OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE 311

Anaerobic Biodegradability of Organic Compounds in Digested Sludge: ~~Method Bb~~y Measurement of Gas Production

INTRODUCTION

1. There are a number of screening tests for assessing aerobic biodegradability of organic chemicals (OECD 301 A-F; 302 A-C; 303 A) (1)(2) and the results of applying these have been successfully used to predict the fate of chemicals in the aerobic environment, particularly in the aerobic stages of waste water treatment. Various proportions of water-insoluble chemicals, as well as of those~~,~~ which adsorb on to sewage solids, are also dealt with aerobically, since they are present in settled sewage. However, the larger fractions of these chemicals are bound to the primary settled sludge, which is separated from raw sewage in settlement tanks before the settled, or supernatant, sewage is treated aerobically. The sludge, containing some of the soluble compounds in the interstitial liquid, is then passed to heated digesters for anaerobic treatment. As yet there are no tests in this series for assessing anaerobic biodegradability in anaerobic digesters and this test is targeted to fill this gap; it is not necessarily applicable to other anoxic environmental compartments.

2. Respirometric techniques that measure the amounts of gas produced, mainly methane (CH_4) and carbon dioxide (CO_2) under anaerobic conditions have been used successfully for assessing anaerobic biodegradability. Birch et al (3) reviewed these procedures and concluded that the work of Shelton and Tiedje (4), based on earlier studies (5)(6)(7), was the most comprehensive. The method (4), which was further~~,~~ developed by others (8) and has become the American standards (9)(10), did not resolve problems related to the differing solubilities of CO_2 and CH_4 in the test medium and to the calculation of the theoretical gas production of a test substance. The ECETOC report (3) recommended the additional measurement of the dissolved inorganic carbon (DIC) content of the supernatant liquid, which made the technique more widely applicable. The ECETOC method was subjected to an international calibration exercise (or ring test) and became the ISO Standard, ISO 11734 (11).

3. This Guideline, which is based on ISO 11734 (11), describes a screening method for the evaluation of potential anaerobic biodegradability of organic chemicals under a specific condition (i.e. in an anaerobic digester at a given time and range of concentration of micro-organisms). Because a diluted sludge is used with a relatively high concentration of test substance and the duration of the test typically is longer than the retention time in anaerobic digesters, the conditions of the test do not necessarily correspond to the~~optimal~~ conditions in aerobic digesters,~~allowing maximal biodegradation~~ nor is it applicable for the assessment of anaerobic biodegradability of organic chemicals under different environmental conditions. Sludge is exposed to the test substance for up to 60 days, which is longer than the normal sludge retention time (25 to 30 days) in anaerobic digesters, though at industrial sites retention times may be much longer.

PRINCIPLE OF THE TEST

4. Washed ~~anaerobically~~ digest~~ed~~ing sludge [1], containing low (<10 mg/L) concentrations of inorganic carbon (IC), is diluted about ten-fold to a total solids concentration of 1 g/L to 3 g/L and incubated at 35 ± 2°C in sealed vessels with the test substance at 20 to 100 mg C/L for up to 60 days. Allowance is made for measuring the activity of the sludge by running parallel blank controls with sludge inoculum in the medium but without test substance.

5. The increase in headspace pressure in the vessels resulting from the production of carbon dioxide and methane is measured. Much of the CO_2 produced will be dissolved in the liquid phase or transformed into carbonate or hydrogen carbonate under the conditions of the test. This inorganic carbon is measured at the end of the test.

6. The amount of carbon (inorganic plus methane) resulting from the biodegradation of the test substance is calculated from the net gas production and net IC formation in the liquid phase in excess ~~over~~of blank control values. The extent of biodegradation is calculated from total IC and methane--C produced as a percentage of the measured or calculated amount of carbon added as test substance~~compound~~. The course of biodegradation can be followed by taking intermediate measurements of gas production only. Additionally the primary biodegradation can be determined by specific analyses at the beginning and end of the test.

INFORMATION ON THE TEST SUBSTANCE

7. The purity, water solubility, volatility and adsorption characteristics of the test substance should be known to enable correct interpretation of results to be made. The organic carbon content (% w/w) of the test substance needs to be known either from its chemical structure or by measurement. For volatile test substances, a measured or calculated Henry's law constant is helpful in deciding whether the test is applicable. Information on the toxicity of the test substance for anaerobic bacteria is useful in selecting an appropriate test concentration, and for interpreting results showing poor biodegradability. It is recommended to include the inhibition control unless it is known that the test substance is not inhibitory to anaerobic microbial activities (see paragraph 21 and ISO 13641-1 (12))

APPLICABILITY OF THE METHOD

8. The test may be applied to water-soluble chemicals; it may also be applied to poorly soluble and insoluble chemicals, provided that a method of exact dosing is used e.g. see ISO 10634 (1~~2~~3). In general, a case by case decision is necessary for volatile substances. Special steps may have to be taken, for example, not releasing gas during the test.

REFERENCE SUBSTANCES

9. To check the procedure, a reference substance~~compound~~ is tested by setting up appropriate vessels in parallel as part of normal test runs. Phenol, sodium benzoate and polyethylene glycol 400 are examples and would be expected to be degraded by more than 60% theoretical inorganic carbon (ThIC) within ~~21~~60 days (3)(14).

[1] Digested sludge is a mixture of the settled phases of sewage or activated sludge, which have been incubated in an anaerobic digester at about 35°C to reduce biomass and odour problems and to improve the dewater-ability to the sludge. It consists of an association of anaerobic fermentative and methanogenic bacteria producing carbon dioxide and methane (11).

REPRODUCIBILITY OF TEST RESULTS

10. In an international ring test (1~~2~~4) there was good reproducibility in gas pressure measurements between triplicate vessels. The relative standard deviation (coefficient of variation, COV) was usually below 10 % ~~not significant~~, although this value often increased to >20% in the presence of toxic chemicals or towards the end of the 60-d incubation period. Higher deviations were also found in vessels of volume <150 ml. Final pH values of the test media were in the range 6.5-7.0.

11. The following results were obtained in the ring test.

Test Substance	Total data n_1	Mean degradation (of total data) (%)	Relative Standard deviation (of total data) (%)	Valid data n_2	Mean degradation (of valid data) (%)	Relative Standard deviation (of valid data) (%)	Data >60% degradation in valid tests n_3
Palmitic acid	36	68.7 ± 30.7	45	27	72.2 ± 18.8	26	19 = 70%*
Polyethylene Glycol 400	38	79.8 ± 28.0	35	29	77.7 ± 17.8	23	24 = 83 %*

* Proportion of n_2

12. The coefficients of variation of the mean for all values obtained with palmitic acid and polyethylene glycol 400 were as high as 45% (n = 36) and 35% (n = 38) respectively. When values of <40% and >100% were omitted (the former being assumed to be due to sub-optimal conditions, the latter due to unknown reasons), the COVs were reduced to 26% and 23%, respectively. The proportions of "valid" values attaining at least 60% degradation were 70% for palmitic acid and 83% for polyethylene glycol 400. The proportions of the percentage biodegradation derived from DIC measurements were relatively low but variable. For palmitic acid the range was 0-35%, mean 12% with COV~~c.v.~~ of 92% and for polyethyleneglycol 400 0-40%, mean 24%, with COV~~c.v.~~ of 54%.

DESCRIPTION OF THE TEST METHOD

Apparatus

13. 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 nominal size 0.1 litre to 1 litre, each fitted with a gas-tight septum, capable of withstanding about 2 bar. The headspace volume should be about 10% to 30% of the total volume. If biogas is released regularly, about 10% headspace volume is appropriate, but if the gas release is made only at the end of the test 30% is appropriate. Glass serum bottles, of nominal volume 125ml, total volume around 160ml, sealed with gas-tight silicone septa~~butyl rubber serum caps~~ and crimped aluminium rings are recommended when the pressure is released at each sampling time. If no pressure release is intended, bigger bottles (e.g. nominal volume 500 ml) should be used;

(c) Pressure-measuring device, for example, a pressure meter (Annex 1) connected to a suitable syringe needle; a 3-way gas-tight valve facilitates the release of excess pressure. The device should be used and calibrated according to the manufacturer's instructions. 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.~~;~~

~~Note: As an optional method, the pressure measuring device meter will be calibrated by injecting known volumes of gas into pressure control chambers (prepared in the same manner as the blank controls) which have been equilibrated to atmospheric pressure. A standard curve of the mL injected versus time will be generated and a linear regression will be performed to determine the line equation. Pressure meter readings will be converted to mL of gas produced using the standard curve. The number of moles (n) of gas in the headspace of each chamber will be calculated by dividing the cumulative gas production (mL) by 25286 mL/mole, which is the volume occupied by one mole of gas at 35 ° C and atmospheric pressure. Since 1 mole of CH_4 and 1 mole of CO_2 each contain 12 g of carbon, the mass of carbon (mg) in the headspace (CH) is given by: $m_h = 12 \times n \times 10^3$.~~

Warning ~~—~~ Take care~~Care should be taken~~ to avoid needle-stick injuries when using micro-syringes.

(d) Carbon analyser, suitable for the direct determination of inorganic carbon in the range of 1 mg/L to 200 mg/L;
(e) Syringes of high precision for gaseous and liquid samples;
(f) Magnetic stirrers and followers (optional);
(g) Glove box (recommended).

Reagents

14. Use ~~A~~analytical grade reagents ~~should be used~~ throughout.

Water

15. Distilled or deionised water (if necessary, de-oxygenated by sparging with nitrogen gas), containing less than 2 mg/L DOC~~, should be used~~.

Test medium

16. Prepare t~~T~~he dilution medium ~~should~~ to contain the following constituents at the stated amounts;

Anhydrous potassium dihydrogen phosphate (KH_2PO_4)	0.27 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	1.12 g
Ammonium chloride (NH_4Cl)	0.53 g
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.075g
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	0.10 g
Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$)	0.02 g
Resazurin (oxygen indicator)	0.001g
Sodium sulphide nonahydrate ($Na_2S.9H_2O$)	0.10 g
Stock solution of trace elements (optional, paragraph 18~~7~~)	10 ml
Add de-oxygenated water (paragraph 15)	to 1 litre

NOTE: Freshly supplied sodium sulphide should be used or it should be washed and dried before use, to ensure sufficient reductive capacity. The test may be performed without using a glove box (see paragraph 26). In this case, the final concentration of sodium sulphide in the

medium should be increased to 0.20 g of $Na_2S.9H_2O$ per litre. Sodium sulphide may also be added from an appropriate anaerobic stock solution through the septum of the closed test vessels as this procedure will decrease the risk of oxidation. Sodium sulphide may be replaced by titanium (III) citrate, which is added through the septum of closed test vessels at a final concentration of 0.8 to 1.0 mmol/~~l~~L. Titanium (III) citrate is a highly effective and low-toxicity reducing agent, which is prepared as follows: Dissolve 2.94 g of trisodium citrate dehydrate in 50 ml of de-oxygenated water~~oxygen-free deionised water~~ (to result in a solution of 200 mmol/~~l~~L) and add 5 ml of a 15% (w/v) titanium (III) chloride solution. Neutralise to pH 7 + 0.2 with mineral alkali~~sodium carbonate~~ and dispense to an appropriate vessel under a stream of nitrogen. The concentration of titanium (III) citrate in this stock solution is 164 mmol/~~l~~L.

17. The components in the test medium except the reducing agent (e.g. sodium sulphide) should be mixed and spiked ~~To achieve anoxic conditions, the medium should be sparged~~ with nitrogen gas for about 20 min immediately before use to remove oxygen. A volume of a freshly prepared solution of the reducing agent (prepared in de-oxygenated water) should be added just before use of the medium. The pH of the medium should be adjusted, if necessary, with dilute mineral acid or alkali to 7 ± 0.2.

Stock solution of trace elements (optional)

18. It is recommended that the test medium should contain the following trace elements to improve anaerobic degradation processes, especially if low concentrations (e.g. < 1g/L) of inoculum are used (11).

Manganese chloride tetrahydrate ($MnCl_2.4H_2O$)	50 mg
Boric acid (H_3BO_3)	5 mg
Zinc chloride ($ZnCl_2$)	5 mg
Copper (II) chloride ($CuCl_2$)	3 mg
Disodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	1 mg
Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	100 mg
Nickel chloride hexahydrate ($NiCl_2.6H_2O$)	10 mg
Disodium selenite (Na_2SeO_3)	5 mg
Add de-oxygenated water (paragraph 15)	to 1 litre

Test substance~~compound~~

19. Add ~~T~~the test substance ~~is added~~ as a stock solution, suspension, emulsion or directly, as solid or liquid or as absorbed on to glass-fibre filter to give a concentration of no more than 100 mg/L organic carbon. If stock solutions are used, a suitable solution should be prepared with water (paragraph 15) (previously de-oxygenated by sparging with nitrogen gas) of such a strength that the volume added is less than 5% of the total volume of reaction mixture. Adjust ~~T~~the pH of the stock solution ~~should be adjusted~~ to pH 7 ± 0.2 if necessary. For test substances which are insufficiently soluble in water, consult ISO 10634 (1~~2~~3) ~~should be consulted~~. If a solvent is ~~needed to~~ used, an additional control, with the solvent only added to the inoculated medium, should be prepared. ~~such o~~ Organic solvents which are known ~~strongly~~ to inhibit methane production, such as chloroform and carbon tetrachloride, should be avoided. In this case, an additional control with the solvent only added to the inoculated medium should be prepared.

Warning - Toxic test substances, and those whose properties are not known, must~~should~~ be handled with care.

Reference substances

20. Reference substances such as sodium benzoate, phenol and polyethylene glycol 400 have been used successfully to check the procedure, being biodegraded by more than 60% within ~~21~~60 days. Prepare ~~A~~a stock solution (in de-oxygenated water) of the chosen reference substance ~~should be prepared~~ in the same way as for the test substance~~compound~~ and adjust to pH 7 ± 0.2 if necessary.

Inhibition control (conditional~~optional~~)

21. In order to obtain information on the toxicity of the test substance to anaerobic micro-organisms, add the test substance~~compound~~ and reference substance ~~should be added~~ to a vessel containing the test medium (see paragraph 16), each at the same concentrations as added, respectively (see paragraphs 19 and 20 and see also ISO 13641-1 (13)).

Digested~~ing~~ sludge

22. Collect ~~D~~ddigested~~ing~~ sludge ~~should be collected~~ from a digester at a waste water treatment plant which treats predominantly domestic sewage. The sludge should be fully characterised and its background information should be reported (see paragraph 54~~49~~). If use of adapted inoculum is intended, digested~~ing~~ sludge from an industrial sewage treatment plant may be considered. Use ~~W~~wide-necked bottles constructed from high-density polyethylene or a similar material, which can expand,~~ should be used~~ for the collection of the digested~~ing~~ sludge. Add ~~S~~sludge ~~should be added~~ to ~~the bottle~~ within about 1cm of the top of the bottles and seal tightly, preferably with a safety valve. After transport to the laboratory, the collected sludge may~~should~~ be used directly or placed in a laboratory-scale digester. Release ~~E~~excess biogas by opening bottles of sludge carefully~~should be released~~. Alternatively, laboratory-grown anaerobic sludge may be used as a source of inoculum but its spectrum of activity may have been impaired.

Warning - Digested~~ing~~ 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 ~~should not be used~~ for collecting sludge.

23. In order to reduce background gas production and to decrease the influence of the blank controls, pre-digestion of the sludge may be considered. If pre-digestion is required, the sludge should be allowed to digest without the addition of any nutrients or substrates at 35°C ± 2°C for up to 7 days. It has been found that pre-digestion for about 5 days usually gives an optimal decrease in gas production of the blank without unacceptable increases in either lag or incubation periods during the test phase or loss of activity towards a small number of substances tested.

24. For test substances~~compounds,~~ which are, or are expected to be poorly biodegradable, consider pre-exposure of the sludge to the test substance ~~may be considered~~ to obtain an inoculum~~,~~ which is better adapted. In such a case, the test substance should be added at an organic carbon concentration of 5 mg/L to 20 mg/L to the digested sludge and incubated for up to 2 weeks. The pre-exposed sludge should be washed carefully before use (see paragraph 25) and conditions of the pre-exposure should be indicated in the test report.

Inoculum

25. Wash ~~T~~the sludge (see paragraphs 22 to 24) ~~should be washed~~ just prior to use, to reduce the IC concentration to less than 10 mg/L in the final test suspension.~~,~~ The sludge should be centrifuged ~~by first centrifuging~~ in sealed tubes (e.g. 3,000 g during 5 min) and the supernatant discharged~~ at a relatively low force (e.g. 3,000g) for up to 5 min~~. ~~–~~The resulting pellet should be suspended in de-oxygenated~~ free~~

medium (paragraph 16 and 17), ~~then~~ the suspension ~~should be~~ re-centrifuged and the supernatant liquid discharged.~~discard the washings.~~ If the IC has not been sufficiently lowered, the washing procedure of the sludge could ~~should~~ be repeated ~~washed up to~~ twice as a maximum~~more~~. This does not appear to affect the micro-organisms adversely. Finally, suspend the pellet ~~should be re-suspended~~ in the requisite volume of test medium and determine the concentration of total solids ~~should be determined~~ [e.g. ISO 11923 (145)]. The final concentration of total solids in the test vessels should be in the range of 1 g/L to 3 g/L (or about 10% of that in undiluted digest~~ed~~ing sludge). Conduct ~~T~~the above operations ~~should be conducted~~ in such a way that the sludge has minimal contact with oxygen (e.g. use a nitrogen atmosphere).

TEST PROCEDURE

26. Perform ~~T~~the following initial procedures ~~should be performed~~ using techniques to keep the contact between digested sludge and oxygen as low as practicable, for example, it may be necessary to work within a glove box in an atmosphere of nitrogen and/or purge the bottles with nitrogen (4).

Preparation of test and control assays

27. ~~At least in triplicate of test vessels (see paragraph 13-b) for the test compound, blank controls, the reference substance, and the inhibition control (conditional) should be prepared (see paragraphs 19 to 21). Blank controls can be used for several test substances in the same test. The diluted inoculum should be prepared (see paragraph 25) before adding it to the vessels. Aliquots of the well-mixed inoculum should be added so that the concentration of total solids is between 1 g/L and 3 g/L and the same in all vessels. Stock solutions of the test compound and of the reference substance should be added both previously adjusted to pH 7 ± 0.2, if necessary. The test concentration of organic carbon should normally be 100 mg/L. In the case of toxic test compounds, it may be reduced to 20 mg/L of organic carbon, or even less if only primary biodegradation with specific analyses is to be tested. It should be noted that the lower the test concentration, the higher the variation of test results is likely to occur.~~ At least triplicate test vessels (see paragraph 13-b) should be prepared for the test substance, blank controls, reference substance, inhibition controls (conditional) and pressure control chambers (optional procedure) (see paragraphs 7, 19 to 21). Additional vessels for the purpose of evaluating primary biodegradation using test substance specific analyses may also be prepared. The same set of blank controls may be used for several test substances in the same test as long as the headspace volumes are consistent.

28. The diluted inoculum should be prepared before adding it to the vessels e.g. by the means of a wide-mouthed pipette. Aliquots of well-mixed inoculum (paragraph 25) should be added so that the concentration of total solids is the same in all vessels (between 1 g/L and 3 g/L). Stock solutions of the test and reference substance should be added after adjustment to pH 7 ± 0.2, if necessary. The test substance and the reference substance should be added using the most appropriate route of administration (paragraph 19).

29. The test concentration of organic carbon should normally be between 20 and 100 mg/L (paragraph 4). If the test substance is toxic, the test concentration should be reduced to 20 mg C/L, or even less if only primary biodegradation with specific analyses is to be measured. It should be noted that the variability of the test results increases at lower test concentrations.

~~28~~30. For blank vessels~~In the case of blank vessels~~, an equivalent amount~~s~~ of the carrier used to dose the test substance ~~de-oxygenated water (paragraph 15)~~ should be added instead of a stock solution, suspension or emulsion. If the test substance was administered using glass fiber filters or organic solvents, then the blank should contain a filter or an equivalent volume solvent that has been evaporated. An extra

replicate with test substance~~compound~~ should be prepared for the measurement of the pH value. The pH should be adjusted to 7 ± 0.2, if necessary, with small amounts of dilute mineral acid or alkali. The same amounts of neutralising agents should be added to all the test vessels. These additions should not have to be made since the pH value of the stock solutions of the test substance~~compound~~ and reference substance have already been adjusted (see paragraphs 19 and~~to~~ 20). If primary biodegradation is to be measured, an appropriate sample should be taken from the pH-control vessel, or from an additional test vessel~~mixture~~, and the test substance~~compound~~ concentration should be measured using specific analyses. Covered magnets may be added to all the vessels if the reaction mixtures are to be stirred (optional).

~~29~~31. ~~In order to~~ Eensure that the total volume of liquid V_l and the volume of headspace V_h are the same in all vessels; note and record the values of V_l and ~~V_h should be recorded~~. Each vessel should be sealed with a gas septum and transferred ~~them~~ from the glove box (see paragraph 26) into the incubator (see paragraph 13-a).

Insoluble test ~~compounds~~substances

~~30~~32. Add ~~W~~weighed amounts of substances, which are poorly soluble in water, ~~are added~~ directly to the prepared vessels. ~~The use of solvents is not recommended (see paragraph 19), however, W~~when the use of a solvent is necessary (see paragraph 19), the test substance solution or suspension should be poured into the empty vessels. Where possible, ~~T~~the solvent is evaporated by passing nitrogen gas through the vessels and then the other ingredients should be added, namely, diluted sludge (paragraph 25), and de-oxygenated water ~~and medium (paragraph 16)~~ as required. An additional solvent control should also be prepared (see paragraph 19). For other methods of adding insoluble ~~compounds~~substances, ISO 10634 (12) can be consulted. Liquid test substances may be dosed with a syringe into the ~~otherwise~~ completely prepared sealed vessels, if it is expected that the initial pH will not exceed 7 ± 1, otherwise dose as described above (see paragraph 19).

Incubation and gas pressure measurements

~~31~~33. Incubate~~T~~ the prepared vessels ~~should be incubated~~ at $35°C \pm 2°C$ for about 1h to allow equilibration and release excess gas to the atmosphere, for example, by shaking each vessel in turn, inserting the needle of the pressure meter (paragraph 13-c) through the seal and opening the valve until the pressure meter reads zero. If at this stage, or when making intermediate measurements, the headspace pressure is less than atmospheric, nitrogen gas should be introduced to re-establish atmospheric pressure. The valve (see paragraph 13-c) should be closed and incubation continued ~~to incubate~~ in the dark, ensuring that all parts of the vessels are maintained at the digestion temperature. The vessels should be observed after incubation for 24 to 48h. If the contents of the vessels show a distinct pink coloration in the supernatant liquid, they should be removed, i.e. if R~~r~~esazurin (see ~~see~~ paragraph 16) has changed colour indicating the presence of oxygen. While small amounts of oxygen may be tolerated by the system, higher concentrations can seriously inhibit the course of anaerobic biodegradation.

~~32~~34. Carefully mix ~~T~~the contents of each vessel ~~should be carefully mixed~~ by stirring or by shaking for a few minutes at least 2 or 3 times per week and soon before each pressure measurement. Shaking re-suspends the inoculum and ensures gaseous equilibrium. All pressure measurements should be taken quickly, since the test vessels could be subject to lowering of temperature, leading to false readings. While measuring pressure the whole test vessel including the headspace should be maintained at the digestion temperature. The gas pressure is~~should be~~ measured, for example, by inserting through the septum the syringe needle (paragraph 13-c) connected to the pressure-monitoring meter. Care should be taken to prevent entry of water into the syringe needle; if this occurs the wet parts should be dried and a new needle fitted. The pressure should be measured in millibars (see paragraph 42~~38~~). ~~Shaking re-suspends the inoculum and ensures gaseous equilibrium. While measuring pressure, the whole vessel including the gas~~

~~in the headspace should be maintained at the digestion temperature, but care should be taken to prevent entry of water into the syringe needle. If this occurs, the wet parts should be dried and fit a new needle.~~ If this occurs, the wet parts should be dried and a new needle fitted. ~~For readings of gas pressure, t~~The gas pressure in the vessels may~~should~~ be measured ~~either~~ periodically e.g. weekly, and optionally the excess gas is released to the atmosphere~~. or a~~ Alternatively the pressure is measured only at the end of the test to determine the amount of biogas produced.

~~33~~35. It is recommended that intermediate readings of gas pressure be made, since pressure increase provides guidance as to when the test may be terminated and allows the kinetics to be followed (see paragraph 6).

~~34~~36. ~~The test is n~~Normally end the test~~finished~~ after an incubation period of 60 days unless the biodegradation curve obtained from the pressure measurements has reached the plateau phase before then;~~it,~~ that is the phase in which the maximal degradation >60% has been reached and the biodegradation curve has ~~r~~levelled out. This indicates that a sufficient degree of biodegradation ~~(>60%)~~ has been reached for the test to be ~~has been~~ finished earlier than 60 days. If the plateau value is <60% interpretation is problematic: only part of the molecule has been mineralised, 60% is too high a value of CO_2 production for the particular test substance, or an error has been made. If at the end of the normal incubation period, gas is being produced but a plateau phase is obviously not reached the test should be prolonged until it is reached.

Measurement of inorganic carbon

~~35~~37. At the end of the test after the last measurement of gas pressure, the sludge should be allowed to settle. Each vessel should be opened in turn and immediately a ~~direct~~ sample is taken for the determination of the concentration (mg/L~~l~~) of inorganic carbon (IC) in the supernatant liquor. Neither centrifugation nor filtration should be applied to the supernatant liquor, since there would be an unacceptable loss of dissolved carbon dioxide. If the liquor cannot be analysed on being sampled, it should be stored in a sealed vial, without headspace and cooled to about 4°C for up to 2 days. After the IC measurement, the pH value should be measured and recorded.

38. Alternatively, the IC in the supernatant may be determined indirectly by release of the dissolved IC as carbon dioxide that can be measured in the headspace. Following the last measurement of gas pressure, the pressure in each of the test vessels is adjusted to atmospheric pressure. The IC in the supernatant is determined after acidifying the liquid to pH 1 by adding of concentrated mineral acid (e.g. H_2SO_4) through the septum of the sealed vessels. The vessels are shaken and incubated at 35°C ± 2°C for approximately 24 hours and the gas pressure resulting from the evolved carbon dioxide is measured by the pressure meter."

39. Similar readings should be carried out for the corresponding blank, reference substance and, if included, inhibition control vessels (see paragraph 21).

~~36~~40. In some cases, especially if the same control vessels are used for several test substances, measurements of intermediate IC concentrations in test and control vessels should be~~are~~ considered, as appropriate. In this case, a sufficient~~enough~~ number of vessels should be prepared for all the intermediate measurements. This proceeding should be preferred to taking all samples from~~in~~ one vessel only. The latter~~It~~ can only be made if the required volume for DIC analysis is not deemed to be too high. The DIC measurement should be made after measuring the gas pressure without release of excess gas as described below:

- as small a volume as possible of supernatant samples is taken with a syringe through the septum without opening the vessels and IC in the sample is determined;

- after having taken the sample the excess gas is released, or not;
- it should be taken into account that even a small decrease in the supernatant volume (e.g. about 1%) can yield a significant increase in the headspace gas volume (V_h);
- the equations (see paragraph 4440) are corrected by increasing V_h in equation 3, as necessary.

Specific analyses

3741. If primary anaerobic degradation (see paragraph 3028) is to be determined, an appropriate volume of sample is taken for specific analyses at the beginning and at the end of the test from the vessels containing the test compoundsubstance. If this is done, it should be noted that the volumes of headspace (V_h) and of the liquid (V_l) will be changed and it should be taken into account when calculating the results of gas production. Alternatively samples may be taken for specific analyses from additional test mixtures previously set up for the purpose (paragraph 3028).

DATA AND REPORTING

Treatment of results

3842. For practical reasons, the pressure of the gas is measured in millibars (1 mbar = 1h Pa = 10^2 Pa; 1 Pa = 1 N/m^2), the volume in litres and temperature in degrees Celsius.

3943. Since the biogas evolved is mainly[2] methane and carbon dioxide and since 1 mol of methane and 1 mol carbon dioxide each contain 12 g of carbon, the mass of carbon in a given volume of evolved gas may be expressed as:

$$m = 12 \times 10^3 \times n \qquad \text{Equation [1]}$$

where:

m = mass of carbon (mg) in a given volume of evolved gas;
12 = relative atomic mass of carbon;
n = number of moles of gas in the given volume.

4044. From the gas laws n may be expressed as:

$$n = \frac{pV}{RT} \qquad \text{Equation [2]}$$

where:

p = pressure of the gas (Pascals);
V = volume of the gas (m^3)
R = molar gas constant [8.314J/(mol./K)]
T = incubation temperature (Kelvins).

By combination of equations [1] and [2] and rationalising to allow for blank control production of gas:

[2] For example, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (C/N = 1/2) is reported to be degraded into N_2O except CO_2 and CH_4 with municipal anaerobic sludge (16).

$$m_h = \frac{12000 \times 0.1(\Delta p \cdot V_h)}{RT}$$ Equation [3]

where:

m_h = mass of net carbon produced as gas in the headspace (mg);
Δp = mean of the difference between initial and final pressures in the test vessels minus the corresponding mean in the blank vessels (millibars);
V_h = volume of headspace in the vessel (L~~litres~~);
0.1 = conversion for both newtons/m^2 to millibars and m^3 to litres.

Equation [4] should be used for the normal incubation temperature of 35°C (308 K):

$$m_h = 0.468(\Delta p \cdot V_h)$$ Equation [4]

Note: As an optional method, the pressure-measuring device will be calibrated by injecting known volumes of gas into pressure control chambers (prepared in the same manner as the blank controls) which have been equilibrated to atmospheric pressure. A standard curve of the mL injected versus meter reading will be generated and a linear regression will be performed to determine the line equation.

Pressure meter readings will be converted to mL of gas produced using the standard curve generated by plotting mL injected versus meter reading. The number of moles (n) of gas in the headspace of each chamber will be calculated by dividing the cumulative gas production (ml) by 25286 ml/mole, which is the volume occupied by one mole of gas at 35 °C and atmospheric pressure. Since 1 mole of CH_4 and 1 mole of CO_2 each contain 12 g of carbon, the amount of carbon (mg) in the headspace (C_h) is given by Equation [5]:

$$C_h = 12 \times 10^3 \times n$$ Equation [5]

The mass of net carbon (m_h) produced as gas in the headspace is given by Equation [6].

$$m_h = C_h test - mean C_h blank controls$$ Equation [6]

~~41~~45. The course of biodegradation can be followed by plotting the cumulated pressure increase Δp (millibars) against time, if appropriate. From this curve, the lag phase (days) should be identified and recorded. The lag phase is the time from the start of the test until significant degradation starts (for example see Annex 2). If intermediate samples of supernatant were taken and analysed (see paragraphs 40~~36~~, 46~~42~~ and 47~~43~~), then the total C produced (in gas plus that in liquid) may be plotted instead of only the cumulative pressure.

Carbon in the liquid

~~42~~46. The amount of methane in the liquid is~~should be~~ ignored since its solubility in water is known to be very low. The mass of inorganic carbon in the liquid of the test vessels should be calculated using equation [~~5~~7]:

$$m_l = C_{net} \times V_l$$ Equation [~~5~~7]

where:

m_l = mass of inorganic carbon in the liquid (mg);
C_{net} = concentration of inorganic carbon in the test vessels minus that in the control vessels at the end of the test (mg/L);
V_l = volume of liquid in the vessel (L~~litre~~).

Total gasified carbon

~~43~~47. The total mass of gasified carbon in the vessel should be calculated using equation [~~6~~8]:

$$m_t = m_h + m_l \qquad \text{Equation [~~6~~8]}$$

where:
m_t = total mass of gasified carbon (mg);
m_h and m_l are as defined above.

Carbon of test substance

~~44~~48. The mass of carbon in the test vessels derived from the added test substance should be calculated using equation [~~7~~9]:

$$m_v = C_c \times V_l \qquad \text{Equation [~~7~~9]}$$

where:
m_v = mass of test ~~compound~~ substance carbon (mg);
C_c = concentration of test substance~~compound~~ carbon in the test vessel (mg/L)
V_l = volume of liquid in the test vessel (L~~litre~~).

Extent of biodegradation

~~45~~49. The percentage biodegradation from headspace gas should be calculated using equation [~~8~~10] and the total percentage biodegradation using equation [~~9~~11]:

$$D_h = (m_h / m_v) \times 100 \qquad \text{Equation [~~8~~10]}$$
$$D_t = (m_t / m_v) \times 100 \qquad \text{Equation [~~9~~11]}$$

where:
D_h = biodegradation from headspace gas (%);
D_t = total biodegradation (%);
m_h, m_v and m_t are as defined above.

Validity of results

Maintenance of anaerobic conditions

~~46~~50. Pressure readings should be used only from vessels, which contain no oxygen, i.e. which do not show pink coloration. Contamination by oxygen is minimised by the use of proper anaerobic handling techniques.

~~**Inhibition of degradation**~~

~~47~~51. It should be considered that the test is~~to be~~ valid if the reference substance reaches a plateau which represents >60% biodegradation.[3]

52 If the pH at the end of the test has exceeded the range 7 ± 1 and insufficient biodegradation has taken place, the test should be repeated with increased buffer capacity of the medium.

Inhibition of degradation

~~48~~53. Gas production in vessels containing both the test ~~compound~~ substance and reference substance should be at least equal to that in the vessels containing only reference substance; otherwise, inhibition of gas production is indicated. In some cases gas production in vessels containing test ~~compound~~ substance without reference substance will be lower than that in the blank controls, indicating that the test ~~compound~~ substance is inhibitory.

Test Report

~~49~~54. The test report must include the following information:

Test substance:

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

Test conditions:

- volumes of diluted digester liquor (V_l) and of the headspace (V_h) in the vessel;
- description of the test vessels, the main characteristics of biogas measurement (e.g. type of pressure meter) and of the IC analyser;
- application of test substance and reference substance to test system: test concentration used and any use of 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, etc.), concentration, any information necessary to substantiate this and information on any pre-treatment of the inoculum (e.g. pre-digestion, pre-exposure)
- incubation temperature;
- number of replicates.

Results:

- pH and IC values at the end of the test;
- concentration of test substance at the beginning and end of the test, if a specific measurement has been performed;
- all the measured data collected in the test, blank, reference substance and inhibition control vessels, as appropriate (e.g. pressure in millibars, concentration of inorganic carbon (mg/L) in tabular form (measured data for headspace and liquid should be reported separately);
- statistical treatment of data, test duration and a diagram of the biodegradation of test substance, reference substance and toxicity control;

[3] This should be re-evaluated if adsorptive and insoluble reference substances are included.

- precentage biodegradation of test substance and reference substance;
- reasons for any rejection of the test results;
- discussion of results.

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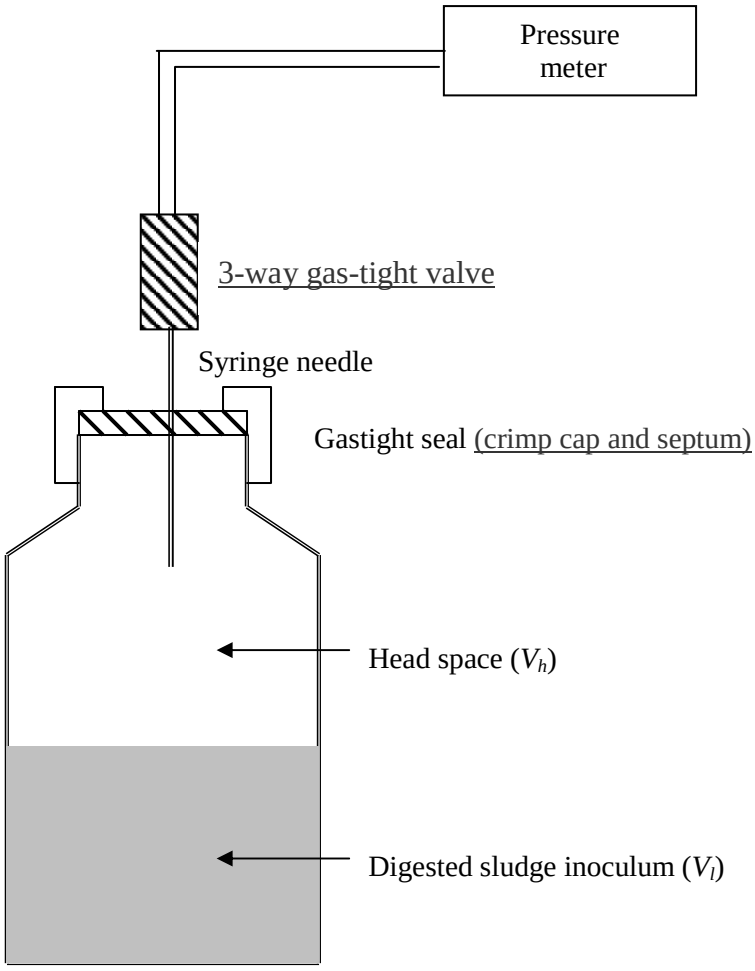
ANNEX 1

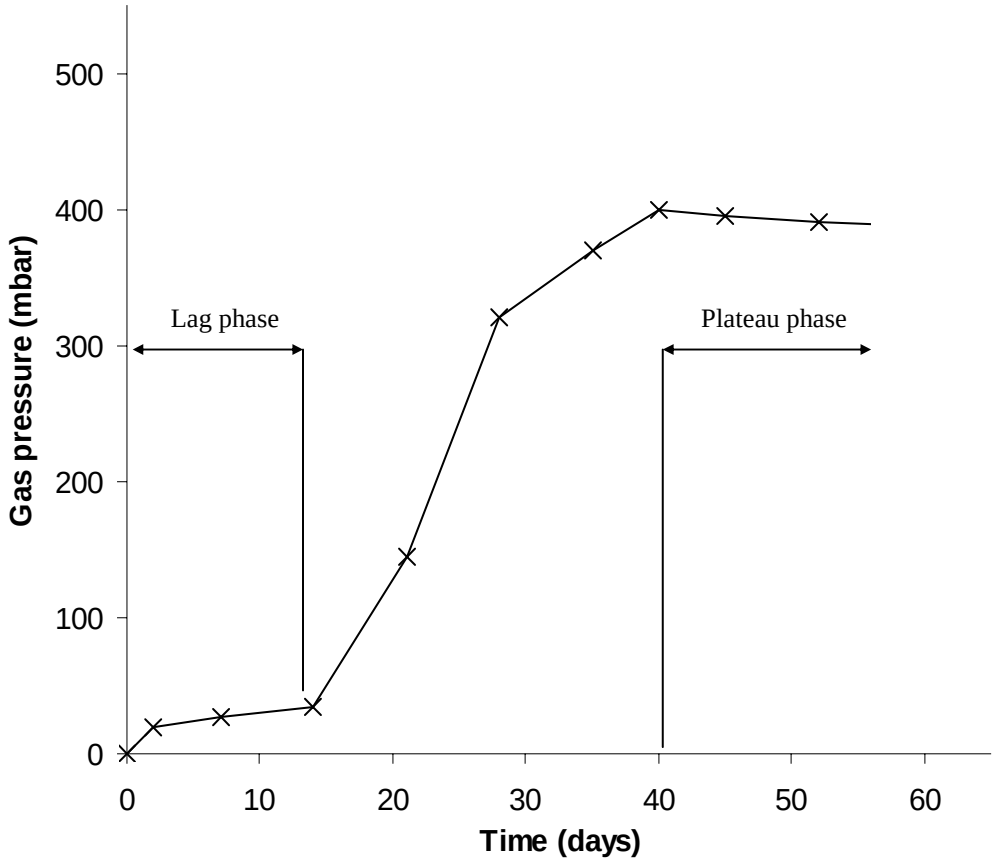
Example of an apparatus to measure biogas production by gas pressure



Test vessels in an environment of~~to~~ 35 °C ± 2 °C

ANNEX 2

Example of a degradation curve (cumulativecumulated net pressure increase)



ANNEX 3

Example of data sheets for the anaerobic biodegradation test – Data sheet for the test substance

Laboratory: ..... Test substance: ..... Test No.: .....
Test temperature:(°C) Volume of headspace (V_h): .... (L~~iters~~) Volume of liquid (V_l) : .... (liters) Carbon in test substance $C_{c,v}$: ..... (mg/l) m_v[1]: ..... (mg)

Day	p_1 (test) (mbar)	p_2 (test) (mbar)	p_3 (test) (mbar)	p (test) mean (mbar)	p_4 (blank) (mbar)	p_5 (blank) (mbar)	p_6 (blank) (mbar)	p (blank) mean (mbar)	p (net) test - blank mean (mbar)	Δp (net) cumulative~~ed~~ (mbar)	m_h headspace C[2] (mg)	D_h Biodegradation[3] (%)
	$C_{IC,1}$ test (mg)	$C_{IC,2}$ test (mg)	$C_{IC,3}$ test (mg)	C_{IC} test mean (mg)	$C_{IC,4}$ blank (mg)	$C_{IC,5}$ blank (mg)	$C_{IC,6}$ blank (mg)	C_{IC} blank mean (mg)	$C_{IC,net}$ test -blank mean (mg)	m_l liquid C[4] (mg)	m_t total C[5] (mg)	D_t Biodegradation[6] (%)
IC (end)												
pH (end)												

[1] Carbon in test vessel, m_v (mg): $m_v = C_{C,v} \times V_l$
[2] Carbon in headspace-, m_h (mg) at normal incubation temperature (35 °C)~~"~~: $m_h = 0.468 \Delta p \times V_h$
[3] Biodegradation calculated from headspace gas, D_h (%): $D_h = (m_h \times 100) / m_v$
[4] Carbon in liquid, m_l (mg): $m_l = C_{IC,net} \times V_l$
[5] Total gasified carbon, m_t (mg): $m_t + m_l$
[6] Total biodegradation, D_t (%): $D_t = (m_t \times 100) / m_v$

ANNEX 3 (continued)

Example of data sheets for the anaerobic biodegradation test – Data sheet for the reference substance

Laboratory: ..... Reference substance: ..... Test No.: .....
Test temperature:(°C) Volume of headspace (V_h): .... (L~~iters~~) Volume of liquid (V_l) : .... (liters) Carbon in reference substance $C_{c,v}$: (mg/l) m_v[7]: ..... (mg)

Day	p_1 (ref.) (mbar)	p_2 (ref.) (mbar)	p_3 (ref.) (mbar)	p (ref.) mean (mbar)	p_4 (inhib.) (mbar)	p_5 (inhib.) (mbar)	p_6 (inhib.) (mbar)	p (inhib.) mean (mbar)	p (ref.) ref. - blank (mbar)	Δp (ref.) cumulative~~ed~~ (mbar)	m_h headspace C[8] (mg)	D_h Biodegradation[9] (%)
	$C_{IC,1}$ ref. (mg)	$C_{IC,2}$ ref. (mg)	$C_{IC,3}$ ref. (mg)	C_{IC} ref. mean (mg)	$C_{IC,4}$ inhib. (mg)	$C_{IC,5}$ inhib. (mg)	$C_{IC,6}$ inhib. (mg)	C_{IC} inhib. mean (mg)	$C_{IC,net}$ ref. - inhib. (mg)	m_l liquid C[10] (mg)	m_t total C[11] (mg)	D_t Biodegradation[12] (%)
IC (end)												
pH (end)												

[7] Carbon in test vessel, m_v (mg): $m_v = C_{C,v} \times V_l$
[8] Carbon in headspace , m_h (mg) at normal incubation temperature (35 °C)": $m_h = 0.468 \Delta p \times V_h$
[9] Biodegradation calculated from headspace gas, D_h (%): $D_h = (m_h \times 100) / m_v$
[10] Carbon in liquid, m_l (mg): $m_l = C_{IC,net} \times V_l$
[11] Total gasified carbon, m_t (mg): $m_t + m_l$
[12] Total biodegradation, D_t (%): $D_t = (m_t \times 100) / m_v$