C. 10. BIODEGRADATION

ACTIVATED SLUDGE SIMULATION TESTS

1. METHOD

1.1. Introduction

1.1.1. General remarks

The method is applicable only to those organic substances which, at the concentration used in the test:

-are soluble in water to the extent necessary for the preparation of the test solutions,

-have negligible vapour pressure under the test conditions,

-are not inhibitory to bacteria.

Information on the relative proportions of the major components of the test material will be useful in interpreting the results obtained, particularly in those cases where the results are low or marginal.

Information on the toxicity of the substance to micro-organisms is desirable for the interpretation of the low results and in the selection of appropriate test concentrations.

1.1.2. Determination of ultimate biodegradability (DOC/COD analysis)

The purpose of the method is to determine the ultimate biodegradability by the measurement of the removal of the substance and any metabolites in an activated sludge plant model at a concentration corresponding to > 12 mg DOC/litre (or approximately 40 mg COD/litre); 20 mg DOC/litre seem to be optimal. (DOC = Dissolved Organic Carbon; COD = Chemical Oxygen Demand).

The organic carbon content (or the chemical oxygen demand) of the test material must be established.

1.1.3. Determination of primary biodegradability (specific analysis)

The purpose of the method is the determination of the primary biodegradability of a substance in an activated sludge plant model, at a concentration of about 20 mg/litre, using a specific analytical method (lower or higher concentration can be used if analytical method and consideration of toxicity permits). This allows the assessment of the primary biodegradability of the substance (disappearance of the parent chemical structure).

The purpose of this method is not the determination of the mineralization of the tested substance.

An adequate analytical method for the determination of the tested substance must be available.

1.2. Definitions and units

1.2.1. DOC/COD analysis

The degree of removal of the substance is given by:

$$DR = \frac{T - (E - E_o)}{T} x100\% \qquad [1(a)]$$

where:

DR = degree of removal in percent DOC (or COD) within the given mean retention time with respect to the test material,
T = concentration of the test material in the influent in mg DOC/litre (or mg COD/litre),
E = DOC (or COD) concentration in the effluent of the test unit in mg DOC/litre (or mg COD/litre),
E_o = DOC (or COD) concentration in the effluent of the blank unit in mg DOC/litre (or mg COD/litre).

The degradation is stated as the percentage DOC (or COD) removal within the given retention time with respect to the test material.

1.2.2. Specific analysis

The percentage elimination of the tested substance from the aqueous phase (R_w) within the given mean retention time is given by

$$R_W = \frac{C_I - C_o}{C_I} \times 100\% \qquad [1(b)]$$

where:

C_I = concentration of the substance in the influent of the test unit (mg substance/litre, determined by specific analysis),
C_o = concentration of the substance in the effluent of the test unit (mg substance/litre, determined by specific analysis).

1.3. Reference substances

In some cases when investigating a new substance, reference substances may be useful; however, specific reference substances cannot yet be recommended.

1.4. Principle of the test methods

For the determination of ultimate biodegradability, two activated sludge pilot units (OECD confirmatory test or porous pot units) are run in parallel. The test substance is added to the influent (synthetic or domestic sewage) of one of the units, while the other receives the sewage alone. For the determination of primary biodegradation with specific analysis in the influent and effluent, only one unit is used.

The DOC (or COD) concentrations are measured in the effluents, or the substance concentrations are determined by specific analysis.

The DOC due to test material is not measured but simply stated.

When DOC (or COD) measurements are performed, the difference in mean concentrations between the test and the control effluents is assumed to be due to undegraded test material.

When specific analyses are preformed, change in the concentration of the parent molecule can be measured (primary biodegradation).

The units may be operated following the 'coupled units mode', by a transinoculation procedure.

1.5. Quality criteria

The starting concentration of the substance depends on the type of analysis performed and its limitation.

1.6. Description of the test method

1.6.1. Preparation

1.6.1.1. Apparatus

A pair of units of the same type are needed except when specific analyses are performed. Two types of device may be used:

OECD confirmatory test

The equipment (Appendix 1) consists of a storage vessel (A) for synthetic sewage, dosing pump (B), aeration vessel (C), separator (D), air-lift pump (E), to recycle activated sludge, and vessel (F) for collecting the treated effluent.

Vessels (A) and (F) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; any suitable system may be used, providing that input flow and concentration are assured. During normal operation the height of separator (D) is so fixed that the volume contained in the aeration vessel is three litres of mixed liquor.

A sintered aeration cube (G) is suspended in vessel (C) at the apex of the cone. The quantity of air blown through the aerator may be monitored by means of a flow meter.
Air-lift pump (E) is set so that the activated sludge from the separator is continually and regulariy recycled to aeration vessel (C).

'Porous pot'

The porous pot is constructed from sheets of porous polyethylene (2 mm thick, maximum pore size 95 µm), which are made into cylinders 14 cm in diameter with a conical base at 45° (Figures 1 and 2 of Appendix 2). The porous pot is contained in an impervious vessel of suitable plastic 15 cm in diameter with an outlet at a height of 17,2 cm on the cylindrical part, which determines the volume (3 litres) in the pot. There is a rigid supporting ring made of suitable plastic around the top of the inner vessel, so that there is an effluent space of 0,5 cm between the inner and outer vessels.

The porous pots may be mounted in the base of a thermostatically controlled water-bath. There is an air supply to the base of the inner vessel on which are placed suitable diffusers.

Vessels (A) and (E) must be of glass or suitable plastic and hold at least 24 litres. Pump (B) must provide a constant flow of synthetic sewage to the aeration vessel; any suitable system may be used, providing that input flow and concentration are assured.

Spare inner porous pots are required to replace any which may block in use; blocked pots are cleaned by 24-hour immersion in hypochlorite solution followed by thorough washing in tap water.

1.6.1.2. Filtration

Membrane filtration apparatus and membrane filters with a pore size of 0,45 µm. Membrane filters are suitable if it is assured that they neither release carbon nor adsorb the substance in the filtration step.

1.6.1.3. Sewage

Either suitable synthetic feed or domestic sewage may be used.

Example of synthetic feed

Dissolve in each litre of tap water:

Peptone:	160 mg,
Meat extract:	10 mg,
Urea:	30 mg,
NaCl:	7 mg,
$CaCl_2 .2H_2O$:	4 mg,
$MgSO_4 .7H_2O$:	2 mg,
K_2HPO_4:	28 mg.

Domestic sewage

This should be collected freshly each day from the overflow of the primary settlement tank of a treatment plant treating predominantly domestic sewage.

1.6.1.4. Stock solution of test material

A solution of test material, e.g. 1% , should be prepared for addition to the test unit. The concentration of the material must be determined, so that the appropriate volume to be added to the sewage or directly to the unit via a second pump to give the required test concentration is known.

1.6.1.5. Inoculum

Remark: When domestic sewage is used, there would be no point in using an inoculum of low bacterial concentration, but activated sludge may be used.

A variety of inocula may be used.

Three examples of suitable inoculum are given:

(a) Inoculum from secondary effluent

The inoculum should be obtained from a secondary effluent of good quality collected from a treatment plant dealing with predominantly domestic sewage. The effluent must be kept under aerobic conditions in the period between sampling and use. To prepare the inoculum, the sample is filtered through a coarse filter, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the day of collection. At least 3 ml are to be used for inoculation.

(b) Composite inoculum

Inoculum from secondary effluent:

See description above.

Inoculum from soil:

100 g of garden soil (fertile, not sterile) are suspended in 1000 ml chlorine-free drinking water. (Soils with an extremely large fraction of clay, sand or humus are unsuitable). After stirring, the suspension is allowed to settle for 30 minutes. The supernatant is filtered through a coarse filter paper, the first 200 ml being discarded. The filtrate is aerated immediately and until use. The inoculum must be used on the day of collection.

Inoculum from a surface water:

A further partial inoculum is drawn from a mesosaprobic surface water. The sample is filtered through a coarse paper, the first 200 ml being discarded. The filtrate is kept aerobic until used. The inoculum must be used on the, day of collection.

Equal volumes of the three partial inoculum samples are united, mixed well, and the final inoculum drawn from this mixture. At least 3 ml are to be used for inoculation.

c) Inoculum from activated sludge

A volume (not more than 3 litres) of activated sludge (suspended solids content of up to 2,5 g/litre) taken from the aeration tank of a plant treating predominantly domestic sewage may be used as an inoculum.

1.6.2. Proredure

The test is performed at room temperature; this should be kept between 18 and 25 °C.

If it is appropriate, the test may be performed at a lower temperature (down to 10 °C); if the substance is degraded then no further work is normally required. If, however, the substance is not degraded, the test must be conducted at a steady temperature between 18 and 25 °C.

1.6.2.1. Running-in period: Sludge formation/stabilization of the units

The sludge growth/stabilization period is the period during which the concentration of the activated sludge suspended solids and the performance of the units progress to a steady state under the operating conditions used.

The running-in period is the period which lasts from the time the test substance is first added to the time when its removal reaches a plateau (relatively constant value). This period must not exceed six weeks.

The evaluation period is a three weeks period, three weeks from the time that the removal of the test substance reaches a relatively constant, and ususally high, value. For those substances which show little or no degradation in the first six weeks, the evaluation period is taken as the following three weeks.

Initially, fill the unit(s) needed for one test with the inoculum mixed with influent.

The aerator (and air lift (E) in the case of the OECD confirmatory test units) and dosing device (B) are then set in operation.

Influent without substance to be tested must pass through the aeration vessel (C) either at the rate of one litre per hour or a rate of one-half litre per hour; this gives a mean retention time of either three or six hours.
The rate of aeration should be regulated so that the content of vessel (C) is kept constantly in suspension while the dissolved oxygen content is at least 2 mg/litre.

Foaming must be prevented by appropriate means. Anti-foaming agents which inhibit the activated sludge must not be used.

The sludge which has accumulated around the top of the aeration vessel (C) (and, in the case of the OECD confirmatory test units, in the base of the settling vessel (D), and in the circulation circuit) must be returned to the mixed liquor at least once each day by brushing or some other appropriate means.

When sludge fails to settle, its density may be increased by addition of 2 ml portions of a 5% solution of ferric chloride, repeated as necessary.

The effluent is collected in vessel (E or F) for 20 to 24 hours, and a sample is taken after thorough mixing. Vessel (E or F) must be carefully cleaned.

In order to monitor and control the efficiency of the process, the chemical oxygen demand (COD) or the dissolved organic carbon (DOC) of the filtrate of the accumulated effluent is measured at least twice weekly, as well as that of the filtered influent (using a membrane of pore size 0,45 µm, the first 20 ml (approximately) of the filtrate are discarded).

The reduction in COD or DOC should level off when a roughly regular daily degradation is obtained.

The dry matter content of the activated sludge in the aeration tank should be determined twice a week (in g/litre). The units may be operated in one of two ways: either the content of dry matter in the activated sludge should be determined twice a week, and, if it is more than 2,5 g/litre, the excess activated sludge must be discarded, or 500 ml of mixed liquor is wasted from each pot daily to give a mean sludge retention time of six days.

When the measured and estimated parameters (efficiency of the process (in COD or DOC removal), sludge, concentration, sludge settleability, turbidity of the effluents, etc.) of the two units are sufficiently steady, the test substance may be introduced in the influent of one of the units, following 1.6.2.2.

Alternatively, the test substance may be added at the beginning of the sludge growth period (1.6.2.1), especially when sludge is added as the inoculum.

1.6.2.2. Test procedure

The operating conditions of the running-in period are maintained and sufficient stock solution (approximately 1 %) of the test material is added to the influent of the test unit so that the desired concentration of test material (approximately 10 to 20 mg DOC/litre or40 mg COD/litre) in the sewage is obtained. This can be done by mixing the stock solution to the sewage daily or by means of a separate pumping system. This concentration may be reached progressively. If there are no toxic effects of the test substance on the activated sludge, higher concentrations can also be tested.

The blank unit is fed only with influent without added substances. Adequate volumes of the effluents are taken for analysis and filtered through membrane filters (0,45 µm) the first 20 ml (approximately) of filtrate being discarded.

The filtered samples have to be analysed on the same day, otherwise they must be preserved by any suitable method, for example, by using 0,05 ml of a 1% mercuric chloride ($HgCl_2$) solution for each 10 ml of filtrate or by storing them at 2 to 4 °C up to 24 hours, or below -18 °C for longer periods.

The running-in time, with addition of test substance, should not exceed six weeks and the evaluation period should not be shorter than three weeks, i.e. about 14 to 20 determinations should be available for calculation of the final result.

Coupled units mode

The coupling of the units is achieved by interchanging 1,5 litres of mixed liquor (including sludge) from the activated sludge aeration vessels between the two units once a day. In the case of strongly

absorbing test materials, 1,5 litres of supernatant liquid only are drawn from the settling vessels and poured into the activated sludge vesselof the other unit.

1.6.2.3. Analysis

Two kinds of analyses may be performed in order to follow the behaviour of the substance:

DOC and COD

The DOC concentrations are performed in duplicate with the carbon analyser and/or the COD values according to reference (2).

Specific analysis

The concentrations of the tested substance are determined by a suitable analytical method. When possible, specific determination of the substance absorbed on sludge should be performed.

2. DATA AND EVALUATION

2.1. Coupled units mode

When using 'coupled units mode', the daily degrees of removal, DR are calculated according to 1.2.1.

These daily degrees of removal DR are corrected to DRc for the material transfer due to the transinoculation procedure with equation [2] for a three-hour or equation [3] for a six-hour mean retention time.

$$DRc = \frac{8}{7} DR - \frac{100}{7} \qquad [2]$$

$$DRc = \frac{4}{3} DR - \frac{100}{3} \qquad [3]$$

The mean of the series of DRc values is calculated and in addition the standard deviation according to equation [4]

$$^{s}DRc = \sqrt{\frac{\sum_{i=1}^{n} \left(\overline{DRc} - DRc_i\right)^2}{n-1}} \qquad [4]$$

where:

^{s}DRc = standard deviation of the series of DRc values,
$\overline{DR}$ c = mean of DRc value,
n = number of determinations.

Outliers of the DRc series are eliminated according to a suitable statistical procedure, e.g. Nalimov (6), atthe 95% probability level and the mean and the standard deviation of the outlier free DRc data set are recalculated.

The final result is then calculated with equation [5] as

$$DRc = \overline{DRc} \pm \frac{t_{n-1;\alpha}}{\sqrt{n}} {}^{s}DRc \qquad [5]$$

where:

t_{n-1} ;α = table value of t for n value pairs of E and E_o and statistical confidence P (P =1-α) whereby P is at 95% (1).

The result is stated as the mean with tolerance limits at the 95% probability level, the respective standard deviation and the number of data of the outlier-free DRc data set, and the number of outliers, e.g.

DRc = 98,6 ± 2,3% DOC removal,
s = 4,65% DOC removal,
n = 18,
x = number of outliers.

2.2. Non-coupled units mode

The performance of the units may be checked as follows:

$$\text{percentage removal of COD or DOC} = \frac{\text{CODorDOCsewage} - \text{CODorDOCeffluent}}{\text{CODorDOCsewage}} \text{x100}$$

These daily removals may be plotted graphically to reveal any trends, e.g. to acclimatization.

2.2.1. Using COD/DOC determinations

The daily degree of removal DR is calculated according to 1.2.1.

The mean of the series of DR values is calculated; in addition, its standard deviation is calculated according to:

$$^{s}DR = \sqrt{\frac{\sum_{i=1}^{n} \left(\overline{DR} - DR_i\right)^2}{n-1}} \qquad [6]$$

where:

^{s}DR = standard deviation of the series of DR_i values,
$\overline{DR}$ = mean of DR_i values,
n = number of determinations.

Outliers of the DR series are eliminated according to a suitable statistical procedure, e.g. Nalimov (6), at the 95% probability level, and the mean and the standard deviation of the outliers-free DR set are recalculated.

The final result is then calculated with equation [7] as

$$DR = \overline{DR} \pm \frac{t_{n-1;\alpha}\, ^{s}DR}{\sqrt{n}} \qquad [7]$$

where:

$t_{n-1};\alpha$ = table value of t for n value pairs of E and E_o and statistical confidence P (P = 1-α) whereby P is set at 95% (1).

The result is stated as the mean with tolerance limits at the 95% probability level, the respective standard deviation and the number of data of the outlier free DR data set, and the number of outliers, e.g.

DR = (98,6 ± 2,3) % DOC removal,
s = 4,65)% DOC removal,
n = 18,
x = number of outliers.

2.2.2. Using specific analysis

The percentage of elimination of the tested substance from the aqueous phase (R_w) is calculated according to 1.2.2.

3. REPORTING

3.1. Test report

The test report shall, if possible, contain the following:

-the formsheet given in Appendix 3, showing the operating conditions for the test,

-which apparatus was chosen (OECD confirmatory test or porous pot),

-which operating mode was chosen: coupled units mode or not,

-which sewage. synthetic or domestic -in the case of domestic sewage, date and location of sample,

-which inoculum, with date and location of sample,

-a statement with description of the analytical method if specific analyses were performed,

-plot of COD or DOC removal versus time, including running-in and evaluation period,

-analytical recovery of the test substance as COD of DOC in the stock solution,

-if specific analyes were performed, plot of the percentage removal of the tested substance from the aqueous phase versus time (running-in and evaluation period),

-the mean removal of DOC or COD of test substance and standard deviation are calculated from the results of the evaluation period; i.e. when there is a steady removal of test material or period of steady operation,

-plot of activated sludge concentration versus time,

-any remark concerning the activated sludge (discard of excess sludge, presence of bulking, $FeCl_3$ etc.),

-concentration of the substance used in the test,

-any results concerning analysis done on the sludge;

-all information and experimental results concerning the test substance and the reference substance if used,

-scientific reasons for any changes of the procedure.

3.2. Interpretation of results

Low removal of the tested substance from the aqueous phase may be due to inhibition of micro-organisms by the test substance. This may also be revealed by lysis and loss of sludge, giving a turbid supernatant, and by a decrease of the COD (or DOC) removal efficiency of the pilot plant.

Physico-chemical adsorption can sometimes play a role. Differences between biological action on the molecule and phyisico-chemical adsorption may be revealed by analysis performed on the sludge after an adequate desorption.

Further tests are necessary if a distinction is to be drawn between biodegradation (or partial biogradation) and adsorption.

This can be done in a number of ways, but the most convincing is to use the supernatant as inoculum in a base-set test (respirometric test preferably).

If high DOC or COP removals are observed, then this is due to biodegradation while, at low removals, biodegradation is indistinguishable from elimination. For example, if a soluble compound exhibits a high adsorption constant of 98% and the surplus sludge wastage rate is 10% per day, an elimination of up to 40% is possible; at a surplus sludge wastage rate of 30% elimination due to adsorption on and removal with surplus sludge may amount to up to 65% (4).

When using specific analysis, attention should be paid to the relationship between the structure of the substance and the specific analysis used. In this case, the phenomenon observed cannot be interpreted as a mineralization of the substance.

4. REFERENCES

(1) OECD, Paris, 1981, Test Guideline 303 A, Decision of the Council C(81) 30 final.

(2) Annex V C9 Degradation Test -Chemical Oxygen Demand, Commission Directive 84/449/EEC, Official Journal of the European Communities, No L 251,19.9.1984.

(3) Painter, H. A., King, E. F., WRC Porous-Pot method for assessing biodegradability, Technical Report TR70, June 1978, Water Research Centre, United Kingdom.

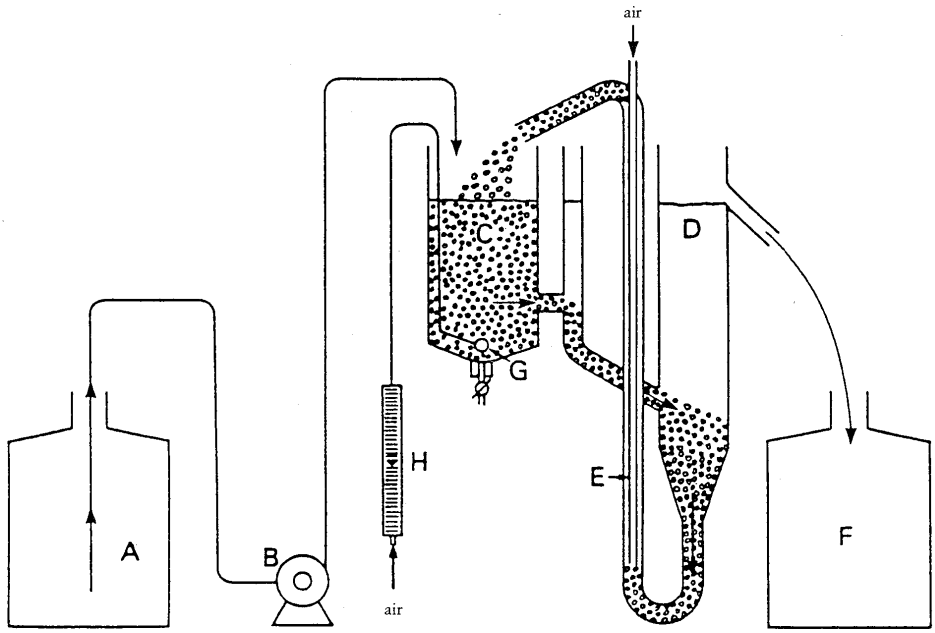
(4) Wierich, P., Gerike, P., The fate of soluble, recalcitrant, and adsorbing compounds in activated sludge plants, Ecotoxicology and Environmental Safety, vol. 5, No 2, June 1981, pp. 161 to 171.

(5) Council Directives 82/242/EEC and 82/243/EEC, Official Journal of the European Communities, No L 109, 22. 4. 1982, amending Council Directives 7Y404/EEC and 73/405 /EEC on biodegradability of detergents, Official Journal of the European Communities, No L 347, 17. 12. 1973.

(6) Streuli, H., Fehlerhafte Interpretation und Anwendung von Ausreßertests, insbesondere bei Ringversuchen zur Oberprüfung analytisch-chemischer Untersuchungsmethoden, Fresenius-Zeitschrift für Analytische Chemie, 303 (1980), pp. 406 to 408.

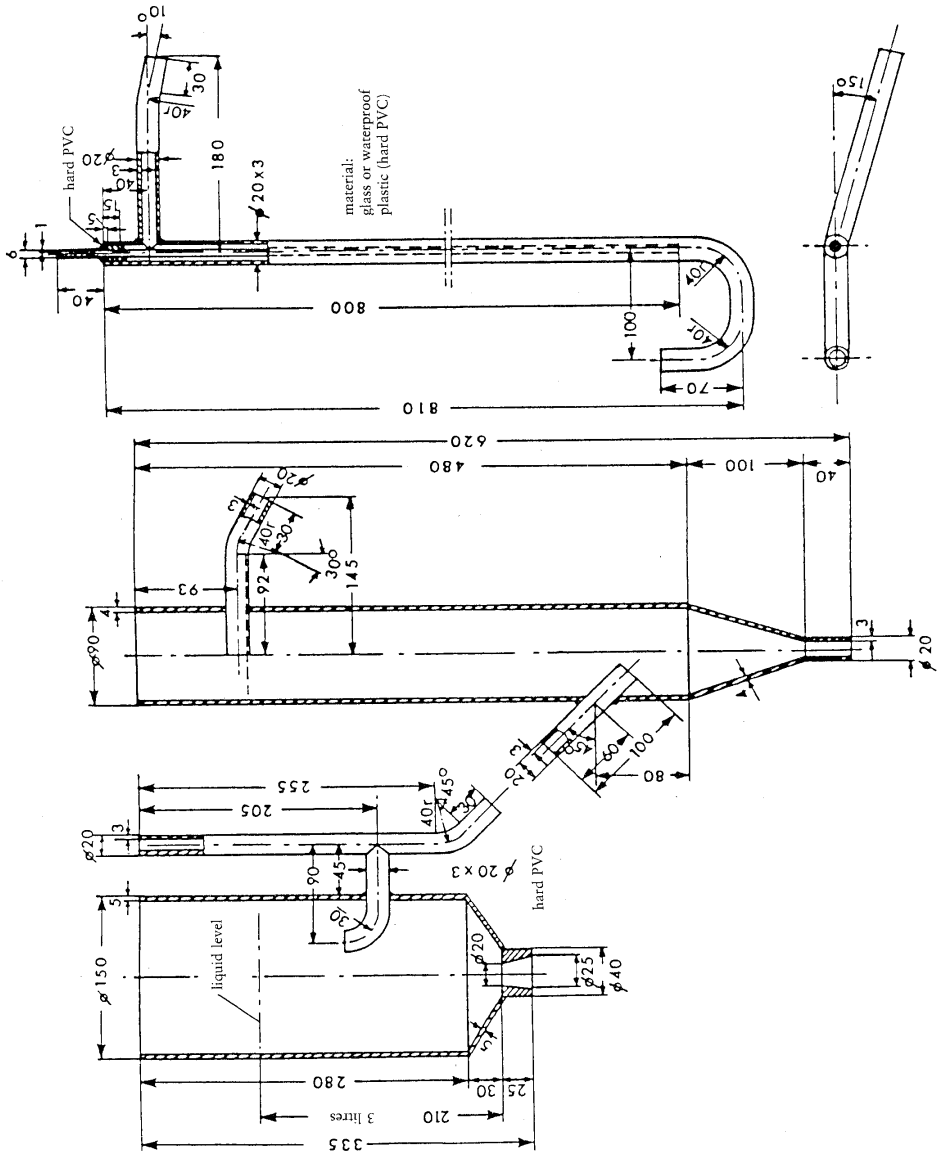
APPENDIX 1

Figure 1



Key: A = storage vessel;
B = dosing device;
C = aeration chamber (3 l capacity);
D = settling vessel;
E = air lift;
F = collector;
G = aerator;
H = air flow meter (optional).

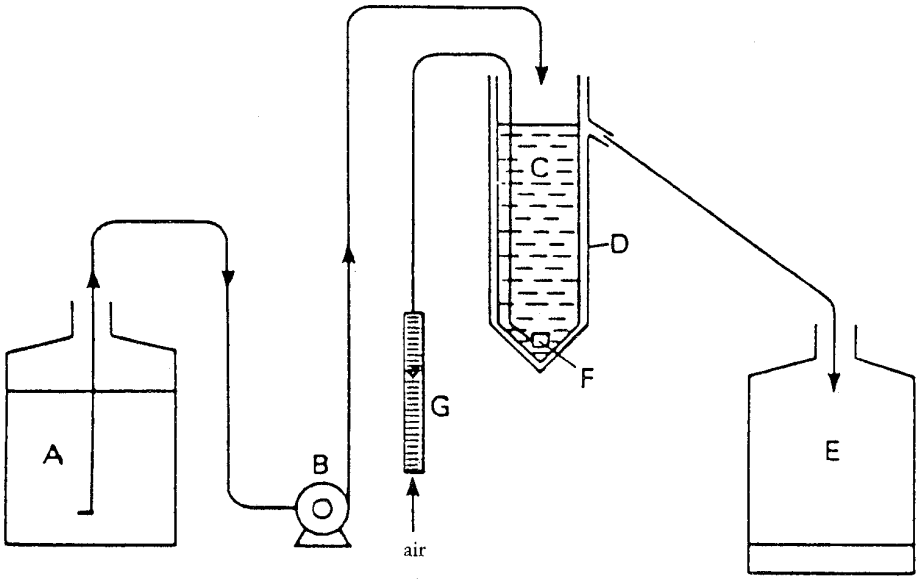
Figure 2



APPENDIX 2

Figure 1

Equipment used for assessing biodegradability



Key: A = storage vessel;
B = dosing pump;
C = porous aeration vessel;
D = outer impermeable vessel;
E = effluent collection vessel;
F = diffuser-stone aerator;
G = rotameter (optional).

Figure 2

Details of three-litre porous-pot aeration vessel