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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., break-down 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 biodegrada-tion 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.

$$C_nH_aO_b + (n - \frac{a}{4} - \frac{b}{2})H_2O = (\frac{n}{2} - \frac{a}{8} + \frac{b}{4})CO_2 + (\frac{n}{2} + \frac{a}{8} - \frac{b}{4})CH_4$$
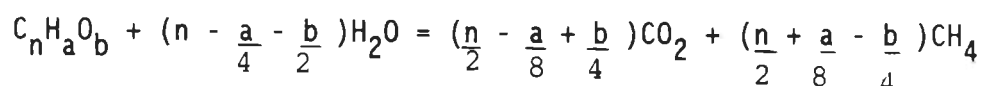
2.2 Nature of Chemicals reaching the Anaerobic process

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/1 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.1 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.