Anaerobic Biodegradation, Activity and Inhibition (ABAI) Task Group Meeting 9th to 10th October 2006, in Prague

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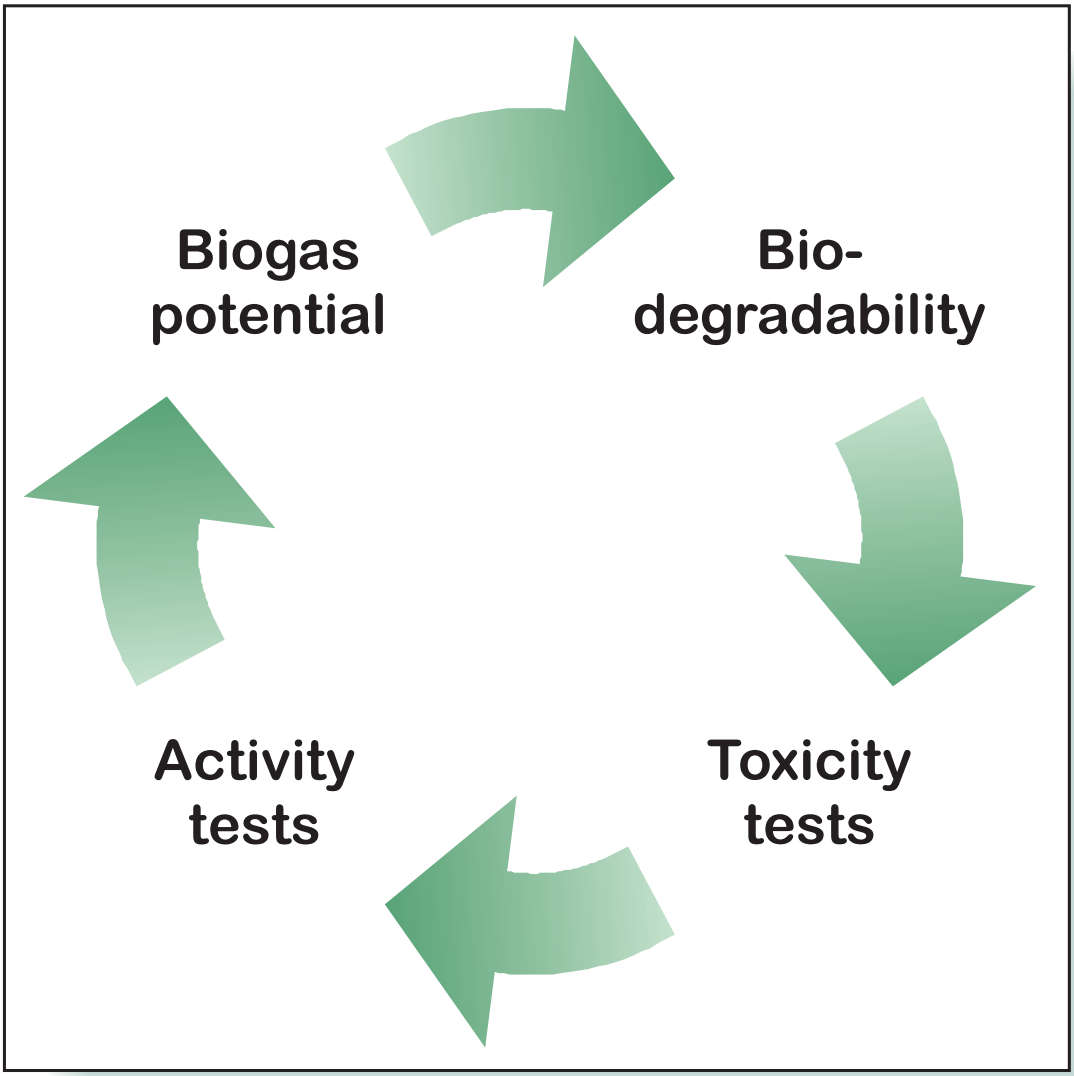

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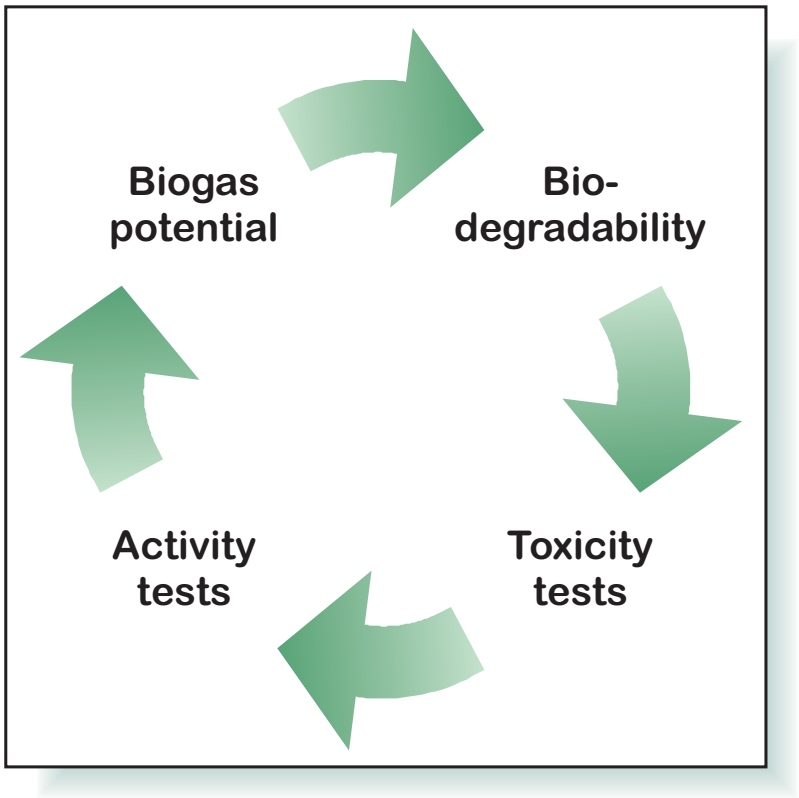

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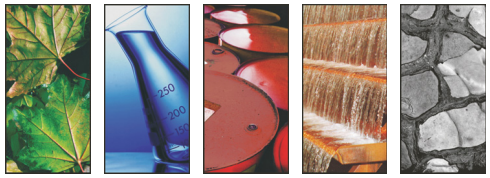

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1 Preface

Interest in renewable energy sources has grown dramatically during the recent decades. Biogas produced by anaerobic digestion (AD) processes is a renewable energy source and can be used for the generation of electricity, heat and as vehicle fuel. Apart from the energy production point of view, AD is also seen as an effective biotechnological method for organic waste and wastewater treatment. Current national environmental regulations and other policies governing land use and waste disposal have increased interest in anaerobic digestion. The EU countries have agreed on a directive requiring that the amount of biodegradable organic waste deposited in land fills to be decreased by 65% by 2016 (CD 1999/31/EC). These environmental and political pressures have increased the need for harmonisation of the anaerobic biodegradation assays.

Huge development both in basic knowledge of AD processes but also in technological aspects have been occurring. Along with development, many different methodologies for assessment of the anaerobic biodegradability of waste and wastewater and the activity of anaerobic reactors have come into use. However, the technical approaches in terms of pre-treatment of the sample, inoculum, gas measurement technique and incubation vary significantly among published methods. As a result of that need, a task group (TG) on Anaerobic Biodegradation, Activity and Inhibition (ABAI) was proposed to be created during the 2001 AD conference at Antwerp. The main initiative and force was proposed by Prof. Alberto Rozzi.

The objective of the TG has been to harmonise anaerobic biodegradability, activity and inhibition assays. In order to harmonize ABAI assays, it is important to identify the common made mistakes and propose a set of rules that are important to follow for performing assays in an acceptable way. Alternative methods used by different groups around the world need to relate to the common rules identified by the ABAI TG group.

Due to the untiring efforts of Prof. Alberto Rozzi, a workshop on Harmonization of Anaerobic Biodegradation, Activity and Inhibition Assays was held on June 7th and 8th 2002 at Lake Orta (Italy).

Four years later, on the 9 to 10th of October 2006, the next ABAI workshop was held in Prague with Institute of Chemical Technology Prague as host. At the workshop the Task Group (TG) discussed limitations and concerns of existing assays on Biodegradation, Activity and Inhibition. The conclusions of the discussions were summarised in this report. However, it was clear from the TG that proper standarisation and harmonisation of the Biodegradation, Activity and Inhibition was a huge time consuming task which can only be worked in the frame of a specific financed project.

2 Role of ABAI Taskgroup

2.1 Long-term objectives: development of standardised/recognised tests for testing waste, wastewater constituents

The long term objectives of the ABAI TG are to define standardised tests for the determination of anaerobic biodegradability, activity and inhibition of various wastes, co-products and chemicals. Currently the international standards (ISO 11734) for anaerobic biodegradability, activity and inhibition are not widely used, due to inaccuracy and user unfriendly equipment. As a consequence, many different procedures have been reported and used to overcome these weaknesses. This has, however, made comparison of results between different methods and techniques difficult.

The main objective of the TG is to define assays harmonising existing protocols, materials and detection systems to support the implementation of important legislation pieces such as EU Landfill Directive 1999/31/EC, draft REACH document (Registration, Evaluation and Authorisation of Chemicals), the 'dangerous substances and preparations' (76/464/EEC), IPPC (96/61/EC) and the Water Framework directive (2000/60/EC). In addition to testing for compliance with such legislation, standard tests are required for the determination of anaerobic treatment performance and the effect of materials and chemicals of anaerobic treatment efficiency.

Specific objectives of taskgroup: development of standards

- Biodegradability test
- Activity test
- Inhibition tests (for different types of inhibitors)
- Sludge stability test

3 Anaerobic Biodegradability Assay

3.1 Background

Although the biodegradability of thousands of different materials in both aerobic and anaerobic conditions have been performed over the last 50 years, comparison of biodegradability data in the literature is very difficult.
Classical anaerobic digestion is the conversion of organic compounds in the absence of any inorganic electron acceptors to the most reduced and oxidised form of carbon, namely to methane and carbon dioxide, while partial conversion of the organic compounds to intermediates is referred to as transformation or primary biodegradation. Anaerobic biodegradation is a highly complex process, involving a consortium of several microbial groups and interrelated biochemical processes. The anaerobic process involves several steps such as enzymatic extracellular hydrolysis of high molecular weight carbohydrates, fats and/or proteins into soluble oligomeric and monomeric compounds. This is followed by acidogenesis which is the conversion of single sugars, amino acids and high molecular weight organic acids into low molecular weight organic acids, alcohols, H_2 and CO_2. During acetogenesis, volatile

fatty acids (VFAs) and alcohols are then converted to acetic acid by the H_2-producing acetogenic bacteria and finally methanogenic archaea convert acetic acid and H_2 gas into CO_2 and CH_4. The stability of the process is dependent on the critical balance that exists between the symbiotic growth rates of the principal metabolic groups of microorganisms i.e. acid forming bacteria, obligate hydrogen producing acetogens and methanogens.

Anaerobic biodegradability assays are used to establish anaerobic biodegradability, for determination of the ultimate methane potential of wastes, but are also used for determination of the rate of this biodegradation in general.

Methods based on product formation measure either the gaseous end product (biogas) or liquid phase production of intermediates such as volatile fatty acids. Most methods in the literature are based on monitoring biogas production. Biogas production is measured either as volume increase under constant pressure (volumetric methods), or measurement of pressure increase in constant volume (manometric methods), or measurement of methane formation by gas chromatography. Gas chromatography is used to measure content of methane and carbon dioxide of the biogas that ends up in headspace of closed vials.

Gas chromatographic methods can be further divided in two groups:

- Using a GC with thermal conductivity detection (TCD) where both methane and carbon dioxide are measured. By using a reference gas e.g. nitrogen in the headspace and regular sampling the volume of biogas can be estimated based on the molar fractions of CH_4, CO_2.
- Using a GC with flame ionisation detection (FID), where only methane is measured. The measurement is compared with methane standard with the known methane content.

The GC method is simple and fast; one methane measurement takes less than a minute (FID) up to a couple of minutes (TCD), thus, many samples can be tested with relatively low time consumption.

Methods based on substrate depletion, where more complex analysis is used to measure substrate depletion such as via chemical analysis, spectrophotometry, or gas chromatography. Substrate depletion can be determined either as non specific parameters such as volatile solids (VS), chemical oxygen demand (COD), dissolved organic carbon (DOC), or direct analysis of the specific compound being used as substrate.

Methods using volume or pressure for determination of biogas production in a closed vessel are inaccurate unless the methane content in the produced volume is monitored. A quite important problem that pH strongly influences the CO_2 (and thus methane) content in the head space of the closed vials. Table 1 summarizes different techniques commonly used to measure biogas production and methane composition in biodegradability assays

Table 4.1 – Summary of techniques used to measure biogas production in biodegradability assays.

Method	Comments
Volumetric	Inaccuracy due to variations of atmospheric pressure Inaccuracy can be significant due to variations inorganic carbon in the liquid phase (influenced by pH). Evaporation of water in displacement systems Simple and cheap
Manometric	Manometric transducers have limited range of accuracy Inaccuracy can be significant due to variations inorganic carbon in liquid phase Hand held pressure transducers more convenient than automated multiport sampling valves due to increasing leakage probability Improved sensitivity and user friendly systems with a dedicated pressure transducer monitoring at regular intervals can be used for biodegradability comparisons.
GC-TCD	Gas chromatograph needed 2 to 3 minutes per sample Many simultaneously Direct measurement, precise
GC-FID	Gas chromatograph needed Fast, 20 seconds per sample Many simultaneously Direct measurement, precise

3.2 Limitations and concerns of existing standard methods for anaerobic biodegradability

A number of different assays have been developed over the last 20 years with a variety of experimental set ups. In addition there is a considerable difference in the protocols employed. The various methods reported as standards can be placed in two main groups: those that are designed to address the definition of anaerobic biodegradability of chemical compounds or plastic (ISO 14853 -1999; ASTM D 5511 – 1994; ASTM 5210 – 1992; ASTM E 2170- 2001; ISO 15473 – 2002) and those that aim to quantify the ultimate biodegradability of complex organic substrates and biogas production (ISO 11734 – 1995; ISO/DIS 14853 – 1999; UK Environment Agency 2005).

Common to these displacement methods is the use of either a highly acid or saline barrier solution to avoid the diffusion of CO_2 in the liquid manometer. However, the assumption that all the CO_2 produced as a result of biodegradation is measured is incorrect, as the high ionic strength does not completely prevent carbon dioxide from dissolving in the liquid being displaced. Such methods would be better used to measure methane produced directly, by using an alkaline solution instead, which effectively absorbs all the carbon dioxide in the off gas.

Although the ECETOC (1988), HMSO, (1988) and ISO 11734, (1995) defined the basic protocol for anaerobic biodegradability tests, many researchers have sought to

improve the methods usability by developing automatic operated instruments. Critical to all gasometrical determination of anaerobic biodegradability is the accurate measurement of biogas production at low headspace pressure to prevent errors associated with CO_2 solubility.

3.3 Suggested standard protocol

3.3.1 Objective

The objective of the protocol is to provide important experimental guidelines to carry out an accurate assessment of the anaerobic biodegradability of any compound or material to methane and carbon dioxide.

3.3.2 Inoculum type

The inoculum should be "fresh" i.e. inoculum that is not stored for periods longer than a few days, from any type of active anaerobic reactor e.g. sludge reactors, manure reactors or sludge bed reactors, such as UASB.

The inoculum should have a "broad trophic" microbial composition in order to ensure that the anaerobic conversion of different substrates is not limited. Where the inoculum originates from a reactor which is fed with a simple feed composition, different inocula should be mixed with the sampled inoculum, e.g. sewage sludge inoculum together with granules. Both mesophilic and thermophilic inocula can be used; however, inocula originating from reactors treating similar substrates are preferable, as it reduces the risk for inhibition due to non adapted microbial populations.

3.3.3 Treatment of the inoculum

The inoculum should be "degassed" i.e. pre-incubated in order to deplete the residual biodegradable organic material. The pre-incubation should be carried out at the same temperature as the process from which the inoculum originated. Degassing should be performed until no further significant methane production, typically this point may take between 2 to 5 days of incubation. In some cases, for example when the inoculum is taken from a reactor fed with relatively high fat/oil concentration, higher periods of pre-incubation may be required, in order to eliminate all the residual (adsorbed/entrapped) substrate.

The inoculum should be a close representation of the one sampled from the reactor, and **SHOULD NOT** (as described on previous ISO 11734, ASTM E 2170 (2001)) be washed to remove residual substrate material and IC compounds.

3.3.4 Activity of the inoculum

The quality of inoculum could be examined by performing activity tests on acetate and cellulose, immediately preceding the biodegradability test (see below activity tests). Activity test of the inoculum is not necessary, if the inoculum is originated from a "healthy" reactor, as indicated by a high biogas production rate and low volatile fatty acids level.

3.3.5 Amount of Inoculum

The volume of inoculum can be from 10 to 80% of the total test volume, depending on the activity and biomass concentration of the inoculum. For instance an inoculum taken from a manure digester has typically 2 to 3% VS and a drained granular sludge has typically 10%VS or more. Furthermore, in granular sludge, the most of the VS consists of microbial biomass, whilst in manure, the inoculum VS is mainly represented by recalcitrant lignocellulosic residues and not active microbial biomass. Therefore, inoculation according to a specific amount of VS of inoculum is inappropriate, unless the ratio of microbial biomass to recalcitrant VS is known.

General recommendations for the type and concentration of inoculum that should be used in anaerobic biodegradability assays are as follows.

The inoculum concentration in the test vessel should be high (typically be 70% to 80% of the total liquid used in the test) if relatively low activity inocula such as digested manure or sludge are used (SMA around 0,1g COD-CH4/gVSS/day). For highly active inocula such as granular biomass (SMA around 0.5 g COD-CH4/gVSS/day, the inoculum concentration should be relatively low (10% to 20% of the total liquid used in the test). The duration of the test will be dependant on strength of the inoculum and, in both cases, the higher the inoculum concentration, the fastest the anaerobic conversion of the substrate will occur and the quicker the test will be completed.

3.3.6 Medium

Necessary nutrients/micronutrient/vitamins are required for optimal performance of anaerobic microorganisms. Nutrient medium containing macro- & micronutrients, buffers, vitamins etc. should be added, unless it can be documented that are present in inoculum or substrate. An example of a basic anaerobic medium in given in table 4.2.

Table 4.2. Basic Anaerobic Medium (Angelidaki and Sanders 2004).

Description of Anaerobic Basic Medium
The basic medium is prepared from the following stock solutions, (chemicals given below are concentrations in g/l, in distilled water). (A) NH_4Cl, 100; NaCl, 10; $MgCl_2$ $6H_2O$, 10; $CaCl_2$ $2H_2O$, 5 (B) K_2HPO_4 $3H_2O$, 200 (C) resazurin 0.5 (D) trace-metal and selenite solution: $FeCl_2$ $4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2$ $2H_2O$, 0.038; $MnCl_2$ $4H_2O$, 0.05; $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2$ $6H_2O$, 0.05; $NiCl_2$ $6H_2O$, 0.092; ethylenediaminetetraacetate, 0.5; concentrated HCl, 1 ml; Na_2SeO_3 $5H_2O$, 0.1 (E) vitamin mixture (componets are given in mg/l): Biotin, 2; folic acid, 2; pyridoxine acid, 10; ridoflavin, 5; thiamine hydrochloride, 5; cyanocobalamine, 0.1; nicotinic acid, 5; P-aminobenzoic acid, 5; lipoic acid, 5; DL-pantothenic acid. To 974 ml of distilled water, the following stock solutions should be added (A), 10 ml; (B), 2 ml; (C), 1 ml; (D), 1 ml and (E), 1 ml. The mixture is gassed with 80% N_2 - 20% CO_2. Cysteine hydrochloride, 0.5 g and $NaHCO_3$, 2.6 g, are added and the medium is dispensed to serum vials and autoclaved if necessary. Before inoculation the vials are reduced with Na_2S $9H_2O$ to a final concentration of 0.025%.

3.3.7 Experimental vessels

The assay is performed in closed vessels (100 ml up to 2 litres) depending on the homogeneity of the substrate i.e. the more homogeneous the substrate the smaller the volume of the vessel is required. (Fig.4. 1).

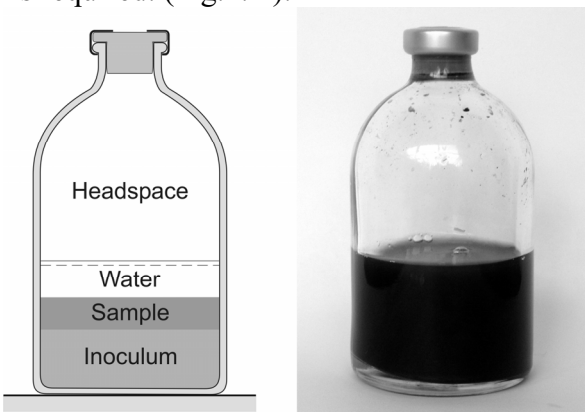

Fig. 4.1. Example of an assay vessel for anaerobic biodegradability test.

3.3.8 Substrate

The characteristics of the substrate to be assayed should be always analysed and the total solids (TS) and volatile solids (VS) should be quantified and controlled. In the case of an acidic waste, for example, putrescible kitchen waste, attention should be given during drying of the sample as volatile compounds can be lost during TS determination. In this case VS can be underestimated due to VFA losses during the analysis of total solids. For materials such as this, the TS determination should be made after increasing the pH of the waste to decrease volatility of volatile fatty acids. Additionally, for very volatile samples, drying during TS determination should be performed at a maximum temperature of 90˚C instead of 105˚C, until constant weight.

3.3.9 Blank and controls

The background methane production from the inoculum, determined in blank assays with medium or water and no substrate, is subtracted from the methane production obtained from the sample assays. The blank and the control should be carried out in triplicate. The control vessels should contain a cellulose standard substrate or gelatine or both depending on the nature of the tested substrate. A cellulose standard should be used for crops or municipal biowaste whilst gelatine should be used when testing meat, fish and other similar wastes.

3.3.10 Assay set up

The assay should be performed at least in triplicate at each dilution used, depending on the complexity (homogeneity) of the substrate and reproducibility of the tests. When using substrates with high heterogeneity more than three replicates should be used.

For new substrates with unknown degradation characteristics, a number of different dilutions of the substrate (with water) are required. Dilutions ensure that the methane potential of the substrates is not underestimated due to overload or potential inhibition. Samples should be tested at concentrations from 5% to 100% (undiluted

samples). When the maximum methane potential is the same in at least two different dilutions of the dilution series, it can be assumed that the inoculum is not overloaded or inhibited. If the specific potential continues to increase with increasing dilution (decreased concentration of substrate), additional dilutions are required.

3.3.11 Assay procedure

Prior to analysis the assay vessels should be flushed continuously with N_2/CO_2 (80/20%V/V) (80/20% gas mixture is resulting in neutral pH) before adding the substrate to the vessel. The substrate can be accurately transferred either by volume or weight, depending on the substrate and consistency and homogeneity. The inoculum is then distributed anaerobically to each assay vessel. The inoculum should be continuously stirred and kept under anaerobic conditions during the process of transfer. Typically N_2/CO_2 (80/20%V/V) is flushed through the headspace of the inoculum storage vessel and the same gas is used to flush the assay vessels. The inoculum can be transferred either directly and diluted with the medium/water (see medium description) or diluted with medium or water prior to transfer.

The transfer of inoculum is typically achieved by using a graduated 100ml syringe with a large orifice to which a suitable length of tubing is fixed to allow the bottom of the inoculum storage bottle to be reached. In the case of granular inoculum, the granules are first drained using a suitable sieve with a sufficient mesh size to retain the granules. Once drained a specific granular volume can be transferred using a spoon, ensuring that the same volume is distributed to each assay vessel. After transferring the inoculum, substrate and medium, the assay vessels are sealed using a thick butyl rubber stopper held in place with an aluminium crimp.

3.3.12 Data collection

In the biodegradability assay, the methane accumulated in the headspace of the closed vessel should be measured by gas chromatography (GC). For that, a sample volume of e.g. 100 µL should be collected with a gas-tight syringe and injected into the GC. Either a Thermal Conductivity Detector (TCD) or a Flame Ionization Detector (FID) can be used. The obtained peak area should be compared to that obtained by injecting the same volume of a standard gas mixture of the known composition. The standard gas mixture should be injected at the atmospheric pressure because if the gas sample is taken with a gas tight over pressurised syringe, and compared with a gas standard injected under atmospheric pressure the methane percentage will be more than 100%.

The volume of methane produced is obtained by multiplying the headspace volume by the % of CH_4 in the headspace as determined by GC analysis. For publication and comparison with other studies, the values are often calculated to STP conditions i.e. converted to 0˚C and 1 atm.

3.3.13 Data interpretation and reporting

The anaerobic biodegradability results should always be accompanied by a clear description of inoculum source, activity and VS or VSS content, medium composition, waste (water) composition or description, and dilutions used. The methane production profiles with respect to time together with the profiles for the blank and control assays should be presented.
In the final report, the following items should be considered:

- date, time of start and end of the test;

- tested substrate, amount or quantity and chemical-physical characteristics;
- inoculum, origin and activity, amount or quantity and chemical-physical characteristics;
- test conditions (temperature, inoculum/substrate ratio, volume of the vessel, number of replicates…);
- results of blank and controls biogas production (report graphics);
- methane production in the triplicate and average and standard deviations;
- specific methane production: volume of CH_4 per gram VS; or CH_4 per gram COD, or CH_4 g sample.

4 Anaerobic Activity test

4.1 Background

Anaerobic activity tests can be used to select an adapted sludge as inoculum, to follow the changes in sludge activities due to a possible build-up of inert materials, to estimate maximum applicable loading rate to certain sludge or to evaluate batch kinetic parameters. These tests can be also used to monitor the biomass composition along with the usual reactor performance evaluation parameters for giving a better insight into the reactor stability and performance.

Characterization of the quality of inoculum is an important in order to design the start up strategy of the anaerobic reactors. Based upon these tests results, the organic loading rates can be either increased or decreased in order to improve performance of the system and to achieve stable conditions. The activity of inoculum is also important for prediction of methanisation characteristics of specific wastes that could be introduced in a specific reactor. Higher methanogenic activities contribute to prevent the acidification during anaerobic batch biodegradation of waste, since the inoculum is able to process a higher flow of metabolites such as hydrogen, acetate and other VFA, preventing their accumulation. To know the activity of inoculum should allow defining the amount of inoculum needed for a batch operated digester.

During no steady state operational periods (start-up or organic and hydraulic shock loads), intermediates such as volatile fatty acids will accumulate in anaerobic reactors as a result of an imbalance between production and consumption. The determination of the microbial activity of the different steps of the anaerobic process is useful for identifying the limiting step and to give information about the maximum organic load which can be applied to the system without causing a loss of its stability. Additionally, aceticlastic, hydrogenotrophic and total methanogenic activity tests provide a simple technique that may aid in evaluating the performance and stability of anaerobic bioreactors.

4.2 Objective

To determine acidogenic, acetogenic and methanogenic activities of a given anaerobic reactor.

4.3 Suggested protocol

4.3.1 Substrates for activity tests

For determination of activities of different trophic groups, model substrates are usually chosen (Table 5.1). As substrates for methanogenic activity, H2/CO2 (1 atm overpressure) or 1 g/l acetic acid are suggested for hydrogenotrophic and aceticlastic methanogenic activity, respectively. For estimation of acetogenic activities, 0.5 g/l propionic and butyric acid are suggested. For determination of acidogenic activity, 1 g/l glucose can be used as substrate, while, for hydrolytic activities, 1 g/l cellulose for cellulolytic activity and 1 g/l casein for proteolytic activity are recommended.

Table 5.1. Suggested model substrates for determination of activites of different trophic groups in a biogas reactor.

Population	Initial substrate concentration proposed
Hydrolytic	1 g amorphous cellulose/L
Acidogenic	1 g glucose/L
Proteolytic	1 g casein/L
Acetogenic	0.5 g propionic/L
	0.5 g n-butyric/L
Acetoclastic	1 g acetic acid /L
Hydrogenotrophic	overpressure of 1 atm of a mixture of H_2/CO_2 (80/20)

4.3.2 Initial biomass concentration

The kinetic behaviour is represented by a zero order model when the inoculum concentration (X_o) is higher than the amount of biomass produced during the activity test ($Y_{SX} \cdot (S_o - S)$). An initial biomass concentration of 2.5 g VSS/L is proposed taking into account the yield coefficients. In this case, VSS is considered to represent bacterial biomass. In cases when a large amount of recalcitrant material is present such as of manure inocula samples, an approximation should be made. i.e. only a (small) part of VSS (or VS) will represent bacterial biomass.

4.3.3 Experimental environment

The activity assays should be carried out under test conditions (e.g. temperature, pH, and mixing) that closely imitate the environment of a given reactor system, e.g. if the reactor system that the inoculum to be tested is operating at thermophilic temperature, the activity tests should be performed at thermophilic temperatures and not mesophilic.

4.3.4 Test methodology

A sample of biomass from biogas reactor is placed into approx. 100 ml serum bottles which headspace is flushed with nitrogen gas. The volume of the headspace/volume of the liquid phase ratio must be adjusted in order to measure the maximum theoretical overpressure expected without need to equalize the pressure to the atmospheric one.

Substrate along nutrients, trace minerals and buffer ($NaHCO_3$) are added to the medium. Then pH is adjusted to optimum pH, which depends on the reactor where the inoculum was taken from by addition or HCl or NaOH. The optimum pH is usually in the range of 7-8. The bottles are closed and the head space and liquid phase is flushed

to achieve anaerobic conditions. Bottles are maintained in an incubator at the temperature which corresponds to the optimum temperature for the biomass. The vials are incubated at the desired temperature, preferably under shaking.

4.3.5 Monitoring parameters for establishing activity

The activity test focuses on methane production and is thus mainly qualified for analysing substrates that are directly converted to methane, especially acetate, H2, methanol and formate. Most other substrates are converted though several steps, thus following methane production is only an indirect indication for the substrate conversion activity,

For testing *methanogenic activity* (from H2-CO2, acetate, methanol or formate), the accumulated methane production is monitored. For test of *acetogenic activity* (from e.g. butyrate, propionate etc.) methane production measurement is not enough, as the methane production rate will only reflect the slowest step of a more complex degradation process. Therefore, in cases that acetogenic activity is tested, the substrate depletion rate should be monitored. For testing *acidogenic activity* (from e. g. glucose), the accumulated product (methane and VFA) production rate should be used for estimation of activity. In order to produce a curve of the accumulated product formation, as unit for VFA, methane equivalents (from the theoretical stoichiometric conversion) can be used.

4.3.6 Data interpretation

The initial rate of methane accumulation is the optimal estimate of methanogenic activity of the biomass. Bacterial growth and adaptation changes the biomass characteristics. Likewise, pH and concentration of substrate and nutrients in the vials are changing during process. Therefore, only the initial linear rate of the methane accumulation curve should be considered to indicate that adaptation, or significant growth of biomass has not take place, during the test period. Therefore, the test period of the assay should be limited to the linear part of the methane production period which is usually depending on the activity and is in the range of 0.5-7 days.

4.3.7 Protocol evaluation (pro-s, con-s, constraints, special situations)

Some cautions must be considered in order to avoid false results:

1) Septa must be capable of withstanding about 2 bars of pressure and, as they are pierced several times with a needle their tightness must be tested before starting the test.
2) The headspace and liquid phase were flushed with N_2 (for pH around 8) or N2/CO2 (80:20) (for pH around 7), to remove the oxygen.
3) pH value of each bottle must be measured after the final pressure measurement.
4) A COD balance can be done to validate the measurements obtained.
5) A suitable headspace volume must be chosen to generate an overpressure according to the sensitivity of a differential pressure transducer.
6) The assays must be carried out in triplicates.

5 Assessment of waste(water) inhibition for anaerobic inoculum

5.1 Background

The potentially inhibiting compounds are ubiquitous in waste(water)s. Majority of these potential inhibitors are not inhibitors at all (frequently even have a stimulatory effect) at low concentrations. The typical examples can include natural methanogenic substrates and their precursors – acetate, H_2, methanol, VFA etc. which become strong inhibitors for anaerobic sludge under elevated concentrations. We will call such inhibitors as **metabolic** ones.

The second group of inhibitors which can be named as **physiological** ones includes compounds which usually do not participate in a main stream of metabolism of anaerobic microorganism but can exert a quite detrimental effect on microbial physiology. The typical examples are (earth)alkali metals as well as other simple ions like ammonium, sulphate, sulphide etc.

The third group of inhibitors exerts a direct lethal effect on biomass. These compounds are not usual in common waste(water). Typical examples are xenobiotics, BTEX, chlorinated hydrocarbons, antibiotics, man-made chemicals etc. We will call these inhibitors as **biocidal** ones.

For inhibitors belonging to the first and second group, the inhibition phenomena can be schematically depicted as in Fig. 1. Biocidal inhibiters belonging to the third group exert an immediate toxicity effect on the biomass (absence of phase 1 and 2 in Fig.6.1).

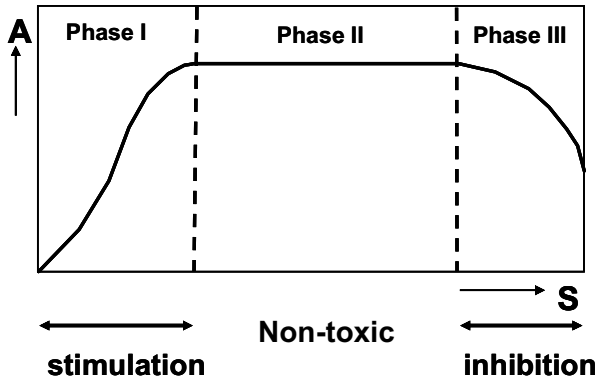

Fig. 6.1. The schematic representation of inhibition phenomenon.

Inhibition phenomena on anaerobic biomass can be reversible or irreversible.

Biomass can fully or partly recover from **reversible inhibition** when the inhibitor is removed from the bulk liquid. Reversible inhibitors belong to the first and partly to the second group of inhibiting compounds. The degree of recovery generally depends on the nature, concentration and duration of exposure. The inhibitors of the 3rd group are usually **irreversible**.

Quantification of inhibition phenomenon of specific compounds is generally done by assessing the IC_{50} value which represents the concentration of inhibitor exerting 50% decrease in conversion capacity of the sludge.

Inhibition phenomena are often coincided with the so-called "adaptation" of the biomass. Adaptation refers to the ability of the sludge to develop a higher tolerance to the inhibitor. In general, **a progressive increase in time** in the methanogenic sludge activity is observed. A typical example of sludge adaptation to ammonia as inhibiting agent is depicted in Fig. 6.2. In contrast to adaptation, **a progressive decrease in time** in the methanogenic sludge activity might be observed. In such situations, an inhibiting compound may exert a toxic effect on the anabolic metabolism (growth) of the microorganisms, meanwhile the catabolic conversions, recorded as methanogenic activity are not affected. Alternatively, an inhibiting compound is accumulating during continuous operation which at a specific moment trespasses the inhibitory level.

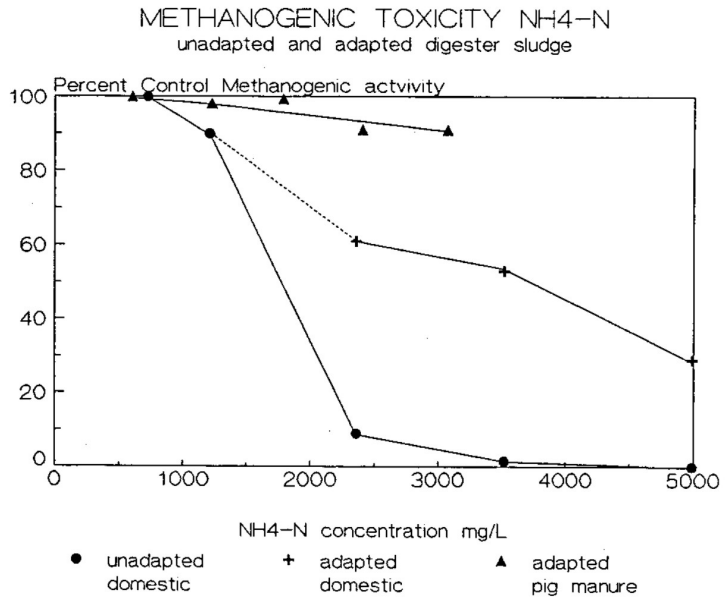

Fig. 6.2. Adaptation of sludge to ammonia

For many inhibiting compounds, the degree of inhibition is dependent on environmental conditions like pH and temperature. Particularly, the ionizable toxicants like NH_3, H_2S, VFA etc usually exert their inhibiting effect when present in the unionized form. Fig. 6.3 shows IC_{50} value for acetate and propionate as a function of the pH. From Fig. 6.3, it can be derived that acidification of the reactor content will accelerate the inhibitory effect of VFA

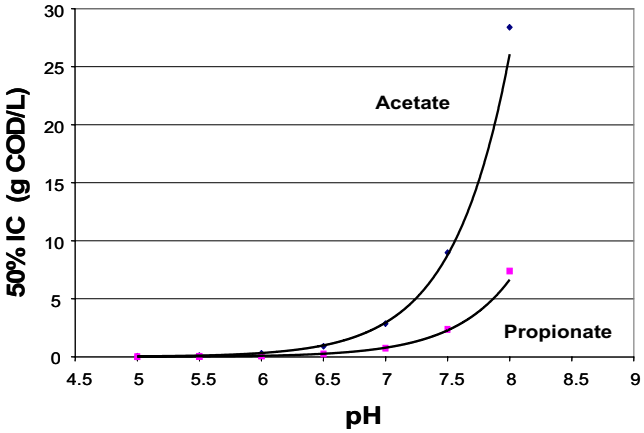

Fig. 6.3. The IC_{50} value for acetate and propionate as a function of the pH

Fig 6.4 shows an example of the impact of temperature on the fraction of a unionized inhibitor (NH_3) in the medium as a function of pH. It is seen that increase in temperature leads to an increase in inhibitor concentration.

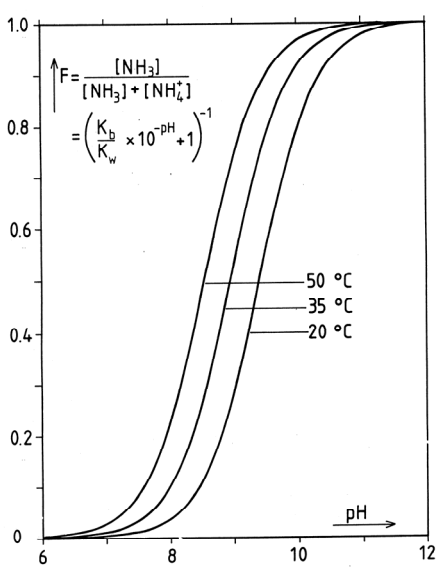

Fig. 6.4. The impact of temperature on the fraction of an unionized inhibitor (NH_3) in the medium as a function of pH

In addition to the physico-chemical conditions, the sludge morphology determines the degree of inhibition of the anaerobic biomass. Granular sludge and/or biofilms are generally less sensitive to inhibitors compared to suspended sludge, which can be attributed to concentration gradients over the granule/biofilm.

The inhibition picture can be further aggravated/ masked by accompanying processes like biodegradation of toxicant, its speciation over different phases (solid/liquid/gas), precipitation, chelation etc., in other words, decreasing the bioavailability of the corresponding inhibitor.

5.1.1 Objective

The main objective of the inhibition test is to assess the degree and the nature of inhibition of the given anaerobic sludge by a specific compound or non-defined waste(water) sample. The additional objectives can also include the assessment of ability of the sludge to recover the activity after inhibition and/or to adapt to the inhibitor.

5.1.2 Suggested protocol (applied in Moscow State University)

The basal medium is the same as for activity test and usually contains (mg l^{-1}): $NaHCO_3$ (5000), NH_4Cl (280), $CaCl_2 \cdot 2H_2O$ (10), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), yeast extract (100), H_3BO_3 (0.05), $FeCl_3 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.05), $CuCl_2 \cdot 2H_2O$ (0.03), $(NH_4)_2SeO_3 \cdot 5H_2O$ (0.05), $AlCl_3 \cdot 6H_2O$ (2), $NiCl_2 \cdot 6H_2O$ (0.05), $CoCl_2$ (0.01), 36% HCl to adjust the pH value – usually to 7.

The inhibition assays are usually performed in 120 ml glass serum bottles containing 25-50 ml of standard medium without stirring or with mild shaking (20-30 rpm) under defined constant temperature. The sludge (final concentration around 2-2.5 g VSS/L) is added to the flasks which are then sealed with rubber stoppers, flushed with inert gas (argon or 70:30 mixture of N_2:CO_2) and incubated overnight or during 24 h to deplete all readily biodegradable organic compounds introduced with the seed sludge.

The inhibition of the target compounds or suspected WW samples are usually studied with regard to their influence on aceticlastic methanogenic activity of anaerobic sludge (the other sludge activities can be assessed in a similar way). Two types of the experiments can be carried out.

a) In the first case, the sludge activity is determined in the presence of an inhibitor. For this purpose, after a 12-24-h sludge starvation in the basal medium and gas space flushing with inert gas for 5 min, an investigated WW sample or suspected compound are added to the flasks in various concentrations (usually 5-6) & the flasks are incubated for 24 h. Thereafter, 1 ml of stock sodium acetate solution (final concentration 2 g COD/L) is added to the same flasks without removal of inhibitor, and the flasks are flushed with inert gas.

b) In a second set of the experiments, the reversibility of the inhibition can be assessed: after 24-h incubation with an inhibitor, the liquid phase is decanted and the sludge is quickly washed by an oxygen-free mineral medium. Immediately after this, a new portion of an inhibitor-free medium containing 2 g COD/L of sodium acetate is added and the flasks are flushed with inert gas.

In both cases, the concentration of CH_4 in the headspace or volumetric CH_4 production is recorded throughout the incubation, and specific aceticlastic activities of the sludge are calculated from the linear segment of kinetic curves of methane production (see Activity assay). The aceticlastic activity of the sludge not treated with a toxicant is taken as a control. The IC_{50} values for the investigated compounds are estimated from the concentration dependencies of the specific activities. All experiments are carried out in triplicates and the results obtained are statistically treated. The example of 2 types of investigation of inhibition is presented on Fig. 6.5.

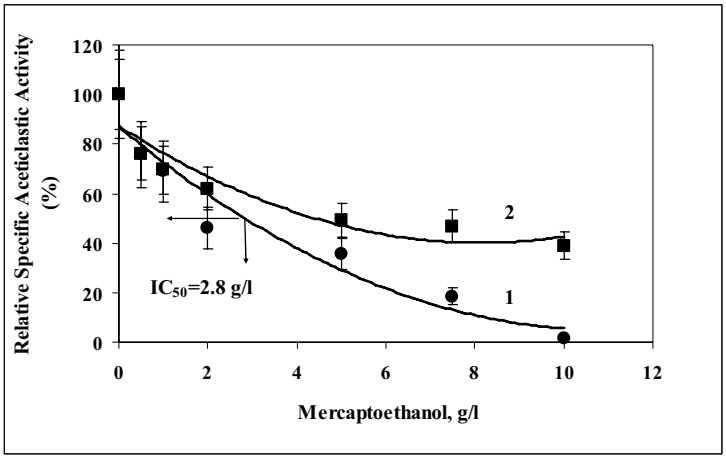

Fig. 6.5. Relative aceticlastic activity of the sludge in the presence (1) and after removal (2) of mercaptoethanol (the activity of the sludge not treated with mercaptoethanol was taken as 100%).
(Sklyar V.I., Mosolova T.P., Kucherenko I.A., Degtyarova N.N., Varfolomeyev S.D., Kalyuzhnyi S.V. Anaerobic Toxicity and Biodegradability of Hydrolysis Products of Chemical Warfare Agents. – Appl. Biochem. Biotechnol., 1999, v.81, N2, p. 107-117)

5.1.3 Protocol evaluation (pro-s, con-s, constraints, special situations)

If the investigator is not sure about the activity of the sludge or its uniform distribution over all flasks (usually they are many in these studies), 2 g COD/L of sodium acetate is added to each flask to assess this activity & to reactivate the sludge in order to have a more-or-less equal sludge activity in all flasks. In this case, the pre-conditioning experiment should last till acetate is completely depleted and a plateau in methane formation is reached. The flasks with the activity which differs more than 20% should be eliminated from the further experiments as non-reproducible.

5.1.4 Approach on inhibition problems, protocols to follow

1) New waste(water) stream, new component(s) in waste(water)

The impact of new, potentially inhibiting compounds is relatively easy to investigate because such an effect will be seen from the concentration dependencies of the specific activities. For example, the IC_{50} for mercaptoethanol is ~2.8 g/l (Fig. 5, curve 1). Thus, the biodegradability assay for this compound should be assessed under lower concentrations. Also, in the case of continuous treatment, the concentration of this compound in the reactor medium should be kept below this value. If the methanogenic sludge has the ability to adapt to the inhibitor, higher concentrations can be allowed in the reactor on due time. Repetition of the IC_{50} assessment after a lengthy period of continuous operation is then advisable.
From Fig. 5 (curve 2), it is seen that mercaptoethanol belongs to mainly reversible inhibitors because even, after incubation with 10 g/l of this compound, the sludge activity can be restored to 40% of that for untreated sludge.
For new wastewaters with unknown compounds, the SMA of the sludge is generally assessed at various concentration of that wastewater. From the specific activities at the various dilutions, a similar IC_{50} value can be derived.

2) Anaerobic reactor starts to suffer

A bigger problem in full scale plants is a slowly deteriorating reactor performance without a direct clear cause. The progressive decrease in SMA might indicate that growth and bacterial yield are affected. Therefore, inhibition experiments must be performed that address both the catabolic and the anabolic metabolism. In most anaerobic high-rate rectors, sludge retentions times reach 100-300 days, explaining why retarded growth becomes visible only after prolonged periods of time. In addition, depending on the WW characteristics, an in-reactor accumulation of potentially inhibiting compounds may occur on the sludge. The latter is sometimes observed with apolar compounds, or compounds with long aliphatic chains. The advised procedure to address the problem is:

- Assessment of IC_{50} values by the inhibition tests described above using both long-term exposed sludge as well sludge that is not (or short-term) exposed to the wastewater.
- The presence of surface proportional inhibitors, such as LCFAs and surfactants, often explains the progressive decrease in the methanogenic sludge activity. SMA assessment at an inhibitory concentration using different sludge concentrations will elucidate whether or not surface proportional inhibitors are present, i.e. when the degree of inhibition decreases with the increase in inoculum size.
- Bacterial growth assessment experiments at various concentrations of the wastewater to be treated. Differences in IC_{50} values for the inhibition-growth curve and inhibition- SMA curve gives an indication for the long-term sludge development.

Practical solutions are often found in preventive actions such as:

- enhanced pre-treatment (e.g. flotation, coagulation)
- increased recirculation over the anaerobic reactor
- dilution of the influent with detoxified effluent
- isolation of disturbing stream
- prevention of batch discharges of inhibitors

6 Stability tests for sewage sludge

6.1 Background

Sewage sludge is produced from the treatment of wastewaters and consists of two basic forms – raw_primary sludge, basically organic material of different origin, and secondary sludge, or biological sludge, a living ‘mixed culture’ of organisms that help remove contaminants from wastewater before it is returned to water bodies. The sludge is transformed into biosolids using a number of complex treatments such as thickening, aerobic or anaerobic digestion, dewatering, drying and lime stabilization.

The treatment process reduces the water content of the sludge, reduces its ability to produce gas and renders it virtually free from harmful organisms. Treated biosolids are therefore easily transportable, less odorous and almost 100% pathogen free. The applied treatments also reduce the amount of waste, as the volatile solids content of treated sludge is lower than that of raw sludge.

After the treatment, biosolids are disposed off at present, typical disposal routes and relative costs are the ones reported in Table1.

Table 7.1. Costs for sludge disposal in Europe (adapted from ISWA and EEA, 1997).

Sludge disposal	Percentage of disposal, %	Disposal costs, €/ton
Agriculture	46	100 – 270
Landfilling	21	150 – 400
Incineration	20	300 – 600
Composting	7	150 – 400

According to data reported in table 7.1 agriculture is the main route for sludge disposal, however, because of new regulations this practice has been reducing and incineration is growing, especially in Switzerland and Germany, while in the rest of Europe and other parts of the world, landfilling is still the main way to dispose of sludge.

The sludge should be always specifically processed before landfilling to comply with legislative standards and limitations for its final disposal. Objectives of sludge processing are to reduce its biodegradability and tendency towards anaerobic digestion and leachate production. Because of the new EU regulations, i.e., Directive n. 31 of 1999, final disposal of sewage sludge will be restricted in Europe by the severe limits on landfill. The Council Decision of 19 December 2002 practically bans a disposal of biodegradable organic wastes in landfill, therefore sludge disposal is a problem of primary importance in waste management.

In fact, the acceptance in landfill for non hazardous waste is restricted to those wastes for which the leaching test shows values lower than the limit of 800 mg of dissolved organic carbon (DOC) per kg of dry substance when carrying out leaching tests with a liquid/solid ratio of 10 (10 litres of water per kg of dry matter).

Preliminary tests carried out by CEN (2002) showed DOC values of 31.500 mg per kg of dry matter for the leachate of sewage sludge.

Test performed in the USA to define the grade of stability of organic waste (municipal solid biowaste and biosolids) by Kelly et al. (2006) showed that both the VS content and the BMP tests declined for the material from old landfills, indicating the stability of the treated material. In particular, the experimentation showed that the VS content ranged between 40 and 80% for fresh material and declined down to 20-40% for stable material, while the BMP values passed from some 100-200 ml CH_4 per gram of dry matter down to less than 50 ml per gram of dry matter for material which stayed in landfills for more than 5 years.

So, the combination of the VS% and the BMP could be considered as good indicators for the waste stability. In general, they found that BMP correlated weakly with all other parameters as its variability was very large. Further, as biowaste in general were treated, the authors also suggested to evaluate parameters like lignin and cellulose and their ratio to define the rate of stability. Probably, in case of evaluation of sludge, which shows relatively constant characteristics, only VS and BMP can be considered for the stability tests.

Kopp and Dichtl (2001) reported that the stability of treated sludge can be evaluated combining the value of the degradation of volatile solids and the post biogas production.
The degree of degradation can be defined as

$$\rho_{VS} = (1 - VS_{AD} / VS_0) x 100$$

where, ρ is degree of degradation of volatile solids, VS_{AD} is the concentration of volatile solids after the digestion treatment and VS_0 is the concentration of volatile solids before the stabilization process.

Expected values are in the range 40-45% for anaerobic digestion and 25-30% for aerobic stabilization.

Another important parameter is the post biogas production: that is the biogas production of digested sludge obtained in batch tests for biogas production. Post-biogas production is then defined as the cumulative gas production during a fixed period (usually 60 days) related to the amount of volatile solids treated. Plotting the degree of degradation versus the post biogas values it is obtained a graph like the one in figure 7.1.

So, according to figure 7.1, a stable sludge shows values of the degree of degradation of 40-45% and a biogas production of around 50 ml/gVS.

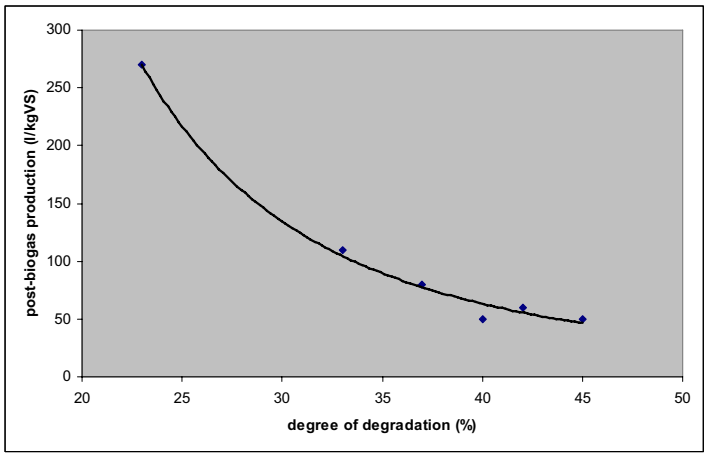

Figure 7.1. Degree of degradation vs biogas

According to these findings, it is opinion of this document that it is necessary to define a test for the determination of the "stability" or "rate of stabilization".

Here, the suggestion for the stability test, which can be performed as modification of BMP protocol, is given.

6.2 Objective

The main goal of the proposed method is the determination of the rate of stabilization in order to define if waste can be accepted in a landfill or not.

6.3 Suggested protocol

6.3.1 Inoculum

The inoculum should be "fresh" from any type of active anaerobic reactor. The suspended biomass from digester for sludge treatment in wastewater treatment plant can be recommended. Granular sludge is in this case less preferable. Both mesophilic and thermophilic inocula can be used.

6.3.2 Treatment of the inoculum

The inoculum should be "degassed", i.e. preincubated in order to deplete the residual biodegradable organic material. The pre-incubation should be done at the same temperature as the process temperature, where the inoculum was originating. Degassing should continue until significant methane production does not occur: typically 2 to 5 days of incubation. In some cases, e.g. when the inoculum is taken from an overloaded reactor or from a reactor fed with relatively high fat/oil concentration, longer periods of pre-incubation may be required, in order to eliminate all the residual (adsorbed/entrapped) substrate. The inoculum should not (as described on previous ISO 11734, ASTM E 2170 (2001)) be washed to remove residual substrate material and IC compounds.

6.3.3 Verification of the activity of the inoculum

The quality of inoculum should be checked by performing activity tests on acetate and cellulose, immediately preceding the stability test (see below activity tests). The inoculum should have a minimum specific activity of 0.1 gCH_4-COD/ gVSS/d.

6.3.4 Amount of inoculum

The volume of inoculum can be from 20 to 80% of the total test volume, depending on the activity and biomass concentration of the inoculum. For instance an inoculum taken from a manure digester has typically 2 to 3% VS and a drained granular sludge has typically 10%VS. Therefore, the inoculum concentration in the test vessel is recommended to fit in the range 1.6 to 2.4% VS, being 2%VS a typical value.
The volume of inoculum can also be estimated by a simple mass balance provided the hydrolysis rate is known or estimated. According to Angelidaki and Sanders (2004), the volume of inoculum can be determined as:

$$Vinoculum = \frac{X_{SS} V_{ww} k_h}{VSS_{inoculum} SMA_{inoculum}}$$

where
X_{SS} is the concentration of hydrolysable substrate in the waste(water) (g/L), V_{ww} is the volume of waste(water) in the assay vessel (L), k_h is the first order hydrolysis constant (day^{-1}), $VSS_{inoculum}$ is the VSS content of the inoculum (gVSS/L) and $SMA_{inoculum}$ is the specific methanogenic activity of the inoculum (g COD-CH_4/gVSS.day).

6.3.5 Medium

Necessary nutrients/micronutrient/vitamins are needed for optimal function of anaerobic microorganisms. Nutrient medium containing macro- micronutrients buffers vitamins etc. should be added, unless it can be documented that are present in inoculum or substrate.

6.3.6 Experimental set-up (this is a repetition from biodegradability test, so, can be shortened)

The assay is performed in closed vessels (100 ml up to 2 litres) depending on the homogeneity of the substrate (Fig. 4.1). The assay vessels should be flushed continuously with N_2/CO_2 (80/20%V/V) before transferring the substrate accurately either by volume or weight, depending on its consistency. Then, the inoculum is distributed anaerobically to the assay vessels. It should be continuously stirred and kept under anaerobic conditions during the process of transfer. Typically N_2/CO_2 (80/20%V/V) is flushed in the headspace of the inoculum storage vessel. Also assay vessels are flushed using the same gas. Inoculum can be transferred directly or diluted with medium or water (see medium description).

The transfer of inoculum is typically achieved by using a graduated 100 ml syringe with a large orifice with attached tubing to reach the bottom of the inoculum storage bottle. In the case of granular inoculum, the granules are first drained using a suitable sieve which has a sufficient mesh size to retain the granules. Once drained of liquid, a specific granular volume can be transferred using a spoon, ensuring that the same volume is distributed to each assay vessel. After transferring the inoculum, substrate and medium the assay vessels are closed with a thick butyl rubber stopper which is hold in place by sealing with an aluminum crimp.

6.3.7 Blank and controls

The background methane production from the inoculum, determined in blank assays with medium or water and no substrate, is subtracted from the methane production obtained in the assays. The control assays are carried out with standard substrate instead of tested material.

6.3.8 Replicates

The number of replicates should be at least three, depending on the complexity of the substrate and reproducibility of the tests.

6.3.9 Measurement

In the stability test, the methane accumulated in the headspace of the closed vessel should be measured by gas chromatography. For that, a sample volume of e.g. 100 µL should be collected with a gas-tight syringe and injected in the GC. Either a Thermal Conductivity Detector (TCD) or a Flame Ionization Detector (FID) can be used. The obtained area should be compared to the one obtained by injecting the same volume of a gas mixture with a known composition. The standard gas mixture should be injected at the atmospheric pressure (Absolute Pressure=1 atm). The volume of methane produced is obtained by multiplying the headspace volume by the % of CH_4 in the headspace.
Besides the final product methane, also intermediates such as volatile fatty acids can be measured when the primary biodegradation of any material is assessed.

6.3.10 Other test parameters

The characteristics of the tested sludge are part of the test. Total solids, total volatile solids and COD have to be controlled to define the rate of stabilization of the tested material.

6.3.11 Data interpretation and reporting

The results of biodegradability experiments should be accompanied by a document which is a clear description of inoculum source, activity and VSS content, medium composition, waste(water) composition or description, dilutions. The graphs, including the blank assays should be presented.
In the final report the following items should be considered:

- date, time of start and end of the test
- tested substrate, amount or quantity and chemical-physical characteristics
- inoculum, origin and activity, amount or quantity and chemical-physical characteristics See annex _II showing the influence of the SMA on the methane production from kitchen waste
- test conditions (temperature, inoculum/substrate ratio, volume of the vessel, number of replicates…)
- results of blank and controls biogas production (report graphics)
- biogas production, CH_4 and CO_2 %, in the triplicate and average and standard deviations
- specific biogas production: volume of CH_4 (or gram CH_4-COD) per gram of chemical compound or volatile (suspended) solid

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