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Evaluation of Anaerobic Biodegradation

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SUMMARY

For certain chemicals information on their biodegradability under anaerobic conditions could be an important factor in the assessment of their environmental impact particularly where consideration of their use and properties suggests they may be associated with sewage solids and/or sediments, .

Methods to determine anaerobic biodegradation described in the literature were examined but it was felt that no protocol could be recommended without further experimental work. Preliminary studies indicated a number of inadequacies in existing techniques. Subsequently a programme of work was undertaken with the aim of developing a test method for the assessment of anaerobic biodegradability.

A number of methods were examined and the influence of key parameters such as concentration of test compound, inoculum concentration and pretreatment, digester design and media composition were investigated. On the basis of the work done, ECETOC concludes that a test method suitable for screening chemicals for anaerobic biodegradation can now be recommended. The technique is simple, requires only a knowledge of the carbon content of the chemical under test, and is also applicable to poorly soluble compounds.

The proposed test guideline provides data of acceptable reproducibility and it is recommended that the method now be examined in more laboratories using a wider range of chemicals. The results indicate that the procedure is capable of demonstrating the ultimate biodegradability of chemicals under the conditions used for the anaerobic treatment of sewage sludge. It should be emphasised that a negative result in this test does not imply that the material will not degrade in the environment but that further investigations are required.

B. INTRODUCTION

Biodegradation of organic chemicals can occur under conditions in which oxygen is present (aerobic) or absent (anaerobic). In the former process organic carbon is oxidised to carbon dioxide while in the latter it may be reduced to methane. Both processes take place in the treatment of the sewage and can also occur in nature.

While many laboratory methods have been developed for studying the aerobic biodegradation of chemicals (cf. OECD Test Guidelines 301A-E, 302A, 303A, 304A; 1981), tests for assessing anaerobic biodegradation have received less attention. Nevertheless, anaerobic biodegradation is considered to be an important process especially for those chemicals which are not aerobically biodegradable or whose physico-chemical properties are such that their occurrence in an aerobic environment is restricted (e.g. chemicals which are strongly adsorbed or insoluble). For these chemicals it is possible that anaerobic degradation is the major process responsible for their breakdown in the environment.

For these reasons it was considered necessary to develop a test procedure capable of screening organic chemicals for anaerobic biodegradability. With this aim in view, ECETOC established a Task Force with the following terms of reference:

> "To set out a detailed programme of practical research for resolving the main problems in assessing the anaerobic biodegradation of industrial chemicals in waste-water treatment systems. This research should ultimately lead to the development of a scientifically reliable and practicable protocol."

All the test procedures described in the literature had some limitations. It was felt that further experimental development was required before a test guideline for the assessment of the anaerobic biodegradability of chemicals could be recommended. This report indicates some of the special problems associated with the measurement of anaerobic biodegradation. It describes the work carried out by ECETOC in establishing a test procedure that could form the basis of a test guideline.

C. BACKGROUND

1. GENERAL

In making an environmental hazard assessment of a chemical the estimation of the likely environmental concentration is essential. The predicted concentration can then be compared with experimentally determined toxic effect levels and the likelihood of adverse effects assessed. Although some substances may be destroyed by specific abiotic processes such as

photodegradation, hydrolysis, oxidation/reduction reactions, etc., breakdown by living organisms or biodegradation is considered to be the major removal process for most organic chemicals likely to reach the aqueous and soil environment in significant concentrations. Heterotrophic micro-organisms which rapidly convert a wide variety of organic substances to simple compounds (e.g. CO_2, CH_4, sulphides, nitrate, ... etc) are by far the most important group of organisms which bring about biodegradation.

Most of the laboratory methods developed so far for studying biodegradation have involved aerobic micro-organisms which utilise molecular oxygen as the hydrogen acceptor during the respiration process. Environmental conditions where molecular oxygen is at very low levels or absent are not uncommon. In these anoxic and hypoxic environments communities of anaerobic micro-organisms can become established in which sulphates, nitrates, carbon dioxide etc. are employed as hydrogen acceptors.

Anaerobic conditions occur naturally in the lower layers of sediments in lakes and ponds, river beds and estuaries and are deliberately maintained during sewage treatment for the processing of sewage sludge. Consequently, any material released to the environment which is slightly soluble in water and/or strongly adsorbs on solids, is likely to become available as a potential substrate for anaerobic organisms.

2. THE ANAEROBIC DEGRADATION PROCESS

Anaerobic biodegradation may be briefly defined as the microbial breakdown of organic matter, in the absence of oxygen, to carbon dioxide and methane. The process is far more complex than this simple definition implies and several features of the anaerobic process have a direct bearing on the design of the test method. A brief description of the process is therefore necessary to clarify the existing problems.

2.1 Steps Involved in the Anaerobic Biodegradation Process in a Sludge Digester

Crude sewage reaching a conventional sewage treatment works is first partially separated into aqueous (settled sewage) and solid (primary sludge) phases. The aqueous phase is treated in an aerobic biodegradation plant. The primary sludge is combined with either waste activated sludge or humus in the case of trickling filter plants and these waste sludges are digested under anaerobic conditions. The purpose of the process is to stabilise the sludge by destroying the water retaining structure, reduce its mass, volume, organic content and control pathogenic organisms.

The anaerobic process is complex but is usually considered to occur in at least three concurrent main stages : the first involves liquefaction (hydrolysis) of complex organic molecules like carbohydrates, proteins and lipids by the action of extracellular enzymes. In the second stage the hydrolysis products are fermented yielding mainly short chain fatty acids, alcohols, hydrogen and carbon dioxide (acidogenic step). Alcohols and acids are subsequently

converted to acetate and hydrogen (acetogenic step). The last degradation step is effected by a group of obligate anaerobes, collectively referred to as methanogenic bacteria (methanogens) utilising acetate and hydrogen to form methane . Thus the ultimate degradation products of digestion are mainly carbon dioxide and methane. The microbiological processes involved are complex, involve several reaction stages and require different species of bacteria. A schematic diagram illustrating the various steps and microorganisms involved is represented in Figure 1 (Donnelly, 1984).

The ratio of methane to carbon dioxide produced varies with the composition of the chemical used as substrate. According to Tarvin and Buswell (1934) materials containing C, H and O are converted to carbon dioxide and methane according to the following empirical formula.

$$C_nH_aO_b + (n - \frac{a}{4} - \frac{b}{2})H_2O = (\frac{n}{2} - \frac{a}{8} + \frac{b}{4})CO_2 + (\frac{n}{2} + \frac{a}{8} - \frac{b}{4})CH_4$$

2.2 Nature of Chemicals reaching the Anaerobic process

The initial separation of the aqueous and solid phases during sewage treatment selects to some degree which chemicals will reach the aerobic and anaerobic processes. This selection will, to a large extent, depend on the physico-chemical properties of the materials. In practice only about 1 % of the incoming volume will reach the anaerobic process. Hence, as a general rule, anaerobic biodegradability data will not be required for soluble compounds which do not adsorb on the solids.

Conversely, chemicals which adsorb strongly on solids or are poorly soluble in water are likely to occur at significant concentrations in the waste sludges. Thus chemicals which are aerobically biodegradable may partially circumvent the aerobic stage and be released to the environment associated with the waste sludge. After anaerobic digestion (cf 2.3 below) waste sludge will usually be disposed of at sea, be used as landfill, or as an agricultural fertiliser or be incinerated. The task of demonstrating environmental safety will be eased if it can be demonstrated that the chemical under consideration is degraded in the anaerobic process.

For these reasons an assessment of anaerobic biodegradability may be useful for chemicals which are insoluble or have a tendency to adsorb on solids. Consequently, any test method must be capable of dealing with these classes of chemicals.

2.3 Specific Parameters influencing Anaerobic Biodegradation

The anaerobic process tends to be self-inhibitory since the hydrolysis of complex substrates such as fats, proteins and carbohydrates results in the formation of volatile organic acids. These, by lowering the pH of the system, may inhibit the growth of the methane producing bacteria unless the system has sufficient buffer capacity.

Inhibition of gas production as a result of the toxicity of the chemical is also possible. For this reason, the ratio of test chemical to inoculum concentrations must be considered when designing a test method (Reynolds et al., 1987), bacteria can acclimatize to changing conditions and adapt to new chemicals, which, although initially recalcitrant, may eventually become susceptible to biodegradation. Although it is expected that in many cases the duration of the test would be sufficient to allow for such a process, use of an acclimatized sludge from a laboratory sewage plant treating the test compound may be necessary.

There are also some indications that certain chemicals e.g. nitrilo-triacetic acid, are only degraded anaerobically when aerobic organisms acclimatized to the test chemical are incorporated in the raw sludge fed to the digesters (Moore and Barth, 1976). Such refinements should not be considered as a part of a screening test but could be included in a simulation test procedure.

D. ESTABLISHMENT OF A TEST METHOD

1. REVIEW AND ASSESSMENT OF EXISTING TEST METHODS

The most straightforward method to investigate anaerobic biodegradability is the use of uniformly 14C labelled test substances. In such tests the detection of radioactive methane and carbon dioxide provides unequivocal evidence of ultimate biodegradation. Although this procedure is well validated, the specialized equipment required, the necessity of preparing costly radio-labelled materials and the difficulty of replicating radio-labelled commercial products makes the technique unsuitable as a screening test for new chemicals.

A more generally applicable test method based on a determination of summary analytical parameters (e.g. DOC,COD,TOC) could be used. The complex composition of the digester sludge and the requirement for a method which is also applicable to strongly adsorbing and/or poorly soluble chemicals, excludes the use of dissolved organic carbon (DOC) or total organic carbon (TOC) as analytical parameters.

In some cases specific analytical methods exist which can be used to assess primary degradation i.e. measurement of the disappearance of the chemical (e.g. for dyes; Brown and Laboureur, 1983). In most cases analysis of a specific chemical in a complex mixture with sludge is difficult. Most methods described in the literature are based on respirometric techniques. They involve measurement of methane and carbon dioxide production which are the final products of anaerobic biodegradation. Of all the methods reported, the work of Shelton and Tiedje (1984) is the most comprehensive. This method is based on earlier studies by Owen et al.(1979), Healy and Young (1979) and Gledhill (1979) who used a gastight syringe and a pressure transducer to monitor gas production. A sample of anaerobic sludge is diluted in a mineral salts medium, and a suitable quantity of test chemical is added. The mixture is digested in a sealed vessel and the net gas production (test -

control) is followed by measurement of the pressure in the headspace above the digesting liquid. The quantity of gas evolved is corrected to allow for carbon dioxide and methane dissolved in the aqueous phase. The required correction factors are determined experimentally.

Although the Shelton and Tiedje method may be considered as a reasonably adequate screening procedure a number of problems are not resolved. A major difficulty in accurately quantifying the gas production arises from the solubility of carbon dioxide in the digesting liquor; the solubility of CO_2 being affected by pressure, pH, ratio of headspace to liquid volume, temperature and the complex thermodynamic equilibria established between carbon dioxide and carbonates/bicarbonates of calcium and magnesium. To evaluate the results it is necessary to have a knowledge of the mole fraction of each gas produced from the chemical, and the distribution of carbon dioxide and methane between the aqueous and gaseous phases. The theoretical quantities of carbon dioxide and methane can be calculated from the Tarvin and Buswell (1934) equation but for this an exact knowledge of the empirical formula of the chemical is required, which for commercial products is not always easy to establish .

Problems may also arise when excessively large quantities of carbon dioxide and methane are produced by the sludge used to inoculate the system. Significant differences in the net gas production (i.e. between test and control digesters) can be obtained by the use of high concentrations of test chemical. This approach is limited in application as the toxicity of many chemicals would be inhibitory at the high concentrations required.

2. REQUIRED CRITERIA FOR A SCREENING TEST

The above review of existing test methods shows that an ideal screening test should have the following characteristics:

i) it should be easy to perform;
ii) it should use readily available equipment;
iii) it should not require a knowledge of gas solubilities;
iv) it should be applicable at concentrations which, for most materials, would be below the toxic inhibitory concentrations (i.e. in the range of 20-50 mg/l as C);
v) it should use an inoculum with a low background gas production (i.e. from control).

Since none of the existing methods completely meet all these requirements a number of alternative procedures were investigated.

3. FACTORS RELEVANT TO ALL ANAEROBIC TEST METHODS

This section will consider those factors which are common to all existing test methods and which comply with the required criteria for a screening test. The principal part, i.e. the determination of the ultimate anaerobic biodegradation by a measurement of the gas production will be considered below.

3.1. Apparatus

The precise design of the apparatus is not critical. The only essential requirements are the use of an airtight vessel and a means of measuring the amount of the gases produced. Satisfactory basic designs are shown in Figure 2 for the measurement of the volume of evolved gas and in Figure 3 for the measurement of gas pressure using a pressure transducer. The total volume of the digestion vessel should be ascertained as well as the volumes of the aqueous and gaseous phases. The volume of aqueous phase should not be less than 100 ml and the headspace above the liquor should be between 10 and 40% of the total digester volume. Vessels which correspond to these requirements and specifically designed for head space analysis are commercially available (e.g. Wheaton bottles). The use of other types, whenever they comply with the above requirements, have also been shown to be suitable.

During the test the temperature should be maintained at 35°C +/-2°C. A suitable thermostatically controlled bath or incubator is required for this purpose.

3.2 Inoculum

3.2.1. Origin. The most appropriate source of micro-organisms is sludge from the anaerobic digester of a sewage treatment plant treating predominantly domestic sewage. This anaerobic sludge is used to inoculate individual tests directly or is used to inoculate a laboratory digester which can then be operated continuously to provide an "in-house" supply of micro-organisms.

If the sludge from a sewage treatment plant is used directly, it is advisable to minimise any thermal shock to the micro-organisms during transport to the laboratory.

3.2.2. Preparation of the Inoculum. In order to use the minimum amount of test chemical necessary to provide a measurable increase in the net gas production, the possibility of pretreating the sludge to reduce the background gas production was investigated.

Predigestion of the sludge in the laboratory to reduce the level of biodegradable compounds in the inoculum and/or washing of the sludge with dilution water prior to use were examined. As in the studies mentioned above (Shelton and Tiedje, 1984), the gas produced by the inoculum was further minimised by the fact that the inoculum concentration in the test system was diluted to about 10% of the sludge concentration in real digesters.

3.3. Dilution Water

The mineral salts medium (dilution water) used was modified from that reported by Shelton and Tiedje (1984) as follows:

a) addition of trace metals was eliminated since it was considered that essential metal elements are likely to be present in sufficient amounts in the inoculum even after washing;

b) addition of sodium sulphide was omitted since it is also present in the inoculum;

c) dipotassium hydrogen phosphate was replaced with the sodium salt to provide a more environmentally relevant sodium/potassium ratio;

d) in methods 2 and 3 described below (cf 4) sodium bicarbonate was omitted in order to reduce the amount of inorganic carbon in the system;

e) oxygen was removed by sparging with pure nitrogen before use.

3.4. Test Chemical Concentrations

Chemicals which inhibit the anaerobic process usually do so at a concentration of about 2 % on dried sludge solids (DSS). The level of dried solids in anaerobic digesters is approximately 3.0 % and if a 10 % inoculum is used then, to limit the possibility of inhibitory effects, the maximum concentration of test compound would be about 60 mg/l. Higher concentrations, which improve the reliability of the method, were used in some laboratories and present no problems other than an increased possibility of inhibition. Based on the present ECETOC experience, test compound concentrations in the range 20 -50 mg.l carbon seem to offer a reasonable compromise between obtaining reliable data and not having to repeat too many tests due to inhibition. Any inhibitory effect will be immediately apparent since the gas production in the controls will exceed that observed in the test vessels.

Detailed descriptions of the above test parameters are given in Appendix 1.

4. TEST METHODS INVESTIGATED

Several techniques to measure the extent of gas production from degradation under anaerobic conditions were examined in some preliminary studies performed by ECETOC. Seven laboratories participated in different experimental exercises to select the most suitable technique. In all methods the net carbon dioxide and methane gas produced by anaerobic biodegradation of the test chemical was obtained by a differential measurement between test and control systems.

4.1. Method 1

This method is based on the measurement of the amount of gas produced during the digestion as described by Shelton and Tiedje (1984). To quantify the gas production it is necessary to make a correction for gas dissolved in the aqueous phase. This requires a knowledge of the relative quantities of methane and carbon dioxide produced from the test chemical; experimentally determined solubility factors can then be applied to allow for the amount of each gas dissolved in the digested liquor.

Provided the chemical formula of the test chemical is known, the theoretical quantities of methane and carbon dioxide produced under anaerobic conditions can be calculated from the Tarvin - Buswell equation. To establish whether these theoretical values are obtained under the test conditions, a series of experiments were performed using 14C labelled compounds. The results obtained are given in Table 1. From these results it is apparent that the ratio predicted by the Tarvin-Buswell equation was not realised on all occasions and that inter- and intra-laboratory results may vary considerably. Possible explanations for the observed discrepancies are :

a) the methanogenic bacteria which convert a part of the carbon dioxide to methane are obligate anaerobes and may be inhibited by the presence of small amounts of oxygen;

b) since the process occurs in a series of discrete reaction steps the predicted carbon dioxide/methane ratio is unlikely be established until all reactions are complete;

c) periodical measurement of gas production followed by venting would result in a permanent disturbance of the gaseous equilibria.

It was concluded that the validity of the correction for the solubilities of the gases is open to doubt. Measurement of the methane concentration in the headspace gas by, for example, gas chromatography is a possible alternative but it complicates the method and again requires determination of the correction factor for the solubility of carbon dioxide. Further, for commercial products, specifically preparations the chemical formulae and composition are not always available.

4.2. Method 2

Since methane is slightly soluble in water a negligible error is introduced by assuming that it is present only in the gaseous phase. The total amount of the carbon mineralised could be assessed if, in addition to the usual gas production measurement, the dissolved carbon dioxide is determined separately. This requires one additional pressure or volume measurement if carbon dioxide is quantitatively released from the aqueous phase by acidification at the end of the digestion period. Experiments were performed to evaluate the procedure. Typical results shown in Table 2 are representative of the experiments performed.

The results show that significant quantities of carbon dioxide remain in solution even at low pH values. Although the dissolved carbon dioxide could be determined by sparging the acidified liquid with nitrogen and passing the gas through suitable traps it was considered that this would unnecessarily complicate the test procedure.

4.3. Method 3

This method is based on a measurement of the amount of gas produced during digestion and of the dissolved inorganic carbon (DIC) in the digested liquor. Since both methane and carbon dioxide contain only a single carbon atom it is possible to quantify the total carbon in the gas phase even though the relative quantities of each gas are unknown. The total test compound carbon converted to carbon dioxide and methane is determined directly and knowledge of the carbon content of the test chemical is the only prerequisite.

In principle this technique had in principle, been used for some time with considerable success in one of the participating laboratories. As difficulties had been experienced with the other two methods. It was selected for a more detailed examination.

The examination included fundamental studies on pre-treatment of the inoculum and optimization of test compound and inoculum concentrations. Techniques for reducing the background levels of gas/DIC while maintaining a low test chemical concentration were investigated since it was considered that the precision of the differential measurement could thereby be improved. The techniques included washing the sludge with the anaerobic mineral salts medium which in some tests was preceded by preincubation of the sludge to reduce the level of anaerobically biodegradable substrates.

4.3.1. Effect of washing of the inoculum. The anaerobic inoculum was washed with mineral salts medium. The results shown in Table 3 illustrate that the background DIC measured with a carbon analyser (cf. Appendix 1) is effectively removed by washing, and that one wash is sufficient to reduce the DIC to an acceptable level.

During the course of anaerobic biodegradation the levels of carbon dioxide and DIC will increase as a result of the transformation of organic carbon initially present in the sludge. The effect of the washing procedure on the background gas and DIC production is shown in Table 4. The results show that control gas and DIC levels are significantly reduced by washing.

Before accepting such a treatment it was necessary to demonstrate that it did not adversely influence the biological activity of the inoculum. Tests using 14C labelled chemicals were inoculated with washed and unwashed sludges to examine the effect of washing on the viability of the micro-organisms. Results shown in Table 5 indicate that the biological activity of the inoculum was not adversely affected by the washing procedure.

4.3.2. Effect of preincubation of the inoculum. Although the background inorganic carbon in the inoculum can be drastically reduced by washing it is not clear if this treatment also reduces its anaerobically degradable organic carbon content. It was thought that preincubation of the sludge could achieve this. A subsequent washing should then further eliminate inorganic carbon.

To evaluate the effect of such treatments, background gas production was measured with inocula preincubated for 2 and 4 weeks respectively and compared with an untreated inoculum. As can be seen in Table 6 the amount of background gas produced was significantly reduced by preincubation. Anaerobic biodegradation experiments performed with fresh and preincubated inocula indicate that such treatment can reduce considerably the rate of biodegradation and increase the lag period. An example for the anaerobic biodegradation using sodium benzoate is shown in Fig. 4.

An obvious consequence of preincubation of the inoculum is a slight increase of the DIC level as organic carbon is converted to inorganic carbon (Table 7). The DIC increase does not create a problem as the sludge after preincubation will always be washed before use.

In subsequent tests about 5 days predigestion seemed to give an optimum decrease in background gas production without unacceptable increases in either the lag or incubation periods.

4.4. Effect of Inoculum concentration

Within certain limits the extent of biodegradation should be independent of the inoculum concentration. Practical experience has demonstrated that the inoculum concentration affects the rate of biodegradation, the occurrence and duration of a lag period and the degree of inhibition resulting from the toxicity of the test compound.

In selecting the optimum inoculum concentration the following factors need to be considered:

i) An increase of inoculum concentration increases the rate of biodegradation and permits a shorter test duration;

ii) A higher inoculum concentration makes the system less susceptible to the inhibitory effects of traces of oxygen;

iii) An increase in inoculum concentration decreases the inhibitory effects of the toxicity of test material on biodegradation;

iv) The quantity of gas liberated in the control digester and the control DIC concentrations increase in proportion with the amounts of solids present in the inoculum.

Some exploratory experiments to examine the influence of the inoculum concentration on anaerobic biodegradation of sodium benzoate and phenol were made. The results given in Fig. 5 for sodium benzoate show the effect of different concentrations of a prewashed inoculum after 5 days preincubation in the laboratory. They indicate that lag period, and the rate and extent of biodegradation were not significantly different at the various inoculum concentrations examined. A small increase in the lag period is apparent as the level of inoculum is decreased. Such an effect would be expected only if the doubling time for reproduction of the bacteria responsible for the biodegradation of the chemical was relatively long. Comparable conclusions were reached when testing phenol at inoculum concentrations of 0.24 and 0.48 % DSS and following the course of degradation by monitoring the gas production.(Figure 6).

Further experiments were made using sodium benzoate to assess the effect of inoculum concentration on the precision of the method. It is apparent from the data presented in Table 8 that as the inoculum concentration is increased, and higher levels of background gas and DIC are produced, the precision of the results is decreased significantly. It is worthy of note that at the washing stage the solids level can be adjusted as required by controlling the volume of test medium used to disperse the sludge pellet. Where test chemicals are known to be or suspected of being toxic the use of higher inoculum levels may be advisable even though some loss of precision may occur.

4.5 Conclusions based on Preliminary Studies

The method finally adopted is in many ways similar to that developed by Shelton and Tiedje but there are a number of important differences which make the procedure more widely applicable.

A separate determination of the dissolved inorganic carbon in the digested liquor eliminates the need for any correction for the solubility of carbon dioxide. Consequently use of the Tarvin and Buswell equation, which in turn requires a knowledge of the chemical formula of the test chemical, is also unnecessary. Only a knowledge of the carbon content of the chemical is needed. Further, conversion of carbon dioxide to methane in the quantities predicted by the above equation is no longer essential. Since this depends on the viability of methanogenic bacteria which are obligate anaerobes, the requirement for strictly anoxic conditions is reduced.

The reduction in the background gas and DIC production in the controls achieved by washing the sludge used to inoculate the tests would be expected to improve the precision of the method. Experiments showed that this was so, but for sludges with low initial DIC the improvement might only be marginal. The use of high sludge concentrations, although having some advantages, further exacerbates the problem of high background gas production.

At present it is not possible to make a firm recommendation as to the test duration but, based on the results obtained once a plateau has

been reached the test can be terminated. For a number of chemicals degradation can be complete in a reasonable period. In addition to the nature of the test material, the kinetics of degradation will also depend on the quality of the inoculum and how rigouroulsy oxygen is excluded when setting up the test. Since both of these factors are difficult to control it is recommended that intermediate measurements are made and the test terminated when the net gas production reaches a plateau.

The dilution medium recommended by Shelton and Tiedje was simplified without any apparent problems. To maintain the inorganic carbon at a low level no bicarbonate was added to the medium and for similar reasons the carbon dioxide/nitrogen mixture used to sparge out oxygen was replaced by pure nitrogen. The buffer capacity of the medium was found to be sufficient to maintain the pH of the digested liquor in the range 6.7 - 7.1.

From a practical standpoint the use of the Wheaton bottles sealed with butyl rubber serum caps and crimped aluminium rings have a number of advantages as does the use of a pressure transducer for the measurement of gas production. However, a wide variety of digester designs, which included the more conventional volumetric methods of assessing gas production, were used. As might be expected the results demonstrated that the size and design of the digester had little effect on the final results.

E. EVALUATION OF THE TEST METHOD

Preliminary studies established that the measurement of gas production combined with the determination of DIC and pretreatment of the inoculum could lead to the establishment of a tentative test procedure. A detailed description of the method which was evaluated with a number of different chemicals is given in Appendix 1.

Although, for the reasons discussed above, data for soluble compounds would not routinely be required, initial studies were confined to chemicals in this class to avoid any uncertainty due to difficulties in introducing insoluble compounds into the test system. Results for the soluble materials examined are given in table 9A. The range of results obtained for each test compound are generally satisfactory with the notable exception of potassium phthalate. Although a longer test period (77days) was used to obtain the positive result it should be pointed out that in the laboratory that failed to degrade the compound other materials examined in the same run were extensively mineralised in 28 days. On the basis of these observations it was not possible to reject either result. Possible explanations for the discrepancy are differences in the nature of the sludges used to inoculate the tests or that the organisms responsible for the degradation of phthalate grow extremely slowly. It should be noted that two of the compounds included in Table 9 A i.e. the sodium salts of palmitic and benzoic acids, may have been precipitated as their calcium and magnesium salts during the course of the test.

Having established the validity of the technique, a limited number of poorly soluble materials were examined using different methods to introduce the chemical into the test. The results given in table 9B suggest no difficulties are likely to be experienced with the less soluble compounds provided they can be introduced into the system in a suitably dispersed form. (de Morsier et al., 1987).

In Table 9C results are presented where a sufficient number of replicates were used to allow reliable statistical evaluation of the data. The results indicate that the procedure is capable of demonstrating the anaerobic biodegradability of materials with a precision of approximately +/- 10% (cf. Appendix 2).

In those cases where similar chemicals were used the results agreed with the conclusions reported by other workers. (Tarvin and Buswell, 1934; Moore and Barth, 1976; Healy and Young, 1979; Boyd et al, 1983; Shelton and Tiedje, 1984).

F. RECOMMENDATIONS

1. The proposed test guideline should be further validated by the involvement of more laboratories working with a wider range of chemicals paying particular attention to those chemicals which are poorly soluble or adsorbing strongly on solids.

2. The observed biodegradation of chemicals in full scale digesters should be compared with data obtained in the laboratory to assess further the environmental relevance of the method.

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TABLE 1

COMPARISON OF THEORETICAL AND OBSERVED

CARBON DIOXIDE/METHANE RATIOS

Chemical	Carbon Dioxide/Methane Ratio	
	Theoretical	Observed
Glucose	1.00	1.71
Glucose	1.00	1.85
Glucose	1.00	1.75
Glucose	1.00	1.09
Glucose	1.00	1.13
Phenol	0.71	1.07
Phenol	0.71	1.14
Phenol	0.71	1.02
PEG 400	0.60	0.61
PEG 400	0.60	0.64

TABLE 2

DETERMINATION OF QUANTITY OF DISSOLVED CARBON DIOXIDE

LIBERATED AFTER ACIDIFICATION

CO_2 in solution $mg.l^{-1}$	reduction in concentration of CO_2 after acidification to pH = 1 with HCl mg.l-1	CO_2 evolved %
16.0	8.8	55
23.1	8.8	38
20.0	5.7	30
9.4	5.5	28
10.6	6.4	60

TABLE 3

INFLUENCE OF WASHING OF THE INOCULUM ON DIC* CONTENT

No. of washes	No. of determinations	Mean DIC $mg.l^{-1}$	% Removal
0	3	867	-
1	3	26	97.0
2	3	6	99.3
3	3	3	99.7

* DIC: dissolved inorganic carbon

TABLE 4

INFLUENCE OF WASHING OF THE INOCULUM

ON NET GAS AND DIC PRODUCTION

		unwashed sludge	Washed sludge (1 wash)
Gas formed (ml)	blank	22.0	0.5
	test	29.0	7.5
	net gas	7.0	7.0
DIC ($mg.ml^{-1}$)			
- start	blank	70.0	1.1
	test	70.0	1.5
- end	blank	74.0	2.7
	test	77.0	7.5
- net DIC		3.0	4.4

TABLE 5

INFLUENCE OF WASHING ON THE BIOLOGICAL ACTIVITY OF THE INOCULUM

	% Biodegradation	
Test Compound	Unwashed Sludge	Washed Sludge
Glucose	68.8	79.2
Stearylalcohol ethoxylate	68.0	67.4

TABLE 6

INFLUENCE OF PRE-INCUBATION OF INOCULUM ON BACKGROUND GAS FORMATION

Time (days)	mean gas production (ml)		
	fresh sludge	after 2 weeks predigestion	after 4 weeks predigestion
7	3.60	0.84	0
14	8.44	2.01	0.49
21	16.40	5.14	2.71
28	20.37	8.85	5.86
35	23.40	10.98	7.34*

* after 33 days

TABLE 7

INFLUENCE OF PREINCUBATION ON DIC FORMATION

IN UNWASHED SLUDGE.

Duration of Preincubation (days)	DIC ($mg.l^{-1}$)
0	540
7	587
14	660

TABLE 8

EFFECT OF SOLIDS CONCENTRATION ON PRECISION

Test Compound : Sodium Benzoate

Solids Concentration %	Carbon in Controls mg	Biodegradation %	Standard Deviation	Number of Replicates
0.69	10.3	91.5	2.03	7
1.08	16.9	86.9	5.21	6
1.38	21.9	86.2	9.37	7

TABLE 9A : RESULTS OBTAINED USING PROPOSED METHOD - SOLUBLE COMPOUNDS

Test Cpd	% DSS	Cpd/DSS(%)	Inoculum	Test Period (days)	Replicates	% Biodeg'n	Range
Glucose	0.4	1.3	P1:W	34	4	78.5	63 - 99
	0.2	5.2	P2:W	28	5	68.0	
	0.1	5.5	:W	21	3	63.0	
	0.1	4.7	:W	10	3	97.3	
	0.1	11.7	:W	10	2	98.6	
	0.1	4.7	:W	10	3	96.0	
	0.1	11.7	:W	10	2	98.2	
	0.1	4.7	:U	28	3	82.9	
	0.1	11.6	:U	28	3	82.7	
	0.1	11.6	:U	10	3	94.0	
	0.1	4.6	:U	10	3	89.8	
	0.1	11.6	:U	10	2	89.9	
Phenol	0.2	2.1	P1:W	62	4	73.7	45 - 74
	0.5	1.1	P1:W	62	4	66.8	
	0.1	6.1	:U	28	3	45.3	
Na palmitate	0.4	1.3	P1:W	22	4	79.8	80 - 94
	0.2	3.0	P2:W	28	5	94.0	
Na benzoate	0.4	1.4	P1:W	56	5	96.5	42 - 97
	0.3	1.9	P1:W	35	5	91.9	
	0.4	1.3	P1:W	61	4	49.8	
	0.2	3.6	P2:W	28	5	74.0	
	0.3	1.9	:W	35	5	95.3	
	0.4	1.3	P2:W	47	4	60.5	
	0.4	1.3	P4:W	33	4	42.5	
PEG 400	0.4	1.2	P1:W	35	5	>60.0	60 - 80
	0.2	3.8	P2:W	28	5	80.0	
E phthalate	0.4	1.5	P1:W	77	5	60.7	0 - 61
	0.1	4.1	:U	28	3	0.2	
	0.1	10.2	:U	28	3	1.5	
Alkyl sulphate	0.3	1.5	P2:W	56	5	87.8	
NTA	0.3	1.8	P2:W	56	5	<10.0	
Metanilic acid	0.3	1.8	P2:W	56	5	0.0	
	0.2	5.0	P2:W	28	5	0.0	
Ethanol	0.2	4.0	P2:W	28	5	78.0	
Na acetate	0.1	10.6	:U	28	2	82.1	
	0.1	26.4	:U	28	3	83.3	

Pn : Predigested n weeks. W : Washed sludge. U : Unwashed sludge
% DSS - percentage of dried sludge solids in the medium
Cpd/DSS % - percentage of test compound on dried sludge solids

TABLE 9B : RESULTS OBTAINED USING PROPOSED METHOD - INSOLUBLE COMPOUNDS

Test Cpd	% DSS	Cpd/DSS(%)	Inoculum	Test Period (days)	Replicates	% Biodeg'n	Range
Glycerol Monostearate	0.4	1.2	:W	51	7	73.8(i)	74 - 108
	0.4	1.2	:W	51	8	108.3(ii)	
Glycerol Trioleate	0.4	1.2	:W	51	8	106.0(iii)	63 - 106
	0.4	1.2	:W	51	8	63.0(i)	
Benzoic acid	0.3	1.9	:W	35	5	89.5	89 - 111
	0.1	3.2	:U	21	3	110.5	

(i) Added as an ether solution and solvent evaporated
(ii) Added as an aqueous suspension
(iii) Added directly to digester using a microlitre syringe

Table 9C Results obtained using Proposed Method - Intra-Laboratory Reproducibility

Test Cpd	% DSS	Cpd/DSS(%)	Inoculum	Test Period (days)	Replicates	% Biodeg'n	Std Dev
Glucose	0.7	2.8	P3:W	77	10	76.3	4.3
	1.3	3.1	P3:W	50	8	93.3	3.3
Phenol	1.3	1.6	P3:W	50	8	80.1	3.6
Na palmitate	0.7	1.5	P3:W	77	10	78.8	4.3
Na benzoate	0.7	4.6	P1:W	28	7	94.5	2.1
	1.1	3.0	P1:W	28	6	90.1	5.4
	1.4	2.3	P1:W	28	7	89.0	11.7
	0.3	1.7	:U	20	10	99.7	5.2
	0.4	1.3	:W	20	10	90.8	4.3
PEG 400	0.7	2.2	P3:W	77	10	66.8	4.3
Ethanol	1.3	2.4	P3:W	50	8	110.1	5.3
Glycerol Monostearate	0.4	1.2	:W	51	7	73.8(i)	8.0
	0.4	1.2	:W	51	8	108.3(ii)	7.8
Glycerol Trioleate	0.4	1.2	:W	51	8	106.0(iii)	12.1
	0.4	1.2	:W	51	8	63.0(i)	7.5

Pn : Predigested n weeks. W : Washed sludge. U : Unwashed sludge

% DSS - percentage of dried sludge solids in the medium

Cpd/DSS % - percentage of test compound on dried sludge solids

FIGURE 1.

SUBSTRATE DISSIMILATION IN ANAEROBIC BIOLOGICAL REACTORS

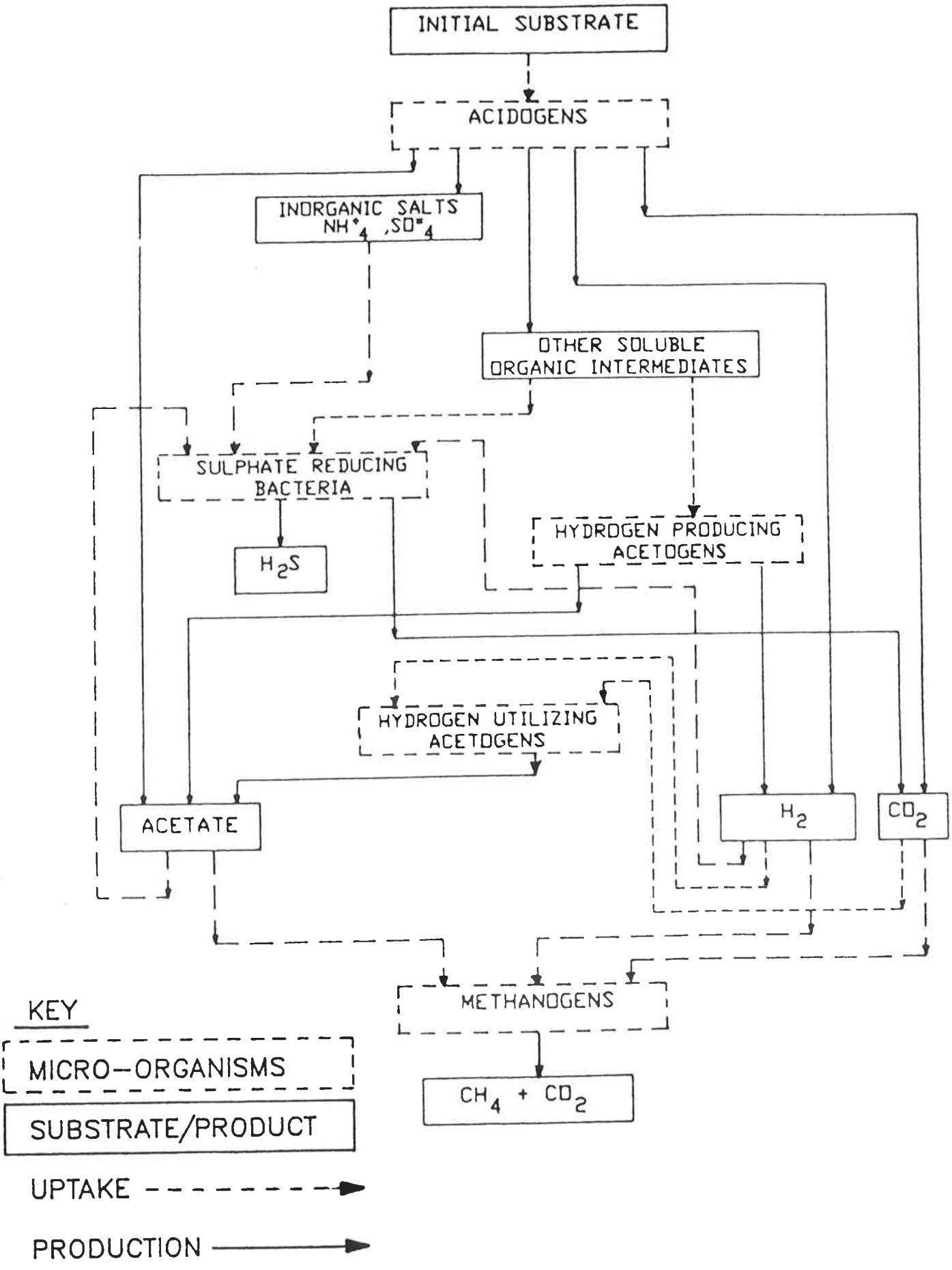


FIGURE 2.

APPARATUS FOR THE MEASUREMENT OF GAS VOLUME

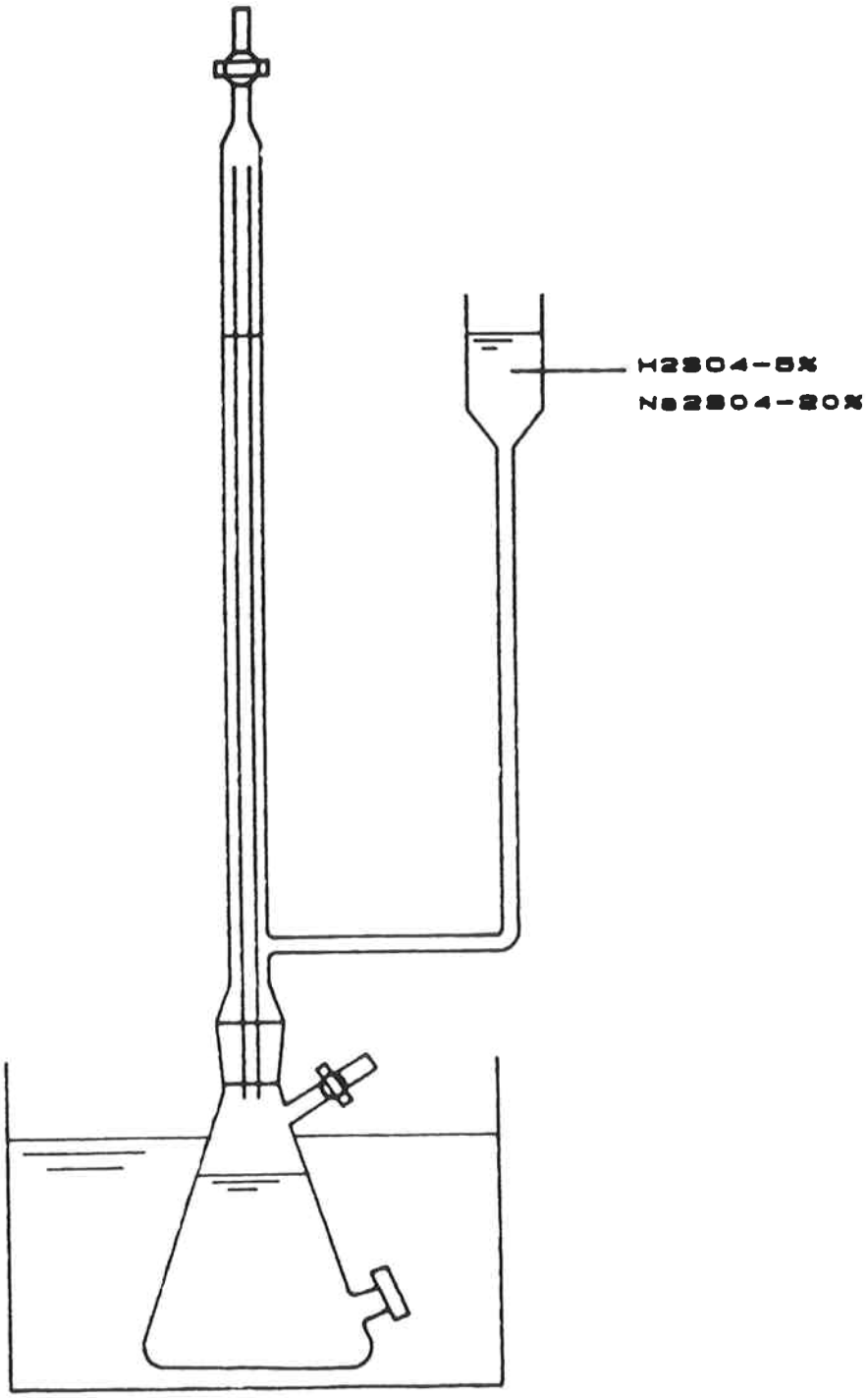


FIGURE 3.

APPARATUS FOR THE MEASUREMENT OF GAS PRESSURE

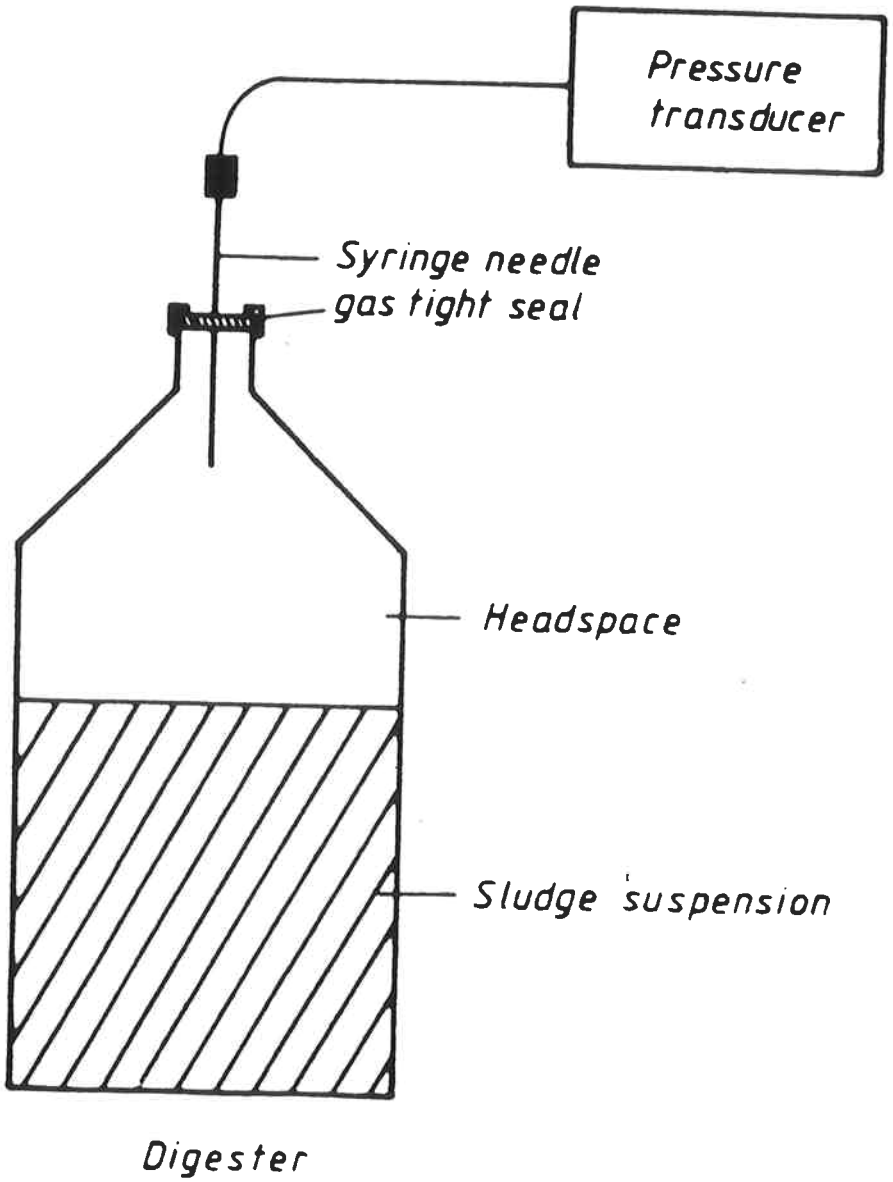


FIGURE 4. EFFECT OF PRE-DIGESTION OF INOCULUM ON RATE OF BIODEGRADATION.

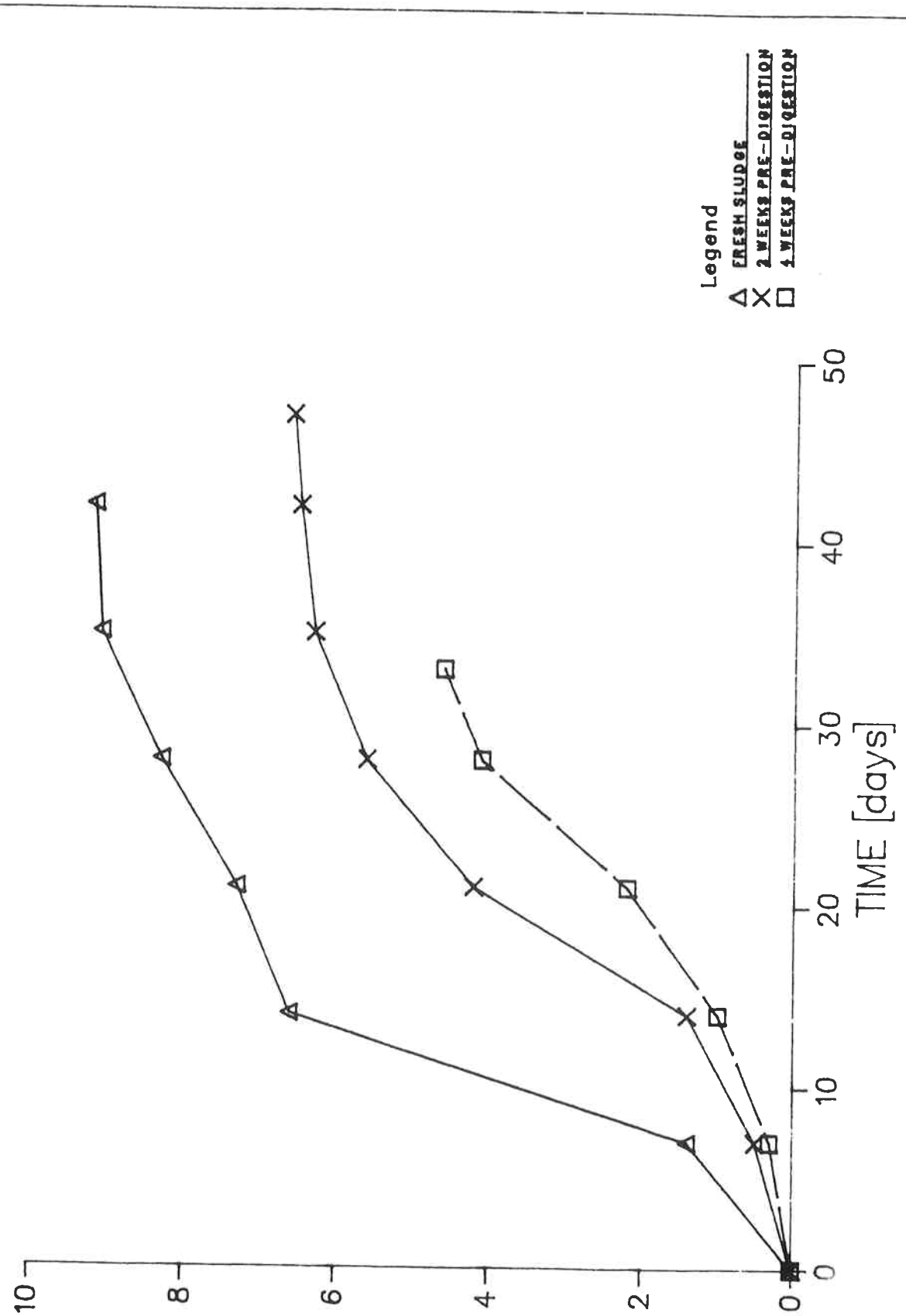


FIGURE 5. EFFECT OF INOCULUM CONCENTRATION ON RATE OF BIODEGRADATION – TEST COMPOUND SODIUM BENZOATE

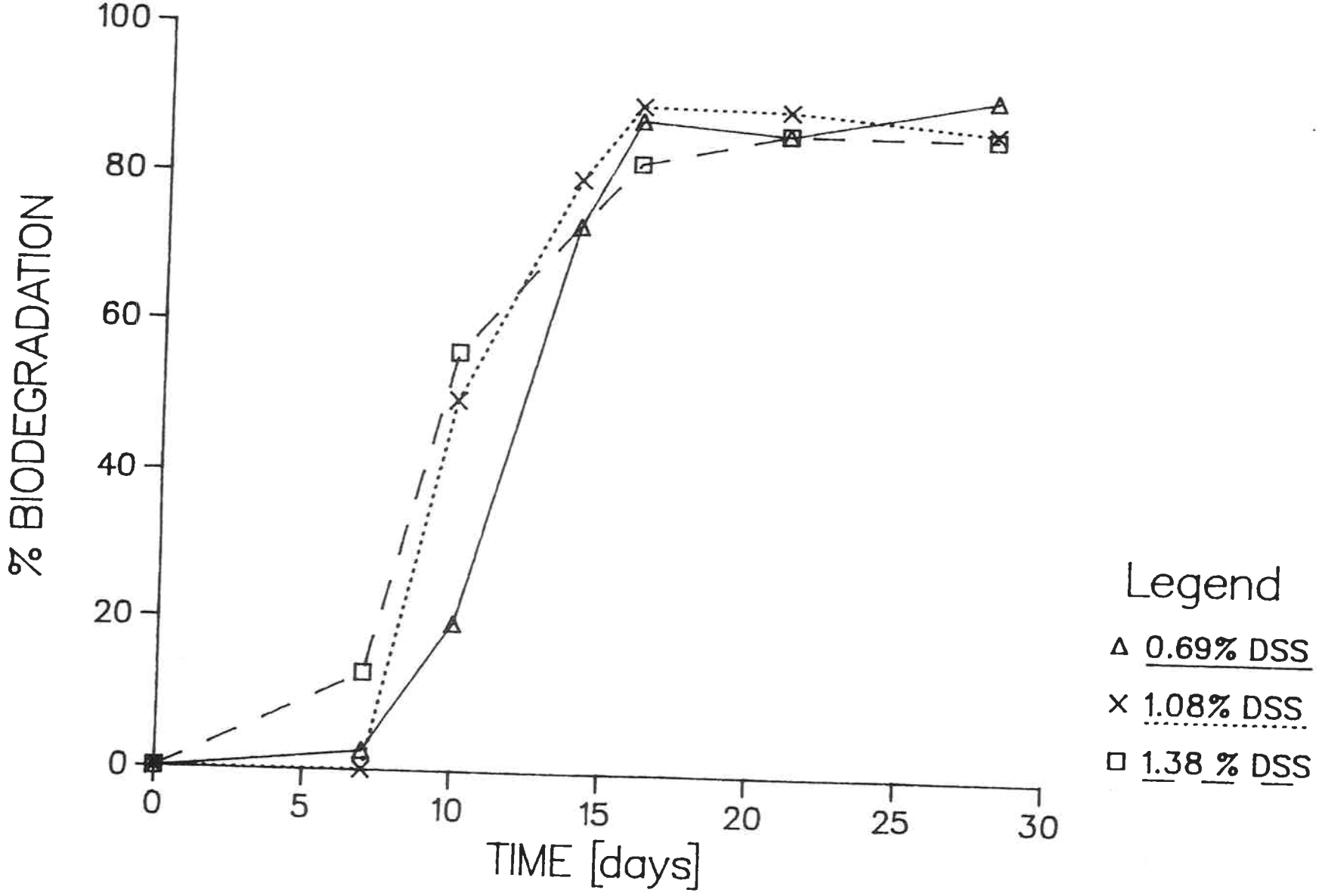
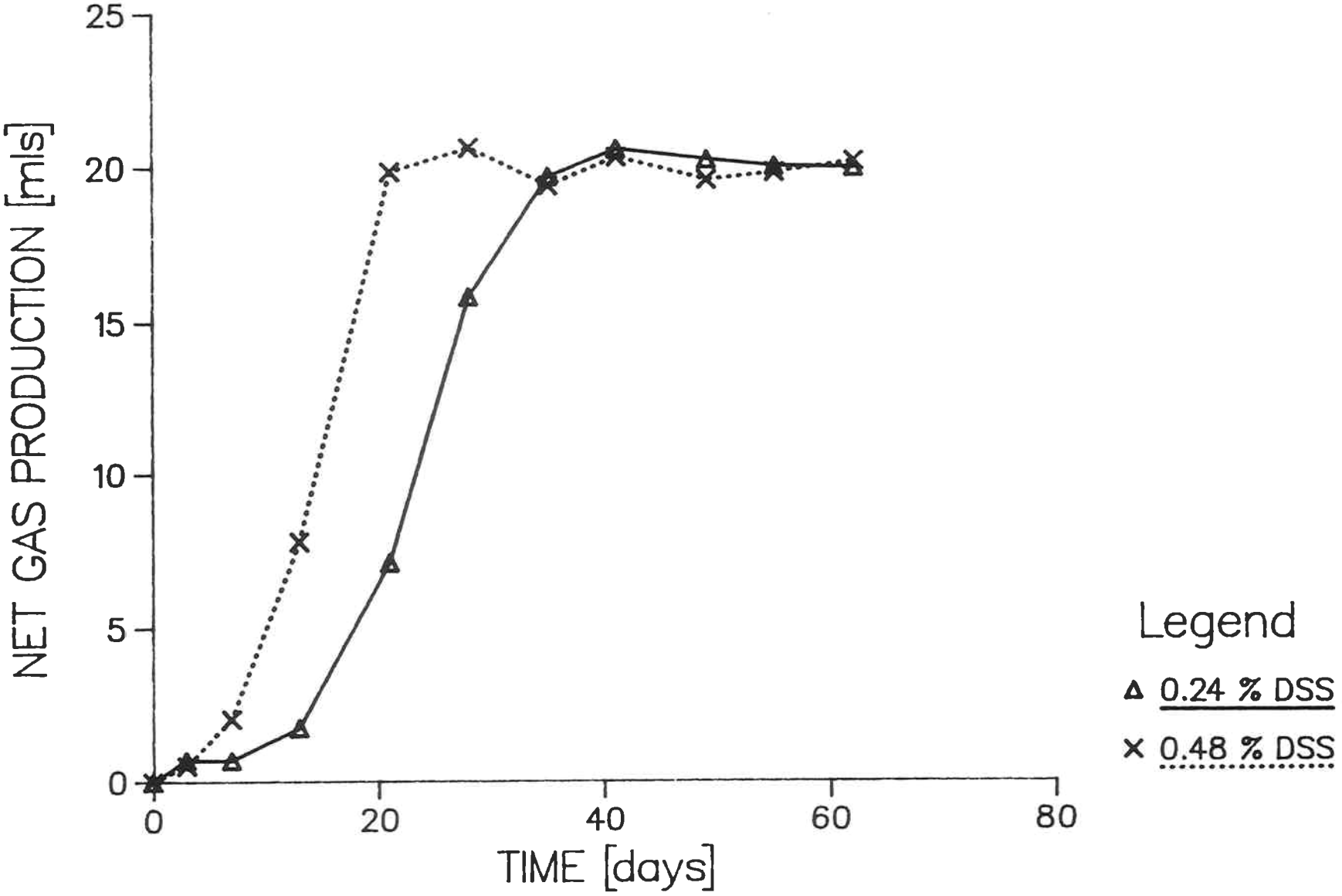


FIGURE 6. EFFECT OF INOCULUM CONCENTRATION ON RATE OF GAS PRODUCTION
TEST COMPOUND PHENOL



J. APPENDICES

APPENDIX 1

GUIDELINE FOR SCREENING OF CHEMICALS FOR ANAEROBIC BIODEGRADABILITY

1. INTRODUCTORY INFORMATION

° Prerequisites

- Carbon content of the test compound.

° Guidance Information

Vapour pressure,
Structural formula of test compound,
Water solubility,
Chemical stability,
Purity of the test substance,
Concentration at which the substance inhibits anaerobic degradation.

° Qualifying Statements

Materials having a high vapour pressure should be checked to establish their volatility from aqueous solution.

The test vessels must be maintained at a constant temperature during the entire test period.

The incubation temperature and the volume of the headspace must be known since the final calculation involves the use of gas laws.

Precautions are necessary to minimise oxygen contact with the sludge.

The possibility of partitioning of the test compound into materials of the test apparatus (eg biphenyl into the septa) should be considered.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TESTS

The method is intended to provide a simple and reliable screening test for the ultimate biodegradability of chemicals under anaerobic conditions. The method is applicable to soluble and poorly soluble organic chemicals. In principle the method can also be used to measure primary anaerobic biodegradability provided a suitable specific analytical method is available.

The results are most relevant to the assessment of anaerobic biodegradation in digester systems operated at approximately 35°C.

However, due to the relatively stringent test conditions (limited time allowed for acclimatization of the microorganisms; lower level of solids compared with conventional digesters) a positive result indicates that anaerobic degradation also takes place under less favourable conditions. The method should be considered as a screening test and consequently a negative result in this test should not be regarded as proof that the material is anaerobically non-biodegradable in the environment. In this case additional investigations are necessary to assess anaerobic biodegradability.

The method is limited to materials which are not inhibitory to the micro-organisms at the concentrations used (20 -50 mg/l as carbon or about 1% as organic carbon on dried sludge solids) and which are not significantly volatile from aqueous solution. Materials which are of low toxicity can be tested at higher concentrations but in principle low concentrations are preferred wherever possible to avoid inhibitory effects. A suitable approach for the determination of inhibition is described in the literature. (Reynolds et al.,1987).

If gas production in test digesters is significantly lower than that produced in the controls then inhibition by the test chemical is the most probable cause. In the latter case the test may be repeated at lower test concentrations. If no gas is produced in the controls then the results should be treated with caution since even sludges subjected to long periods of predigestion produce significant quantities of gas during the course of the test. In such cases, inhibition by excessive formation of acid or by ingress of oxygen are likely causes.

° D e f i n i t i o n s

Anaerobic Biodegradation - the microbial degradation of organic compounds in the absence of oxygen. It is effected by bacterial consortia which degrade the compound in a stepwise process yielding organic acids, carbon dioxide and hydrogen and, ultimately, methane and carbon dioxide.

Dissolved Inorganic carbon (DIC) - the total soluble carbonate species, including dissolved carbon dioxide, present in aqueous solution.

Net DIC Formation - the difference in the level of DIC in the test digester compared with the level in the control digester.

Dissolved Organic Carbon (DOC) - the total soluble organic carbon content present in aqueous solution.

Pressure Transducer - A device for converting gas pressures into electrical signals.

Headspace - the volume in a sealed vessel not occupied by the liquid phase.

Gas Production - the gas produced, principally carbon dioxide and methane, by the action of anaerobic micro-organisms on organic compounds.

Net Gas Production - the difference in gas production between the test and control digesters.

Digester - sealed vessel in which anoxic conditions can be maintained and any gases evolved can be collected and measured.

°Reference Substances

No reference substance is currently recommended. Suitable potential materials are listed below and can be used as control substances to establish the viability of the inoculum:

- sodium benzoate or sodium palmitate;
- PEG 400 (polyethylene glycol, MW = 400);
- meat extract (example of a complex substrate).

°Principle of the Test Method

During the anaerobic biodegradation of organic materials, a mixture of gases, principally methane and carbon dioxide, are the usual final decomposition products although at some intermediate stages some cell growth may occur. The ratio of the two gases produced is dependent on the chemical composition of the compound and the conditions and duration of the test.

A known volume of anaerobic sludge, (corresponding to 10% of the sludge concentration in a real digester), suspended in an oxygen free medium, is placed in a suitable vessel leaving headspace into which any gases produced may be evolved. Prior to sealing a small amount of test compound is added.

The vessels are incubated at constant temperature and pH for a period of up to 8 weeks. The headspace pressure resulting from the production of gas is measured, and the DIC content of the digesting liquid determined. From the measured values of net gas production and the net DIC formation the extent of biodegradation is calculated. If an indication of the kinetics of degradation is required then additional intermediate measurements are made at suitable intervals during the course of the test.

° Conditions for the Validity of the Test

The viability of the inoculum is indicated by gas production in the controls. Further, gas production in the test vessels should at least equal that in the controls; if the gas production in the controls exceeds that observed in the test vessels by a significant amount then inhibition of the system by the test compound is indicated. If a reference compound is included then extensive biodegradation (e.g. > 70%) of the material is probably the best indication of a valid test.

B. DESCRIPTION OF THE TEST PROCEDURE

° Preparations

Equipment

In addition to the normal laboratory facilities the following specialised equipment is required:

- incubator or thermostatically controlled water or sand bath.
- pressure resistant glass vessels, nominal size 0.1 -1.0 litre, fitted with gas tight septa, capable of withstanding 2 atmospheres.
- pressure measuring device, e.g. pressure transducer, connected to a syringe needle (gauge 12 - 18 or 2.65 - 1.24 mm O.D), three way gas tight valve which also facilitates the release of excess pressure but this is not essential. To measure the gas pressure the needle is inserted through the septa into the headspace.
- carbon analyser, suitable for the direct DIC determinations in range 0 - 200 mg DIC/l.
- a suitable apparatus is required if sludge is to be predigested or grown in the laboratory. A flask which can be thermostatically controlled and provided with a low speed stirrer is required for this purpose. A gas collection tube or any other form of gas metering device is advantageous as it allows the course of digestion to be followed but this is not essential.
- barometer - for measurement of atmospheric pressure.
- glove box - to allow test to be set up in oxygen free atmosphere (optional).

° Chemicals/reagents/materials

Test Medium

Mineral salts medium prepared in deionised water to contain in 1 litre each :

potassium dihydrogen phosphate,	KH_2PO_4	0.27g
disodium hydrogen phosphate,	$Na_2HPO_4.12H_2O$	1.12g
ammonium chloride,	NH_4Cl	0.53g
calcium chloride,	$CaCl_2.2H_2O$	0.075g
magnesium chloride,	$MgCl_2.6H_2O$	0.10 g

ferrous chloride, (freshly prepared)	$FeCl_2.4H_2O$	0.020g
resazurin (redox indicator),		0.001g optional

The pH of the medium should be 7 +/- 0.2 and should be adjusted with dilute mineral acid or alkali if required.

Oxygen is removed from the medium by purging with oxygen free nitrogen for approximately 1 hour immediately before use.

Inoculum

A sufficiently large sample of digester sludge is obtained from a sewage plant treating predominantly domestic sewage. The volume of sludge required will depend on the number of test flasks to be used and the volume of test liquor used in each flask. The sludge is transported to the laboratory and used directly, or placed in a laboratory scale digester and the course of gas production measured. Alternatively, a laboratory grown sludge can be used as an inoculum source. If predigestion is required the sludge is allowed to digest without the addition of substrate at 35 +/- 2 ° C for 2 to 7 days. This reduces the level of anaerobically biodegradable substrates in the sludge.

Prior to use in the test, the sludge is washed to reduce the inorganic carbon content to an appropriate concentration (< 10 mg DIC/l) in the final test solution). The washing procedure consists of centrifuging the sludge in sealed tubes at a relatively low speed (e.g. 3000 g) for up to 5 minutes not lower than room temperature (cooled centrifuges should be avoided). All steps should be performed as quickly as possible to minimise ingress of air but rigorous exclusion of oxygen is not necessary.

The recommended washing procedure is as follows:

i) a sample of sludge is centrifuged, the supernatant liquor discarded and the sludge pellet carefully re-suspended in mineral salts medium (e.g. by means of a glass bar or a spatula).

ii) the sludge is again centrifuged, the washings are discarded and the washed pellet resuspended in a suitable volume of the mineral salts medium.

If more than one washing is required step ii) is repeated but a maximum of 3 washings is recommended to avoid adverse effects on the micro-flora.

At stage ii) it is possible to adjust the inoculum solids concentration to any desired level. This is achieved by reducing the volume of medium used in the final step and determining the solids level before dilution to the required volume. The final dried solids

level should normally be in the range of 1 - 5 g.l but DSS levels outside this range can be used.

Test Substance

The test material may be dosed in the form of a solution, solid, suspension, or emulsion, and where applicable the strength of the test solution/suspension should be adjusted so that the volume added is a maximum of 5% of the total liquid volume. In addition the final concentration of the test substance should be in the range 20 - 50 mg C/l. In the absence of inhibition higher concentrations can be used. All solutions should be prepared with water that has previously been purged with oxygen free nitrogen. Poorly soluble materials can be added directly or as a suspension or dissolved in an organic solvent (de Morsier et al., 1987) which is evaporated from the test vessel before the addition of the test medium. Organic solvents known to inhibit anaerobic digestion (e.g. chloroform) should be avoided if possible. Substances having a high volatility from aqueous solution cannot be tested since loss of the test material when setting up the test cannot be avoided.

Performance of the test

Performance of the following initial procedures are best carried out in an atmosphere of nitrogen contained within a glove box, but this is not essential.

Measured aliquots of well mixed inoculum and the test substance (or an equal volume of water in the case of the control), are mixed into a vessel previously flushed with nitrogen. If the digester contents are to be magnetically stirred the bar magnet must be added at this stage. In all test flasks the total volume of liquid and the concentrations of the solid sludge dry matter should be the same. The volume of the head space should be between 10 and 40% of the total digester volume and should be the same for all test vessels. The vessels should be sealed with an inert gas septa, incubated at 35 +/- 2 ° C for about 1 hour to allow equilibration and the excess gas pressure then released to atmosphere. If at this stage or when making intermediate measurements the headspace pressure is less than atmospheric then nitrogen must be introduced to re-establish atmospheric pressure in the headspace. Considerable care should be taken to ensure that the entire vessel is maintained at the digestion temperature. At least 3 replicate vessels are required for each test material and ideally the number of controls should be twice the number of replicates used per test compound.

At the start of the test, the date, time, DIC, pH, atmospheric pressure and digester temperature are recorded. In some cases the test compound can change the pH of the medium. In such cases a check of the pH at the start of the test is necessary and if required the pH adjusted with mineral acid or alkali.

Anaerobic conditions must be maintained throughout the whole of the test period. Resazurin, if used, should remain colourless throughout

the test period. Occasionally a slight pink coloration will be observed in some vessels at the start of the test but in most cases it will have disappeared by the following day. The vessel contents need to be mixed carefully by stirring or shaking for a few minutes at least 2-3 times per week and before pressure measurement to resuspend the inoculum and ensure gaseous equilibrium. Even sludges subjected to long periods of predigestion produce significant quantities of gas during the course of the test. If gas production in the test digesters is significantly lower than that produced in the controls then inhibition by the test chemical is the most probable cause. In the latter case the test should be repeated at lower test compound concentrations.

Gas pressure is measured by inserting a syringe needle through the septa connected to a pressure monitoring apparatus. The internal volume of the pressure transducer, tubing and valve should be minimised so that errors, introduced by neglecting the volume of the apparatus, are insignificant. Precautions should be taken to prevent entry of water into the syringe needle. Great care must be taken to ensure that the gases in the head space are maintained at the incubation temperature when pressure measurements are made. This may be achieved by almost total immersion of the test vessel in the sand or water bath. It is strongly recommended that intermediate readings be made since this allows the kinetics of the gas production to be followed and also provides guidance as to when the test can be terminated. Time intervals of 1 week between the intermediate readings have proved to be sufficient and suitable. When making intermediate measurements excess gas can be released to the atmosphere if it seems likely that subsequent readings will be outside the range of the pressure transducer. Whenever gas is vented to atmosphere the remaining pressure in the vessel must be recorded.

At the end of the test, following measurement of the gas pressure, the sludge is allowed to settle, the digesters opened and the DIC content of the clear supernatant liquid determined immediately. The supernatant liquid must not be centrifuged or filtered at this stage since either procedure will result in an unacceptable loss of dissolved carbon dioxide. Alternatively, a sample may be stored refrigerated for 3 - 4 days in a suitable sealed vial without any headspace. The pH of the liquor is measured and recorded. Corresponding data for the controls must be obtained at the same time.

° <u>C a l c u l a t i o n o f r e s u l t s</u>

The number of moles of gas in each test vessel is calculated from the gas laws assuming ideal behaviour, thus:

$$n = \frac{dP \,.\, V}{R \,.\, T}$$

where

n = moles of gas in headspace,

dP = pressure difference between initial and final readings (in atms.). When intermediate measurements are performed the individual pressure differences should be totalled to give the total increase in pressure.

V = headspace volume (in litres),

T = temperature (degrees Kelvin),

R = gas constant (0.08205 litres. atms/moles/deg).

Since 1 mole of methane and 1 mole of carbon dioxide each contain 12g carbon then carbon in headspace(C_h) is given by:

$$C_H = 12 \times n \times 10^3 \text{ mg}$$

The carbon in the liquid phase (C_L) is given by:

$$C_L = DIC \times V_L$$

where

V_L = Volume of digesting liquor in litres.

DIC = Concentration of dissolved inorganic carbon in the liquor in mg.l

For each vessel the total mineralised carbon (C_T),is given by:

$$C_T = C_H + C_L$$

and the extent of biodegradation by:

$$\% \text{ Biodegradation} = \frac{\text{mean C (test) - mean C (control)}}{\text{mg test compound carbon added}} \times 100$$

3. DATA AND REPORTING

Treatment of results

Test report

The test report should contain the following information:

- Test substance, chemical identification data,
- Inoculum, source, concentration and pretreatment,
- Test conditions,
- Digestion temperature,
- Test compound concentration ($mg.l^{-1}$),
- Digester volume,

- Headspace volume,
- Solids content of digestion liquor,
- Percentage test compound on dried sludge solids,
- Percentage test compound (expressed as carbon) on dried sludge solids,
- Initial pH (optional) and final pH (mandatory),
- Atmospheric pressure, initial, intermediate (when applicable), and final pressure values,
- Digestion period,
- All pressure readings,
- Number of replicates,
- For pressure transducers all pressure data to be presented as raw data (eg. volts, ohms,millibars) with appropriate conversion factors,
- Mean and (if at least 4 replicates are used) standard deviation of head space pressure of control and test vessels,
- All DIC measurements,
- Mean and (if at least 4 replicates are used) standard deviation of DIC measurements of control and test vessels,
- Mean and (if at least 4 replicates are used) standard deviation of observed percentage biodegradation values for each material examined.

Ideally information on the kinetics of biodegradation can be obtained by determining both gas production and increase in DIC at intermediate times, but essentially similar information may be obtained by pressure measurements alone. Calculation and statistical treatments of results are discussed more fully in Appendix 2 .

4. L I T E R A T U R E

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- de Morsier, A., Blok, J., Gerike, P., Reynolds, L., Wellens,H., and Bontinck, W.J.(1987). Biodegradation tests for poorly-soluble compounds. Chemosphere, 16 (4), 833.

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APPENDIX 2

CALCULATION AND STATISTICAL TREATMENT OF RESULTS

The precision of the results will obviously be improved by increasing the number of replicates. Even a simple statistical treatment of test results such as a calculation of a standard deviation requires at least 4 replicates. The results obtained indicate that a standard error of about 10% should be achieved when 6 test and 12 control replicates are used. Further, in tests of this type where one set of controls are often used to evaluate data from a number of tests added confidence in the blank values is highly desirable. From a statistical standpoint it is therefore recommended that the number of control replicates be at least twice that used for any one test chemical.

Increasing the number of measurements on a single digester will increase the precision of the data regarding that unit but not that of the overall test.

The main purpose of intermediate analysis is to provide information on the progress of the biodegradation process and to give an indication when to terminate the test. Ideally the intermediate measurements should include determination of DIC but this precludes any further measurements being made on the digester used. An adequate indication of the course of the degradation can be obtained from the gas pressure measurement alone, the test being terminated when the difference in gas production between the control and test digesters (= net gas) reaches a constant value.

Several methods can be used to calculate the extent of biodegradation and to assess the confidence limits of the result. The following method of calculation is provided as an example :

Change in DIC in digester $C_L = (DIC_f - DIC_i).\ Vol$ mg

where :

DIC_f and DIC_i : final and initial DIC concentrations in the digester in mg/litre;

Vol : volume of digesting liquor in litres.

Carbon in headspace $C_H = \dfrac{(P_f - P_i) \times HSvol}{RT} \times 12 \times 10^3$ mg

where :

P_f and P_i : final and initial gas pressures in the headspace (atmospheres);

HSvol : volume of the headspace in litres;

T : digester temperature in °Kelvin;

R : gas constant in litre-atmospheres per degree per mole.

Where intermediate measurements are made any excess is vented to atmosphere then the above calculation is repeated for each time interval and the total gaseous carbon produced at the completion of the test, (C_H), is obtained by summation of the intermediate measurements of gas production. Irrespective whether intermediate measurements are made or not, when :

$$C_t = C_H + C_L$$

where C_t is the total amount of carbon in the digester converted to either carbon dioxide or methane during the course of digestion. The above calculation is made for the individual digesters in each group i.e. control and test digesters, and the mean total carbon and the standard deviations are calculated in the usual manner.

The mean % biodegradation, standard errors and the 95 % confidence limits are then calculated as follows:

$$\% \text{ Biodegradation} = \frac{[C_t(\text{test}) - C_t(\text{control})]}{C_i} \times 100$$

where :

C_i is the amount of carbon added to the test digesters as test compound.

$$\text{Error (\% Biodegradation)} = s = \frac{100}{Ci} \sqrt{\frac{St^2}{n_1} + \frac{Sc^2}{n_2}}$$

Where :

St and Sc : the standard deviations for the total carbon in the test and control digesters ;

n_1 and n_2 : number of replicate test and control digesters respectively.

95% Confidence Limits = % Biodegradation +/- t x s

where :

t is the t-distribution value for 95 % probability with $(n_1 + n_2 - 2)$ degrees of freedom.

APPENDIX 3

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APPENDIX 4

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