding about 2 bar or 2×10^5 Pa (for coating use e.g. PTFE = polytetrafluorethene). Glass serum bottles of nominal volume 125 mL, with an actual volume of around 160 mL, sealed with serum septa² and crimped aluminium rings are recommended; but bottles of total volume between 0.1 and 1 litre may be used successfully;

(c) Precision pressure-meter³ and needle attachment

Total gas production (methane plus carbon dioxide) measured by means of a pressure-meter adapted to enable measurement and venting of the gas produced. An example of a suitable instrument is a hand-held precision pressure-meter connected to a syringe needle; a three-way gas-tight valve facilitates the release of excess pressure (ANNEX 1). It is necessary to keep the internal volume of the pressure transducer tubing and valve as low as possible, so that errors introduced by neglecting the volume of the equipment are insignificant;

(d) Insulated containers, for transport of digesting sludge;

(e) Three-way pressure valves;

(f) Sieve, having a 1 mm square mesh;

(g) Reservoir, for digesting sludge, a glass or high-density polyethylene bottle, capacity about 5 litre, fitted with a stirrer and facilities for passing a stream of nitrogen gas (see paragraph 13) through the headspace;

(h) membrane filters (0.2 μm) for sterilising the substrate;

(i) micro syringes, for the gas-tight connection of the pressure transducer (see paragraph 12(c)) to the headspace in the bottles (see paragraph 12(b)); also for adding insoluble liquid test materials into the bottles;

(j) glove box, optional but recommended, with a slight positive pressure of nitrogen.

¹ The recommended size is 0.1 litre to 1 litre.

² The use of gas-tight silicone septa is recommended. It is further recommended that the gas-tightness of caps, especially butyl rubber septa, be tested 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 under the conditions of the test.

- Gas tight coated septa are recommended and must be used for volatile substances (some commercial septa are relatively thin, less than 0.5 cm, and do not stay gas tight after piercing with syringe needle);
- Butyl rubber septa (about 1 cm) are recommended, if the test substances are not volatile (these normally stay gas tight after piercing.)
- Prior to the test it is recommended that the septa are carefully examined for their ability to stay gas tight after piercing.

³ The meter should be used and calibrated at regular intervals, according to the manufacturer's instructions. If a pressure-meter of the prescribed quality is used e.g. capsulated with a steel membrane, no calibration is necessary in the laboratory. It should be calibrated by a licensed institute at the recommended intervals. The accuracy of the calibration can be checked in 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), conversion is recommended over the total range at regular intervals (ANNEX 2).

Reagents

13. Use analytical grade reagents throughout. Nitrogen gas, of high purity with a content of less than 5µL/L oxygen, should be used throughout.

Water

14. If dilution is necessary at any stage, use deionised water previously de-aerated. Analytical controls on this water are not necessary, but ensure that the deionising apparatus is regularly maintained. Use deionised water also for the preparation of stock solutions. Prior to the addition of the anaerobic inoculum to any solution or dilution of test material, make sure that these are oxygen-free. This is done either by blowing nitrogen gas through the dilution water (or through the dilutions) for 1 hour before adding the inoculum, or alternatively by heating the dilution water to the boiling point and cooling to room temperature in an oxygen-free atmosphere.

Digested Sludge

15. Collect actively digesting sludge from a digester at a wastewater treatment plant, or alternatively, from a laboratory digester, treating sludge from predominantly domestic sewage. Practical information regarding sludge from a laboratory digester can be found elsewhere (11). If use of an adapted inoculum is intended, digesting sludge from an industrial sewage treatment plant may be considered. Use wide-necked bottles constructed from high-density polyethylene or a similar material, which can expand, for sludge collection. Add sludge to the sample bottles to within about 1 cm from the top of the bottles, seal them tightly, preferably with a safety valve (paragraph 12(e)), and place in insulated containers (paragraph 12(d)) to minimise temperature shock, until being transferred to an incubator maintained at 35 °C ± 2°C. When opening the bottles, take care to release excess gas pressure either by cautiously loosening the seal, or by means of the three-way pressure-release valve (paragraph 12(e)). It is preferable to use the sludge within a few hours of collection, otherwise store at 35 °C ± 2°C under a headspace of nitrogen for up to 3 days, when little loss of activity normally occurs.

Warning – Digesting sludge produces flammable gases which present fire and explosion risks: it also contains potentially pathogenic organisms, so take appropriate precautions when handling sludge. For safety reasons, do not use glass vessels for collecting sludge.

Inoculum

16. Immediately prior to use, mix the sludge by gentle stirring and pass it through a 1 mm² mesh sieve (paragraph 12(f)) into a suitable bottle (paragraph 12(g)) through the headspace of which a stream of nitrogen is passed. Set aside a sample for measurement of the concentration of total dry solids (see e.g. ISO 11 923 (13)). In general, use the sludge without dilution. The solids concentration is usually between 2% and 4% (w/v). Check the pH value of the sludge and, if necessary, adjust to 7 ± 0.5.

Test substrate

17. Dissolve 10 g nutrient broth (e.g. Oxoid), 10 g of yeast extract and 10 g of D-glucose in deionised water and dilute to 100 mL. Sterilize by filtration through a 0.2 µm membrane filter (paragraph 12(h)) and use immediately or store at 4 °C for not longer than 1 day.

Test substance

18. Prepare a separate stock solution for each water-soluble test substance to contain, for example, 10 g/L of the substance in oxygen-free dilution water (paragraph 14). Use appropriate volumes of these stock

solutions to prepare the reaction mixtures containing graded 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 concentration. The pH of the stock solutions should be adjusted to 7 ± 0.2 if necessary.

19. For test substances which are insufficiently soluble in water, consult ISO 10 634 (12). If an organic solvent is needed to be used, avoid solvents such as chloroform and carbon tetrachloride, which are known strongly to inhibit methane production. Prepare a solution of an appropriate concentration of water-insoluble substance in a suitable volatile solvent, for example, acetone, di-ethylether. Add the required volumes of solvent solution to the empty test bottles (paragraph 12(b)) and evaporate the solvent before the addition of sludge. For other treatments use ISO 10 634 (12) but be aware that any surfactants used to produce emulsions may be inhibitory to anaerobic gas production. If it is thought that the presence of organic solvents and emulsifying agents causes artefacts, the test chemical could be added directly to the test mixture as a powder or liquid. Volatile compounds and water-insoluble liquid test compounds may be injected into inoculated serum bottles, using micro-syringes (paragraph 12(i)).

20. Add test substances to the bottles to give a geometric series of 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 toxicity is not known from similar substances, first carry out a preliminary range-finding test with concentration of 1000 mg/L, 100 mg/L and 10 mg/L to ascertain the appropriate range.

Reference substance

21. Prepare an aqueous solution of 3,5-dichlorophenol (10 g/L) by gradually adding the minimum amount of 5 mol/L of sodium hydroxide solution to the solid, while shaking, until it has dissolved. Then add de-oxygenated dilution water (paragraph 14) to the required volume; sonication may aid dissolution. Other reference substances may be used when the average range of the EC_{50} has been obtained in at least three tests with different inocula (different sources or different times of collection).

INTERFERENCE/ERRORS

22. Some constituents of sludge presumably could react with potential inhibitors making them unavailable to micro-organisms so giving lower, or no, inhibition. Also, if the sludge already contains a substance which is inhibitory, erroneous results would be obtained when that substance was subjected to the test. Apart from these possibilities, there are a number of identified factors which can lead to false results. These are listed in ANNEX 3, together with methods of eliminating or at least reducing errors.

TEST PROCEDURE

23. The number of necessary replicates depends on the degree of precision required for the inhibition indices. If the bottle seals are sufficiently gas-tight over the duration of the test, set up just one batch (at least triplicates) of test bottles at each concentration required. Similarly, set up one batch of bottles with reference substance and one set of controls. However, if the seals of the bottles are reliable for only one or a few piercings, set up a batch (e.g. triplicates) of the test bottles for each interval (t) for which results are required for all concentrations of a test substance to be tested. Similarly, set up 't' batches of bottles for the reference substance and for the controls.

24. The use of a glove box (paragraph 12(j)) is recommended. At least 30 minutes before starting the test, start a flow of nitrogen gas through the glove box containing all the necessary equipment. Ensure that the temperature of the sludge is within $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ during handling and sealing of the bottles.

Preliminary Test

25. If the activity of the sludge is unknown, it is recommended to carry out a preliminary test. Set up controls to give, for example, concentrations of solids of 10 g/L, 20 g/L and 40 g/L plus substrate but use no test substance. Also, use different volumes of reaction mixture in order to have three or four ratios of volume of headspace to volume of liquid. From the results of gas volumes produced at various time intervals, the most suitable conditions which allow two daily measurements yielding significant volumes of gas and release of pressure per day at optimal sensitivity⁴ without fear of explosions.

Addition of test substances

26. Add water-soluble test substances to empty test bottles (paragraph 12(b)) as aqueous solutions (paragraph 18). Use at least triplicate sets of bottles for each of a range of concentrations (paragraph 20). In the case of insoluble and poorly soluble test substance, inject solutions of these in organic solvents using a micro-syringe into empty bottles to give replicate sets of each five concentrations of test substance. Evaporate the solvent by passing a jet of nitrogen gas over the surface of the solutions in the test bottles. Alternatively, add insoluble solid chemicals as weighed amounts of the solid directly to the test bottles.

27. If insoluble and poorly water-soluble liquid test substances are not added using a solvent, add them directly by micro-syringe to the test bottles after addition of inoculum and test substrate (see paragraph 30). Volatile test substances may be added in the same way.

Addition of inoculum and substrate

28. Stir an appropriate volume of sieved digesting sludge (see paragraph 16) in a 5 litre bottle (paragraph 12(g)), while passing a stream of nitrogen gas through the headspace. Flush test bottles, containing aqueous solutions or evaporated solvent solutions of test substances, with a stream of nitrogen gas, for about two minutes to remove air. Dispense aliquots, e.g. 100 mL, of the well-mixed sludge into the test bottles using a large-tipped pipette or a measuring cylinder. It is essential to fill the pipette in one step to the exact volume of sludge required because of the ease of settlement of sludge solids. If more is taken up, empty the pipette and start again.

29. Then add sufficient substrate solution (paragraph 17) to give a concentration of 2 g/L of each of the nutrient broth, yeast extract and D-glucose in the mixture, while nitrogen is still flushing through. The following is an example for test batches.

⁴ This applies to the experimental set-up and experimental conditions whereby the volumes of gas produced – from control blanks and from vessels indicating 70 - 80 % inhibition – may be estimated with acceptable margins of error.

Final mass concentration of test substance in test bottles (mg/L)	Volume of test substance (mL)		Reagents and media (mL)		
	Stock solution a) 10 g/L para. 18	Stock solution b) 1 g/L para. 18	Dilution water para. 14	Inoculum para. 16	Substrate para. 17
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

Total volume of bottle = 160 mL. Volume of liquid = 103 mL

Gas volume = 57 mL, or 35.6% of total volume.

30. Similarly flush out with nitrogen gas sufficient empty test bottles to deal with any volatile and insoluble liquid test substance (see paragraph 27).

Controls and reference substance

31. Set up at least triplicate sets of bottles, containing sludge and substrate only, to act as controls. Set up further replicate bottles containing sludge and substrate plus sufficient stock solution of the reference substance, 3,5-dichlorophenol (paragraph 21) to result in a final concentration of 150 mg/L. This concentration should inhibit gas production by about 50 %. Alternatively, set up a range of concentrations of the reference substance. In addition, set up four extra bottles for pH measurement which contain sludge, de-oxygenated water and substrate. Add the test substance to two bottles at the highest concentration being tested and add de-oxygenated water to the remaining two bottles.

32. Ensure that all bottles – test and reference substances, and controls – contain the same volume (V_R) of liquid; where necessary, add de-oxygenated deionised water (paragraph 14) to make up the volume. The headspace should be between 10 % and 40 % of the bottle volume, the actual value being selected from the data obtained from the preliminary test. After adding all constituents to the bottles, remove the needle supplying the gas and seal each bottle with a rubber stopper and an aluminium cap (Paragraph 12(b)) moistening the stopper with a drop of deionised water to aid insertion. Mix the contents of each bottle by shaking.

Incubation of bottles

33. Transfer the bottles to the thermostatically controlled incubator, preferably equipped with a shaking device, and maintained at $35\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The bottles are incubated in the dark. After about 1 hour, equalise the pressure in the bottles to atmosphere by inserting the syringe needle, attached to the pressure-meter (paragraph 12(c)), through the seal of each bottle in turn, open the valve until the pressure-meter reads zero and finally close the valve. The needle should be inserted at an angle of about 45° to prevent gas leaking from the bottles. If the bottles are incubated without shaking facility, shake manually twice

each day during the total incubation period to equilibrate the system. Incubate the bottles and invert them to prevent any loss of gas through the septum. Inversion is, however, not appropriate in cases in which insoluble test substances may adhere to the bottom of the flask.

Pressure measurement

34. When the bottles have reached $35\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$, measure and record the pH of the contents of two of the four bottles set up for the purpose and discard the contents; continue incubating remaining bottles in the dark. Measure and record the pressure in the bottles twice a day over the following 48 hours to 72 hours by inserting the needle of the pressure-meter through the seal of each bottle, in turn, drying the needle between measurements. Keep all parts of the bottle at the incubation temperature during the measurement, which should be carried out as quickly as possible. Allow the pressure reading to stabilise and record it. Then open the valve for ventilation and close it when the pressure reads zero. Continue the test usually for 48 hours from the time of first equalising the pressure, designated 'time 0'. The number of readings and ventilations should be limited for volatile chemicals to one (at the end of incubation) or two to minimise loss of test substance (10).

35. If the pressure reading is negative, do not open the valve. Moisture sometimes accumulates in the syringe needle and tubing, 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.

pH measurement

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

DATA AND REPORTING

Expression of results

37. Calculate the sum and average of the pressures recorded at each time interval for each set of replicate bottles and calculate the mean cumulative gross gas pressure at each time interval for each set of replicates. Plot curves of mean cumulative gas production (Pa) against time for control, test and reference bottles. Select a time on the linear part of the curve, usually 48 hours, and calculate the percentage inhibition (I) for each concentration from equation [1]:

$$I = (1 - P_t/P_c) \times 100 \text{ - - - - [1]},$$

where

- I = percentage inhibition;
- P_t = the gas pressure produced with test material at selected time, in Pascal (Pa);
- P_c = the gas pressure produced in the control at the same time, in Pascal (Pa).

It would be advisable to draw both plots, i.e. Plot I against concentration and also against logarithm of the concentration so that the curve which is nearer to linearity may be selected. Assess the EC_{50} (mg/L) value visually or by regression analysis from that curve nearer to linearity. For comparative purposes it may be more useful to express the concentration of the substance as mg substance/g of total dry solids. To obtain this concentration, divide the volumetric concentration (mg/L) by the volumetric concentration of dry sludge solids (g/L) (paragraph 16).

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

39. Convert the mean pressure of the gas produced in the control (PcPa) to the volume by reference to the pressure-meter calibration curve (ANNEX 2) and from this calculate the yield of gas, expressed as the volume produced in 48 hours from 100 mL undiluted sludge at a solids concentration of 2 % (20 g/L) to 4 % (40 g/L).

Validity criteria

40. Results from the ISO inter-laboratory trial (5) showed the reference substance (3,5-dichlorophenol) caused 50% inhibition of gas production in a range of concentrations of 32 mg/L to 510 mg/L mean 153 mg/L (paragraph 10). This range is so wide that firm limits for inhibition cannot confidentially be set as validity criteria; this should be possible when developments have shown how to produce more consistent inocula. The volumes of gas produced in control bottles in 48 hour ranged from 21 mL/g sludge dry matter to 149 mL/g (mean 72 mL/g). There was no obvious relation between volume of gas produced and the corresponding EC₅₀ value. The final pH varied between 6.1 and 7.5.

41. The test is considered to be valid when an inhibition of greater than 20% is obtained in the reference control containing 150 mg/L of 3,5-dichlorophenol, more than 50 mL of gas per g of dry matter is produced in the blank control and the pH value is within the range of 6.2 to 7.5 at the end of the test.

Test Report

42. The test report must include the following information:

Test substance:

- common name, chemical name, CAS number, structural formula and relevant physico-chemical properties;
- purity (impurities) of test substance.

Test conditions:

- volumes of liquid contents and of headspace in test vessels;
- descriptions of the test vessels and gas measurement (e.g. type of pressure-meter);
- application of test substance and reference substance to the test system, test concentrations used and use of any solvents;
- details of the inoculum used: name of sewage treatment plant, description of the source of waste water treated (e.g. operating temperature, sludge retention time, predominantly domestic sewage or industrial waste, etc.), concentration of solids, gas production activity of anaerobic digester, previous exposure or possible pre-adaptation to toxic substances or site of collection of mud, sediment etc;
- incubation temperature and range;
- number of replicates.

Results:

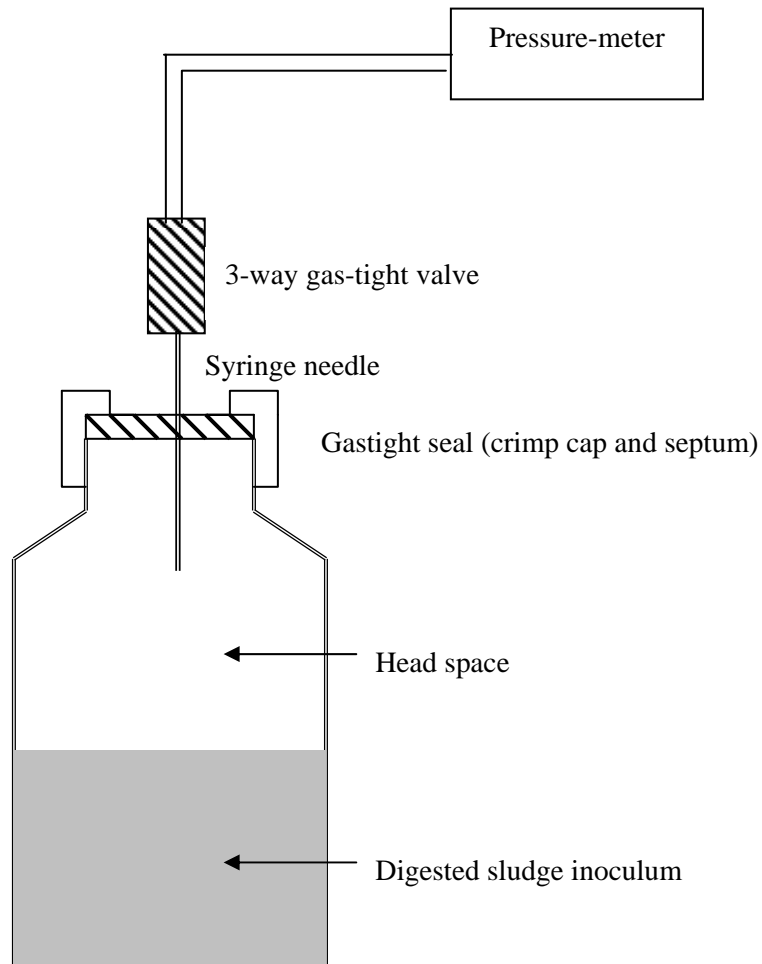
- pH values at end of test;
- all the measured data collected in the test, blank and reference substance control vessels, as appropriate (e.g. pressure in Pa or millibars) in tabular form;
- percentage inhibition in test and reference bottles, and the inhibition-concentration curves;
- calculation of EC₅₀ values, expressed as mg/L and mg/g;
- gas production per g sludge in 48 hours;
- reasons for any rejection of the test results;

- discussion of results, including any deviations from the procedures in this guideline and discuss any deviations in the test results due to interferences and errors from what would be expected;
- address also whether the purpose of the test was to measure the toxicity to either pre-exposed or non pre-exposed microorganisms.

LITERATURE

- (1) OECD Guidelines for testing chemicals (1984). 209: Activated Sludge, Respiration Inhibition Test. Organisation for Economic Co-operation and Development, Paris.
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- (3) International Organisation for Standardisation (2003) ISO 13 641-1 Water Quality – Determination of inhibition of gas production of anaerobic bacteria – Part 1: General Test.
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- (5) ISO (2000) Ring test of ISO 13 641-1 and ISO 13 641-2. Determination of inhibition of activity of anaerobic bacteria. BL 6958/A. Evans MR, Painter HA. Brixham Environmental Laboratory, AstraZeneca UK Ltd., Brixham, TQ5 8BA UK.
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- (11) Kawahara K, Yakabe Y, Chida T, and Kida K (1999). Evaluation of laboratory-made sludge for an anaerobic biodegradability test and its use for assessment of 13 chemicals. *Chemosphere*, 39 (12), 2007-2018.

- (12) International Organization for Standardization (1995) ISO 10 634 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.
- (13) International Organization for Standardization (1997) ISO 11 923 Water Quality - Determination of suspended solids by filtration through glass-fibre filters.

ANNEX 1**Example of an apparatus to measure biogas production by gas pressure**

Test vessels in an environment of $35\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$

ANNEX 2**Conversion of the Pressure-meter**

The pressure-meter readings may be related to gas volumes by means of a standard curve and from this the volume of gas produced per g dry sludge per 48 hours may be calculated. This activity index is used as one of the criteria by which to assess the validity of test results. The calibration curve is produced by injecting known volumes of gas at $35\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in 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 $35\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ into five serum bottles. Seal the bottles and place in a water bath at $35\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 1 hour to equilibrate;
- Switch on the pressure-meter, allow to stabilise, 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 $35\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ into each bottle. Insert the needle (on the meter) through the seal of one of the bottles and allow the pressure reading to stabilise. Record the pressure, open the valve until the pressure reads zero and then close the valve;
- Repeat the procedure with the remaining bottles;
- Repeat the total procedure 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 conversion curve of pressure (Pa) against gas volume injected (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 3**Identified factors which can lead to false results**(a) Quality of the bottle-caps

Different types of septa for the serum bottles are available commercially; many of them, including butyl rubber, lose tightness when pierced with a needle under the conditions of this test. Sometimes the pressure falls very slowly once the septum has been pierced with the syringe needle. The use of gas-tight septa is recommended to overcome leaks (paragraph 12(b)).

(b) Moisture in the syringe needle

Moisture sometimes accumulates in the syringe needle and tubing, and is indicated by a small negative pressure reading. To rectify this remove the needle and shake the tubing, dry with a tissue and fit a new needle (paragraphs 12(c) and 35).

(c) Oxygen contamination

Anaerobic methods are subject to error from contamination by oxygen, which can cause lower gas production. In this method this possibility should be minimized by the use of strictly anaerobic techniques, including use of a glove box.

(d) Gross substrates in sludge

The anaerobic gas 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 still often contains recognizable matter like hair and plant residues of cellulose, which tend to make it difficult to take representative samples. By sieving the sludge gross insoluble matter can be removed, which makes representative sampling more likely (paragraph 16).

(e) Volatile test substances

Volatile test substances will be released into the headspace of the test bottles. This may result in the loss of some of the test material from the system during venting after pressure measurements, yielding falsely high EC₅₀ values. By suitable choice of ratio of headspace volume to liquid volume and by not venting after taking pressure measurements, the error can be reduced (10).

(f) Non-linearity of gas production

If the plot of mean cumulative gas production against incubation time is not approximately linear over the 48h period, the accuracy of the test may be lowered. To overcome this, it may be advisable to use digesting sludge from a different source and/or to add an increased concentration of the test substrate-nutrient broth, yeast extract and glucose (paragraph 29).

ANNEX 4**Application to environmental samples of low biomass concentration – anaerobic muds, sediments, etc.****Introduction**

A.1 In general, the specific microbial activity (volume of gas produced per g dry solids) of naturally occurring anaerobic muds, sediments, soils, etc, is much lower than that of anaerobic sludge derived from sewage. Because of this, when the inhibitory effects of chemicals on these less active samples are to be measured some of the experimental conditions have to be modified. For these less active samples there are two general course of action possible:

- (a) Carry out a modified preliminary test (paragraph 25) with the undiluted sample of mud, soil, etc at $35\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ or at the temperature at the sample site of collection, for more accurate simulation (as in Part 1 of ISO 13 641);
- (b) Or make the test with a dilute (1 in 100) digester sludge to simulate the low activity expected from the environment sample, but maintain the temperature at $35\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ (as in Part 2 of ISO 13 641).

A.2 Option (a) may be achieved by following the method described here (equivalent to Part 1 of ISO 13 641), but it is essential to make a preliminary test (paragraph 25) to ascertain optimal conditions, unless these are already known from previous testing. The mud or sediment sample should be thoroughly mixed, e.g. in a blender, and, if necessary, diluted with a small proportion of de-aerated dilution water (paragraph 14) so that it is sufficiently mobile to be transferred by a coarse-tipped pipette or a measuring cylinder. If it is considered that nutrients may be lacking, the mud sample may be centrifuged (under anaerobic conditions) and re-suspended in the mineral medium containing yeast extract (A.11)

A.3 Option (b). This reasonably mimics the low activity of environmental samples but lacks the high concentration of suspended solids present in these samples. The role of these solids in inhibition is not known, but it is possible that reaction between the test chemicals and constituents of the mud, as well as adsorption of the test chemicals onto the solids, could result in a lowering of toxicity of the test chemical.

A.4 Temperature is another important factor: for strict simulation, tests should be made at the temperature of the sample site, since different groups of methane-producing consortia of bacteria are known to operate within different temperature ranges, namely thermophiles ($\sim 30\text{-}35^{\circ}\text{C}$), mesophiles ($20\text{-}25^{\circ}\text{C}$) and psychrophiles ($<20^{\circ}\text{C}$), which may display different inhibitory patterns.

A.5 Duration. In the general test, Part 1, using undiluted sludge, the production of gas in the 2-4 days was always sufficient, while in Part 2 with one-hundred diluted sludge insufficient gas, if any, was produced in this period in the ring test. Madsen et al (1996), in describing this latter test, say at least 7 days should be allowed.

Testing with low biomass concentration (Option b)

The following changes and amendments should be made, adding to or replacing some existing paragraphs and sub-paragraphs of the main text.

A.6 Add to Paragraph 6: Principle of the test;

“This technique may be used with 1 in 100 diluted anaerobic sludge, partially to simulate the low activity of muds and sediments. The incubation temperature may be either 35 °C or that of the site from which the sample was collected. Since the bacterial activity is much less than in undiluted sludge, the incubation period should be extended to at least 7 days.”

A.7 Add to paragraph 12 (a):

“the incubator should be capable of operating down to temperatures of 15 °C.”

A.8 Add an extra reagent after Paragraph 13:

“Phosphoric acid (H₃PO₄), 85% by mass in water.”

A.9 Add at end of Paragraph 16:

“Use a final concentration of 0.20± 0.05 g/L of total dry solids in the test.”

A.10 Paragraph 17. Test substrate

This substrate is not to be used, but is replaced by yeast extract (see paragraphs 17; A.11, A.12, A.13).

A.11 A mineral medium, including trace elements, for diluting anaerobic sludge, is required and for convenience the organic substrate, yeast extract, is added to this medium.

Add after Paragraph 17:

“(a) Test mineral medium, with yeast extract.

This is prepared from a 10-fold concentrated test medium (paragraph 17 (b); A.12) with a trace element solution (paragraph 17 (c); A.13). Use freshly supplied sodium sulphide nonahydrate (paragraph 17 (b); A.12) 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 (paragraph 12 (j)), the concentration of sodium sulphide in the stock solution should be increased to 2 g/L (from 1 g/L). Sodium sulphide may also be added from an appropriate stock solution through the septum of the closed test bottles, as this procedure will decrease the risk of oxidation, to obtain a final concentration of 0.2 g/L. Alternatively titanium (III) citrate (paragraph 17 (b)) may be used. Add it through the septum of closed test bottles to obtain a concentration of 0.8 mmol/L to 1.0 mmol/L. Titanium (III) citrate is a highly effective and 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 (paragraph 14) (which results in a 200 mmol/L solution) and add 5 ml of a titanium (III) chloride solution (15 g/100 L dilution water). Neutralize to pH 7± 0.5 with sodium carbonate and dispense to an appropriate serum bottle under a stream of nitrogen gas. 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.

A.12 (b) Tenfold concentrated test medium, prepared with the following:

anhydrous potassium dihydrogenphosphate (KH_2PO_4)	2.7 g
Disodium hydrogen phosphate (Na_2HPO_4) (or 11.2 g dodecahydrate)	4.4 g
ammonium chloride (NH_4Cl)	5.3 g
calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	0.75 g
magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)	1.0 g
iron (II) chloride tetrahydrate ($\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$)	0.2 g
resazurin (redox indicator)	0.01 g
sodium sulphide nonahydrate ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$)	1.0 g
(or titanium (III) citrate) final concentration	0.8 mmol/L to 1.0 mmol/L
trace element solution (see paragraph 17 (c); A.13)	10.0 mL
yeast extract	100 g
Dissolve in dilution water (paragraph 14) and make up to:	1000 mL

A.13 (c) Trace element solution, prepared with the following:

manganese (II) chloride tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$)	0.5 g
ortho-boric acid (H_3BO_3)	0.05 g
zinc chloride (ZnCl_2)	0.05 g
copper (II) chloride (CuCl_2)	0.03 g
sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$)	0.01 g
cobalt (II) chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$)	1.0 g
nickel (II) chloride hexahydrate ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$)	0.1 g
disodium selenite (Na_2SeO_3)	0.05 g
Dissolve in dilution water (paragraph 14) and make up to:	1000 mL”

A.14 Paragraph 25: Preliminary test

It is essential that a preliminary test is made as described in paragraph 24, except that the concentration of sludge solids should be one hundredth of those given, that is 0.1g/L, 0.2g/L and 0.4g/L. The duration of incubation should be at least 7 days.

NOTE: In the ring test (5) the headspace volume was much too high at 75% total volume; it should be in the recommended range of 10%-40%. The relevant criterion is that the volume of gas produced at around 80% inhibition should be measurable with acceptable precision (e.g. $\pm 5\%$ to $\pm 10\%$).

A.15 Paragraph 26 to 30: Addition of test substance, inoculum and substrate.

The additions are made in the same way as described in these paragraphs, but the substrate solution (paragraph 17) is replaced by the test medium plus yeast extract substrate (A.11).

Also, the final concentration of dry sludge solids is reduced from 2 g/L - 4 g/L to 0.2 ± 0.05 g/L (A.9). Two examples of the addition of components to the test mixture are given in Table A.1, which replaces the table in paragraph 29.

A.16 Paragraph 33: Incubation of bottles

Because of the expected lower rate of gas production, incubation is carried on for at least 7 days.

A.17 Paragraph 34: Pressure measurements

The same procedure for measuring the pressure in the headspace of the bottles is used as described in paragraph 34 if the amounts in the gaseous phase are required. If total amounts of CO₂ plus CH₄ are to be measured, the pH of the liquid phase is reduced to about pH 2 by the injection of H₃PO₄ into each relevant bottle and measuring the pressure after 30 minutes shaking at the temperature of the test. However, more information on the quality of the inoculum may be obtained by measuring the pressure in each bottle before and after acidification. For example when the rate of CO₂ production is much higher than that of methane, the sensitivity of the fermentative bacteria may be altered and/or methanogenic bacteria are preferentially affected by the test substance.

A.18 Paragraph 36: pH measurement

If H₃PO₄ is to be used some extra bottles, to which no H₃PO₄ is added, would have to be set up especially for the pH measurement.

Reference: Madsen, T, Rasmussen, HB; and Nilsson, L (1996), Methods for screening anaerobic biodegradability and toxicity of organic chemicals. Project No.336, Water Quality Institute, Danish Environment Protection Agency, Copenhagen.

Table A.1. Examples of the test set-up for test batches

Reaction Mixture components	Example 1	Example 2	Normal order of addition
Concentration of prepared inoculum (g/L)	0.42	2.1	-
Volume of inoculum added (mL)	45	9	4
Concentration of inoculum in test bottles (g/L)	0.20	0.20	-
Volume of test medium added (mL)	9	9	2
Volume of dilution water added (mL)	36	72	3
Concentration of yeast extract in test bottles (g/L)	9.7	9.7	-
Volume of test substance stock solution (mL)	3	3	1
Total liquid volume (mL)	93	93	-