CO-DIGESTION OF HIGH STRENGTH / TOXIC ORGANIC EFFLUENTS IN ANAEROBIC DIGESTERS AT WASTEWATER TREATMENT WORKS

E. U. Remigi and C. A. Buckley

Pollution Research Group, School of Chemical Engineering
University of KwaZulu-Natal, Durban

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EXECUTIVE SUMMARY

The province of KwaZulu-Natal is relatively well endowed with water resources and has thus attracted water intensive industries such as the textile industry. Increasing population and development has increased the pressure on the resource which together with proposed waste discharge charges has lead to increased costs for water supply and wastewater treatment. This has lead to the increasing adoption of various management tools, such as waste minimisation and water conservation.

These methods have proved effective in reducing both the water consumption and the discharge of large volumes of wastewaters. As a consequence, the residues have generally become more concentrated in organic pollutants which are not ideally suited for conventional aerobic treatment systems.

The existence of underutilised anaerobic digesters in the region (Bell, 1997) has suggested the option of anaerobically treating municipal sludge in combination with those high strength industrial effluents which are potentially toxic for the microbial consortia if digested in isolation.

The aims of this project were to:

1. *Illustrate that high-strength or toxic organic liquid effluents can be disposed in conventional sewage works at the anaerobic digestion stage.*
2. *Provide a protocol for the evaluation of liquid effluents for disposal in conventional wastewater treatment digesters.*
3. *Provide an alternative treatment system for high-strength liquid effluents that are currently being disposed to landfill*

It is generally accepted that when industrial effluents, which are characterised by a high content of organic matter, are fed to an anaerobic digester, an *organic overload* could seriously impair the performance of the process, particularly of the methanogenic stage, and possibly lead to the failure of the digester.

Industrial effluents may also contain compounds which display a *toxic effect* on the anaerobic consortium. This effect may be temporary, i.e. providing that the micro-organisms are exposed to sufficiently small concentrations of the compounds for a sufficiently long period of time, they can eventually adapt to their presence in the feed and possibly even degrade them to a certain extent.

But the toxic effect may be permanent and therefore result in worsened performance, or, in more severe cases, in the failure of the digester.

That is why there is a real need for a method to assess whether an industrial effluent can be safely treated in an anaerobic digester; or if it requires to be mixed with another readily biodegradable effluent in order to be simultaneously digested (co-digestion); or else if it is not amenable to be anaerobically digested at all.

The primary objective of this project was therefore to establish such a screening protocol.

We believe that the main characteristics that such a protocol should be:

- it must use simple analytical and technical methods (possibly established), because these are generally also relatively inexpensive, so that on the one hand it is affordable to small companies and on the other hand the impact on the cost of disposal would not be excessive;
- it must be reliable and provide meaningful information regarding the effect of the test material on the digestion process;
- it must be quick, so as to be able to give a response within a time which is compatible with the requirements of an industrial process.

The serum bottle method (Annexure B) is based on the determination of the volume of gas produced in a sealed vial, as a measure of the activity of the anaerobic sludge. This method is certainly simple, as it makes use of small glass vials (125 mℓ-volume were used in this research) which do not require any stirring or feeding device or other engineered tool: a serum bottle is sealed immediately after all components are poured inside and thereafter conducted in a batch mode and occasionally shaken to ensure adequate homogenisation of the components. The only variable which is regularly measured is the volume of biogas produced. This is done manually by puncturing the rubber septum of the vial with the needle of a glass syringe and allowing the plunger to move freely until equilibrium is reached between the overpressure inside the vial and the atmospheric pressure. The gas collected in the syringe is wasted, to prevent an excessive build-up of pressure.

To ensure that the method is accurate, the composition of the gas is analysed by gas chromatography, as methane is the ultimate sink of the organic carbon in the anaerobic digestion process. Here is no extraction of liquid samples for practical considerations and to keep the method simple, the only indirect indicator of the progression and of the stability of the process is the fraction of carbon dioxide in the headspace.

We demonstrated that highly accuracy results can be achieved by regularly quantifying the volume and the composition of the biogas produced (Figure 1).

The two assays, originally developed by Owen et al. (1979) to separately address the toxicity and the biodegradability have been combined in a single test termed AAT, Anaerobic Activity Test, which enables the simultaneously assessment of the inhibitory effect on the methanogenic biomass and the biodegradability of the test material as well as the ability of the biomass to adapt to the test material and therefore to overcome the initial inhibition.

A second method that has been considered is relatively more recent and more specific.

The pH-stat titrimetric method consists of compensating the change in pH caused by a physico-chemical or biological process within a suspension, by the controlled addition of an appropriate solution which neutralises the *excess* acidity or alkalinity produced or consumed.

The anaerobic digestion process seems ideally suited for such measuring principle, as the pH of the suspension decreases during the acid stage when the organic substrates are converted to volatile fatty acids and then increases during the methanogenic phase. However, it is the overlap of the two phases which complicates the application of a pH-stat titrimetric method. Nevertheless, the original application whereby a single (acidic) control unit was used to monitor aceticlastic methanogenesis has proven reliable and very quick in assessing the toxicity of a test material (Rozzi et al., 2001). This can be used as a valid alternative to the serum bottle method for a quick toxicity assessment and it is proposed as a preliminary step prior to the AAT. However, it may not be as inexpensive as the serum bottle method, unless a computer controlled titrator is *ad-hoc* constructed, because at present these pH-stat titrimetric sensors are still at a prototype stage.

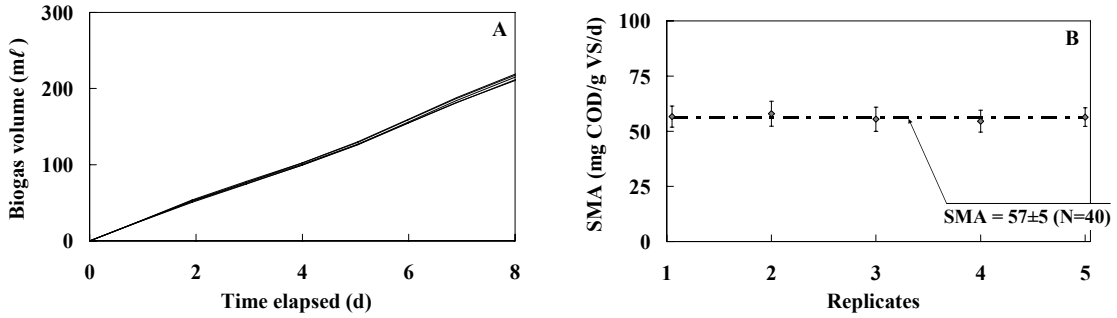

*Figure 1 – Repeatability and accuracy of the estimates of a serum bottle activity test: biogas volume (**A**) and methanogenic activity calculated (**B**).*
The test lasted 8 days and comprised 5 replicates. Symbols denote the average Specific Methanogenic Activity (SMA) calculated for each replicate; error bars represent the standard deviation. The dashed line indicates the

overall average SMA on 40 individual determinations.

The experimental work conducted during this project is extensively presented in Chapter 4 and in Annexure G (as explained later on). The following exercises were carried out.

- serum bottle toxicity (anaerobic toxicity assay – ATA) and biodegradability tests (biological methane potential - BMP) on landfill leachates and textile effluents;
- a serum bottle activity test on a synthetic dyestuff;
- serum bottle tests on distillery, size and scour effluents;
- co-digestion serum bottle assays on mixtures of distillery and size effluents and of scour and synthetic dye effluents;
- a laboratory-scale experiment of anaerobic digestion of the distillery effluent; off-line serum bottle activity tests were discontinuously used as a tool for monitoring the methanogenic activity of the sludge in the digester.

▪ The toxicity and biodegradability tests on the landfill leachates and textile effluents were conducted according to the conventional procedure and the quality of the experimental data is only sufficient to draw qualitative conclusions. More importantly this experience enabled us to develop an improved experimental protocol that was applied in the following tests.

▪ The activity test on the synthetic dyestuff was aimed at studying the potential of adaptation of the anaerobic biomass: it clearly showed that although initially an almost complete inhibition of methanogenesis occurs, activity is resumed after 30 to 40 days of adaptation, the duration of this lag-phase being a function of the concentration of the effluent.

Besides the specific experimental indications, this test provides a guideline for the execution of similar type of long-term assessments and enabled us to refine the experimental procedure for the anaerobic activity test (AAT).

▪ Two activity tests were conducted to assess the biodegradability and toxicity of the distillery, size and scour effluents, at volumetric ratios ranging from 1 to 40 % v/v (corresponding to COD concentrations of 7 to 54 g/ℓ for the distillery effluent; of 6 to 35 g/ℓ for the size effluent; and 4.9 to 5.4 g/ℓ for the scour effluent).

This shows the first methodological limitation that one faces when dealing with diluted (or low organic strength) effluents, such as the scour effluent: ideally, one should set-up a serum bottle test to examine the concentration of organic matter rather than at the volumetric ratio, as design parameter, but this may result in large and impractically volumes.

However, the scour effluent although characterised by low organic strength seems to be highly inhibitory for methanogens (Figure 28).

Both the distillery and size effluents proved partially biodegradable and not inhibitory, except at high concentration.

The biodegradability potential of a compound is expected to be concentration-independent, although relatively high concentrations may inhibit the methanogenic process due to the presence of organic substrate in excess –specifically, to the accumulation of acidic intermediates. The distillery and size effluents were degraded by 30-40 % at intermediate concentrations, but seemed to be very poorly degradable at low and high concentrations (Figure 39).

- The serum bottle co-digestion tests were aimed at investigating the effect of digesting two effluents in combination (co-digestion) as opposed to individually. A quantitative methodology to conduct the comparison was developed: the *effectiveness* of the co-digestion is the ratio between the volume of methane generated by the mixture and that calculated assuming that the individual contributions of the two components were independent (equation 15).

  This rigorous approach indicates that low concentrations (0.8 g COD/ℓ) of a 1:1 mixture of distillery and size effluents is actually preferable than the two components in isolation –at least from a biodegradability point of view (Figure 31); but as the organic concentration and the proportion of size effluent in the mixture increase, the benefit of the co-digestion gradually reduces (Figure 35).

  A similar quantitative approach can be developed to characterise the relative activity (or inhibition potential) of the biomass in presence of the test material and to ultimately assess the appropriateness of co-digestion. However the experimental data obtained were insufficient to validate this.

- The guidelines for the transposition of the knowledge obtained through serum bottle activity tests to (semi)continuous experiments at the laboratory-scale, i.e. for the identification of tentative values for the hydraulic retention time and organic loading rate, are discussed formally in Section 4.2.1.

- A completely stirred laboratory-scale digester was set-up using a 5 ℓ-volume flask connected to a variable-volume headspace that ensures the gas-tightness of the system and to a semi-automatic liquid displacement system to measure the biogas production. The digester was run for almost 150 days. This is a relatively long period of time for a start-up; however, the

operating conditions had been repeatedly changed in order to verify hypotheses or counteract potentially dangerous situations that could lead to a complete failure of the digester.

Nine experimental periods are individually presented in Section 4.2.2, which include three hydraulic retention times (20, 35 and 80-85 days) and four organic loading rates (1, 2, 2.5 and 3.4 g COD/ℓ/d).

An overview of the variables measured and parameters calculated for the entire experimental period is presented in Figure 52.

More than quantitative conclusions, this exercise enabled us to identify a few methodological issues that may be of interest for similar studies.

1) The liquid displacement system to measure the gas production is very delicate and can provide very inaccurate indications, if it is not checked and calibrated frequently.
2) The gas composition, i.e. the molar fractions of carbon dioxide and methane, turned out to be the most accurate and reliable indicators of process performance; the alkalinity and volatile fatty acid (VFA) concentration are just as accurate and reliable, but more labour-intensive, as they require that a liquid sample is withdrawn and carefully manipulated and the manual titrimetric determination needs to be undertaken with care and precision.
3) A set of operating conditions, specifically of hydraulic retention time and organic loading rate, should be maintained for sufficiently long periods of time, to enable the characterisation of the performance of the system at steady-state or relatively stable conditions. Otherwise the biomass may be repeatedly stressed, and also the performance indicators that one calculates may be not reliable and difficult to interpret (Figure 53).
4) A procedure to conduct off-line activity tests on sludge samples extracted from an anaerobic digester has been developed (Section 4.2.2.3) that enables one to accurately determine the methanogenic activity of the biomass; the degree of inhibition of the methanogenic biomass; and the non-readily biodegradable COD of the test material.

A few semi-quantitative results can be isolated from this exercise.

a) The distillery effluent undergoes a relatively rapid acidification that can cause a temporary accumulation of volatile acids in the suspension (reflected in the carbon dioxide molar fraction in the gas phase) which can impair the activity of methanogens: this limits the organic loading rate, to about less than 2 g COD/ℓ/d.
b) The distillery effluent is biodegraded by 50-60 % at a HRT of 35 d without significantly reducing the methanogenic activity of the biomass (Figure 54; 60-70 mg COD/g VS/d).

Much better performance (90 % biodegradation) seem to be attainable at very long retention times (80 days), which however are of practical interest only when sludge can be retained in the digester.

The **screening protocol** is illustrated in Annexure A.

The protocol consists of a sequence of assays which employ the serum bottle methodology.

A first step of the procedure is aimed at rapidly estimating whether the effluent is potentially toxic to the methanogenic biomass and in what concentration. The second step is a more extensive screening, aimed at precisely characterising the toxicity of the effluent, the extent of biodegradation that can be achieved, as well as at establishing whether a potential for adaptation of the biomass exists upon exposure. If the sample passes the screening stage, the same serum bottle method is then used to conduct a series of batch co-digestion experiments aimed at evaluating a convenient volumetric ratio between the test material and the readily biodegradable substrate.

Finally, a laboratory-scale co-digestion trial is used to simulate the full-scale process, thus enabling the selection of appropriate operating conditions for the start-up of the full-scale implementation.

Furthermore, the protocol includes a monitoring phase whereby the characteristics of the effluent source (which often vary as a consequence of changes in the industrial production process) and the performance of the co-digestion process are periodically verified. A sampling campaign at the co-digestion facility monitors the activity of the sludge and the methanogenic toxicity of the effluent, so that action can be taken to ensure the continuous economical viability and environmental safety of the operations.

An extensive discussion of the **serum bottle method** to perform AAT is presented in Annexure B.

A serum bottle AAT comprises (Table B.2) two control sets (which allow for the quantification of the baseline e.g. endogenous activity of the seed inoculum and of the reference activity, respectively) and a number of test units, in replicates, in which the anaerobic sludge is exposed to a variety of conditions. The activity is assessed at varying concentrations of the test material.

The crucial aspect that one should bear in mind when designing an activity test using serum bottles is that for the period between two subsequent re-equilibrations, the vials are

pressurised as a result of gas production. This is the *measuring principle* upon which the test relies; therefore, one would want that the signal (i.e. the pressure differential) to be sufficiently large. However, the pressure increase inside a vial will also alter the gas/liquid equilibrium, particularly of carbon dioxide. As the pressure in the headspace increases, an increasing fraction of carbon dioxide will dissolve in the suspension, ultimately causing the pH to decrease, possibly to such an extent that it actually inhibits the methanogenic biomass (Figure B.1).

An excessive pressure can be due to either of the following causes: a large (or inherently highly active) seed inoculum; a high concentration of the organic substrate; or a large liquid volume (or low gas-to-liquid volume ratio).

There are two modes of conducting a serum bottle AAT: a conventional batch mode and a fed-batch mode. The former requires that all the components, i.e. the seed inoculum, the test material and the reference substrate, are poured into the vial prior to sealing it; thereafter, the degradation of the initial dose of substrate is monitored. The fed-batch mode consists in batch feeding the vials with the reference substrate, until a steady-state in methane production is reached (control); thereafter, both the reference substrate and the test material are spiked (test unit). The drawback of such technique is that the duration of the two phases is limited by volume that can be added into the vial.

As mentioned above, the objective of an activity test is the *combined* determination of the biodegradability and toxicity of the test material, by respectively quantifying the portion of the organic matter contained in the test material that is converted to methane and by comparing the methanogenic activity of the sludge exposed to the test material to the activity in the absence of it. This effect is a function of the concentration of the test material, so that generally the effect of a compound ranges from *no-effect*, at low concentrations, to *full inhibition*, at high concentrations. This effect-to-concentration relationship is formally described by an inhibition curve.

The protocol for the conduction of serum bottle activity tests that we propose enables one to assess both the short- and the long-term inhibition which are quantified immediately after starting the test and in correspondence to the maximum methanogenic activity attained by the biomass respectively.

Acetate or other *direct* precursors of methane, e.g. propionate, are preferred to e.g. glucose as reference substrates because the drop in pH which follows the initial break down of these substrates could result in a temporary reduction in methanogenic activity, that would hide the

actual effect of the test material, i.e. it would be interpreted as inhibitory effect. This could result in the inhibition caused by the test material to be over calculated, or worse, interpreted as irreversible when in fact it was actually only temporary.

Irrespective to the ultimate outcome of the assay, the key-point is the accurate mass balance on the organic carbon. This is calculated step-by-step, according to equation B.4:

$$\Delta v_{CH,t} = f_{CH,t} \cdot (\Delta v_t + V_{t-\Delta t}) - f_{CH,t-\Delta t} \cdot V_{t-\Delta t}$$

where f_{CH} indicates the molar fraction of methane (at time t and $t-\Delta t$ respectively); $V_{t-\Delta t}$ is the headspace volume at time $t-\Delta t$ (for simplicity, it can be considered constant throughout the test, unless a fed-batch regime is adopted); and Δv is the volume of gas produced in the interval $(t-\Delta t, t)$.

The Sludge Methanogenic Activity (SMA, in g COD/g VS/d or g COD/g VSS/d) is calculated according to equation B.6:

$$SMA = \frac{R_{CH}}{X_0}$$

where R_{CH} is the *net* rate of methane production; and X_0 is the biomass initially present in the vial. R_{CH} is calculated as the first derivative of the *net* volume of methane.

Finally, the ultimate biodegradation (B) of the test material and the inhibition effect (E) are calculated according to equations B.5 and B.7:

$$B = \frac{V_{CH,\infty}}{V_{CH,theory}} \text{ and } E = \frac{SMA}{SMA_{ref}}$$

where $V_{CH,theory} = f_T \cdot 0.350 \cdot COD_0$ (f_T is a temperature correction factor: $f_T = \frac{T}{273}$); and SMA_{ref} is the baseline activity, in the control unit.

In the course of this project, the suitability of a non-conventional method of assessing biological activity to effectively screen an industrial effluent, namely the **pH-stat titrimetric method**, was also investigated.

The principle of pH-stat titration has proven to enable the accurate determination of the biological activity of several microbial populations (Ficara et al., 2003) and specifically of acetotrophic methanogens: however, as the process under investigation gets more complicated, the method may prove unreliable, as extensively discussed in Annexure C.

The principle of pH-stat titration can be summarised in three key features: *i*) it detects the change in concentration of protons [H^+] and compensates this change by adding an equivalent

amount of the appropriate titrating solution; *ii*) it maintains the pH of the suspension constant, within a limited range; and *iii*) it relates the microbial activity to the rate of addition of a titrant solution. An additional feature in particular cases is that the titrating solution can not only provide the protons to maintain the pH value constant, but also the substrate which is consumed by the micro-organisms, hence the substrate concentration is maintained constant.

The application of the pH-stat titrimetric method for the assessment of activity and inhibition of the aceticlastic methanogens has proven very successful, as the CO_2/HCO_3 equilibrium between the liquid and the gas phase is the only source of interference, which can be effectively controlled (Rozzi et al., 2001).

An automated titrimetric sensor has been used for this research. The hardware and software set-up of this instrument have evolved to the latest version, equipped with two independent titration lines, multiple channels for secondary measurements and an advanced Windows®-based interface. The detailed procedure to set-up the instrument and start the activity test outlined in Section C.2 is intended to provide a general guideline and to be applicable to either version of the instrument. A key factor is the selection of the working (or set-point) value of pH, which is related to the molar fraction (hence partial pressure) of carbon dioxide in the headspace (Figure 6) and which influences the inherent sensitivity of the measurement (Figure C.4).

Two distinct types of activity test can be conducted, at *decreasing* or *constant* substrate concentration respectively.

The measuring principle (which is valid irrespective to the particular application) is that the biological activity is proportional to the rate of titrant addition (Section C.3).

The application of a two-unit pH-stat titration sensor, which could monitor simultaneously the acidogenic and methanogenic stages, thus enable the monitoring of the anaerobic digestion process, is currently under evaluation (Remigi and Ficara, 2005; in Annexure I). This new set-up will enable activity tests equivalent to serum bottle assays, to be undertaken with the advantages of a shorter duration and automated data acquisition.

Annexure D provides a brief discussion of concepts related to the **operation of laboratory-scale anaerobic digesters**. Key features for a simple completely stirred reactor are the stirring device, the temperature control and the set-up for the feeding, extraction and sampling ports; whereas the operating parameters that can be independently set are limited to the hydraulic retention time (or feed flow rate) and the organic loading rate (Section D.1).

Monitoring the performance of a digester requires the estimation of the *state* of the system in response to the *input* and operating conditions (Figure D.1).

Our experience suggests that the variables that one can measure in order to reliably assess the state of an anaerobic process are (Table D.1):

- the gas flow rate and composition, possibly limited to the detection of carbon dioxide and methane, which are indicative of the acidity hence potential instability of the process and for the extent of degradation of the organic matter respectively;
- the pH, alkalinity and volatile acids concentration, which are related to the acidity of the process and to the imbalance between the acidification and methanisation of the organic substrate; and
- the COD and solids concentration, which are related to the overall concentration of the organic matter and of the biomass respectively.

The knowledge of the pH, alkalinity, VFA concentration and carbon dioxide molar fraction enable the calculation of the *reserve bicarbonate alkalinity*, which should be regarded as an accurate monitoring parameter, when more sophisticated monitoring and control devices are not available (Section D.2.).

Ultimately, the rigorous mass balance of the organic matter is essential to assess the performance of the process, which, among others parameters, can be quantified in terms of removal efficiency as outlined in Section D.2.7.

In our experience, organic overloads are the most frequent incidents that may occur in the start-up phase of a laboratory-scale digester: a possible way of dealing with such events is discussed in Section D.3.

The experience accumulated in the course of this project suggested that the use of a simple spreadsheet was not the most efficient way of managing the large and often non-homogenous amount of data deriving from the operation of an anaerobic digester, particularly when, for research purposes more than one unit has to be operated. In fact, when sophisticated devices for online monitoring and control are not available, the operator can only rely on an accurate day-by-day acquisition and analysis of the data collected.

We therefore oriented our interest to the development of a **database application** (Annexure E).

This database consists of the user-interface and of the underlying database.

The main page of the log-book is showed in Figure E.2 and consists of a commands bar; a navigation bar and a main body. This latter enables the user to:

- *identify* each set of data, by inputting the date, time and name of the unit to which that particular determination is referred; and by specifying the type of data entered, i.e. a reading of gas volume or composition (which does not affect the liquid volume); a liquid sample (which can result in a value of pH, COD, alkalinity, VFA or solids concentration); or a feed (which results in a volume or flow-rate entering the reactor and the respective organic load);
- specify the volume of the feed or of the sample; and
- enter the actual analytical and instrumental data recorded, e.g. pH, gas volume, COD, etc.

Most of the parameters are *manipulated* quantities which result from the raw data, e.g. replicated measurements which contribute to a single averaged value. Conventionally this would be carried out on a separate spreadsheet, whereas a database application allows multiple levels of input to interact within the same underlying database. This translates in each set of raw data to be managed through a secondary input form (which is dedicated to that particular data type, as data types are generally non-homogenous): this enables to *i*) store all raw data in a fashion that they can be retrieved at a later stage; *ii*) perform all routine calculations (including consistency checks) in the background; and *iii*) display the elaborated result of each analytical or instrumental determination on the main form (which corresponds to the log-book).

Furthermore, a number of parameters which characterise the process (e.g. hydraulic and solids retention time) or which are used as indicators of process performance (e.g. methanogenic activity and alkalinity-to-VFA ratio) are calculated internally and displayed on the main page of the log-book. Other possible indicators could be the gas flow-rate, the methane-to-carbon dioxide ratio. Clearly more than the current values of these indicators, it is of greater interest their trend over a certain predefined period of time. A more efficient way of displaying these indicators would be to generate a secondary form (or a report) in which a series of data can be viewed over a selected period of time.

The technical aspects of the experimental work are discussed in details in Annexure F. These include the description of the mineral salts and nutrients stock solution; the characteristics and the calibration procedure of the gas-chromatograph that has been employed for the determination of the gas composition; the two-point titration method to measure alkalinity and volatile fatty acids concentration; and the methodology for the calculation of the organic

carbon content of a test compound and of the composition of the biogas resulting from its anaerobic biodegradation.

The GOW MAC 350 gas chromatograph (equipped with a thermal conductivity detector and a Haysep D stainless steel column) was able to ensure very low detection limits (3 μℓ) and a high accuracy (> 99.9 % in the range 10 to 60 μℓ) for both carbon dioxide and methane.

The two-point titration method enables a very quick determination of the alkalinity (titration point: pH 5.1) and of the volatile fatty acids concentration (titration point: pH 3.5): the calculation has been implemented in a pre-formatted spreadsheet (as well as in the database).

When the gas composition is simultaneously measured, one can calculate the fraction of carbon dioxide dissolved in the liquid phase and ultimately assess the stability of the process, based on the concept of residual alkalinity (Section D.2.).

The stoichiometric calculation of the COD of the test material and of the expected composition of the off-gas (when the elemental composition of the test material is known) enables one to calculate a mass balance and to gain a better insight of the process (e.g. a gas composition different from the one expected could indicate that the process is unbalanced).

The objective of Annexure G is threefold.

- It is intended to complement Chapter 4. The results of the different experiments are summarised in Chapter 4, both for clarity and to illustrate the ultimate objective of each experiment rather than the technicalities of some specific aspects. For instance, for a serum bottle activity test, the curves of the biogas volumes are presented, since this is the primary measured quantity of those tests. Then the outcome of each test is described according to the methodology developed (e.g. a mass balance is calculated based on the carbon dioxide and methane molar fractions measured in the off gas) and the end results are presented in form of summary tables and/or plots.

  Additional/intermediate experimental results discussed in Chapter 4 are presented in this Section: typically, for a serum bottle activity test, the curves of biogas production *measured*, the curves of net methane production *calculated*, the curves of nitrogen, carbon dioxide and methane molar fractions *measured* and finally the curves of specific methanogenic activity *calculated* for each individual unit are reported.
- This background information is organised in a consistent format throughout this Section, which is proposed as the standard way one should analyse and discuss the outcome of a serum bottle activity test.

Such a format facilitates the visual comparison between replicates of the same unit; between units at different concentration of the same test material and between units containing different test materials; and enables an analysis of the progression of the degradative process in time and therefore the comparison of the time of occurrence of particular events of relevance.

- Furthermore, this Section presents the discussion of some aspects which may be considered secondary to the main objective of a particular experiment and yet are relevant to substantiate the main results or to validate a methodology or to achieve a better understanding of the process.

This Annexure comprises two main Sections, the screening tests with the serum bottle methodology and the laboratory-scale simulation.

The serum bottle methodology was used in two separate periods and different test materials were tested, therefore the results, hence the discussion presented, are not homogeneous.

A first group of assays were conducted following the *conventional* procedure as outlined in Owen et al. (1979) which can be summarised as follows:

- two separate assays (termed BMP and ATA) target biodegradability and toxicity respectively;
- the duration of a toxicity assay and of a biodegradability assay are fixed at 7 d and 30-60 d respectively;
- the volumes of the components in a vial are to some extent predetermined (e.g. it is generally agreed that an aliquot of 2 mℓ of a mixture of acetate and propionate be used as the reference organic substrate);
- no specific requirements are indicated as to the frequency of gas composition determination: typically, a single analysis is undertaken at the end of a toxicity assay and a few during a biodegradability assay.

To the best of our knowledge, no indication as to how various aspects such as the volume of the syringe used to re equilibrate the headspace, the frequency of the gas composition determination, etc. can influence the accuracy of the overall mass balance and ultimately the outcome of the assays is available in literature.

Furthermore, these experiments were conducted in the *early* stage of the research and much of the information that would have been necessary to reprocess the results using the current understanding of the serum bottle methodology (Remigi and Foxon, 2005) were not properly recorded at the time.

The results of these early experiments are presented in Chapter 4.1.1 and Sections G.1.1 and G.1.2, under the heading *Conventional toxicity and biodegradability tests*. The test materials were leachates from the Shongweni, Aloe and Holsfontein landfill sites and effluents of the textile industry.

A second group of assays was conducted in the final year of the research when the rigorous serum bottle methodology was being developed (and actually were instrumental to the understanding of the methodology). These comprise:

- a first activity assay in which the toxicity and biodegradability of a synthetic dye effluent and the potential of the biomass to adapt upon exposure were simultaneously tested, that represents the first step towards the definition of the improved serum bottle methodology (Chapter 4.1.2 and Section G.1.3);
- two anaerobic activity tests, in which the biodegradability and toxicity of the test material are simultaneously assessed by supplementing all units with the reference substrate (acetate) and by monitoring the gas production until the microbial activity becomes negligible, i.e. reaches the endogenous level (Chapter 4.1.3; Sections G.1.4 and G.1.5); and
- three anaerobic activity tests, aimed at assessing the simultaneous digestion of mixtures of the test materials (Chapter 4.1.3; Sections G.1.6 to G.1.8). The set-up of these tests differs from the general methodology in that no external reference substrate is supplemented; the reason being that the objective was to quantify the effect of mixing a supposedly not fully biodegradable test material with a readily biodegradable effluent, more than to assess the characteristics of each one individually where a term of reference might be needed. However, the aspect is debatable and still needs further investigation.

The test materials were effluents from a distillery and from a textile industry, i.e. scour and size effluents.

An interesting discussion is proposed in Section G.1.9 which compares the outcome, i.e. gas production and composition and (endogenous) activity of the control units of the five independent activity tests mentioned above, as a mean of validating the serum bottle method and therefore the specific response obtained for each test material.

Section G.1.10 presents the application of a method which enables the correction of the volume of gas measured through multiple insertions of the needle in a serum bottle. Under these circumstances, all but the last determination are in fact conducted at a pressure higher than atmospheric, thus resulting in an underestimation of the actual volume of gas released with each re-equilibration.

The second section of Annexure G is dedicated to the laboratory-scale simulation and is subdivided into batch-phase, semi-continuous phase and off-line activity tests.

A series of off-line activity tests were carried out on samples withdrawn from the laboratory-scale reactor to monitor the specific methanogenic activity of the sludge. The output of such tests although inevitably delayed in time from the moment when the sample was extracted from the reactor, has proven to convey a great deal of information, when the general methodology is modified as presented in Section G.2.3:

- during a first period, lasting a few days, the sludge sample is monitored under batch conditions as the residual organic substrate present at the time of extraction is consumed: the specific methanogenic activity at the time of extraction and the portion of the residual substrate which is further biodegradable given a longer exposure time (which simulates a longer solids retention time) are evaluated;
- during a second period, pure acetic acid is spiked daily to enable the evaluation of the *reference* specific methanogenic activity of the sludge, i.e. the aceticlastic activity: by comparing this information over time, it is possible to further estimate the methanogenic fraction of the active biomass;
- a subsequent period can be used to simulate experimental conditions of interest, using a *clone* of the laboratory-scale digester: e.g. different loading rates to that currently applied can be tested; the correction of pH can be optimised by the addition of alkalinity; the lack of micronutrients as possible cause of low methanogenic activity can be ascertained by supplementing aliquots of nutrient and mineral salts solution; etc.

A few specific outputs of Annexure G can be summarised as follows.

- The concentration of the external reference substrate in an anaerobic activity test is to be sufficiently low that it does not result in a temporary inhibition of methanogenesis (as this would interfere with the assessment of the biodegradability and toxicity of the test material). A concentration of 1 g COD/ℓ is recommended.
- The concentration of the external reference substrate is to be lower than the concentration of the test material, in order for the gas production (and composition) to be mainly driven by the degradation of the test material. In other words, one would want that the output measured, i.e. the gas production (and composition) to be mostly due to the degradation of the test material for the biodegradability assessment to be reliable and the contribution of the reference substrate negligible and entirely exhausted within the early stages of the assay.

- When the seed inoculum is withdrawn from a well-operated anaerobic digester, a nutrients and minerals medium may be unnecessary, as all macro- and micronutrients are generally available in a healthy digester.
- The endogenous specific methanogenic activity of an anaerobic sludge can be estimated in a range of 2 to 3 mg COD/g VS/d.

The **publications** (1 refereed, 4 conference proceedings and 1 paper in preparation) which have originated from this project are collected in Annexure H.

The **capacity building and transfer of knowledge** took the form of:

- theses (5) written by former students who collaborated to the project, being part of the Research Team at some stage;
- courses (4) which mainly contributed to the dissemination of knowledge on mathematical modelling applied to wastewater treatment processes, and
- co-operations with national (1) and international institutions (4), which have been particularly fruitful in creating long-distance collaborative links as well as in attracting students to work with Pollution Research Group for their post-graduate degrees.

These outputs are presented in Annexure I.

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Mr G. N. Steenveld	Water Research Commission (Chairman)
Prof G. N. Tivchev	Durban Institute of Technology (Steve Biko Campus)
Prof B. Loveday	University of KwaZulu-Natal
Mr F. B. Stevens	Durban Metro Wastewater Services, DMWS
Mr W. Rössle	East Rand Water Care Company, ERWAT
Mr L. R. Gravelet-Blondin	Department of Water Affairs and Forestry, DWAF
Mr D. Nozaic	Umgeni Water
Mr D. H. Goodes	Durban Institute of Technology (ML Sultan Campus)
Mr L. J. Strachan	eThekwini Solid Waste
Mr P. de Mattos	Institute of Waste Management of SA, (IWMSA)
Mr P. Canton	SAUR RSA (Pty) Ltd
Ms S. Chetty	Water Research Commission (Committee Secretary)

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Dr Valerie Naidoo	Ms Sithembile Dlamini
Ms Mapaseka Petlane	Mr Zipathele Chamane
Ms Thabisile Rakgotho	Ms Katherine Foxon
Mr Dinesh Naidoo	

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GLOSSARY

-clastic	(suffix; Greek *klastos*: broken) it indicates 'destruction of' something: e.g. aceticlastic denotes a process or a group of micro-organisms which *degrade* acetate. Synonym: -trophic (Greek *trophe*: food, nourishment) is more specifically related to the feeding process: e.g. acetotrophic denotes a process or group of micro-organisms which *utilise* acetate as substrate.
Acclimation	The adaptation of a microbial community to degrade an inherently *recalcitrant* compound through prior exposure to that compound.
Adaptation	A change in the microbial community that increases the rate of transformation of a test compound as a result of prior exposure to that test compound.
Anaerobic digestion	The microbial degradation of an organic compound in the absence of oxygen. It is carried out by anaerobic micro-organisms which degrade the compound in a step-wise process, yielding organic acids, carbon dioxide and hydrogen and, ultimately, methane and carbon dioxide.
Anaerobic Toxicity Assay (ATA)	It allows for the determination of the *toxic* or *inhibitory* potential of a test compound, which is reflected in a reduced initial rate of gas production (compared to a reference system, not exposed to the compound), in proportion to the relative volume of compound added.
Batch culture	A close culture environment in which conditions are continuously changing, according to the metabolic state of the microbial culture.
Biodegradability	The property of a substance, primarily dependent on the molecular structure, that refers to its susceptibility to undergo a biologically mediated degradation.
Biogas	The gas produced, principally methane and carbon dioxide, by the action of anaerobic micro-organisms on organic compounds.

Biological Methane Potential (BMP) A measure of the portion of organic material in a waste stream which can be anaerobically converted to methane. Units: g COD/ℓ (or %).

Chemical Oxygen Demand (COD) A measure of the total amount of organic material in a waste stream. The following relationship holds: $COD \geq BMP$. Units: g O_2/ℓ.

Co-digestion Term used to describe the treatment of high-strength or toxic organic effluents with municipal sewage sludge whereby the *organic load* to an anaerobic digester can be increased by the addition of a concentrated wastewater, without compromising the overall performance of the system.

Co-metabolism Phenomenon whereby complex organic compounds which would normally persist undegraded in the environment due to their inherent toxicity and/or very low (trace) concentrations, undergo a biodegradation process in presence of an external source of energy, which acts as the *primary* substrate. This type of process generally does not result in biomass growth.

Confidence interval It is associated with a percentage α (typically, 95 %) and indicates the probability that the estimates of the parameters falls within the interval. It is calculated by extending either side of the estimate of the parameter (as average) by some multiple of the standard error (which depends on the probability distribution for the population parameter).

Effluent A stream flowing from a sewage tank or industrial process.

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

Hydraulic Retention Time (HRT) Ratio between the reactor volume and the incoming flow rate. It defines the window of opportunity afforded the microbes to accomplish their task (Speece, 1996). Units: hours or days.

Inhibition Impairment (generally, irreversible) of the bacterial functions. *See also* Toxicity.

Labile	Readily biodegradable.
Methanogens	Bacteria which utilise volatile organic acids as substrate and produce methane and carbon dioxide.
Mineralisation	Microbial decomposition of an organic compound to inorganic constituents such as carbon dioxide, methane and water.
Organic load	Measure of the amount of organic matter fed to a digester. Also: Organic Loading Rate (OLR). Units: kg COD/m^3/d or kg VS/m^3/d.
pH-stat	The operating conditions whereby the pH in the reaction vessel is maintained at a constant value, by the controlled addition of an acidic or alkaline solution.
Recalcitrant	Resistant to microbial degradation.
Sludge	The general term applied to the accumulated solids segregated from waste water. A large portion of the sludge material in a digester consists of bacteria which are responsible for its decomposition and ultimately *mineralisation*.
Sludge Retention Time (SRT)	Ratio between the reactor volume and the outgoing flow rate of solids. It determines which organisms can replicate and predominate and what biomass inventory can be maintained (Speece, 1996). Units: hours or days.
Toxicity	Temporary (generally, non-lethal) adverse effect on bacterial metabolism. *See also* Inhibition.
-trophic	(suffix; Greek *trophe*: food, nourishment) see -clastic.
Variation coefficient	It is a measure of the standard deviation *relative* to the mean: $VC = \frac{StDev}{Mean}$.
Volumetric load	Measure of the amount of sludge fed to a digester. Units: m^3/d.
Warning (parameter)	A parameter which *i*) is sufficiently sensitive to the characteristics of the influent and *ii*) responds fast enough to enable the operator to

	intervene on the characteristic of the feed *before* the upsetting conditions become irreversible.
Xenobiotics	(prefix; Greek *xenos*: foreign, stranger) synthetic organic chemicals or natural chemicals present in unnatural concentrations, which is 'foreign' to micro-organisms and therefore they do not possess the enzymatic machinery to biodegrade it.

ABBREVIATIONS

AAT	Anaerobic Activity Test.
ATA	Anaerobic Toxicity Assay.
BMP	Biological Methane Potential.
COD	Chemical Oxygen Demand.
EC_{50}	50 %-Effect Concentration.
GC	Gas-chromatograph.
HRT	Hydraulic Retention Time.
NMS	Nutrients and Minerals Salts solution.
OLR	Organic Loading Rate.
PRG	Pollution Research Group.
SCP	Sustainable Production and Consumption
SMA	Specific Methanogenic Activity.
SRT	Sludge Retention Time.
STP	Conditions of Standard Temperature and Pressure, i.e. 0 °C and 1 atm
VFA	Volatile Fatty Acids.
VS, VSS	Volatile Solids and Volatile Suspended Solids.

THE RESEARCH TEAM

A block diagram of the people that have been involved in the project over the four-year period is presented in the Figure 2: blocks with a dark background and a thick frame indicate completed theses; blocks with a light background indicate an on-going work that will eventually result in a thesis.

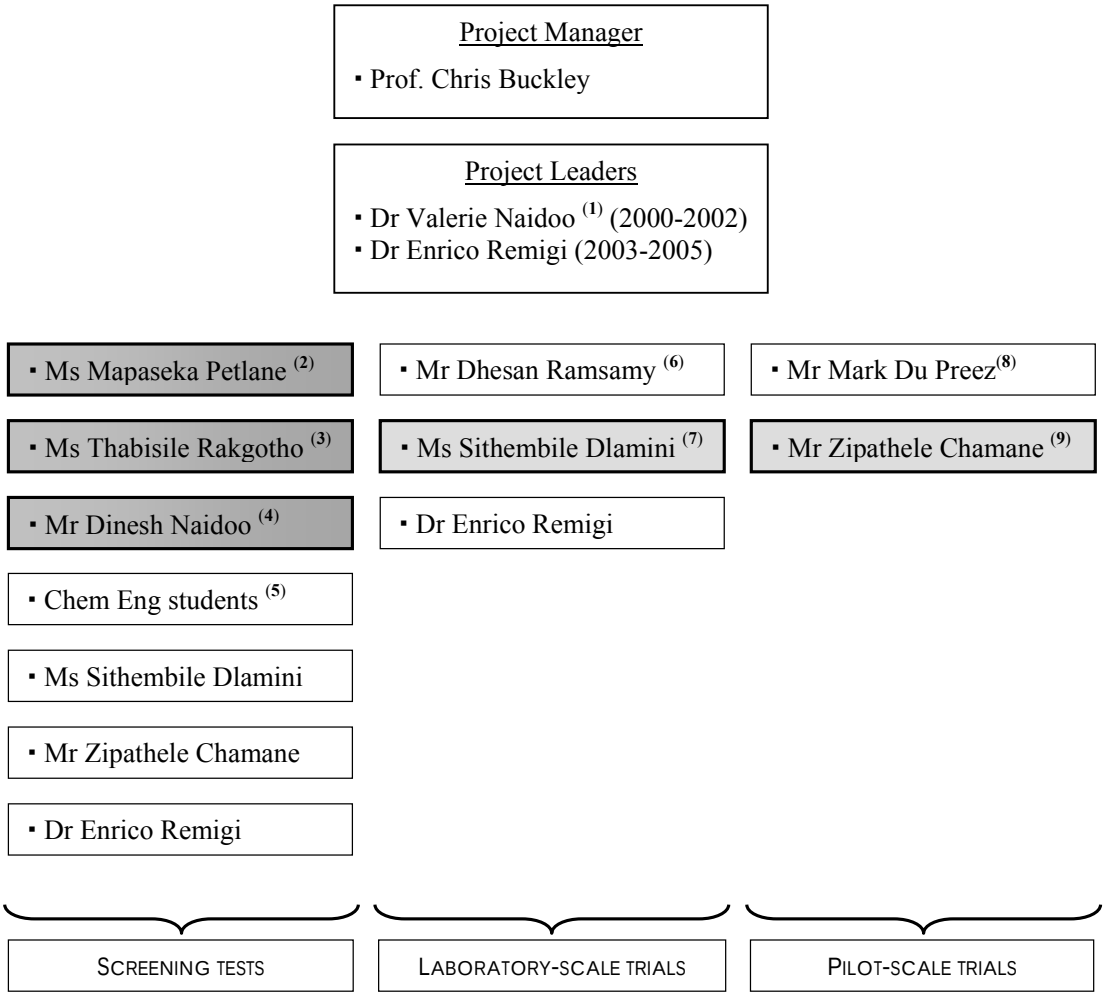

Figure 2 – The Research Team.

(1) Dr Valerie Naidoo resigned to join Unilever.

(2) Ms Mapaseka Petlane completed her BSc(Hon) degree in 2002 thereafter she undertook research on another WRC project as an MSc student (Geographic and Environmental Science).

(3) Mrs Thabisile Rakgotho completed her MTech with Durban Institute of Technology in June 2005. She took up a post with Department of Water Affairs and Forestry in Pretoria in

2001 and in August 2002, transferred to the DWAF Durban offices. She is presently working at the DWAF Water Quality Management (Gauteng South).

(4) Mr Dinesh Naidoo completed his MSc Eng (Chemical) degree in June 2003. He worked as an environmental/process engineering consultant for Environmental Business Strategies CC (May 2003 to February 2005) in Johannesburg. He has recently joined Nuclear Consultants International (NCI) based in Cape Town (April 2005). Mr Naidoo is currently on secondment at NNC Ltd in Manchester, United Kingdom (April 2005 to October 2006). Upon his return he is contracted to work as a Nuclear Engineer for NCI for a 3 year contract.

(5) Ms Nirasha Sewpersad and Mr Dheshen Naidoo worked under the supervision of Mr D. Naidoo when they were 4th year Chemical Engineering students. They conducted experimental work using the serum bottle and the pH-stat titrimetric techniques, for biodegradability and for aceticlastic activity/inhibition assessment, respectively. Mr Anban Moodley worked on biochemical methane potential and anaerobic toxicity tests for brewery effluent when he was 4th year Chemical Engineering student. He is currently employed at Sasol.

Mr Deric Dignon worked under the supervision of Mr. David D'Ambrosio (exchange student from Politecnico di Milano, Italy) when he was 4th year Chemical Engineering student. He used the pH-stat titrimetric sensor to conduct activity and inhibition tests, to evaluate the kinetics of different sludges.

(6) Mr Dhesan Ramsamy curtailed his research in April 2003 to take up full time employment at Unilever. He has not completed his dissertation, nor made available to the rest of the team his experimental results.

(7) Ms Sithembile Dlamini initially registered as an MSc Eng student with the PRG at the University of KwaZulu-Natal; she subsequently transferred her registration to an MTech at Durban Institute of Technology.

(8) Mr Mark du Preez was an MSc Eng student: he resigned in January 2003.

(9) Mr Ziphatele Chamane is currently research assistant with the PRG at the University of KwaZulu-Natal and an MTech student at Durban Institute of Technology.

CHAPTER 1

INTRODUCTION

This chapter provides a brief background to the application of anaerobic treatment to industrial effluents, with particular regard to the South African situation. It also outlines the scope of this project and the manner in which the report has been organised.

1.1 THE GEOGRAPHIC AND LEGISLATIVE CONTEXT

South Africa lies in a semi-arid region: the climate varies from desert and semi-desert in the West, to sub-humid along the eastern coastal area; the average rainfall for the country is as low as 450 mm per year, well below the World average, i.e. 800 mm per year, while evaporation is comparatively high (DWAF, 1986). As a result, water across the country is unevenly distributed and South Africa is regarded as a water scarce country that is poorly served with natural water systems.

1.1.1 Water regulation

According to the new Constitution of the Republic of South Africa *everyone has the right to an environment that is not harmful to their health or well-being and to have the environment protected, for the benefit of the present and future generations, through responsible legislative and other measures that prevent pollution and ecological degradation and secure ecologically sustainable development*.

This Bill of Rights of the Constitution gives effect to the fundamental objective outlined in the National Water Policy and the National Water Act of achieving a sustainable use of water, i.e. the efficient and effective use of water for the optimal social and economic benefit.

▪ The National Water Act, 1998 (Act 36 of 1998)was formulated to ensure that South Africa's water resources are protected, used, developed, conserved, managed and controlled in a sustainable and equitable manner, for the benefit of all persons. It states that it is not practical nor realistic to avoid all impacts to water quality otherwise the country will not be able to grow economically and we will not be able to alleviate poverty or to have social development. This Act requires a balance between using and protecting water resources to be achieved: this is the principle of sustainability (Table 1).

Table 1 – Relevant factors to achieve a sustainable development.

▫ To avoid (or minimise) the pollution and the degradation of the environment;
▫ To avoid the wastage of water, or to minimise and reuse or recycle, if possible; otherwise to dispose of used water in a responsible manner.

▪ The National Environmental Management Act (Act No. 107 of 1998) is the reference for managing the activities that might have detrimental impact on the water resources environment. It states that the government must secure ecologically sustainable development and the use of natural resources while promoting justifiable economic and social development.

▪ Activities such as industrial processes, and waste and sewage disposal are amongst those activities which will probably have a detrimental effect on environment and hence control of such activities needs to be implemented (Environmental Conservation Act, Act 73 of 1989).

1.1.2 Current disposal practices

Currently, high strength industrial effluents are sent to landfill, or to marine outfall (Bell, 1997).

The option of effluent disposal to landfill sites has great environmental implications, as liquid effluents will enhance the formation of leachate that might ultimately contaminate the aquifer. Leachate is formed when water is allowed to percolate through the decomposing waste. It contains a variety of chemicals and residues which result from the degradation process which takes place within the landfill body. Its physico-chemical characteristics vary, according to the age of the site, i.e. to the extent of stabilisation progressively reached.

In regions where there is a net surplus of rainfall, landfill sites have a greater potential of polluting the groundwater. Many landfill sites that are built, are not designed for saturated soil conditions.

Marine disposal of wastewater relies on the ability of the sea to disperse the effluents and to biodegrade many organic wastes; and on the binding properties of sea sediments. Marine disposal is an inexpensive and widely used disposal option in South Africa.

The Department of Water Affairs and Forestry created seawater quality guidelines (Oelofse, Viljoen, et al., 2004) which forms the basis for the planning of sea outfalls and for the environmental assessments of areas subjected to pollution loads: maximum allowable concentrations are stipulated for the discharge of effluents to the sea.

Even stricter standards are set for discharge into rivers due to the more immediate risk posed to public health.

1.1.3 A new strategy

To ensure sustainable development, i.e. to prevent the deterioration of water quality in rivers, estuaries and oceans, a pollution prevention approach to control hazardous pollutants has been adopted, and the end-of-pipe approach has been replaced by a hierarchy approach (Figure 3), which encompasses the following:

- the need to extend an acceptable level of waste management services to all communities and to ensure appropriate treatment of hazardous wastes prior to disposal in an adequately designed waste disposal facility.
- the implementation of cleaner production and/or waste minimisation measures: the former entails the continuous application of an integrated preventive environmental strategy, applied to processes, products and services to increase eco-efficiency and reduce risks to humans and to the environment.

The implementation of such practices at the effluent source, will lead to the generation of a larger number of small streams of high strength and/or toxic liquid effluents that will be treated by biological, chemical or physical methods prior to disposal in the environment.

In anticipation of the move to cleaner production it is necessary to have pro-active strategies to deal with residual concentrates. These concentrated effluents can then be treated through biological, chemical or physical methods to reduce the pollution load in the fresh water.

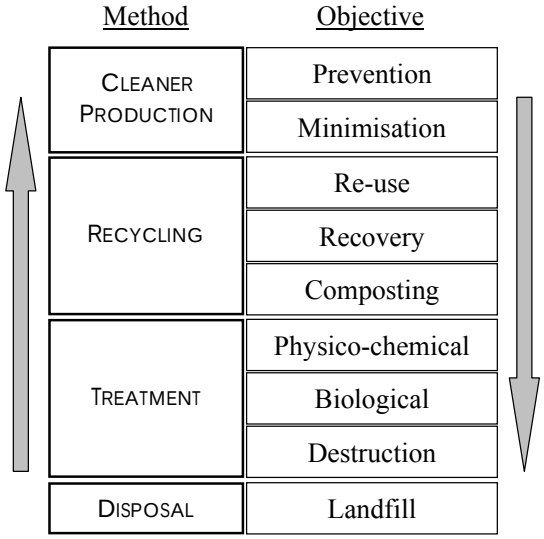

Figure 3 – The waste hierarchy.

1.1.4 A survey in the KwaZulu-Natal province

Several of the wastewater treatment plants in KwaZulu-Natal have anaerobic digestion facilities which are under-utilised i.e. digesters with spare hydraulic and/or organic capacity. A digester with spare hydraulic capacity is one which is receiving a smaller *volumetric load* than its design specifications while spare organic loading capacity implies that a digester can accept a greater *organic load* without experiencing an overload or shock loading.

A survey conducted by Bell (1997) identified several digesters for the treatment of industrial wastewater. For instance, the Amanzimtoti Wastewater Treatment Works had a total of six anaerobic digesters and only three were operational: these off-line digesters could, therefore, be utilised for the treatment of industrial effluents produced in the vicinity. The Amanzimtoti Works is located near to the Prospecton industrial area and approximately 50 % of its volumetric load is composed of industrial effluent. Therefore, the biomass within the anaerobic digester has been acclimatised to a certain extend to industrial wastes. Southern Works is also an ideal candidate for receiving industrial wastewater because it is located in an area containing several industries. Phoenix is currently a residential area but has been targeted for industrial development in the near future.

The study by Bell (1997) also identified plants that contained anaerobic digesters that were operating below design capacity. Anaerobic digesters at treatment plants such as Mpumalanga, Northern and Phoenix were found to have as much as 50 % available capacity. These digesters could receive wastewater from surrounding industries without causing a hydraulic and/or organic overload. Mpumalanga WTP is ideally situated to receive industrial waste from the Hammarsdale industrial area. The digesters at Umbilo and Northern treatment works were found to be healthy and operating at 25 % and 39 % below design capacity, respectively. It should be noted that New Germany no longer operates its anaerobic digesters: the sludge from this plant is now piped to Northern Wastewater Treatment plant for digestion (Fortmann, pers comm, 2001).

This survey demonstrated that several wastewater treatment plants in KwaZulu-Natal had available anaerobic digester capacity, which could be used to treat high strength or toxic industrial wastewater. The industrial wastewater could be transported to the treatment plants by tankers. These transport costs would be similar to cost that industries are paying currently to transport waste to landfills and marine outfalls.

Table 2 – Anaerobic digester availability and capacity information (source: Bell, 1997).

Treatment plant	Fraction of industrial waste (%)	Idle digesters (No.)	Available capacity (%)
Amanzimtoti	50	3	23
Darvil	10	1	8
KwaMashu	10	0	14
Mpophomeni	0	1	0
Mpumalanga	0	0	51
Newcastle	10	3	Decommissioned
New Germany	30	3	Decommissioned
Northern	4	0	39
Phoenix	0	1	56
Scottburgh	0	0	51
Southern	40	2	Decommissioned
Umbilo	20	0	25

1.2 ANAEROBIC BIOTECHNOLOGY FOR INDUSTRIAL WASTEWATER

Anaerobic digestion processes have been used for the treatment of concentrated municipal and industrial wastewaters for over a century (McCarty, 1971). It is a multi-step process of parallel and sequential reactions in which complex substrates are degraded by different micro-organisms within the anaerobic microbial consortia to simple monomers that are finally converted into methane, a fuel that can yield a net energy gain from process operations.

The anaerobic conversion of organic waste to methane (and carbon dioxide) occurs in the absence of oxygen, and consequently requires no direct energy input other than organic substrate to effect the biochemical conversions.

The common alternative to anaerobic biotechnology for treatment of industrial wastewater is the aerobic process. The major factors for comparison are (Lettinga, 1995):

- unlike activated sludge processes which require oxygen, the electrical power usage is low;
- the formation of methane gas, which is a source of energy; and
- excess microbial cell production, which has an associated disposal cost: the growth rates of micro-organisms are relatively slow, resulting in a low production of waste sludge.

Moreover, the digested sludge is a good soil conditioner. However, low growth rates of the microbial populations mean that high biomass retention times are required to achieve practical treatment rates. Rarely is the value of the methane product from a given effluent sufficient to be the sole justification for selecting anaerobic biotechnology: rather, the reduced costs of excess cell disposal or reduced electricity consumption are the contributing factors favouring adoption of anaerobic biotechnology (Speece, 1983).

Because of advances in treatment technology and knowledge of process microbiology, applications for the treatment of industrial wastewaters have become extensive.

There are numerous industrial wastewaters, which may be toxic to the anaerobic biomass due to the presence of *xenobiotics* or recalcitrant molecules. Fortunately, it has been shown that acclimated cultures can be induced to biodegrade highly toxic, or recalcitrant, compounds which had previously impaired metabolism. The time required for *acclimation* depends on the substrate structure, the inoculum source and the environmental conditions (Speece, 1996).

Initially, anaerobic digestion was applied primarily to complex feed stocks, such as municipal sludges, which contained a wide range of nutrients and alkalinity sources. Other candidate feed stocks considered for anaerobic treatment were food-processing wastewater, such as effluent from meat-packing plants and sugar beet operations. It was found that these effluents

contain readily biodegradable organics and that the carriage water has a normal complement of inorganic ions.

Anaerobic digestion has historically not enjoyed a good reputation, because of its poor record with respect to process stability.

1.2.1 Co-digestion

Municipal sludge or generally *labile* substrates can be treated together with industrial effluents, which are characterised by a high organic matter content (thus termed: *high strength* effluents) and/or which contain substances that would be toxic for the microbial consortia if anaerobically digested in isolation or discharged to a wastewater treatment works or the aquatic environment.

This treatment option, in which different types of wastes are treated together and compensate for their respective deficiencies, is termed: *co-digestion*. It allows the disposal of an otherwise difficult effluent (e.g. due to the inhibitory effect of some agents, the lack of some components, etc.), without compromising the performance of the digestion process to an unacceptable extent.

The main advantages of this approach are outlined by Angelidaki and Ahring, (1997):

- it is expected to cost less than the separate treatments;
- it can improve the digestibility of a highly concentrated effluent (e.g. high organic content, or high solid fraction), by dilution with other wastes;
- it can counteract the presence of inhibitors (e.g. ammonia or *xenobiotic* compounds) or the lack of nutrients, by dilution;
- it can also favour the rise of *co-metabolism*, which is often the only way to achieve detoxification of specific organic compounds.

Co-metabolism describes the phenomenon whereby complex organic compounds are partially degraded or completely mineralised by various consortia of micro-organisms. No one organism could utilise these potentially recalcitrant molecules as a sole carbon and energy source and all require a co-substrate for growth. Hence, in a dual substrate ecosystem, co-metabolism is defined as the transformation of a non-growth associated substrate (*secondary* substrate) by micro-organisms which grow on a *primary* substrate; or by resting micro-organisms in the absence of a growth substrate (Criddle, 1993). A growth-associated substrate is capable of providing carbon and energy for microbial growth and maintenance.

Many co-metabolic enzymes and cofactors are induced by the utilisation of the primary substrate: the secondary substrate is biotransformed by these enzymes, but cannot be used by the biomass to support growth.

Several investigations into the potential of co-digestion have been conducted and are summarised in (Section 2.2).

1.2.2 Industrial wastewater

Special industrial wastewater, such as olive mill effluents, in Mediterranean countries (Angelidaki and Ahring, 1997), textile and dye manufacturing effluents in KwaZulu-Natal (Bell, 2002) are often produced in large amounts in concentrated areas. These wastes pose great environmental hazard due to the very high organic COD loads (102 g COD/ℓ) and/or the presence of potentially inhibitory compounds.

As regulations become ever more stringent, the need for more technically and economically efficient means for the disposal of industrial effluents grows more acute.

In isolation, these effluents can be anaerobically biodegraded to some extent: 80 % organic removal was achieved for olive mill effluents (Angelidaki and Ahring, 1997); an overall colour removal ranging from 78 to 98 % is reported by Beydilli et al (1998).

However, at high feed concentrations, the process may prove to be unstable due to inhibition effects of other compounds, such as polyphenols (Angelidaki and Ahring, 1997), anthraquinone dye (dos Santos et al., 2005), dye metabolites (Shaw et al., 2002), salts, etc.; due to the lack of ammonia; or to a low alkalinity. Furthermore, dilution with water results in unnecessary large effluents volumes and addition of chemicals is not economical and environmentally desirable.

Textile effluents

Wastewater originating from the textile industry is a complex mixture of potentially polluting substances consisting of textile dyes, heavy metals associated with dyes and other auxiliaries used during the dyeing process. Increasingly strict environmental legislation has led to textile finishing industries being labelled as high priority industry with respect to pollution (Sacks and Buckley, 1999) and with specific regard to toxicity originated from high salt, recalcitrant COD and heavy metals.

During processing, up to 15 % of the total dye remains unreacted and is lost in the effluent (O'Neill et al., 1999).

Although the dyes contribute little to the biological/chemical oxygen demand of these effluents, as compared to other colourless organic substances, they cause aesthetic

deterioration and impede the penetration of light into natural water bodies; furthermore, some of the dyes, dye precursor and dye degradation products are reported to be carcinogenic and mutagenic in nature (Henderson et al, 1997).

Although different physico-chemical treatments are being used for colour removal from wastewater (e.g. chemical coagulation/flocculation and activated carbon adsorption: Sheng and Ming, 1997; advanced oxidation processes: Georgiou et al., 2002), they concentrate the pollution into solids or liquid side streams, requiring additional treatment or disposal.

Under low redox potential conditions, such as those maintained during microbial anaerobic processes, the azo bond undergoes cleavage, therefore permanently destroying colour (Beydilli et al., 1998).

Size effluents represent the main components of the organic load of the effluents from textile finishing mills. The traditional sizing agent used to be starch which resulted in an effluent that was a high-strength organic wastewater. The sizing agents comprise substances which are predominantly polar in nature. These polar organic pollutants pose problems because they are non biodegradable and their elimination in incomplete: when mixed with the remainder of the mill effluent, they increase the COD of the final effluent.

Sizing recipes can be very diverse. Three families of sizing agents are mostly used: polysaccharides (e.g. starch and cellulose), synthetic vinyl (e.g. PVA) and protein-based sizes (e.g. the derivatives of gelatine and casein).

Due to the high content of readily biodegradable matter, sizing wastewater has been considered for anaerobic digestion process (Vandevivere et al., 1999)

Landfill leachate

Sanitary landfilling is the most common way to eliminate municipal and industrial solid wastes. Besides its economic advantages (Lema et al., 1988), landfilling minimises adverse environmental effects and other risks while allowing waste to decompose under controlled conditions until its eventual transformation into relatively inert, stabilised material. However, little attention has been paid to the collection and treatment of landfill leachates which has now been recognised as a significant problem associated with landfills.

Leachate is formed when water filters and/or percolates through the dumped waste and transports the organic and inorganic products from both physical extraction and hydrolytic and fermentative processes. The mixture which results from this process is characterised by a highly variable content of organic and inorganic substances and micro-organisms which

depends on several factors, including the hydrogeology and climate of the site, the type of residues accumulated and the type of pre-treatment that had been undertaken, the engineering of the landfill site, particularly in terms of isolation from the ground, diversion of the incoming water and collection of the leachate produced. The characteristics of a leachate greatly vary with the age of the landfill, i.e. with the degree of stabilisation of the waste: as a landfill ages, the biodegradable fraction of the leachate will become smaller thus the leachate will be more recalcitrant.

Analysis of the organic fraction of leachates shows that volatile fatty acids contribute the majority for the high COD levels. Other organic fractions include proteins, carbohydrates and hydroxylated aromatics. The presence of these aromatic compounds has been found to be possible source of toxicity and inhibition when biological treatment is applied (Field and Lettinga, 1987).

Strategies for the treatment of leachates are hindered by their great diversity, which results in techniques successfully developed for one site not necessarily being applicable elsewhere.

Various ways, e.g. physical and chemical methods, are being used to treat landfill leachate and regarded as separation processes. The disadvantage with these methods is that they are expensive whereas biological treatment methods are cost-effective and have been proven to reduce pollutants in landfill leachate (Reinhart and Pohland, 1991).

An argument in favour of a combined treatment of leachates and domestic sewage is that since the former contains an excess of nitrogen and the latter an excess of phosphorous, neither of these nutrients need to be supplied at the treatment plant (Lema et al., 1988).

1.3 PROJECT OUTLINE

Problem formulation

- The sustainable consumption and production tools (SCP) which have been implemented result in concentrated effluent being discharged, which are characterised by a high organic content and by the presence of toxic compounds.
- The province of KwaZulu-Natal attracts water-demanding industry.
- In KwaZulu-Natal, several anaerobic digesters at wastewater works are presently underutilised and would therefore be the ideal candidates to receive concentrated industrial effluents.

Aims of the project

- ✓ To investigate the feasibility of the co-digestion of toxic and/or high organic strength effluents with municipal effluent in existing digesting units, at wastewater treatment works.
- ✓ To establish a protocol whereby an industrial effluent can be tested prior to be co-digested and which is capable of indicating to what extent the effluent (termed: *test material*) would affect the overall performance of the process.

The ultimate objective of this project was to provide an alternative treatment system for high-strength liquid effluents that are currently being disposed to landfill by establishing whether an industrial effluent of inherently difficult characteristics can be combined with a municipal effluent (or sludge) and be anaerobically treated, with no, or not significant, worsening of the overall performance of the treatment process.

A detailed overview of how this Report is structured in accordance to the general objective of the Project is presented in Figure 4.

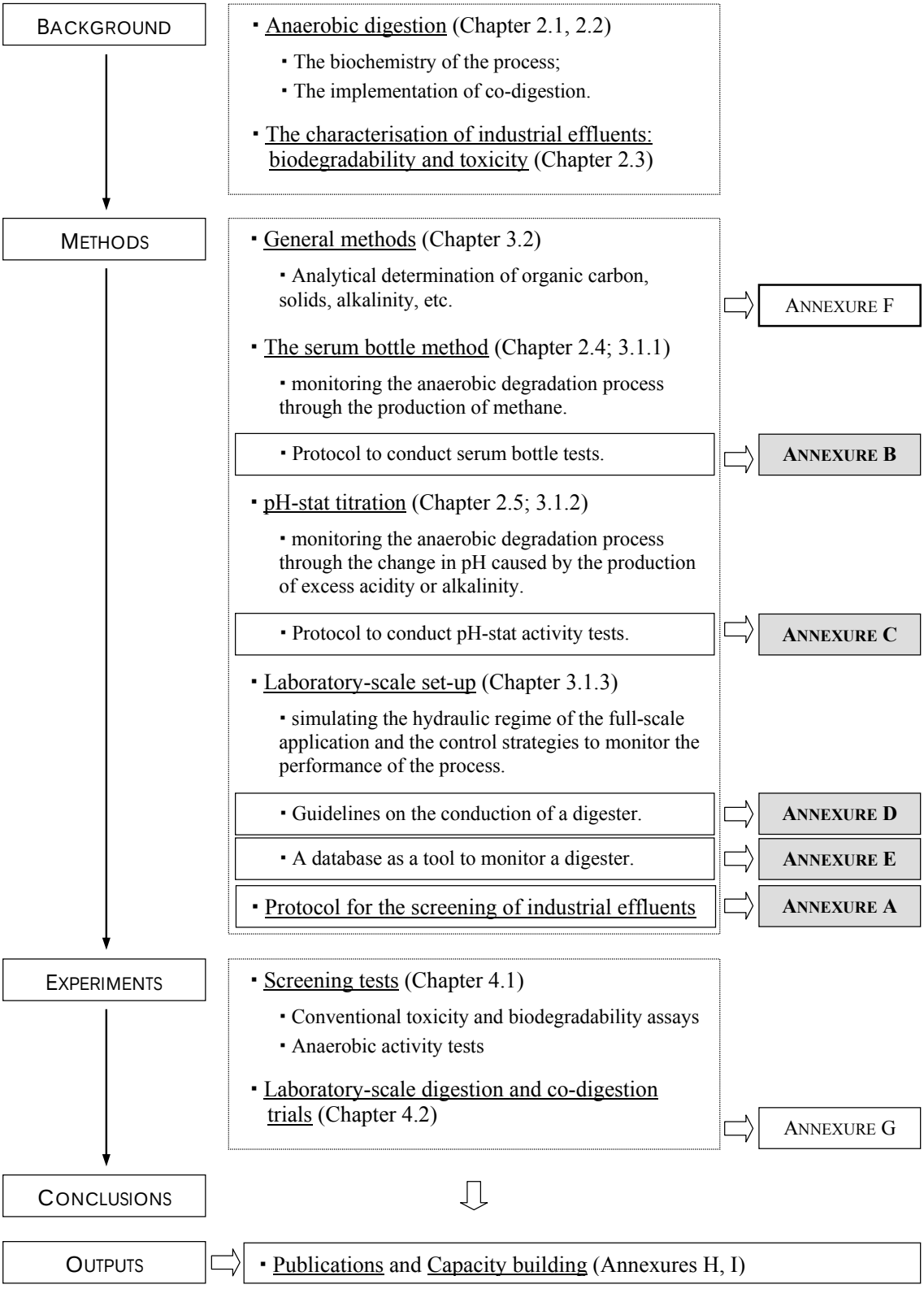

Figure 4 – Synopsis of this report.
The four main sections (the boxes on the left) are detailed in the centre of the diagram. The outputs (at the bottom and in the framed boxes on the right) are in the form of self-containing Annexures. The dark background indicates the deliverables of the project.

CHAPTER 2

LITERATURE SURVEY

This chapter gathers a general body of information which constitutes the background of the Project. The process of anaerobic digestion is outlined in Section 2.1. Several successful experiences on co-digestion are reported in literature and are briefly summarised in Section 2.2. The concepts of biodegradability, activity and toxicity (or inhibition) are crucial when assessing the characteristics of an effluent (Section 2.3). A relatively quick and inexpensive method to screen an effluent makes use of small, sealed vessels, maintained in batch conditions (Section 2.4). An alternative approach that is proposed in this project is the use of a pH-stat titration device to assist in the screening of industrial effluents prior to co-digestion (Section 2.5).

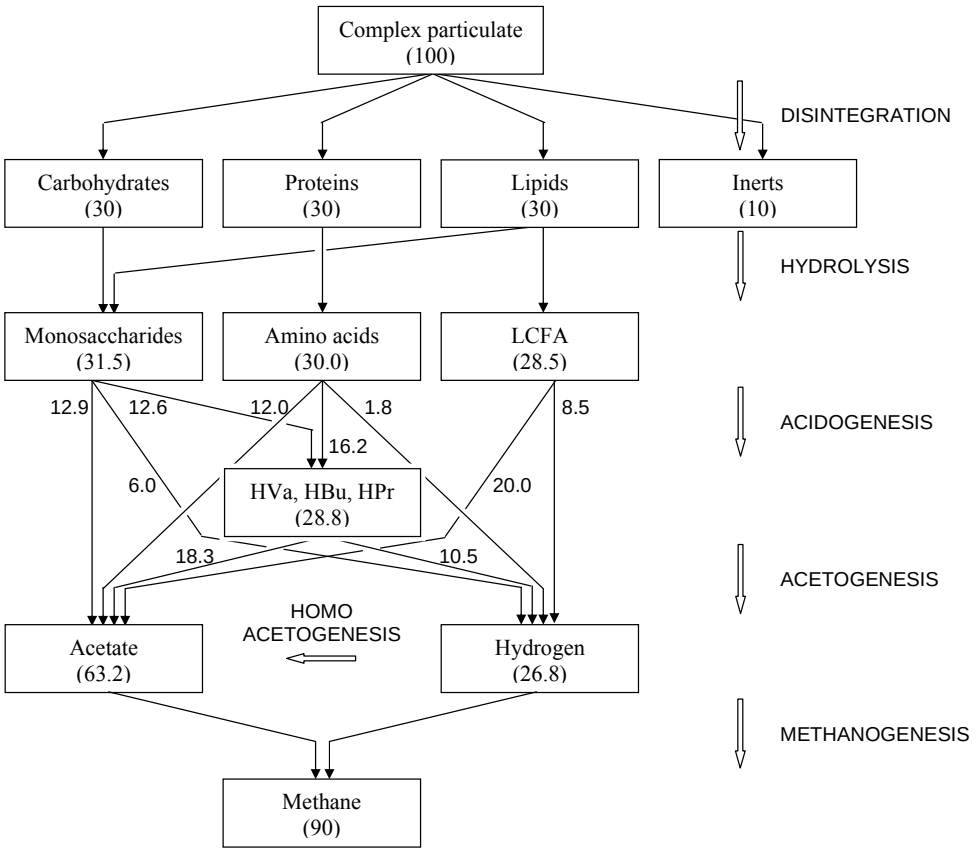

Figure 5 – Flow-diagram for the anaerobic degradation of a composite particulate material. Valerate (HVa), Butyrate (HBu) and Propionate (HPr) are grouped for simplicity. Figures indicate COD fractions (from Batstone et al 2002*).*

2.1 THE ANAEROBIC DIGESTION PROCESS

Anaerobic digestion is commonly visualised as a biochemical chain reaction (Figure 5), where the product of one process is the reactant of the next; however, in reality, it is a complex system of microbiologically mediated and physico-chemical sub-processes, with feedback loops of substrate and product regulation and inhibition. In fact, there is a relatively narrow range of environmental or operating conditions in which all the sub-processes of the digestion can function simultaneously.

2.1.1 Biochemistry and microbiology

Biochemical processes may be subdivided into extracellular processes, effected by enzymes (e.g. disintegration and hydrolysis) and intracellular processes.

- Disintegration and hydrolysis: Complex materials such as large molecular weight proteins, carbohydrates and lipids are hydrolysed extracellularly by enzymes excreted by hydrolytic bacteria, with some release of energy. This process can be divided into the *disintegration* of composite particulate material into smaller carbohydrate, protein and lipid fractions and the *hydrolysis* of large molecular weight compounds to *long chain fatty acids* and the monomers of sugar and protein compounds; to simple sugars; and to *amino acids*.
- Anaerobic oxidation: *Long chain fatty acids* are oxidised to simple organic acids in a process termed *anaerobic oxidation*, in which the carbon chain is sequentially shortened by two carbon atoms at a time. The final product of fatty acids with an even number of carbon atoms is *acetate* only; when the fatty acid has an odd number of carbon atoms, one mole of *propionate* is produced per mole of substrate. Relatively large amounts of dissolved hydrogen are released in this process.
- Acidogenesis: *Amino acids* and *simple sugars* are fermented by the next category of bacteria, termed *acidogenic* or "acid formers" that produce simple organic acids such as acetic, propionic, butyric and lactic acids. The end product of acidogenesis is determined by the environmental conditions (Mosey, 1983). Different amounts of di-hydrogen are produced depending on the acidogenesis end product.
- Acetogenesis: A further category of bacteria (*acetogenic*) ferment *propionic, butyric and lactic acids* to *acetic acid*. In most cases, each group of acetogens can only ferment one type of organic acid.

- Aceticlastic methanogenesis: The final stage in anaerobic digestion is the conversion of *acetic acid* to *methane* and *carbon dioxide* by a group of *Archaea* known as *aceticlastic methanogens*. The conversion to methane is the only strictly anaerobic step which results in the removal of chemical oxygen demand from the liquid to the gas phase.
- Hydrogenotrophic methanogenesis: *Hydrogenotrophic methanogenesis* is the production of methane from *dissolved hydrogen* and *carbon dioxide* by a select group of slow-growing methanogens. This process can account for up to 30 % of the methane produced by anaerobic digestion of an organic waste.
- Homoacetogenesis: *Homoacetogenesis* is the generation of acetic acid from *dissolved hydrogen* and *carbon dioxide*. Homoacetogens are one of the most versatile physiological groups among the anaerobic bacteria. They utilise and transform one-carbon compounds and can carry out incomplete oxidation of reduced fermentation products released by other fermenting bacteria. Homoacetogens can use various substrates sequentially or simultaneously and may constitute an energy link from hydrogen, via acetate to heterotrophic methanogens.
- Sulphate reduction: The presence of any sulphate in the waste will result in *sulphidogenesis*, the generation of sulphide from sulphate. As with methanogenesis, this process can be either aceticlastic or hydrogenotrophic. Organisms reducing sulphur can obtain the electrons directly by oxidising organic acids, or by oxidising the hydrogen produced by acetogens. Additionally, organic acids are used as a carbon source, and as a result, organisms reducing sulphur compete with the majority of groups in anaerobic digestion (Kalyuzhnyi and Fedorovich, 1998). Further complicating the effect on anaerobic systems, the reduced product, sulphide, is inhibitory to different extent for almost all microbial groups. Reduced sulphide has a similar acid-base system to the inorganic carbon system. Hydrogen sulphide is also a gas phase component, with a relatively high solubility.
- Denitrification: Dissimilatory nitrate reduction, a form of anaerobic respiration, is the reduction of nitrate (NO_3^-) to nitrogen oxides, such as NO_2^-, NO, N_2O, and N_2. Denitrification has a higher yield per unit of substrate consumed than methanogenesis and competes for the same substrate. Denitrification intermediates have also been found to inhibit methanogenesis. This results in a decrease in methane production and an increase in alkalinity. In an overall methanogenic system, nitrate reduction can have

significant impact on both the carbon and electron flow, on microbial competition and inhibition, and on gas composition.

2.1.2 Physico-chemical processes

The physicochemical system can be defined as non-biologically mediated processes that commonly occur in anaerobic reactors. There are three broad types, listed below:

- Liquid-liquid processes: this mainly refers to ion association and dissociation with hydrogen and hydroxide ions. There are a number of important compounds, which have dissociation constants close to the operating pH of anaerobic systems.
- Gas-liquid processes, i.e. gas liquid transfer of carbon dioxide, methane, hydrogen, hydrogen sulphide and nitrogen gases.
- Liquid-solid processes, i.e. precipitation/solubilisation of ions.

Important physico-chemical subsystems include inorganic carbon, organic acids dissociation, sulphate, sulphite, sulphide, ammonia/ammonium, oxidised nitrogen, phosphate, and the gas liquid interaction hydrogen and methane gases.

2.1.3 Thermodynamics and self-regulation

From a thermodynamic point of view, the energy change per unit (mole) of substrate fermented in anaerobic digestion is very small; moreover, many organic compounds are fermented to methane in a step-wise process by different groups of micro-organisms, therefore the low energy yield from the overall conversion must be divided into even smaller packets. The extent to which bacterial growth occurs is a function of the energy released by the electron transfer and the efficiency of the energy utilisation by the micro-organism mediating the transfer. The low amount of energy released in anaerobic transformations results in a low growth yield for all types of anaerobic micro-organism, although the individual growth yields for each type of micro-organism depend on the energy release by the reaction that they mediate and the conditions under which they operate. McCarty (1971) uses a thermodynamic approach to understand which of the many possible reactions are likely. The dominant micro-organisms in a consortium will be those that can harvest the available energy in any substrate most rapidly and efficiently in a given environment. These species will outcompete other micro-organisms that utilise the same substrate.

This has consequences for process stability since upsets in environmental conditions can reduce the Gibbs free energy of a certain sub-process to the point where it is no longer thermodynamically feasible, or only at dramatically reduced rates. This in turn interrupts the

supply of substrate and therefore energy to all subsequent processes that occur later in the metabolic chain. For example, the harmony between propionate oxidation, acetate decarboxylation and hydrogen oxidation is critical for a stable anaerobic digestion process. The optimal range for all three reactions (i.e., they must all be exergonic) is very narrow, and is mainly controlled by free propionate, hydrogen and acetate concentrations. (Gujer and Zehnder, 1983).

The peculiarity of the anaerobic digestion process is that most of the control is undertaken directly by the micro-organisms themselves: i.e., pH regulation by the formation and removal of short chain fatty acids and regulation of redox potential by formation and removal of trace concentrations of dissolved hydrogen (Mosey, 1983). Andrews (1969) suggested that instability and failure of such an ecosystem might be caused by substrate inhibition: high concentrations of volatile fatty acid produced by the fast growing acidogenic bacteria inhibit the metabolism of the slowly growing methanogens. In fact, the specifics of pH and hydrogen regulation have been shown to be very complicated, and are not fully understood: product and substrate inhibition of several of the anaerobic sub-processes have been observed, resulting in feedback loops which assist in maintaining pH and redox conditions in ranges which allow anaerobic digestion to proceed. It is however possible to increase the range of operating conditions and stability to a certain extent by a physical separation of acidogenic and methanogenic bacteria in serial reactors since they are sufficiently different with respect to their physiology and nutritional requirements (Van den Heuvel and Zoetemeyer, 1982).

2.2 CO-DIGESTION

The term co-digestion indicates the anaerobic digestion of two effluents, whereby a readily biodegradable stream is mixed with a more recalcitrant one in order to enhance the digestibility of the latter.

Some agricultural products processing industries generate wastewaters which can vary greatly in quantity and characteristics throughout the year, but are generally characterised by a high organic content. The seasonal nature of these effluents makes the application of individual treatment units not feasible, due to the long start-up periods required by anaerobic digestion; not to mention that it can be prohibitively expensive for small-scale industries.

For these reasons, effluents generated from various sources, either a single multiproduct processing company or multiple specialised small-size companies distributed in an area, are ideally suited for centralised co-digestion treatment works.

Olive mill or dairy wastewater are typical seasonal effluents, in the Mediterranean area and contain high concentrations of lipids and carbohydrates respectively.

An economically viable option is reported by Gavala et al. (1996) and consists in co treating these effluents with piggery effluent, which is continually produced and is rich in proteins. The individual streams all have a high organic content (i.e. 44, 105 and 60 g COD/ℓ, piggery, olive mill and dairy wastewater respectively) and in combination give their own contribution to the mixture in terms of lipids, carbohydrates and proteins content. The Authors adapted three separate batches of anaerobic sludge to the three effluents, for a period of one year. They subsequently used piggery effluent adapted sludge as seed inoculum for three batch co-digestion experiments, in which the olive mill and dairy effluents were digested with the piggery effluent. The results were used to estimate the kinetic parameters of a dynamic mathematical model describing the co-digestion process.

Farm animal manure is also becoming a major environmental issue for developed and developing countries. Huge amounts are produced in limited areas which then require urgent treatment and disposal solutions, because ammonia and greenhouse gases may cause air pollution and the improper application of nitrogen and phosphorous to land may result in eutrophication of surface water bodies and pollution of soils and groundwater.

On a farm site, the biogas produced by anaerobic digestion of animal waste can be used as fuel for boilers, in replacement of natural gas: it can be burned in generators to produce electricity for on-farm use. The residual sludge is stabilised, as soil fertiliser.

Increasing cost of landfilling and energy taxes on fossil fuels encourage the exploitation of renewable energy sources, thus making anaerobic digestion a highly competitive alternative for the treatment of animal manure (Salminen and Rintala, 2002).

Angelidaki and Ahring (1997) also studied the co-digestion of olive mill effluent (high concentrations of lipids, low concentrations of alkalinity and ammonia, and presence of polyphenols) with cattle manure, household waste or sewage sludge. The Authors conducted a preliminary screening to ascertain the feasibility and performance of the co-digestion, using a batch experiment in which the olive mill effluent with one of the readily biodegradable effluents was tested separately in a serum bottle test, at different dilutions of olive mill effluent with water. Subsequently, the manure and the household effluents were selected as preferential co-substrates for a laboratory-scale investigation. Conventional completely stirred anaerobic digesters were maintained at a hydraulic retention time of 13 d and at a temperature of 55 °C. During the start-up phase, which lasted two months, the labile effluents only were fed to the digesters; thereafter, the olive mill effluent was added to the feed and different dilutions tested. The utilisation degree (calculated as in equation B.5) of the olive mill effluent ranged from 55 to 75 %.

This study demonstrated that the high buffering capacity contained in the manure together with the content of several nutrients, makes possible to degrade an effluent which is lacking these compounds, without previous dilution –which would result in large effluent volumes– nor addition of chemicals, e.g. alkalinity or a nitrogen source –which is generally not economical nor environmentally desirable–.

Güngor-Demirci and Demirer (2004) studied the co-digestion of cattle (53 g COD/ℓ) and broiler manure (12 g COD/ℓ) in batch reactors and specifically the influence of various conditions on the process performance, i.e. initial organic and solid content, digestion temperature and degree of acclimation of the seed inoculum. The batch reactor type was chosen because in agriculture, complex process configurations may result in technical and operational problems.

A study by Lin et al. (1998) reported a case of co-digestion of septage and landfill leachate. Septage and leachate were mixed at different ratios: as increasing fractions of septage were used, a marked improvement in the removal efficiencies of total COD was observed.

Co-digestion is frequently used for the co-disposal of urban solid wastes too.

Peres et al. (1992) carried out a study aimed at treating primary sewage sludge and municipal solid waste. Results showed a total solids destruction of 54 to 57 % and a volatile solids destruction of 58 %.

Purcell and Stentiford (2000) investigated the co-digestion of biodegradable supermarket wastes and sewage sludge. Experiments were conducted in completely stirred reactors, operated at 35 °C. A total solids destruction of 28 % was reported for the control cells, while the co-digestion cells achieved significantly higher destruction of 54 %. The destruction of volatile solids in the co-digestion unit was again much higher than the control units, i.e. 63 % and 37 % respectively.

The advantages of co-digestion are that *i*) it is generally less expensive than the separate treatments; *ii*) due to the dilution effect, it can improve the digestibility of a highly concentrated effluent as well as it can counteract the presence of inhibitors or the lack of nutrients; and *iii*) it can also favour the rise of co-metabolism, which is often the only way to achieve detoxification of specific organic compounds (Angelidaki and Ahring, 1997).

2.3 CHARACTERISATION OF EFFLUENTS: BIODEGRADABILITY, ACTIVITY, INHIBITION

Numerous methods have been developed over the past 30 years, since Van den Berg et al. (1974) illustrated a *manometric* method based on the measurement of the gas production to assess methanogenic activity.

Methanogenesis is often the rate limiting step of the entire process and since the quantification of gas flow rate is relatively easy to perform, most of the methods reported in literature monitor the **production of biogas**. These methods are referred to as *volumetric* or *manometric* methods, as the volume of biogas produced or the pressure increase due to gas production inside a close vessel are assessed, respectively. However, this same concept can be employed to assess activity or inhibition of individual metabolic steps preceding the methanogenic one, providing that they are rate limiting for the whole process.

Other direct or indirect methods, targeting physico-chemical or microbiological parameter exist and have been investigated by various authors.

- **Biodegradability** indicates the inherent property of a test material, which depends primarily on its molecular structure and refers to its susceptibility to undergoing a biologically mediated degradation. The extent of such degradation can be further referred to as (Battersby, 2000):
  - *ultimate*, if the organic material is converted into inorganic by-products (associated with the specific metabolic process) that cannot be further biologically degraded; or
  - *primary*, if the chemical structure of the parent compound is altered to an extent that results in the loss of a specific property, forming products that may also be biodegradable.

  An organic material can also be referred to as *inherently biodegradable* when it is potentially biodegradable, if and only if specific actions are taken, such as pre-exposure of the inoculum to the substrate, increased test duration and/or higher micro-organism-to-substrate ratios.

  Biodegradability is generally expressed as the mass of the test substance converted within a given period of time, as compared to the theoretical mass that could be stoichiometrically converted to methane, i.e. based on the measured (or calculated) chemical oxygen demand of the test material (equation B.1).
- **Activity** indicates the inherent ability of a microbial group to undertake the degradation of the test material. It is measured as the specific rate of substrate consumption or product generation (equation B.6), referred to either the total biomass or the targeted microbial

population. Bioassays have been established since the 1970s, that employ serum bottles and measure the pressure increase or the volume of liquid displaced by gas production and/or analyse the composition of the gaseous phase. Other more refined methods have recently been developed.

Activity can be assessed under non-limiting or limiting substrate concentration. The two approaches are equivalent if anabolism (i.e.: growth) and catabolism (i.e.: energy generation) are assumed to be coupled by a proportionality factor, as occurs in steady-state conditions, but the behaviour of the microbial culture in transient conditions may be more complicated. Biomass growth can influence the assessment, to an extent that also depends on the designated method of analysis: it generally induces a significant increase in the (absolute) rate of substrate consumption (relative to the steady-state value), that can be revealed by plotting the logarithm of the specific activity vs. time (Coates et al., 1996; Figure C.7).

- **Inhibition** indicates a detrimental effect that the test material exerts on the activity of a microbial population. The use of the terms *inhibition* and *toxicity* may generate some confusion; however, Speece (1996) remarks that inhibition denotes an impairment of the bacterial functions, that is generally irreversible; whereas, toxicity is a temporary, non-lethal adverse effect on bacterial metabolism.

  Inhibition is assessed by comparison to a reference baseline activity, measured under optimal conditions, e.g. non-limiting substrate concentration; therefore, it is generally expressed as percentage reduction of the specific activity (equation B.3).

  Two *forms* of inhibition can be distinguished, i.e.: short- and long-term, based upon the exposure time: for the purpose of assessing the fate of organic substances in the environment, the short-term inhibition is generally to be reported, although this does not necessarily imply that the compounds cannot be biodegraded, under appropriate conditions.

  The effect of exposure time on inhibition of methanogenesis has been investigated by many authors (Yang and Speece, 1986; Campos and Chernicharo, 1991).

  The experimental techniques for assessing microbial inhibition do not differ in principle from the ones designed to measure microbial activity, although the test conditions may be different. If a batch system is used, the base activity and the inhibitory effect are separately assessed: in a first run, activity is measured at a given concentration of the reference substrate; subsequently, the same concentration of substrate is applied to a

number of vials, that are each subjected to different concentrations of the inhibitory substance and the reduced activity is measured. If a continuous system is used, the culture is fed with the reference substrate at a constant load, until the gas production (or another performance indicator) is stable; thereafter the inhibitory substance is spiked to the reactor or added to the feed.

Although the term respiration strictly applies to a sequence of metabolic steps whereby aerobic or anoxic micro-organisms obtain energy and carbon using oxygen as the terminal electron acceptor, the term respirometer is widely used in anaerobic applications to designate a device whereby biodegradability or inhibition are assessed through the measurement of the biogas production. In the particular case of hydrogenotrophic methanogenesis, the consumption of hydrogen and carbon dioxide can be measured.

In a respirometer for anaerobic assays, gas production (or consumption) can be quantified either as volume, at constant (atmospheric) pressure or as pressure, at constant volume. Both *volumetric* and *manometric* techniques may use manual or automated devices. Early applications are reported by Valcke and Verstraete (1983) and by Shelton and Tiedje (1984), respectively.

2.3.1 Volumetric methods

In a volumetric respirometer, the volume of biogas produced is displaced into an external container for measurement, prior to discharge. This may be a lubricated syringe in which the piston expands to balance the overpressure generated inside the reactor to the atmospheric pressure. The needle of the syringe is inserted into the cap of the reactor (Owen et al., 1979) or else the syringe can be used as reactor itself (Cohen, 1992). The same two set-ups can be automated.

In a different arrangement, the biogas produced inside the reactor moves into a suitable external vessel, containing a barrier solution and displaces an equivalent volume of liquid, which can be manually or automatically measured. A Mariotte flask or a *eudiometer* (ISO 11734, 1995) are widely used for the manual measurement. The former consists of a closed vessel with an inlet tube inserted deep into the barrier solution and an outlet tube at the same level: this ensures that a constant head is maintained throughout the duration of the test, and therefore a constant discharge per unit volume is produced. The latter consists of a metered gas collecting pipe, mounted on top of the reactor and of a reservoir tank; biogas production pushes the barrier solution towards the reservoir tank thus altering the two levels. After the reading, the reservoir tank is lowered to reset the levels, i.e. to bring the

overpressure back to the atmospheric value. A conceptually similar device is described by Valcke and Verstraete (1983) and makes use of a reversed cylinder or burette (Figure C.3).

An automated device for the measurement of the volume displaced typically consists of a small column containing the barrier solution; a sensor for the detection of the level and a three-way solenoid valve: the barrier solution is pushed inside the column until it reaches a pre-set level, where the sensor actuates the valve that discharges the excess biogas and resets the system.

Some automatic volumetric methods may be considered as mixed volumetric/manometric systems where the pressure is allowed to increase to a pre-set level until a valve is opened which allows the gas to be discharged.

Since methane is slightly soluble in water whereas a considerable fraction of carbon dioxide can dissolve (Stumm and Morgan, 1996), it is preferable that the latter is confined either in the gaseous phase (by using an acidic or saline barrier solution) or in the liquid phase (by using an alkaline barrier solution): correspondingly, the volume displaced will account for a mixture of the two components (in a definite ratio) or for methane only. It is more convenient to use an alkaline solution which enables to directly measure the sole methane fraction, as this is directly related to the COD conversion and thus makes mass balances easier to be carried out. A possible drawback arises when the amount of sludge tested is relatively small and/or exhibits low specific methanogenic activity, and when the gaseous composition in the headspace of the vessel changes during the test (James et al., 1990).

2.3.2 Manometric methods

In a manometric respirometer, the biogas produced is confined into the bioreactor and hence generates a proportional overpressure.

An early manometric respirometer for anaerobic applications was derived from the Warburg respirometer (Umbreit et al., 1964), which makes use of a differential manometer.

The measurement of the pressure build-up can be automated by using a pressure transducer permanently fitted to the bioreactor (Concannon et al., 1988).

When multiple bioreactors are run simultaneously, a more viable option consists in using a single very sensitive transducer and a rotating unit with multiple inlets, which sequentially connects each vessel to the transducer (Cohen, 1992). The results obtained on a similar device were validated against the conventional gas-chromatographic measurement of the biogas composition (Angelidaki et al., 1998).

The output of a pressure transducer can be extremely accurate but the procedure requires particular care: the pressure increase, the temperature and the gas-to-liquid volume ratio can have a strong influence on the assessment.

The main drawback of dealing with a pressure increase in a closed system is that the solubility of the gas components changes, depending on the particular physico-chemical properties; this in particular affects the carbon dioxide in solution, which in turn affects the pH.

James and co-workers (1990) indicate that for a biogas mixture CH_4:CO_2 of 3:1, errors can be as high as 1 % and 30 % for methane and carbon dioxide respectively (up to 100 % for the latter, if bicarbonate is taken into account). Angelidaki et al (1998) report that under poor mixing and low biogas production conditions, super saturation of the liquid with carbon dioxide is likely to occur.

To avoid large increase of the pressure, the gas accumulated inside the vessel should be regularly vented.

Variations in temperature create background fluctuations that reduce the sensitivity of a pressure transducer (Sufl Oelofse, SHH, Viljoen, P, Taljaard, S and Botes, WAM (2004) ita and Concannon, 1995).

The inaccuracy of the assessment that would result from disregarding dissolved carbon dioxide can be counteracted by comparing the initial dissolved inorganic carbon (DIC) concentration to the final one (ISO 11734, 1995): this allows a more accurate mass balance to be performed. It is also recommended that the sludge be washed prior to use, in order to lower its initial DIC, with a carbonate-free solution and flush it using pure nitrogen.

2.4 THE SERUM BOTTLE METHOD

A serum bottle assay is a small-scale batch test, whereby an aliquot of anaerobic sludge is placed into a gas-tight glass vial (100-250 mℓ), is supplemented with an organic substrate and is diluted (if necessary) with a solution containing nutrients and minerals, to support the metabolism of the microbes. The vial is then sealed with a rubber septum and an aluminium crimp and stored in suitable conditions (e.g. controlled temperature, in the dark) for a given period of time and regularly monitored by the operator. The biogas produced by microbial activity accumulates in the headspace and causes the build-up of the pressure inside the vial and must be regularly released, by puncturing the rubber cap with a glass lubricated syringe and allowing the plunger to move freely and re-equilibrate to atmospheric pressure. The excess gas is generally wasted.

It is a very simple laboratory technique, which does not require sophisticated devices (other than a gas-chromatograph); it is inexpensive and relatively immediate; moreover, a large number of replicates at different conditions (e.g. concentrations of the test substance) can be carried out simultaneously, thus providing comprehensive and sufficiently reproducible results.

Owen and co-workers (1979) modified a conventional technique for growing anaerobic cultures and introduced the use of serum bottles to conduct Biological Methane Potential (BMP) and Anaerobic Toxicity (ATA) assays.

In a biodegradability assay, the anaerobic sludge is exposed to a given substance (termed, the *test material*) as the sole organic substrate (unless a co-substrate is required). The aim of the assay is to determine the fraction of the test material that the biomass is able to convert to methane: this is expressed as the ratio between the ultimate volume of methane produced and the amount of organic matter present at the beginning. This ratio is termed Biological Methane Potential (BMP), as it indicates the fraction of the total organic matter which is *biologically* degradable. The test is carried out until the gas production is negligible, hence the curve of the cumulative gas volume levels off.

In a toxicity (or inhibition) assay, the anaerobic sludge is exposed to a readily degradable organic substrate and to a given substance. The objective of the assay is to assess the effect of the test material on the 'efficiency' of degradation of the substrate: this is expressed as the ratio between the *undisturbed* activity of the sludge, i.e. exposed to the sole substrate and the activity of the sludge in presence of the test material. The test is carried out for a period of time sufficient to obtain a reliable estimate for the biomass activity, i.e. the slope of the

cumulative gas (or methane) volume. Since under general conditions, the methanogenic step is the most vulnerable to toxicant, acetate is mostly used as substrate and aceticlastic activity measured.

The serum bottle method (and more generally, any manometric technique including more sophisticated devices, such as pressure transducers) is widely employed as a screening tool to measure biodegradability and toxicity of various substances. Healy and Young (1979) used serum bottles to simulate strict anaerobic environment, in which they developed microbial cultures capable of degrading several aromatic lignin derivatives. Conventional activity and toxicity tests are used by Sponza (2003) to study the treatability of carbon tetrachloride and tetrachloroethylene, two halogenated compounds which are widely used in industry and agriculture: in this work, the serum bottle technique is used to quantify activity, toxicity and acclimation of the biomass to the chemicals. Aceticlastic and hydrogenotrophic activity tests are used by Hollingsworth et al. (2005) to study the susceptibility to anaerobic biodegradation of effluents of the metal industry containing copper and other chemicals.

2.5 THE pH-STAT TITRATION METHOD

A pH-stat titrimetric sensor is an instrument whereby the in change in pH originated by a physico-chemical or biological process within a suspension is compensated by the controlled addition of an appropriate solution which neutralises the *excess* acidity or alkalinity produced or consumed.

Unlike a simple pH controller, such instrument enables to measure the reaction rate as well as the amount of reactant converted through the rate of titration and the amount of titrant dosed respectively (Figure 7). In principle, this technique is applicable to any reaction affecting the protons concentration, i.e. which converts neutral reactants into acidic or alkaline products, or which consumes acidic or alkaline reactants to make neutral products.

A comprehensive discussion of these concepts can be found in Ficara et al. (2003).

Several processes of interest in environmental engineering are known which have an effect on the pH of a biological suspension: nitrification and denitrification produce and consume protons respectively; the heterotrophic degradation of organic substrates produces carbon dioxide; aceticlastic methanogenesis produces bicarbonate; the growth of chemolitotrophic organisms consumes carbon dioxide; the anaerobic oxidation of ammonia consumes carbon dioxide and generates bicarbonate.

There has been a long debate on the specialised literature regarding the most appropriate definition of a pH-stat titrimetric *sensor, applied to biological processes*: some authors (Rogers and Gerlach, 1996) maintain that the term ***bio*sensor** should be used, as the instrument is applied to the monitoring of a biological process and detects a biological effect; others suggest that ***bio*assay** is more rigorous, as the term biosensor is generally reserved to devices which produce a quantifiable response based on the action or reaction of a biological or biomimic element (e.g. enzymes, proteins, whole cells, etc.), which is integrated or located immediately adjacent to a physical/chemical (e.g. electrochemical, optical, etc.) transducer detection system. According to Rogers and Gerlach (1996), biosensors are centrally located in a continuum from analytical technologies to bio-analytical assays; what differentiates a biosensor from a bioassay is, thus, the intimate contact between the biological material and the physico-chemical transducer.

We believe that the general principle underlying the specific set-up should be the focus, which is a titration method applied to maintain the pH of the solution constant; therefore the specific

process to which such *sensor* is applied becomes of secondary importance, i.e. should it be a pH-stat *bio*- or a pH-stat *chemo*-sensor.
For this reason, we prefer the term **pH-stat titration sensor**.

A pH-stat titrimetric method was originally designed by Lee and co-workers (1987) and further improved by Ramadori et al. (1980): on this latter concept, a research team at Politecnico di Milano (Italy) patented a sensor which consisted in a thermostated reaction vessel, that accommodates a pH and a temperature probe and a titration unit, comprising the storage vessels and the dosing system for the titrating solutions. The titration unit is interfaced through an Input/Output module to a computer for data logging and processing.

2.5.1 General layout of the instrument

The titration unit consists of two independent lines for the acidic and alkaline solution respectively; each line is coupled to a three-way micro valve which is actuated by the control software. Mariotte flasks are used as reservoirs for the titrating solutions, because they can maintain a constant head above the micro valves throughout the test (Figure C.2), thus ensuring that the flow rate delivered by the micro valves is only a function of the opening time. Since the opening time is set by the software, the volume of titrant delivered per opening is constant, except for variations of the temperature, which affect the viscosity of the fluid: the temperature of the titrants is monitored through a probe (independent from the one monitoring the temperature in the reaction vessel) and a correction function is implemented in the control software.

2.5.2 The first application to the nitrification process

The first pH-stat titration biosensor (Ramadori et al., 1980) was developed to evaluate the activity of the micro-organisms responsible of the first step of nitrification:

$$NH_4^+ + 1.5O_2 \rightarrow 2H^+ + NO_2^- + H_2O \qquad (1)$$

The activity of ammonia oxidisers was therefore measured as the production of acidity (protons) rather than as oxygen or ammonia depletion, as commonly proposed in literature.
This concept was later improved to measure ammonia concentration at the same time as nitrification activity and patented under the name of ANITA, Ammonia and NITrification Analyser) (Massone et al., 1998).
The method proved to be simple and quick; no analytical determinations are involved.

Depending on the initial concentration of substrate and on the nitrification capacity of the activated sludge sample, an activity test would last between 30 and 60 min.

The same set-up was then tested to measure the nitrifiable nitrogen in wastewater (Yuan and Bogaert, 2001), similarly to the Biological Oxygen Demand (BOD) assay for the determination of the organic carbon in a wastewater; and further to determine conversion rates and substrate depletion by other acidifying micro-organisms, such as phenol oxidisers, which produce carboxylic acids as metabolites (Rozzi et al., 1997 a).

An obvious extension of the application to the nitrification process was the measurement of inhibiting effects on nitrifiers, either to check the potential toxicity of the influent to a plant, or to carry out eco-toxicological assessments on chemicals to be released into the environment.

The test is performed by dosing ammonium at non-limiting concentration and measuring the nitrification rate that will be regarded as *baseline* activity. Thereafter, a potentially inhibitory compound is spiked (several additions can be done, in order to progressively reach the desired final concentration) and the reaction rate after each addition is compared to the baseline value and plotted against the corresponding concentration of the inhibitor substance. This 'dose-response' plot enables the estimation of the EC_{50} concentration for the tested toxic substance. The procedure is very quick compared to the standard ISO 9509 standard and does not require analytical determinations (Ficara, 2001).

2.5.3 The denitrification process

A somehow obvious follow-up of the first application was the study of denitrification (Massone et al. 1996), which is an alkalising reaction:

$$6NO_3^- + 5CH_3OH \rightarrow 3N_2 + 6OH^- + 5CO_2 + 7H_2O \qquad (2)$$

A wastewater containing nitrates is added to a vessel with denitrifying activated sludge and readily biodegradable organic carbon (rbCOD) at non-limiting concentrations: denitrifying activity and nitrate concentrations are respectively measured by monitoring the acid consumption rate needed to keep the pH in the vessel constant, and the acid required to neutralise the alkalinity until the reaction is completed. In this case, pH control is more difficult than during nitrification because there is no aeration flow to keep the partial pressure of carbon dioxide constant in the vessel. To overcome this problem, it is advisable to sparge, at reduced flow rates, a N_2/CO_2 mixture containing some 1-5% CO_2.

This application of the pH-stat titrimetric sensor was named DENICON, DENitrification CONtroller.

This methodology was applied to evaluate the readily biodegradable organic carbon and the volatile fatty acids concentrations, as follows: a sample of wastewater is added to the reaction vessel, containing a denitrifying biomass and nitrates in excess; since the biodegradation of the rbCOD is coupled to the reduction of nitrate, if the stoichiometry of the process (i.e. the ratio COD-to-nitrate) is known, the alkalinity generated in the process can be used to estimate the amount of rbCOD initially present (Rozzi et al., 1998).

The amount of nitrates needed to biologically oxidise a given mass of organics in anoxic conditions has been defined as the Biochemical Nitrate Demand (BND), and this could be used to estimate the denitrification potential of a wastewater or a given chemical product.

Volatile fatty acids are generally considered a crucial monitoring and control variable in anaerobic digesters; however, their determination can be (expensive and time-consuming) particularly for on-line applications. As VFA make up the main fraction of rbCOD in anaerobic reactor mixed liquors and effluents, it could be possible to monitor them by measuring their BND (Rozzi et al., 1997 b).

2.5.4 The aceticlastic methanogenesis process

The same concept was applied to the study of aceticlastic methanogenesis, as the degradation of acetate generates alkalinity, due to both the production of bicarbonate and the release of methane, which reduces the concentration of carbon dioxide in the liquid phase:

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^- \qquad (3)$$

As a result, there is an increase in pH of the sludge due to a shift in the CO_2/HCO_3 equilibrium.

This application was named MAIA, Methanogenic Activity and Inhibition Analyser and is reported in Rozzi et al. (2001).

The alkalinity generated by the process is compensated by the addition of an acidic titrant, e.g. hydrochloric or acetic acid: in the latter case, not only the pH is maintained constant, but also the substrate (see Annexure C).

The main advantage of the use of the pH-stat titrimetric sensor is that it provides the possibility of measuring the conversion rate of a methanogenic culture while keeping the substrate concentration and pH constant as long as required. Generally, the assessment of the methanogenic activity takes 24 to 48 h.

Numerous tests (Murugen et al., 1999; Rozzi et al., 2002) have shown that this instrument enables easier and more accurate evaluation of maximum activity, kinetics and inhibition of aceticlastic methanogens as compared to other existing volumetric or manometric procedures.

CHAPTER 3

METHODS

This Chapter presents the methods used for the experiments discussed in Chapter 4, Results and Discussion. It is structured in two main sections:

- Section 3.1 illustrates the various set-ups used for culturing the anaerobic sludge and assessing its performance under various conditions; and
- Section 3.2 gives an overview of the analytical procedures that were used to measure the various parameters.

A more comprehensive discussion of the same issues is contained in the pertaining annexures, according to the following schematic:

Section	*Details in:*
3.1.1 The serum bottle method	Annexure B
3.1.2 The pH-stat titration method	Annexure C
3.2 Analytical methods	Annexure F

3.1 APPARATUS

Three types of reactor units have been used for the experiments described in this report:

- small-scale units, consisting of 125 mℓ glass vials, were used to conduct Anaerobic Activity Tests (AAT) mostly in batch conditions;
- laboratory-scale units, consisting of 2 to 5 litres completely stirred glass vessels, were used to conduct co-digestion experiments; and
- a pilot-scale 3.5 m3 glass fibre vessel, operated as a completely stirred reactor by internal recycling of the mixed liquor, was placed at the New Germany Wastewater Treatment Plant and was aimed at starting up a large-scale co-digestion demonstration.

The advantages and disadvantages of these set-ups are compared in Table 3.

Table 3 – Comparison of pros and cons of different set-ups for the study of anaerobic digestion processes.

Set-up	Pros	Cons
Serum bottle	▪ a large number of replicates can be carried out simultaneously; ▪ different operating conditions can be simulated, by using replicate units in parallel; ▪ bottles can be sacrificed for autopsy of total contents; ▪ it is possible to calculate a very accurate mass balance (Annexure B); ▪ the reproducibility between replicates is generally very high (Figure 1); ▪ it is possible to simulate 'extreme' conditions, even leading to failure, without risking the same consequences on a larger unit.	▪ generally limited to batch operation and steady conditions; ▪ great care should be taken in ensuring that the small aliquots of seed inoculum and of the other components are representative; ▪ limited possibility of monitoring the process (the sampling of the mixed liquor for conventional analysis may not be feasible); ▪ limited to monitoring reactions which directly results in the production of gas; ▪ the supervision is entirely manual and may be time-consuming.
Laboratory-scale	▪ possibility of more closely simulate real applications, both in terms of reactor configuration and of operating regimes; ▪ different operating modes can be used, i.e. batch, fed-batch, continuous; transient and upset conditions can be simulated; ▪ possibility of carrying out a limited number of replicates; ▪ several parameters can be monitored; ▪ possibility of (semi)automated operation and control: the supervision may result less labour-intensive.	▪ the construction and operation can be expensive; ▪ the start-up is the critical phase, as on real scale; ▪ failure can be an expensive event; ▪ maintenance is required for all the components, e.g. pumps, sensors, etc.; ▪ ensuring the gas-tightness of the reactor at all times can be difficult (leaks can likely happen); ▪ possible leaks and frequent necessity of recalibrating the gas measuring device may result in inaccurate mass balance.
Pilot-scale	▪ it is generally the final step before a real scale implementation: it allows for the tuning of operational conditions and ultimately to correctly design the full-scale plant; ▪ the supervision can be very limited, both because all operations are generally automated (i.e. feed, recycling, etc.) and because this stage is seldom used for research purposes; ▪ industry is almost exclusively interested in the outcome of this stage.	▪ it requires a considerable knowledge of the process, that must have been achieved through extensive experimentation at a smaller scale; ▪ large equipment is needed and mechanical problems can become predominant; ▪ the possibility of carrying out replicates is generally limited; ▪ measuring the gas composition can be very difficult (unless sophisticated on-line sensors are used): an averaged gas sample can be collected in a gas sampling bag and analysed off-site. This inevitably limits the frequency of sampling and the sensitivity of the determination; ▪ gas flow rate can be relevant, therefore safety becomes an issue.

3.1.1 The serum bottle method

A serum bottle anaerobic activity test comprises a 'control' set –which allows for the quantification of background conditions– and a number of 'test' units, in which the anaerobic sludge is exposed to a variety of conditions. All units are generally carried out in replicates, to ensure the repeatability of the results.

The composition of each set varies depending on the type of test to be conducted (Table B.1) and activity is assessed at varying concentrations of the substrate (for a biodegradability test) or of the test material (for a toxicity test).

The general procedure for conducting a serum bottle assay was developed by Owen and co-workers (1979). An extensive discussion is reported in Annexure B and summarised hereafter.

- All the components are poured into the vials, ideally handling the sludge under strict anaerobic conditions. Should this be not feasible and as further safety prescription in all case, each vial is flushed with a mixture of oxygen-free gas, e.g. nitrogen, pure or mixed with carbon dioxide.
- The vials are then sealed, with rubber septa and aluminium crimps, and stored at the appropriate temperature.
- After sealing, the pressure inside the vials is likely to increase due to physico-chemical adjustment; therefore, it must be re-equilibrated to the atmosphere, prior to starting the test.
- Thereafter, the excess pressure generated by the microbial activity is periodically re-equilibrated and the volume of gas produced measured using a glass syringe (Section 3.2.5.1); gas composition should also be determined by gas chromatographic analysis, to enable accurate mass balances to be carried out.

The following measurements should be performed prior to starting a test, to optimally set-up the units:

- solids concentration: volatile solids (g VS/ℓ) are generally used to report microbial activity;
- organic content (g COD/ℓ) of the seed inoculum: ideally, the residual substrate in the inoculum should be negligible compared to the substrate added, so that its contribution to the biogas production is small;
- concentration of the substrate and of the test material (g COD/ℓ): it can be measured or calculated stoichiometrically (see Annexure F.4);

- pH of the sludge and of the test substance: the nutrients and minerals solution is *by definition* neutral; sufficient alkalinity and a neutral pH should be ensured, in order to adequately buffer the carbon dioxide and volatile acids production in the vials.

The determination of the organic content of the seed inoculum can be difficult as the turbidity of the suspension is likely to interfere with the colorimetric determination of the end point of the open-reflux method, and ultimately result in an inaccurate quantification of the COD.

A simplification which is generally accepted is that the greatest contribution to the readily biodegradable COD of the sludge is dissolved COD. Therefore, one can discard the solid fraction through filtration and quantify the COD concentration of the liquid fraction only. This approach was adopted in this research. However, the contribution of the solid fraction of the sludge to the total COD, either adsorbed onto the particles, stored inside the cells or granules, etc. may not be negligible.

A more rigorous and direct method is to use a serum bottle activity test in analogy to a conventional BOD test for activated sludge and aerobic processes (Section 3.2.2.1).

Similar determinations should be performed at the end of the test, i.e. solids and COD concentration; other parameters, such as the concentration of some metabolites or end-products, would provide additional data for conducting accurate mass balances.

The main outputs of a serum bottle assay are the *biodegradability* of the test material and/or the *inhibitory effect* of the test material on the methanogenic biomass.

3.1.1.1 Assessing the biodegradability of the test material

The biodegradability of the test material is the ratio between the actual extent of degradation achieved (and quantified as the volume of methane produced) and the content of organic matter of the test material, as follows:

$$B = \frac{V_{CH}(u) - V_{CH}(b)}{V^{*}_{CH}(u)} \qquad (4)$$

where B is the biodegradability; $V_{CH}(u)$ and $V_{CH}(b)$ indicate the volume of methane (in mℓ), produced by the test and the control units; and $V^{*}_{CH}(u)$ indicates the stoichiometric volume of methane (in mℓ) which corresponds to the organic carbon in the test compound (see Annexure F.4).

The quantities V_{CH} can equally be expressed in COD units.

Equation 4 holds if $V_{CH_4}(u) > V_{CH_4}(b)$; otherwise, the test compound is inhibitory.

3.1.1.2 Assessing the toxicity of the test material

The inhibitory effect of the test material is generally referred to the methanogenic step, since this is generally the most vulnerable step of the entire process. Therefore, the methanogenic activity measured in presence of the test material is compared to that measured in optimal methanogenic condition, i.e. in presence of excess acetate, as follows:

$$I = \frac{A(b) - A(u)}{A(b)} \tag{5}$$

where I is the degree of inhibition; $A(u)$ and $A(b)$ indicate the activity measured in the test unit and the baseline methanogenic activity (i.e. that in the control unit).

The activity A is calculates as:

$$A = \frac{1}{X}\frac{d}{dt}V_{CH} \tag{6}$$

where X indicates the amount of biomass present in the system (in grams of volatile or volatile suspended solids).

Generally, the initial biomass is used, unless a significant growth has happened and a more accurate value can be estimated. For a short-term test, the average of the initial and final values could be used.

3.1.2 The pH-stat titration method

It is believed that a pH-stat titration activity test could constitute a rapid screening stage within the experimental protocol (Annexure A). The application named MAIA was used to conduct toxicity and biodegradability assays (Naidoo, 2003).

The general layout of a pH-stat titration sensor has been illustrated in Section 2.5. It consists of the following components.

- The bioreactor: this is a 1.2 litre-volume glass custom-made vessel. It has six ports that accommodate the pH and temperature probes, a liquid sampling port and a gas outlet; and two inlets for titrant dosing. Continuous stirring is provided by a magnetic stirrer. The appropriate temperature is maintained by water circulation in a soft plastic tubing that encircles the flask or by placing the instrument in a temperature controlled room.
- The gas measuring system: this is an external unit, not controlled through the software and consists of a Mariotte flask containing a suitable barrier solution (NaOH 1 N was used).
- The titration unit, comprising two solenoid electro valves (SIRAI, Model 301) and the corresponding reservoir tanks, which maintain a constant head over the electro valves

regardless of the consumption of the titrant. Hence, the flow rate of the titrant is simply a function of the temperature, since temperature affects the viscosity of liquids.

- The control unit consists of a software package which translates pH and temperature signals from the respective probes and actuates the appropriate valve when the pH deviates from the set-point by a differential set by the operator (generally, 0.02 units).

Either acetic acid or hydrochloric acid can be used as titrants (see Annexure C): the former is preferred when the targeted process is aceticlastic methanogenesis (e.g. toxicity tests), as it maintains the concentration of the substrate nearly constant throughout the test (Figure C.6 B). Hydrochloric acid is preferred when the entire process of anaerobic degradation of an organic substrate is monitored (e.g. biodegradability tests), as it only maintains the pH constant while the substrate is depleted (Figure C.6 A).

The experimental conditions must be accurately set in order to obtain meaningful titration results. The main operating conditions are discussed in Rozzi et al. (2001): they are briefly summarised hereafter.

- Liquid-gas equilibrium. The dissolved carbon dioxide should ideally be in equilibrium with the carbon dioxide in the gas, throughout the test. When aceticlastic methanogenesis is the targeted process, since the biogas released is made of an equimolar mixture of carbon dioxide and methane, the molar fraction of carbon dioxide in the headspace must be set to 0.5 prior to starting the activity test. When the entire biodegradation process is monitored, the composition of the biogas will vary during the course of the test: this will inevitably interfere with the titration process, despite the initial partial pressure of carbon dioxide.
- pH and alkalinity. The selection of the set-point pH value is critical, as the pH is related to the alkalinity of the solution and to the sensitivity of the titration process. In the pH range suitable for methanogenesis (i.e. 7 to 8), the buffering capacity almost exclusively depends on the concentration of bicarbonate ions. The dissociation equilibrium of the inorganic carbon can be written as:

$$H_2O + CO_2 \leftrightarrow H^+ + HCO_3^- \tag{7}$$

$$K_1 = \frac{[H^+]\cdot[HCO_3^-]}{[CO_2]} \quad \rightarrow \quad \frac{[HCO_3^-]}{[CO_2]} = 10^{-pK_1+pH} \tag{7.a}$$

This shows that *i*) at constant pH, a change in bicarbonate concentration is reflected in an equivalent change in carbon dioxide concentration; and *ii*) in a plot $[CO_2]_{liq}$ vs. $[HCO_3^-]$, the pH-stat condition is a straight line through the origin (Figure 6)

In a system where the molar fraction of carbon dioxide in the gas is constant, the bicarbonate buffer capacity does not have a maximum for $pH = pK_{CO_2/HCO_3}$, unlike in closed systems: instead, it increases with pH (Stumm and Morgan, 1996). Therefore, the higher the pH, the higher the buffer intensity. In a pH-stat titration system though, the buffer capacity must be maintained relatively low (compatible with the requirements of the biomass), to ensure that the control system be sensitive.

This is why a set-point value of 6.8 to 7.0 is generally adopted, to compromise between a high enough value for methanogenesis and a sufficiently low value for the buffering capacity of the system (Figure C.4).

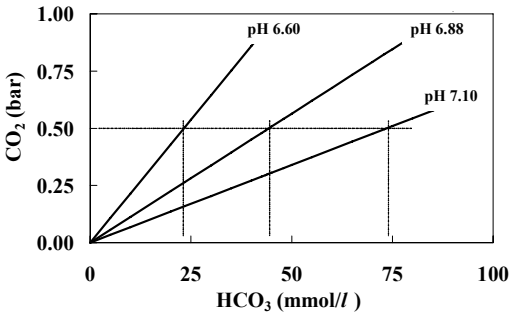

Figure 6 – Dissociation equilibrium of carbon dioxide, in an open system: graphical representation of the pH-stat condition (T 35 °C).

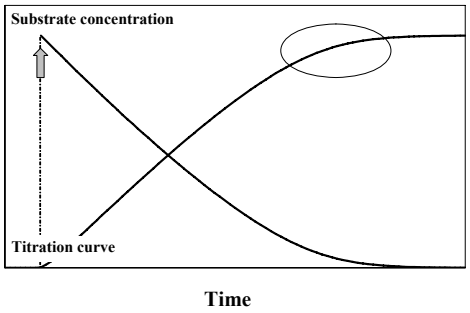

Figure 7 – The principle of pH-stat titration: the rate of substrate depletion is proportional to the titration rate.
The arrow indicates the addition of substrate. The circle denotes the sharp change in the slope of the titration curve, in proximity of the K_S value.

The detailed procedure for conducting a pH-stat titrimetric activity test is illustrated in Annexure C.

The control system records pH, cumulative acid titration volume, cumulative alkaline titration volume and temperature in the reaction vessel at regular time intervals. The main output of an activity test is the cumulative volume of titrant solution dosed, termed titration curve (Figure 7). The microbial activity is proportional to the slope of the titration curve: until the substrate concentration is much higher than the semi-saturation constant of the targeted reaction (K_S), the titration line is basically a straight line; when the substrate concentration approaches zero, the titration line levels off, reaching an asymptotic value, which is proportional to the initial amount of substrate supplemented. A simple stoichiometric conversion enables to back-calculate the profile of the substrate concentration from the titration curve (and calculate a mass balance; Figure 7).

The latest set-up of the instrument (SPES Fabriano, Italy) is named MARTINA, Multiple Analysis Reprogrammable TItriatioN Analyser. Notable changes are *i*) the substitution of the bulky I/O modules with a printed motherboard; *ii*) the improvement of the original DOS-based interface, with a Windows-based interface coded in Labview® that enables an easier manipulation of the instrument; *iii*) the use of a peristaltic pump, instead of the Mariotte flask, to maintain a constant flow rate of the titrants; and more importantly *iv*) the ability of controlling two independent reaction vessels, on two process parameters simultaneously, e.g. pH and dissolved oxygen (DO). However, for our purposes, the DO probe could quite simply be substituted by another probe, e.g. a redox potential electrode.

3.1.3 The laboratory-scale reactors

The 5 ℓ laboratory-scale set-up for batch and fed-batch experiments is depicted in Figure 8. The glass flask was sealed with a rubber bung in which two holes accommodated the gas outlet (**A**) and a 5 mm compression fitting holding a Polyurethane tubing PHHU (OD 6 mm x ID 4 mm; Parker) (**B**) which was used to feed the reactor and to sample the mixed liquor. Continuous mixing was provided by a magnetic stirrer (**C**). The gas outlet was connected to a gas sampling port, consisting of a butyl septum screwed onto a modified compression fitting (**D**), a compensation volume of 1 ℓ (**E**) and terminated at the gas measuring device.

The gas sampling port (**D**) consisted of a T-compression fitting, which housed a 9.5 mm butyl septum (Supelco); a 100 μℓ gas-lock syringe (SGE syringe, Supelco) was used to puncture the septum and to withdraw 40-80 μℓ of gas for GC analysis (a pressure-lock syringe was used at times).

The compensation volume consisted of a 1 ℓ Tedlar bag (Supelco). This is normally used on larger scale equipment to collect gas samples and transport them to the laboratory. It is equipped with a special fitting and a butyl septum. The former can be locked when pulling the bag off the gas line, thus sealing the gas sample inside the bag. The septum allows the extraction of a sample of the gas. The Tedlar bag was used to provide a variable volume headspace which enabled a constant pressure to be maintained inside the reaction vessel when the liquid volume changed, during the liquid sampling and feeding operations: when a liquid sample is withdrawn, gas flows into the reactor and the bag deflates; reversely, when the feed is pumped into the reactor, the gas flows back into the bag which inflates. Both operations leave the gas meter unaffected.

The gas measuring device consisted of a plastic U-shaped tube (45 mm-diameter), filled with acidified water (H2SO4 1 %) to the level of the minimum level probe (Section 3.2.5.2).

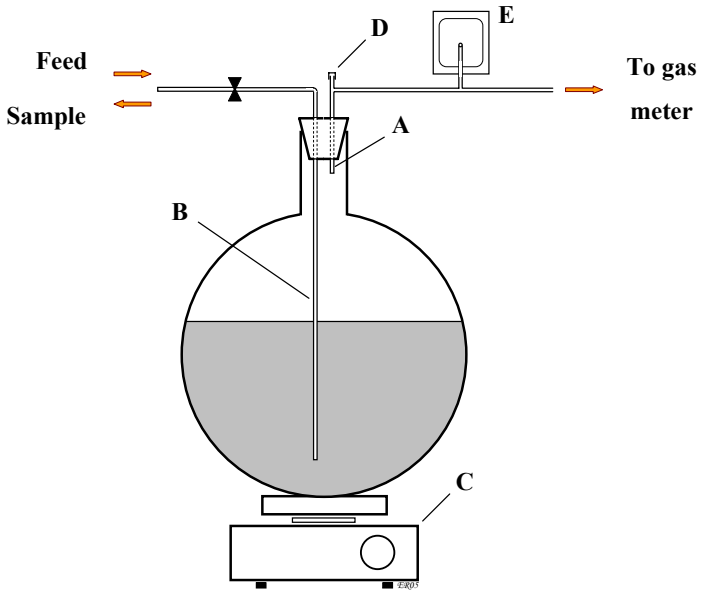

Figure 8 – Schematic of the laboratory-scale batch or fed-batch completely stirred reactor.
***A**, gas outlet; **B**, feeding and mixed liquor sampling port; **C**, magnetic stirrer; **D**, gas sampling port; **E**, compensation volume (Tedlar bag).*

3.1.4 The pilot-scale reactor

The reactor consists of a large fibreglass tank (**A**, Figure 9) with a total working volume of 3,500 ℓ (Ø 1.4 m, H 2.3 m). An external Mono pump (**B**) recirculates the sludge through a coil, which runs through a heating bath (**C**): the recycle stream serves both to mix and to heat the contents of the digester. Two holding tanks are used to store the feed for the reactor: a drum pump is used to take the feed from these tanks into a feeding cone (**D**), which is situated in a room above the reactor to allow the effluent to be fed to the digester by gravity.

Reactor operation. The pilot plant is operated in a semi-batch mode. In order to bleed the reactor of the digested effluent the re-circulation pump is switched off and the valve on the line that leads from the reactor to the pump is shut. The content is allowed to settle until the supernatant level reaches the level from which the effluent bleed outlet is situated. The bleed valve is then opened and the supernatant is allowed to drain out into the effluent storage drum. A vacuum breaker is present to decrease the internal suction pressure when the effluent supernatant is being bled. Simultaneously during this phase, the feed is taken from the drums by means of a drum pump which transfers it into the feed cone. The effluent bleed valve is then closed and the valve at the base of the feed cone is opened. The recycle pump is switched on. This draws the fresh feed from the feed cone into the reactor. There is an automatic shut-off level control, which ensures that the feed in the cone is not exhausted.

Once the correct amount of fresh feed is in the reactor, the pump is switched off and the feed cone valve is closed. The valve on the line that leads from the reactor to the pump is now opened and the recycle pump is switched on.

Fresh effluent should be trucked in weekly. The contents of the reactor must be turned over every 24 h, in order to provide adequate mixing of the contents.

Because the reactor is run in a semi-batch mode, mixing is stopped during supernatant and sludge withdrawal. Sampling ports in the reactor will enable the evaluation of the mixing. If the solids concentration does not vary more than 10 % and the temperature more than 2 °C, then the reactor can be considered to be fully mixed. The reason behind the semi-batch mode is mainly to increase the solid retention time of the biomass. An external settler was considered in a continuous operation but biomass loss in gravity clarifier overflow can be excessive if a poorly settling biomass develops. This condition is not suitable for the stable performance of the granule synthesis, in anaerobic biological processes.

As the pilot-plant makes use of a recycle loop for mixing, care must be taken that the shear created by the pump does not disintegrate the granular structure: this could cause troubles in the settling phase.

The gas flow rate is measured volumetrically, using the device presented in Section 3.2.5.2.

The heating bath contains water roughly at 42 °C and is closed off to prevent evaporative losses. A coiled pipe runs through the heated water bath and holds the re-circulating sludge. The pipe has an internal diameter of 10 mm and a wall thickness of 1 mm. There are six coils, each with a diameter of 250 mm to increase the area for heat transfer and to increase the turbulence of the liquid inside the tube. The bath is monitored by a control loop, which ensures that the temperature of the liquid inside the reactor remains at 35 °C.

Operating conditions. The mean hydraulic retention time (HRT) can be set at 30 d for these pilot-scale studies: this translates in a feed rate of effluent mixture of about 100 to 120 ℓ/d. The HRT may be reduced in a subsequent phase, if results are promising.

In this study, the biomass (solids) is not recycled to the digester; however, the sludge and hydraulic retention times are in fact uncoupled because the process is run in a semi-batch mode, as explained above, and the sludge is allowed to settle prior to withdrawal.

The organic loading rate (in g COD/ℓ/d) into the digester is given as:

$$OLR = Q_{in} \cdot C_{in} \cdot \frac{1}{V_R}$$

where Q_{in} and C_{in} denote the feed flow rate (ℓ/d) and the concentration of organic matter (g COD/ℓ) in the feed respectively; and V_R is the volume of mixed liquor in the digester.

Acclimation is necessary when the biomass is confronted with a substrate which requires additional enzymes, metabolic pathways, or environmental conditions not encountered prior to that phase of bacterial growth. This aspect is to be specifically ascertained prior to the start-up of the pilot-scale simulation, in order to enable stable operations of the digester.

Routine analyses will be carried out on the supernatant effluent, i.e. COD, alkalinity and VFA concentration; pH; solids concentration, preferably as volatile and volatile suspended solids. Gas composition will also be analysed frequently, by unplugging the Tedlar bag from the gas line and taking it to the laboratory for GC determination.

As discussed later on (Table 18), pH, alkalinity, VFA concentration and gas composition are the primary indicators of process stability: a change in any of these parameters alone may not be significant but adverse changes in a few of them simultaneously signal upset. The usual pH range for anaerobic treatment is from 6.5 to 7.5. Volatile acids and alkalinity affect the pH. Processes can be operated over a wide range of volatile acid concentrations. The ratio of volatile acids to alkalinity is more important than the values of either parameter (Speece 1996): increases of the ratio above 0.3 to 0.4 indicate upset and the necessity for corrective action; when the ratio exceeds 0.8, pH depression and inhibition of methane production occurs, resulting in process failure.

The methane content of the gas is typically in the range of 65-75 %, with carbon dioxide comprising of the bulk of the remainder. However, the composition of the off-gas is a function of the carbon/organic content of the feed (Figure F.4).

Care needs to be taken in ensuring that the pH and alkalinity measurement of a sample is a true measure of the condition inside the reactor: if the sample is allowed to stand exposed to the air for a few minutes, carbon dioxide will escape, causing the pH to rise. In order to measure the pH of a sample accurately, it should be capped with negligible headspace and the pH measured promptly to achieve minimum loss of carbon dioxide (Section F.3.1).

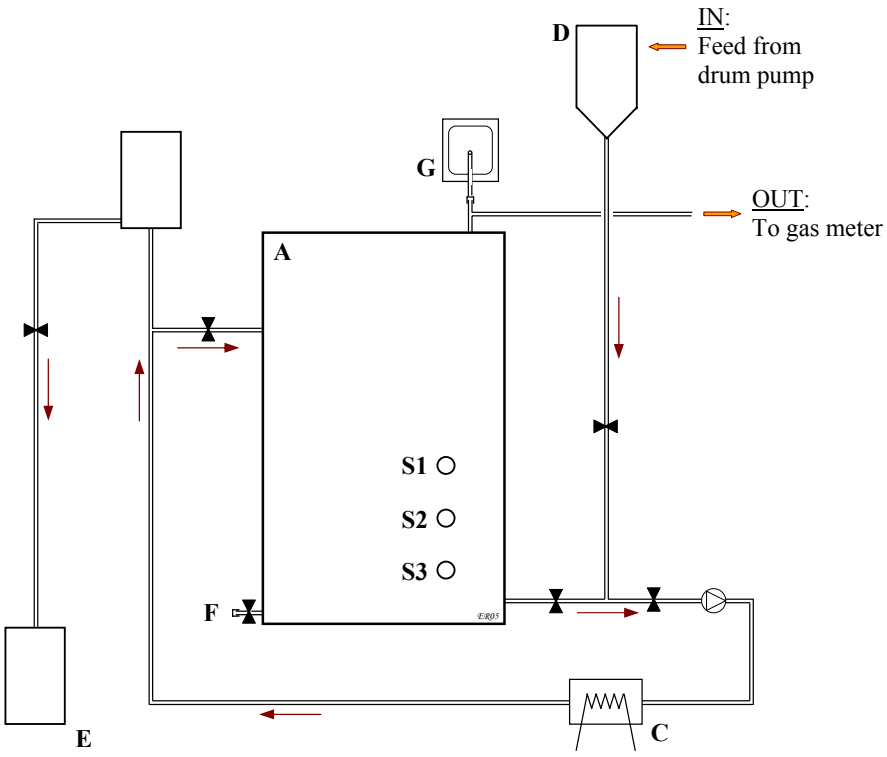

*Figure 9 – Schematic of the pilot-scale anaerobic digester, situated at the New Germany Wastewater Works. **A**, reaction vessel; **B**, recirculation pump; **C**, heating unit; **D**, feeding cone; **E**, effluent storage tank; **F**, sludge extraction port; **G**, biogas outlet and sampling port (fitted with a Tedlar bag); **S1**, **S2**, **S3**, sampling ports.*

3.2 ANALYTICAL METHODS

Analysis included the determination of the concentration of solids, organic carbon, volatile fatty acids and alkalinity; the determination of the pH value and the determination of the volume and compositions of the biogas.

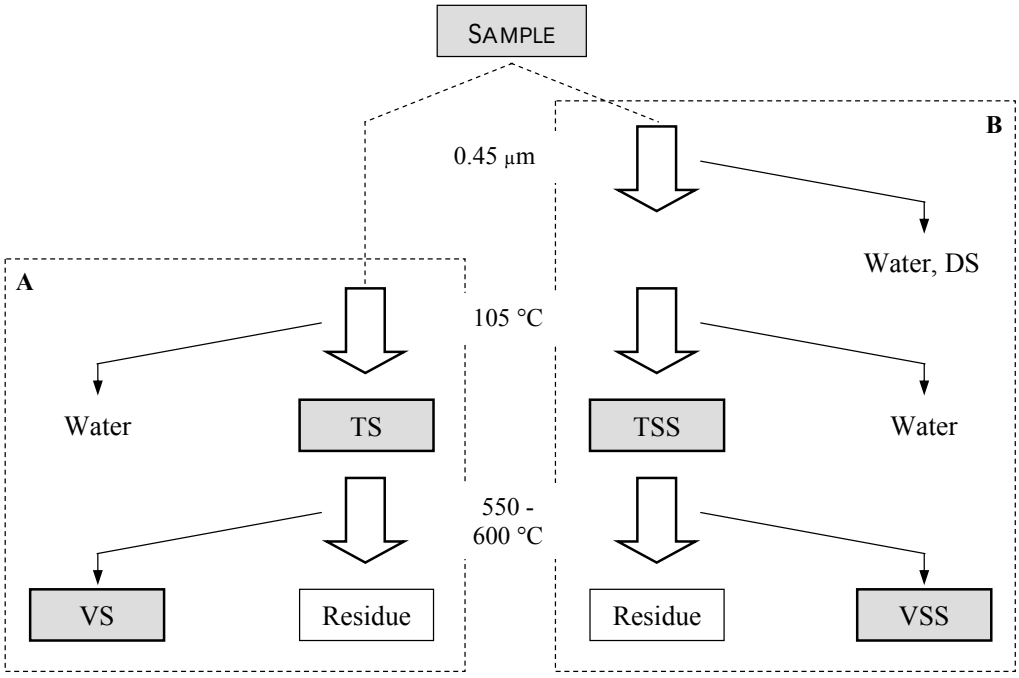

Figure 10 – Schematic representation of the method for measuring solids concentration.

3.2.1 Determination of solids

A Standard Method (American Public Health Association, 1989) was used for determination of solids in anaerobically digested sewage sludge (Figure 10). Total solids (TS) and volatile solids (VS) are determined on a raw sample of the sludge. Total suspended (TSS) and volatile suspended solids (VSS) require that the sludge be filtered, through a glass-fibre filter and the liquid fraction discarded. The solid content of a sludge is generally reported as mass of solids per unit volume of sludge, i.e. g TS/ℓ (for granular sludges, g TS/g is also used).

- Total solids. A crucible is placed in a muffle furnace at 550-600 °C for approximately 60 min. The crucible is cooled in a desiccator, weighed and stored until used. A well-mixed sample is transferred to the weighed dish and evaporated to dryness in a drying oven at 105 °C. The dish is then cooled in a desiccator and weighed. The difference in weight represents the total residue (equation 8).
- Volatile solids. The residue from the total solids test is then ignited in a muffle furnace (pre-heated to 600 °C) for 60 min. The dish is partially cooled in air and then

transferred to a desiccator for final cooling. The dish is weighed once completely cooled. The loss of weight on ignition is reported as the volatile solids (equation 9).

- Total and volatile suspended solids. A similar procedure is used for the determination of the suspended fractions of the solids, in which the role of the evaporation dish is taken by the glass-fibre filter (equations 10 and 11). Great care must be put in handling the filter, since the accuracy of the determination can easily be impaired, e.g. by humidity taken up from fingers or atmospheric particulate matter.

Since there is a great difference in the ranges of weight involved in the determination of total and suspended solids (typically, three orders of magnitude), a *technical* scale and a *precision* scale must be used.

Table 4 – Determination of the solids concentration.

Parameter	**Formula**	
Total solids	$TS = \frac{W_{105} - W_0}{M}$	(8)
Volatile solids	$VS = \frac{W_{105} - W_{600}}{M}$	(9)
Total suspended solids	$TSS = \frac{W^*_{105} - W^*_0}{M}$	(10)
Volatile suspended solids	$VSS = \frac{W^*_{105} - W^*_{550}}{M}$	(11)

- W denotes the weight (g) of the crucible or the filter (indicated by the superscript *).
- M denotes the size of the initial sludge sample, as volume (ℓ) or mass (g).
- the subscripts 105, 550 and 600 indicate the temperatures; 0 denotes the tare.

3.2.2 Determination of organic carbon

The measurement of the Chemical Oxygen Demand (COD) is a simple, relatively inexpensive method, which gives an accurate indication of the electron equivalence. It is based on a closed or open reflux method (Standard Methods, 1995), or on a calorimetric method. Chemical oxygen demand is the amount of oxygen needed to stabilise organic components.

In general, a strong oxidant is used to determine this value. The oxidant must be of sufficient strength to oxidise any organic component that may be present in the sample. The oxidant most commonly used is dichromate ($Cr_2O_7^{-2}$). The principle of the test is the observation that most organic matter is destroyed by a boiling solution of chromic and sulphuric acid. The amount of oxidisable organic matter is proportional to the dichromate consumed.

The Standard Method is suitable for wastes where a large volume of concentrated sample is available. The sample to be analysed is diluted in a volumetric flask, to reach a concentration comprised within the detectable range of the method, i.e. not exceeding 900 mg/ℓ. A 10 mℓ aliquot of the diluted sample is placed in a 250 mℓ refluxing flask. A small amount of mercuric sulphate, several glass beads and 15 mℓ of sulphuric acid reagent are added to the flask. A 5 mℓ aliquot of potassium dichromate solution (0.0417 M) is then added. The solution is mixed and allowed to cool. The flask is then attached to the condenser, the cooling water turned on and the mixture refluxed for 2 h. A blank consisting of 10 mℓ distilled water, instead of the sample, is refluxed in the same way. The samples are cooled and diluted with 80 mℓ of distilled water. Thereafter, they are titrated with ferrous ammonium sulphate solution (FAS) using ferroin indicator.

The COD (in g/ℓ) of the sample is evaluated as follows:

$$COD = \frac{(V_B - V_S) \cdot C_{FAS}}{v} \cdot \alpha \qquad (12)$$

where V_B and V_S denote the volumes of FAS (mℓ) used to titrate the blank and the sample, respectively; C_{FAS} denotes the concentration of the FAS (mol/ℓ); v denotes the volume of the sample (mℓ); and $\alpha = 8000$ is the equivalent weight of oxygen, multiplied by 1000 (mℓ/ℓ).

Problems associated with COD measurements are:

- halogens can be oxidised;
- aromatic carbohydrates and some aromatic heterocyclic compounds are not oxidised;
- volatile straight-chain aliphatic compounds are not oxidised to any appreciable degree;
- reduced inorganic compounds e.g. ferrous iron, are oxidised quantitatively under the species.

Nevertheless, the open reflux method for organic carbon measurement is still considered reliable and accurate.

3.2.2.1 Experimental determination of the organic carbon content of the sludge

The COD of an anaerobic sludge can be experimentally determined by measuring the amount of methane generated in a serum bottle activity test, which only contains an aliquot of the sludge (the set-up is identical to that of a control unit of a conventional activity test, Table B.2). If no external substrate is supplemented, the biogas produced will only come from the residual organic matter, irrespective of the form in which this is present in the sludge, i.e. particulate or dissolved.

Ideally, a plot of the specific methanogenic activity vs. time would show two distinct slopes (Figure 11 A), i.e. a first steep(er) segment, approximately linear, followed by a sharp change in slope. If one assumes that the discontinuity at time t^* corresponds to the exhaustion of the readily biodegradable COD, which was present in the sludge and that thereafter the methanogenic activity is purely endogenous, then one can back-calculate the initial COD concentration (Figure 11 B).

The key assumption that the COD concentration at time t^* is zero may be seen as arbitrary, in that it is not analytically measured, but it is possibly as arbitrary as the assumption that the COD measured on the filtered sample is actually representative of the whole.

This methodology to quantify the COD of a sludge is still under investigation.

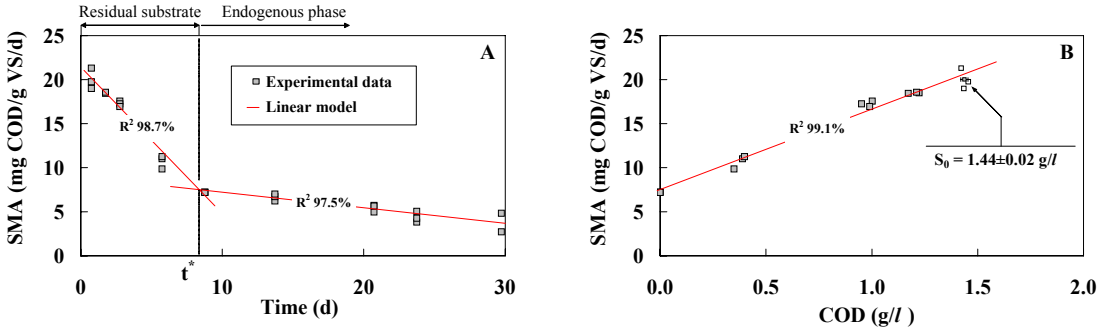

*Figure 11 – Example of the method for the determination of the COD of an anaerobic sludge. Experimental data of the specific methanogenic activity vs. time (**A**; N=3). Regression of the initial COD concentration (**B**).*

3.2.3 Determination of the pH

Methanogenic micro-organisms prefer nearly neutral pH conditions and although methanogenesis is observed to occur at pH values as low as 6.0, the rate of the process is reduced. A suitable and well-maintained pH assists in process stability. A generally accepted optimum pH range is between 6.5 and 8.2 (Speece, 1996). Within this range, methanogenesis proceeds at high rates. It is essential for the system to contain adequate buffering capacity to accommodate the production of volatile fatty acids and carbon dioxide that might dissolve at the operating pressure.

Measurement of pH have been generally done *off-line*, using a pH electrode (Toledo series 400), manually immersed into the sample of sludge or effluent. Care must be taken in ensuring that the pH determination of a sample is a true measure of the pH in the system. If the sample is allowed to stand exposed to air, carbon dioxide will escape causing the pH to rise. Similarly, by stirring the sample while measuring the pH, favours the stripping of carbon dioxide and therefore alters the pH value (Section F.3.1).

On the pH-stat titration sensor, a pH electrode is connected to a data acquisition module and pH values are automatically recorded.

3.2.4 Determination of the alkalinity and volatile acids concentration

Alkalinity is a measure of the capacity of a solution to neutralise acids and is primarily due to the salts of weak acids (Sawyer et al., 1994). It controls the pH and thus is a measure of the capacity of an aquatic system to buffer the pH in presence of unbalanced acids (Speece, 1996).

Alkalinity is comprised of different species of the salts of weak acids, so it is conventionally expressed as its $CaCO_3$ equivalence.

$CaCO_3$ equivalent of acetic acid: example of calculation.

- The *equivalent weight* (EW, in g/equivalent) of a chemical species is defined as:

$$EW = \frac{MW}{valence}$$

where MW is the molecular weight (in g/mol).

- The *$CaCO_3$ equivalent* of a substance is calculated as the ratio between the equivalent weight of $CaCO_3$ and that of the species:

$$EW(HAc) = \frac{60}{1} = 60 \text{ g/equivalent}; \quad EW(CaCO_3) = \frac{100}{2} = 50 \text{ g/equivalent}.$$

- Hence, the $CaCO_3$ equivalent of acetic acid is: $\frac{50}{60} = 0.83$ g $CaCO_3$/g HAc.

Anaerobic digesters operate optimally at neutral pH conditions in which the bicarbonate ion is the dominant weak acid species, therefore bicarbonate alkalinity is an important parameter. It is crucial that one distinguishes between *bicarbonate* and *total* alkalinity. The former refers to the total alkalinity minus the VFA equivalent alkalinity (due to neutralised VFA): however, VFA alkalinity is transient since the VFA concentration varies and therefore cannot be consistently relied upon. A conservative approach is generally adopted of ignoring the VFA alkalinity and monitoring the bicarbonate fraction only.

A *two-point titrimetric* method was developed by Anderson and Yang (1992) which enables a precise determination of bicarbonate alkalinity and total volatile fatty acids concentrations. A modification of this method was followed throughout this project (Annexure F.3).

The concept of *reserve bicarbonate alkalinity* is indicated by Speece (1996) as the concentration of bicarbonate alkalinity which is available to neutralise *additional* free VFA; or equally, as the increase in VFA concentration which can be tolerated before the pH is depressed below a safe threshold value (typically, 6.2 to 6.5). Therefore, the reserve

bicarbonate alkalinity is quantified by titrating the sample of mixed liquor with a standardised solution of acetic acid to the desired minimum threshold pH.

In the practice, the ratio of VFA concentration to total alkalinity (TA) is commonly used as control parameter: however, as discussed earlier, the alkalinity of the salts of VFA is not actually available to neutralise additional VFA. For this reason, a more accurate monitoring parameter is the reserve alkalinity (Speece, 1996).

3.2.5 Determination of the gas volume and composition

Gas volume was measured manually with a glass syringe or automatically using a liquid displacement device. The former method was used for the serum bottle assays; the latter, for the laboratory-scale and pilot-scale digesters.

The composition of the biogas was determined by manually withdrawing a sample with a gas- or pressure-lock 100 μℓ syringe and immediately injecting it into a GowMac gas chromatograph (Series 350) equipped with a thermal conductivity detector, which detects methane, carbon dioxide and nitrogen. The carrier gas used was helium. A packed column was used for the separation of the gases. The specifications of the GC and the calibration procedure are detailed in Annexure F.2.

3.2.5.1 Determination of the gas volume for serum bottle assays

A glass syringe (Popper and Sons Inc.) fitted with a disposable needle was used to sample the headspace of the serum bottle. The syringe must be lubricated with distilled water prior to sampling in order to allow the free movement of the plunger. It is then inserted into the butyl rubber septum and held horizontally, to allow the plunger to equilibrate between the bottle and atmospheric pressure: the reading is verified by drawing the plunger past the equilibrium point and releasing to ensure that the plunger returned to the original volume.

To continue the assay, gas is re-injected into the bottles without contamination or loss, or vented as necessary to prevent gas leakage and overpressure effects (this latter option is generally preferable).

3.2.5.2 Determination of the gas volume for laboratory- and pilot-scale digesters

A volumetric liquid displacement device was constructed in our laboratory and mounted on a rack which can host eight units; each of those in connected to an analogue counter which enables to semi-automatically record the volume of biogas produced in the respective digester. The measuring unit is depicted in Figure 12 and consists of a U-tube (**A**), filled with an appropriate barrier solution; a three-way solenoid electro valve (**B** or **B'**) and a three-level

electrical sensor (consisting of an earth probe and a minimum and maximum level sensors; **C**) which triggers the valve to release the excess pressure.

The principle of the measuring device is the following:

- the biogas produced in the digester enters one arm of the tube, through the solenoid valve (which is in the state **B**), causing the level of the barrier solution in the other arm to rise;
- when the level in the second arm comes into contact with the maximum level sensor, the electrical circuit triggers the valve, changing its state to **B'**;
- the excess biogas that was 'trapped' inside the tube is pushed back through the valve and vented to the atmosphere;
- the state of the valve is restored to **A**, when the level of the barrier solution in the second arm loses contact to the minimum level sensor.

When the solenoid valve is opened, a signal is passed to an analogue event counter which registers a count.

The volume of gas measured (v , mℓ) for each opening of the electro valve (i.e. per count on the analogue event counter) can be simply calculated as:

$$v = \frac{\pi}{4} \cdot D^2 \cdot h \cdot 10^3 \qquad (13)$$

where D is the diameter (mm) of the tube; and h the distance (mm) between the minimum and maximum level sensors.

An acidified or alkaline solution (e.g. H2SO4 or NaOH, 1 to 2 N) can be used as barrier solution. The former prevents the solubilisation of carbon dioxide (at least after equilibrium is reached between the barrier solution and the biogas: Rozzi and Remigi, 2004). Therefore the entire volume of the biogas produced is measured: the analytical determination of the gas composition is imperative, in order to quantify the amount of COD actually converted to methane.

A more accurate option is the use of an alkaline solution which enables the direct measurement of the volume of methane produced.

The sensitivity of the device can be tuned to the desired level by adjusting the minimum and maximum levels, or by using tubes of smaller or larger diameters.

It is very important for the long term reliability of the device, that the correct set-up for the solenoid valve is used. This type of valve (SIRAI, Model 301) is commercially available in two set-ups termed 'normally open' and 'normally closed': both can be used, providing that the loading phase, i.e. when the biogas flows into the U-tube and likely the most frequent

and/or longer lasting condition corresponds to the 'normal' state of the valve: this ensures a minimal overheating of the valve, which occurs in the excited state. This is schematically depicted in Figure 13, in which the loading condition corresponds to the 'normal' state of the electro valve: the upper and lower cases represent the normally open and normally close set-ups respectively (**R**, reactor; **U**, U-tube).

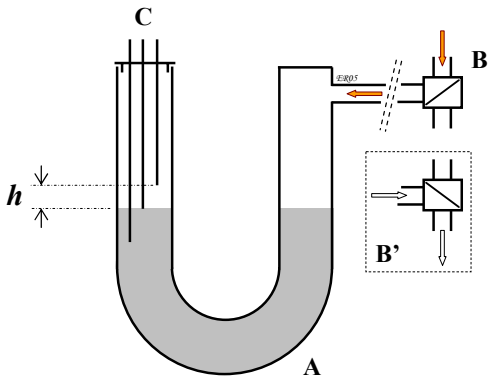

Figure 12 – Volumetric device to measure the volume of biogas produced in a digester.
*U-tube (**A**), filled with the barrier solution (in grey); three-way solenoid valve (**B**, **B'**, loading vs. venting state respectively); three-level sensor (**C**). The unit volume of gas measured in proportional to the distance **h**.*

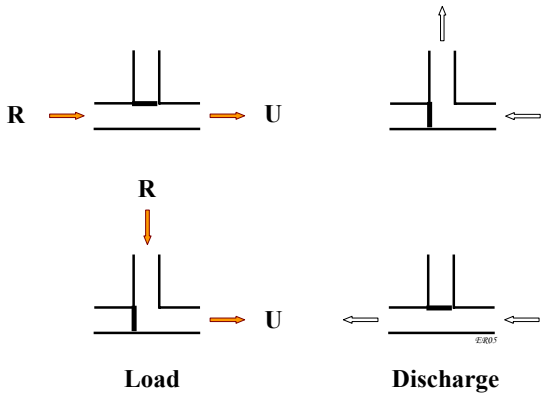

Figure 13 – Position of the three-way solenoid valve within a volumetric gas measuring device.
*The valve is connected to the digester on one side (**R**) and to the volumetric device on the other side (**U**).*
The arrows indicate the direction of the flow of biogas.
Two set-ups are available, termed normally open (top) and normally closed (bottom).
Two arrangements are possible for the Load (left) and Discharge (right) conditions.

CHAPTER 4

RESULTS AND DISCUSSION

The experimental results are organised in two main sub-sections.

The first one (Section 4.1) presents the application of the serum bottle method to the objective of characterising a test material in terms of its biodegradability and toxicity (as outlined in Annexure A). This methodology was improved in the course of the project, so that a first group of experiments was conducted using the *conventional* toxicity (ATA) and biodegradability (BMP) tests, according to the procedure outlined by Owen and co-workers (1979), i.e. with *predetermined* amounts of the different components, a discontinuous determination of the gas composition and a test duration of 7 and 30 to 60 days respectively. The results of these applications are discussed in Section 4.1.1.

A third group of results was obtained using the *modified* anaerobic activity test (AAT) procedure, outlined in Annexure B. This method does not differentiate rigidly between the set-up for the assessment of toxicity or biodegradability, in that it prescribes that both the reference substrate and the test material are added to the test vials and requires a closer monitoring of the gas evolution. These results are discussed in Section 4.1.3.

This modified procedure was developed through several attempts n which the main concern was to obtain the greatest understanding possible, given the basic set-up of a serum bottle assay. An example is discussed in Section 4.1.2, that was aimed at studying the adaptation potential of an anaerobic sludge to a dyeing effluent upon long-term exposure.

The second section of this chapter (Section 4.2) presents the results obtained in a laboratory-scale experiment which used the distillery effluent that was screened in the previous stage.

Each Section is organised as an independent unit, which consists of an **Objectives**, a **Materials and Methods**, a **Results and Discussion** and a **Conclusions** sections. For clarity, the details, e.g. specific plots and tables, of each test have been grouped in the pertinent section of Annexure G, whereas the *general* outcome only is discussed in this chapter.

The experience gained from these exercises has led to the elaboration of the main outputs of this project (Figure 4), i.e. a protocol for the screening of industrial effluents; a protocol to conduct an anaerobic activity test using the serum bottle method; a protocol to conduct pH-stat titrimetric toxicity tests; and a series of recommendations on the start-up of a laboratory-scale digester. Each one of these outcomes is organised in a separate annexure (Figure 4).

4.1 SCREENING TESTS

With the term *screening tests*, we indicate those small-scale assays that are aimed at assessing the amenability of an industrial effluent to be treated in an anaerobic digester. As explained in Annexure A, we decided to use the serum bottle method because it is sufficiently simple and inexpensive that any institute or authority should be able to afford it.

4.1.1 Conventional toxicity and biodegradability assays

Objectives

Toxicity and biodegradability assays on leachates, textile effluents and on individual components of a specific textile recipe were conducted according to the ATA and BMP methodologies.

Materials and methods

Landfill leachate. Leachates originating from three landfill sites were tested.

- Holfontein landfill site is located in Gauteng on the border to Mpumalanga. It deals with hazardous industrial wastes and was the first engineered high hazardous commercial waste disposal site, designed and permitted according to the *minimum requirements* of the Department of Water Affairs and Forestry. Domestic wastes are co-disposed of in the site to provide the necessary substrates that sustain the anaerobic biodegradation process.

  The landfill consists of several cells, lined with layers of clay and isolated from the ground by a polypropylene liner to prevent contamination of the aquifer.
- Shongweni landfill site is located in KwaZulu-Natal and is classified as low hazardous site: it treats low hazardous domestic and commercial waste; co-disposal of liquid streams and sludge waste is carried out in controlled excavated trenches.
- Aloes tip site is located north of Port Elisabeth and is classified as highly hazardous. A first unlined waste tip has been closed and is being rehabilitated. Unpleasant odours originating from the leachate pond have been reported and concern has been raised regarding a possible contamination of the watercourses.

Sizing effluents. Three textile effluents were synthesised in the laboratory, based on recipes obtained from Frametex (New Germany), as follows (figures indicate grams per 100 mℓ of distilled water):

- Recipe 1: starch (3.3) and wax (8.8);
- Recipe 2: maize starch (42.0), wax (1.7) and polyvinyl alcohol (25.0); and
- Recipe 3: starch (6.6), wax (3.3) and Elvanol (42.0).

For recipes 1 and 3, Kollotex was used as starch.

The following components of sizing recipes were investigated (their organic content is reported in Table 5).

- Starch: starch granules consist of α and β amylase with the former being insoluble in water. The qualities that give starch its usefulness as a sizing agent are its ability to form a pliable film and the ability to provide a good coating without excess penetration into the yarn.
- Wax: this term includes most lubricants of a solid nature. The chemical composition varies widely but is generally based on a long chain hydrocarbon molecule or a derivative.
- Polyvinyl alcohol (PVA): it is a synthetic polymer resin produced by acid or alkaline hydrolysis of polyvinyl acetate; it is an excellent textile warp size because of its strength, adhesion, flexibility and film-forming properties.

Table 5 – Organic content (g COD/ℓ) of the components and of the complete recipes of the textile effluents synthesised in the laboratory.

	Starch	**Wax**	**PVA**	**Elvanol**	***Total***
Recipe 1	0.131 [(a)]	9.4	-	-	7.7
Recipe 2	39 [(b)]	4.4	51	-	81
Recipe 3	105 [(a)]	9.6	-	164	193

(a) Kollotex.
(b) Maize starch.

The toxicity of the effluents and of the individual components was first studied (sections 4.1.1.1 and 4.1.1.3). Thereafter, suitable concentration levels for the mixture and for the components were selected to assess their biodegradation potential (sections 4.1.1.2 and 4.1.1.4).

This is the approach that one should apply when investigating industrial effluents consisting of a mixture of components, in the endeavour to identify the component responsible for the toxicity effect of the effluent to anaerobic sludge. Based on the outcome, one could examine managing options that would divert the component(s) from the main stream and dispose of it separately, using a suitable and possibly more effective treatment option.

All experiments were conducted in 125 mℓ serum bottles: the detailed composition is summarised in Tables G.2, G.3, G.4 and G.5. For the assessment of toxicity, six levels of concentration were tested for each test material, ranging from 0.04 % to 40 % v/v. The level of concentration which showed the least toxicity was selected for the assessment of biodegradability.

Stock solutions were prepared, by diluting the test materials in distilled water and a fixed volume of 40 mℓ added to the vials.

A mixture of acetate and propionate was used as the reference substrate for the assessment of toxicity: 2 mℓ of a stock solution containing 75 mg of acetate and 25.6 mg of propionate per 100 mℓ was added to the vials.

The seed sludge was obtained from the Umbilo Wastewater Treatment Plant.

The toxicity assays lasted 7 days and were monitored daily, by measuring the volume of gas produced; gas composition was determined only at the end of the test. The biodegradability assays lasted 30 to 60 days and were periodically monitored, by measuring the volume and the composition of gas. The excess gas was re-injected into the vials, until the overpressure exceeded 0.5 atm that under the operating conditions corresponded to approximately 12 mℓ of gas. All tests were performed in triplicate.

Results and discussion

The results of this series of ATA and BMP experiments are illustrated in the following pages, using a consistent format.

▪ Toxicity tests. A plot of the activity of the sludge vs. the concentration of organic matter is presented for each test material; a semi-logarithmic scale seemed more appropriate to show the progressive change of the relative activity on the wide intervals of concentrations that was examined.

The conventional methodology does not prescribe that the composition of the gas be determined except at the end of the test, to enable a mere overall mass balance (which however may be largely inaccurate if the gas composition had not reached equilibrium). Therefore it has not been possible to calculate the true specific methanogenic activity because the fraction of methane in the off-gas was not quantified. A very important consequence is that if a test material had caused an organic overload to the methanogenic biomass at relatively high concentration, the pattern of gas composition could be completely different from that obtained for low(er) concentration levels, although the overall gas production might not differ significantly: clearly, by appraising the activity of the biomass in terms of overall gas production would be completely misleading (most likely the inhibitory effect at high concentration would be underestimated because the low production of methane would be shadowed by the overproduction of carbon dioxide).

This makes the quantitative comparison between the responses obtained for different test materials totally unreliable.

This observation is one of the reasons that led us to propose a modified methodology for the conduction of toxicity assays.

The quantitative outputs are summarised in a table that reports the cumulative volume of biogas generated (mℓ), the maximum sludge activity (mℓ/d), which indicates the (local) maximum of the microbial activity (calculated as the step-by-step rate of gas production) and the lag-phase (d). All are indicated as average of the three replicates (except few occasions); the lag-phase is visually appraised as the period of time before the gas production took off.

▪ Biodegradability tests. A similar layout is adopted to present the results of the biodegradability assays, i.e. a set of plots, followed by a table in the parameters of interest are summarised. A single volumetric ratio was tested for both the landfill leachates and the textile effluents, so that an inter-comparison of the extent of biodegradation attained by the three leachates that were tested at different concentrations may be poorly meaningful; certainly the concentration effect could not be evaluated.

Generally, the curves of the cumulative net volume of methane are presented in this chapter, followed by a summary bar plot indicating the ultimate biodegradability of the test materials. Other plots are assembled in Sections G.1.1 and G.1.2 for reference.

The quantitative results of the BMP assays are reported in a table at the end of the respective sections, which lists five parameters that have been selected to comprehensively summarise the outcome of a biodegradability assay.

These tests too were conducted following the conventional protocol which, in particular, does not require the gas composition to be regularly determined in combination with the volume of gas. Gas samples were extracted for gas chromatographic analysis on a weekly basis, which is sufficient for the objective of assessing biodegradability but does not enable one to calculate a step-by-step mass balance on the methane produced (equation B.4) and therefore the specific methanogenic activity (as pointed out above). However, since the test is aimed at assessing the ultimate biodegradability only, it is the overall mass balance the parameter of interest. This can be calculated by assuming that the volume of methane produced in a time interval is proportional to the differential volume of biogas only (through the measured methane molar fraction). This method temporarily neglects the variation of the volume of methane in the headspace, which however is only relevant in the early stages of the assay. The methane volume in the headspace is calculated at the end of the test, by adding the following quantity to the cumulative volume calculated:

$$\Delta V_{CH,H} = V_H \cdot f_{CH,end} \qquad (14)$$

where V_H is the volume of the headspace and $f_{CH,end}$ is the final value of the methane molar fraction in the off-gas. This is based on the reasonable assumption that, at the end of the test, the composition of the gas produced is practically constant, hence identical to the composition of the headspace.

A discontinuous determination of the gas composition may be sufficient to calculate the ultimate conversion of the organic matter to methane but does not enable one to calculate a meaningful value for the methanogenic activity. Therefore the sludge activity is reported in the summary tables.

4.1.1.1 Toxicity assessment of landfill leachates

The activity of the sludge exposed to the Holfontein leachate was practically unaffected up to a volumetric ratio of 4 % v/v (equivalent to a concentration of 3.6 g COD/ℓ). In fact, the sludge activity seems to be slightly enhanced as relatively to that of the reference units; however, this cannot be taken as a conclusive evidence because the greater biogas production might have consisted largely of carbon dioxide. At higher concentration levels, the activity of the sludge progressively decreases and ceases completely at the highest concentration tested (Figure 14 A).

The Aloes leachate too left the sludge activity unaffected up to a volumetric ratio of 4 % v/v (equivalent to a concentration of 1.6 g COD/ℓ) and rather slightly enhanced by about 10 % to the control units. The next level of concentration tested (10 % v/v) showed a clear drop in activity, which then, unexpectedly raised at 20 % v/v (Figure 14 B). We believe though that this has to be attributed to an experimental error or inaccuracy in the determination and is to be disregarded. Otherwise, the very low residual activity (10 % only of the control) that was measured at the highest concentration would appear an unrealistically abrupt change from no-inhibition to almost full-inhibition, although the COD concentration almost doubles. Furthermore this pattern would result qualitatively similar to that of the Holsfontein leachate (Figure 14 D).

The activity of the sludge exposed to the Shongweni leachate shows a consistent increase up to a volumetric ratio of 10 % v/v (1.1 g COD/ℓ), thereafter it decreases to a value comparable to that of the control at the highest concentration tested.

The missing information on the composition of the gas generated would have allowed a better understanding of this pattern which seems not very realistic: being the concentration of the reference substrate (the mixture of acetate and propionate) not limiting nor excessively high to

cause inhibition of the methanogenic biomass (0.9 g COD/ℓ; Table G.2), one would eliminate the hypothesis that the control units were inhibited and the presence of an alternative organic substrate had a beneficial effect on the biomass (a similar situation occurred in tests No 1 and No 2, discussed in sections 4.1.3.1 and 4.1.3.2). The only possible explanation is therefore that the biogas produced was in fact composed mainly of carbon dioxide and other fractions generated by the rapid fermentation and acidification of the test material.

This is a fundamental methodological observation which demonstrates that the response of a serum bottle test could be completely misleading when the determination of the gas volume is not accompanied by that of the gas composition.

A concentration of 4 % v/v for the Holfontein and Aloes leachates and of 10 % v/v for the Shongweni leachate were selected for the determination biodegradability.

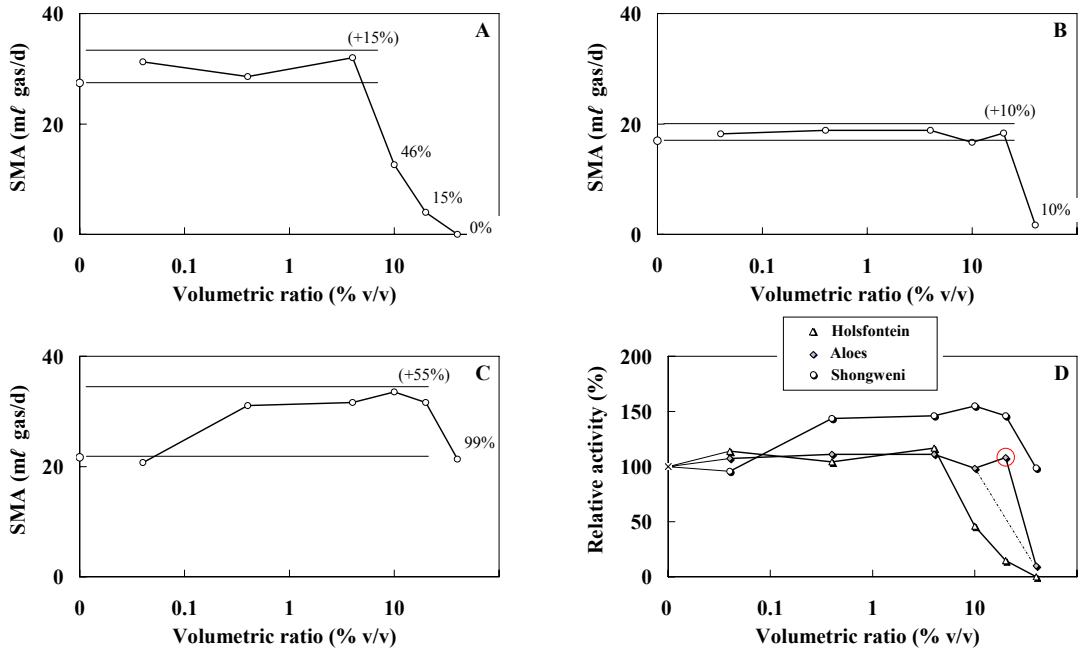

*Figure 14 - Maximum sludge activity for the Holfontein (**A**), Aloes (**B**) and Shongweni (**C**) leachates vs. volumetric ratio (% v/v, logarithmic scale).*
*Figures indicate activity, compared to the control (reference unit). Plot **D** presents a synthesis of the relative activity vs. volumetric ratio.*

Table 6 – Summary of the results of the toxicity assay on the leachates.

Parameter		Control	0.04 %	0.4 %	4 %	10 %	20 %	40 %
Holsfontein leachate								
Ultimate gas volume	mℓ	66±2	87±4	83±4 [(a)]	102±3 [(a)]	59±2 [(a)]	10±0	1±0
Max sludge activity	mℓ/d	27±0	31±1	29±0 [(a)]	32±2 [(a)]	13±1 [(a)]	4±1	[(b)]
Lag-phase	d	0	0	0	0	> 1	> 3	[(b)]
Aloes leachate								
Ultimate gas volume	mℓ	56±1	57±3	59±1 [(a)]	62±1	60±2	58±2	3±1
Max sludge activity	mℓ/d	17±0	18±1	19±0 [(a)]	19±1	17±1	18±2	[(b)]
Lag-phase	d	0	0	0	0	0	≈ 1	[(b)]
Shongweni leachate								
Ultimate gas volume	mℓ	67±0	67±1	104±1	104±0	100±1	100±1	98±1
Max sludge activity	mℓ/d	22±1	21±1	31±1	32±1	34±2	32±1	21±1
Lag-phase	d	0	0	0	0	0	< 1	1

Figures refer to three replicates (N=3).
[(a)] number of replicate considered in the calculation: N=2.
[(b)] not determined: the volume of gas produced at the highest concentration of Holfontein and Aloes was negligible after 7 days and did not allow a meaningful quantification.

4.1.1.2 Biodegradability assessment of landfill leachates

The main observation that one can draw from the results of the biodegradability assessment of the landfill leachate relates to methodological issues.

Firstly, the volume of biogas shows a step-like progression which is generally consequence of a very low activity of the sludge that results in a very low volume of gas produced between two re-equilibrations; or of a limited mixing of the suspension that can favour the temporary accumulation of the gas and only release discrete volumes discontinuously.

Neither of these circumstances could be ascertained because the test was not repeated under different conditions (e.g. sludge concentration) and/or using a refined methodology.

A consequence of this apparently erratic behaviour of the gas production is that the general reproducibility of the assay is poor (Table 7). This is possibly worsened by the relatively low volume of gas that is generated in the test units, in excess to that generated in the controls, which inevitably translates into a low sensitivity of the detection of the 'signal', i.e. the pressure generated inside the vials. This is a crucial aspect that has to be evaluated when planning a serum bottle test.

A second aspect worth noting is that the gas composition too does not show a consistent, gradual variation: particularly, the methane fraction in the test units containing the Shongweni leachate (Figure G.12) shows an unrealistic decrease between the second and the fifth week (which is mirrored by a similar increase in the nitrogen fraction); the control units of the test on the Aloes leachate (Figure G.10) show a sudden peak towards the end of the experiment, which is clearly an outlier compared to the adjacent values. This brings us to another methodological recommendation, i.e. one single analysis on one individual unit (particularly when the sampling frequency is as low as once a week) is not sufficient to identify possible faulty units or unrealistic patterns that, cannot be corrected if backup data from a replicate analysis, a replicate unit, or a precedent or subsequent determination are not available. This results in totally unreliable mass balances.

A third aspect is to be reported that is the low concentration of carbon dioxide in the headspace, which was consistently in the range of 10-15 % (Figures G.8, G.10 and G.12).

This is generally the consequence of flushing pure nitrogen through the suspension prior to starting the test, which causes most of the dissolved carbon dioxide to strip, thus progressively unbalancing the carbonate equilibrium, in the direction of consuming bicarbonate alkalinity. These circumstances are to be avoided (by only flushing the headspace, Annexure B), because the suspension is left with very little, if any, buffering capacity to counteract a potentially excessive production of volatile acids.

The overall results of this exercise are reported in Table 7. Surprisingly, although the ultimate fraction of methane was relatively similar in all test units and certainly within a methanogenic range (50-60 %), the activity of the sludge was generally very low and comparable to that of the controls and the extent of biodegradation attained by the three leachates was very low. It is worth noting that although the ultimate gas production in the units containing the Shongweni leachate was only 5 mℓ larger than that in the control units, this corresponded to almost 70 % biodegradation.

This figure is totally unreliable and can be explained with the observation on the sensitivity of the detection, discussed above: when the difference between the outcomes of the test units and of the controls is so little and at the same time corresponds to a large fraction of the organic carbon of the test material, a small inaccuracy in the manual determination of the gas volume would dramatically affect the outcome (Remigi and Foxon, 2005).

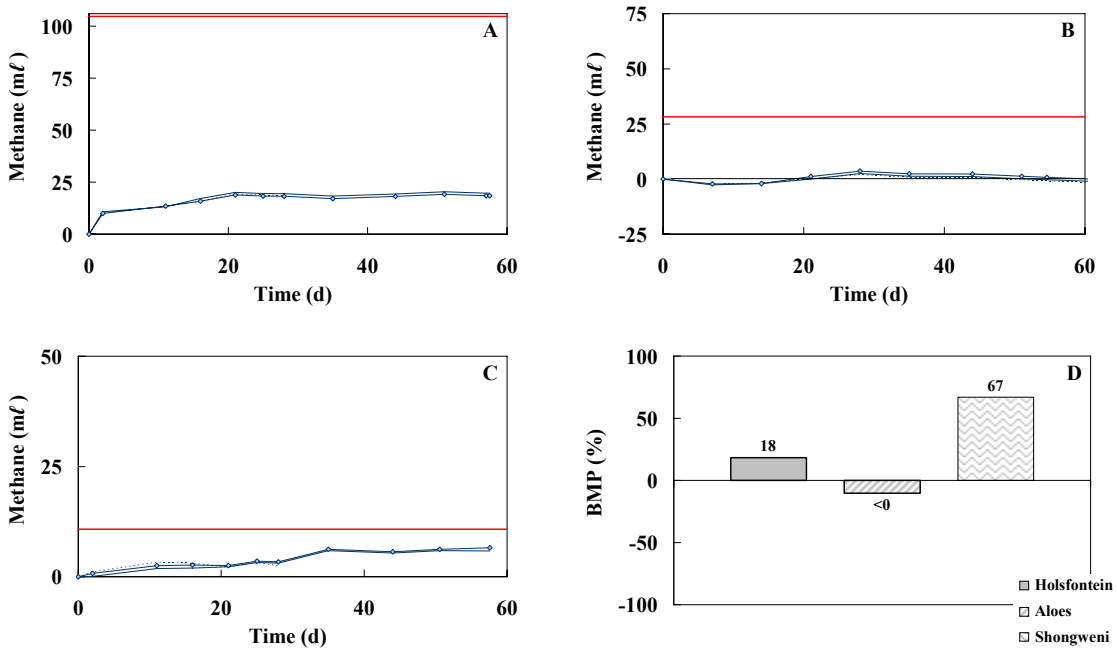

*Figure 15 - Net methane production for the Holsfontein (**A**), Aloes (**B**) and Shongweni (**C**) leachates; overview of the biodegradability potential (BMP) of the three landfill leachates (**D**).*

Table 7 – Summary of the results of the biodegradability assay on the leachates.

Parameter		Control	Test units (*)
Holfontein leachate			
Ultimate CH_4 fraction	%	55 – 58 (N=1)	54 – 56 (N=1)
Ultimate gas volume	mℓ	31 (N=1)	50±1 (N=2)
COD-to-CH_4	%	-	18±1 (N=2)
Max sludge activity	mℓ/d	3±1 (N=3)	15±1 (N=3)
Lag-phase	d	0	0
Aloes leachate			
Ultimate CH_4 fraction	%	61 – 62 (N=1)	58 – 59 (N=1)
Ultimate gas volume	mℓ	38±2 (N=3)	36±1 (N=3)
COD-to-CH_4	%	-	< 0
Max sludge activity	mℓ/d	2±1 (N=3)	7±0 (N=3)
Lag-phase	d	0	0
Shongweni leachate			
Ultimate CH_4 fraction	%	55 – 58 (N=1)	55 – 59 (N=1)
Ultimate gas volume	mℓ	32±3 (N=2)	37±1 (N=2)
COD-to-CH_4	%	-	67±5 (N=2)
Max sludge activity	mℓ/d	3±1 (N=3)	3±1 (N=3)
Lag-phase	d	0	0

(*) Holfontein and Aloes were tested at 4 % v/v; Shongweni at 10 % v/v.

4.1.1.3 Toxicity assessment of textile effluents

The methodological weakness in conducting serum bottle (toxicity) assays that was pointed out for the previous test on landfill leachates is even more evident in the outcome of this experiment on the sizing effluent labelled as Recipe No 2 and on the individual components, i.e. starch, wax and PVA.

The rationale of comparing the biodegradability and toxicity of a composite effluent to that of its individual components is to possibly identify the component(s) which contribute(s) the most to the poor performance of the mixture in an anaerobic digester. Based on this knowledge, one could pre-expose the sludge to that particular component to favour the adaptation of the biomass and ultimately improve the degradability of the mixture.

When the individual components of the mixture are characterised by very different COD concentrations, such is the case of starch, wax and PVA (Table 5), the set-up of the serum bottles is to be adapted to each test material, bearing in mind that the parameters of interest are the total concentration of organic matter and the proportion of organic matter deriving from the test material (Remigi and Foxon, 2005). Those parameters are to be consistently maintained across the groups of vials to ensure meaningful results.

On the contrary, a consistent volumetric ratio (Table G.4) will almost certainly result in a poor reproducibility, because a given volume of test material might be appropriate for a component with high COD, but could be insufficient for a component with low COD to ensure a gas production sufficiently different from the baseline (i.e. a clear signal, as discussed previously).

▪ Unexpectedly, the control units too showed a poor reproducibility (Figure G.13) with an ultimate variation coefficient as high as 13 % (Table 8). This circumstance alone indicates the failure of a serum bottle test, because such a large uncertainty on the baseline activity makes inevitably meaningless the quantification of the relative activity, hence inhibition effect, of the test materials.

A tentative mass balance can be carried out to verify the reliability of the output of the control units. One would expect that the mixture of acetate and propionate be fully degradable within the experimental period of 7 days. The stoichiometric methane production for the 2 mℓ of the acetate-propionate solution is 33 mℓ. The ultimate methane fraction can be calculated (approximated by excess), as follows:

$$f_{CH} \approx \frac{v^{*}_{CH}}{\Delta v_{end} + V_0} = \frac{33 m\ell}{(38+25) m\ell} = 52\%$$

where v^*_{CH}, Δv_{end} and V_0 denote the stoichiometric volume of methane, the volume of gas generated and the volume of the headspace.

Considering that the substrate is acetate and propionate, this methanogenic fraction seems low. One could probably hypothesize that, rather than the reference substrate not being entirely degraded, the set-up was not very accurate.

▪ The sizing effluent showed a progressively larger biogas production up to a volumetric ratio of 10 % which however decreased at higher volumetric ratios. It is interesting noting the progression of the shape of the cumulative gas production curves (Figure G.13): at 4 % v/v, the gas production seems to level off after 4-5 days, as the biodegradable fraction of the effluent had been fully degraded; at 10 % v/v, the gas production seems to be continuing at a rather constant rate beyond the experimental period; at 20 % v/v, it levels off temporarily after 4 days and starts off again on day 5; at 40 % v/v, it seems to have levelled off after 4 days but does not start off before the end of the test. This qualitative picture is compatible to an easily hydrolysable and fermentable organic substrate, which however as the concentration increases, requires a progressively longer time for the methanogenic biomass to be able to process the excess acidity generated. Therefore the 10 % volumetric ratio seems to be the safest dilution at which the effluent does not affect (even only temporarily) the methanogenic step. Most likely, the determination of the gas composition would have showed a progressive increase in the carbon dioxide molar fraction in the units at 20 % v/v, until day 4-5.

The maximum biomass activity too increased up to a concentration of 10 % v/v and then levelled off (Figure 16 A): the value indicated by the arrow is probably due to an inaccurate measurement.

▪ The maize starch contributes the most to the total organic content of the sizing effluent. One would therefore expect that the outcome of the test with starch as the test material is at least qualitatively comparable to that of the effluent. At 4 % v/v, the gas production started off after approximately 1 d. Since starch is expected to be readily biodegradable, this apparent lag-phase is very likely caused by a transient accumulation of carbon dioxide as a result of the prompt conversion of starch to fatty acids. The gas production seems to have levelled off by the end of the experimental period. Unexpectedly, this initial lag-phase does not appear in the test units which received larger volumes of starch.

At 10 % v/v, the rate of gas production is initially high, then decreases after 2 d and starts off again after 4-5 days.

In general, the reproducibility of these units too was not very good and resulted in a large degree of uncertainty. Particularly at 20 % v/v, the activity of the sludge seems unrealistically high as compared to that at 10 % and 40 % v/v and is very likely due to an error in the manual determinations (Figure 16 B)

▪ The first aspect that is to be noted for the test units supplemented with wax is that up to a concentration of 10 % v/v the stoichiometric volume of methane, due to the wax, was only 17 mℓ (52 %) in excess to that due to the reference substrate. As discussed above, these circumstances indicate a very low reliability of the test due to the potentially very high sensitivity of the manual determination of the gas. This could have been avoided when designing the test, by considering that being the organic strength of the stock solution of wax only 1/20 of that of the sizing effluent, the same dilution rates could not be successfully applied. The activity of the sludge calculated for volumetric rates higher than 10 % v/v showed a consistent increase with the concentration (Figure 16 C) which could be in agreement with this explanation.

In fact, the replicate units showed uneven behaviours, particularly at 4 % and 40 % v/v which make the calculation of any meaningful summary parameter very difficult (Table 8). Qualitatively, one can only remark that the gas production at all dilutions was very low, which could possibly indicate that wax is the most critical component of the sizing mixture.

▪ The outcome of the test units containing PVA as test material is somewhat opposite to that of wax: although the organic strength of PVA is of the same order of magnitude of that of the sizing effluent (and of starch), the reproducibility of the replicates was poor (particularly at 4, 10 and 20 % v/v; Figure G.19); and the cumulative gas production after 7 d is relatively low at all concentration levels, thus suggesting a poor biodegradability potential for PVA (Table 8).

The ‘weakness of the signal’ in the case of PVA cannot be invoked as the cause of the poor reproducibility; instead it is possible that the suspension supplemented with PVA was not homogeneous, thus hampering the release of the gas generated.

Unlike with the composite effluent or with the starch, the activity of the sludge is not remarkably affected by PVA (Figure 16 D).

The overall conclusion that can be drawn from this exercise is inevitably only qualitative (and more importantly would have to be confirmed by repeating the assessment and accurately quantifying the fraction of methane in the gas):

- the sizing effluent is not inhibitory to the anaerobic biomass, possibly due to the prevailing proportion of starch in its matrix;
- the wax could be a critical component of the effluent, particularly in terms of inhibitory potential;
- PVA could be critical due to its low biodegradability or to a relatively long period of adaptation required by the biomass to actively degrade it.

Therefore, if one ought to improve the performance of an anaerobic digester treating the size effluent, a valuable option could be that of pre-exposing the sludge to wax and/or PVA.

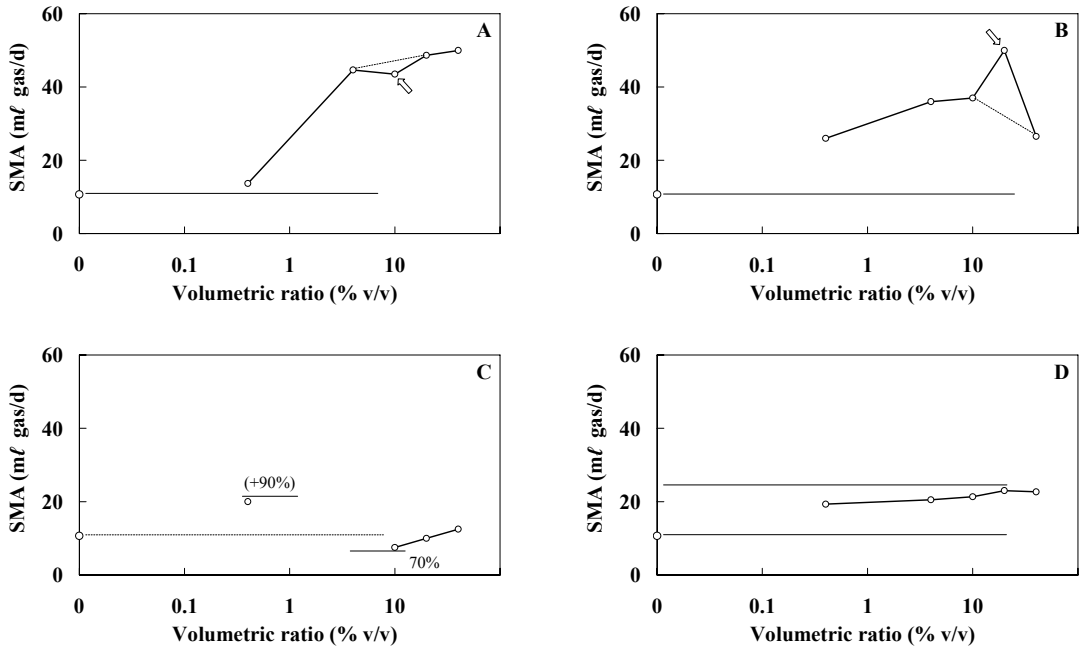

*Figure 16 – Maximum sludge activity for the sizing textile effluent, recipe No 2, vs. volumetric ratio (% v/v, logarithmic scale): mixture (**A**) and components, i.e. starch (**B**), wax (**C**) and polyvinyl alcohol (**D**). Figures indicate activity, compared to the control (reference unit). Arrows indicate probable outliers.*

Table 8 – Summary of the results of the toxicity assay on the sizing effluent 'Recipe No 2'.

		Control	0.4 %	4 %	10 %	20 %	40 %
Sizing effluent (mixture)							
Ultimate gas volume	mℓ	38±5	49±5	157±3	160±4	127±2	111±9
Max sludge activity	mℓ/d	11±1	14±3	45±2	44±1	49±2	50±0
Lag-phase	d	-	0	0	0	0	0
Starch							
Ultimate gas volume	mℓ	38±5	72±3	132±1 [(a)]	147±7 [(a)]	131±1 [(a)]	114±5 [(a)]
Max sludge activity	mℓ/d	11±1	26±2	36±3 [(a)]	37±4	50±0 [(a)]	27±1 [(a)]
Lag-phase	d	-	≈ 1	0	0	0	0
Wax							
Ultimate gas volume	mℓ	38±5	57±6	n.a.	28±1 [(a)]	37±1 [(a)]	39±3 [(a)]
Max sludge activity	mℓ/d	11±1	20±2	n.a.	8±1 [(a)]	10±0 [(a)]	13±1 [(a)]
Lag-phase	d	-	n.a.	n.a.	n.a.	n.a.	n.a.
PVA							
Ultimate gas volume	mℓ	38±5	53±6	56±2 [(a)]	63±7	60±1 [(a)]	69±5
Max sludge activity	mℓ/d	11±1	19±2	21±1 [(a)]	21±1	23±1 [(a)]	23±1
Lag-phase	d	-	0	0	0	≈ 1	0

Figures refer to three replicates (N=3).
[(a)] number of replicate considered in the calculation: N=2.
[(b)] not determined: the volume of gas produced at the highest concentration of Holsfontein and Aloes was negligible after 7 days and did not allow a meaningful quantification.

4.1.1.4 Biodegradability assessment of textile effluents

The observations reported for the toxicity assay on the sizing effluent are largely confirmed by the outcome of this biodegradability experiment, which lasted 30 days.

▪ The quality of the data of gas volume is poor and the frequency of the data of gas composition is too low. This translated in an overall reproducibility of the outcome of the serum bottle test which is insufficient to obtain reliable quantitative information on the biodegradability of the effluent and its individual components. The following discussion will be therefore mainly qualitative. However, it is interesting noting that the accuracy of the determination of the gas volume is significantly higher for the test units containing the effluent than for the control units (Figure 17).

This can be due to the low sensitivity of the syringe used for the manual re-equilibration: as discussed in Annexure B, the volume of the syringe should be selected in function of the actual gas production of the serum bottles. In this case, a medium size syringe (e.g. 10-

20 mℓ) was obviously appropriate for a high rate of gas production (in the test units) and a small size one (e.g. 2 mℓ) should have been used for the reference units.

▪ It has also been pointed out in the previous section that the relative proportions of the different components that are added to a serum bottle are to be chosen taking the concentration of organic matter and of solids, hence their ratio, as a reference. This is particularly evident by looking at the composition of the vials for this test (Table G.5), in which the test units containing the wax are a clear example of incorrect set-up: by taking the dilution rate of the test materials as reference, the substrate-to-biomass ratio S-to-X for these units as about 20 times smaller than that of the other test units.

Besides the objectively low biodegradability of the wax, this factor could have contributed the poor overall biodegradability attained by the wax. Moreover, the stoichiometric volume of methane for these units, due to the organic matter supplemented as wax, was only 7 mℓ, which explains the low sensitivity of the determination on these units.

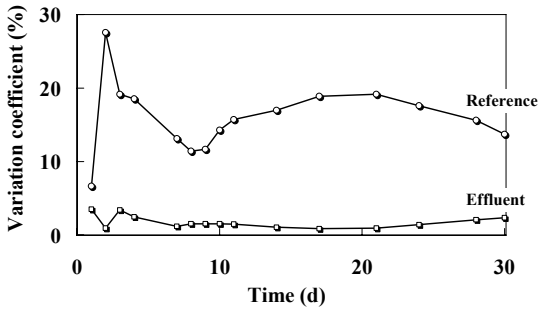

Figure 17 – Variability of the cumulative volume of gas measured on the reference units and on the test units containing the sizing effluent.

▪ Unexpectedly, the mass balance conducted on the reference units too indicates a low *recovery* of the COD supplemented as acetate and propionate mixture (Table 9). A similar unrealistic situation has been reported for the 7-day toxicity test. This can only be explained by a poor quality of the data, both of volume and composition of the gas generated, as discussed earlier.

However, the other test units not being supplemented with the reference substrates, the outcome of these units does not invalidate the whole experiment.

▪ The units containing 4 % v/v of the sizing effluent showed a good reproducibility between replicates and attained both a relatively high biodegradation and maximum sludge activity (Table 9).

An interesting observation can be done by examining the plot of the sludge activity that shows a temporary abrupt drop of the calculated value of activity from 4 mℓ/d to zero, between days 9 and 10 (Figure 18 A). This is clearly to be attributed to an experimental error, most likely in the determination of the gas volume, since the similar values of activity before and after the transient indicate that the rate of gas production at that stage of the process was nearly constant (dashed line in Figure 18 A). This ultimately suggests that the value of BMP calculated for the sizing effluent is underestimated. If one corrects the values of activity for the period between day 9 and 10 (e.g. using a simple linear regression), and re-calculates the gas production and methane production from the values of activity (data not shown), the ultimate volume of methane generated is 86 ± 2 mℓ which translates in a slightly better biodegradation figure, i.e. 42 ± 1 %.

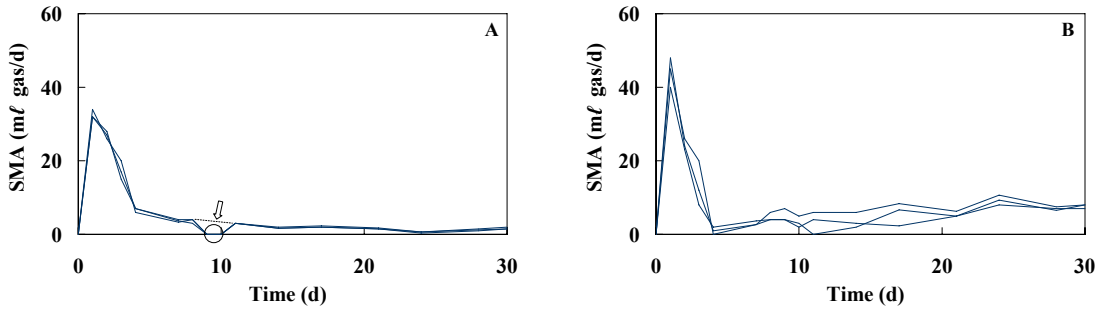

*Figure 18 – Biomass activity, calculated as the rate of biogas production in the test units containing the sizing effluent (**A**) and containing the starch (**B**).*

▪ The outcome of the test units containing 4 % v/v of wax is meaningless for the reasons exposed above: the combination of excessively low substrate available for the biomass (hence a low S-to-X ratio) with an inherently low biodegradability (and possibly high inhibitory potential) of the wax, resulted in an ultimate gas production and in a maximum sludge activity only slightly greater that those of the controls. But the poor reproducibility of these units does not allow one to trust these outputs.

▪ Unlike the units containing wax, those containing 10 % v/v of PVA received a sufficient amount of organic matter that corresponds to a stoichiometric volume of methane of 196 mℓ, which should be clearly detectable. The outcome of these units can surely be trusted in that it indicates an almost negligible biodegradability of the PVA and a very low activity attained by the sludge (Table 9).

▪ The test units containing 10 % v/v of starch showed generally poor reproducibility between the replicates, because the gas production in one of the vials shot up after 7 d and continued almost parallel to the other two vials (*Figure* 19). However the extent of biodegradation attained by starch (and calculated on two replicates only) is slightly better than that of the sizing effluent and the maximum activity of the sludge has significantly improved (Table 9).
This suggests that the starch contributes the most to the biodegradability of the sizing effluent. It also suggests that the low activity attained by the sludge when exposed to the effluent is mostly due to the other components of the recipe which must therefore have a true inhibition potential (although the poor quality of the data has not allowed us to quantify such potential).
It is interesting noting the particular patter of the sludge activity (Figure 18 B) which seems to suggest a two-stage degradation process: for the first 4 d, the readily biodegradable fraction is degraded and the activity of the sludge peaks on day 1; thereafter, the activity drops, then resumes (day 8) and stabilises at lower values of 5-10 mℓ/d throughout the experimental period, thus indicating that a slowly degradable fraction of the substrate is being metabolised.
A tentative mass balance can be carried out to verify the reliability of the two estimates of biodegradability of the effluent and of the starch. Taking into consideration that 50 % (f_{starch}) of the mixture is starch and assuming that the biodegradability of wax and PVA is negligible, one could calculate the *expected* biodegradability of the starch based on that *measured* for the mixture as follows:

$$BMP_{starch} = \frac{BMP_{mix}}{f_{starch}} = \frac{39}{0.50} = 78\%$$

This value is significantly higher than that actually calculated on the test units containing starch and would suggest that the vial that has been disregarded in the calculation could be in fact the most accurate of the set.

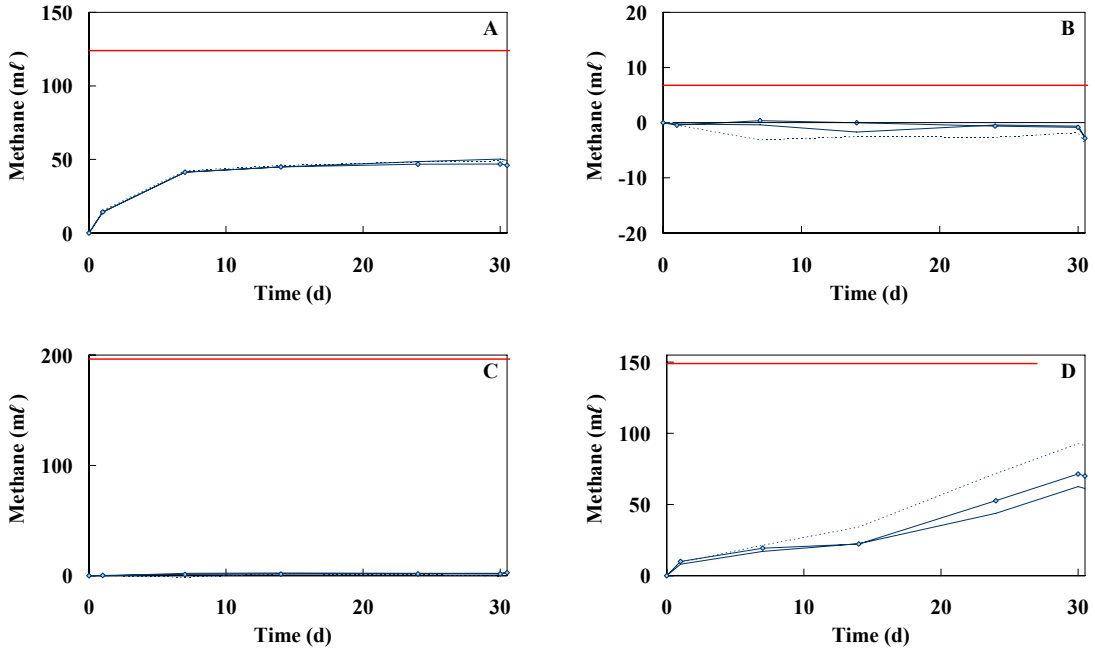

*Figure 19 – Net methane production for the sizing textile effluent: mixture (**A**), wax (**B**), polyvinyl alcohol (**C**) and starch (**D**). The solid lines indicate the stoichiometric methane production.*

Table 9 – Summary of the results of the biodegradability assay on the sizing effluent 'Recipe No 2'.

Parameters		**Control**	**Reference**	**Mixture**	**Wax**	**PVA**	**Starch**
Ultimate CH_4 fraction	%	54 – 59 [(a)]	70 – 71 [(a)]	55 – 56 [(a)]	48 – 52 [(a)]	56 – 63 [(a)]	52 – 54 [(a)]
Ultimate gas volume	mℓ	41±2	55±8	129±3	44±3	45±1 [(b)]	210±14 [(b)]
COD-to-CH_4	%	-	**63±10** [(b)]	**39±1**	**< 0**	**2±0** [(b)]	44±4 [(b)]
Max sludge activity	mℓ/d	4±1	15±1	33±1	6±1	7±1	44±4
Lag-phase	d	-	0	0	0	0	0

[(a)] number of replicate considered in the calculation: N=1.
[(b)] number of replicate considered in the calculation: N=2.

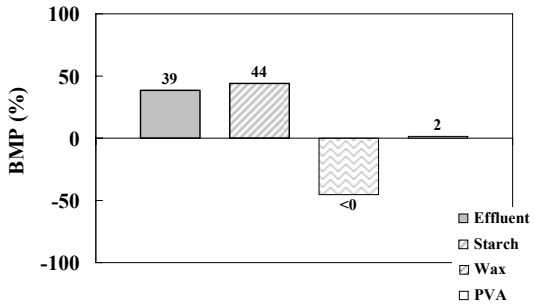

Figure 20 – Overview of the biodegradability potential (BMP) of the sizing effluent and its components.

4.1.1.5 Conclusions

The following methodological remarks can be derived from this exercise.

- It is mandatory that gas composition be determined for the toxicity assessment too, because a situation of organic overload may easily go undetected if the sole gas production is measured.
- The determination of gas composition is to be carried out simultaneously to the re-equilibration of the vials and not discontinuously; to improve the reliability of the determination, which is crucial for mass balance purposes, one single gas chromatographic analysis on one individual serum bottle is largely insufficient.
- Although biodegradability is theoretically concentration-independent, multiple levels of concentration should be examined: this would enable to validate the estimates and to detect possible reduction of biodegradation with increasing concentration of the test material, which indicates the overloading of the biomass.
- It is crucial that a sufficiently 'clear signal' is ensured when performing a serum bottle test: in other words, it is necessary that the volume of gas produced in the test units is significantly larger than that of the controls (and secondarily, that the differential gas production between re-equilibrations is sufficiently greater than the volume of the syringe used to quantify the volume). These circumstances are to be ascertained when setting up the test by ensuring that the residual organic substrate in the seed sludge, hence the baseline gas production, is sufficiently low; that the differential contribution to the organic carbon coming from the test material is sufficiently high; or by using a relatively low biomass concentration.
- When composite mixtures are tested, the total COD concentration and the fraction of COD deriving from the test material should be the key parameter for designing the assay. Test materials characterised by low COD content might need to be screened using unrealistically large volumes, to ensure the sensitivity of the serum bottle method.

4.1.2 Improved toxicity and biodegradability assay

Objectives

A first attempt to improve the methodology of serum bottle assays, in the direction of maximising the information that one can obtain was started as a conventional toxicity test aimed at studying the effect of a Navy shaded synthetic dye effluent.

Materials and methods

The set-up comprised reference units with acetate as substrate and two levels of concentration of the effluent, i.e. 10 % and 25 % v/v respectively. The effluent was prepared in the laboratory, according to a recipe provided by Frametex, by mixing the following stock solutions components in distilled water (figures indicate millilitres per 100 mℓ of distilled water): 1 % m/v Drimarene Navy (30), 1 % m/v Drimarene Red (6.6), 1 % m/v Drimarene Yellow (1.9), 10 % v/v Tebolan (2), 10 % v/v Dekol (6) and 10 % v/v Avcoson (8). The original recipe included 10 mℓ of 20 % m/v sodium carbonate stock solution, which was not used for this specific test.

All units were prepared in triplicate (Table G.6).

The headspace was re-equilibrated (i.e. the gas volume and composition determined) daily, for the first 10 days; thereafter, less regularly every 2 or 3 days.

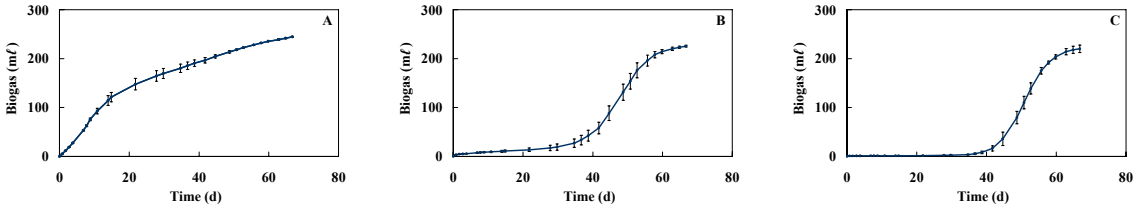

*Figure 21 – Average gas production in the reference units (**A**) and in the test units with 10 % (**B**) and 25 % v/v (**C**) of the synthetic dye effluent.*

Results and discussion

If one assumes the concepts summarised in (Figure B.1) as guidelines for the preparation of a serum bottle activity test, it is evident that two major mistakes were done.

- Sufficient care was not taken in flushing the vials with gas prior to starting the test. The appropriate nitrogen and carbon dioxide mixture was not available at the time, so that pure nitrogen had to be used. In these circumstances, it is recommended (Section B.2) to flush the headspace only, without passing the gas through the suspension; otherwise, the

dissolved carbon dioxide would be stripped out, leaving a low alkalinity in the solution, which would therefore be exposed to the risk of acidification. This had clearly not been the case (Figure G.25), since the carbon dioxide molar fraction ranged from 6±0 to 9±0 in the reference units; from 5±0 to 8±1 in the test units at 10 % v/v concentration; and from 3±0 to 6±0 in the test units at 25 % v/v concentration.

- A relatively high seed inoculum was used (100 mℓ) which resulted in a small headspace volume. As explained in Section B.1, it is always preferable to maintain a relatively large headspace-to-liquid volume ratio to avoid that the gas pressure builds up excessively between two subsequent re-equilibrations. These circumstances translate in an underestimation of the actual volume of gas produced, inversely proportional to the headspace-to-liquid volume ratio (Remigi and Foxon, 2005).

Table 10 – Summary of the results of the toxicity and biodegradability assay on the synthetic dyestuff.

Parameter		Controls	10 %	25 %
Ultimate CH_4 fraction	%	91±1	92±1	93±1
Ultimate CH_4 volume	mℓ	228±1	215±1	215±6
COD-to-CH_4	%	**126±0**	**< 0**	**< 0**
Max SMA	mg COD/g VS/d	38±3	42±2	65±2
Lag-phase	d	< 1	≈ 30	40-45

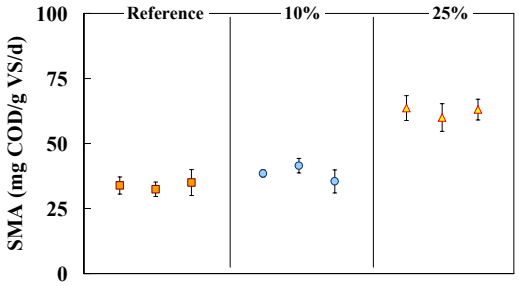

Figure 22 – Overview of the maximum Sludge Methanogenic Activity for the test on the synthetic dye effluent.

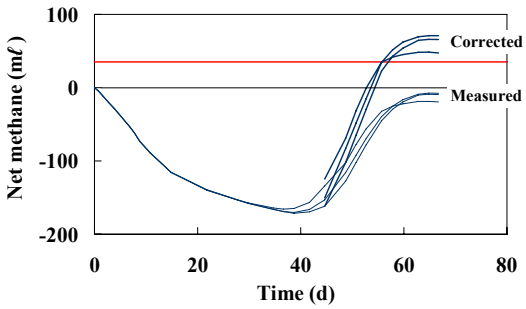

Figure 23 – Effect of the correction of the volume of gas measured, when the excessive overpressure is corrected through a mass balance.

The effect of the first procedural mistake is almost impossible to account for, in the endeavour of post-processing the experimental data, because the fraction of carbon dioxide left in solution, hence of alkalinity, depends on a variety of factors among which the duration and effectiveness of the nitrogen flow through the suspension. Furthermore, an insufficient alkalinity would risk to invalidate the outcome of the assay, in that the consequences of the permanent acidification of the suspension due to the low buffering capacity, would be

interpreted as irreversible inhibition effect of the test material, whereas in normal conditions, acidification would be promptly resumed or not even occur. Therefore the depletion of dissolved carbon dioxide (and alkalinity) should be avoided altogether.

In contrast, the effect of a small headspace-to-liquid volume ratio can be taken into account by correcting the determinations of gas volume (Remigi and Foxon, 2005). This however requires that the operator records each individual insertion of the needle. Since this is seldom the case, post-processing of the data would have a certain degree of uncertainty (as discussed hereafter): it is therefore preferable to ensure a large headspace-to-liquid volume ratio as prescribed in Section B.1.

The results of the test are presented in Figure 21.

Qualitatively one can observe that the gas production in the reference units does not reach a plateau but after 10 d it gradually decreases and continues at a much lower rate until the end of the experiment. This is reflected in the corresponding specific methanogenic activity (Figure G.26) that shows a clear decline in activity that eventually stabilises at a relatively constant value of 6±2 mg COD/g VS/d (N=42).

This may be explained by hypothesizing that a certain amount of residual organic matter was present in the seed inoculum, which gets utilised by the biomass once the acetate supplemented as reference substrate is exhausted. In fact, a similar trend in the gas production curves (Figure 21) and a similar baseline activity (Figure G.26) are shown in the test units.

A very long lag-phase was observed before the gas production in the test units started off. This may in fact be regarded as unusually long due to the low buffering capacity of the suspension: should the dyestuff have actually undergone a partial biodegradative process which resulted in a relatively large amount of volatile acids being produced, these may have temporarily inhibited the methanogenesis for a long time.

As a general remark, one can observe that the match between the replicate gas curves is very good, indicating an excellent reproducibility of the test (the variation coefficient on the ultimate volume of methane produced is as low as 0.5-2.5 %, Table 10).

Although the biomass required a long period to adapt to the test material, once the methanogenic activity resumed, it attained values higher than those of the reference (Figure G.26). This could indicate that the biomass had grown, and therefore that the specific methanogenic activity calculated using the initial biomass concentration is overestimated; or

that the SMA measured in the reference units in the early stages of the test was in fact inhibited by the excess acidity not buffered in the suspension.

If one observes the absolute values of the maximum SMA, these do not differ too much from the value calculated in the validation experiment (Figure 1 B) which can be assumed as most accurate estimation of the actual aceticlastic methanogenic activity, i.e. 57±5 mg COD/g VS/d. This could therefore suggest that the biomass had grown; however, the solid concentration was not determined at the end of the test and no definite conclusions can be drawn.

Another methodological observation can be made with reference to Figure 22: the symbols represent the maximum specific methanogenic activity (SMA) in each unit and the error bar the standard deviation, calculated as explained in Section B.3.3. The deviation between the maximum and two adjacent values is very small in all cases, thus indicating that the frequency of re-equilibration was adequate, hence the determination of SMA accurate.

The other parameter of interest in a serum bottle activity test is the assessment of the extent of biodegradability that the test material can achieve, which is represented by the ultimate volume of methane produced compared to the initial organic matter available. In Table 10, the ultimate volume of methane indicates the total volume of methane measured at the end of the test in a specific unit. The COD-to-CH_4 conversion factor is calculated taking only into consideration the substrate targeted in each unit, i.e. acetate for the reference group and the dyestuff effluent for the test units and is to be regarded as the acetate recovery and the actual biodegradability of the test material respectively.

If one looks at the ultimate volume of methane, the difference between the reference group and the two test units is very small. This would suggest that the organic matter present in the synthetic dye effluent (i.e. 36 and 91 mg COD in the 10 % and 25 % v/v test units, corresponding to a stoichiometric additional volume of methane of 14 and 35 mℓ respectively) has not been converted to methane (although a partial degradation to intermediate products cannot be excluded). This is the reason for the negative values of COD-to-CH_4 in the test units.

A value greater than 100 % for the acetate recovery in the reference units is generally indicative of the presence of residual organic matter in the seed inoculum. This had not been quantified prior to start the test and assuming that the acetate supplemented was the only source of carbon is a clear underestimation that has lead to the unrealistic value of the recovery.

As pointed out at the beginning of this discussion, a factor that could have heavily influenced the outcome of the test in the low headspace-to-liquid volume ratio which might have resulted in the underestimation of the volume of gas produced. Should this have occurred, the result depicted by the negative COD-to-CH_4 ratio may change favourably.

Unfortunately, this was a case where the operator had not recorded every insertion of the needle, so that a rigorous mass balance is not possible. Nonetheless we wanted to validate the underlying principle and the daily cumulative volumes of gas measured, together with the knowledge of the glass syringe used are sufficient to the scope. The general practice had been to re-equilibrate the headspace of each vial using the 10 mℓ syringe repeatedly until the last re-equilibration, when the smallest syringe available (2 mℓ) was used. Under these assumptions, we considered for the test units at 25 % v/v of the dye effluent the individual gas volume determinations that exceeded 12 mℓ (raw data, available on the CD; Annexure J): as a threshold value, we set 13-15 mℓ, since the error associated is negligible and post-processing those data would not be worthwhile. In fact, several daily cumulative determinations exceeded 30 mℓ, which generate an underestimation of the actual volume of gas produced of about 28 % (on a 25 mℓ-volume headspace).

We implemented a macro in Excel that automatically re-calculates the correct volume of gas produced, given the cumulative volume measured and the volume of the syringe used (it assumes that the last determination is done with a 2 mℓ-volume syringe).

The result of this exercise is depicted in Figure 23. In this particular case, by only adjusting a few gas volume determinations, the previous conclusion is overturned, in that the synthetic dye effluent seems to be completely biodegradable (COD-to-CH4 ratio = 195±10 %, N=2). Furthermore, the volume of methane generated in excess to the stoichiometric (33±4 mℓ) is relatively close to the corresponding quantity in the reference units (63±1 mℓ) –taking into consideration the inaccuracy inherent to this approach. This would indicate that a certain amount of organic matter was present in the seed inoculum and resulted in a consistent unaccounted volume of methane in all units; the acetate supplemented as substrate in the reference units was stoichiometrically recovered as methane; the dye effluent at a concentration of 25 % v/v may in fact be completely biodegradable after a lag-phase of 40 d.

The purpose of this exercise was not to quantitatively compensate for the effect of the small headspace-to-liquid volume ratio: inevitably, when a number of crucial information is missing (e.g. occasionally a 50 mℓ-volume syringe was used), such method of post-processing data

may be not rigorous but is certainly sufficient to give an idea of the magnitude of the error that repeated insertions of the needle, ultimately due to excessive overpressure, can cause.

However, following the observation that although the biomass activity resumed after a lag-phase the ultimate methane produced indicates that no degradation of the dyestuff had occurred, an interesting aspect to ascertain would have been whether other forms of dyestuff utilisation had occurred, e.g. decolourisation, by measuring the absorbance of a filtered sample at the end of the test. The most obvious follow-up of such assay would be to harvest the biomass that had been exposed to the test material and use it as seed inoculum for another test (ideally using the same concentration levels), to verify whether the biomass had adapted (e.g. shorter or non-existing lag-phase).

In this event, a further aspect that one could examine is the way in which the biomass has adapted. For instance, using microbiological tools, one could verify whether a change in the population dynamics has occurred, in favour of more specialised groups or if the existing population has actually developed the ability of processing the test material.

Conclusions

This test showed the substantial reliability of a serum bottle activity test, as a tool to simultaneously assess the toxicity and biodegradability of a test material and the adaptation of the biomass to the test material. It has also provided valuable indications as to how an incorrect set-up can affect the outcome of the assessment. It further enabled us to validate the mass balance to account for the excessive build-up of pressure inside the vials.

Ultimately, although none of the objectives of a study on biomass adaptation could be pursued, they form a possible guideline for the application of the serum bottle method to such investigations.

4.1.3 Modified anaerobic activity tests

This 'hybrid' between a toxicity and a biodegradability test originated from a pure toxicity test, in which the effect of the presence of a test material on the degradation of a methanogenic precursor (e.g. acetate) was investigated. However, we realised that, by simply monitoring the process for longer times, much information about the impact of the test material on the anaerobic digestion could be obtained. Moreover, by simultaneously testing two test materials, in isolation and combined, the co-digestion process could be effectively simulated on a small scale.

4.1.3.1 Test No 1 – individual components

Objectives

This test was aimed at evaluating *i*) the effect of the addition of the Nutrient and Minerals salts Solution (NMS) on the performance of the seed sludge; and *ii*) the response of the biomass to the presence of the test materials. The following aspects were evaluated:

- the toxicity of the test materials on the methanogenic stage;
- the biodegradability of the test materials; and
- if a toxic effect had been displayed in the early stages of the process, whether the biomass was able to adapt to the test material, after a sufficient period of time.

Table 11 – Chemical oxygen demand (g/ℓ) of the industrial effluents screened by the anaerobic activity tests.

Distillery	Scour	Size	Synthetic dye
122±3 (N=6)	4±0 (N=6)	76±2 (N=6)	12±1 (N=6)

Materials and methods

The industrial effluents tested were distillery, scour, size and dye effluents. They were characterised in terms of their chemical oxygen demand (Table 11).

To answer the first question, i.e. whether the use of a NMS could improve the performance of the seed sludge, three controls groups were prepared, each consisting of three replicate units:

- *group A* (termed control) contained only the sludge (40 mℓ): it provided the background gas production, hence activity, due to residual organic matter and/or endogenous decay. It is expected to be relatively low.
- *group B* (termed reference-no NMS) contained the sludge (40 mℓ), supplemented with acetate (3.5 g/ℓ) to trigger the methanogenic activity of the biomass. It is expected to display a gas production significantly higher than that of group A.

- *group C* (termed reference) contained the sludge (40 mℓ), acetate (3.5 g/ℓ) and the NSM (10 mℓ). If the seed sludge was originally lacking some nutrient or growth factor, this group would display a faster onset of gas production and possibly a larger volume of methane would be produced.

The ultimate volume of methane that one could expect from groups B and C was calculated to be 105 mℓ (equation B.2).

To answer the second question, i.e. to evaluate the effect of the test materials on the methanogenic activity of the sludge, three levels of concentration were prepared for each test material, containing 9 %, 23 % and 41 % v/v of the test material, therefore indicated as 'low', 'medium' and 'high' concentration levels respectively. Each group consisted of three replicates. All groups contained the seed inoculum, acetate as reference substrate and NMS: therefore, their gas production was compared to that of group C of the controls.

The detailed composition of the vials is summarised in Table G.7.

The experiment lasted 81 days and was monitored daily (with few exceptions), by measuring the volume of gas produced and gas composition, according to the procedure outlined in Annexure B. The excess gas was wasted, after quantification.

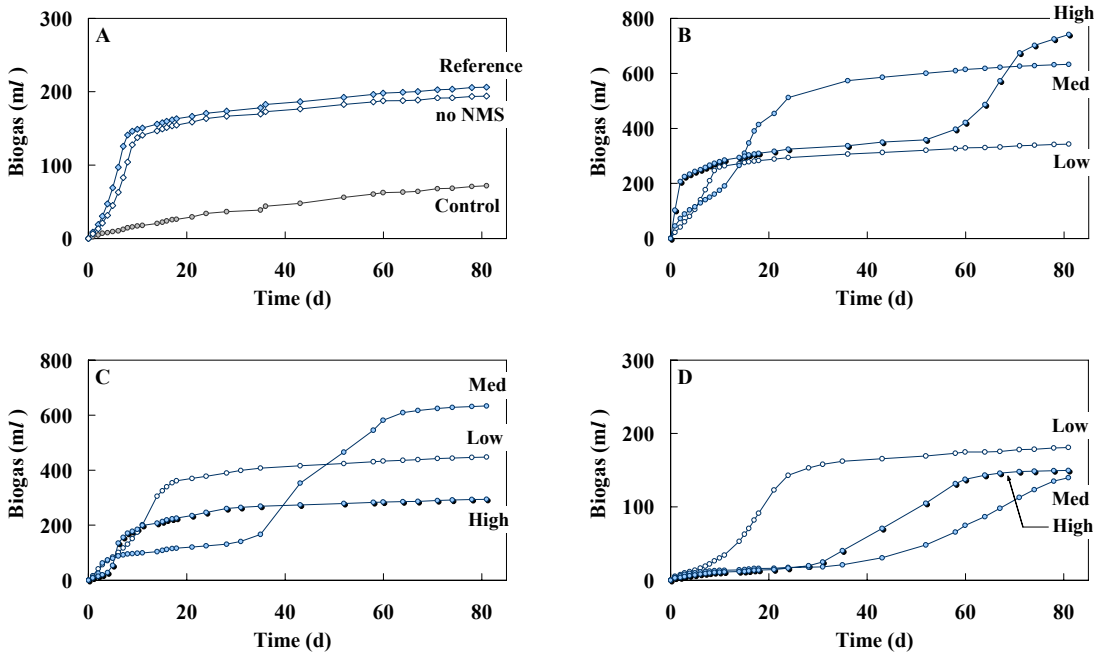

*Figure 24 –Average gas production in the control units (**A**), in the units with size effluent (**B**), with distillery effluent (**C**) and with scour effluent (**D**).*

Results and discussion

The cumulative biogas production curves for the controls, size, distillery and scour units are reported in Figure 24. The units which received the synthetic dye did not display any appreciable gas production, therefore are not shown. Each curve in the figure represents the average of the three replicates. Error bars are not reported for clarity; however, the variation coefficients were constantly in the range 1-6 % (except for the scour units, values as high as 10 % towards the end of the experiment were calculated): this indicates a very good reproducibility between replicates.

The background gas production (Figure 24 A), on the residual organic matter possibly present in the seed sludge and/or on the endogenous decay of the biomass, was low (with an ultimate volume of methane as low as 47±1 mℓ, Table 12): this indicates that the outcome of the test can be trusted, since the external organic substrate has not significantly affected the gas production.

The addition of a nutrients and mineral supplement to the sludge does not seem to be necessary in this case, possibly because the seed inoculum was withdrawn from a well-operated full-scale anaerobic digester and likely contained those elements in excess. The difference in the ultimate gas production of the control with and without nutrients (Table 12) is statistically non-significant, as it falls within the range of variability of the results. A possible effect of the addition of the NMS could be seen in the slightly faster onset of biomass activity and a smaller overall conversion of the initial COD added as acetate (Table 12). The methane and carbon dioxide fractions at the end of the test, in the control groups A and C were 37±1 % and 66±1 % respectively.

The shape of the gas production curve for the units containing the size effluent is very informative (Figure 24 B). At the lowest concentration, the curve levelled off after only 10 d and correspondingly the gas composition is constant thereafter: this indicates that the biodegradable material (i.e. acetate + the organic fraction of the size effluent) was exhausted relatively quickly. Interestingly, the pattern of carbon dioxide concentration displays a short surge in the first two days, which is rapidly compensated thereafter (Figure G.33). At the intermediate concentration, the degradation process seems to occur in two partially overlapping steps and the gas production curve levelled off much later, well after 40 d. The gas composition profile is extremely interesting, as it shows that the concentration of carbon dioxide increases up to 70 % in the first week and gradually declines to 40 % after 15 d. This clearly indicates that the size effluent undergoes a relatively quick fermentation which results

in the accumulation of volatile acids and therefore in a larger fraction of carbon dioxide in the headspace and probably in a lower pH in the solution. This might temporarily delay the subsequent degradation phase, which only takes place when the conditions are favourable to the methanogenic biomass (second peak in Figure G.34). At the highest concentration tested, the volume of gas produced emphasized the two-stage process discussed for the intermediate concentration: after only 2 d, more than 200 mℓ of biogas were produced, almost exclusively consisting of carbon dioxide. Thereafter, the gas production drastically dropped and levelled off to values close to those of the lowest concentration; between day 50 and 55, the gas production resumed, at a rate comparable to that of the second stage of the intermediate concentration (26±1 and 31±2 mg COD/g VS/d respectively) and eventually exceeded the ultimate gas production displayed by this latter. Unfortunately, the test was stopped before the gas production had reached a plateau. The gas composition for this level of concentration (Figure G.33) shows that the carbon dioxide fraction reached values as high as 90-94 % within the first days: the concomitant reduction of the pH in the solution must arguably have inhibited the methanogenic biomass for a long time; the methane fraction steadily increased, but at a very low rate. After 60 d, one of the replicates displayed a sudden drop in methane concentration, which is believed to be due to factors not related to the microbial process (e.g. leaks through the septum or inaccurate gas sampling) and therefore should be disregarded. The methane and carbon dioxide fractions in the units at low and intermediate concentration of size, which actually reached a plateau, were in the same range as those of the reference units, i.e. 66±0 % and 33±1 % respectively (*Table* 12). The addition of alkalinity in the initial feed would have resulted in a lower pH inhibition and a more rapid treatment.

A similar discussion is applicable to the test units containing the distillery effluent (Figure 24 C), in which the progression of the two phases is barely visible, at low concentration and becomes evident at intermediate and high concentration levels.

The concentration of carbon dioxide in the units at low concentration increased during the first 4 days and gradually decreased thereafter: by day 7, methanogenesis becomes predominant and in 20 days, the biogas production levelled off. At the intermediate concentration, the surge in the concentration of carbon dioxide, and supposedly the inhibition of methanogenesis due to an excessively acidic environment, lasted over 35 days; thereafter, the microbial activity was resumed until the (degradable) substrate was exhausted after about 65 days. The bell-shaped surge in the concentration of carbon dioxide was even more marked at the highest concentration tested, preventing any methanogenic activity for over 20 days

(Figure G.37). Thereafter, the methane concentration gradually increases, indicating that some methanogenic activity was occurring; however, the volume of gas produced in this period is very small and the overall biogas production is significantly lower than that at the low concentration: this suggests that a high concentration of distillery effluent might result in a permanent inhibition of the process.

The inhibitory effect of the scour effluent is evident from Figure 24 D: the units containing the intermediate and high concentration of scour effluent displayed an overall biogas production lower than that displayed by the units at low concentration and also showed a considerably longer lag-phase of 30 to 40 days. Unexpectedly, the units containing the intermediate concentration showed a lower gas production than the units with the highest concentration. However, due to the much lower COD content of the scour effluent as compared to the other effluents tested, the ranking based on the *volumetric* concentrations is in fact reversed if one considers the *organic* loads (see Table G.7).

Similarly to what has been observed for the other test units, methane is readily produced at low concentrations of scour effluent, whereas progressively longer times are needed for the carbon dioxide fraction to be compensated at higher concentrations (Figure G.41).

Conclusions

The qualitative discussion that has been presented so far is summarised hereafter.

Table 12 reports the following quantities (averages and standard deviations are indicated):

- the ultimate fraction of methane, measured at the end of the test;
- the ultimate volume of methane produced: this is calculated through the step-by-step mass balance, discussed in Annexure B (equation B.4);
- the fraction of the COD initially available that was converted to methane: it is calculated by comparing the *net* methane production to the COD equivalent of the test material. This method assumes that the biomass will preferably degrade the readily biodegradable substrate (i.e. acetate) and only as a second option the test material;
- the maximum methanogenic activity: since the manual measurement can lead to locally incorrect values, the use of the local maximum may be equally arbitrary. The quantity plotted in Figure 25 for each unit has been calculated according to the procedure outlined in Section B.3.3. The values reported in the Table are the average of the average values across the three replicates; and
- the lag-phase is visually assessed as the period of time required to achieve 10 % of the ultimate methane production.

Figure 25 shows an overview of the activity and biodegradability estimates. The average values of SMA for the individual units are plotted (full and dotted lines separate the different groups of test materials and the different concentration levels within each group, respectively). The error bars indicate the variation of SMA between the local maximum and the two immediate adjacent values, according to the method proposed in Section B.3.3. As this is a function of the frequency of the manual detection, it should be regarded as qualitative information only. Nevertheless, the results clearly indicate that:

- the size effluent is inhibitory (ca. 42 % inhibition), only at the highest concentration tested (31 g COD/ℓ) and it greatly enhances activity at lower concentrations;
- the distillery effluent is also inhibitory, at the intermediate and high concentration levels (28 and 50 g COD/ℓ respectively): ca. 42 % and 93 % inhibition; and
- the scour effluent is highly inhibitory, at all concentrations tested (> 0.4 g COD/ℓ).

Figure 25 B depicts the fraction of COD converted to methane, i.e. the biodegradability of the test materials:

- the size effluent shows an unexpected behaviour, in that the intermediate concentration appears to be more efficiently biodegraded than the lowest concentration;
- the distillery effluent shows a clear concentration effect on biodegradability, which is a valuable indication for the co-digestion trial, suggesting that a load not exceeding 15 g COD/ℓ/d (equation A.1) should be maintained or alkalinity added, in order to prevent a severe drop in pH due to the solubilisation of carbon dioxide and to ensure that a sufficient fraction of the test material is converted to methane; and
- the scour effluent seems not only highly inhibitory (as discussed above) but also recalcitrant, at least in isolation.

The ‘design variable’ in this test was the volumetric ratio rather than the organic content of the test material: in other words, the same volumetric ratio yielded different organic loads for the different materials, e.g. the 9 % v/v concentration corresponded to 7, 11 and 0.4 g COD/ℓ for the size, the distillery and the scour effluents respectively. As the volume of seed sludge added to each vial was the same, the substrate-to-biomass ratios also varied greatly (Table G.7), so that if a conclusion can be drawn by comparing the outcomes of the same test material at different concentrations, the comparison of the outcomes of different test materials at the same concentration is not so immediate.

Table 12 – Summary of the results of the serum bottle assay on industrial effluents (No 1).

	Parameter		Group A	Group B	Group C
Controls	Ultimate CH_4 fraction	%	67±0	37±1	66±1
	Ultimate CH_4 volume	mℓ	156±1 (N=2)	47±1	153±1
	COD-to-CH_4	%	**104±0** (N=2)	n.a.	**101±1**
	Max SMA	mg COD/g VS/d	58±5	2±0 [(a)]	45±5
	Lag-phase	d	0	0	0
Size	Ultimate CH_4 fraction	%	66±1	66±0	50-60 [(b)]
	Ultimate CH_4 volume	mℓ	218±1 (N=2)	357±6	214±59
	COD-to-CH_4	%	**27±0** (N=2)	**38±0**	**≈ 2** (N=1)
	Max SMA	mg COD/g VS/d	69±3	67±1	26±1 (N=2)
	Lag-phase	d	**≈ 2**	**≈ 15**	n.a.
Distillery	Ultimate CH_4 fraction	%	66±1	57±1	45±2
	Ultimate CH_4 volume	mℓ	291±6	282±7	35±4
	COD-to-CH_4	%	**40±2**	**16±0** (N=2)	**< 0**
	Max SMA	mg COD/g VS/d	64±4	26±6	3±1 [(a)]
	Lag-phase	d	**≈ 12**	**≈ 45**	n.a.
Scour	Ultimate CH_4 fraction	%	66±1	67±4	69±1
	Ultimate CH_4 volume	mℓ	134±3	103±5	102±6
	COD-to-CH_4	%	**< 0**	**< 0**	**< 0**
	Max SMA	mg COD/g VS/d	20±3	7±1	7±1
	Lag-phase	d	n.a.	n.a.	n.a.

Group A, B and C indicate the low, medium and high concentrations respectively (for the test units, with the industrial effluents); the controls with nutrients and acetate, sludge only and without nutrients respectively. Three replicates have been considered for the calculations (N=3), unless otherwise indicated.

[(a)] The SMA did not show a distinct maximum: this value indicates the average calculated over time.

[(b)] The repeatability of the three test units was very poor: a reliable average value could not be calculated.

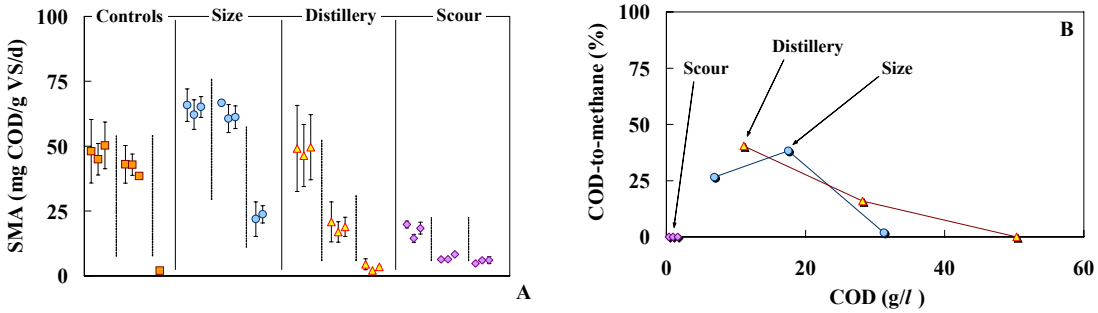

*Figure 25 – Maximum methanogenic activity (**A**) and fraction of COD converted to methane vs. initial COD (**B**), for the different industrial effluents tested (test No 1).*

4.1.3.2 Test No 2 – individual components

Objectives

A second test was conducted, using the same test materials (at lower levels of concentrations) and the same seed sludge as those used for test No 1, both of which had been stored at 4 °C since the first test was started.

The objectives of the second test do not differ from those discussed for test No 1.

Moreover, the information on the biomass activity and on the biodegradability of the test materials at lower ranges of concentrations could enable one to evaluate a relationship between effect and concentration, which will be of great interest for the design of a larger-scale experiment.

Materials and methods

A set-up similar to that of test No 1 was adopted, consisting of three groups of controls and three levels of concentration for each test material. All sets were done in triplicate.

- *Group 1*, *2* and *3* were the same as for the previous test.
- *Group A*, *B* and *C* contained the 'low', 'intermediate' and 'high' concentration levels respectively. These were 1, 5 and 9 % v/v for the size, the distillery and the scour effluents; and 0.2, 0.5 and 1.0 % v/v for the synthetic dye.

The detailed composition of the vials is summarised in Table G.8.

The experiment lasted 36 days and was monitored daily (with few exceptions) by measuring the volume of gas produced; the gas composition was determined for two out of the three replicates in each group, following the rotation *ab, bc, ca, ab, ...* The excess gas was wasted, after quantification.

Results and discussion

The cumulative biogas production curves for the size, distillery, scour and synthetic dye units are reported in Figure 26. The same convention as that of test No 1 applies, that each curve represents the average of the three replicates (except in the case of the intermediate concentration level of the synthetic dye effluent, in which one unit was clearly an outlier and has therefore been disregarded) and error bars are not shown (the variation coefficient ranged from 2 to 5 %).

The background gas production (Figure G.43) was low with an ultimate volume of methane of 34±3 mℓ (Table 13), sufficiently similar to the figure found in test No 1 to conclude that the performance of the seed inoculum was consistent and therefore the outcomes of the two

assays can be compared. A more accurate comparison of the response of the anaerobic sludge used as inoculum is proposed in Section G.1.9.

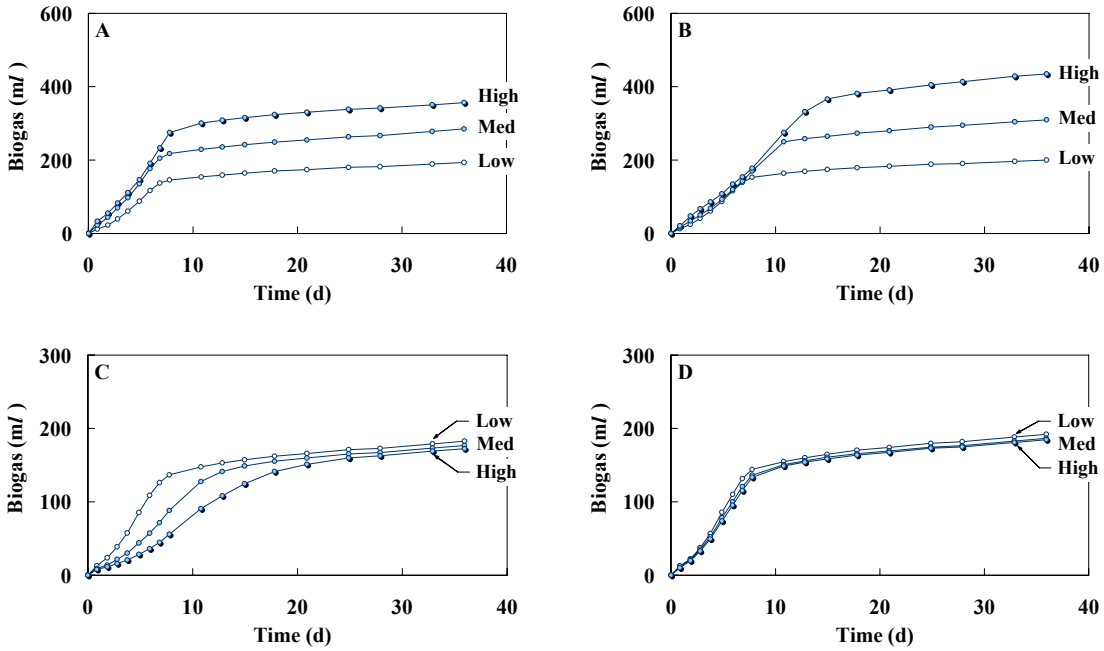

*Figure 26 – Average gas production in the units with size effluent (**A**), distillery effluent (**B**), scour effluent (**C**) and synthetic dye effluent (**D**).*

The moderately negative effect that the addition of nutrients and minerals showed in terms of maximum SMA in the previous test is confirmed here (Table 13; Figure G.46); however, the ultimate methane molar fraction and volume produced and the overall conversion (recovery) of acetate are the same for the two groups with and without NMS (Table 13). A possible explanation of the lower maximum SMA in this latter group of vials is that nutrients and minerals being available in the sludge sample withdrawn from the digester, the addition of NMS might have resulted in an excessive concentration of salts which in turn may partially inhibit the biomass activity. This supposition is consistent with the smoother shape of the SMA curves calculated for the units supplemented with NMS (Figure G.46).

Increasing concentrations of size effluent resulted in a progressively larger volumes of gas generated (Figure 26 A). However, most of the methane produced seems to originate from the acetate, thus resulting in an ultimate COD conversion which compared well to the figures obtained in the previous test, at the intermediate and high concentration levels (Table 12). At the lowest concentration level though, a much lower degradation is achieved (Table 13): this is a realistically inconsistent outcome, since the biodegradability of an organic substrate is concentration-independent, i.e. it should be nearly constant until an upper limit of

concentration at which the excessive substrate may cause the inhibition of the microbial activity and the apparent decrease of biodegradability.

This counter-intuitive result can be explained by looking at the relative availability of the reference substrate and of the test material at the lowest level of concentration: the former (5.4 g COD/ℓ) would account for most of the methane that can be stoichiometrically generated, i.e. 96 mℓ (at STP) vs. 13 mℓ from the test material (0.75 g COD/ℓ). This relatively very high concentration of acetate, associated with a relatively low concentration of the test material translated in a low sensitivity of the final assessment. Therefore the low biodegradability calculated should be regarded as largely underestimated. This is obviously a drawback of the methodology proposed (i.e. the use of a reference substrate for the assessment of biodegradability), which needs to be taken into account when setting up an activity test, so that the reference substrate does not interfere with the assessment of biodegradability while still enabling the simultaneous assessment of inhibition (Annexure B).

Furthermore, one should observe that the concentration of the reference substrate was not only *relatively* high compared to that of the test material(s) in both tests (this one and test No 1), but possibly too high altogether, thus resulting in the initial inhibition of the biomass activity which is clearly visible in all units, particularly in the reference units: the bell-shape of the curves of the specific methanogenic activity suggests that the micro-organisms need an unexpectedly long time to attain the maximum SMA (between 5 and 9 days). This again is counter-intuitive, if one considers that the sludge had been sampled shortly before being used to seed the serum bottles from a well-balanced anaerobic digester and can be explained by the high acetate concentration in the reference units that largely exceeded the range of operating concentration reported by Speece (1996).

This is an evident methodological error which could have contributed to the low biodegradation displayed by the size effluent at the lowest concentration. However, it is not clear how higher concentrations of the size effluent could have not been influenced by the same inhibition effect, unless the presence of larger amounts of an organic substrate other that acetate could somehow compensate that inhibition, e.g. by triggering the activity of other microbial groups which ultimately results in the release of the pressure on methanogens.

Similarly to what has been pointed out in the previous test, the degradation of the size effluent resulted in an initial production of carbon dioxide hence a transient increase of its molar fraction in the off-gas: however, at lower concentration levels compared to test No 1, the magnitude of such increase becomes negligible (Figure G.49). This indicates that no

inhibition had occurred and is confirmed by the net methane production curves (Figure G.48) which are constantly positive.

A similar discussion applies to the units supplemented with the distillery effluent: increasing concentrations resulted in progressively larger volumes of gas being produced (Figure 26 B); the extent of degradation of the organic matter achieved at the intermediate and high concentration levels matches well with the figures obtained at the lowest concentration level in the previous test (Table 12); the lowest concentration displays an inconsistently low degradation potential which can be ascribed to the effect of the relatively high concentration of acetate discussed above.

The maximum SMA does not differ in the three groups of serum bottles (Table 13); however, the abundant production of carbon dioxide in the early stage of the degradation results in a progressively longer delay in the attainment of methanogenic conditions at the intermediate and high concentrations of distillery effluent (Figure G.54). The units with the high concentration, although seemingly poorly reproducible in this initial stage of the test, experienced a temporary inhibition, before day 10, as showed in Figure G.52 where the net methane production curves become negative.

The curves of biogas production for the units with the scour effluent (Figure 26 C) do not show an increase in the volume of gas generated at increasing concentrations of the test material. Furthermore, the ultimate volume of methane produced does not differ significantly (at least, at the lowest concentration level) from that of the reference units (Table 13). In another situation, this would indicate that the scour effluent is totally undegradable. In this case though, the relatively low contribution of the test material to the total organic matter as compared to the contribution of the acetate supplemented is most likely the cause of such pattern. In fact, due to the low organic strength of the scour effluent (Table 11) and to the methodological approach adopted for this (and the previous) test, i.e. to maintain constant the dilution rate rather than the COD concentration across the different effluent tested, the volumes of scour effluent added to the respective units corresponded to a stoichiometric volume of methane of 0.7; 3.5 an 7 mℓ respectively. These small volumes represent a negligible fraction of the methane deriving from the acetate supplemented (143±3 mℓ) and are likely to fade in the background noise of the process.

It is true however that the scour effluent proved to be increasingly inhibitory (Table 13). This is particularly evident at the intermediate and high concentration that showed a negative net methane production (Figure G.56) for most of the experimental period. Furthermore, since no

significant increase in the molar fraction of carbon dioxide was detected (Figure G.57) and the actual concentration of the test material was very low, one can certainly conclude that the scour effluent is highly inhibitory (Figure 28).

Based on these remarks, it is evident that the scour effluent would be the ideal candidate test material to investigate both the potential of the biomass to adapt upon exposure and of the presence of a labile substrate to enhance its biodegradability.

The concentration levels tested for the synthetic dye effluent were too small to obtain a meaningful response of the assay (Figure 26 D). The effect described for the scour effluent is even stronger for the synthetic effluent that contributed for than 2 mℓ at the highest concentration to the overall methane generated. The ultimate methane volume produced does not significantly differ from that of the reference units to conclude that the test material was fully degraded (so the 100 % COD conversion to methane reported in Table 13 is not to be trusted). However, the methanogenic activity seemed to be slightly enhanced. This could indicate once more an unspecified effect of the presence of a secondary substrate besides acetate, when this latter is present at excessively high concentrations.

Conclusions

This test differed from the previous one for the lower levels of concentration used; all other conditions were identical.

It is therefore interesting, particularly from a methodological perspective, to compare the outcomes of the two tests. However, a better understanding will be achieved when the information regarding the interaction of the same test materials obtained in the following co-digestion experiments will complement the picture. A comprehensive discussion will be presented thereafter (Section 4.1.3.6). A few aspects can be summarised here.

- The effect of the addition of nutrients and minerals seems to be negligible, since no evident benefit in terms of methanogenic activity nor in terms of overall COD conversion to methane was shown by the reference units containing acetate as substrate. On the contrary, it seems that the presence of the nutrient medium may have slowed down the methanogenic activity: the units which had been supplemented with the NMS reached a slightly lower maximum SMA than the units not supplemented with the NMS. A tentative explanation of this outcome is that nutrients and minerals were present in sufficient concentration in the sludge that had been sampled from a well-balanced anaerobic digester shortly before being used to seed the serum bottles: an excessive concentration of salts in particular could have been the cause of the poor performances.

- In the range of concentration tested, the size and distillery effluents undergo a biodegradation to an extent of 30-35 % and 40 % respectively and do not seem to severely affect the methanogenic activity, except at high concentrations (i.e. 30-35 g COD/ℓ). Otherwise the scour effluent seems not biodegradable and inhibitory to the methanogens, at all concentrations tested.

  These quantitative results however might be significantly affected by two methodological aspects which had not been adequately taken into consideration prior to starting the test: the concentration of organic substrate, particularly the reference substrate and the relative concentration of the reference substrate and test material.
- The concentration of acetate was clearly excessive as one can argue from the shape of the SMA of the reference units (with and without NMS): since the sludge was withdrawn from a digester and carefully manipulated shortly before being distributed to the vials, the methanogenic activity should have been high at the beginning of the test and only gradually decrease to level-off at the endogenous level when acetate was exhausted. However, the SMA consistently showed a bell shape and reached its maximum after 7-9 days.

  This does not only affect the activity of the biomass which in turn could possibly alter its ability of metabolising the test materials thus deteriorating the reliability of the assessment, but also unproductively increases the duration of an activity test.
- The relative concentration of the reference substrate and test material also affects the response of the test, on another level i.e. by reducing the sensitivity of the method. In fact, when the fraction of organic substrate coming from the test material is significantly lower than that deriving from the reference substrate (irrespective of whether this latter is not excessive to cause inhibition of the methanogenic biomass), the 'signal' i.e. the volume of methane generated by the degradation of the test material will be too weak to be accurately detected. In this situation, if the overall degradation of the test material would result in few millilitres of methane, a little inaccuracy in the determination of the gas volume and/or of the gas composition would result in larger variation in the estimates of the biodegradability and specific methanogenic activity.

Table 13 – Summary of the results of the serum bottle assay on industrial effluents (No 2).

	Parameter		Group A	Group B	Group C
Controls	Ultimate CH_4 fraction	%	67±1	28±2	65±1
	Ultimate CH_4 volume	mℓ	143±3	34±3	143±6
	COD-to-CH_4	%	**104±3**	nod.	**104±6**
	Max SMA	mg COD/g VS/d	60±7	3±1 [(a)]	52±3
	Lag-phase	d	0	0	0
Size	Ultimate CH_4 fraction	%	67±0	64±1	65±0
	Ultimate CH_4 volume	mℓ	147±4	180±1	209±1
	COD-to-CH_4	%	**6±3**	**24±1**	**33±1**
	Max SMA	mg COD/g VS/d	64±4	75±3	93±7
	Lag-phase	d	0	0	0
Distillery	Ultimate CH_4 fraction	%	68±0	68±1	67±0
	Ultimate CH_4 volume	mℓ	155±3	215±5	276±6
	COD-to-CH_4	%	**9±3**	**32±3**	**39±2**
	Max SMA	mg COD/g VS/d	64±6	64±4	68±9
	Lag-phase	d	0	0	0
Scour	Ultimate CH_4 fraction	%	66±1	66±1	67±1
	Ultimate CH_4 volume	mℓ	140±3	134±1	129±2
	COD-to-CH_4	%	**< 0**	**< 0**	**< 0**
	Max SMA	mg COD/g VS/d	59±7	41±2 (N=2)	27±2
	Lag-phase	d	0	0	0
Synthetic dye	Ultimate CH_4 fraction	%	67±1	67±0 (N=2)	67±0
	Ultimate CH_4 volume	mℓ	150±2	146±5 (N=2)	144±3
	COD-to-CH_4	%	**≈ 100** [(b)]	**≈ 100** [(b)]	**n.d.** [(b)]
	Max SMA	mg COD/g VS/d	61±5	59±6 (N=2)	56±1
	Lag-phase	d	0	0	0

Group A, B and C indicate the low, medium and high concentrations respectively (for the test units, with the industrial effluents); and the controls with nutrients and acetate, sludge only and without nutrients respectively. Three replicates have been considered for the calculations (N=3), unless otherwise indicated.

[(a)] The SMA did not show a distinct maximum: this value indicates the average calculated over time.

[(b)] These figures are unrealistic, since the organic matter of the test material only accounts for a negligible portion of the overall methane volume, as discussed in the text.

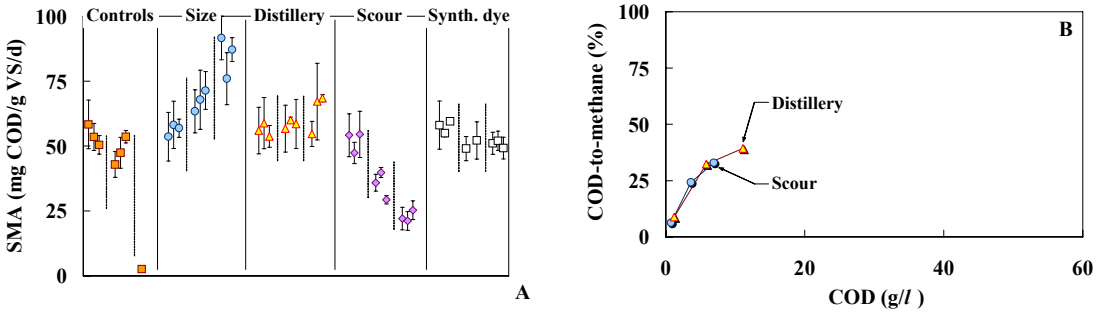

*Figure 27 – Maximum Sludge Methanogenic Activity (**A**) and fraction of COD converted to methane vs. initial COD (**B**), for the different industrial effluents tested.*

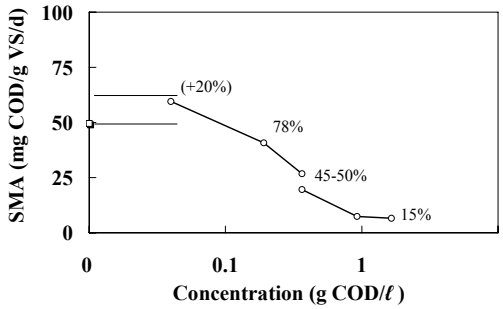

Figure 28 – Experimental toxicity curve for the scour effluent.

4.1.3.3 Test No 3 – co-digestion

Objectives

This test was aimed at simulating the combined digestion of two industrial effluents, namely the distillery and size effluents, in order to determine if the performance of the process would benefit from the simultaneous presence of the two test materials.

In this type of test, besides the usual parameters that have an influence on the outcome of the test, the volumetric ratio of two test materials must also be taken into account. Ultimately, the results of this type of AAT will be analysed in function of two parameters, i.e. the organic load and the volumetric ratio.

Materials and methods

The composition of the vials used for this test is reported in Table G.9. The set-up comprised:

- a control group, to quantify the baseline gas production in the absence of external substrate;
- three groups containing the size effluent as external substrate, at increasing concentrations;
- three groups containing the distillery effluent as external substrate (the same concentrations as the size effluent were used);
- three groups containing a 1:1 mixture of size and distillery, on a COD basis, i.e.: being the overall COD concentration the same as the other test units, the volumes of the two test materials were chosen so that each one contributed to half of that COD concentration.

The experiment lasted 44 days. The same procedure applied to monitor test No 2 was adopted here.

Assessment of the effectiveness of the combined digestion of two test materials

In principle, two approaches can be used to present the results of co-digestion exercises.

- The simplest method assumes the concentration of the *total* organic matter as the 'independent variable': it therefore compares the biodegradability (or activity) attained by the test material in isolation at a certain COD concentration, to that attained by the test material in the presence of another substrate, at the same *total* COD concentration of the mixture.
- The second method looks at the contribution of each one of the test materials separately, i.e. assumes the *partial* COD concentration deriving from one of the two, preferably the recalcitrant effluent, and neglects the other one.

The first approach can be interpreted as follows: if we *split* the *total* COD between the two test materials based on a given volumetric ratio, how does the fraction of COD converted to methane change ? Therefore if a fixed COD concentration results in a higher volume of

methane produced than that produced by same amount of COD deriving from one of the two test materials only, one would conclude that the use of the mixture is preferable in terms of performance of the process (see for example, Figure 30 A).

The second approach would be interpreted as follows: if we take a given concentration of organic matter, how does the volume of methane produced change if no additional substrate is present (i.e. the recalcitrant effluent is present alone) or if the labile effluent is also present (resulting in additional COD being supplemented) ? In this second case, for a given COD concentration one should compare the volume of methane produced by the one test material in isolation, to the portion of the total volume of methane produced when both test materials are present which is due to the test material alone (since in the latter test condition the overall volume of methane will obviously be larger).

This method needs a further manipulation of the data and requires that if a concentration c_1 and a concentration c_2 are respectively tested for the two materials in isolation, a concentration equal to the sum thereof is tested when the two are used in combination. In the test presented in this section, these circumstances are applicable to one case only (Table G.9), i.e. we compare the two intermediate concentrations of size and distillery, to the highest concentration of the mixture as follows:

	Units	Size effluent	Distillery effl.	Mixture
Concentration	g COD/ℓ	3.62	3.80	7.13 (1:1) [(a)]
Net volume of methane produced	mℓ	47	52	99
Stoichiometric volume of methane	mℓ	73	76	149

[(a)] The mixture contained 3.51 and 3.63 g COD/ℓ of size and distillery effluent respectively, as one can calculate from Table G.9.

The volume of methane that one would expect be produced by the mixture of the two test materials is calculated as follows:

$$v^* = 47 \cdot \frac{3.51}{3.62} + 52 \cdot \frac{3.63}{3.80} = 95 \text{ m}\ell$$

This enables a realistic calculation of the actual extent of biodegradation of the mixture, in that one does not compare the volume of methane measured to the theoretical volume expected on the basis of the COD supplemented, but takes into consideration the knowledge of the actual biodegradability of the two components of the mixture.

We propose a more rigorous formulation of this methodology that is based on the following observation: if testing the two effluents in isolation will provide an estimate of their inherent biodegradability, testing them in combination is meant to provide an indication of the *synergistic effect*, if any. In other words, one is to compare the actual biodegradation (volume

of methane generated) to the volume of methane that would be generated if the presence of one test material was not affecting the other (i.e. if there was no interaction between the two test materials). For this approach, the previous knowledge of the actual biodegradability of the two test materials in isolation is essential.

Let $V_{CH,mix}$, $V_{CH,1}$ and $V_{CH,2}$ be the net volumes of methane generated (measured) in the units containing the mixture, effluent 1 and effluent 2 respectively. Let f_1 and f_2 be the volumes (or the amounts) of effluent 1 and effluent 2 combined to form the mixture, compared to the volumes (or amounts) used for their respective biodegradability assessment in isolation.

The net volume of methane that would be generated if neither effluent would benefit from the presence of the other one can be calculated as:

$$V^*_{CH,mix} = f_1 \cdot V_{CH,1} + f_2 \cdot V_{CH,2} \quad (15)$$

(i.e. the two contributions would be purely additive).

The *effectiveness* of the co-digestion option can be defined as:

$$E = \frac{V_{CH,mix}}{V^*_{CH,mix}}$$

being $V_{CH,mix}$ the actual net volume of methane generated. Ultimately, the response of a co-digestion assay can fall into one of the following categories:

- $E < 1$: the co-digestion is <u>not</u> convenient;
- $E \approx 1$: the co-digestion is not detrimental; and
- $E > 1$: the co-digestion is <u>a valid option</u>.

Clearly, should this approach be transposed to the assessment of a full-scale co-digestion application, a safety factor should be used which takes into account the inherent inaccuracy of the experimental determination of biodegradability as well as other factors that might play a role in the scale-up of these results. At this stage, such margin is difficult to define, but 10 % could be a reasonable compromise.

Results and discussion

The cumulative biogas production curves for the control units, for the size and distillery effluent and for the 1:1 mixture test units are reported in Figure 29: each curve represents the average of the three replicates (except for the intermediate concentration of the distillery effluent and for the low and intermediate concentrations of the mixture, which are slightly out of range) and error bars are not shown for clarity. The variation coefficients ranged from 0.5 to 8 %, which indicates a high reproducibility between the replicates.

The background gas production reached a value of approximately 50 mℓ (Figure 29; Table 14), which is slightly higher than the one reported in the previous tests: this might be interpreted as a larger concentration of residual organic matter in the sludge sample (this was collected at the same wastewater treatment plant but a few months later).

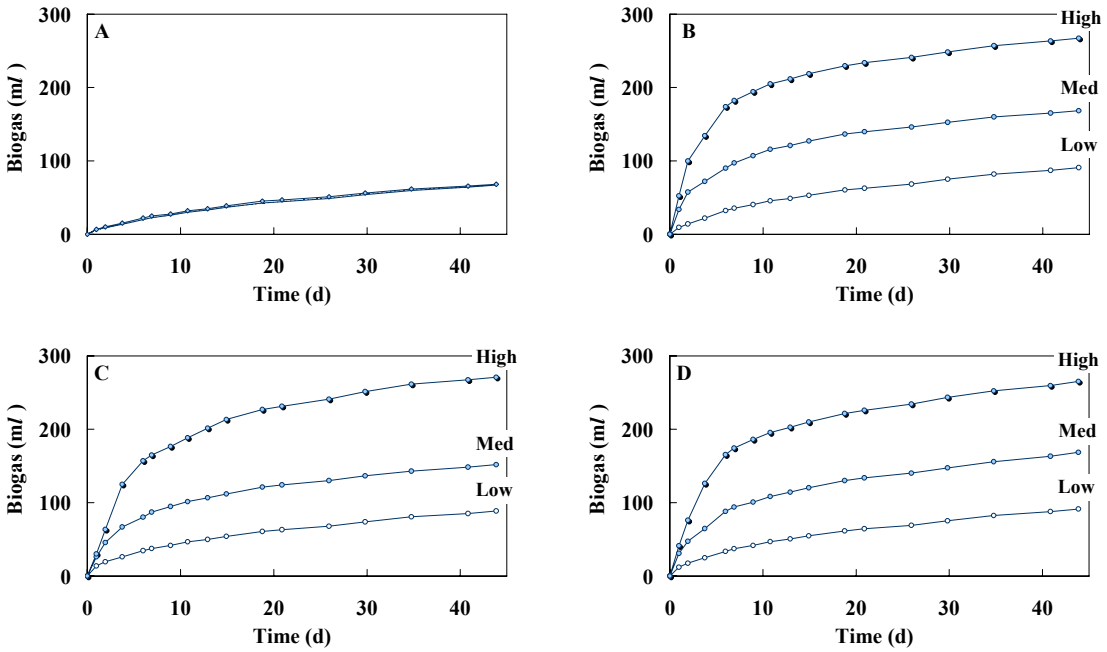

*Figure 29 – Gas production in the control units (**A**) and in the test units with size effluent (**B**), distillery effluent(**C**) and the 1:1 mixture (**D**).*

Unlike the previous two tests in which the same test materials were used, in this case the concentration effect is much clearer, i.e. the gas production is progressively higher at increasing concentrations of the test material. Furthermore, no significant lag-phase can be reported in any of the cases studied, as one can observe in terms of methanogenic activity (Figures G.70, G.74 and G.78) which invariably started off at the highest value and gradually levelled-off to the baseline (endogenous) value after 10-15 days. This is obviously due to the absence of an excessively high, hence inhibitory, concentration of the reference substrate that caused the initial transient observed in tests No 1 and No 2. This however must not lead to the conclusion that the option of not using the reference substrate is preferable, otherwise one loses the benefits of the combined assessment of toxicity and biodegradability that an AAT ensures. On the contrary, it should stress the importance of the appropriate choice of the relative concentration of reference substrate and test material that has been discussed in the previous section:

- the concentration of reference substrate (e.g. acetate) should not exceed a level that might inhibit the activity of methanogens; and
- the concentration of the test material should be sufficiently high to generate a volume of gas (or methane) significantly larger than that generated in the reference units, so that the differential contribution of the test material emerges from the inevitable background noise of the serum bottle method.

The absence of acetate and also the lower concentration of organic matter used for this test (ranging from 0.8 to 8 g COD/ℓ, as opposed to 5 to 54 and 5 to 16 g COD/ℓ for tests No 1 and No 2 respectively) had generally resulted in a progressive increase of the methane fraction in the off-gas, with a negligible accumulation of carbon dioxide for a very short period and for the highest concentration levels tested only (Figures G.69, G.73 and G.77).

The suitability of the co-digestion option to dispose of two effluents is to be evaluated by looking at two aspects, i.e. the extent of biodegradation that can be achieved as well as the reduction of the toxic effect of the recalcitrant effluent. In other words, we compare the situation in which the two effluents are digested in combination to a 'baseline' situation in which they are digested separately. In this respect, the test units which contain the two individual effluents can be regarded as *controls*, similarly to the control units in a simple AAT aimed at studying a single test material.

At the range of concentrations tested, the size and the distillery effluents in isolation showed a consistent biodegradability potential (Table 14), except the lowest concentration of the distillery effluent. This finding confirms the accuracy and repeatability of the methodology of the serum bottle and indirectly substantiates the hypothesis that the relatively low biodegradation achieved by these test materials at low COD concentration in the previous two tests was actually caused by the interference due to a superimposed toxicity effect of acetate. It is however possible that at concentrations as low as 0.8 g COD/ℓ, the distillery effluent is actually more biodegradable (ca. 83 % vs. 68 %).

This could be due to the presence of some compounds that at higher concentrations exerts an inhibitory action on the methanogenic biomass, thus preventing it from extensively degrading the organic matter.

At this stage, the experimental data obtained are not sufficient to draw any definite conclusion in this respect. Nevertheless, this does not seem to be an erroneous experimental outcome, since a similar pattern can be observed for the 1:1 mixture of the two effluents (Table 14),

which can be explained as a proportionally larger contribution of the distillery fraction of the mixture at low concentration than at higher concentrations.

These results are summarised in Figure 30 A. The first approach proposed above for the analysis of a co-digestion assay has been adopted in the figure, i.e. the biodegradability of the components at certain concentration is compared to the biodegradability of the mixture at the same COD concentration. The line of the mixture lies above the lines of both the individual components, at the low and intermediate concentrations, but crosses the line of distillery at the highest concentration tested. This outcome can be interpreted by saying that the digestion of the two effluents in combination improves the effectiveness of COD conversion to methane up to a concentration of ca. 3.7 g COD/ℓ; beyond this level, the effectiveness deteriorates slightly but in a situation where the size effluent was the concern, the co-digestion option would still be a good choice, because the loss in biodegradability of the distillery effluent side would be more than compensated on the size effluent side.

The alternative approach discussed, i.e. to actually 'isolate' the contribution of each component of the mixture and tentatively assess the 'type of interaction' between them, as depicted in Figure 31. The following numerical example may clarify the rationale.

Let us take the intermediate concentration levels for the size and the distillery effluents, as reference to calculate the actual methane volume that can be generated per unit COD (this equates to the quantities f_1 and f_2 in equation 15). We obtain:

$$f_1 = \frac{47}{3.62} = 12.98 \text{ m}\ell\text{/g COD; and}$$

$$f_2 = \frac{52}{3.80} = 13.68 \text{ m}\ell\text{/g COD.}$$

By substituting these factors in equation 15and using the respective concentrations of the size and distillery effluents, one can calculate the expected volume of methane at the different concentrations of mixture tested.

For the high concentration level, the effectiveness of the co-digestion can be calculated as:

$$E = \frac{99}{12.98 \cdot 3.51 + 13.68 \cdot 3.63} = \frac{99}{95} = 1.04$$

This figure suggests that the digestion of the distillery and size effluents in combination at 7 g COD/ℓ may be convenient from a biodegradation point of view, but the margin of 4 % only may be too small to trust this conclusion.

The reference substrate not being supplemented to the test units, does not allow one to assess the suitability of co-digesting the two effluents from the point of view of methanogenic

activity or inhibition. Nevertheless, a qualitative evaluation is still possible. With reference to Figure 30 B, one can observe that at the low and intermediate concentrations, the maximum SMA displayed in presence of the mixture is not lower that the corresponding values displayed when the effluents are digested individually; on the contrary, it is significantly higher than that in presence of the size effluent in isolation. This supports the conclusion that, in a situation where the size effluent was the concern, the option of co-digesting it in combination with the distillery effluent is certainly recommended.

At high concentrations though, the maximum SMA displayed in presence of the mixture is lower than the corresponding values attained in presence of the components alone. This suggests that a concentration of 7 g COD/ℓ would possibly affect the methanogenic biomass thus making co-digestion no longer convenient.

Ideally, a further use of the data of specific methanogenic activity could be that of estimating the kinetics of the digestion process, for the components in isolation and mixed. The range of concentrations tested and the number of data points are certainly insufficient to obtain a reliable estimate, at least or the effluents alone. A very simplistic approach however can be applied to the mixture. The generally acknowledged Michaelis-Menten model can be used to interpolate the set of experimental data. The result is shown in Figure 32.

More than a quantitative, experimental outcome, this should be regarded as a further potential response that one could obtain from a well planned and executed serum bottle co-digestion test.

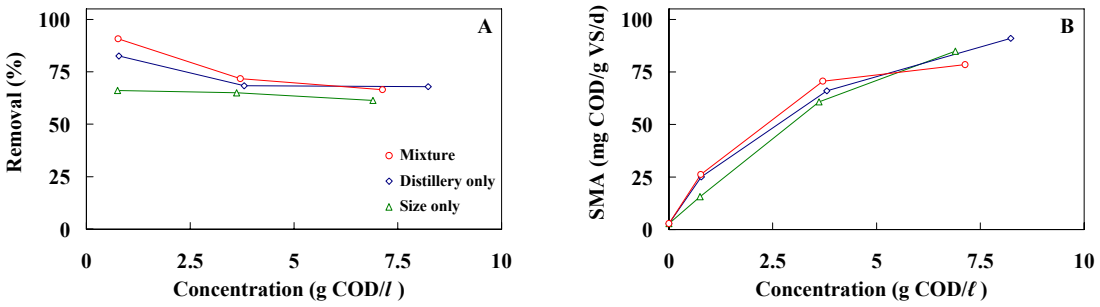

*Figure 30 – Summary of the outcome of the activity test (No 3) and graphical comparison between the performance of the individual effluent and of the mixture, in terms of biodegradability (**A**) and the methanogenic activity (**B**).*

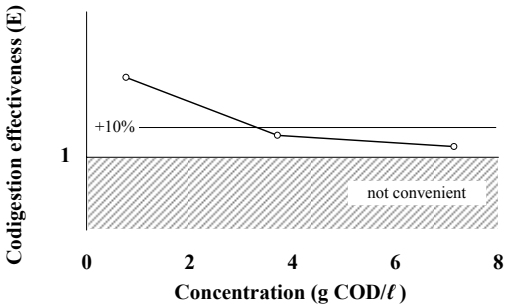

Figure 31 – Effectiveness of the co-digestion option for a 1:1 mixture of size and distillery effluents.

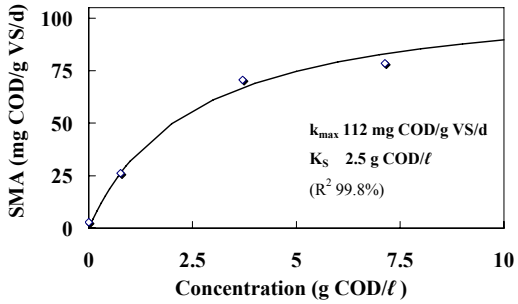

Figure 32 – Evaluation of the kinetics of the co-digestion process for the 1:1 mixture of size and distillery effluents.

Table 14 – Summary of the results of the serum bottle assay on industrial effluents (No 3).

	Parameter		Group A	Group B	Group C
Controls	Ultimate CH_4 fraction	%	38±3	---	---
	Ultimate CH_4 volume	mℓ	43±4	---	---
	COD-to-CH_4	%	n.d.	---	---
	Max SMA [(b)]	mg COD/g VS/d	3±0 [(a)]	---	---
	Lag-phase	D	0	---	---
Size	Ultimate CH_4 fraction	%	43±2	50±1	54±0
	Ultimate CH_4 volume	mℓ	54±3	90±1	133±1
	COD-to-CH_4	%	**66±7** (N=2)	**65±1**	**61±3**
	Max SMA [(b)]	mg COD/g VS/d	16±3	61±10	85±5
	Lag-phase	d	0	0	0
Distillery	Ultimate CH_4 fraction	%	44±1	53±1	59±0
	Ultimate CH_4 volume	mℓ	56±1	95±2	159±3
	COD-to-CH_4	%	**83±10**	**68±3**	**68±2**
	Max SMA [(b)]	mg COD/g VS/d	25±6	66±1	91±5
	Lag-phase	d	0	0	< 1
Mixture 1:1	Ultimate CH_4 fraction	%	44±1	53±1	56±1
	Ultimate CH_4 volume	mℓ	56±0	97±4	143±0
	COD-to-CH_4	%	**88±6**	**72±3**	**66±2**
	Max SMA [(b)]	mg COD/g VS/d	26±2 (N=2)	71±1 (N=2)	78±2 (N=2)
	Lag-phase	d	0	0	< 1

Group A, B and C indicate the low, medium and high concentrations respectively. Three replicates have been considered for the calculations (N=3), unless otherwise indicated.

[(a)] The SMA did not show a distinct maximum: this value indicates the average calculated over time (N=12).

[(b)] This test was not conducted according to the procedure outlined for AAT, specifically in that the reference substrate was not supplied to the test units. Therefore, the SMA of the control units and of the test units are not 'homogeneous' (or comparable) in that the former indicates the endogenous activity and the latter the activity on a substrate other than acetate (hence no inhibition assessment can be obtained with this test).

Conclusions

The outcome of this test, particularly the extent of biodegradation achieved by the test materials in isolation, will be discussed in Section 4.1.3.6 in conjunction with the corresponding results of the other activity assays.

This experiment was started as a conventional biodegradability test, in that no reference substrate was supplemented to the control units (and therefore to the test units). This has certainly eliminated the two aspects that proved critical in test No 2, i.e. the risk of overloading the methanogenic biomass with an excessive concentration of acetate as well as of creating an interference between the 'signal' of the methane generated by the degradation of acetate and that of the test material.

Since the combined digestion of the two effluents may affect both the extent of biodegradation of the organic matter and the activity of the sludge in the digester, the effectiveness of the co-digestion option is to be evaluated by examining both the parameters for the mixture of the test materials. However, the absence of acetate has prevented the assessment of the potential inhibitory effect of the test materials and therefore the effectiveness of the combined digestion of the two was only assessed in terms of biodegradability potential.

Another methodological difference from the previous experiments was that a COD concentration rather than a volumetric ratio approach was adopted: the level of concentration for the test materials and for the mixture were almost the same irrespective to the difference in the initial organic strength of the two effluents. This is preferable because it ensures the direct comparison between the different test units and concentration levels.

Based on these remarks, we recommend that a lower limit of organic matter of 1 g COD/ℓ be used to ensure reliable detection of the biogas production. This equates to ca. 20 mℓ of methane at 35 °C, assuming a volume of 50 mℓ of the suspension.

This is to be regarded as a rule-of-thumb only, as it obviously depends on the characteristics of the seed inoculum, i.e. its residual organic concentration. Therefore a preliminary activity test with a set of controls (and reference units) would enable one to correctly adjust the operating conditions, particularly the concentration of the test material.

Specifically, this experiment showed that at a concentration of ca. 1 g COD/ℓ and possibly up to 3 g COD/ℓ, the co-digestion of the size and distillery effluents at a volumetric ratio of 1:1 would result in a beneficial effect both in terms of conversion of organic matter to methane and in reduction of the inhibitory effect of the size effluent on the methanogenic biomass.

However a broader range of concentrations (and volumetric ratios) would probably need to be examined, if a more reliable and specific response is to be given regarding the suitability of co-digestion for the size and distillery effluents. This is certainly imperative for the actual implementation of a screening protocol, as discussed in Annexure A.

Ultimately, this exercise has demonstrated the feasibility of performing a co-digestion assay using the serum bottle technique and illustrated a rigorous methodology to quantify the effectiveness of digesting two effluents in combination as opposed to in isolation.

A side result of this test is discussed in Section 4.1.3.6 for clarity and deals with the comparison of the biodegradability estimates for the size and the distillery effluents which can be carried out based on the outcome of the three AAT discussed so far.

4.1.3.4 Test No 4 – co-digestion

Objectives

This co-digestion assay is the ideal continuation of test No 3, in which the suitability of the size and distillery effluents to be co-digestion at a volumetric ration of 1:1 was studied. The results, particularly from a methodological point of view, were promising. Therefore it was decided to investigate the same two effluents at volumetric ratios of 1:2 and 2:1 of size and distillery respectively.

Ideally, this test should have been performed simultaneously to the previous one: this would have enabled us to save the control units and the size and distillery units.

We therefore recommend that a co-digestion assay include at least three sets of test units in which the same combination of test materials is investigated at different volumetric ratios. This approach has been adopted in the final co-digestion assay, presented in Section 4.1.3.5.

Materials and methods

The composition of the vials is reported in Table G.10. The set-up comprised:

- a control group, to quantify the baseline gas production (no acetate was supplemented);
- three groups containing the size effluent and three groups containing the distillery effluent as sole substrates: as explained earlier, these units act as 'secondary' controls in that they provide a reference activity and biodegradability against which the effectiveness of the test units is to be assessed; and
- two sets of test units which contained the mixture of size and distillery effluents; each set comprised three groups in which different initial organic concentrations were obtained for the same volumetric ratio.

Two concentration levels were maintained from the previous test to enable comparing the outcomes of the co-digestion assays at the different volumetric ratios. A higher concentration level was also tested, so that an organic range from 1 to 13 g COD/ℓ could ultimately be screened.

All the groups were done in triplicate, resulting in a total of 39 individual serum bottles that were monitored for 63 days. The general experimental procedure was adopted, i.e. gas volume determination and simultaneous gas composition analysis on two out of three replicates per group on a daily basis, for the first week. Thereafter the frequency of re-equilibration was progressively reduced: the last three determinations, when the degradation process was almost complete, were carried out at a week distance.

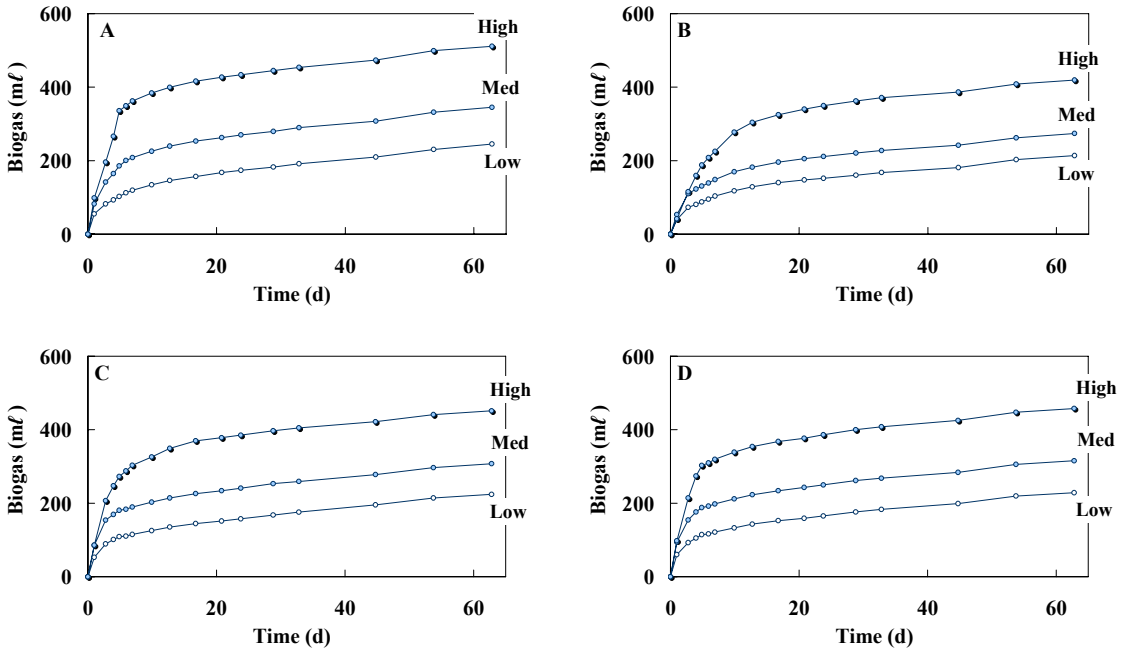

*Figure 33 – Gas production in the test units with size (**A**) and distillery effluents (**B**) and with the 1:2 (**C**) and 2:1 mixtures (**D**).*

Results and discussion

The cumulative biogas production curves for the size and distillery *reference* units and for the two mixtures are reported in Figure 33.

As a general rule, each curve represents the average of the three replicates, unless one differed significantly from the other two and was therefore disregarded in the calculations. In this test, all replicates displayed a very low deviation from the average gas production (Figures G.83, G.87, G.91 and G.95), thus indicating a very high reproducibility at all concentrations tested. The only exception is the group containing the size effluent at low concentration, in which one vial was accidentally broken on day 21.

The absence of acetate as reference substrate constitutes a methodological error, according to the general protocol for activity test (Annexure B) and has been discussed in the previous section.

Both test materials, in isolation and combined, were degraded to an extent ranging from ca. 50 % to 70 % and showed a relatively high methanogenic activity (Table 15).

The biomass was quantified as volatile suspended solids, therefore the figures of SMA reported in the table are not directly comparable to those obtained in the previous tests.

A few qualitative remarks can be made by simply observing the results summarised in Table 15.

- All units containing the test materials, i.e. the *reference* units with the size and distillery effluent alone and the test units with the two mixtures, are characterised by a higher methane fraction in the headspace than the control units; moreover, this fraction seems to increase slightly with the increasing concentration tested.
- The distillery effluent seems to be degradable to a lower extent than the size effluent; furthermore, the methanogenic activity of the sludge exposed to the size effluent is higher than when exposed to the distillery effluent. This suggests that the higher organic strength of the distillery effluent (Table 11) may be associated with a larger proportion of slowly- or undegradable organic matter. The former may require intermediate steps to actually become accessible to methanogens. A further clue in favour of this hypothesis is that carbon dioxide hardly accumulated in the units containing the size effluent (Figure G.85) as opposed to the units containing the distillery effluent (Figure G.89). Moreover, the highest concentration tested for the distillery effluent, unlike the other two concentration levels, showed a short lag-period and the maximum SMA was attained between day 3 and day 4. Most likely, the distillery effluent contains a larger fraction of non-degradable organic matter which may exert an inhibitory effect on the methanogenic population: in fact, for the same COD concentration (ca. 13 g /ℓ), the pattern of SMA for the units containing the size effluent showed a second peak on day 5 which may indicate that a more slowly degradable fraction was being converted to methane whereas a much smaller peak is present in the unit containing the distillery effluent, only on day 10.
- These differences disappear when a mixture of the size and distillery effluents is tested: the biodegradation achieved by both mixtures is very similar (the statistical difference in the values estimated is negligible) and so is the methanogenic activity displayed by the sludge exposed to either one.

These observations are summarised in Figure 34, which presents the two quantities of interest, i.e. the extent of biodegradation achieved (termed the removal) and the maximum specific methanogenic activity calculated.

One should conclude that there is hardly any difference in the COD removal obtained using different proportions of the two test materials in the mixture (Figure 34 A); at the lowest concentration tested though the biodegradability of the test materials in combination is higher

than that of the two in isolation. The only apparent effect is that the biodegradability of the combined effluents is more concentration-dependent.

On the contrary, the effect of the combination of the size and distillery effluents on the methanogenic activity of the sludge is evident (Figure 34 B), particularly at the low and intermediate concentration tested, where the maximum SMA was enhanced by between 20 % and 45 %.

The effect of the co-digestion option was quantified using the methodology described in the previous section: the result is depicted in Figure 35.

The only combination that improves the biodegradability is the low concentration of the 1:2 mixture. The 2:1 size-to-distillery volumetric ratio is the worst and at high COD concentration it achieves an effectiveness lower than 1, which indicates an antagonistic effect of the two test materials. It is particularly interesting noting that, although the size effluent performed better than the distillery effluent in isolation, the mixture containing two thirds of size effluent showed a lower biodegradability.

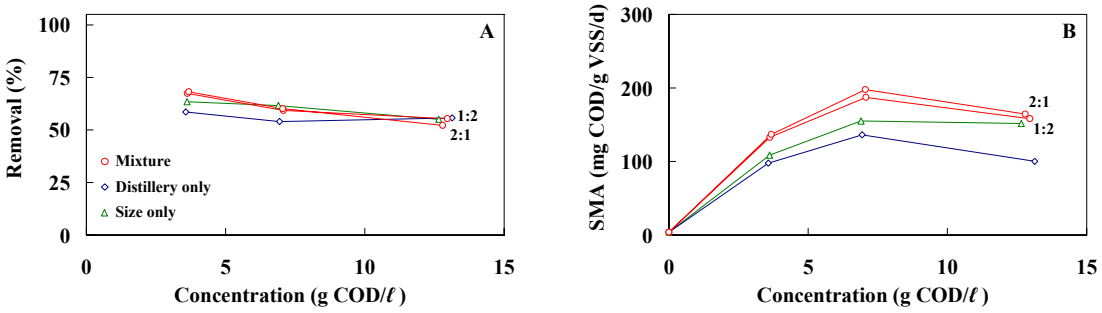

*Figure 34 – Summary of the outcome of the activity test (No 4) and graphical comparison of the performance of the individual effluents and of the mixtures, in terms of biodegradability (**A**) and methanogenic activity (**B**).*

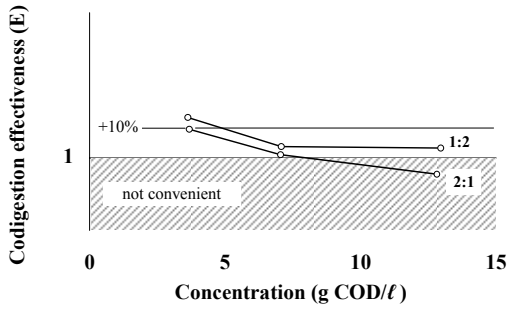

Figure 35 – Effectiveness of the co-digestion option for the mixtures of size and distillery effluent.

Table 15 – Summary of the results of the serum bottle assay on industrial effluents (No 4).

	Parameter		Group A	Group B	Group C
Controls	Ultimate CH_4 fraction	%	50±0	---	---
	Ultimate CH_4 volume	mℓ	83±2	---	---
	COD-to-CH_4	%	n.a.	---	---
	Max SMA	mg COD/g VSS/d	4±1 [(a)]	---	---
	Lag-phase	d	0	---	---
Size	Ultimate CH_4 fraction	%	53±1 (N=2)	54±0	55±0
	Ultimate CH_4 volume	mℓ	129±2 (N=2)	173±5	242±6
	COD-to-CH_4	%	**63±3** (N=2)	**62±4**	**55±2**
	Max SMA	mg COD/g VSS/d	109±11 (N=2)	155±2 (N=2)	152±4 (N=2)
	Lag-phase	d	0	0	0
Distillery	Ultimate CH_4 fraction	%	53±0	56±1	58±1
	Ultimate CH_4 volume	mℓ	124±1	159±4	240±8
	COD-to-CH_4	%	**59±2**	**54±3**	**56±2**
	Max SMA	mg COD/g VSS/d	98±7 (N=2)	136±3 (N=2)	100±4 [(b)]
	Lag-phase	d	0	0	≈ 1
Mixture 1:2	Ultimate CH_4 fraction	%	55±1	57±0	57±1
	Ultimate CH_4 volume	mℓ	128±2	167±4	238±4
	COD-to-CH_4	%	**67±3** (N=2)	**59±3**	**55±1**
	Max SMA	mg COD/g VSS/d	133±4 (N=2)	187±3 (N=2)	158±8
	Lag-phase	d	0	0	< 1
Mixture 2:1	Ultimate CH_4 fraction	%	54±1	55±1	57±1
	Ultimate CH_4 volume	mℓ	126±5	164±7	230±7
	COD-to-CH_4	%	**68±1** (N=2)	**60±1** (N=2)	**52±3**
	Max SMA	mg COD/g VSS/d	137±15 (N=2)	198±3 (N=2)	164±14 (N=2)
	Lag-phase	d	0	0	0

Group A, B and C indicate the low, medium and high concentrations respectively. Three replicates have been considered for the calculations (N=3), unless otherwise indicated.

[(a)] The SMA did not show a distinct maximum: this value indicates the average calculated over time (N=19).

[(b)] The pattern of the methanogenic activity is not homogeneous across the replicates, in the initial phase of the test (Figure G.90): this is possibly caused by an inaccurate determination of the gas composition. Therefore the maximum SMA reported here is calculated using unpaired data, obtained between day 3 and 5.

Conclusions

This test provided a set of data on the characteristics of the seed inoculum (e.g. its endogenous methanogenic activity), on the biodegradability of the size and distillery effluents and of their mixtures which can be compared with the other sets of data obtained so far, as well as interesting hints of debate. These aspects will be discussed jointly later on (see Sections 4.1.3.6, G.1.9 and G.1.10).

The qualitative observation that the methanogenic activity seems to actually benefit from the combined digestion of the two effluents contrasts with the (quantitative) assessment of the effectiveness of the process in terms of conversion of the organic matter. This proves that co-digestion should be assessed by looking at both the biodegradability and methanogenic activity (or inhibition) and supports the methodology proposed in Annexure B of simultaneously test both parameters in a modified serum bottle activity test.

4.1.3.5 Test No 5 – co-digestion

Objectives

This final co-digestion assay was aimed at testing 'realistic' combinations of the four industrial effluents.

The activity and co-digestion assays presented so far, may have not been conclusive in providing quantitative answers to the initial questions, i.e. to what extent is each test material biodegradable ? how severely is the methanogenic activity of the biomass affected in presence of each test material ? and ultimately, how do these two indicators change when a combination of two effluents is digested simultaneously ? However they clearly indicated:

- that the size and distillery effluents are biodegradable to a considerable extent (> 60 %) and do not inhibit the microbial activity for concentrations lower than 13 g COD/ℓ; and
- that the scour and synthetic dye effluents are not biodegradable and do inhibit the methanogenic activity at relatively low concentrations.

Therefore a realistic scenario would be to study whether the combinations of size and scour and of distillery and synthetic dye can actually improve the biodegradation of the recalcitrant components without causing an excessive deterioration of the methanogenic activity.

Materials and methods

The composition of the vials is reported in Table G.11. The setup comprised:

- a control group to assess the baseline gas production (no acetate was supplemented);
- four distinct sets of *reference* groups, each one containing one effluent as the sole organic substrate; two concentration levels of each test material were tested in parallel to provide the reference activity and biodegradability against which the effectiveness of the respective test units is to be assessed;
- three sets of test units, containing the first combination, i.e. the size and the scour effluents; each set corresponded to a volumetric ratio, i.e. 1:2, 2:1 and 1:1; and each volumetric ratio was tested at two levels of concentration; and
- three sets of test units, containing the combination of distillery and synthetic dye effluents; the same volumetric ratios and concentration levels were assessed.

All the individual groups were done in triplicate: a total of 63 serum bottles were monitored for over 70 days and the general experimental procedure adopted, i.e. a daily re-equilibration on two out of three replicates per group for the first week; thereafter a progressively decreasing frequency of re-equilibration was maintained.

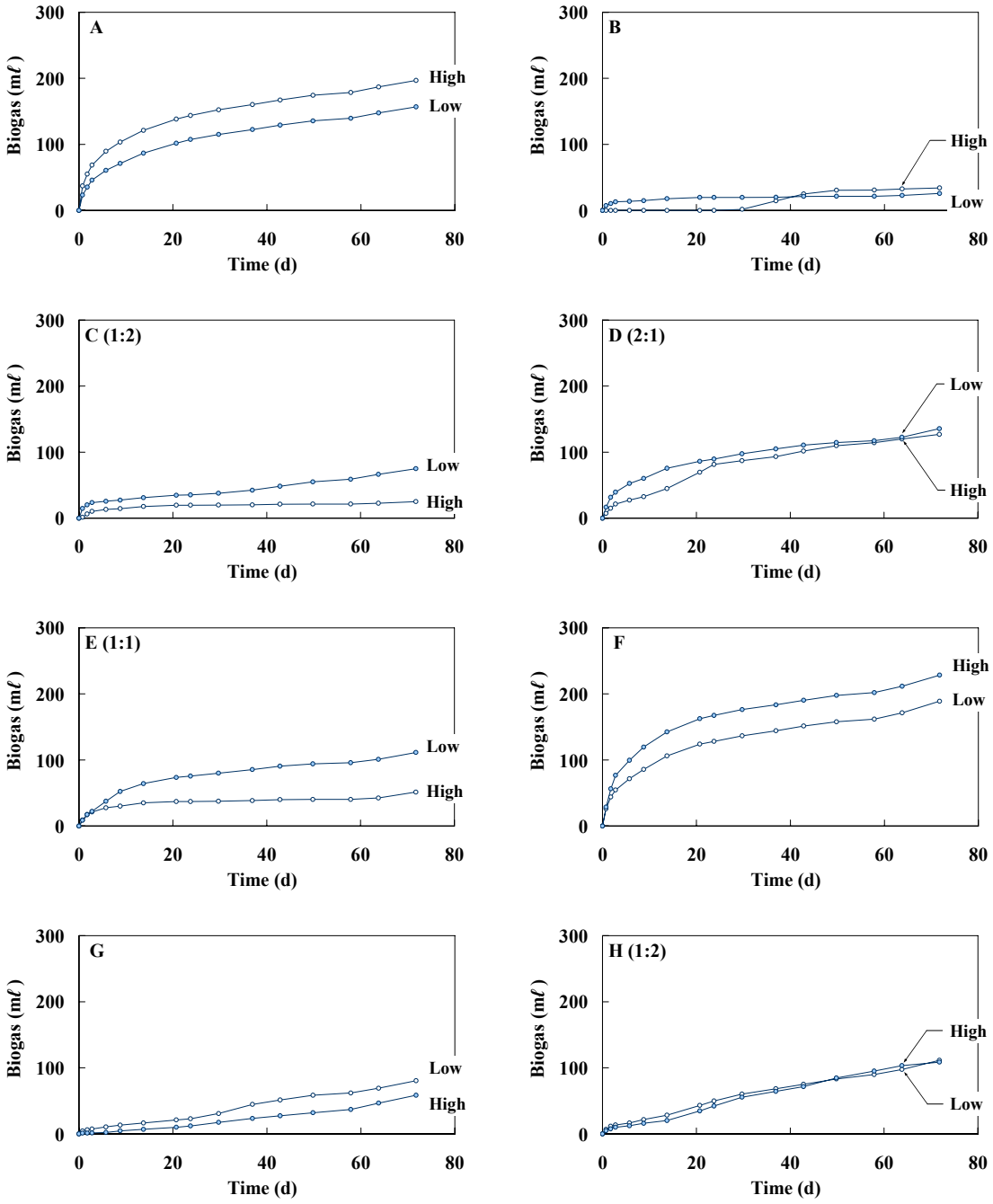

*Figure 36 – Average gas production in the reference units, containing the size (**A**), scour (**B**), distillery (**F**) and synthetic dye effluents (**G**); average gas production in the test units, containing the mixtures of size and scour effluents (**C**, **D** and **E**) and of the distillery and synthetic dye effluents (**H**, **I** and **J**) (cont.).*

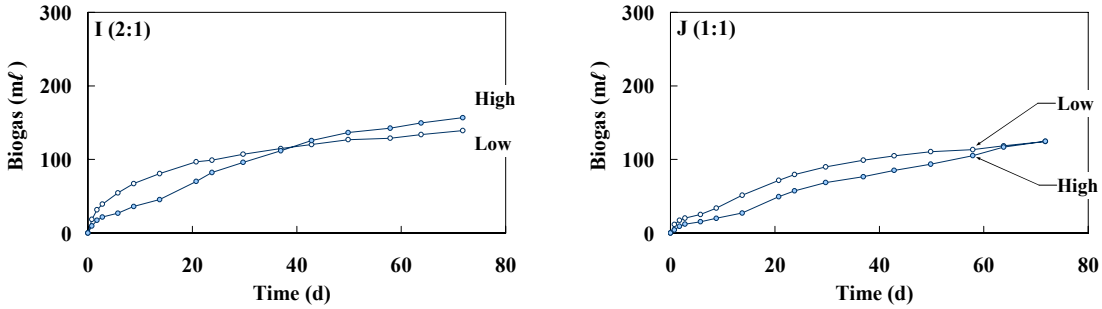

*Figure 36 – (continued) Average gas production in the reference units, containing the size (**A**), scour (**B**), distillery (**F**) and synthetic dye effluents (**G**); average gas production in the test units, containing the mixtures of size and scour effluents (**C**, **D** and **E**) and of the distillery and synthetic dye effluents (**H**, **I** and **J**).*

Results and discussion

The cumulative biogas production curves for the *reference* and test units are reported in Figure 36. Each curve is obtained as the average of three replicates, unless one differed significantly from the other two and was therefore disregarded in the calculations.

Generally, the gas production and composition in all replicates matched well (with only few exceptions).

Combination of size and scour effluents.

▪ The two test materials in isolation confirmed the expectations (Table 17) in that the size effluent resulted biodegradable to an extent of 70 to 85 % (which in fact is greater than the figures calculated in the previous experiment and possibly overestimated; Figure 39) and the methanogenic activity is in good agreement with the results obtained earlier; whereas the scour effluent is undegradable and likely inhibitory for the methanogenic biomass. The volume of methane produced at both concentration levels was smaller than that generated in the controls (Figure G.108); interestingly, at the lowest concentration (1.2. g COD/ℓ), the biomass seemed to partially resume a (low) methanogenic activity, after a period of ca. 20 days (Table 16), whereas the methanogenic activity at the highest concentration remained close to zero throughout the test, resulting in a methane fraction in the headspace much lower than that of the controls.

Unlike the case of a high organic strength effluent which may cause an organic overload to the biomass, due to the excessively large production of acidity (and carbon dioxide) in the early stage of the degradation process, the scour effluent appears to exert a truly inhibitory effect on the biomass since no accumulation of carbon dioxide occurs at the highest

concentration that can explain the poor methanogenic activity. However, it seems that some activity may be resumed, even at high concentration, after day 60 (Figure G.107); the test was interrupted shortly thereafter and no sufficient evidence is available to support this hypothesis.

▪ The response of the co-digestion units containing the size and scour effluents in combination confirms this general picture: the mixture with the largest proportion of scour effluent (1:2) had the poorest performance indicators (Table 17), i.e. no biodegradation, progressively lower methane fraction in the headspace and negligible methanogenic activity throughout the experimental period; a low methanogenic activity though, comparable to that of the controls, is resumed at the lowest concentration, after a lag-period of ca. 20 days (Table 16). As the proportion of scour in the mixture lessens, the performances improved: the 1:1 mixture still showed no biodegradation but the methanogenic activity was not totally impaired; the 2:1 mixture showed a moderate biodegradability (in the range of 5 to 30 % only), a low methanogenic activity (possibly lower than the reference activity on acetate, thus still indicating inhibited conditions, but this could not be ascertained) and a relatively large fraction of methane in the headspace. After a period of between 5 and 25 days (this variability being due to the unusually scattered data on gas composition), the units at low concentration resumed a low methanogenic activity (Table 16).

Incidentally, one should note that the determination of the gas composition in the units containing the mixture of size and scour effluents was generally poorly repeatable (Figures G.113, G.117 and G.121).

These observations are summarised in Figure 38 A which shows that the only combination of the two effluents that reaches a non-negative effectiveness is that with two thirds of the size effluent, i.e. of the labile component. This combination however lies in the region where co-digestion is not convenient, due to the obviously high inhibitory potential of the recalcitrant substrate which prevents the biomass from degrading the labile substrate (and reciprocally, the biodegradability potential of this latter must not be sufficient to promote the degradation o the former).

Nevertheless it is possible that more diluted combinations (i.e. containing a smaller proportion of the scour effluent) may pass the threshold set at E=1.

<u>Combination of distillery and synthetic dye effluents</u>.

▪ The two test materials in isolation confirmed the expectations (Table 17). The distillery effluent was degraded to an extent of 70 to 90 % (which is too a little higher than the figures estimated in the previous experiment, Figure 39) and the methanogenic activity is in the same

range as that displayed in the units supplemented with the size effluent, i.e. in excess to 100 mg COD/g VSS/d; unlike in the previous experiments, no significant accumulation of carbon dioxide was detected in the early stages of the process.

The synthetic dye effluent proved not biodegradable, although methanogenic activity was not completely inhibited: biogas was actively produced at both concentrations tested (with only a lag of ca. 5 d, at the highest concentration); the methane fraction became relevant only after approximately 20 d and levelled off at values lower than 40 % at high concentration. This suggests that the synthetic dye effluent is acutely inhibitory to methanogens but adaptation is likely to occur upon relatively long exposure times –this could be an aspect worth being investigated for a real-scale application.

At both concentration levels, the SMA resumed slightly after a period of ca. 30 d and reached values comparable to those of the controls (Table 16).

▪ A similar pattern can be reported for the units containing the distillery and synthetic dye effluents in combination, in that as the proportion of the former, more labile effluent increases, the overall biodegradability of the mixture and the methanogenic activity of the biomass improve (Table 17).

Interestingly, one third of distillery effluent in the mixture is sufficient to attain an overall biogas production equivalent to that of the controls (Figure G.132) and a methane fraction in the headspace close to 50 %, although the highest concentration level showed a delay in the onset of methanogenic activity of over 10 d (Figure G.133).

In the 1:1 mixture, the lag is gradually disappearing, although the high concentration still showed a significant methane production only after 8 d (Figure G.141); and the overall volume of methane produced exceeded that in the controls (Figure G.140) although after only 55 d for the units at high concentration, thus indicating a biodegradability in the range of 10 %. The mixture containing one third of synthetic dye did not show any delay in the onset of methanogenic activity nor showed any accumulation of carbon dioxide at the lowest concentration tested (Figure G.137); and reached an ultimate biodegradation of ca. 30 %, irrespective of the initial COD concentration.

It is worth noting that all units exposed to the synthetic dye effluent showed a similar pattern of the SMA: the units containing the effluent in isolation displayed a very low activity for a long period and after ca. 30 d a small peak in the methanogenic activity was observed; the units containing synthetic dye and distillery effluent started off with a relatively high activity (in fact, the maximum increases with the increasing proportion of distillery effluent) that

progressively decreased to negligible values and then displayed a second smaller peak after 15 to 20 d (Table 16). This behaviour suggests that, in presence of the synthetic dye effluent, the digestion process might occur through two distinct phases, i.e. the readily biodegradable fraction is metabolised first and only when the biomass has adapted to the more recalcitrant fraction this latter is eventually degraded.

These observations are summarised in Figure 38 B. One can observe that although none of the combinations tested proved entirely effective for being digested, the performances improve rapidly as the proportion of the distillery effluent increases: the 2:1 mixture reached values of E in the range of 0.55 to 0.60, twofold the effectiveness of the 1:1 mixture and significantly higher than the corresponding mixture of size and scour effluents.

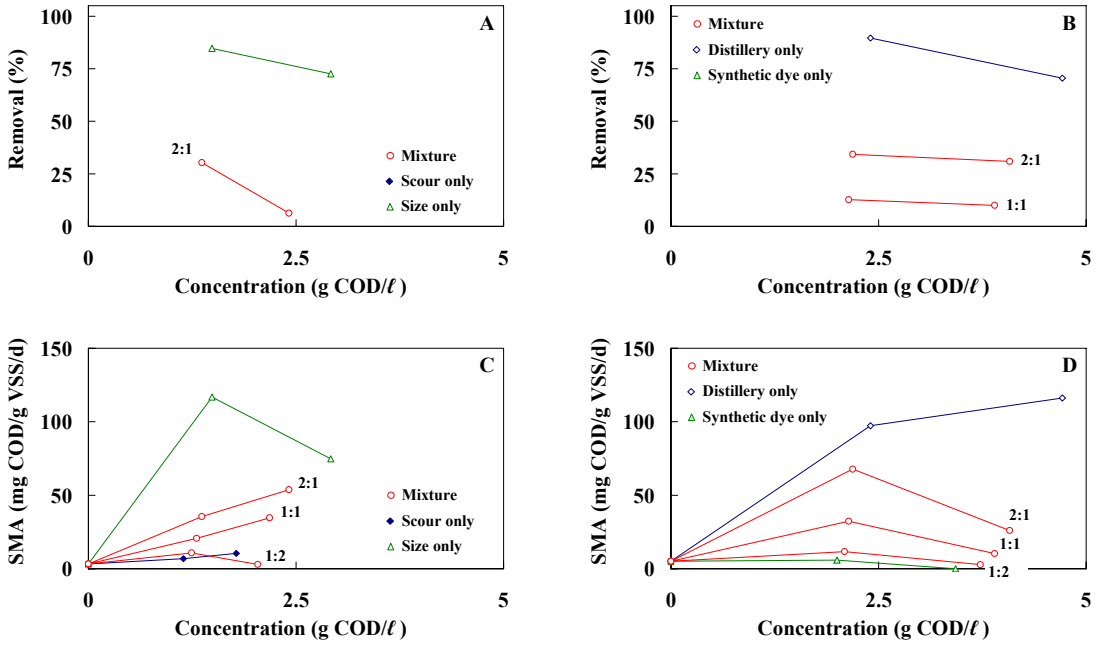

*Figure 37 – Summary of the outcome of the activity test (No 5): graphical comparison of the performance of the individual effluents and of the mixtures, in terms of biodegradability (**A**, **B**) and methanogenic activity (**C**, **D**).*

Conclusions

The inaccuracy of this test as a whole is slightly larger than that of the previous experiments, in that a few groups of replicates showed a non perfectly homogeneous behaviour.

It is certainly difficult to identify the cause of the lower quality of the response of this test: to answer this question one would probably need to set-up a specific experiment, which is not

within the scope of this research. However, a possible reason lies in the unusually large number of vials that were tested simultaneously.

If we consider that monitoring one bottle, i.e. determination of gas volume and gas chromatographic analysis of the gas composition, takes at least 8 minutes (the GC run lasts approximately 2 min and triplicate injections were done to ensure the reliability of the analysis), the operator needed a net of 5.6 h (equivalent to two thirds of 63 units) to monitor the entire experiment.

If one takes into account the time often required for instance to clean the gas-lock syringe which gets easily blocked with biological material when repeatedly inserted in the rubber septa, that figure may result largely underestimated. Furthermore, a key methodological aspect that is often neglected (Remigi and Foxon, 2005) is the necessity that the gas volume and gas composition for an individual serum bottle, and consequently for the (two) replicates monitored daily, are determined simultaneously or within the shortest period of time. This translates into the necessity that as soon as the gas volume is quantified in a set of replicates, their gas composition is also analysed. Otherwise a considerable delay might occur between the determination of the volume of gas produced in the vials monitored last and the analysis of the composition of the respective headspaces. This delay is obviously greater, the larger the number of serum bottles comprised in the test. This has the very important consequence that the two independent measurements are uncoupled, thus introducing a potentially large error in the mass balance (equation B.4) and particularly in the calculation of the SMA.

Ultimately these remarks provide a twofold methodological indication.

- The number of vials that a single operator can accurately monitor is limited: in our experience, assays requiring more than 35 to 40 units should be avoided.
- The accuracy of the response of an activity test depends on the simultaneous determination of the gas volume and composition in each vial.

The following specific conclusions can be derived from this exercise.

- The digestion of the size effluent, which is readily biodegradable, in combination with the scour effluent, which is non biodegradable and likely inhibitory, seems promising although only the 2:1 mixture reached a non-negative value of effectiveness, ranging from 0.30 to 0.40.
- The digestion of the distillery effluent (labile), in combination with the synthetic dye effluent (recalcitrant) has proven progressively more effective as the proportion of the former increases: with two thirds of the mixture consisting of distillery effluent, the

effectiveness was still insufficient (E<1) to ensure the profitability of the process, but it appears that more diluted mixtures of the synthetic dye effluent are likely to pass the threshold.

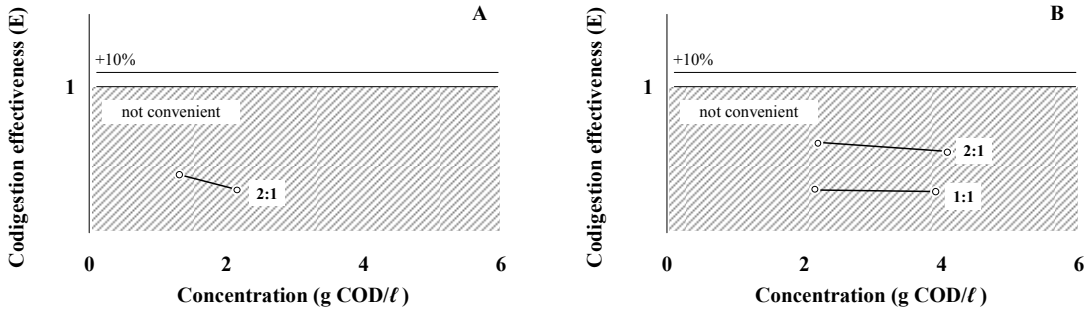

*Figure 38 – Effectiveness of the co-digestion option for the mixtures of size and scour effluents (**A**) and of distillery and synthetic dye effluents (**B**).*
The mixtures are labelled in terms of volumetric ratio, v_1:v_2, of the labile and recalcitrant components respectively (negative values of E are meaningless and therefore not represented in the plots).

Table 16 – Two-phase degradation process: lag-phase (visually estimated) and maximum methanogenic activity.

	Initial SMA		**Maximum SMA after lag-period**	
	Low	High	Low	High
Scour	< 1	10±2	≈20 d; 7±0 (6)	---
Mix 1:2	20±2 (2)	11±1 (2)	≈20 d; 3±1 (15)	---
Mix 2:1	35±6 (2)	54±6 (2)	5-25 d; 7±3 (11)	---
Synthetic dye	6±1 (2)	< 1	≈30 d; 7±1 (6)	≈30 d; 4±1 (6)
Mix 1:2	12±2 (2)	4±1 (6)	≈20 d; 6±0 (5)	≈20 d; 8±1 (8)
Mix 2:1	68±0 (2)	26±2 (2)	---	≈20 d; 12±2 (6)
Mix 1:1	32±2 (2)	10±0 (2)	≈14 d; 10±1 (8)	≈20 d; 11±0 (4)

Note: Units of SMA are (mg COD/g VSS/d); *Low* and *High* denote the concentration levels tested; the number in brackets indicates the population size used to calculate the respective average and standard deviation.

Table 17 – Summary of the results of the serum bottle assay on industrial effluents (No 5) (cont.).

	Parameter		Group A	Group B
Controls	Ultimate CH_4 fraction	%	48±1	---
	Ultimate CH_4 volume	mℓ	72±1	---
	COD-to-CH_4	%	n.a.	---
	Max SMA	mg COD/g VSS/d	3±1 [(a)]	---
	Lag-phase	d	0	---
Size	Ultimate CH_4 fraction	%	53±0	55±1
	Ultimate CH_4 volume	mℓ	95±2	114±0
	COD-to-CH_4	%	**85±1** (N=2)	**73±1**
	Max SMA	mg COD/g VSS/d	75±1 (N=2)	117±1 (N=2)
	Lag-phase	d	0	0
Scour	Ultimate CH_4 fraction	%	55±5	13±3
	Ultimate CH_4 volume	mℓ	37±1	9±1
	COD-to-CH_4	%	**< 0**	**< 0**
	Max SMA	mg COD/g VSS/d	7±0	10±2
	Lag-phase	d	≈ 20	n.a.
Mixture 1:2	Ultimate CH_4 fraction	%	44±2 [(b)]	10±1 [(b)]
	Ultimate CH_4 volume	mℓ	43±4	7±1
	COD-to-CH_4	%	**< 0**	**< 0**
	Max SMA	mg COD/g VSS/d	20±2 (N=2)	11±1 (N=2)
	Lag-phase	d	≈ 20 [(c)]	n.a.
Mixture 2:1	Ultimate CH_4 fraction	%	54±1 [(b)]	47±0
	Ultimate CH_4 volume	mℓ	81±3 (N=2)	74±2
	COD-to-CH_4	%	**30±9** (N=2)	**6±2** (N=2)
	Max SMA	mg COD/g VSS/d	35±6 (N=2)	54±6 (N=2)
	Lag-phase	d	0	0
Mixture 1:1	Ultimate CH_4 fraction	%	46±4	16±1
	Ultimate CH_4 volume	mℓ	66±5	14±1
	COD-to-CH_4	%	**< 0**	**< 0**
	Max SMA	mg COD/g VSS/d	35±0	21±1 (N=2)
	Lag-phase	d	0	n.a.

Group A and B indicate the low and high concentrations respectively. Three replicates have been considered for the calculations (N=3), unless otherwise indicated.

Note: the SMA values reported in this table indicate the maximum methanogenic activity, irrespective to the lag-phase indicated, if any (see Table 16).

[(a)] The SMA did not show a distinct maximum: this value indicates the average calculated over time (N=12).

[(b)] The three replicates showed a very poor repeatability.

[(c)] The three replicates showed a very poor repeatability and the methanogenic activity started between day 10 and day 37.

Table 17 – (continued) Summary of the results of the serum bottle assay on industrial effluents (No 5).

	Parameter		Group A	Group B
Distillery	Ultimate CH_4 fraction	%	54±0	55±1
	Ultimate CH_4 volume	mℓ	114±2	138±4 (N=2)
	COD-to-CH_4	%	**90±3**	**71±4** (N=2)
	Max SMA	mg COD/g VSS/d	97±1 (N=2)	116±3 (N=2)
	Lag-phase	d	0	0
Synthetic dye	Ultimate CH_4 fraction	%	43±1	37±1
	Ultimate CH_4 volume	mℓ	50±1	35±2
	COD-to-CH_4	%	**< 0**	**< 0**
	Max SMA	mg COD/g VSS/d	7±1 (N=6)	4±1 (N=6)
	Lag-phase	d	≈ 20	≈ 20
Mixture 1:2	Ultimate CH_4 fraction	%	48±1	52±2
	Ultimate CH_4 volume	mℓ	67±1	65±6
	COD-to-CH_4	%	**< 0**	**0±1**
	Max SMA	mg COD/g VSS/d	12±2 (N=2)	8±1 (N=8)
	Lag-phase	d	0	≈ 15
Mixture 2:1	Ultimate CH_4 fraction	%	53±1	59±1
	Ultimate CH_4 volume	mℓ	87±1	100±1 (N=2)
	COD-to-CH_4	%	**34±1**	**31±1** (N=2)
	Max SMA	mg COD/g VSS/d	68±0 (N=2)	26±2 (N=2)
	Lag-phase	d	0	≈ 10
Mixture 1:1	Ultimate CH_4 fraction	%	51±0	56±1
	Ultimate CH_4 volume	mℓ	77±1	81±1
	COD-to-CH_4	%	**13±3** (N=2)	**10±1**
	Max SMA	mg COD/g VSS/d	32±0 (N=2)	11±0 (N=4)
	Lag-phase	d	≈ 8	≈ 15

Group A and B indicate the low and high concentrations respectively. Three replicates have been considered for the calculations (N=3), unless otherwise indicated.

Note: the SMA values reported in this table indicate the maximum methanogenic activity, irrespective to the lag-phase indicated, if any (see Table 16).

4.1.3.6 Summary of the findings on the biodegradability of the test materials

One of the main outputs of this project is the evaluation of a protocol that enables the conduction of screening tests aimed at assessing the biodegradability and inhibitory potential of an industrial effluent (under anaerobic conditions).

Functional to this primary objective is the evaluation of the reliability of the method that we recommend as the preferential tool to conduct the screening, i.e. the serum bottle method.

A method is reliable when it is able to picture the phenomenon targeted as close as possible to the reality. This concept of reliability implies two other concepts, i.e. the accuracy and the reproducibility of the method which apply to two distinct levels.

The accuracy deals with the ability of replicates of the same conditions to give a consistent response: it is therefore evaluated by comparing the replicate units within a single experiment.

On the other hand, the reproducibility deals with the ability of independent tests to respond consistently under the same conditions.

So far it has been demonstrated that each activity assay was inherently *accurate*, in that the variation (both in the volume and in the composition of gas produced) between the replicate units within the same experimental group was generally very small, with only few exceptions.

The *reproducibility* of the serum bottle method can be appraised by comparing the response of independent activity tests on the same test material under homogeneous 'boundary conditions'.

This is the objective of this section.

The biodegradability potential of the size and distillery effluents was repeatedly assessed through the activity tests described earlier in this chapter.

The output of these tests is summarised in Figure 39.

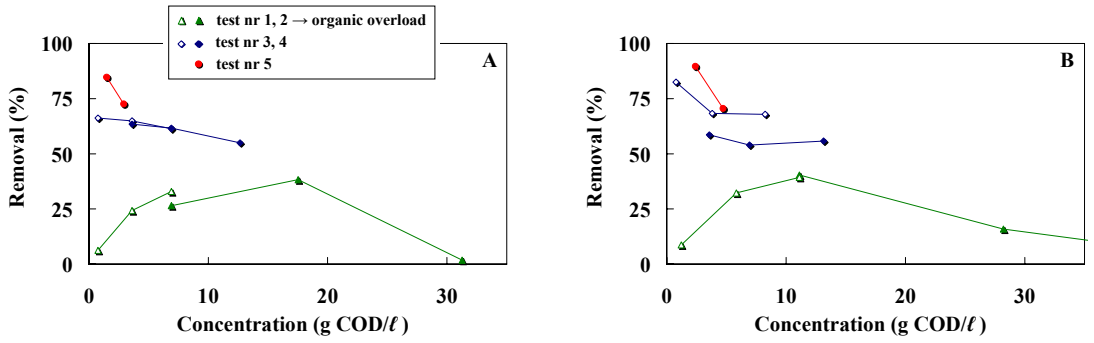

*Figure 39 – Comparison of the biodegradability potential for the size (**A**) and distillery effluents (**B**), assessed in the five serum bottle activity tests performed.*

Not all the experiments conducted were homogeneous: tests No 1 and No 2 were conducted according to the methodology of a combined activity and biodegradability test proposed in Annexure B, i.e. an external reference substrate was supplemented along with the test materials; tests No 3 to 5 were conducted without external substrate as pure biodegradability tests. More importantly, an excessively large amount of acetate was supplemented in tests No 1 and No 2 which most likely resulted in the inhibition of the methanogenic biomass.
Therefore a comparison is possible within the two groups separately.
The values of biodegradability for both the size and the distillery effluent obtained in test No 1 and No 2 match quite well. In fact, one concentration level only was replicated in the two tests (7 and 11 g COD/ℓ respectively) and the corresponding estimates are sufficiently close for the size effluent and almost identical for the distillery effluent. However, the general pattern described by the values calculated at different concentrations is intuitively correct, because the removal is progressively decreasing at low and at high concentrations, due to a detection limit and to the inhibition of the biomass respectively; and it is significantly lower than that calculated under non-inhibited conditions, i.e. the other group of tests.
The reproducibility of tests No 3 and No 4 for the size effluent is very high since the values of biodegradability calculated at 3.6 and 6.9 g COD/ℓ are matching almost perfectly. The biodegradability of the size effluent gradually declines with the concentration.
For the distillery effluent, the reproducibility of these tests is clearly poorer; however, one can agree that unaccounted factors such as the residual overpressure during the re-equilibration (we demonstrated that the biodegradability may be underestimated by 15-20 %) can be responsible of this result.
Unexpectedly, test No 5 indicates a much higher biodegradability of both the size and distillery effluents at 1.5 and 2.4 g COD/ℓ respectively (whereas at 2.9 and 4.7 g COD/ℓ, the value is closer to that of the previous tests).
The reason for this is unknown and would possibly need to be investigated.

Ideally, the same type of analysis should be conducted on the other parameter quantified through a serum bottle activity test, i.e. the inhibitory effect of the test material. However, the available set of data is insufficient, since tests No 3, 4 and 5 did not contain acetate, thus do not allow a comparison of the biomass activity to a reference activity.

In conclusion, the serum bottle method seems to be sufficiently reproducible although a quantitative assessment of this property (similar to the variation coefficient of the replicates, for the assessment of the accuracy) was not possible due to a generally poor homogeneity of the different tests performed.

4.2 LABORATORY-SCALE EXPERIMENTS

The objective of this experimental phase at the laboratory-scale was ultimately to feed a digester in a continuous mode with a mixture comprising a labile and a recalcitrant effluent in appropriate proportions, for a period of time sufficient to assess the performance of the process under a variety of operating conditions. This clearly requires that the entire system is technically reliable and that the performance of the process is preliminarily evaluated under reference conditions against which the effectiveness of the co-digestion option will then be evaluated.

For the system to be reliable, the set-up is to be verified, particularly the reliability of the device that measures the biogas flow rate and the functionality of the feeding and sampling systems. One should also elaborate a monitoring and controlling strategy that enables the reliable detection of the conditions of the process and the effective control of potentially dangerous situations (e.g. incoming acidification).

This is equivalent to the necessity of setting-up a group of reference units along with the test units in a serum bottle activity test. Since the digester is to be conducted in a feed-and-draw mode, that will eventually become a continuous mode, and since monitoring manually more than one unit is unpractical, these two parallel conditions, i.e. the reference and the test units, change into two sequential phases within the same reactor.

Therefore it was decided to start-up a completely stirred reactor, inoculated with anaerobic sludge extracted from an operating full-scale anaerobic digester, and to feed it with one of the labile effluents, i.e. the size or the distillery effluent, for a certain period of time. This first phase would have provided the reference conditions for the final assessment of the effectiveness of the co-digestion process but would have also enabled us to optimise the set-up and the monitoring program.

Although both the size and the distillery effluents proved to be biodegradable (up to 60-65 % and 55-65 %, respectively) in batch conditions and were therefore the obvious candidates of labile substrates (as opposed to the recalcitrant scour and synthetic dye effluents), the distillery effluent had consistently shown that a fraction of the readily biodegradable organic matter was rapidly converted to volatile acids, thus resulting in an initial accumulation of carbon dioxide in the headspace that might create inhibitory conditions for the methanogens.

This can be a delicate issue in a continuous mode when the loading rate (and retention time) must not allow the long-term accumulation of fatty acids in the reactor (and of carbon dioxide in the off-gas).

It was however decided to use the distillery effluent as labile substrate to feed the digester, in order to enable the microbial consortium to develop the enzymatic machinery that was necessary to effectively degrade the organic fraction of the labile substrate and to cope with the possible accumulation of acidity in the suspension hence of carbon dioxide in the gas phase.

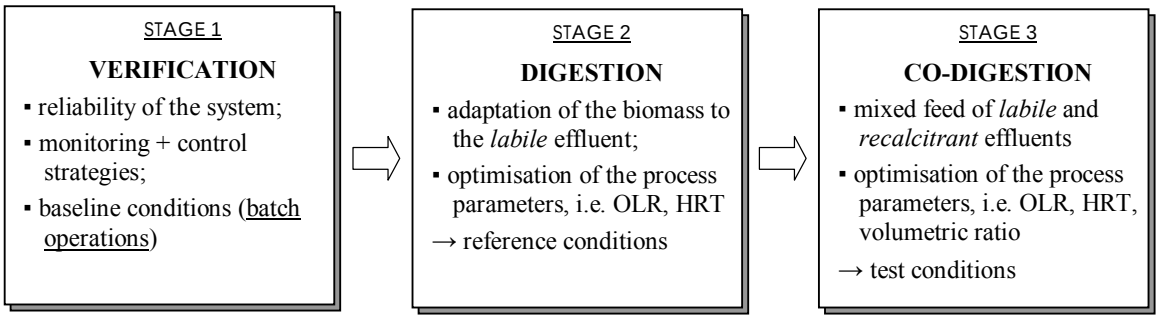

Figure 40 – Experimental plan of the laboratory-scale investigation.

The exercise would have comprised the following stages (Figure 40).

- Batch conditions. The seed sludge is discontinuously supplemented with an easily degradable substrate, typically acetate, to promote a stable methanogenic activity prior to the addition of more complex substrates that would generate acidic intermediates. This is done manually by spiking an appropriate dose of the substrate in the digester e.g. daily, based on the knowledge of the SMA of the seed sludge –ideally this is to be assessed in advance with a serum bottle activity test.

  This phase does not differ in principle from a serum bottle activity test in that it allows to verify the reliability of the gas measuring device and gas tightness of the digester, through mass balances, based on the assumption that the cumulative volume of methane generated must be equivalent to the amount of acetate fed.

- Semi-continuous (or fed-batch) conditions. The actual test effluent is used as the sole source of organic matter to feed the sludge; an appropriate volume of effluent is to be selected that will not result in an organic (or toxic) shock for the biomass and sufficient nutrients must be provided (e.g. by diluting the effluent with a NMS prior to feeding the reactor). The rationale of the semi-continuous regime is to give the biomass a sufficient period or time (reaction time) to degrade the organic substrate, and overcome any transient event, under pseudo-batch conditions before the following feeding operation.

  This phase is obviously a step forward in the direction of a continuous operation in that the micro-organisms are basically working in batch conditions (like in a serum bottle test) but

are periodically subjected to a dose of substrate and therefore can operate in a limited period of time, between two subsequent dosages.

- Continuous feeding. Once the biomass has been exposed to the substrate and has proved able to degrade it, the influent can be fed to the digester continuously.

  Under these conditions, the parameters of interest are the organic loading rate (OLR) and hydraulic retention time (HRT). The ultimate objective is to optimise the performance of the process, i.e. maintaining the desired organic removal efficiency at the highest possible OLR (hence lowest possible HRT).

  Thereafter a co-digestion exercise can be started in which the recalcitrant effluent is added to the feed (e.g. by 'splitting' the OLR set in the previous phase between the labile and the recalcitrant components of the mixture in appropriate proportions) and the performances are evaluated (and possibly optimised) against the performances of the digestion of the labile substrate in isolation.

The full experimental plan could not be completed in the course of this project, due to a variety of technical problems and to the unexpectedly long duration of the semi-continuous phase.

It was therefore decided to start a second unit, that was to be fed with the size effluent as labile substrate and to perform the co-digestion exercise under semi-continuous conditions, avoiding the continuous phase altogether.

This chapter illustrates the results obtained in the batch and semi-continuous phases on the first digester that treated the distillery effluent; the results of the second digester and of the – co-digestion experiments that are partially on-going at present will be reported separately (Dlamini, in preparation).

4.2.1 What information can a serum bottle activity test provide for the start-up of a semi(continuous) experiment ?

When some understanding as to how the test material behaves under anaerobic conditions is obtained through a series of serum bottle tests, the question is how can one apply that information to effectively stat-up an anaerobic digester.

The parallel between a serum bottle and a continuously fed reactor is twofold: in a batch system, the biomass is initially exposed to a relatively high concentration of the substrate; thereafter it has virtually unlimited time to adapt if necessary, hence to overcome temporary inhibitory conditions and ultimately to degrade the substrate; in a continuous system, the biomass is constantly exposed to a certain concentration of the substrate (which is set by the ability of the biomass itself to degrade it) and the time for adapting and processing the feed is limited by the hydraulics of the system.

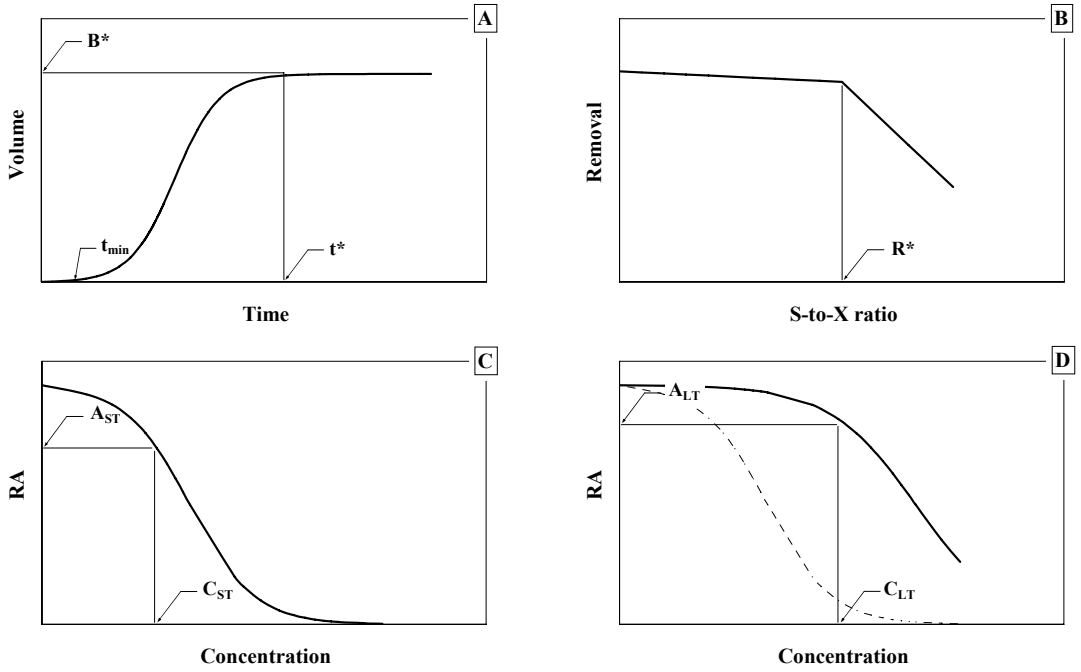

Figure 41 – Schematic outcome of a series of serum bottle activity tests, on a generic test material and relevant parameters to be transferred to the start-up of an anaerobic digester.

With reference to Figure 41, a tentative guideline can be provided.

- If t^* is the period of time (days) needed for the micro-organisms to degrade the test material (to an extent B^*) and t_{min} the period of time needed to adapt to the test material (e.g. to overcome overloading or inhibitory conditions), the HRT can be ideally set to equal t^*.

Should t^* be excessively long, hence HRT be not feasible, a lower retention time could be set that would result in a lower removal rate. A lower limit for HRT should be set at t_{min}, below which it is likely that the test material would leave the reactor undegraded.

- If R^* is the limit of the substrate-to-biomass ratio (g COD/g VS/d) that ensures a sufficient (or desired) level of biodegradation, the OLR can be set accordingly, i.e.

$$OLR = \frac{R^*}{HRT} \cdot X \qquad (9)$$

where X is the biomass concentration in the digester.

- A combined biodegradability and toxicity assay can provide information on both the short- and long-term inhibitory effect of the test material, the difference being that the former is calculated with reference to the SMA immediately after the test is started; the latter, with reference to the maximum SMA which will likely occur after a period of adaptation.

  The question is that although the test material might be partially biodegradable, it can also negatively affect the methanogenic activity temporarily, e.g. because it is rapidly converted to acidic intermediates, or permanently. In practice, a concentration should be chosen that only results in a limited, or even nought reduction in the relative activity (RA) of the biomass.

  One can interpret the reduction of RA as the reduction of (active) methanogenic biomass from X to $X' < X$ (which can be real or apparent) which in turn limits the OLR that can be applied, according to equation 9.

  In this calculation, one has to refer to the long-term inhibition (Figure 41 D), because the corresponding value of the retention time should be sufficient for the biomass to attain its maximum activity.

4.2.2 Anaerobic digestion of the distillery effluent

A 5 ℓ-volume flask was used to set-up the completely stirred laboratory-scale reactor, to ultimately undertake a long-term co-digestion experiment that used the distillery effluent as the labile substrate.

Background information

As illustrated earlier in this chapter, the serum bottle method (or another equivalent small scale assessment) should be used to provide specific information regarding a 'safe' set of operating parameters which will constitute the starting point for a larger scale investigation.

The AAT described in section 4.1.3 can be summarised as follows:

- the distillery effluent is biodegradable to an extent of about 40 % at a concentration of 10-12 g COD/ℓ (overloaded conditions) or 55-70 % in a range of concentration of 4 to 14 g COD/ℓ;
- it is not inhibitory on the long-term to an unacclimated biomass for concentrations as high as 10-11 g COD/ℓ; a transient reduction in methanogenic activity (40-60 %) occurs on the short-term at the same level of concentration (although these figures refer to overloaded conditions);
- the maximum methanogenic activity of the sludge exposed to the distillery effluent ranges from 65 to 90 mg COD/g VS/d; and
- at relatively high concentration (i.e. higher than 8 g COD/ℓ), the rapid acidification of the organic fraction of the effluent may result in a temporary decrease in pH, hence in an organic overload of the methanogenic biomass: in batch conditions, this transient lasted 2 to 3 days at most.

Objectives

This experiment was conducted primarily to start-up and maintain a stable anaerobic digestion process, in which the effluent from a distillery factory can be effectively converted to methane. This required the evaluation of the appropriate operating conditions (e.g. retention time, loading rate, etc.) which would ensure a sufficient (if not optimal) performance of the process.

This in turns translates in the necessity of closely monitoring the process, to identify the 'performance indicators' which are most sensitive.

The indicators which are commonly used are (Table 18):

- the gas flow rate, or preferably the flow rate of methane (ℓ/d);
- the gas composition, i.e. the fraction of carbon dioxide and methane in the headspace (%);

- the methanogenic activity (ℓ CH4/g VS/d);
- the COD removal efficiency (%) or the COD concentration (g/ℓ) in the effluent;
- the solids concentration (g VS/ℓ) in the sludge;
- the alkalinity and/or the VFA concentration (mol/ℓ) or their ratio; and
- the pH value of the suspension.

The determination of other parameters, e.g. hydrogen concentration in the liquid and/or in the gas phase, VFA speciation, etc., would certainly assist in obtaining a better monitoring and control of the process, but established methods were not available and would certainly not be available routinely on site at a wastewater work.

Table 18 – Operational parameters which can be used as indicators of performance and/or as early warnings of an incipient upsetting condition.

Quantity	Indicator	Warning (*)
Gas (or methane) flow rate	✓	✓
Gas composition	×	✓
SMA	✓	(✓)
COD concentration	✓	×
Solids concentration	✓	×
Alkalinity, VFA concentration	×	✓
pH value	✓	(✓/×)

(*) A parameter suitable to provide an early warning must *i*) be sufficiently sensitive to the characteristics of the influent and *ii*) respond fast enough to enable the operator to intervene on the characteristic of the feed *before* the upsetting conditions become irreversible.

Materials and methods

Three litres of sludge were withdrawn from the anaerobic reactor at the Umbilo Wastewater Treatment Plant and transferred in a 5 ℓ glass vessel that was immediately closed with a rubber cap. No further manipulation of the sludge was done, to prevent any possible contamination with air. The reactor was placed in a temperature controlled room, maintained at 31 ± 2 °C throughout the experimental period.

- The volume of gas produced was automatically measured, by a liquid displacement (volumetric) system (Figure 12). Readings of the gas volume and analysis of the gas composition were done at least once a day.
- Feed and samples extractions were done manually using a 50 mℓ plastic syringe.
- COD, solids concentration, alkalinity, VFA concentration and pH were determined as discussed in Chapter 3, all on a daily basis except solids that were measured weekly.

- The methanogenic activity of the sludge was evaluated weekly by means of an off-line AAT (in duplicate): an aliquot of mixed liquor (30 to 50 mℓ) was poured into a serum bottle, immediately after extraction; flushed with a 1:1 N_2:CO_2 gas mixture for one minute, then sealed; no external substrate nor NMS were added and the SMA on the residual substrate (i.e. distillery) was evaluated for the two subsequent weeks.

Results and discussion

The experimental period can be subdivided into two phases:

- phase No 1 (days 0-11): the reactor was maintained in a batch-fed mode: acetic acid was spiked daily in the attempt of favouring the methanogenic populations; the last four days of operations, the distillery effluent was also added, at increasing volumes (*Table* 19), to evaluate whether it had a significant impact on the 'baseline' methanogenesis. The anaerobic process was closely monitored through gas production and composition: readings of the gas counter and gas-chromatographic analysis of gas samples were done several times, after the addition of the reagents.

Table 19 – Composition of the feed during the 10-day batch phase.

Day No.	Acetic acid (mℓ)	Distillery effluent (mℓ)	Organic load (*) (g COD/d)	NMS (mℓ)
1-5	5	0	5.5 (0)	0
6	7.5	0	8.3 (0)	100
7	0	20	2.4 (100)	0
8	5	40	10.3 (46)	0
9	5	80	15.1 (63)	0
10	5 + 5	80+80	30.3 (63)	0

(*) The fraction of distillery effluent on the total organic load in indicated in brackets (%).

- phase No 2 (days 12-141): the regime was switched to semi-continuous, that consisted in feeding the reactor and withdrawing a sample of the mixed liquor every day (except occasionally, when feeding and/or sampling were discontinued; *Table* 20).

  Being the laboratory-scale digester a completely stirred tank which had not been coupled to a settler for sludge thickening and recirculation, the only option to retain the sludge in the digester upon sampling would have been to stop the mixing for some time prior to extracting the sample, to allow the sludge to settle within the digester.

  We were concerned that the liquid sample extracted would not adequately represent the conditions inside the digester. However, it is certainly advisable for a possible follow-up

of this study (e.g. for the continuous phase) that some simple modifications be incorporated in the basic layout of the digester, in order to enable the independent sampling of the supernatant and of the mixed liquor, thus to uncouple the hydraulic and the sludge retention times (Section D.1).

This will become clear in the following discussion, since the best performance in terms of biodegradation of the distillery effluent (and yet as low as 40 % organic removal) were obtained at unrealistically long hydraulic retention times (70-80 d).

For this semi-continuous experiment though, the sludge retention time could only be extended by lowering the feed flow rate (i.e. HRT = SRT; *Table* 20) and ultimately no significant growth of new biomass was observed (Figure 52 D).

During this phase, not only gas production and composition were constantly monitored, but also pH, alkalinity, VFA concentration and COD were measured daily (with few exceptions); solids were quantified on a weekly basis and off-line activity tests using serum bottle were carried out.

The flow rate and composition of the feed were changed a few times, so that this phase can be subdivided into eight distinct experimental periods (*Table* 20).

Table 20 – Hydraulic conditions during the semi-continuous phase.

Period	**Feed (mℓ)**			**Other substrates (g)**		**HRT**	**OLR**
(d)	**Acetic ac.**	**Effluent**	**NMS** [*]	**Glucose**	**Yeast**	(d)	(g/ℓ/d)
I (12-16) [1]	0	100	0	0	0	34	3.4±0.1
II (17-41) [2]	0	100	100	0	0	18	3.4±0.2
III (42-69) [3]	0	25	75	0	0	35	1.0±0.1
IV (70-75)	0	50	50	0	0	35	1.9±0.1
V (76-93)	0	50	50	1.0	2.0	35	2.5±0.2
VI (94-105) [4]	0	50	50	1.0	2.0	≈ 80	≈ 1.0
VII (106-116) [5]	0	0	0	0	0	-	-
VIII (117-141) [6]	0	50	50	1.0	2.0	≈ 87	≈ 1.0

[*] Concentrated nutrient and minerals stock solution (10 x).
[1] On day 12, 5 mℓ of acetic acid were dosed in the digester.
[2] The feed was discontinued between day 28 and day 32, according to the following scheme (acetic acid:effluent: NMS, figures in mℓ): days 28-29, 5:100:95; day 30, 0:100:100; day 31, 0:0:50; day 32, 0:0:150.
[3] On days 47 and 48, acetic acid (3 and 5 mℓ respectively) was added to the feed.
On days 58 and 59, the feed was discontinued. On days 66 to 69, the NMS was discontinued and the distillery effluent was mixed with tap water.
[4] The reactor was fed every 2 d, except on days 100 and 102 when feed was discontinued.
[5] No feed.
[6] The reactor was irregularly fed: the HRT and OLR are calculated as average on the entire period.

4.2.2.1 Batch phase

During the fed-batch phase, the only variables monitored were the gas production and composition.

Initially (days 0-5), the digester was fed with acetic acid (glacial, 35.4 %): a single dose of 5 mℓ was spiked daily in order to favour the (start up) of methanogenesis. The dose of 5 mℓ had been chosen based on a previous experience on serum bottle tests, in which doses of 30 to 50 μℓ of acetic acid (or ethanol) were spiked in the vials containing 50 mℓ of seed sludge: that procedure was proven to be successful in maintaining a stable methanogenic activity for long period of times, without affecting the composition of the biogas produced.

A relatively constant biogas flow rate was maintained for the first five days (3.5±0.6 ℓ/d; Figure 42 A) and the gas composition seemed to be reaching an equilibrium, with a methane molar fraction of about 60 % (Figure 43 A).

If we consider that a dose of 5 mℓ/d of acetic acid equates to a stoichiometric volume of methane of 2.1 ℓ/d, the measured flow rate corresponds to a recovery close to 100 %.

Another evidence that the sludge was performing well, and therefore that it could have been safe to feed the digester with the distillery effluent, comes from a rough estimation of the specific methanogenic activity. Data are not as accurate as for a serum bottle activity test; in particular, we only measured the biomass concentration on day 14 (16 g VS/ℓ) and the amount of sludge poured in the reactor was not exactly quantified prior to starting the test, so that the actual biomass present in the digester can be a little inaccurate.

Given these limitations however, the SMA is:

$$2.1\frac{\ell CH_4}{d}\cdot\left(0.350\cdot 1.1\frac{\ell CH_4}{gCOD}\right)^{-1}\cdot\left(16\frac{gVS}{\ell}\cdot 3.4\ell\right)^{-1}=100\frac{mgCOD}{gVS\cdot d}$$

which compares well to the reference value of 57±5 mg COD/g VS/d (Figure 1).

On day 6, a larger dose of acetic acid was added together with 100 mℓ of concentrated NMS, in the attempt of supplementing the culture with nutrients and minerals, should these have been depleted by the degradation of the previous doses of acetic acid.

No apparent improvement in the biomass activity was detected (Figure 42 A): this was assumed as an indication of relatively good health of the micro-organisms. It was therefore decided that was safe to add a dose of the test material to the feed.

The following three days (days 7 to 9), single doses of acetic acid mixed to increasing volumes of the distillery effluent were added once a day. On day 10, two doses were added approximately 12 h apart. Being this batch feeding phase potentially delicate (due to the risk

of overloading the biomass), the gas composition was monitored several times per hour. This procedure enabled us to identify a consistent trend in the composition of the biogas (an example is shown in *Figure* 43 B): one can observe that immediately after the feed is spiked in the digester, the test material undergoes a relatively fast fermentation, which generates carbon dioxide in excess; thereafter, methanogenic conditions are gradually resumed within a day. This same behaviour was observed consistently in the serum bottle activity tests reported earlier in this chapter. The general trend in the methane fraction seems promising, as it progressively increased from day 7 to day 10 (Figure 43 A).

During the same period, the volume of gas produced increased remarkably (Figure 42 A).

One can tentatively plot the gas flow rate against the organic load, to verify whether the increasing doses of distillery effluent had an impact on the efficiency of the digestion. Figure 42 B shows that the activity of the biomass improved; surprisingly, a double dose of distillery effluent on day 10, boosted the biomass activity. The values of sludge activity are calculated taking 4.5 h after the digester had been feed as reference, based on the observation that the activity peaks for a short period (less than 0.5 h) immediately after feed (Figure G.147 A), then it levels off and remains relatively constant.

Conclusions

The following indications can be derived from this short batch experiment:

- a transient accumulation of carbon dioxide (hence volatile acids) shortly after feed is to be expected, when the distillery effluent is fed to the digester; and
- a loading rate of approximately 4.5 to 9.0 g COD/ℓ/d, corresponding to 40 to 80 mℓ/d of distillery effluent is expected to have a negligible impact on the methanogenic biomass.

It may be worth reporting that a considerably deep layer of foamy sludge was noticed on the liquid surface, which possibly prevented the biogas generated to escape. This condition is not unusual when digesters are subjected to high organic loads (Speece, 1996), but it should be avoided since it might cause, if persistent, the acidification of the suspension.

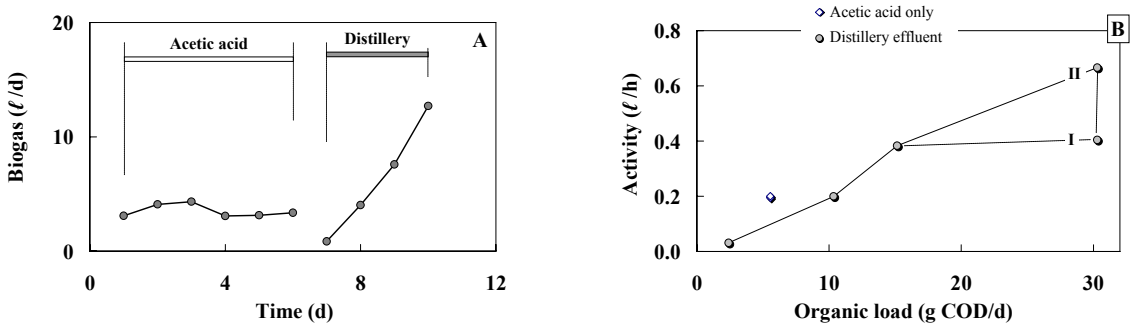

*Figure 42 – Average daily flow rate of biogas (**A**) and average sludge activity, as a function of the organic load (**B**). (I), (II) first and second doses of feed supplemented on day 10.*

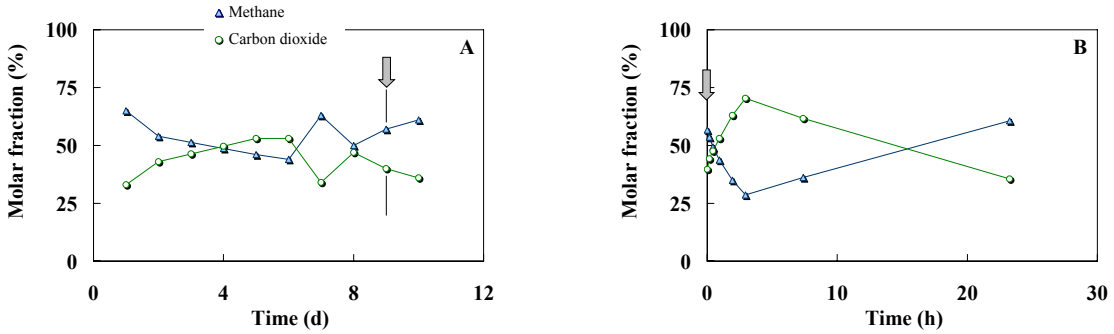

*Figure 43 – Progression of the composition of the biogas throughout the experimental period (**A**) and after the 40 mℓ-dose of distillery effluent, on day 9 (arrow) (**B**).*

4.2.2.2 *Semi-continuous phase*

Period-I

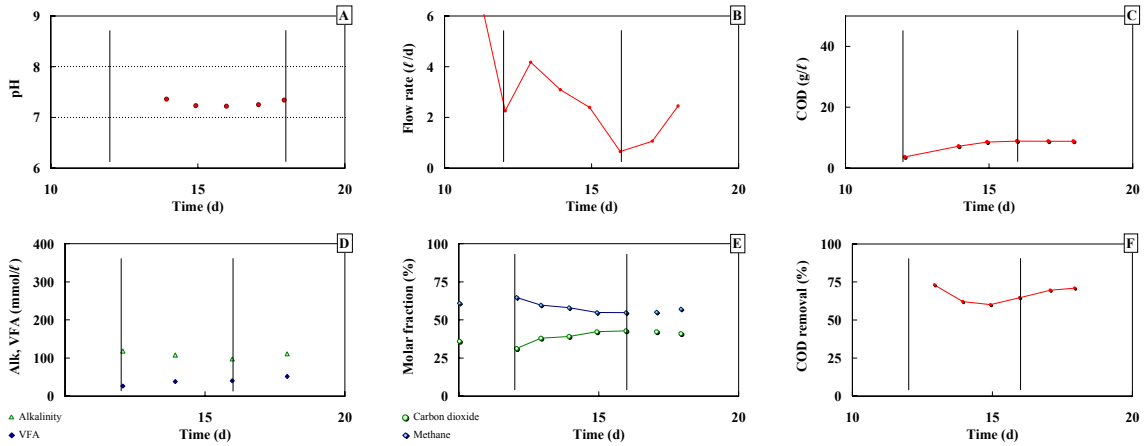

*Figure 44 – Overview of period-I of the semi-continuous phase: pH (**A**); biogas flow rate (**B**); COD concentration in the effluent (**C**); alkalinity and VFA concentration (**D**); molar fraction of methane and carbon dioxide in the off-gas (**E**); COD removal efficiency (η_{II}) (**F**).*

Undiluted distillery effluent was fed to the reactor (Q = 0.1 ℓ/d), except on the first day when a dose of acetic acid (5 mℓ) was also spiked.

All the variables monitored seem to indicate a progressive deterioration of the performance of the biomass: the biogas flow rate dropped from 4.2 to 0.7 ℓ/d (Figure 44 B); the gas composition drifted towards a greater carbon dioxide-to-methane ratio (Figure 44 E); the pH and alkalinity concentration decreased, from 7.4 to 7.2 and from 118 to 98 mmol/ℓ respectively (Figure 44 A and D); the concentration of volatile acids increased from 26 to 40 mmol/ℓ (Figure 44 D); the concentration of organic matter in the digester doubled, from 3.7 to 8.9 g COD/ℓ (Figure 44 C).

Based on these observations, we concluded that the biomass needed means of counteracting the acidity generated by the fermentation of the organic matter in the distillery effluent, as well as the nutrients and minerals that during continuous operations would have been depleted. It was therefore decided to feed the digester with a 1:1 mixture of distillery effluent and concentrated NMS.

This however proved to be wrong decision that could possibly have been avoided if the same experimental conditions had been maintained for a sufficiently longer period of time.

In fact, compared to the subsequent periods, all the variables seem to be levelling off, rather than increasing (or decreasing) indefinitely; and the removal efficiency of COD was pretty

high (Figure 53): *it is therefore probable that the system was evolving towards a new steady-state and that the change of regime was premature.*

Period-II

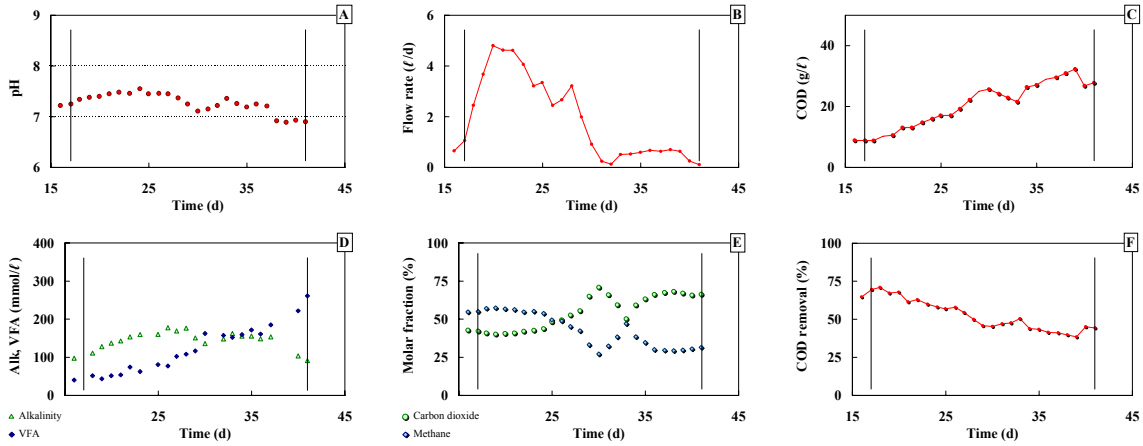

*Figure 45 – Overview of period-II of the semi-continuous phase: pH (**A**); biogas flow rate (**B**); COD concentration in the effluent (**C**); alkalinity and VFA concentration (**D**); molar fraction of methane and carbon dioxide in the off-gas (**E**); COD removal efficiency (η_{II}) (**F**).*

A feed consisting of the distillery effluent and the concentrated NMS at a volumetric ratio of 1:1 was used throughout this experimental period (with few interruptions), at a flow rate of 0.2 ℓ/d; the organic loading rate remained unchanged relative to period-I whereas the retention time was halved (*Table* 20). These conditions were maintained for a sufficiently long period of time to enable a few conclusive observations to be done.

The biogas flow rate initially increased until day 20, then steadily decreased to as low as 0.1 ℓ/d; between days 34 and 39, it seemed to have attained a relatively stable condition at 0.6-0.7 ℓ/d, but towards the end of the period, it decreased to 0.1 ℓ/d (Figure 45 B).

The gas composition was relatively stable until day 23 (the methanogenic fraction ranged from 54 to 57 %), then rapidly deteriorated until day 30, thus indicating a progressive acidification of the suspension (methane fraction, 27 %). For a short period, between days 30 and 33, when the feed was discontinued, i.e. no distillery effluent was fed (*Table* 20), the trend in the composition of the gas phase was reversed and the methane fraction increased to 47 %. As soon as the feeding regime was resumed, the methane and carbon dioxide molar fractions seemed to attain a steady-state at 29-32 % and 66-68 % respectively (Figure 45 E).

The other indicators of process performance, i.e. pH, alkalinity concentration and VFA concentration confirm the diagnosis that the digester was evolving towards acidification.

The pH initially increased to values as high as 7.5; then dropped to 7.1 on day 30; after a transient (days 30-33), it eventually precipitated to 6.9 at the end of the period (Figure 45 A).
The evolution of the alkalinity was obviously similar to that of pH, i.e. it increased from 98 to 170 mmol/ℓ (day 28), then temporarily stabilised, between days 30 and 37, and eventually decreased to 90 mmol/ℓ on day 41 (Figure 45 D). The concentration of volatile acids steadily increased throughout the experimental period, from as low as 40 to 260 mmol/ℓ (Figure 45 D), which clearly indicates that a considerable fraction of the organic matter was accumulating inside the reactor, partially degraded to acidic intermediates. This picture is in agreement to the conclusions of the serum bottle activity tests on the distillery effluent.
Accordingly, the COD concentration too increased steadily throughout the experimental period, from 9 to 32 g COD/ℓ (Figure 45 C).
The interruption of the feed, between days 30 and 33 was consistently reflected in all the process variables, thus indicating that a retention time of 18 d is surely insufficient, at least in the start-up phase when the biomass is not fully established. This is evident from the decrease in the biomass concentration, from 16 to 10 g VS/ℓ (Figure 52 D).
Ultimately, the lumped parameter COD removal decreased from as high as 70 % to 45 %.
Although the general conditions deteriorated, a clear quantitative response on the operation of the digester could be obtained: a COD removal of 40 % can be achieved at a retention time of 18 d and an organic loading rate of 3.1 g COD/ℓ/d (Figure 53).

Period-III

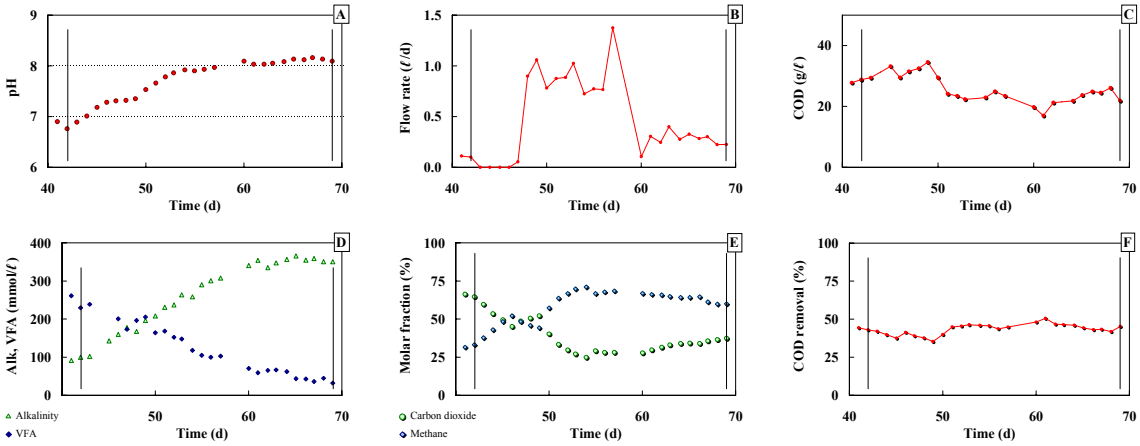

*Figure 46 – Overview of period-III of the semi-continuous phase: pH (**A**); biogas flow rate (**B**); COD concentration in the effluent (**C**); alkalinity and VFA concentration (**D**); molar fraction of methane and carbon dioxide in the off-gas (**E**); COD removal efficiency (η_{II}) (**F**).*

In the attempt of halting the acidification of the digester and the risk of washing out the whole biomass, it was decided to reduce both the organic loading rate and the flow rate. The feed consisted of distillery effluent and concentrated NMS at a volumetric ratio of 1:3 (*Table* 20).
As anticipated, all the variables monitored indicated a consistent improvement in the process performances.
The biogas flow rate dropped to barely detectable values. This circumstance was initially misinterpreted as actually reflecting a negligible methanogenic activity, due to the organic overload. However, being the gas composition improving (Figure 46 E), it was soon discovered that the gas measuring device was not functioning, in that the solenoid valve was permanently open, thus continuously venting the biogas generated. On days 47 and 48, two single doses of acetic acid were spiked, to enhance the methanogenic activity. This however, only resulted in a relatively long transient, during which the biogas flow rate increased to a range between 0.7 and 1.0 ℓ/d (Figure 46 B). The feed was temporarily discontinued on days 58 and 59; thereafter, the biogas flow rate went back to the usual levels of 0.25-0.30 ℓ/d.
The gas composition showed a very clear pattern, shifting from the previous steady-state at 35 % of methane and 65 % of carbon dioxide, to a new steady-state at 65 % of methane and 35 % of carbon dioxide (day 66; Figure 46 E). However, two major "disturbances" occurred which altered this evolution, i.e. the two doses of acetic acid, which resulted in a clearly identifiable transient; and the substitution of tap water to the concentrated NMS, to dilute the distillery effluent, from day 66 onwards. This had the immediate effect of increasing the carbon dioxide fraction in the gas phase, thus suggesting that the nutrients delivered through the NMS might be actively depleted and need to be supplemented at least periodically.
The success of the choice of reducing the organic loading rate, while extending the retention time is evident in the evolution of pH, alkalinity and VFA concentration (Figure 46 A and D): both the pH and the alkalinity concentration steadily increased, reaching a steady-state of 8.0-8.2 and of 355-365 mmol/ℓ respectively; the VFA concentration does not seem to have levelled off at the end of period-III (ca. 30 mmol/ℓ).
The COD concentration seems to have benefited the most from the two doses of acetic acid, in that it moved from a level of 30-35 g COD/ℓ to a lower level of 22-25 g COD/ℓ (Figure 46 C). The solid concentration was relatively stable throughout the experimental period, at 9 g VS/ℓ (Figure 52 D).
The cumulative COD removal efficiency ranged between 42 and 45 % (Figure 46 G).

This may not appear as a great improvement in the performances of the digester, relative to those of period-II. However, the removal efficiency depicted in Figure 46 G is calculated on the *cumulative* amount of organic matter (according to equation D.5) and is obviously not a sensitive enough parameter. A more accurate estimation is presented in Figure 53, based on each individual experimental period and actually indicates that the performances improved from 40 % to 49 % COD removal (HRT = 38 d; OLR = 1 g COD/ℓ/d).

Period-IV

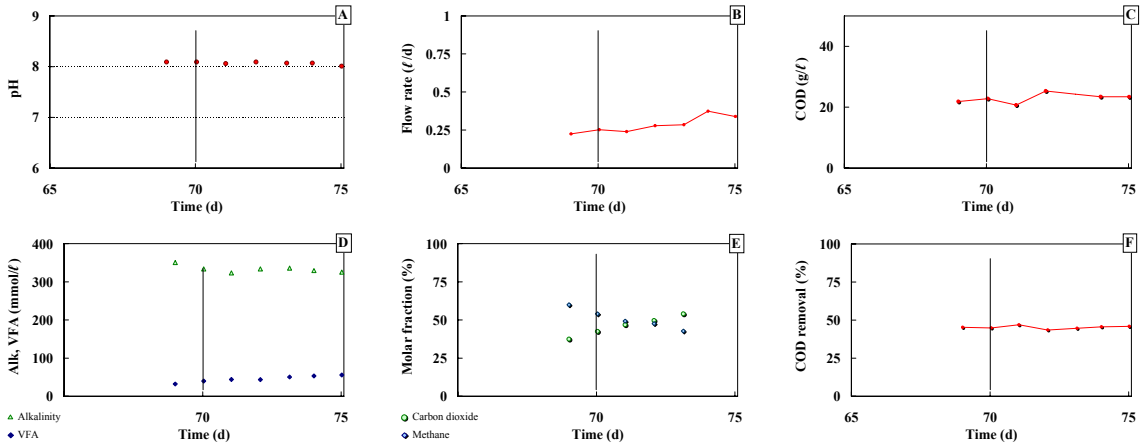

*Figure 47 – Overview of period-IV of the semi-continuous phase: pH (**A**); biogas flow rate (**B**); COD concentration in the effluent (**C**); alkalinity and VFA concentration (**D**); molar fraction of methane and carbon dioxide in the off-gas (**E**); COD removal efficiency (η_{II}) (**F**).*

The objective of this experimental period was initially to investigate the performance of the digester at a higher organic loading rate. The same flow rate of period-III was maintained (Q = 0.1 ℓ/d) but the feed consisted of equal proportions of distillery effluent and concentrated NMS (*Table* 20). As one could expect, the conditions gradually worsened which is the reason why after only 6 d it was decided to supplement the biomass with a more readily biodegradable substrate such as glucose and with yeast extract (period-V) to promote the microbial activity, although this inevitably resulted in an even higher organic loading rate.

The biogas flow rate seems to have increased from 0.2 to 0.3 ℓ/d (Figure 47 B); however, the composition of the gas phase proved to be a very sensitive indicator in that the molar fraction of carbon dioxide immediately increased from 37 % to 60 % (Figure 47 E).

Do to the larger buffer, the liquid phase reacted more slowly to the change in the feeding regime: the pH decreased slightly; 25 mmol/ℓ of alkalinity were consumed and 34 mmol/ℓ of volatile acids accumulated (Figure 47 A and D).

The sensitivity of the analytical method of COD determination was obviously not sufficient to detect any significant change (Figure 47 C).

Interestingly though the cumulative COD removal efficiency remained practically unaffected (Figure 47 F), whereas that calculated on this period only improved slightly from 49 % to 52 % (Figure 53).

Period-V

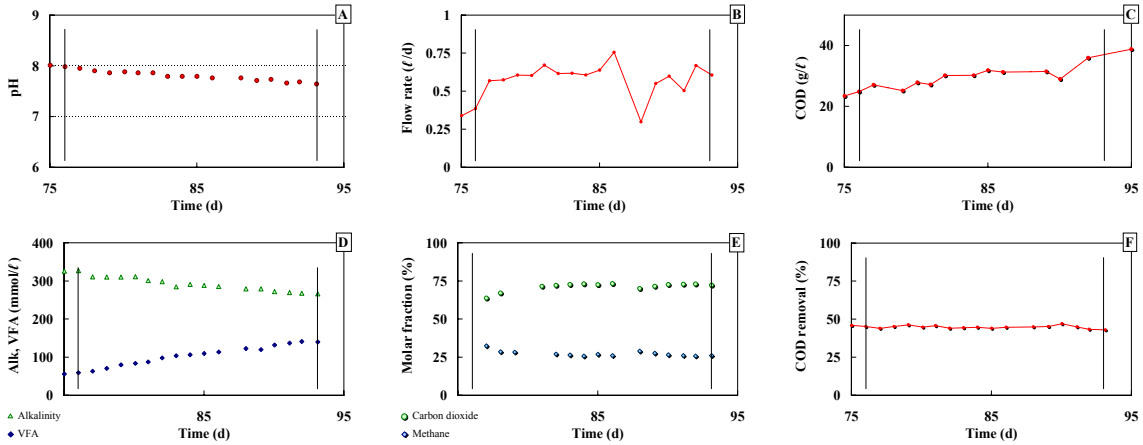

*Figure 48 – Overview of period-V of the semi-continuous phase: pH (**A**); biogas flow rate (**B**); COD concentration in the effluent (**C**); alkalinity and VFA concentration (**D**); molar fraction of methane and carbon dioxide in the off-gas (**E**); COD removal efficiency (η_{II}) (**F**).*

For 18 days, a flow rate of 0.1 ℓ/d of a 1:1 distillery effluent to concentrated NMS was maintained and single doses of 1 g of yeast extract and 2 g of glucose were added to the feed in the attempt of promoting the activity of the sludge (*Table* 20).

This was achieved to some extent, but the limiting factor was clearly the insufficient retention time. This is the reason why in the subsequent three experimental periods the composition of the feed was maintained but the hydraulic regime modified: the digester was fed every second day (periods-VI and VIII) or the feed discontinued for 10 d (period-VII).

The biogas flow rate reacted promptly to the higher loading rate and different composition of the feed, in that it shifted steadily to values of about 0.6 ℓ/d (Figure 48 B).

The composition of the gas seemed to attain a steady-state, at 25 % and 75 % molar fraction of methane and carbon dioxide respectively which is clearly indicative of predominantly acidic conditions and partially inhibited methanogenesis (Figure 48 E). This would suggest that the addition of glucose had been beneficial exclusively to the fermentative populations, but probably put more pressure (in terms of organic load) on the methanogenic biomass.

This impression is confirmed by the pH, alkalinity and VFA indicators. The pH decreased steadily from 8.0 to 7.6 (Figure 48 A); the alkalinity concentration from 325 to 265 mmol/ℓ (Figure 48 D); and the VFA concentration almost doubled from 75 to 140 mmol/ℓ (Figure 48 D). This actually suggests that a significant portion of organic matter was readily converted to acidic intermediates and accumulated in that form being the methanogenic activity insufficient or the methanogenic biomass not sufficiently developed to cope with that acid production rate. The COD concentration too increased steadily from 23 to 37 g COD/ℓ (Figure 48 C). The biomass concentration seems to have decreased, although this is not a fast-reacting variable and would only be indicative of the actual performance of the digester if the same operating conditions were maintained for sufficiently long periods of time.

Expectedly, the cumulative efficiency of COD removal reacted slightly to the organic overload and partial accumulation of undegraded intermediates, decreasing from 46 % to 43 % (Figure 48 F). The removal efficiency calculated on this period only (Figure 53) shows correctly a significant deterioration in the performance of the biomass from 52 % to 34 %.

Period-VI

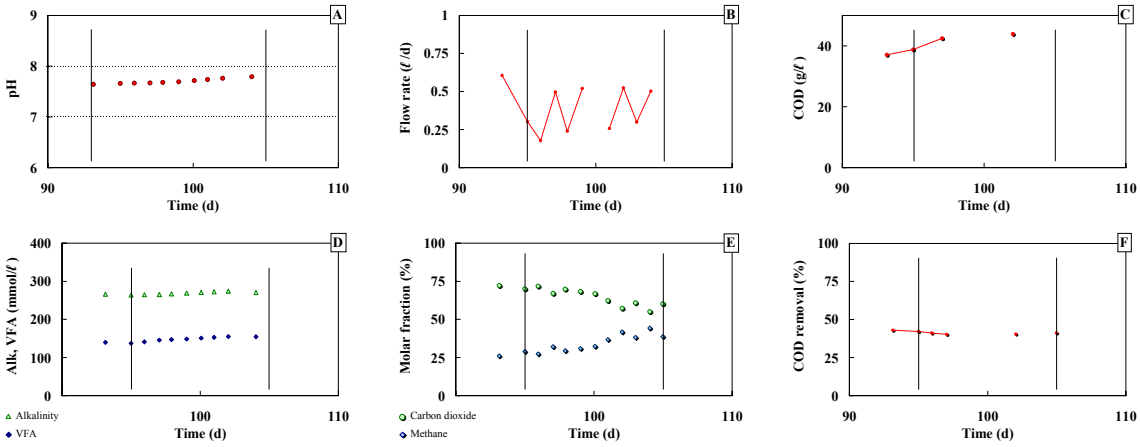

*Figure 49 – Overview of period-VI of the semi-continuous phase: pH (**A**); biogas flow rate (**B**); COD concentration in the effluent (**C**); alkalinity and VFA concentration (**D**); molar fraction of methane and carbon dioxide in the off-gas (**E**); COD removal efficiency (η_{II}) (**F**).*

The same composition of the feed was maintained but the digester was only fed every two days: this resulted in an average flow rate and organic loading rate of 0.05 ℓ/d and 1.0 g COD/ℓ/d respectively (*Table 20*).

The biogas flow rate shifted to a lower range, oscillating between 0.25 and 0.50 ℓ/d (Figure 49 B). The gas composition responded promptly to the variation of the operating conditions, moving steadily towards methanogenic conditions (the methane fraction increased

from 26 % to 40 %, Figure 49 E). The pH and the alkalinity concentration closely mirror the picture suggested by the composition of the gas phase: the pH increased from 7.6 to 7.8 and the alkalinity concentration from 260 to 274 mmol/ℓ (Figure 49 D). Unexpectedly though the concentration of VFA continued rising and reached a value of 155 mmol/ℓ at the end of the period (Figure 49 D), thus indicating that the acidic intermediates were still accumulating and most likely increasing the pressure on the methanogenic biomass.

In fact, the COD concentration too continued rising, although it seems to level off at 44 g COD/ℓ (Figure 49 C). The cumulative efficiency of COD removal is progressively sensing the overloaded conditions, as it decreased to 41 % (Figure 49 F) while the efficiency calculated on this period only reached the lowest level of the entire semi-continuous phase, at 18 %.

This can probably be interpreted by considering that although the organic loading rate had been halved as compared to the previous period, the biomass was not able to cope with the residual organic matter inside the digester, which largely consisted of volatile acids.

For this reason, we decided to discontinue the feed in the next period and conduct the digester in batch conditions for 10 days.

Period-VII

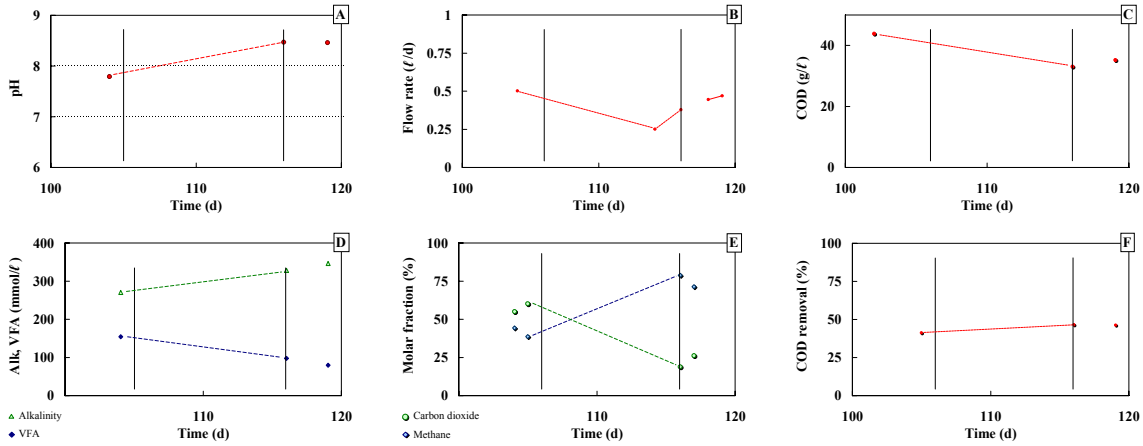

*Figure 50 – Overview of period-VII of the semi-continuous phase: pH (**A**); biogas flow rate (**B**); COD concentration in the effluent (**C**); alkalinity and VFA concentration (**D**); molar fraction of methane and carbon dioxide in the off-gas (**E**); COD removal efficiency (η_{II}) (**F**).*

During the batch period, the digester was not monitored, i.e. no liquid samples were taken to be analysed for alkalinity, VFA or COD concentration; no gas sample was analysed for gas composition; nor regular readings of gas counter were done. Therefore, we can only assess

the effect of the interruption of the feed, by comparing the state of the digester before and after this interval (*Figure* 50).

Both the pH and the alkalinity concentration were displaying an increasing trend before the feed was discontinued; the pH increased further from 7.8 to 8.5, reaching a level that maintained after the feed had been resumed (period-VIII) and that is possibly excessively alkaline. The alkalinity concentration too further increased from 270 to 330 mmol/ℓ.

The biomass seemed to be able to partially recover, from the organic overload, in that 57 mmol/ℓ of volatile acids were consumed and the COD concentration was reduced from 44 to 33 g COD/ℓ. The composition of the gas phase which throughout the semi-continuous experiment has proven to be the most sensitive indicator of the state of the digester, evolved from a methane-to-carbon dioxide ratio of 40:60 to a ratio of 80:20 which is clearly indicative of highly methanogenic conditions.

The cumulative COD removal (which on the contrary is a very slow-response indicator) increased from 41 % to 47 %.

According to the gas counter, only 1.8 ℓ of methane were produced during the batch period, although the mass balance on the COD removed from the suspension would require that over 13 ℓ should have been generated. This observation makes extremely unreliable the calculation of the gas flow rate and of the removal efficiency based on the amount of methane produced (Figure 53). We did not have time to examine this issue in detail, to find out whether it was related to a malfunctioning component of the device or an inherently poor reliability of our liquid displacement set-up.

Period-VIII

For the final 25-day experiment, the digester was irregularly fed, so that only the average retention time and organic loading rate can be reported (*Table* 20): the average flow rate for the period was 0.04 ℓ/d; the feed consisted of a 1:1 mixture of distillery effluent and concentrated NMS with an additional dose of yeast extract (1 g) and glucose (2 g).

The very long retention time associated with the low organic loading rate had a generally positive impact on the state of the process (Figure 53): the cumulative COD removal efficiency steadily improved reaching 52 % at the end of the period; the COD concentration seems pretty erratic but approximately oscillating in the range 30-33 g COD/ℓ; the biomass concentration too reached a steady-state at 9 g VS/ℓ.

Being characterised by a faster response to variations in the feed, the other indicators show the evolution of the system towards stable methanogenic conditions.

The profile of methane and carbon dioxide molar fractions in the gas phase clearly shows that the gas composition should be selected as monitoring parameter since it immediately reacts, within a few hours time, to any change in the feeding conditions and/or characteristics. The carbon dioxide molar fraction shows a consistent pattern, in that it increases immediately after feeding then gradually decreases until the following feed (segments in Figure 51 E). The general underlying trend indicates that the gas composition moved from a methane-to-carbon dioxide ratio of 80:20 to a more balanced ratio of 60:40. The pH decreased only slightly, whereas the alkalinity concentration increased from 330 to 400 mmol/ℓ and the VFA concentration seems to attain a relatively stable level of 40 mmol/ℓ. This very low concentration of acidic intermediates associated with a still relatively high COD concentration suggests that the residual COD is the fraction of distillery effluent which is more resistant to biodegradation.

*The removal efficiency calculated on this period only (*Figure 53*) is extraordinarily high at 90 %. These operating conditions, possibly due to the long pre-exposure of the sludge to the distillery effluent, would constitute an ideal starting point for a follow-up study.*

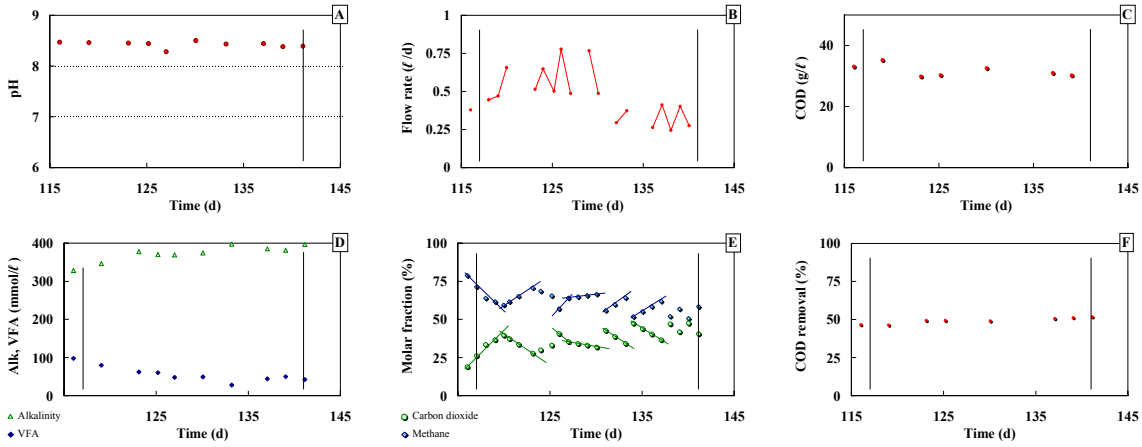

*Figure 51 – Overview of period-VIII of the semi-continuous phase: pH (**A**); biogas flow rate (**B**); COD concentration in the effluent (**C**); alkalinity and VFA concentration (**D**); molar fraction of methane and carbon dioxide in the off-gas (**E**); COD removal efficiency (η_{II}) (**F**).*

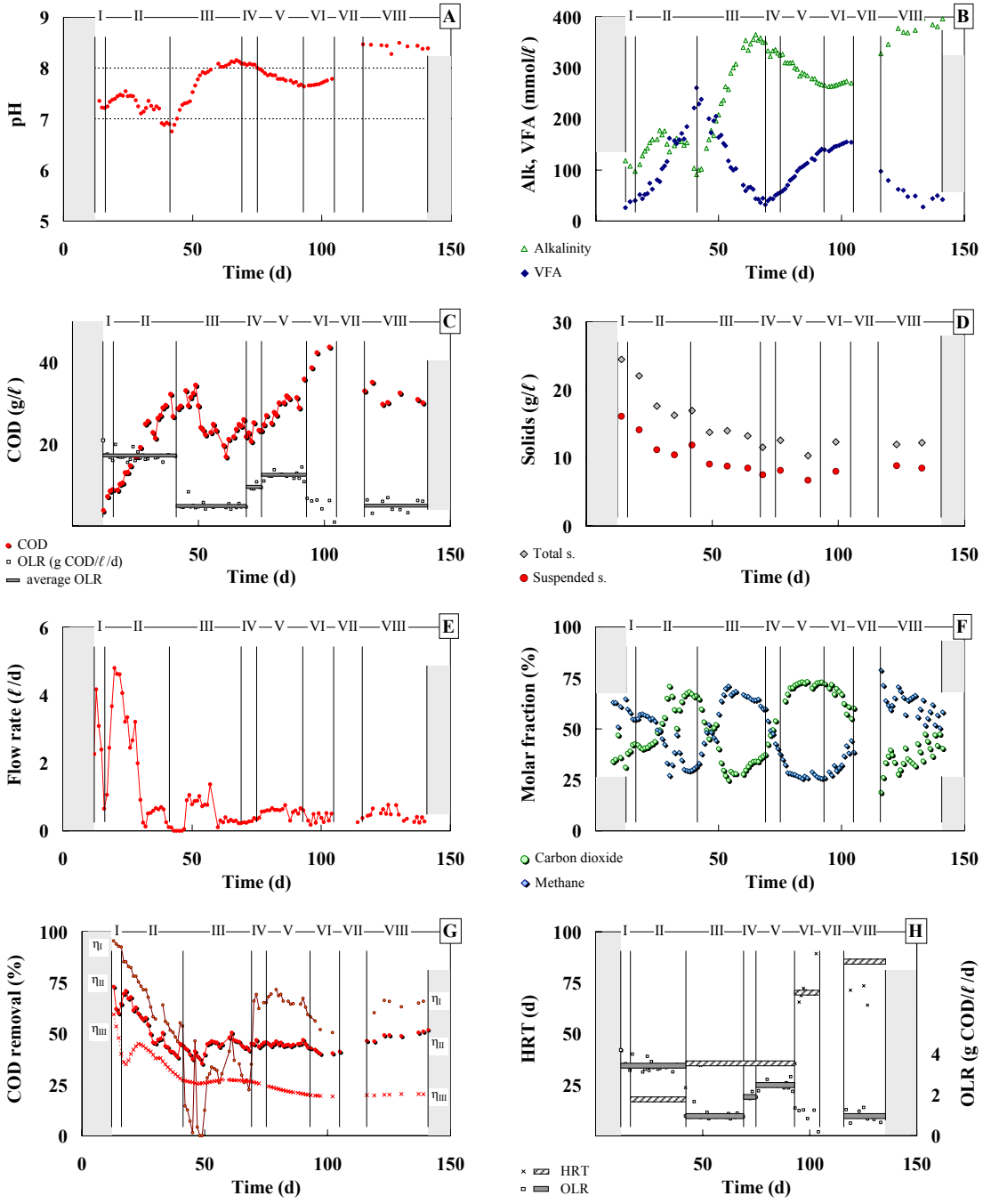

*Figure 52 – Overview of the semi-continuous phase: pH (**A**) and alkalinity and VFA concentration profiles (**B**); COD concentration in the effluent and organic loading rate (**C**); total and volatile solids concentration (**D**); biogas flow rate (**E**); molar fraction of methane and carbon dioxide in the off-gas (**F**); COD removal efficiency (**G**); and hydraulic retention time and organic loading rate (**H**).*

The removal efficiency is a function of both the retention time and the organic loading rate simultaneously, but the data available from this experiment are not sufficient to conduct a meaningful multivariate analysis. We therefore only tentatively interpreted the relationship between the removal efficiency and the two parameters, separately, using the approach of equation D.5.

A general trend can be observed which is indicated by the straight lines in Figure 53 B and C. Interestingly, one can note that a few data points do not fit into this simplified univariate analysis (empty symbols in Figure). We can tentatively explain this as being due to overload conditions (periods V and VI), initial start-up (period-I) and recovering conditions following a period of batch operation (period-VIII).

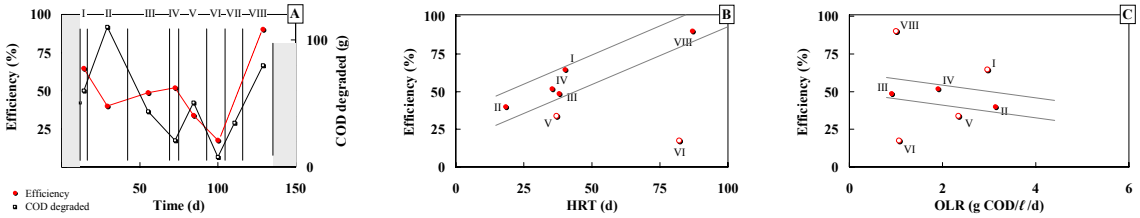

*Figure 53 – Efficiency of COD removal as a function of time (**A**), hydraulic retention time (**B**) and organic loading rate (**C**).*

Conclusions

As a preliminary investigation on the feasibility of a laboratory-scale digestion and co-digestion of the industrial effluents, this exercise was certainly successful in that it enabled us to identify a number of issues that would be of great help for a follow-up study.

These can be summarised as follows.

- The best option of monitoring variable, given the facilities in our laboratory, is the composition of the gas phase: this determination can be reliably undertaken within 10 min from the extraction of the gas sample and unlike alkalinity or VFA concentration does not require the extraction of a liquid sample.
- The liquid displacement gas measuring device is not reliable. We have not been able to conclusively identify the problem, but this is surely a key issue that one will have to address prior to starting any larger-scale and/or long-term investigation, as the determination of the volume of gas generated is crucial for conducting mass balances.
- The distillery effluent can definitely be regarded as partially biodegradable and does not inhibit the methanogenic biomass, except indirectly and for a limited period of time due to a

rapid degradation of the organic matter to acidic intermediates. In this respect, the main risk to avoid is organic overloading.

- The addition of a NMS to the feed seemed to be beneficial in that it helped avoiding the depletion of alkalinity. Whereas the addition of a more readily degradable complex substrate such as glucose has not proven very successful as it can promote the acidogenic activity of the sludge rather than the methanogenic activity, thus further enhancing the conditions of substrate inhibition for the methanogens.
- As a general procedural recommendation, one should maintain a feeding regime for a sufficiently long period of time of at least one retention time, unless the stability of the digester was at risk. This will ensure a set of meaningful data that cannot be obtained if the operating conditions are varied frequently.

4.2.2.3 Assessing the methanogenic activity of the sludge

The methanogenic activity of the sludge is an ideal indicator of the performance of a digester: it complements the information on the efficiency of conversion of the organic matter to methane (i.e. the COD removal, Section D.2.7) in that it specifically quantifies the performance of the methanogenic micro-organisms which convert acetate to methane.

Formally, it is calculated (equation B.6) as the ratio between the rate of methane production (litres of methane per day) and the amount of biomass present in the system (g VSS, or g VS).

The former variable can be easily determined in a semi-continuous way, by measuring the biogas flow rate (ℓ of gas/d), e.g. using a liquid displacement or an equivalent system, and by analytically determining the gas composition, i.e. the molar fraction of methane.

Although online indirect techniques exist to measure solids concentration (e.g. flow cytometry), these are also very expensive and therefore not viable. On the other hand, it is generally not feasible to measure solids off-line too often. Therefore the assessment of the methanogenic activity of the sludge is necessarily a discontinuous process variable too.

For practical purposes, the following options are equivalent:

- to estimate a daily value for SMA, using the measured methane flow rate and the last measured solids concentration; or to predict a daily solids concentration, based on previous measurements of solids concentration (a simple regression model is sufficient); or
- to calculate a value for SMA in correspondence to the measurement of solids concentration, using the current measured flow rate; or an average methane flow rate.

Since the liquid displacement system proved not reliable, an alternative method is the use an aliquot of the sludge withdrawn from the digester, as the seed inoculum for a serum bottle AAT. No external substrate should be supplemented, nor NMS: the sludge is to be poured in the vials, purged with a nitrogen-carbon dioxide mixture (or equivalent gas) and immediately sealed. Our experience indicates that purging for 1 to 2 minutes is sufficient to thoroughly flush the headspace: the molar fraction of nitrogen and carbon dioxide, one or two hours after sealing the vials, were 50±2 % (N=9) when a N_2:CO_2 1:1 mixture was used and 97±2 % and 3±2 % (N=15) respectively when pure nitrogen was used.

Therefore, starting this type of AAT (two replicates are generally sufficient) is very quick.

The methanogenic activity of the sludge can be assessed immediately after one day, according to the mass balance in equation B.4.

The advantage of this method of assessing the methanogenic activity of the digester off-line is that a number of vials will be available when the assessment has ended, which clone the

digester on a smaller scale and may be used for testing other conditions or operations, while the digester remains undisturbed.

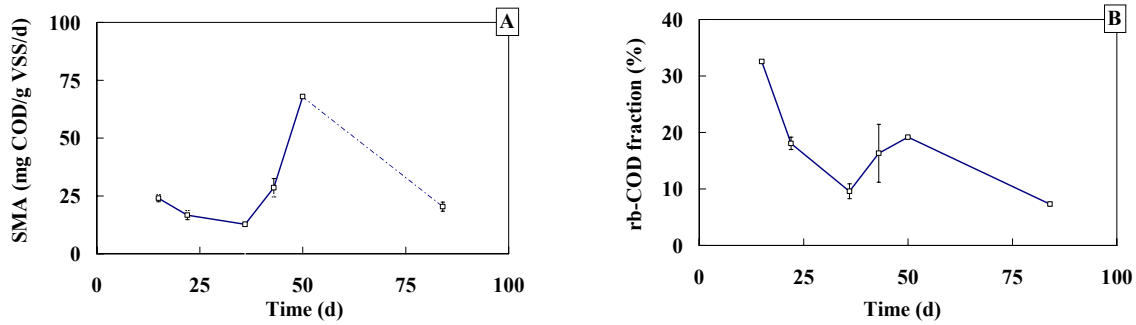

*Figure 54 – Methanogenic activity (**A**) and rbCOD fraction (**B**) during the semi-continuous phase, evaluated by the off-line activity tests.*

The procedure that was used in this research comprised the following steps.

- Activity assessment: day 0 to day 1.
- Biodegradability assessment: day 1 until the gas production levels off. The non-readily biodegradable fraction of the test material can be calculated as the ratio between the ultimate gas production and the initial COD (which is equal to the COD measured in the 'mother' digester prior to withdrawal). This is valuable information in that it can suggest whether an increase in the retention time of the digester would significantly improve the removal efficiency.
- Activity assessment: until a constant activity is attained. An aliquot of acetic acid is spiked daily to evaluate the methanogenic activity under optimal conditions. A volume of 30 µℓ of acid 35 % in 30-50 mℓ of sludge does not affect the chemistry of the system to an irreversible extent.

  An accurate mass balance during this phase can provide very useful indications on the actual degradation process. Ideally the entire aliquot of acetate fed should be stoichiometrically converted to methane. Should an excess of methane be detected, this would suggest that the presence of the readily biodegradable substrate allows the further degradation of the test material (Table G.13, day 36).

  Moreover, this phase prepares the sludge for the next one, by creating a baseline optimal activity.
- Biodegradability assessment: the availability of small scale clones of the digester enables to investigate changes in the operating conditions without distressing the digester. For

example, by spiking a dose of the test material and NMS or a dose of a labile substrate, conditions of nutrients deficiency or of co-substrate requirements could be identified, respectively.

Six AAT were conducted on the sludge of the laboratory-scale digester, according to this procedure. The results are summarised in Table G.13.

The sludge methanogenic activity at the operating conditions was evaluated as the average of the first one or two values for each duplicate vial. These estimates, throughout the experimental period, are plotted in Figure 54 A: the initial slow decrease in the methanogenic activity mirrors the general slow worsening of the performance of the digester that has been discussed above; subsequently, a remarkable almost three-fold increase in activity was observed, which indicates that the digester is eventually approaching a steady operation. Between day 50 and day 84, no off-line AAT was conducted due to various technical problems in the laboratory. In the same period, the organic loading rate of the digester was gradually increased. This resulted in a general worsening of the performance of the process, which is reflected in the severe drop in the methanogenic activity.

A somewhat similar trend was obtained for the estimated in-reactor residual biodegradable COD. This is calculated, as for a biodegradability assessment, by comparing the ultimate volume of methane produced at the end of the first period, to the initial COD content at the time of the withdrawal. In the first period of operation of the digester (Figure 54 B), the rbCOD was as high as 30 %. Thereafter it remained in the interval 10-20 % (the last value measured is approximately 7 %). A relatively high rbCOD certainly indicates that the operating conditions (particularly the OLR) are possibly causing a stress to the biomass which is not able to effectively degrade the organic matter that is fed to the reactor. However, a low value of rbCOD alone should not be interpreted as a sign of improving performance of the process. If we look at the last value measured, the low rbCOD is associated with a low value of SMA: this is rather indicative of a highly overloaded biomass.

The first AAT started on day 15 (period-I) completed the three stages, i.e. on the residual organic matter (batch mode), on acetic acid and on distillery effluent (fed-batch mode).

The results are plotted in Figure 55: the curve of the COD concentration is obtained through a mass balance on the methane produced from the COD concentration initially measured. The curve of the COD recovery (Figure 55 B) is obtained through a mass balance (equation D.6),

based on the substrate pertaining to the specific period, that is the residual COD in the first period; the acetic acid dosed in the second period; and the distillery effluent added in the third period.

At the end of the test, the volume of the mixed liquor, pH, alkalinity, VFA concentration, COD and solids were determined.

- The final volume was 58 mℓ, against an expected value of 59.4 mℓ, resulting in a negligible error of only 2.4 %.
- pH, alkalinity and VFA concentration were 7.79; 214 mmol/ℓ and 14 mmol/ℓ respectively.
- The final COD was 9.2±0.0 g/ℓ, against an expected value of 8.4 g/ℓ (-9.2 % error).
- The solids concentration was 16±0 g/ℓ and 10±0 g/ℓ of total suspended and volatile suspended solids respectively. A considerable amount of solids was also found attached to the internal wall of the vial. Small glass beads were poured into the empty vial and 30 mℓ of distilled water added; the vial was capped and vigorously shaken, until most of the biofilm was removed from the wall. This portion of solids was determined separately and yielded the following results: TSS 0.11 g and VSS 0.03 g, which account for the 11.9 and 5.2 % of the total and volatile suspended solids respectively.

The *reference* sludge methanogenic activity calculated for the second AAT (day 22, period-II) is very low compared to the other values calculated and the recovery of the acetate at the end of the second period is only 40-50 % (Table G.13): this possibly indicates that the sludge was highly inhibited.

As per the off-line AAT procedure outlined above, the addition of acetic acid was suspended for one day between the second and the third experimental period. When the latter phase was due to start, it was noted that a considerable gas production had occurred, so the addition of the mixture of the distillery effluent and the NMS was postponed. Such 'residual' gas production was observed for the following ten days: this clearly prevented the start of the third phase, but it further substantiated the hypothesis that the sludge was previously inhibited. In fact, while not being fed, both units produced a volume of methane equivalent to 1.3-1.4 times the amount of the residual acetate at the time the spikes were stopped (data not shown), therefore accounting for a portion of the residual COD from the distillery effluent.

A similar conclusion (i.e. of an overloaded digester) can be drawn if one looks at the acetate recovery of the second period of the third AAT started on day 36 (period-III, Table G.13): the

mass balance, in excess of 115 % suggests that the addition of acetate might have enhanced the degradation of the residual effluent.

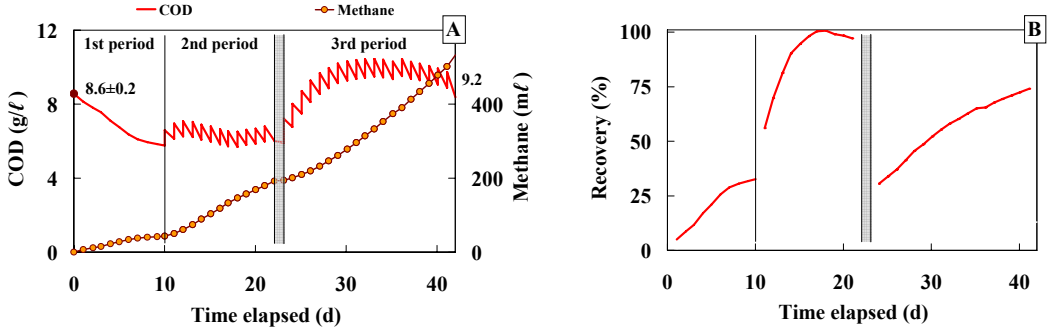

*Figure 55 – Off-line AAT (day 15; period-I): calculated COD concentration vs. measured methane production (**A**) and COD recovery (**B**) for the three periods.*

Conclusions

An off-line AAT aimed at assessing the actual and the optimal methanogenic activity of the sludge and the residual biodegradable organic matter is certainly not a viable option for monitoring the performance of the digester, since it lasts several days. Nonetheless, being a relatively inexpensive test it could be of help in the feasibility assessment phase, or as a research tool.

If it is used merely to estimate the activity under the operating conditions, a reliable estimate can be obtain within 24 hours and it could therefore be performed regularly.

4.3 A MATHEMATICAL MODEL FOR A TWO-UNIT PH-STAT TITRATION SENSOR

Objective

To develop a mathematical model of the pH-stat titration sensor, which can be used to interpret the results of activity and toxicity tests, and possibly of a biodegradability test too. Remigi (2001) reported applications to the assessment of aceticlastic methanogenic activity and inhibition; of obligate hydrogen producing acetogens; and of anammox micro-organisms. Complete biodegradability tests have not been extensively performed to date, although preliminary results seemed promising (Remigi, 2001). The reason for this is the inherent inability of a pH-stat titrimetric sensor of monitoring simultaneous pH-affecting processes, such is the case of the acidogenic/methanogenic sequence of the anaerobic digestion process. However, *i*) a general list of recommendations has been derived from the experience gained in the course of this project and *ii*) an extensive experimental campaign has been planned and will constitute the object of a joint effort of the PRG and the laboratory of Sanitary and Environmental engineering of Politecnico di Milano (Annexure I).

Materials and methods

A mathematical model of a pH-stat titration sensor should incorporate the following:

- an appropriate description of the hydraulics of the sensor;
- a general model of the anaerobic digestion process; and
- a model of the control system, i.e. the titration units.

The latter has been the specific output of this modelling exercise.

The mathematical model has been implemented in AQUASIM, a commercial software specifically designed for modelling aquatic systems (Reichert, 1998).

Reasons that lead to the choice of this particular software were:

- the relatively low price,
- the possibility of the user to implement a system from scratch, with hardly any restriction and more importantly with no need for writing code,
- the fact that it is a widely used software among sanitary engineers, as it allows to define small-scale, experimental set-ups.

On the other hand, AQUASIM may not be particularly easy in handling multiple interconnected systems and full-scale set-ups and also requires that all variables, parameters and processes of the model are manually inputted by the user (e.g. it does not incorporate a library of predefined equations or models of general interest). However, these are usually not perceived as restrictions in a research environment.

4.3.1 Specific features of Aquasim

A mathematical model in AQUASIM consists of a system of differential and/or algebraic equations, which deterministically describe the evolution of a set of state variables.

Two types of processes can be defined, termed dynamic and equilibrium processes.

A dynamic process requires a *stoichiometry* and a *kinetic* equation to be defined; a system of dynamic processes is therefore mathematically described in form of a set of *differential equations*, which is conventionally represented using a *tableau* format, termed Peterson matrix. In such matrix, the cell (i, j) indicates the stoichiometric coefficient for the conversion of the j-*th* variable within the i-*th* process; the rate of change of the i-*th* process is specified as rate r_i. The transformation rate of the i-*th* substance (ρ in equation 19) is calculated by summing all the contributions of the *m* processes in which the component is involved, each weighted with the stoichiometric coefficient $\alpha_{i,j}$:

$$\rho_i = \sum_{i=1-m} r_i \cdot \alpha_{i,j} \qquad (16)$$

If all the dynamic variables are expressed using the same units, the mass conservation principle translates in the condition that the sum of the all the stoichiometric coefficients within each process (i.e. the sum on a row of the matrix) equals zero:

$$\forall i \quad \sum_{j=1-n} \alpha_{i,j} = 0 \qquad (17)$$

An equilibrium process is assumed to have a relatively fast kinetics compared to that of a dynamic process, so that it can be neglected. Typically, physico-chemical reactions are modelled as equilibrium processes. Since the kinetics of these processes is neglected a system of *algebraic equations* serves to model a set of equilibrium processes. For such system to be univocally solved the number of equilibrium processes must equate the number of equilibrium state variables.

Various types of compartments can be described. The one used in this modelling exercise is the *mixed reactor*, which implements spatially homogeneous systems (i.e. stirred reactors).

The reactor is fully defined when the following elements are specified.

- The active processes and variables: in general, different processes and variables can be independently activated in different compartments (but they are all defined within the appropriate module).
- The initial conditions: these are necessary for the initialisation of the algorithm. It is particularly important that the initial conditions specified for the equilibrium variables are

sufficiently close to the actual values, to avoid numerical instabilities that could lead the simulation to fail. This can be obtained in two ways, i.e. either by manually solving the system of algebraic equations or by running a first simulation which is only aimed at setting all the state variables (dynamic and equilibrium ones) at their respective steady-state. The first option is generally preferable for the simulation of the pH-stat sensor, because the initial pH and molar fraction of carbon dioxide in the gas phase are set by the operator and are sufficient to fully determine the physico-chemical system (see later in this Section).

- The inputs, which include the feed flow rate and the loads (i.e. mass per unit time) of each individual component.

The hydraulics of a mixed reactor compartment is described by the following mass balance:

$$\frac{d}{dt}V_R = Q_{in} - Q_{out} \tag{18}$$

and the temporal change in the concentration of the i-*th* substance, dissolved or suspended in the liquid phase, is given by the following equation:

$$\frac{d}{dt}C_i = \frac{Q_{in} \cdot C_{i,in}}{V_R} - \frac{Q_{out} \cdot C_i}{V_R} + \rho_i \tag{19}$$

where V_R is the reactor volume (ℓ); Q_{in} and Q_{out} are the in- and out-flowing flow rates respectively (ℓ/d); $C_{i,in}$ and C_i are the concentrations of the i-*th* substance in the feed and in the reactor respectively (mg/ℓ); and ρ is the transformation rate of the i-*th* substance (g/ℓ/d). Units are merely indicative, as any other set of units can be used, providing that internal consistency is ensured.

Two compartments can be connected using two types of links, termed advective and diffusive links. The former describes the flow of water and the advective transport of material between the compartments. An advective link does not only connect compartments directly, but enables one to build splits and junctions.

A diffusive link represents diffusive boundary layers or membranes between compartments which can be diffusively permeated by substances. The mass flux (L_i, mass per unit time) of the i-*th* substance, from compartment 1 to compartment 2, is given by the equation:

$$L_i = k_{La,i} \cdot \left(f_i \cdot C_{i,1} - C_{i,2}\right) \tag{20}$$

where $k_{La,i}$ denotes the exchange coefficient (usually given as product of the surface area of the interface and a mass transfer coefficient; 1/d); and f_i is a conversion factor used to describe the phase transition. If the gas and the liquid phase of a digester are modelled using

two mixed reactor compartments, the conversion factor is the inverse of the non-dimensional Henry's law coefficient (H), defined as follows:

$$f_i = \frac{1}{H_i} = \frac{1}{K_{H,i} \cdot R \cdot T} \qquad (21)$$

where $K_{H,i}$ is the Henry's law coefficient of the i-*th* substance (mol/ℓ/bar); R is the universal gas constant (ℓ·bar/K/mol) and T the temperature (K).

4.3.2 The mathematical model

The general structure of the model is derived from the ADM1 (Batstone et al., 2002), but the model was simplified and only comprises three microbial steps, i.e. fermentation (in which the organic substrate is converted to volatile acids, described as acetate equivalents); and acetotrophic and hydrogenotrophic methanogenesis. The fermentation results in a net production of acidity, whereas the conversion of acetate and hydrogen to methane results in a net production of alkalinity.

A Michaelis-Menten function, which incorporates the growth of biomass too, is used to describe the kinetics of the three uptake processes. The decay of each microbial group has been implemented individually, using a first-order kinetics.

The chemistry of the system and particularly the calculation of pH, which is the key variable of this application, is defined by the dissociation equilibria of acetic acid and water, the equilibrium of inorganic carbon (i.e. between bicarbonate and dissolved carbon dioxide) and the balance of the charges (anions and cations). The liquid phase equilibrium of inorganic carbon is actually modelled as a dynamic process.

Four gas components are explicitly modelled, i.e. carbon dioxide, methane, hydrogen and nitrogen; this latter does not take part in any biological reaction but it is generally used to flush the headspace prior to starting the test and can be gas chromatographically determined.

The interaction between the liquid and the gas phase is described by a diffusive link, i.e. through the Henry's law and the gas-liquid exchange rate, for the equilibrium and the dynamics portions respectively.

The on-off pH-control is the original contribution of this exercise. It simulates the flow of an alkaline and an acidic titrant, thus enabling the generation of titration plots equivalent to those obtained experimentally.

4.3.3 The hydraulics

Two mixed reactor compartments were used to model the pH-stat sensor.

The first unit mimics the liquid phase. It is modelled as a *variable volume* compartment, with no outflow and an inflow which accounts for the titration volume. This description translates the fed-batch regime, in which the volume of the mixed liquor increases as the degradation process evolves, due to the addition of the titrant solutions. This dilution effect can be relevant in activity tests of long duration (and makes unrealistic the otherwise acceptable approximation that the test in conducted at constant substrate concentration).

If manual sampling and/or feeding are to be simulated, the respective contributions can be added to the outflow and/or inflow.

The second unit mimics the gas phase; it too is modelled as a variable volume compartment, with an outflow equal to the sum of the gas flow rate and the titration volume and an inflow equal to the gas flow rate.

As one can observe, the overall mass balance on the entire system comprised of the liquid and gas phase is conserved, being the titration flow rate the only external contribution, whereas the gas flow rate represents an internal transfer.

This system definition allows the realistic description of the increasing volume of the liquid phase and the equivalent decrease in the volume of the gas phase, as the activity test progresses.

All biochemical reactions occur within the liquid phase and the gas is virtually generated in the same compartment; the gas components are then transferred to the gas phase, at a rate and to an extent governed by the gas-liquid exchange and by the Henry' law respectively, implemented as a diffusive link. Although a pH-stat titrimetric activity test is conducted at atmospheric pressure (otherwise the solubility of the gas components and particularly of carbon dioxide would vary, thus affecting the titration process), the generation of the biogas is modelled via the calculation of the excess pressure inside the gas compartment.

The pressure results from the combination of the individual partial pressures of each gas component; ultimately, the gas flow is calculated as proportional to the pressure generated in excess to the atmospheric pressure, as follows:

$$Q_{gas} = V \cdot \frac{p - p_{atm}}{p_{atm}} \cdot 10^3 \qquad (22)$$

where V is the volume of the headspace (ℓ) and 10^3 (d^{-1}) is an empirical factor which acts as the 'gain' tuning the response of the system in releasing a unit of titrant as the consequence of unit increase in pressure (the higher the factor, the faster is the response).

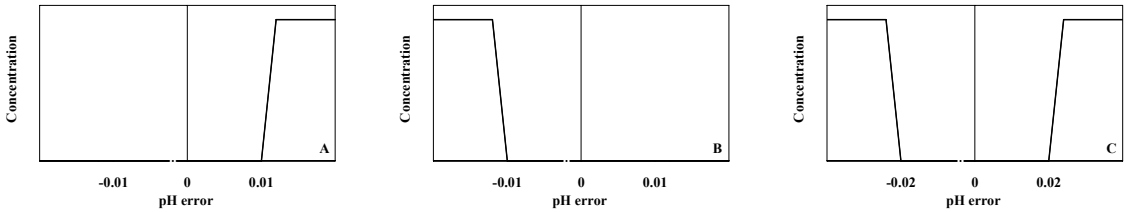

*Figure 56 – Proportional controller for the simulation of a pH-stat titrimetric sensor: acid (**A**) and alkali (**B**) concentrations and titrant flow rate (**C**).*

4.3.4 The titration unit

The control system is based on the 'pH error', that is the difference between the current pH and the set-point value. Each titration unit (i.e. acidic and alkaline) is simulated as a proportional controller:

$$\Phi = q \cdot c \qquad (23)$$

where Φ (mol/h) is the action of the titration unit; q is the titration flow rate (ℓ/h) and c the concentration of the titrant (mol/ℓ). The flow rate and concentration of the titrants are in fact the two variables that enable the operator to adjust the sensitivity of the control system.

The pH control module is as follows:

Variable	Switching condition	Value	
Titrant concentration	$pH_{err} \geq 0.01$	$C_{Ac} = \hat{C}_{Ac}$	(Figure 56 A)
	$pH_{err} \leq -0.01$	$C_{Bs} = \hat{C}_{Bs}$	(Figure 56 B)
Titrant flow rate	$pH_{err} \leq -0.02$	$q_{titr} = \hat{q}_{titr}$	(Figure 56 C)
	$-0.02 < pH_{err} < 0.02$	$q_{titr} = 0$	
	$pH_{err} \geq 0.02$	$q_{titr} = \hat{q}_{titr}$	

where $\hat{C}_{Ac}$, $\hat{C}_{Bs}$ and $\hat{q}_{titr}$ are the concentrations of the acidic and alkaline titrants and the flow rate of the titrant respectively. This latter is assumed to be proportional to the absolute pH error, through a gain of 10^3; for simplicity, no differentiation is made between the two titration units, although in the practice they are independently calibrated prior to starting the test.

In order to incorporate the effect of temperature on the physico-chemical equilibria and on the metabolism of the microbial populations, the Henry's law constant, the dissociation constants for carbon dioxide and the uptake rates have been modelled as temperature dependent (Remigi and Ficara, 2005, in Annexure H).

4.3.5 The selection of equilibrium vs. dynamic state variables

The choice of implementing a mathematical model that uses a mixed differential algebraic system, as opposed to a pure differential system, may be advantageous. The peculiarity of this approach though is that the equilibrium and the dynamic variables are independently determined through the algebraic and differential components of the model respectively. In other words, the equilibrium variables will act as parameters in the system of differential equations and reciprocally the dynamic variables will be used as parameters in the system of algebraic equations.

This translates in the necessity of carefully assigning each species within a dissociation equilibrium to one or the other category. Being all 3-component systems, consisting of the undissociated species and the two ions, we adopted the convention of taking the ionised forms as the equilibrium variables.

This has another important consequence in this particular application, in which acidic and alkaline titration are the key processes: being the hydrogen and hydroxyl ions equilibrium variables, they cannot be used to define the inputs coming from the titration units (by definition, an input can only be set using a dynamic variable). Therefore, the acidic and alkaline titrant solutions are to be modelled using the respective anion and cation counterparts respectively. This has been done with the following inputs, added to the liquid phase:

- acidic titration: $L_{an} = C_{Ac} \cdot q_{titr}$
- alkaline titration: $L_{cat} = C_{Bs} \cdot q_{titr}$

The same rationale applies to the simulation of the titration with acetic acid (which can be an option to conduct pure methanogenic activity tests). The total acetate (undissociated acid plus acetate ion) has been used as the dynamic state variable and the acetate ion as the equilibrium state variable. The definition of a third variable, that represents the undissociated acid to model the titrant solution would be physically correct but conceptually redundant in that within the 3-component mass balance of acetate ($[\text{Tot.acetate}] = [\text{HAc}] + [\text{Ac}^-]$), the third variable is a linear combination of two independent variables. Therefore, the titration with acetic acid is implemented by using two equivalent fluxes, i.e. of total acetate and of anions: the former

brings the acetate in the system; the latter indirectly generates acidity within the system, by pushing the chemical equilibria towards the hydrogen ion.

4.3.6 Setting the initial conditions

The initial conditions for the physico-chemical sub-system have to be precisely indicated, so that the algorithm does not simulate a very fast titration process to compensate for the unbalanced system of algebraic equations, thus altering the actual initial conditions.

This is done by manually solving the dissociation equilibrium of the inorganic carbon in combination with the gas-liquid transfer of carbon dioxide, governed by the Henry's law:

$$\begin{cases} [HCO_3^-] = [CO_2]_{liq} \cdot 10^{pH-pK} \\ [CO_2]_{liq} = K_H \cdot p_{CO_2} \end{cases}$$

where K and K_H are the dissociation and Henry's law constants for carbon dioxide and p_{CO_2} denotes the partial pressure of carbon dioxide.

A typical set of initial conditions, at a temperature of 35 °C (and assuming a set-point pH of 6.80 and a carbon dioxide partial pressure of 0.5 atm) is as follows (units: mol/ℓ):

S_{H+}	S_{HCO3}	S_{OH-}	S_{Cat+}	S_{An-}
$1.585\ 10^{-7}$	$39.97\ 10^{-3}$	$1.318\ 10^{-7}$	$39.97\ 10^{-3}$	$2.67\ 10^{-8}$

As a further safety measure, it is recommended to temporarily switch off the control unit for an initial transient, to avoid unrealistic patterns in the titration process simulated, which might be caused by numerical unbalances. The transient must be very short compared to the duration of the test, e.g. 1-2 min for a 24-h test.

The parameters used in the model are reported in Table 22 and the Petersen matrix of the dynamic processes is represented in Table 23.

Table 21 – Algebraic system of the equilibrium processes of the mathematical model of the pH-titrimetric sensor

Process	**Equation**
Charge balance	$0 = S_{H+} - S_{OH-} - \frac{S_{Ac-}}{64} + S_{Cat+} - S_{An-} - S_{HCO3}$
Acetate dissociation equilibrium	$0 = K_{diss,Ac} \cdot S_{Ac} - (K_{diss,Ac} + S_{H+}) \cdot S_{Ac-}$
Water dissociation equilibrium	$0 = S_{OH-} - \frac{K_{diss,W}}{S_{H+}}$

Table 22 – Parameters of the mathematical model of the pH-titrimetric sensor.

Description	Symbol	Units	Value
Decay rate for all microbial groups	b	d^{-1}	0.1
Carbon content of glucose	C_{Glu}	mol/g COD	6/192
Carbon content of acetate	C_{Ac}	mol/g COD	2/64
Carbon content of methane	C_{CH4}	mol/g COD	1/64
Carbon content of biomass	C_{bio}	mol/g COD	0.03
Fraction of acetate from soluble substrate	f_{Ac}	-	0.75
Fraction of aceticlastic methanogens on the biomass	f_{XAc}	-	0.15
Fraction of hydrogenotrophic methanogens on the biomass	f_{XH2}	-	0.15
Henry's law constant for methane (298 K)	H_{CH4}	-	0.0315
Henry's law constant for carbon dioxide (308 K)	H_{CO2}	-	0.625
Henry's law constant for hydrogen (298 K)	H_{H2}	-	0.0162
Dissociation constant for acetate ($-\log_{10} K_{diss,Ac}$)	$pK_{diss,Ac}$	-	4.76
Dissociation constant for carbon dioxide ($-\log_{10} K_{diss,CO2}$)	$pK_{diss,CO2}$	-	6.362
Dissociation constant for water ($-\log_{10} K_{diss,W}$)	$pK_{diss,W}$	-	13.641
Gas-transfer coefficient	K_{La}	d^{-1}	100
Rate of the pseudo-equilibrium for inorganic carbon	$k_{eq,CO2}$	d^{-1}	10^8
(Asymptotic) maximum uptake rate for fermentation (*)	$\hat{k}_{max,Su}$	g COD/g VS/d	20
Maximum uptake rate for aceticlastic methanogenesis	$k_{max,Ac}$	g COD/g VS/d	10
Maximum uptake rate for hydrogenotrophic methanogenesis	$k_{max,H2}$	g COD/g VS/d	5
Half-saturation constant for acidogens	$K_{S,Su}$	g COD/ℓ	0.5
Half-saturation constant for aceticlastic methanogens	$K_{S,Ac}$	g COD/ℓ	0.2
Half-saturation constant for hydrogenotrophic methanogens	$K_{S,H2}$	g COD/ℓ	$5\cdot10^{-5}$
Concentration of the titrant solutions	m	mol/ℓ	0.5
Atmospheric pressure	p_{atm}	bar	1.013
Gas law constant	R	ℓ·bar/K/mol	0.082
Lag-period for slow fermentation	t_{lag}	d	0.2
Time constant for slow fermentation	t_f	-	0.1
Yield of acidogenic biomass	Y_{Su}	g COD-VS/g COD	0.1
Yield of aceticlastic methanogens	Y_{Ac}	g COD-VS/g COD	0.005
Yield of hydrogenotrophic methanogens	Y_{H2}	g COD-VS/g COD	0.024

(*) $$k_{max,Su} = \begin{cases} t < t_{lag} : \hat{k}_{max,Su} \cdot \left[1 - \exp\left(-\frac{t_f}{t_{lag} - t} \right) \right] \\ t \geq t_{lag} : \hat{k}_{max,Su} \end{cases}$$

Table 23 – Petersen matrix for the dynamic processes of the pH-titrimetric sensor.

	Particulate species			**Soluble species**						
Processes	X_{Su}	X_{Ac}	X_{H2}	S_{Su}	S_{Ac}	S_{H2}	S_{CO2}	S_{CH4}	S_{HCO3}	**Rates**
P1	-1									ρ_1
P2		-1								ρ_2
P3			-1							ρ_3
P4	Y_{Su}			-1	$f_{Ac} \cdot (1 - Y_{Su})$	$(1 - f_{Ac}) \cdot (1 - Y_{Su})$	$C_{Glu} - C_{Ac} \cdot f_{Ac} \cdot (1 - Y_{Su}) - C_{bio} \cdot Y_{Su}$			ρ_4
P5		Y_{Ac}			-1		$C_{Ac} - C_{CH4} \cdot (1 - Y_{Ac}) - C_{bio} \cdot Y_{Ac}$	$1 - Y_{Ac}$		ρ_5
P6			Y_{H2}			-1	$-C_{CH4} \cdot (1 - Y_{H2}) - C_{bio} \cdot Y_{H2}$	$1 - Y_{H2}$		ρ_6
P7							1		-1	ρ_7
	Acidogenic biomass	Aceticlastic methanogens	Hydrogenotrophic methanogens	Fermentable substrate	Acetate	Hydrogen	Carbon dioxide	Methane	Bicarbonate	

Legend:

P1, P2, P3: Decay of the acidogenic biomass; aceticlastic and hydrogenotrophic methanogens $\rho_i = b \cdot X_i$

P4, P5, P6: Fermentation; aceticlastic and hydrogenotrophic methanogenesis $\rho_i = k_{max,i} \cdot X_i \cdot \frac{S_i}{K_{S,i} \cdot S_i}$

P7: Equilibrium of the inorganic carbon $\rho_7 = k_{eq,CO2} \cdot (S_{HCO3} \cdot S_{H+} - K_{diss,CO2} \cdot S_{CO2})$

CHAPTER 5

RECOMMENDATIONS

Specific recommendations regarding technical aspects of the various stages of the protocol are discussed in the pertaining Annexures.

Serious consideration should be made to implementing co-digestion facilities. This project has indicated that it is feasible to establish the treatability of industrial effluents by co-digestion and to determine the state of the digester so as to prevent malfunction.

The most significant contribution of this project to co-digestion, particularly of industrial effluents, is the detailed methodology of the serum bottle activity test.

The discussion presented in Annexure A should enable the operator to obtain highly accurate and reproducible results.

The new variation of an otherwise established method enables one to consider a serum bottle activity test as a fully independent test, of comparable scientific and technical value to a conventional larger-scale approach with a number of particular advantages. The serum bottle test need not be considered as a mere tool, but as being equivalent to any other analytical or instrumental determination that is used to support the findings of a larger-scale experiment.

Furthermore when it is undertaken for regulatory purposes, the application of a screening step before a more extensive experimental stage could result in the reduction of the experimental time with the associated reduction in costs.

However, we believe that the laboratory-scale stage of the protocol would be significantly improved if a more efficient data acquisition and control could be implemented. This is relatively easy.

A first degree of improvement could be ensured by refining the database application that has been developed as a demonstration tool. Aspects that should be addressed are:

- the possibility of automatically processing data in order to generate standard reports on the state of the digester, and to export data into pre-formatted spreadsheets in which standard plots would be automatically updated;
- the implementation of 'flexible' internal calculations, such as the calculation of the specific methanogenic activity or of the removal rate on moving windows of variable width: this in particular would simplify the data analysis, enabling a reliable monitoring of the process;
- the integration of a simplified anaerobic digestion mathematical model to fit to the experimental data.

The next stage could be the development of a proper monitoring software, using commercial packages, that would acquire data from a number of sensors, record them in the appropriate format and display a real-time control panel that would enable the operator to intervene on the digester, e.g. by modifying the feed flow rate, or switching on a sampling pump, etc. Some operations, e.g. gas sampling and analysis would still be done manually, but the related information could be inputted using the same monitoring software and processed to automatically monitor the process. This advancement would clearly necessitate a considerable investment in the hardware, including sensors and input/output modules, but would possibly allow the conduction of several parallel simulations.

As a general remark, we would recommend that an anaerobic master culture (e.g. 20 to 30 ℓ) be maintained in a laboratory-scale completely stirred digester, to provide the seed inoculum for the screening stage and for the subsequent laboratory-scale simulation. This would shorten the experimental time by eliminating the initial transient in a serum bottle activity test; eliminate interference due to the variability of the inocula (and associated residual organic matter); overcome the problems associated with using sludge which had been exposed to variable conditions a full-scale digester at a wastewater treatment works.

CHAPTER 6

CONCLUSIONS

As the research progressed, it became clear to us that we were unlikely to obtain a significant set of experimental data on a particular set of industrial effluents that could prove or disprove the suitability of that material to be co-digested with a labile substrate. In fact, such an output would have been of little benefit for other researchers, because it would inevitably be specific to the experimental conditions and materials.

A lot more valuable would have been a set of **practical tools** that enable a researcher or an operator to experimentally ascertain whether a given effluent is amenable to be co-digested and to what extent and to identify safe operating conditions to start-up a full-scale application. This is the approach used in this report.

The research tackled issues that are particularly urgent in the province of KwaZulu-Natal:

- the relative abundance of rainfall as compared to the rest of the country has attracted water demanding industry, such as the textile industry;
- the implementation of Sustainable Consumption and Production tools has lead to the reduction of the quantity of the emissions, hence more concentrated effluents are generated;
- under- or non-utilised anaerobic digesters exist at Wastewater Treatment Works which could be operated to co-treat those industrial effluents and other more labile ones in a centralised facility.

In pursuing this objective, the first step was to be able to characterise an effluent in a way which is at the same time functional to the ultimate objective and sufficiently simple that can be implemented as a routine decision support tool.

The properties of an effluent that the operator (or the Authority) should target, as immediately related to the suitability of the effluent of being co-digested, are its biodegradability and toxicity to anaerobic micro-organisms. The methodology that selected was the serum bottle method: this has proven to be very accurate and does not require sophisticated infrastructures nor highly specialised skills.

The protocol for screening industrial effluents is to be regarded as the main output of this project (Figure 57), in that it is the ultimate result of the experience the research team has accumulated by conducting the experimental work. The protocol is discussed in detail in

Annexure A and comprises three stages, namely a rapid screening of the test materials, to be conducted through a serum bottle activity test or a pH-stat titrimetric test; a more in-depth study of the biodegradability and toxicity of the test material, using the serum bottle methodology; and a simulation of co-digestion conditions either using the serum bottles or a laboratory-scale set-up.

Furthermore the tools provided by the protocol will assist the operator in the monitoring process of the fully operative application.

Other authors proposed similar concepts, e.g. a protocol to determine the effect of toxic organic chemicals (Young and Tabak, 1993) or co-digestion experiments on serum bottles (Angelidaki and Ahring, 1997). However, our approach is original in that it proposes a wider picture and provides the operator with a set of methodologies to apply which will ensure that the results he obtains are reproducible and accurate.

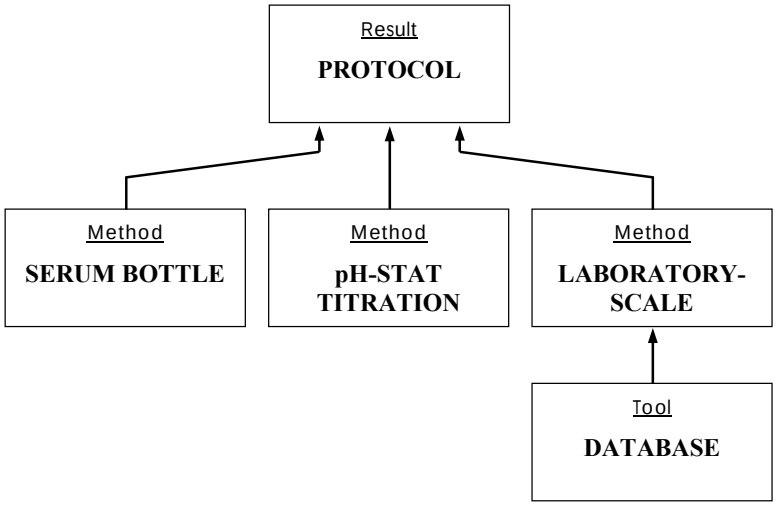

Figure 57 – Schematic of the outcome of the project.

The technical tools that are instrumental to the execution of the protocol are the serum bottles method, to conduct anaerobic activity and co-digestion tests; the pH-stat titrimetric technique to perform rapid methanogenic activity test; and a laboratory-scale set-up which enables a close simulation of a full-scale application (Figure 57).

The serum bottle method is a relatively well established method that has been used for decades to perform biodegradability and toxicity tests. However, unlike other authors, we attempted to obtain from a serum bottle assay the most accurate information and in doing so we developed a very detailed protocol for conducting these tests (Annexure B).

This should be regarded as the major scientific output of this project.

Key aspects to fully exploit the potential of the test are a correct set-up (Figure B.1) and an accurate mass balance: these should be obvious recommendations, but become particularly necessary when the methodology is very simple, hence vulnerable to several factors of interference.

The pH-stat titrimetric technique too is a relatively established method to assess microbial activity and inhibition. Particularly, it has proven as accurate as other methods of measuring methanogenic activity, but competitively more rapid. We therefore would see its application as screening tool in combination with the serum bottle method.

In our opinion, a very promising application of this technique could be the monitoring of the complete anaerobic digestion process (rather than to the final methanogenic stage only). In the course of this project, we started evaluating this possibility, by developing (and partially testing) a mathematical model of the titration process coupled to a simplified anaerobic digestion model. Further investigations and experimental work need to be done in this respect, but it seems that such application is feasible and could become competitive with the serum bottle method.

At this stage though we only propose a detailed protocol for conducting methanogenic pH-stat activity tests (Annexure C) which summarises the knowledge acquired over the years and which would enable an operator to use one of the commercially available pH-stat titrimetric sensors.

A laboratory-scale experiment to simulate a full-scale application is a common practice, because it enables easier monitoring and control hence a better understanding of the process (Table 3).

We conducted several laboratory-scale trials using the same set-up which in fact has proven sufficient to our scope, despite the apparent simplicity. The set-up consisted of 2 to 5 ℓ-volume flasks sealed with a rubber bung and maintained at 35 °C, in a temperature controlled room. Continuous mixing was ensured by a magnetic stirrer. Various options are available to measure the biogas flow rate: a semi-automatic liquid displacement counter was used for our experiments, which we found sufficiently easy to operate although, in the long-term, not very reliable in the response: it seems that frequent recalibration of the level sensors and maintenance of the solenoid valve are crucial in ensuring a consistent response of the device.

We found that a compensation volume that creates a variable-volume headspace can prevent air and biogas leaks during sampling and feeding operations, thus ensuring a better accuracy of the gas measurements.

Routine determinations were conducted:

- COD, alkalinity and VFA concentration; pH; gas flow rate and composition on a daily basis;
- solids concentration, weekly.

Certainly the most sensitive parameter which can be used as early warning indicator (Table 18) is the gas compositions, i.e. the carbon dioxide and methane molar fractions in the off-gas.

The conduction of off-line activity tests on the sludge withdrawn from the reactor has proven a very useful monitoring tool, although the response is inevitably delayed in time. The benefit consists in isolating a sample of the mixed liquor that is representative of the whole reactor at a given moment in time and being able to perform various activity tests on it, while maintaining the regime in the source reactor unaltered. A specific experimental plan is proposed (Section G.2.3) which comprises three stages, namely a first period aimed at evaluating the SMA and whether a significant portion of the residual COD had the potential of being further biodegraded; a second period aimed at evaluating the optimal methanogenic activity (i.e. on acetate); and a third period aimed at evaluating other conditions, e.g. the methanogenic activity on the test material at a particular loading rate.

A tool was specifically developed to assist the operator in the efficient acquisition of experimental data of laboratory-scale simulations (Figure 57). In situations where all operations and analytical determinations are done manually, a large amount of diverse data is to be recorded and analysed. The database (Annexure E) can serve a two-fold objective: it enables the storage of all the details of the different determinations for future reference without preventing an immediate picture of the current state of the digester. In principle, all information is inputted at different levels and the operator can activate the view that is relevant at that moment. Additional benefits of this form of data management are that:

- some routine calculations can be coded, thus ensuring the immediate availability and the correctness of the calculations; and
- quality and consistency checks can be implemented to limit accidental input errors thus enhancing the data quality.

The current version of the database is very basic. Ideally, feedbacks from operators or researchers in the field of co-digestion would enable us to improve the database as to meet the requirements of a potential market.

An overview of the experimental results for the distillery, size and scour effluents is presented in Table 24.

We limited the summary to the results obtained in the last period of the project essentially because the methodology itself is the output of the project, and as such it has been developed gradually and became available toward the end of the project. Therefore, those three test materials were tested under comparable conditions and the results are sufficiently homogenous.

The purpose of this summary is not to present a conclusive set of data which is inevitably of limited interest, rather to show the type of results that one could expect from the protocol developed in this project.

Table 24 – Summary of the results of the project.

SERUM BOTTLE ACTIVITY TESTS, ON INDIVIDUAL TEST MATERIALS

		Distillery	**Size**	**Scour**
Range [1]	g COD/ℓ	0.8-50	0.8-31	1.1-5.4
BMP	%	≈ 40 (≈ *11*) [*]	27-38 (*7-17.5*) [*]	< 0 (*all conc.*) [*]
		55-70 (*4-13*)	62-66 (*1-6.9*)	
RA [2], [*]	%	≈ 62 (*1-11*)	60-70 (*6-22*)	7-57 (*4.9-5.4*)
		≈ 25 (*16*)		
A [3]	mg COD/g VS/d	90-100 (*2.5-13*)	80-120 (*1.5-7*)	7-10 (*1.1-1.8*)

SERUM BOTTLE CO-DIGESITION TESTS

Distillery: Size	**BMP** %	**A** mg COD/g VSS/d	**E** -	**COD** g COD/ℓ
2:1	55-67	133-187	1.03-1.14	3.6-13.0
1:1	66-88	26-78 [#]	1.04-1.27	0.8-7.1
1:2	52-68	137-198	0.94-1.10	3.7-12.8

Size: Scour	**BMP** %	**A** mg COD/g VSS/d	**E** -	**COD** g COD/ℓ
2:1	6-30	35-54	0.29-0.40	1.4-2.4
1:1	< 0	21-35	< 0	1.3-2.2
1:2	< 0	11-20	< 0	1.2-2.0

Distillery: Synth dye	**BMP** %	**A** mg COD/g VSS/d	**E** -	**COD** g COD/ℓ
2:1	31-34	26-68	0.55-0.61	2.2-4.1
1:1	10-13	11-32	0.27-0.29	2.1-3.9
1:2	≈ 0	8-12	< 0	2.1-3.7

ENDOGENOUS METHANOGENIC ACTIVITY

95 % CI	mg COD/g VS/d	2.3 to 2.6 mg COD/g VS/d (N=44) or 3.3 to 4.2 mg COD/g VSS/d (N=32)

LABORATORY-SCALE START-UP

Ranges of HRT and OLR tested: 18-87 d and 1.0-3.4 g COD/ℓ/d respectively. The operating conditions were varied repeatedly so that no steady-state conditions could be attained: alkalinity, VFA concentration and the composition of the gas phase fluctuated; the COD concentration gradually increased (possibly levelled off at 30-35 g COD/ℓ; the overall COD removal reached 50 % (Figures 52 and 53).

Figures in brackets (*italicised*) indicate COD concentrations.
[1] Range of COD tested (figures indicate the *total* COD concentration, i.e. test material + reference substrate).
[2] Relative Activity: it is equivalent to Inhibition, defined according to equation B.7.
[3] Activity, calculated on the sole test material (tests No 3, 4 and 5).
[*] The figures refer to overloaded conditions (tests No 1 and 2).
[#] Activity expressed in mg COD/g VS/d.

CHAPTER 7

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ANNEXURE A

Experimental protocol for the screening of industrial effluent

The primary output of this project was to propose an experimental approach whereby a generic effluent can be assessed for its amenability to being co-digested.

The experimental approach will necessarily have to address the two key characteristics, which determine the behaviour of a test material in respect to an existing operational anaerobic digester: these are its toxicity and its biodegradability (Section 2.3).

- Toxicity denotes a detrimental effect that the effluent exerts on the activity of a microbial population. It indicates the impact of the effluent on the baseline (i.e. prior to the co-digestion trial) performance of the digester. That is to say:

  if we feed this effluent, under certain conditions, to a given well operating anaerobic digester, how will the overall performance of the system worsen, as a consequence of this additional stream ?

  The following conditions have to be ensured: the gas flow rate must not be dramatically reduced, as methane is the primary economical output of the process and represents COD reduction; the stabilisation of the waste (e.g. the extent of the COD removal) needs to be maintained, as this is a prerequisite that the legislation imposes for the final discharge (and disposal of residues); and the overall stability of the digester must not be compromised, as rescuing a failing digester or starting-up a new digester can be very expensive and time-consuming.

  However, a reasonable reduction in the process performance might be acceptable, as the combined treatment will counterbalance the specific losses.

  Methanogenesis is generally the most sensitive stage of the entire process and certainly this is the case for high strength organic effluents that may be relatively easily converted to acidic intermediates but thereafter cause a stress overload for methanogens. Further, methanogenesis is immediately related to the production of methane. It is therefore obvious that the general practice is to address methanogenic toxicity.

- Biodegradability indicates the inherent property of a test material, which depends primarily on its molecular structure and refers to its susceptibility to undergoing a biologically mediated degradation. It is related to the actual conversion of the test material to degradation products (or intermediates) which will likely pose a lesser concern in terms of final disposal. That is to say:

  if we feed this effluent, under certain conditions, to a given well operating anaerobic digester, to what extent will the biomass succeed in degrading the organic constituents to intermediates and ultimately to methane ?

Since our research focused on organic effluents, we adopted the approach whereby if the organic content of the test material is reduced during the anaerobic digestion process and the extent of this reduction is reflected in a stoichiometrically equivalent output of methane, this is sufficient to conclude that the test material had undergone a certain degree of biodegradation.

Limits of the protocol. By no means, this protocol is intended to be a comprehensive and a rigorous instrument which enables one to assess the fate of the test material, simply because other forms of toxicity may in fact be induced by particular chemical species contained in the test material that are not necessarily limited to inhibiting the methanogenic biomass. Similarly it may happen that the degradation intermediates and/or the non-degradable fractions of the test material are in fact equally or even more environmentally dangerous than the original effluent and although it may be seen that the test material is degraded to some extent, the disposal issue persists.

Other more specific analytical tools exist and generally require a more in-depth study of the degradative pathway of the test material.

However these circumstances may be anticipated by a preliminary knowledge of the general composition of the effluent to be treated and possibly of the industrial process that generates it. This step is also indispensable.

Nevertheless, it is believed that the protocol illustrated in this section can be a reliable decision support tool for high organic strength effluents: purposely, a relatively simple and inexpensive method such as the serum bottle method has been adopted, since it is believed that it is sufficiently simple and accurate to enable the characterisation of a test material in a relatively short period of time and without a great amount of laboratory work. Table A.1 *contains a list of the material needed to perform the experimental assessment described hereafter.*

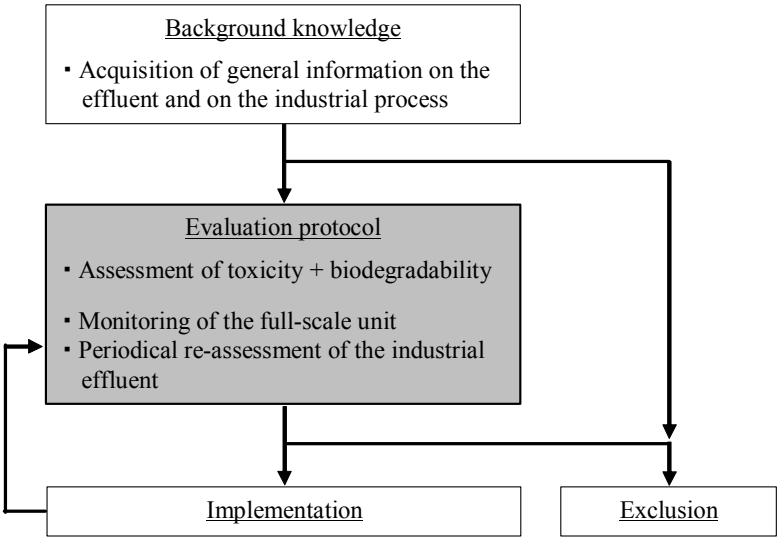


Figure A.1 – Flow diagram of the Protocol for the screening of high strength industrial effluents.

This discussion of concepts can be visualised as in Figure A.1, which summarises the general approach of this research.

Prior to undertaking the experimental assessment, the operator of the co-digestion facility should gather sufficient information regarding the general characteristics of the effluent under study and of the process that generates it. This preliminary step should enable the selection of high strength effluents, which do not pose more specific and possibly dangerous threats to health and environment, other than their high content in organic matter, salts, colour, detergents, etc.

The evaluation protocol serves the double function: it is a tool for the initial assessment of the industrial effluent, aimed at investigating the feasibility of the co-digestion option for its disposal; it is also a tool for the continuous monitoring of both the performance of the digester at the full-scale and of the effluent, should changes in the production process result in varying characteristics of the effluent.

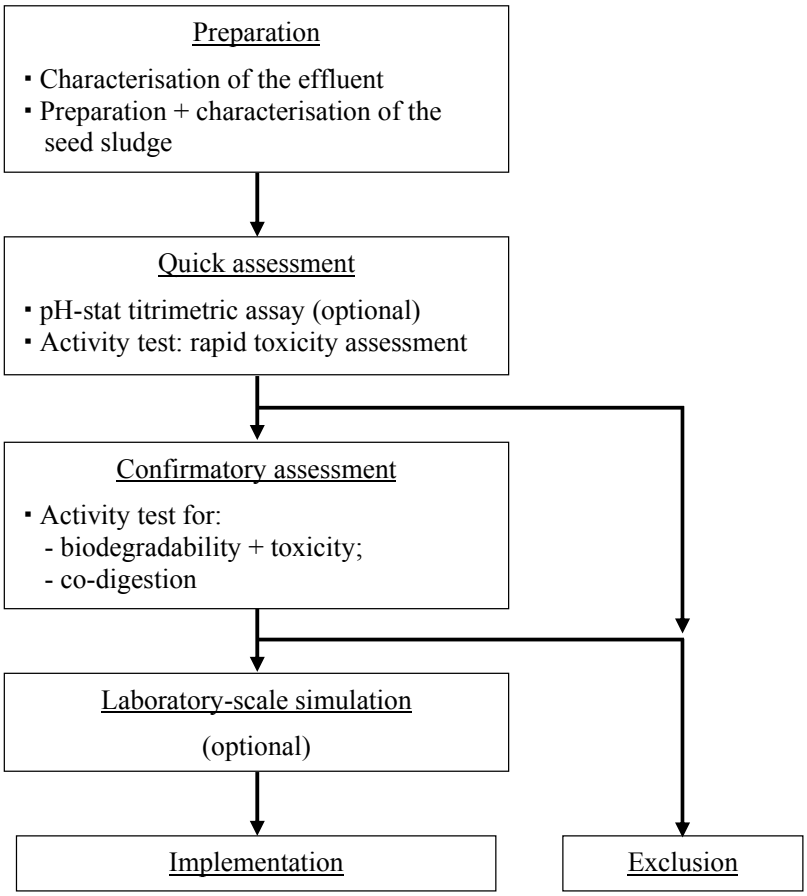


Figure A.2 – Experimental stage of the Protocol for the screening of high strength industrial effluents.

A.1 THE EVALUATION PROTOCOL

Young and Tabak (1993) proposed a comprehensive 3-level protocol which provided a means of determining the fate and the effect of toxic organic chemicals in anaerobic wastewater treatment processes. Level one consists in a 3-step screening protocol to assess toxicity. Level two is a kinetic protocol to assess the kinetics of acetogenesis and methanogenesis under the effect of the toxicant. Ultimately, level three is a protocol to assess the kinetics of the degradation of the toxic compound.

The protocol proposed herein is aimed at assessing the methanogenic toxicity and the biodegradability of the effluent under study (the test material). Insight in the kinetics of the degradation process can be obtained, although it is not a primary objective. It therefore corresponds to the level-one stage of the earlier protocol and is depicted in Figure A.2.

A.1.1 Characterisation of the test material and preparation of the seed sludge.

The test material will be characterised in terms of its organic content (as COD), pH and alkalinity and solids concentration (total and volatile solids).

For specific applications additional parameters, that may be relevant to the anaerobic degradation process and yet relatively simple and inexpensive to obtain, can be determined: for instance, it might be of interest to assess to what extent the colour can be removed through the anaerobic (co)digestion of the test material.

The characterisation of the effluent must be carried out within the shortest possible time after collection, in order to minimise alterations that may occur due to the incorrect handling of the effluent or to biochemical or physical degradation processes that may intervene. Once the characterisation is completed, the test material must be stored at low temperature (4 °C), in the dark, in a sealed container.

A generic, i.e. unadapted, sludge must be used for the small-scale assessments (which include the Quick and the Confirmatory assessment stages; Table A.2), in order to *objectively* assess the toxicity and the biodegradability of the test material in a manner which is independent from the specific application and therefore comparable to other similar assessments as well as to evaluate a possible adaptation of the biomass to the test material upon long-term exposure. The unadapted sludge could be sampled at a wastewater works and maintained for a certain time on a readily biodegradable substrate, e.g. acetate.

For the laboratory-scale simulation, a well balanced digester will be used in order to accelerate the start-up phase. The sludge must be characterised in terms of its solids content and have a low residual organic content. This can be done by subjecting it to a period of starvation prior to starting the assessment.

A.1.2 Quick toxicity assessment

The objective of this first stage is to characterise the test material as potentially inhibitory or readily biodegradable and to identify a concentration range that will be extensively investigated in the following step.

A first activity test (AAT) will be conducted by using three levels of concentration of the test material. The indications which can be given here are inevitably qualitative, as the actual concentrations will vary from case to case taking into account primarily the strength of the test material and also the technical viability of a particular dilution.

- A very high strength effluent will need to be significantly diluted, so that the highest concentration level does not exceed the value reported in Table B.3; a higher COD concentration is likely to negatively affect the performance of the biomass and should therefore be avoided *at this stage*;
- An excessive dilution of the test material may not be of practical interest at the real scale, because it would not meet the rate of production of the test material itself and/or would require storage capacity that might be not available. This is clearly a less precise limit that the former one, but it should similarly be avoided, *at this stage*.

Tentatively, we could take as the *high*, *intermediate* and *low* concentrations the values of 100, 50 and 10 % v/v respectively (these volumetric ratios obviously refer to the aliquot of liquid volume available for the test material). It is important to stress that discretion should be applied in ensuring the primary objective which must always be that the outcome of the AAT is meaningful.

This AAT will comprise **15 units** (6 controls, three containing the seed inoculum only and three supplemented with the reference substrate; and 3 replicates per concentration level: Table A.2) and should be monitored for not less than 7 d. Based on the outcome of this test, the next step will be set-up, according to the following rationale.

a) If no significant inhibition is visible, even at the high concentration level: the test material is likely to be readily biodegradable with no or negligible harm for the digestion process. In the following step, higher concentrations may be investigated or the current test extended for a longer time.

b) If the low concentration level shows a significant inhibition: the test material is likely to result in a strong impairment of the digestion process, unless the biomass would be able to adapt upon exposure. In the following step, if lower concentrations are still technically of interest, lower concentration ranges could be investigated in order to identify a concentration range of low toxicity.

A.1.3 Confirmatory assessment

This experimental step is aimed at characterising the biodegradability and/or the toxicity of the test material: the former will be expressed as the fraction of the organic matter content of the test material which is converted to methane. The latter, will be given as inhibition curve and, if possible, as 50 %

inhibition concentration. In order to define a meaningful inhibition curve (or segment), three or four data points are required.

Further, if the test material shows to be inhibitory in the early stage of the incubation, the test period can be extended until signs of biomass adaptation are seen. In our experience, we believe that if after 60 to 90 d, no gas production has occurred, one should conclude that biomass adaptation is not feasible.

Should the test material be amenable to be digested, two further objectives could be achieved at this stage: *i*) the simulation of the co-digestion treatment and *ii*) the identification of a set of 'safe' operational parameters to start-up the laboratory-scale experiment or to directly implement the same treatment option on a full scale.

This AAT will comprise **15 units** (3 controls, with the seed inoculum and the readily biodegradable substrate; 3 controls, with the seed inoculum and the test material; and 3 replicates per concentration level) and should be monitored *until the methanogenic activity decreases to zero*.

A second level of AAT could be started specifically aimed at testing different volumetric ratios between the test material and the designated readily biodegradable effluent that will be used in the co-digestion treatment. It is beyond the scope of this protocol to actually screen various options of readily biodegradable effluents, unless specifically required.

A.1.3.1 Data interpretation

An anaerobic activity test will provide the Specific Methanogenic Activity (SMA) of the seed inoculum, which indicates the (maximum) activity attainable, i.e. the rate of substrate uptake (g COD/g VSS/d), and the Biodegradability (B) of the test material, which indicates the COD fraction which is actually converted to methane (g COD/g COD).

A tentative value for the Organic Loading Rate (OLR, g COD/ℓ/d) to apply to a laboratory-scale digester can be estimated as:

$$\mathrm{OLR} = \frac{\mathrm{SMA}}{\mathrm{B}} \cdot \mathrm{X} \qquad \text{(A.1)}$$

where X is the concentration of solids in the reactor (g VSS/ℓ).

A.1.4 Laboratory-scale simulation

This phase is aimed at verifying the findings of the screening phase, i.e. the extent of biodegradation, the inhibitory effect to the methanogenic biomass, the potential for adaptation and the optimal volumetric ratio effluent-to-substrate, and at simulating the full-scale co-digestion process. It is crucial that the operation of the laboratory-scale unit is closely monitored, to enable the optimisation of the co-digestion performance. The composition of the biogas is relatively easy to determine and is a very sensitive parameter that could serve as main indicator of the process performance.

A.1.5 Monitoring

Once the co-digestion process has been implemented at the full-scale, the same methodology underlying the *screening* protocol can be used to off-line monitor the performance of the process and to periodically re-assess the characteristics of the industrial effluent.

A serum bottle AAT can be carried out at regular intervals (e.g. monthly) on a sludge sample withdrawn from the digester and incubated in the vials with no addition of other components. During a first period, the activity of the sludge is monitored until the residual organic substrate is depleted. Thereafter a dose of reference substrate is spiked into the vials daily (e.g. 30 μℓ of acetic acid) and the activity monitored until it levels off. The determination of the maximum (or potential) methanogenic activity on acetate, as opposed to the *actual* methanogenic activity on the mixed substrate inside the digester, enables to semi-quantitatively estimate the active methanogenic biomass which provides a direct indication of the health of the digester.

The periodical re-assessment of the industrial effluent is a necessity to ensure that its characteristics do not significantly deviate from those initially assessed, as the result of changes in the production process. The set-up described for the AAT of the Confirmatory Assessment of the protocol (Table A.2) will be used; conditions close to the actual conditions in the co-digestion facility (e.g. effluent concentration, substrate-to-biomass ratio, etc.) will also be used as reference. Should the response be significantly different to the initial one, the option of diverting the effluent until an extensive assessment has been completed must be considered.

CONCLUSIONS

An experimental protocol based on a very simple methodology and requiring little additional laboratory work has been developed to screen high strength effluents prior to being co-digested. The same concept is applied to monitor the performance of the co-digestion process, once implemented at the full-scale.

Table A.1 – List of material required to execute the Protocol for screening industrial effluents.

- Temperature controlled room (or equivalent device, e.g. thermostated cabinet).
  The assays are ideally to be conducted in a mesophilic temperature range, i.e. at 35 °C, and the temperature must be maintained constant throughout the test.
- Material to conduct anaerobic activity tests using the serum bottle method (Annexure B).
  Glass vials (125 mℓ), butyl rubber septa and aluminium crimps (Separation Pty Ltd.), glass syringes (various sizes, e.g. 2, 10 and 50 mℓ), glass syringe (100 μℓ) for gas sampling.
- Gas-chromatograph, equipped with a column capable of detecting carbon dioxide and methane (Annexure F).
- Nutrients and mineral salts stock solution (Annexure F).
- Apparatus for the determination of COD, solids, alkalinity and VFA (Annexure F).

Table A.2 – Protocol guidelines, detailing the number of tests and the time needed to complete the experiment.

- Characterisation of the test material (Overall time: 2 days)

  Perform the following analytical determinations.

  - COD: 1 to 3 determinations, with 4 replicates each (time: 2 days)
  - Alkalinity and VFA: 1 determination, with 3 replicates (time: negligible)
  - Solids: (total and volatile, unless otherwise needed) 1 determination, with 4 replicates (time: 1 day)

- Preparation of the seed sludge (Overall time: 1 day)

  The following steps are to be undertaken.

  - Collect a sample of the sludge from the plant (or from a laboratory-scale reactor).
  - Characterise the sample. As per 'Characterisation of the test material', except for the item *Solids*, where total and volatile suspended solids should be undertaken.
  - Our experience indicates that the sludge sample should not exceed the 30 to 50 g TS/ℓ, to ensure that a representative sample can be obtained.

- **Stage 1** Quick toxicity assessment (Overall duration: 8 days)

  Perform 1 test, using 15 vials.

  - preparation of the test, e.g. preparation of the Nutrient and Mineral salts Solution (1 day).
  - set-up of the test: add all components to the vials; purge with inert gas for 1 to 2 min; seal the vials; re-equilibrate after 1 h and GC determination of the gas composition at time zero (1 day).
  - monitoring the test: every day, determine the gas volume and composition (recommended at least 2 replicates per gas sample; 0.75 day).
  - analytical determinations at the end of the test: pH, COD, Alkalinity and VFA; as per 'Characterisation of the test material', to be conducted on one replicate per group of vials (2 days)

- **Stage 2** Confirmatory assessment - AAT (Overall duration: 30 to 90 days)

  Perform 1 test, using 15 vials.

  Follow the same indications of the 'Quick toxicity assessment': monitor the test every day, until the conditions reach equilibrium; thereafter, once every 3 days or every week.

- Confirmatory assessment - co-digestion (Overall duration: min 8 days)

  Perform 1 test, using 15 vials.

  Follow the same indications of the 'Quick toxicity assessment'. When the methanogenic activity decreases to zero, the operational mode can be switched to fed-batch by spiking one dose of the substrates, for a follow-up experiment. The duration is unknown, because it is function of the biodegradability of the test material (combined with the readily biodegradable substrate), but unlikely less than a week.

- **Stage 3** (optional) Laboratory-scale simulation (Overall duration: variable)

  The set-up should resemble the actual configuration of the co-digestion facility. Otherwise, use a basic completely stirred vessel (e.g. 3 to 5 litre-volume). Feeding and effluent collection can be done manually. A variable volume headspace (e.g. a gas sampling bag) can prevent gas losses and air contamination during these operations. It is imperative to determine the biogas flow rate (a basic liquid displacement device can be used).

  Perform the following determinations:

  - daily: pH, alkalinity and VFA, COD, gas volume and composition;
  - weekly: solids concentration.

  Maintain the same operating conditions for approximately 1 HRT.

ANNEXURE B

The conduction of anaerobic activity tests using the serum bottle method

The serum bottle technique for the cultivation of anaerobic micro-organisms was described by Miller and Wolin (1974).

A serum bottle is a closed vessel, in which the **sample** (i.e. the compound to be tested), a **defined medium** and a **seed inoculum** (the anaerobic sludge) are incubated at the desired temperature. The gas generated inside the vial is periodically released and quantified using a syringe. Owen and co-workers (1979) developed two serum bottles assays to evaluate biodegradability and toxicity, which they termed Biological Methane Potential (BMP) assay and Anaerobic Toxicity Assay (ATA) respectively. Both tests comprise *i*) a control set, which enables to quantify the *baseline* conditions and *ii*) a number of test units, in which the micro-organisms are exposed to a variety of conditions. To ensure the reproducibility of the determination, each unit carried out in replicates (generally three). The composition of each set varies depending on the type of test to be conducted and is summarised in Table B.1.

The key difference between the two assays is the variable targeted. A biodegradability test is aimed at evaluating the susceptibility of a compound to undergo a microbial degradation; therefore, the test compound acts as the substrate for the anaerobic micro-organisms. A toxicity test is aimed at evaluating the negative effect of a compound on the microbial activity under 'normal' conditions, i.e. in the presence of a labile substrate, which therefore must be supplemented in all the bottles: in a toxicity test, the substrate and the test compound are *antagonistic*.

Table B.1 – Set-up of the serum bottles assays.

Type of assay		Sludge	Medium	Sample	Objective of the test
BMP	- sterile control	No	Yes	No	(optional) to verify the existence of non-biological degradation potential.
	- control units	Yes	Yes	No	To quantify the baseline gas production, due to e.g. residual organic matter in the sludge.
	- test units	Yes	Yes	**Substrate** [(1)]	To assess the biodegradability of the substrate and possible influence of substrate concentration.
ATA	- control units	Yes	Yes	Substrate [(2)]	To quantify the 'reference' activity, in the absence of the inhibitory test substance.
	- test units	Yes	Yes	Substrate [(2)] + **Test compound**	To assess the inhibitory effect of the test compound at different concentrations.

In bold, the parameter which is varied in each test.
[(1)] If the test substance is recalcitrant and requires a co-substrate to be biodegraded, both species must be present.
[(2)] Reference, readily degradable substrate: e.g. acetate, propionate, etc.

B.1 TEST PREPARATION

The procedure outlined in this annexure was established in the course of this project, by progressively refining the methodology of Owen and co-workers (1979).

Although the principle underlying a serum bottle test is very simple, several factors of seemingly little importance may in fact influence the outcomes.

We present a generalised procedure which applies to both a biodegradability and a toxicity test, since we believe that *i*) the key variable which is measured in both cases is the microbial activity and that *ii*) similar information can be obtained from either test. Therefore, the term Anaerobic Activity Test (AAT) could be used, to unify the two concepts.

A serum bottle AAT (Table B.2) comprises two control sets (which allow for the quantification of the baseline i.e. endogenous activity of the seed inoculum and of the reference activity, respectively) and a number of test units, in replicates, in which the anaerobic sludge is exposed to a variety of conditions. The activity is assessed at varying concentrations of the test material.

Table B.2 – Set-up of the serum bottle activity test.

Set	Composition	Objective
Control units	▪ Seed inoculum (only)	To quantify the baseline gas production, due to e.g. residual organic matter in the sludge.
Reference units	▪ Seed inoculum ▪ Reference substrate	To quantify the reference activity, in the absence of the inhibitory test substance.
Test units	▪ Seed inoculum ▪ Reference substrate ▪ Test material	To assess the inhibitory effect of the test material at different concentrations and its biodegradability.

Several aspects must be taken into account *prior* to performing a serum bottle test (Figure B.1).

▪ Test duration

The common practice is to say that a biodegradability test should last at least 30 d, or until the biogas production becomes negligible, whereas a toxicity assay should last 7 d.

We propose that both (therefore an AAT) are continued until the microbial activity becomes negligible: this enables to observe slow biodegradation processes as well as to discriminate between acute and permanent inhibitory effects.

It is therefore proposed that the main quantity to be monitored is microbial activity, i.e. the rate of biogas production.

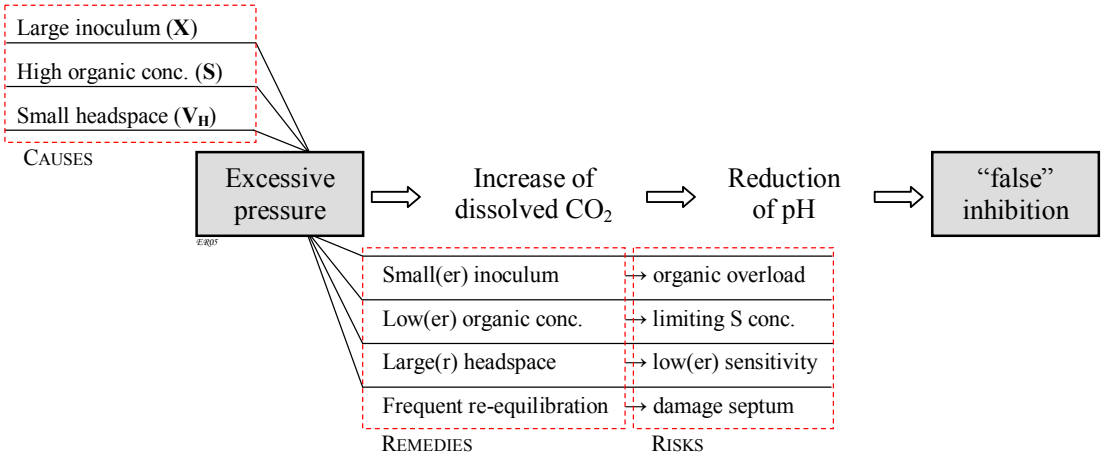


Figure B.1 – Schematic of the cause-effect interaction between the relevant aspects in the design of a serum bottle AAT (source: Remigi and Foxon, 2005).

▪ Total liquid volume in a vial (or: **headspace-to-liquid volume ratio**)

When the test materials are diluted, relatively large liquid volumes may be necessary to obtain a sufficient concentration of the organic matter. At the other extreme, if the test materials have a high COD strength, large liquid volumes are also necessary to lessen the concentration of organic matter.

Low gas-to-liquid volume ratios enhance the sensitivity of the determination, as a higher overpressure is generated for a given amount of biogas produced. However, large liquid volumes leave little space to the biogas produced to expand. This requires that the manual release of the gas is sufficiently frequent to ensure that the pressure does not build up to an extent that the increased solubilisation of carbon dioxide may result in a drop in pH which in turn is detrimental to the activity of methanogens.

▪ Frequency of pressure re-equilibration

The frequency of pressure re-equilibration primarily influences the physico-chemical equilibrium of the liquid and the gas phases; and also the long-term resistance of the seal. If the rate of gas production between two subsequent re-equilibrations is too high, the pressure might exceed the resistance of the butyl rubber septum causing it to bulge to an extent that gas may leak through previous punctures. Since the rate of gas production is a function of the inherent activity of the biomass, of the amount of biomass present and of the amount of organic substrate supplemented, these three variables should be carefully chosen in order to ensure a gas production which can be handled.

Clearly this issue is less important when an automated device (e.g. a pressure transducer) is used, as it is possible to set a threshold of pressure that triggers a vent which re-equilibrates the pressure.

▪ Inoculum type and concentration

The volume of sludge used to seed a serum bottle and its inherent specific activity will affect the rate of gas production and in turn the frequency of pressure re-equilibration, as discussed above.

Speece (1996) states that the cardinal rule in conducting a biodegradability assay is that the seed sludge has been acclimated to the test materials. However, in a first attempt of assessing biodegradability, the inoculum will inevitably be unacclimated to that particular test material; thereafter, the same sludge could be re-exposed to the test material to verify if acclimation has occurred or not.

However, in a batch process, the use of a large inoculum or of a highly active inoculum, can prevent the accumulation of volatile fatty acids that would negatively affect the activity of methanogens.

▪ Substrate (or, test material) concentration

Excessively low or high substrate concentrations are equally detrimental to the accuracy of an AAT: in the former case, the activity displayed by the biomass will be low and the inherent inaccuracy of the manual gas volume determination may be of the same order of magnitude of the gas production. The consequences of a high rate of biogas production have been discussed above. In addition, at high substrate concentrations, the availability of nutrients and other 'growth factors' may become limiting. If the concentration of substrate is too high, it may even become inhibitory to the biomass (e.g. resulting in a fast production of volatile acids that inhibits methanogenesis). The **substrate-to-biomass ratio** has proven to be a crucial factor in batch conditions, which influences the kinetics of the process (Moreno et al., 1999).

▪ Number of units

To verify that the outcome of an AAT is reliable, it is recommended that three replicates per unit are prepared. If more accurate insight in the progression of the reaction is desired, a larger number of replicates must be prepared, so that some vials can be sacrificed at given intervals and their content analysed, e.g. to measure the residual COD. It is recommended that all determinations are conducted at least in triplicate, to calculate a meaningful average and standard deviation of the measurements: therefore, three additional replicates should be considered for each unit that is to be sacrificed.

Table B.3 – Tentative guidelines for the set-up of an activity test.

Parameter	Units	Value or interval
Headspace-to-total volume ratio (V_H/V_{TOT})	-	0.2 – 0.6
Substrate (preferably, acetate)	g COD/ℓ	3 – 6
Organic matter (i.e. substrate + test material)	g COD/ℓ	< 30
Solids (i.e. seed inoculum)	g VS/ℓ	10 – 20
Substrate-to-biomass ratio (S/X)	-	0.2 – 0.3
Re-equilibration period	d	1 – 2
Volume of gas per re-equilibration	mℓ	5 – 30

B.2 EXECUTION OF THE ACTIVITY TEST

A step-by-step guide for the execution of an AAT is illustrated hereafter.

Step 1: Prepare the following components:

- Nutrients and minerals stock solution (NMS): refer to Section F.1.
- Healthy, active and homogenised sludge (the seed inoculum). This must be characterised in terms of its solids content (as volatile or volatile suspended solids) and organic content (as COD).
- Test material: this is to be characterised in terms of its pH, alkalinity and organic content (as COD).
- Organic substrate (for toxicity assays, only), either as concentrated stock solution or pure reagents. Acetate, propionate or a mixture thereof are commonly used. The type of test can be slightly modified, i.e. from a strictly batch mode to a semi-continuous mode; and pure acetic acid or ethanol can also be used.

Step 2: Equilibrate all components to the working temperature.

Step 3: Pour the components into the vials.

- Ideally, the components should be anaerobically transferred into the vials. However, it has been demonstrated that by manipulating them with care, i.e. trying to minimise the exposure to air and thoroughly flushing the vials with oxygen free gas for 1 to 2 min prior to sealing, is sufficient to leave the anaerobic biomass almost unaffected.
- If the substrate (for toxicity tests) or the test material are in solid form (e.g. powder), it might be easier to add them first. In all other cases, they should be added immediately before sealing the vials.

Step 4: Flush with an oxygen free inert gas.

- A nitrogen and carbon dioxide mixture would be ideal in that it removes residues of oxygen while establishing a CO_2 partial pressure above the liquid. If such a mixture is not available, nitrogen can be used to over gas the headspace, but must not be passed through the liquid as it would strip carbon dioxide, thus altering the equilibrium of inorganic carbon.
- Over gas for 5 min at low flow rate; then, immediately seal with butyl rubber septa (20 mm x 3 mm, Separations Pty Ltd.) and aluminium crimp (20 mm, Separations Pty Ltd.).

Step 5: Store at the working temperature, in the dark and motionless.

Step 6: Re-equilibrate the headspace, after 1 to 2 h after preparation.

As the pressure in the serum bottles might have changed as a consequence of the temperature change and of gas/liquid equilibria adjustment, it should be zeroed to atmospheric pressure.

- Puncture each septum with a needle and vent the excess gas that might have accumulated.
- Although the value does not have to be recorded as part of the AAT, quantifying the volume of excess gas could provide useful indications of biological activity. Clearly, all units containing the same volume of liquid are expected to exhibit the same volume of excess gas. Differences from this behaviour could be due to:

i) a prompt start-up of the microbial degradation process, resulting in the immediate production of gas: wasting the excess gas in this circumstance would neglect a portion of microbial activity;

ii) an onset of physico-chemical reactions, in the presence of a test compound which contains volatile fractions; or

iii) non-homogeneous conditions across and/or within each unit; this is indicative of an inaccurate set up of the test: the measurement of the excess gas might help in the identification of the faulty units, which should be discarded.

Step 7: Determine the gas composition, after re-equilibration.

- Although one can assume the initial composition of the headspace, based on the composition of the mixture used to sparge the vials, a measured value is certainly preferable. Moreover, knowing the actual gas composition, could help in understanding the circumstances discussed in Step 6.

Step 8: Record all information, including the starting time on a log sheet (an example is provided in Tables B.4 and B.5).

Step 9: Constantly monitor the progress of the test.

- As mentioned earlier, this consists in the periodical determination of both the volume of gas produced and the composition of the headspace.
- The gas produced should always be wasted to prevent an excessive increase of pressure; unless it is too small to be accurately quantified: in this latter case, it can be re-injected into the vials, so that the pressure can gradually build up to a detectable level. A value lower-than-zero should be recorded on the log sheet, so that it can be taken into account during the data analysis stage.
- Due to the temporary increase in pressure between two subsequent determinations, a fraction of the gas produced remains dissolved in the liquid. The release of the excess gas will bring the pressure back to the atmospheric pressure. However, this will happen too rapidly for the gas/liquid equilibrium to re-establish and the excess dissolved fraction will likely remain undetected. Manually shaking the vials will favour gas/liquid re-equilibration. It is suggested that two measurements of the gas volume be performed, initially on the undisturbed vial, then after shaking. In this way the conditions closed to equilibration will be established, as the 'driving force' will be *from* the liquid *to* the gas phase. Shaking the vial before releasing the excess gas would enhance the internal overpressure to an extent that could make it *practically* impossible to accurately measure the volume of biogas produced, as the excess pressure could force the gas to escape past the plunger. Under particular conditions, this excessive overpressure could also cause a surge of foam that would result in sludge entering the needle and possibly obstructing it.
- A syringe of suitable volume should be used to ensure that the measurement is sufficiently accurate: clearly, if the production is large, a small-volume syringe which is potentially very sensitive, would result in either a loss of gas (due to the leakage past the plunger) or in the necessity of performing

numerous repeated insertions of the needle, that would damage the resistance of the butyl rubber. Conversely, a large-volume syringe would not be sufficiently accurate to quantify a small gas production, due to its low sensitivity.

These circumstances can be anticipated, and avoided to some extent, when preparing the serum bottles and the dilutions.

- The syringe should be lubricated with deionised water, prior to the measurement. The reading of the gas volume (or the last one, in case of multiple punctures) should be done by holding the syringe horizontally, in order to minimise the effect of the weight of the plunger, and by gently twirling it, thus allowing a free movement. Readings are verified, by drawing the plunger past the equilibrium point and released, to verify that it returns to the original equilibrium volume.
- Gas composition should be determined immediately after withdrawal from the headspace. The volume should be small enough to leave the headspace pressure unaffected. With a headspace volume of 50 to 100 mℓ, a sample of 40 $\mu\ell$ would alter the pressure by 4 to 8 mbar. For accurate mass balances, this volume should be recorded and accounted for.
- The frequency of the re-equilibration (and determination of gas volume and composition) should be adjusted to the progress of the reaction. Typically, in the early stages of the process, when the gas production is stronger (and the gas composition changes rapidly), frequent determinations would enable the accurate monitoring of the degradation.

Step 10: Stop the test when the gas production has levelled off and the gas composition is constant.

- If long-term information is required (e.g. when the potential adaptability of the micro-organisms to the test compound is investigated), the test can be continued further. Clearly, the frequency of the manual re-equilibration may be reduced, e.g. once per week, until signs of resumed activity are noted.
- If no follow-up is needed and the bottles can be opened, the following determinations should be done, to provide quantitative data for mass balances:
  - pH, alkalinity and volatile acids concentration;
  - COD concentration; and
  - solids concentration.

A conceptually different way of performing a serum bottle AAT consists in batch-feeding the test units with the reference substrate, until a steady-state in methane production is reached (control); thereafter, both the reference substrate and the test material are spiked into the serum bottle (test unit). The drawback of such technique is that the length of the two phases is inevitably limited by volume that can be poured into the vial. And more importantly, it looks as it defeats the object of a serum bottle assay, since a conventional batch-fed reactor would be definitely more suitable for such regime. However, the following discussion on data interpretation is equally applicable to an AAT that had been performed in this way.

Table B.4 – Front page for a log-book, for serum bottle tests.

Type of test:

☐ Biodegradability

☐ Toxicity

☐ Other (please specify) _____

Unit No	**Type** [1]	**Composition**				**Comments** [3]
		Inoculum	NMS	Test C. [2]	Substrate [2]	

[1] Type of unit, e.g. control, test unit *low* concentration, etc.
[2] Test C., test compound. Type and volume (or concentration) should be recorded.
[3] Record any other information that could be of help in interpreting the results.

Table B.5 – Log-sheet for data capturing, for serum bottle tests.

Test ID _____ Date/Time _____ Day No _____

Conditions (i.e. T, P_{atm}, etc.) _____

Unit No	**Volume** (mℓ)	**Gas composition** (peaks area)				**Comments**
		N_2	CH_4	CO_2	Total	

B.3 DATA INTERPRETATION

The objective of a biodegradability test is to answer the question (see: Section 2.3):

... to what extent is the test material biodegradable ?

This translates into quantifying the portion of the organic matter contained in the test material that undergoes microbial degradation, i.e. that is converted into methane.

This is done by comparing the initial COD added in the serum bottle (COD_0) to the amount of methane produced in the course of the test ($V_{CH,\infty}$), as:

$$B \approx \frac{V_{CH,\infty}}{COD_0} \qquad (B.1)$$

where B denotes the extent of biodegradation.

Suitable units must be used, by converting COD into methane (or vice versa), using the equivalence factor (at standard temperature and pressure, i.e. 273 K and 1 bar):

$$1 \text{ g COD} = 0.350 \ \ell \ CH_4 \qquad (B.2)$$

Note that the extent of biodegradation B can be affected by the concentration of the test compound (or, by the COD-to-biomass ratio): typically, at relatively high substrate concentrations, the activity of the micro-organisms might be inhibited due to excess substrate, whereas at low concentrations, the substrate might be not sufficient to promote the maximum activity.

For this reason (and the lack of an authoritative reference on this matter; Müller and Frommert 2004), it is recommended that more than one level of concentration be assessed.

The objective of a toxicity test is to answer the question (see: Section 2.3):

... how does the test material affect the methanogenic activity of the biomass ?

This translates into comparing the methanogenic activity of the sludge exposed to the test material to the activity in the absence of the test compound.

The reason why acetate or other *direct* precursors of methane, e.g. propionate, are preferred to e.g. glucose as substrates is that the drop in pH which follows the initial break down of the substrate could result in a transient reduction in methanogenic activity, that would hide the actual effect of the test material, i.e. it would be interpreted as inhibitory effect, although the test material was not inhibitory or would worsen the true inhibitory effect of the test material and even make it irreversible, although it was in fact only temporary.

The effect of the test material (E) is quantified as:

$$E \approx \frac{A_{t.m.}}{A_{ref}} \qquad (B.3)$$

where $A_{t.m.}$ is the methanogenic activity in presence of the test material; and A_{ref} is the methanogenic activity on the reference substrate.

Clearly, E is a function of the concentration of the test material, so that generally the effect of a compound ranges from *no-effect*, at low concentrations, to *full inhibition*, at high concentrations.
This effect-to-concentration relationship is formally described by an inhibition curve. For technical applications, the quantity EC_{50}, defined as the concentration of the test material which results in a 50 % reduction in the methanogenic activity as compared to the reference activity, is of more value than the entire curve.

B.3.1 Interpreting biodegradability test

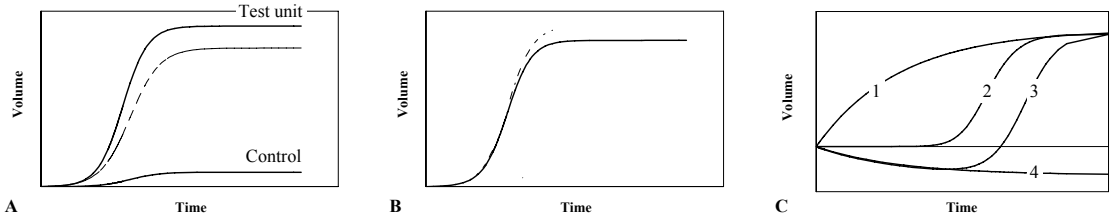


*Figure B.2 – Anaerobic activity test for the assessment of biodegradability. (**A**) Average cumulative volume of biogas, in the test and control units and in the reference units (dashed line). (**B**) Net volume of methane (solid line), in the test units. (**C**) Possible patterns of net methane production (adapted from: Battersby and Wilson, 1988).*

A guide for interpreting the outcome of an AAT aimed at assessing the biodegradability of the test material is illustrated hereafter.

i) Plot the cumulative gas volume for the control and for the test units (Figure B.2, A).

ii) Calculate the range of uncertainty of the curves by calculating the average and standard deviations of each data point. Ideally, the variation coefficient should not exceed 5 to 10 %. If one of the replicates is clearly out of range, it must be disregarded in the further calculations.

iii) Calculate the volume of methane, through a step-by-step mass balance, as follows:

$$\Delta v_{CH,t} = f_{CH,t} \cdot (\Delta v_t + V_{t-\Delta t}) - f_{CH,t-\Delta t} \cdot V_{t-\Delta t} \qquad (B.4)$$

where f_{CH} indicates the molar fraction of methane (at time t and $t-\Delta t$ respectively); $V_{t-\Delta t}$ is the headspace volume at time $t-\Delta t$ (for simplicity, it can be considered constant throughout the test, unless a fed –batch regime is adopted); and Δv is the volume of gas produced in the interval $(t-\Delta t, t)$.

iv) Calculate the *net* volume of methane produced in the test unit, i.e. the methane generated by the conversion of the biodegradable fraction of the test material, by subtracting the volume of methane measured in the control units to the one measured in the test units (Figure B.2, B). Ideally, the gas production in the control should be small compared to the one of the test material to make the determination reliable.

Four cases are possible, which are schematically represented in Figure B.2, C:

- the test material is readily biodegradable (**1**);
- the test material is biodegradable after a lag-phase (**2**);
- the test material is inhibitory in the initial phase of incubation (**3**); or
- the test material is inhibitory throughout the entire period of incubation (**4**).

v) Calculate the ultimate biodegradation (or biodegradability) of the test material, as:

$$B = \frac{V_{CH,\infty}}{V_{CH,theory}} \qquad (B.5)$$

where $V_{CH,theory} = f_T \cdot 0.350 \cdot COD_0$; and f_T is the temperature correction factor, from STP to the working conditions.

vi) To complement the interpretation of the results, one can calculate and plot the Specific Methanogenic Activity (SMA) in g COD/g VS/d (or: g COD/g VSS/d)

$$SMA = \frac{R_{CH}}{X_0} \qquad (B.6)$$

where R_{CH} is the *net* rate of methane production; and X_0 is the biomass initially present in the vial. R_{CH} is calculated as the first derivative of the net volume of methane (Step 4)

The growth rate of the anaerobic micro-organisms is low, so that in many applications it can be neglected. However, over long periods, such those of a biodegradability assay, it can actually result in a measurable increase in biomass concentration. Therefore, the use of the *initial* biomass concentration in equation B.6 would overestimate the SMA of the later stages of the process. If the solids concentration is measured at the end of the test, one can assume a linear increase between the initial and the final values, to obtain a more accurate estimate through equation B.6.

B.3.2 Interpreting toxicity test

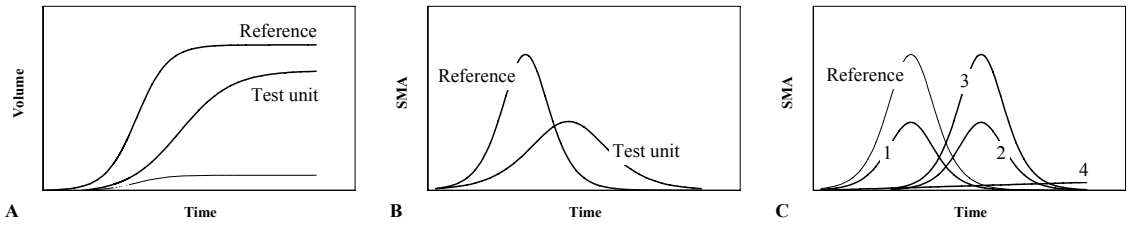


*Figure B.3 – Anaerobic activity test for the assessment of toxicity. (**A**) Average cumulative volume of biogas, in the reference and test units and in the control units (dashed lines). (**B**) Specific Methanogenic Activity, in the reference and in the test units. (**C**) Possible patterns of activity curves.*

A guide for interpreting the outcome of an AAT aimed at assessing the toxicity of the test material is illustrated hereafter.

i) Plot the cumulative gas volume for the control and for the test units (Figure B.3, A).

ii) Calculate the range of uncertainty of the above curves by calculating the average and standard deviations of each data point. Ideally, the variation coefficient should never exceed 5 to 10 %. If one of the replicated is clearly out of range, it must be disregarded in the calculations.

iii) Calculate the volume of methane, according to equation B.4.

iv) Calculate the Sludge Methanogenic Activity, according to equation B.6.

Note that the SMA calculated for a toxicity assay is the rate of methane production in the test units and is conceptually different from the one calculated for a biodegradability assay, which was based on the *net* methane production. The reason being that in a toxicity assay we are interested in the microbial activity *in presence of* the test material, whereas in a biodegradability assay we want to *isolate* the effect of the test material from a baseline.

Four cases of inhibition are possible, which are schematically represented in Figure B.3, C:

- the test material is *partially* inhibitory; however, it does not delay the uptake of the reference substrate (**1**);
- the test material is *partially* inhibitory and requires a period of adaptation before the micro-organisms are able to display a detectable methanogenic activity (**2**);
- the test material is *temporarily* inhibitory: after a lag-phase, the micro-organisms display a methanogenic activity similar to that on the reference substrate (**3**); or
- the test material is *permanently* inhibitory: the biomass is not able to adapt to the presence of the test material (**4**).

v) Calculate the inhibition effect as:

$$E = \frac{SMA_{t.m.}}{SMA_{ref}} \qquad (B.7)$$

where $SMA_{t.m.}$ is the activity in the test unit; and SMA_{ref} is the baseline activity, in the control unit.

As mentioned above, this 'effect' is concentration-dependent and can be conveniently described through an inhibition curve which plots the relationship between concentration of the test material and inhibition. This latter is defined as:

$$I = 1 - E \qquad (B.8)$$

An example of inhibition curve is presented in Figure B.4: the value EC_{50} is found on the horizontal axis in correspondence to $I = 0.5$.

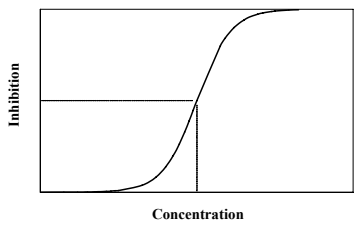


Figure B.4 – Example of inhibition curve.

B.3.3 How does the frequency of re-equilibration affect the calculation of activity ?

The specific methanogenic activity for a serum bottle activity test is calculated as the rate of methane production per unit of biomass (equation B.6):

The accuracy of this calculation is inevitably dependent on the frequency of pressure re-equilibration. Figure B.5 compares the Specific Methanogenic Activity that one can estimate using a *high* (**A**) or a *low* (**B**) frequency of re-equilibration. Ideally, the operator should tune the frequency according to the actual gas production rate, i.e. the differential gas production, rather than maintaining a constant predetermined frequency. In fact, the period of interest for the determination of methanogenic activity (hence inhibition) is the from the beginning of the test until the activity reaches its maximum and a little beyond that point; whereas for the estimation of biodegradability, one is mostly interested in the asymptote, therefore the frequency of re-equilibration after the activity has peaked may be reduced.

The manual re-equilibration is a discontinuous process and so will result the curve of the calculated SMA. This makes virtually (and statistically) impossible to precisely assess the *maximum* specific methanogenic activity, which ultimately is the parameter that is taken as representative of the performance of the seed inoculum under the given operating conditions. For instance, when the toxicity effect of a test material at a given concentration is to be assessed, the *maximum* SMA, calculated when the sludge is exposed to the substance, is compared to the *maximum* SMA, when the sludge is only exposed to the reference substrate.

We propose a way of presenting the estimates of *maximum* SMA which consists in calculating the average and standard deviation of the experimental maximum and the two immediately adjacent values (Figure B.5 C). Clearly, this standard deviation provides a purely qualitative indication of how accurate is the average value calculated.

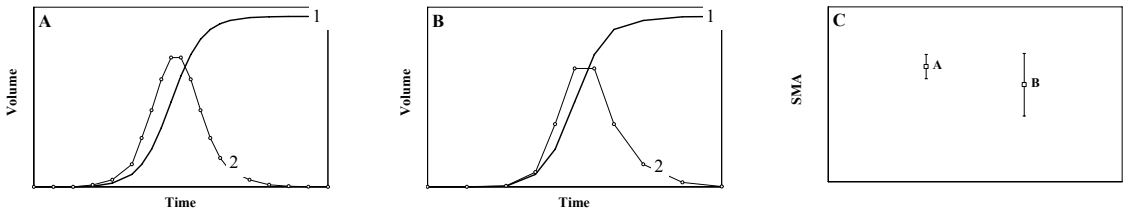


*Figure B.5 – Effect of the frequency of re-equilibration on the accuracy of the calculation of the SMA: high (**A**) vs. low (**B**) frequency; comparison of the two estimates of the maximum SMA (**C**).*

A high standard deviation should be taken as a warning that either of the following has occurred:

- the experimental determination of the methanogenic activity, in correspondence to the actual maximum, was not sufficiently accurate, i.e. the manual determination of the gas volume and/or the analytical determination of the gas composition had been inaccurate this leading to an outlier value of SMA;

- the frequency of re-equilibration, in the vicinity of the actual maximum was too low, thus leading to an apparent steep variation of the SMA.

This approach has been used throughout this report, to present data of SMA (see for instance, Figure 22).

The advantage of this approach is two-fold:

- it provides an experimental ground to the evaluation of the inherent accuracy of a toxicity assessment; and
- it enables to visually compare the activity calculated for different conditions, e.g. at different concentrations of the test material, irrespective to the inherent accuracy of the determination.

CONCLUSIONS

A serum bottle AAT is a convenient, relatively inexpensive and very accurate batch test which enables to assess *i*) the potential negative effect of the test material on an anaerobic sludge as well as *ii*) its biodegradability under anaerobic conditions. This method only requires the manual determination of gas volumes and the analysis of the gas composition, as well as the quantification of the organic matter (as COD) and of the solids content (as VS or VSS).

The detailed protocol for conducting the test and for interpreting the data has been validated in the numerous tests conducted for this research.

The information obtained through an AAT should ideally form the ground for the next experimental stage.

ANNEXURE C

The conduction of anaerobic activity tests using a pH-stat titration method

In Sections 2.5 and 3.1.2 the principle of pH-stat titration has been discussed. In Section 4.3, a mathematical model for the application to the monitoring of the anaerobic digestion of an organic substrate has been presented.

Briefly, the concepts to bear in mind are:

- a **titrimetric method** relates the microbial activity to the rate titrant addition;
- a **pH-stat method** maintains the pH of the solution constant, within a limited range;
- a combined **pH-stat** and **titrimetric method** detects the change in concentration of protons [H^+] and compensates this change by adding an equivalent amount of the appropriate titrating solution;
- incidentally, the titrating solution might not only provide the protons to maintain the pH value constant, but also the substrate which is consumed by the micro-organisms. This additional feature of a pH-stat titrimetric method has been termed RePro-stat (whereby the concentration of a *Re*agent or a *Pro*duct is maintained constant) and offers some very interesting prospects for the development of these instruments (Rozzi and Ficara, 2001).

The above considerations show the obvious limits to the applicability of such method:

i) the targeted process must result in the production or consumption of protons (or equivalent species);

ii) the targeted process must be the only one (or the prevalent one) which acts on the pH of the solution.

To limit the discussion to biological processes, there are several processes which comply with condition (*i*), as illustrated in section 2.5. Condition (*ii*) requires that the contribution to the pH change of all other processes can be quantified separately or is null. The case of monitoring methanogenic activity (i.e. a one-side control) falls into case (*ii*), as the interference generated by the chemistry of inorganic carbon can be effectively controlled by carefully setting the initial conditions of the activity test. In fact, this application has proven very accurate in measuring aceticlastic activity and inhibition (Rozzi et al, 2001; 2002).

The application to the monitoring of the anaerobic degradation of an organic material (i.e. a two-side control) is clearly more complicated and falls into the first case, i.e. unless we are able to separately assess the contribution of the acidification and methanisation phases or to suppress or control one at a time, the pH-stat titration will inevitably provide inaccurate information. This application is currently under investigation and the operating details have not been finalised yet.

The determination of methanogenic activity and inhibition is suitable for the option of maintaining the substrate concentration constant. As illustrated in Section 3.1.2, two species of acids can be used, i.e. acetic and hydrochloric acids, according to the following reactions:

Microbial uptake of acetate:	$CH_3COO^- + H_2O$	$\rightarrow CH_4 + HCO_3^-$	(C.1)
Titration with acetic acid:	$CH_3COOH + HCO_3^-$	$\rightarrow CH_3COO^- + CO_2 + H_2O$	(C.2)
Titration with hydrochloric acid:	$HCl + HCO_3^-$	$\rightarrow Cl^- + CO_2 + H_2O$	(C.3)

The experimental procedure to conduct a methanogenic activity test (termed MAIA, Methanogenic Activity and Inhibition Analyser) has been optimised in the course of this project, to be integrated in the procedure for screening the suitability of an effluent to be co-digested (Annexure A).
This procedure is illustrated hereafter.

C.1 PREPARATION OF THE COMPONENTS

- As common practice, the **sludge** can be stored for relatively long periods of time at temperature below 4 °C. During storage, it is not supplemented with substrate and the activity does not deteriorate significantly.

  However, in order to shorten the lag-phase which would be displayed by a sludge that is recuperating its activity after a period of starvation, it is convenient to maintain an anaerobic culture (in a laboratory-scale digester) which can be sampled to provide a well-active seed sludge for the activity test. Should this not be possible, the sludge must be *at least* conditioned to the operating temperature overnight. This not only allows the gradual resumption of activity; but more importantly, avoids that changes in temperature affect the physico-chemical equilibria particularly that of bicarbonate/carbon dioxide: this would generate an initial transient that would hinder an accurate determination of activity through titration (Figure C.1 A).

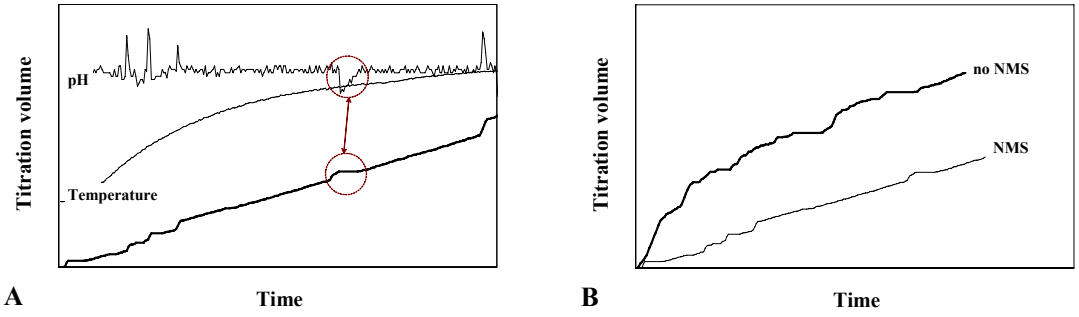


*Figure C.1 – Effects of temperature variations (**A**) and of the use of a NMS (**B**) on the titration curve. The step-increases in the volume titrated are reflected in the local drops in the pH of the suspension (circles) and are an independent phenomenon: they are caused by non-homogenous mixing conditions (Rozzi et al., 2001).*

In any circumstance, the initial period of the test (until a clear steady-operation is achieved, e.g. the titration process is regular) should be closely monitored by the operator, in that the determination of other parameters besides pH could be of help in obtaining a clear understanding of the start-up phase.

Particularly, a frequent determination of the gas composition would allow to detect changes which can severely affect the titration rate.

- The use of a **nutrient and minerals solution** has proven to have a positive effect on the titration process, in that it seems to reduce the background fluctuations (Figure C.1 B). More importantly, it provides the necessary supplements to the seed inoculum and a buffering capacity to the system. As most of these factors are generally present in a well-active sludge, the addition of a NMS might not be necessary. However, the following steps should be undertaken.

  - Measure the solids concentration.

  Our experience indicates that a concentration of approximately 10 g VS/ℓ is ideal to conduct the activity test. However, the "strength of the signal" is clearly a combination of the solids concentration and the inherent activity of the seed inoculum: therefore, this value should be regarded as indicative and should be changed according to the characteristics of the sludge utilised.

  Should it be necessary to dilute the seed inoculum, this should be done using the NMS: deionised water must be avoided as it alters the osmotic pressure in the solution and might severely affect the micro-organisms.

  Should it be necessary to concentrate the inoculum, the sludge sample can be centrifuged and resuspended in the NMS.

  The contribution to alkalinity of the NMS must be taken into account, prior to correcting alkalinity (as explained later in this Section).

  - Prepare the titrating solutions, using either acetic or hydrochloric acid.

  The strength of the solution is immediately related to the sensitivity of the method (Rozzi et al., 2002).

  Based on our experience, a concentration of 0.5 mol/ℓ is the ideal compromise between dilution effect and sensitivity.

C.2 INSTRUMENT PREPARATION

A few advances in the manufacturing of the instrument have been made since the early set-up. This applies both to the hardware and the software.

It is beyond the scope of this report to discuss the details of such modifications, but since future users may operate on instruments which do not resemble the one described hereafter, it is necessary to mention the main changes:

i) the use of a printed board instead of the Input/Output modules;

ii) the DOS-based interface has been substituted with a Windows-based interface;

iii) the gravity delivery of the titrant through a Mariotte bottle has been substituted by a mechanic system, consisting of a peristaltic pump; and

iv) the ability of controlling two independent reaction vessels, on two process parameters simultaneously, i.e. pH and dissolved oxygen (however the DO probe could quite simply be substituted by a redox potential electrode, for instance).

These changes have resulted in user-friendlier control panels, on which the user clicks on buttons rather than typing commands on a blank screen. In particular, the change in the titration system is a great improvement as the calibration and the maintenance of the system has become a lot easier. Particularly, the Mariotte bottle system requires that air bubbles present in the line are carefully removed prior to calibrating (this can make the procedure very time-consuming); also, air can relatively easily seep through, accumulate in the line during the test and make the delivery of the titrant very inaccurate. In this respect, the peristaltic pump ensures more stable performances throughout the test and a quicker calibration. The only drawback is that the tube can deteriorate due to the continuous rotation of the pump.

The following discussion applies to the original DOS-based version, equipped with Mariotte bottles for the delivery of titrants; but the translation to the new instrument should not be difficult.

Step 1: Calibrate the temperature sensors.

The pH-stat titrator is equipped with two sensors (PT100), to detect the temperature inside the reaction vessel and that of the titrant solution respectively.

The temperature of the reaction vessel affects the pH value generated by the signal of the pH electrode: a temperature dependency for the pH is implemented in the source code.

Since a constant head above the electro-valve is ensured by the use of the Mariotte bottle, the temperature of the titrating solution is the only variable which affects the flow rate, by altering the viscosity of the liquid. The flow rate is internally calculated from the number of openings of the electro-valve. It is therefore imperative that the temperature sensors are calibrated before the pH electrode and the electro-valve. The calibration of the temperature sensors though can be performed periodically, as the sensors are more robust and the signal more stable.

Proceed as follows:

- Immerse the first electrode in a container with cold water, e.g. water and ice blocks, and wait until the signal displayed (in mill volts) is stable. Enter the temperature of the bath (a laboratory thermometer can be used) and confirm.
- Repeat the same operation with the hot bath: it is recommended to use temperature of 60 °C or above, to ensure a better accuracy of the calibration process.
- Repeat both steps, for the second sensor.

Note that even if the activity test was carried out in a temperature controlled room, where both the reaction vessel and the titrant reservoir were exposed to the same temperature, both sensors are to be calibrated and connected to the Input/Output module.

Step 2: Calibrate the pH electrode.

It is recommended to perform this step at the beginning of each activity test, as the nature of the anaerobic sludge and the long duration of a test expose the electrode to a relatively fast deterioration of the response. A good practice is to log the results of the calibration in order to be able to promptly identify when it is time to *i*) regenerate the electrolyte or *ii*) substitute the electrode.

Proceed as follows:

- Immerse the electrode in the first pH buffer (e.g. 4) and wait until the signal (in mill volts) is sufficiently stable; then confirm and enter the corresponding pH value, i.e. 4.00.
- Repeat the operation for the second pH buffer (e.g. 10).

It is clearly preferable to use pH 4 and 10 as buffers being the test conducted at a set-point pH of around 7, which falls well between the two limits.

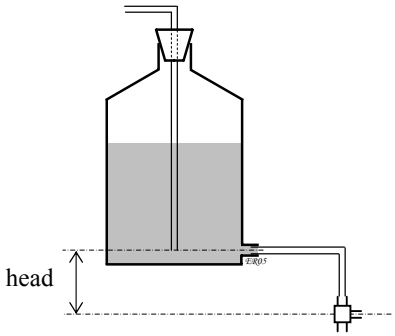


Figure C.2 – Set-up of a Mariotte flask to maintain a constant head above a solenoid electro valve.

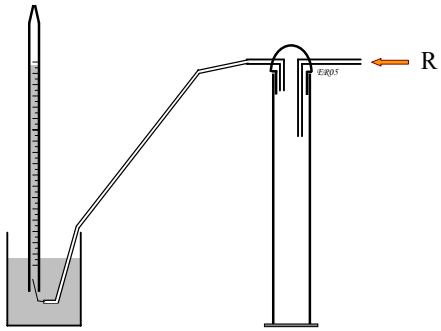


Figure C.3 – Volumetric set-up consisting of a reversed burette, for measuring the biogas production.

Step 3: Set up the electro valve.

This step must also be performed at the beginning of each test and is aimed at removing air bubbles from the line connecting the Mariotte bottle to the electro valve; at correctly setting the head above the electro valve (Figure C.2); and at adjusting the settings of the electro valve to obtain the desired flow rate.

Based on our experience, a flow rate of 10-50 μℓ/pulse ensures a good sensitivity of the control system.

Proceed as follows:

- Set up the opening and closing times of the electro valve. These parameters primarily influence the flow rate of titrant and the sensitivity of the response of the controller, respectively. They are not totally independent however, because the longer the opening time, the smaller is the maximum number of openings in the unit time, hence lower is the overall sensitivity. On the other hand, the shorter the closing time, the higher the sensitivity but also greater the fatigue of the membrane inside the valve, which will overheat and possibly lead to the break down of the electro valve itself.

The default values of 0.2 and 0.5 seconds respectively are generally good.

- Set up the data logging interval.

This step does not strictly relate to set-up of the electro valve, but as mentioned above it indirectly affects the sensitivity of the determination by setting an upper limit to the number of openings of the valve.

The data logging interval does not indicate the frequency of acquisition of the various signals (i.e. pH, temperature, etc.) which are continuously received by the I/O modules, but the frequency on which the signals are averaged and the average value logged as output datum. Clearly, the higher the frequency, the more sensitive is the control action, but the larger is the amount of data hence the size of the output file.

A frequency of 30 seconds is generally a good compromise for long-term tests such as the anaerobic activity test.

- Ensure that the titrant line is bubble-free; set the head above the electro valve and the height of the electro valve above the titrant outlet. This set up must not be changed after the calibration procedure has been completed, otherwise the flow rate will change and the calibration value will be incorrect.

- Place a cylinder underneath the titrant outlet and start the calibration. At any point, stop the valve, enter the exact volume cumulated to the point and confirm. Repeat this step not less than three times (do not exceed the 10 mℓ cumulative volume).

- It is advisable to log the data pairs on a separate spreadsheet and to recalculate the flow rate, using a linear regression. In this way, the titration volumes logged by the software can be validated at the end of the test. Moreover, one can keep a history of the performance of the electro valve (similarly to what suggested for the pH electrode) and diagnose when the electro valve becomes unreliable.

Step 4: Prepare the reaction flask.

Proceed as follows:

- Pour the seed inoculum and the NMS (if needed); insert the temperature sensors and the pH electrode in the respective ports, and seal; insert the top cap with the titrant and substrate addition ports.

- Connect the liquid sample line to the gas bottle with the nitrogen/carbon dioxide mixture.

- Set the biogas outlet in a way that gas can be vented while air is prevented from contaminating the headspace in the reaction vessel (water trap). It is sufficient to let the plastic tube bubble into a beaker with water.

Step 5: Select the appropriate set-point pH.

A crucial aspect to take into account is the sensitivity of the control system: clearly, this depends on the intensity of the *signal* detected, i.e. on the variation in the pH value of the suspension which results from a *unit* change in the concentration of reactants (or products). This in turn depends on the

buffering capacity of the suspension, which can be interpreted as its *ability to resist* to changes in pH. Therefore, the lower the buffering capacity, the higher will be the sensitivity of the titration process hence the accuracy of the activity determination (Figure C.4).

Now, the selection of the set-point value of pH must be a compromise between maintaining a sufficiently low buffering capacity in the system (i.e. a high sensitivity) and ensuring optimal conditions for the methanogens to operate. This latter objective requires the pH to be in the range 6.5 to 7.5. Under such conditions, the buffering capacity of a solution depends almost exclusively on the concentration of bicarbonate ions: the association/dissociation equilibrium of carbon dioxide shows that the condition of constant pH (pH-stat) is a straight line (equation 7.a; Figure 6).

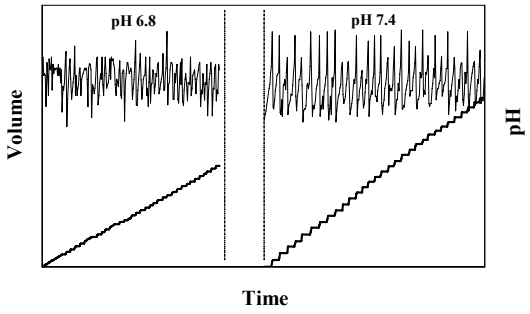


Figure C.4 – Comparison of the sensitivity of the titration unit at different set-point pH.

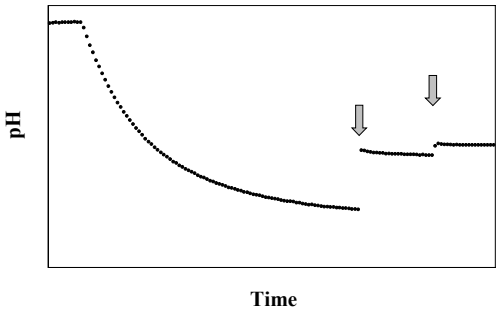


Figure C.5 – pH adjustment prior to starting a pH-stat activity test, by flushing a nitrogen/carbon dioxide mixture and manually correcting alkalinity (arrows).

Step 6: Adjust the pH value and the carbon dioxide molar fraction.

Proceed as follows:

- Start the program which detects the pH but disables the control system: time and pH data will be displayed on the screen, at the preset data logging frequency.
- Let the program run for a couple of minutes, until the reading is stable, then start flushing with the 1:1 N_2:CO_2 gas mixture (Figure C.5).
- Open the bottle and regulate to a low gas flow. The efficiency of gas transfer through the liquid and into the headspace is inevitably limited, because the line for sampling the liquor is used which necessarily has an outlet sufficiently large to ensure a homogeneous sludge sample: a needle would make the gas bubbles much smaller, hence increase the transfer efficiency but would also be rapidly clogged with biomass, when sampling. A gas recycle would improve the transfer efficiency but can be complicated to make.
- As the carbon dioxide diffuses into the liquid and in the headspace, the pH should progressively decrease (Figure C.5) until a second equilibrium point is reached, which corresponds to the conditions of alkalinity of the sludge and to a CO_2 partial pressure of 0.5 bar. If the alkalinity was

accurately measured and corrected (Section C.1) and the temperature constant, this point should be sufficiently close to the desired set-point.

- If the desired set-point is not reached by simply flushing with gas, one can manually correct the pH by dosing concentrated acid or alkaline solutions, through the needle (injection port) on the top rubber cap.

- Once the set-point value is reached (and maintained), turn off the gas, close the liquid sampling line and quit the pH monitoring mode.

Step 7: Set up the gas measuring device.

This step is not actually part of the pH-stat titrator, as the gas production is not logged by the software. However, it has been demonstrated that the information on gas production (thus on COD degraded) can be an ideal complement to the information on the titration rate, to calculate mass balances.

Proceed as follows:

- Connect the gas outlet to the volumetric measuring device. Simple devices can be a Mariotte bottle or a reversed burette (Figure C.3), filled with concentrated alkaline solution, e.g. sodium hydroxide 1 N.

An alkaline solution is preferable to an acidic one, as it absorbs the carbon dioxide fraction of the biogas and the volume displaced is immediately equivalent to the volume of methane produced, which is the ultimate sink of the degraded COD.

- It is very important to hydraulically disconnect the measuring unit from the reaction vessel, to prevent accidental transfer of sodium hydroxide into the reaction vessel. This can be done as in Figure C.3.

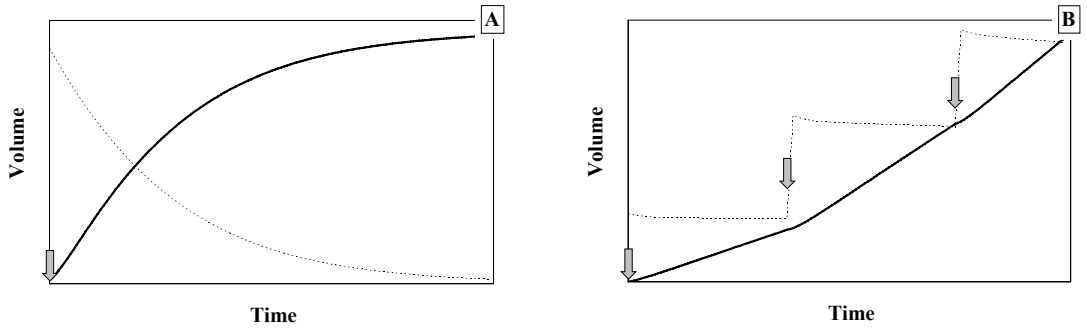


*Figure C.6 – Simulated titration curves: arrows indicate the addition of acetate. Plot **A** refers to the use of hydrochloric acid as titrant and a single dose of substrate. Plot **B** refers to the use of acetic acid and sequential doses of substrate.*

Step 8: Start the activity test.

Proceed as follows:

- Start up the control software and monitor the process for a period of time (e.g. 30 min). If the chemistry of the system has been properly adjusted in the preparatory phase, no titration or gas

production should occur. The addition of the titrant indicates that either the system was not at equilibrium (Rozzi et al., 2001) or that the sludge is endogenously active. The first situation should last few minutes, until the titration system has compensated for the unbalance. The second situation will indicate a baseline activity that is to be quantified at the end of the test and will lower the titration efficiency of the response to some extent.

- Thereafter, add the substrate i.e. sodium acetate (or acetic acid), by spiking it through the top port.

Step 9: Follow-up

- For a pure activity test, aimed at measuring SMA at a non-limiting concentration of acetate, the use of hydrochloric acid is preferable. This mode (termed decreasing substrate concentration) allows replicate injections of acetate to be done and therefore a more reliable estimate to be obtained. A typical titration curve is simulated in Figure C.6 A, for a single addition of substrate.
- For an activity test aimed at assessing the kinetics of the process, either acetic or hydrochloric acid can be used. In the first mode (termed, at constant substrate concentration), multiple spikes of acetate at progressively increasing concentration are to be done and activity monitored for 2 to 3 h after each injection (Figure C.6 B).
- For a toxicity test, acetic acid must be used as titrant to maintain a relatively constant acetate concentration throughout the test: this ensures that the different segments of the test only by the concentration of the test material, the other *relevant* conditions (i.e. pH, substrate concentration and carbon dioxide molar fraction in the gas phase) remain constant.

Step 10: The conduction of the test.

- The key parameter of the test, i.e. the activity of the biomass, is estimated as the titration rate, i.e. the slope of the titration curve. To ensure an accurate estimation of the slope, it is recommended that there is a gap of 2 to 3 h between subsequent additions of substrate or of the test material be maintained. An extensive discussion of the aspects related to the execution of a test is reported in Rozzi et al (2001).
- Regularly monitor the gas production.
- In order to have an accurate and comprehensive overview of the situation, it is recommended to regularly download the data into a spreadsheet and to process the results off-line. To do so, stop the control software for a very short time, copy the log file (*.txt) on a disk and import the data on a spreadsheet. Immediately restart the control software.

C.3 DATA INTERPRETATION

The interpretation of a pH-stat titrimetric activity test is very simple.

The objective parameter to estimate is the *specific methanogenic activity* of the sludge, which is defined as the rate of acetate uptake per unit of biomass. Since one mole of acetate yields one mole of

bicarbonate (equation C.1) and the titrant solution (either acetic of hydrochloric acid) is a monoprotic acid, a 1:1 relationship exists between the moles of titrant dosed and the moles of acetate consumed:

rate of acetate uptake (mol/d) = titrant concentration (mol/ℓ) · rate of titration (ℓ/d)

COD units are generally preferred to moles, to indicate the concentration of organic substrates, therefore:

rate of acetate uptake (g COD/d) = **α** (g COD/mol) · rate of acetate uptake (mol/d)

where **α** = 64 g COD/mol is the COD equivalent of one mole of acetate.

If one assumes that no biomass growth has occurred in the vessel during the test, the activity A can be calculated as:

$$A = \alpha \cdot \frac{C \cdot Q}{X} \text{ (g COD/g VS/d)} \qquad \text{(C.4)}$$

where X is the amount biomass in the seed inoculum (g VS); C and Q are the concentration and flow rate of the titrant respectively.

Although the assumption of absence of growth is reasonable on a short test, it can be verified by plotting the titration flow rate on a semi logarithmic plot (Figure C.7), according to the following rationale.

The dynamics of the biomass concentration is generally described by a first order equation:

$$\frac{dX}{dt} = (\mu - b) \cdot X \qquad \text{(C.5)}$$

where μ (d^{-1}) and b (d^{-1}) are the growth and the decay rates respectively and X the biomass concentration (g VS/ℓ).

The dynamics of the substrate is proportional to the net growth of the biomass:

$$-\frac{dS}{dt} = \frac{1}{Y} \cdot \frac{dX}{dt} \qquad \text{(C.6)}$$

where Y is the biomass yield (g VS/g COD) and S the substrate concentration (g COD/ℓ).

By integrating equation C.5 and substituting into equation C.6, neglecting the biomass decay, one obtains:

$$-\frac{dS}{dt} = \frac{1}{Y} \cdot \mu \cdot X = \frac{1}{Y} \cdot \mu \cdot X_0 \cdot e^{\mu \cdot t} \qquad \text{(C.7)}$$

where X_0 is the initial concentration of biomass.

This can be rewritten as:

$$-\frac{dS}{dt} = \left(\frac{dS}{dt}\right)_0 \cdot e^{\mu \cdot t} \qquad \text{(C.8)}$$

where $\left(\frac{dS}{dt}\right)_0$ is the initial rate of substrate degradation.

As discussed above, the rate of substrate degradation is proportional to the rate of titration; therefore equation C.8 can be rewritten as:

$$\ln Q = \ln Q_o + \mu \cdot t \qquad (C.9)$$

The latter can be plotted as a straight line on a semi logarithmic plot, in which the growth rate is estimated as the slope.

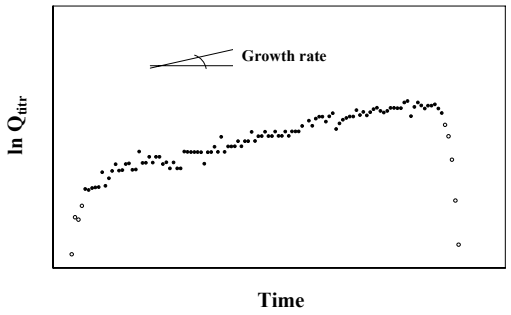


Figure C.7 – The titration data plotted on a semi-logarithmic scale illustrate the growth of the biomass.

CONCLUSIONS

A pH-stat titrimetric sensor can be applied to monitoring the methanogenic step of the anaerobic digestion process, as the degradation of acetate to methane influences the pH of the solution by generating alkalinity.

Two operational modes can be chosen, i.e. a simple pH-stat mode, whereby an inorganic acid is used as titrant to compensate the increase in pH; and a combined pH- and substrate-stat mode, in which acetic acid simultaneously compensates the change in pH and the acetate consumption by the micro-organisms. The latter mode is particularly suited for conducting toxicity tests in which the concentration of the base substrate is maintained nearly constant throughout the test and the test material is spiked at intervals. This application can be proposed as being as reliable as the established serum bottle method, offering the advantages of:

i) a shorter duration of the test: the activity can must be monitored for only few hours after each spike; and

ii) potentially isolating the true effect of the test material on the methanogenic activity from other effects that could be due to a decrease in pH or substrate concentration.

However, the quality of the outcome is highly dependent on the correct start-up of the test: great care must be taken in ensuring that all other pH-affecting processes are neutralised, with particular regard to the bicarbonate/carbon dioxide system that must be equilibrated prior to starting the test, using a 1:1 N_2:CO_2 gas mixture.

The application of a two-unit pH-stat titration sensor, which could monitor simultaneously the acidogenic and methanogenic stages, is currently being studied. This new set-up will enable AAT

equivalent to serum bottle assays, to be undertaken with the advantages of a shorter duration and automated data acquisition.

Table C.1 – Summary of the recommended parameters to conduct a pH-stat titrimetric activity test.

Parameter	**Units**	**Interval or value**
Biomass concentration	g VS/ℓ	10
Alkalinity	mmol/ℓ	29-46 [(a)]
CO_2 partial pressure	bar	0.5 [(a)]
Set-point pH	-	6.7-6.9 [(a)]
Concentration of the titrant	mol/ℓ	0.5
Temperature	°C	30-35
Flow rate of titrant	µℓ/opening	10-50

[(a)] The partial pressure of carbon dioxide is defined by the particular application, as aceticlastic methanogenesis generates an equimolar gas mixture; the set-point value of pH can be selected in the range indicated; the resulting value of alkalinity which ensures that the physico-chemical system is at equilibrium, hence does not interfere with the titration process, is calculated using equation 7.a (or graphically, on Figure 6). The intervals reported in the Table refer to a temperature of 35 °C.

ANNEXURE D

Quick reference guide for the operation of a laboratory-scale digester

The objective of this Annexure is to summarise the experience that the Research Team has accumulated in the operation of laboratory-scale and, to a minor extent, of pilot-scale completely stirred digesters.

This Annexure is structured as a list of topics that can be of help for the researcher in effectively monitoring an anaerobic digester and anticipating potential causes of failure.

This Section is subdivided in the following main subjects:

i) how to **operate** an anaerobic digester;

ii) how to **monitor** the performance of an anaerobic digester; and

iii) how to **assess** the performance of an anaerobic digester.

D.1 OPERATION OF AN ANAEROBIC (CSTR) DIGESTER

The set-up of the laboratory-scale digester that was used for the final experiment is discussed in section 3.1.3 and can be taken as example for a model laboratory-scale reactor.

The essential components are:

- the reaction vessel,
- the stirring device,
- the temperature controlling system,
- the inlet and outlet ports, and
- the gas measuring system.

The size and shape of the reaction vessel have an influence on other characteristics of the set-up and to some extent on the performance of the process itself. However, for general purposes, and certainly in the early stages of an investigation, a conventional cylindrical or conical flask will be adequate.

The use of a magnetic stirring device is obviously limited to small reaction vessel, where the turbulence generated by the rotation of the bar can be effectively transferred to the entire liquid volume. Because the rotation of the bar becomes highly instable as the rotation speed increases, magnetic stirring is generally no longer sufficient for reaction volumes larger than 5 ℓ and a mechanical stirring device is preferable; this may hold two or three paddles to improve the mixing throughout the entire depth of the reactor.

Maintaining a relatively constant temperature is crucial in anaerobic applications because the temperature is a major factor controlling both the chemistry of the system (specifically regulating the solubility of carbon dioxide which in turn has an impact on the pH of the suspension) and the rates of the biological reactions. Furthermore, generic applications are performed in the mesophilic range, around 30 to 35 °C: a drop below or an increase above this interval would be detrimental for the process, resulting in the microbial activity slowing down and ultimately ceasing.

Three systems to control the temperature have been adopted in this research:

- a water bath in which water is heated and pumped into the hollow jacket that constitutes the external wall of the reactor;
- a temperature controlled room; and
- a water bath, similar in principle to the first one, in which the sludge flowing into the recirculation circuit is heated prior to being pumped back into the reactor. This system was used for a larger pilot-scale set-up and illustrated in Figure 9.

The reactor must be equipped with the following ports:

- an inlet port for pumping the feed in the digester; and
- two outlet ports for the extraction of liquid samples and for venting the biogas respectively.

It might be convenient for the inlet port to reach deep into the mixed liquor so that the feed is thoroughly homogenised as it is pumped into the digester: if the inlet is hanging high in the headspace and there is either foam or a thick layer on the surface of the mixed liquor, the feed might not be homogeneously distributed.

To maintain the set-up as simple as possible, the feeding line can be used for the extraction of samples of the mixed liquor for analytical determinations, e.g. COD, solids, etc.

Great care should be taken in both feeding and sampling the digester: when feeding, the entire dose of feed should actually enter the digester and no residual should remain inside the pipe. This can be done by rinsing the tube with some sludge or by reversing the flow a number of times immediately after the feed has been injected. Similarly, when sampling, one should make sure that the sample is representative of the conditions inside the reactor at that time.

The outlet port for gas venting must hang as high as possible above the liquid surface, to prevent the possibility of it becoming clogged with foam.

If the feeding was done mechanically, e.g. using a peristaltic pump, great care should be placed in ensuring that *i*) the feed was well homogenised when added to the digester; that *ii*) no (or very little) oxygen was dissolved in the medium, that would affect the activity of the anaerobic consortium; and that *iii*) the characteristics of the feed stock solution are sufficiently consistent: since the feed contains organic matter, microbial activity would easily establish and alter the characteristics of the feed, if this was not correctly handled and preserved. An option could be to maintain a feed stock solution in a cold bath.

The ideal set-up could be as follows:

▪ immediately before starting the feed operation, the feed stock solution was thoroughly stirred for a period of time (few minutes are sufficient);

▪ the reservoir in which the feed stock solution is stored was sealed and equipped with a variable volume headspace, similar to that described for the actual digester;

▪ immediately after sealing the container, the headspace was flushed with nitrogen: this would ensure that no air was solubilised when the feed stock solution was stirred, nor entered from the environment when the feed was pumped into the digester.

The set-up can be improved to retain the sludge in the digester, thus uncoupling the sludge retention time and the hydraulic retention time. Since a settling unit on the outlet line can be not easy to operate, one can simply stop the mixing prior to extracting the treated effluent. A timer could be used for the following sequential operations:

▫ to stop the mixing a few hours prior to extraction;

▫ to turn on a peristaltic pump that extracts the supernatant; and

▫ to turn on the mixing and (simultaneously) turn on the pump that feeds the digester.

Furthermore, the reaction vessel would have to house two independent sampling lines that reached different depths, i.e. for sampling the supernatant and the settled sludge respectively.

The key factors that should be decided to operate a digester are the **sludge retention time** (SRT) and the **hydraulic retention time** (HRT) and the **organic loading rate** (OLR).

The sludge and the hydraulic retention times coincide if no sludge separation is performed prior to liquid extraction. The simple set-up described in section 3.1.3 does not allow sludge separation, even if stirring was stopped before extraction for a period of time that would allow the sludge to settle, because the pipe used for feeding and for extracting is deep into the mixed liquor.

In general, the SRT should be decided in a way that guarantees the minimum retention time needed for the most vulnerable microbial population.

The HRT will define the feed flow rate, according to the formula:

$$Q = \frac{V}{HRT}$$

where V indicates the volume of the contents of the reactor.

The OLR, being the hydraulic retention time fixed, will define the organic concentration of the feed (C) according to the formula:

$$C = \frac{OLR}{Q} \cdot V$$

D.2 MONITORING A DIGESTION PROCESS

Monitoring the performance of a laboratory- or pilot-scale digester requires the estimation of the *state* of the system in response to the *input* and the operating conditions.

The question therefore is: which variables should be measured in order to reliably assess the behaviour of the digester ?

It is beyond the scope of this report to comprehensively discuss the topic. Our experience however suggests that the following variables are sufficient to obtain an accurate picture of the current state of an anaerobic process.

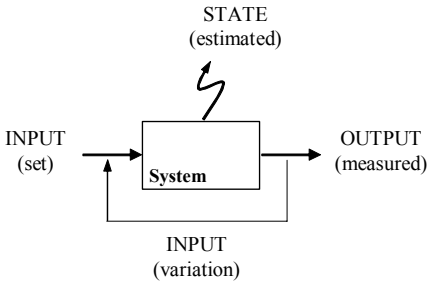


Figure D.1 – The concept of monitoring an anaerobic process: the input variables are set by the operator; the output variables are analytically or instrumentally measured; the state of the system is estimated, based on the observation of output and the knowledge of the input. This ultimately enables to manipulate the input, in order to achieve better performance.

D.2.1 Gas production

This is the most direct output of an anaerobic process; in fact the majority of the existing methods to assess biodegradability of a substance and the activity of a sludge target this variable (Rozzi and Remigi, 2004).

Qualitatively one can say that a high biogas flow rate is indicative of an effective digestion process. However, the knowledge of this variable alone is not sufficient and may even be misleading if not accompanied with the knowledge of the composition of the gas: typically, a high gas flow rate with a low methane fraction may be indicative of a predominantly acidogenic process in which the methanogenic biomass may be inhibited. If these conditions are not detected on time, they may ultimately lead to the failure of the digester.

A simple device for measuring the gas production is by liquid displacement, whereby the gas produced in the digester displaces an equivalent volume of a liquid contained in a separate reservoir: the volume of gas is indirectly quantified by measuring the volume of liquid displaced. Numerous techniques exist: we used a U-tube filled with a barrier solution and equipped with a solenoid valve to release the excess pressure Figure 12.

D.2.1.1 Set-up of the gas line

In order to ensure that the measurement of the volume of gas is accurate and consistent throughout the experimental period, great care should be taken in setting up the gas line.

Besides ensuring that the system is gas-tight, one should set up the line so that the gas measuring device can be disconnected for maintenance and repair without compromising the gas tightness of the reactor. The most frequent incidents that may happen are that the barrier solution evaporates; the solenoid valve fails; or the gas line is blocked (e.g. by biological foam) or leaks.

By installing a manual valve, the operator can isolate the gas line for inspection, without exposing the reactor to air. It goes without saying that the accuracy of the measurement relies entirely on the accuracy of the calibration of the liquid displacement device. *Theoretically*, the system represented in Figure 12 would not require a true calibration, since the unit volume corresponding to one opening of the valve can be geometrically calculated according to equation 13. In practice, it might be difficult to accurately measure the distance between the two levels. It is therefore advisable to actually calibrate the device, e.g. by flushing the headspace of the reaction vessel with inert gas at a constant flow rate. This can even be done on a regular basis, during the experiments without risk of disturbing the process.

A variable-volume gas reservoir can be used to quickly verify that the system is gas-tight and that the device is working properly: the operator can manually compress the bag and verify that the level in the U-tube moves accordingly and that the solenoid valve opens and closes duly. This is a very simple check that can be performed routinely.

D.2.2 Gas composition

In our experience, this is the most sensitive and easy to conduct measurement that the operator can undertake. The gas-chromatographic method does not need to be very sophisticated (the detection of carbon dioxide and methane is sufficient) and the analytical apparatus is not too expensive. The maintenance and calibration procedure for the GC is illustrated in Annexure F. The most common incident is that the syringe gets blocked. This is almost inevitable as the needle is inserted through the septum into a fitting which very likely accumulates biological material.

Nevertheless, we verified that a very high accuracy in determining the composition of the gas con be achieved: we generally take five to eight replicate samples and the variation coefficient has consistently been lower than 2 % (for carbon dioxide and methane).

We cannot recommend a desirable interval for gas composition, as this depends on the composition of the feed (Figure F.4). The operator should consider the variations in the gas composition, particularly increasing fractions of carbon dioxide, as a warning indicator.

D.2.3 pH, alkalinity and VFAs concentration

Unlike the gas flow rate and composition which can be determined almost continuously, these variables are measured on samples of mixed liquor, hence once a day at most. In fact, a lower frequency is sufficient and the operator could measure these variables alternatively to the determination of COD.

The measurement of pH, alkalinity and VFAs concentration only requires very simple equipment, i.e. a pH-meter, a burette and magnetic stirrer. The two-point titration procedure is illustrated in Section F.3.

These variables are strictly dependent on the dissolved carbon dioxide, therefore great care must be taken in carrying out the determination immediately after collecting the sample and in handling the sample properly, i.e. minimising the gas exchange with atmosphere.

This is inherently contradictory, because all determinations require that the sample is homogeneous hence well mixed; however, stirring the sample also greatly enhances the gas exchange between the liquid and the atmosphere, thus enabling carbon dioxide to escape (due to the great different in partial pressure between the atmospheric conditions and inside the digester). In order to minimise this inconvenient, the following rules should be applied:

i. collect the sample in a container which minimises the liquid surface exposed to air, e.g. a cylinder;

ii. measure pH immediately after sampling, directly in the container: stirring is not necessary and pouring the liquid into another container would favour the stripping of carbon dioxide gas;

iii. pour the sample for alkalinity and VFAs determination into a beaker and stir at a very low speed, only sufficient to thoroughly distribute the titrating solution in the entire volume;

iv. use a concentrated rather that diluted titrating solution in order to decrease the titration time. In our experience, a 0.5 mol/ℓ solution is ideal;

D.2.4 COD concentration

The determination of the COD, combined with that of the gas flow rate and composition, is necessary to conduct a mass balance on the organic matter and ultimately calculate the performance of the process. The determination section 3.2.2 is relatively quick and generally accurate; it is recommended to use four replicates to ensure that the average and standard deviation are meaningful.

D.2.5 Solids concentration

The determination of solids in the digester assists in ensuring that the biomass is not washed out of the system, but rather grows on the organic substrate. The determination (Section 3.2.1) is not immediate as it requires the sample to be diluted (if necessary), filtered, dried in the oven and ignited in the furnace and the outcome can only be available one or two days after the sample has been withdrawn.

For this reason and because the growth rate of anaerobic micro-organisms is relatively low, a frequency of one determination per week is generally sufficient. It is recommended to undertake the

measurement of VSS in four replicates. Ideally, one should verify a constant or increasing trend in the concentration of VSS.

The estimation of the methanogenic fraction or activity of the biomass could effectively complement the determination of solids concentration: the method indicated in Section 4.2.2.3 is not immediate but is likely to be sufficiently accurate if a minimum of three doses of acetic acids are added. If the digester is not overloaded, the fed-batch phase of the test can start immediately after the sludge sample has been withdrawn, with no need for a preliminary consumption of the residual substrate. Therefore, the estimation of the methanogenic fraction of the biomass will take *at least* 3 to 4 days.

More importantly, it only provides a *relative* estimate, but is generally sufficient to monitor the process.

D.2.6 Evaluation of the stability of an anaerobic digester

As discussed in Section 4.2, alkalinity and VFA concentrations are commonly used as indicators of the stability of a digestion process. In the practice, the VFA-to-Total Alkalinity ratio is also used.

However, Speece (1996) warns that the *reserve bicarbonate alkalinity* should be regarded as a more accurate monitoring parameter, as it indicates the concentration of bicarbonate alkalinity which is available to neutralise *additional* free volatile acids, before the pH drops below a safe threshold value of 6.2 to 6.5.

A numerical example may help in understanding this concept.

If we consider the situation of the laboratory-scale digester (Section 4.2) on day 56, this looks sufficiently safe, as one can argue from the following figures:

Temperature	pH	Alkalinity	VFA	pCO_2 (bar)
32 °C	7.93	300.4 mmol/ℓ	99.8 mmol/ℓ	0.28

The *reserve bicarbonate alkalinity* (RA) is calculated as:

$$RA = BA - BA_{CO_2} = (TA - TA_{VFA}) - BA_{CO_2} \qquad (D.1)$$

where BA and TA indicate the *bicarbonate* and *total* alkalinity respectively (mol/ℓ); the lower case CO_2 and VFA indicate the alkalinity required to buffer the carbon dioxide and the volatile acids respectively.

The quantities BA_{CO_2} and TA_{VFA} are calculated as follows:

$$BA_{CO_2} = \frac{K_{CO_2} \cdot K_H \cdot p_{CO_2}}{10^{-pH}} \qquad (D.2)$$

$$BA_{VFA} = 0.83 \cdot VFA \qquad (D.3)$$

where K_{CO_2} is the dissociation constant for HCO_3^- / CO_2 (mol/ℓ); K_H is the Henry's law constant for carbon dioxide (mol/ℓ/bar); the factor 0.83 has been explained in Section 3.2.4.

Equation D.2 describes the equilibrium of carbon dioxide in water and between the liquid and the gas phase (Henry's law).

Values for the constants K_{CO_2} and K_H can be calculated as follows (T, temperature in K):

$$pK_{CO_2} = 17052 \cdot T^{-1} + 215.21 \cdot \log T - 0.12675 \cdot T - 545.56$$

$$K_H = 10^{2.6747 - 0.0139 \cdot T}$$

For the conditions existing on day 56, $K_{CO_2} = 4.6 \cdot 10^{-7}$ mol/ℓ and $K_H = 0.0272$ mol/ℓ/atm; therefore:

$$BA = Alk \cdot MW\left(HCO_3^-\right) = 18323 \text{ mg/}\ell$$

$$BA_{CO_2} = 0.299 \text{ mol/}\ell = 18255 \text{ mg/}\ell$$

This clearly leaves a very small safety margin of 68 mg/ℓ only to neutralise additional VFA, which corresponds to 68/0.83 = 82 mg/ℓ of VFA.

D.2.7 Calculating the performance of the process

The ultimate objective of an anaerobic digestion process is to effectively convert the organic carbon of the feed to methane. The efficiency of this process is generally measured in terms of COD removal efficiency as follows:

$$\eta_I = 1 - \frac{COD_{out}}{COD_{in}} \tag{D.4}$$

where COD_{in} and COD_{out} denote the COD concentrations in the feed and in the effluent respectively. However, this approach is correct when the system has reached a steady-state and not before one (or more) retention times have passed, as the efficiency defined through equation D.4 does not account for the dilution effect (function of HRT) that occurs in a completely stirred digester. A mass balance on the COD is actually a better approach in that it compares the *cumulative* amount of organic matter fed, to the *cumulative* amount which residues, as:

$$\eta_{II} = \frac{COD_{removed}}{COD_{fed}} = \frac{\sum C_{in,t} \cdot V_{in,t} - \left[C_{out,t} \cdot V_R + \sum \left(C_{out,t} \cdot V_{out,t}\right)\right]}{\sum \left(C_{in,t} \cdot V_{in,t}\right)} \tag{D.5}$$

where $C_{in,t}$ and $C_{out,t}$ denote the COD concentration in the feed and in the effluent respectively, at time *t*; $V_{in,t}$ and $V_{out,t}$ denote the volume of feed and of mixed liquor respectively, at time *t*; and V_R is the liquid volume.

Ideally, the fraction of COD degraded should be stoichiometrically converted into methane and therefore the efficiency evaluated through equation D.5 should equal the following alternative definition:

$$\eta_{III} \frac{COD_{toCH_4}}{COD_{fed}} = \frac{\sum M_{ch,t}}{\sum \left(C_{in,t} \cdot V_{in,t}\right)} \tag{D.6}$$

where $M_{ch,t}$ is the amount of methane (in g COD) produced at time *t*.

Possible reasons for the η_{II} and η_{III} efficiency not been equal are that:

i) the COD measured analytically is not accurate or not representative of the actual organic matter in the mixed liquor; this is actually possible since the COD is often measured on the filtered mixed liquor, i.e. the portion of COD that has been stored by the biomass is not taken into account; and/or

ii) the quantification of the methane produced is not accurate. In fact, the volume of methane produced is calculated through a mass balance which is based on a number of parameter that must be determined with sufficient accuracy for the overall mass balance to be accurate.

$$\Delta V_{CH} = \Delta V_{CH,out} + \Delta V_{CH,headspace}$$

where $\Delta V_{CH,out}$ denotes the volume of methane that has left the reactor during the interval $(t-1, t)$ with the biogas; and $\Delta V_{CH,headspace}$ indicates the variation of the methane volume in the headspace in the same interval.

The two terms can be calculated as:

$$\Delta V_{CH,out} = \int f_{CH}(t) \cdot Q_{gas}(t) \cdot dt \qquad \text{(D.7.a)}$$

$$\Delta V_{CH,headspace} = V_H \cdot (f_{CH,t} - f_{CH,t-1}) \qquad \text{(D.7.b)}$$

where $f_{CH}(t)$ is the methane fraction in the off-gas (generally a function of time); $Q_{gas}(t)$ is the biogas flow rate (ℓ/d) (also a function of time); $f_{CH,t}$ and $f_{CH,t-1}$ denote the methane fraction, at time *t* and *t-1* respectively; and V_H is the volume of the headspace (generally constant).

For the laboratory-scale experiment described in Section 4.2, the determination of the gas composition was done discontinuously (i.e. once a day) and only the cumulative volume of gas produced was known (V_{gas}). This translates in the practical assumption that *i*) the biogas flow rate is constant and *ii*) the methane fraction varies linearly in the time interval. Therefore, equation D.7.b can be written as:

$$\Delta V_{CH} = \frac{f_{CH,t} + f_{CH,t-1}}{2} \cdot V_{gas} \qquad \text{(D.8)}$$

This calculation is not very accurate and highly dependent on the reliability of the gas measuring device, i.e. on the gas counter –and therefore prone to large underestimation of the actual volume of methane generated.

Table D.1 – Variables that should be measured to monitor a laboratory-scale digester.

Variable	Frequency of determination	Optimal range
Gas flow rate	Continuous (*at least* daily)	▪ Function of the sludge activity and organic loading rate.
Gas composition	*At least* daily	▪ Function of the feed composition and microbial dynamics.
pH	Whenever a liquid sample is collected	6 to 7.5
Alkalinity	As for pH, or every two pH determinations	n.a.
VFA concentration	As for alkalinity	n.a.
COD	Daily or every two days	▪ Should not display a clearly *increasing* trend
Solids	Weekly or every two weeks	▪ Should not display a clearly *decreasing* trend
SMA (*)	As for solids	> 60 mg COD/g VSS/d

(*) The variable measured is the methanogenic activity of the *sludge*, combined with the previous knowledge of the solids concentration. The methanogenic fraction of the biomass is a derived quantity.

Note. These guidelines are meant for the monitoring of the start-up phase and for an experimental digester, on which specific tests are performed, i.e. conditions vary. Steady-state and routinely operated digesters require a much lower frequency of determinations.

D.3 CONTROLLING A DIGESTION PROCESS

A recurrent incident that we experienced during the semi-continuous experiment discussed in Section 4.2 was the organic overload of the biomass that was likely due to a relatively rapid acidification of the organic fraction of the test material, which resulted in the accumulation of large concentrations of volatile acids. These in turn consumed alkalinity and lowered the pH of the suspension. These conditions in the liquid phase were mirrored in the gas phase by an increase in the molar fraction of carbon dioxide.

The increase of the loading rate from 1.0 to 1.9 and to 2.5 g COD/ℓ/d that started on day 70 (period IV) determined a gradual but progressive worsening of the performance of the laboratory-scale digester. In particular, the molar fraction of methane dropped from values above 60 % to values in the range 25 to 30 % (Figure 52 F).

On day 99, it was decided to stop the daily feed for three days, to see whether the system was sufficiently robust to recover on its own. As one can observe in Figure D.2, the gas composition proved to be a very sensitive indicator of the process performance, as it immediately reacted to the cessation of feeding, moving from a ratio CH4:CO2 of 30:70 to a ratio of 42:57 on day 102. Correspondingly, pH, alkalinity and VFAs concentration did not show a significant variation (although

the fact that they appear to be approximately constant suggests that the COD was being progressively degraded and was not resulting in the accumulation of any further acidity in the system).

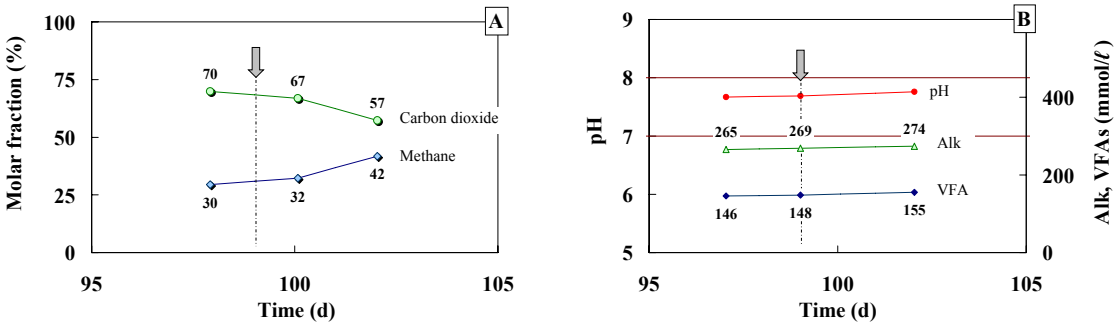


*Figure D.2 – Gas composition (**A**) and pH, alkalinity and VFA concentration (**B**). The arrow indicates the discontinuation of the feed (day 99).*

ANNEXURE E

A database as a tool for monitoring laboratory-scale digestion experiments

Based on the day-by-day experience of laboratory work, we realised that unless an anaerobic digestion unit is highly automated, e.g. with online data acquisition of gas production and composition, pH, automated feeding and sampling procedures, etc., the accurate monitoring of the process can be very time-consuming. Particularly when the number of units to maintain is large (at one stage, due to the repeatedly unsuccessful start-up of the laboratory-scale reactor, we had been manually monitoring four units simultaneously).

Nowadays, the implementation of *ad hoc* control software using commercial packages is relatively simple and inexpensive. However, it still requires time to write the code and for the subsequent debugging. More importantly, this option was still not feasible on the instrumentation side, as the various measuring devices should have been interfaced to the software and not all the devices available in the laboratory were actually designed for that purpose.

For this reason, we focused our efforts in improving the data logging and management step.

A spreadsheet is often not the ideal tool to be used when a large and diverse amount of data, coming from different sources and which need a different type of analysis, are to be manipulated.

Typically, COD measurements are carried out daily and consist in the titration of a certain number of replicates; sometimes, it might be necessary to use two dilutions, to obtain meaningful average values. Therefore, the single datum that is obtained daily is in fact the result of a series of manual determinations.

The determination of alkalinity and VFA concentration using the two-point titration method, a preliminary level of manipulation of the raw data is required before the actual single measurement can be logged on the spreadsheet.

The determination of the gas composition is different. During the course of this project, it has been found to be the most sensitive indicator of process performance. Therefore, frequent gas sampling and analysis were conducted on various occasions and immediately after feeding the reactor (Figure 43). Since the parameter of interest is the gas composition, i.e. the molar fractions of nitrogen, carbon dioxide and methane in the gas phase, the raw output of the GC, i.e. the peak areas, had to be converted into molar fraction (as explained in Section F.2); the same had to be repeated for each replicate of the same sample; then the molar fraction, averaged and sometimes manually corrected (i.e. in some cases, replicates had to be disregarded) before the single datum for gas composition could be inputted on the spreadsheet, in correspondence to the time of sampling. On some occasions, multiple samples (up to 10 to 12) were taken in a day.

It is therefore clear that different levels of pre-treatment of the raw data are necessary prior to logging and that different time scales apply to the different pieces of information. It goes without saying that each one of the data inputting and manipulating steps is inherently exposed to accidental errors, particularly when the same operation is repeated several times. This and the fact that ideally it is good practice to maintain a trace of every step from the actual raw data to the synthetic data at the end of the data-manipulating line, we oriented our interest to the use of a **database application**.

MS-Access was the obligate choice, as it is widely available and as it is ideally suited to interact with MS-Excel, which is still the preferential tool to generate plots.

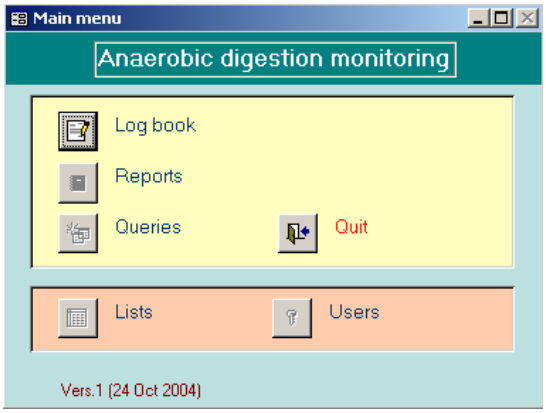


Figure E.1 – Main menu of the application for monitoring anaerobic digestion processes.

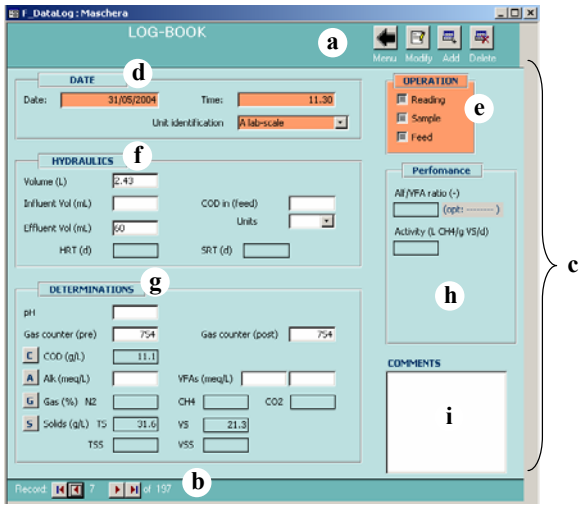


Figure E.2 – Log-book for data inputting.

The application which has been implemented consists of the user-interface and of the underlying database.

The main menu (Figure E.1) is displayed at the start-up. It contains six buttons:

- Log-book: it opens the main sheet of the log-book, through which data will be inputted.
- Reports: it enables the user to make an 'open' selection within a list of predefined sets of data (e.g. the OLR and the gas production rate and composition), that will be displayed, printed or saved as an ASCII file (e.g. to be imported in a spreadsheet for further analysis). This button is not active in the current release, as further debugging is needed.
- Quit: it shuts down the application.
- Lists: this button is not active, but it will enable the user to manage data that can simplify routine calculations, such as molecular weights or COD-equivalents data of the test materials, etc.
- Users: since the application has been used for internal purposes only, the need has not arisen for creating different levels of users with different privileges. This however is generally a good practice when such application is accessed by many users. Typically, to ensure that data are not

accidentally manipulated, only few users are authorised to write data (including modifications and deletions); others are only authorised to query the database and generate reports, etc.

The main page of the **log-book** is showed in Figure E.2 and consists of the following areas:

- commands bar (**a**): it contains four buttons, i.e. Menu (to return to the main menu), Modify (to modify the record which is currently active), Add (to display a blank form and enter a new record) and Delete (to erase the current record).
- navigation bar (**b**), which shows the position of the current record within the database and the total number of data stored; four small buttons with red arrows, i.e. First, Previous, Next and Last, enable to move to the corresponding record of the database.
- the main body (**c**) contains the sub-areas Date, Operations, Hydraulics, Determinations, Performances and Comments.
  - The sub-area Date (**d**) contains the fields Date, Time and Unit ID. As the application is designed to monitor several reactors, each record can be associated to a specific unit, e.g. laboratory-scale A. These three fields are mandatory, to avoid the risk of inputting unrelated data.
  - The sub-area Operations (**e**) is also mandatory and allows the selection of the type of data to be entered: a simple reading (i.e. gas volume and/or gas composition), a liquid sample (which supposedly will provide a value of pH, COD, alkalinity, VFAs and periodically of solids) and feed (which is associated with a volume or flow-rate fed to the reactor, as well as the organic load).
  - The sub-area Hydraulics (**f**) manages the aspects related to the feed and sampling procedures. It also displays, in *read-only* mode, the current hydraulic and sludge retention times, which are internally calculated: it is assumed that the reactor is a completely stirred vessel with no sludge separation, as all the units used in this project; independent calculations of the two could be implemented in a future version.
  - Determinations (**g**) is the sub-area in which the actual analytical and instrumental data are recorded: pH, gas counter (i.e. gas volume), COD, etc. As discussed earlier, most of the parameters are 'manipulated' quantities which result from the elaboration of the raw information, e.g. replicated measurements which contribute to a single averaged value. This was previously done on a separate spreadsheet, whereas here a secondary input form is displayed by clicking on the respective buttons.

    For instance, the Solids button opens the Solids calculation sheet reported in Figure E.3. This form will receive the raw data inputted by the user, elaborate the output (click on Results) and transfer the end result onto the main log-book. The elaboration of the data is not rigid in that it is always possible for the user to control to some extent how the end result is obtained, e.g. an outlier from the set of data can be deactivated, in order to obtain a better estimate (i.e. with a

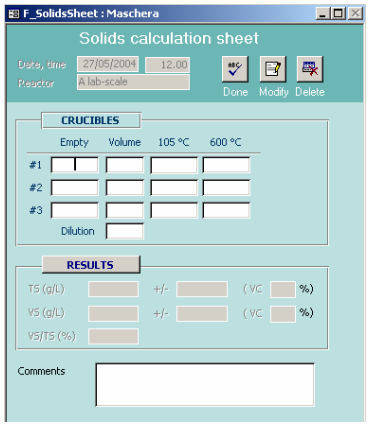
lower variation coefficient). However, all the raw data will be saved in the database, should it ever become necessary to review the past data.

- The sub-area Performances (**h**) displays a range of summary information which is generally used as indicators of process performance, besides the actual values of the measured parameters. At present, only two of such indicators are included, i.e. the Alkalinity-to-VFA ratio and the methanogenic activity.

  Other possible indicators could be the gas flow-rate, the methane-to-carbon dioxide ratio. Clearly more than the current values of these indicators, it is of greater interest their trend over a certain predefined period of time. A more efficient way of displaying these indicators would be to generate a secondary form (or a report) in which a series of data can be viewed for a pre-selected period of time.

- The sub-area Comments (**i**) (present in all the forms) allows the user to input all information that may be considered of interest for a further analysis.

a



b

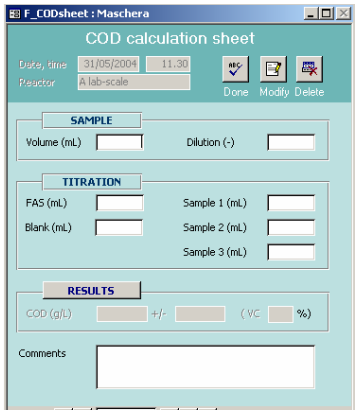


*Figure E.3 – Calculation sheets for Solids (**a**) and for COD (**b**).*

CONCLUSIONS

The database application is far from being an efficient tool for the monitoring of anaerobic digesters, not only because many of the features which are planned have not been implemented yet, but also because a thorough debugging has not been performed. However, it is believed that such tool, when perfected, could be of great help in managing large experimental trials as it would be a full-scale co-digestion trial. In this respect, i.e. when a large amount of data coming from various sources are to be managed (e.g. clinical trials), databases, but not necessarily on Access platforms, have proven very robust and efficient.

ANNEXURE F

Materials and Methods

This Section contains further information on technical aspects encountered during the experimental work: a detailed description of the nutrients and mineral salts medium, which is used to supplement the anaerobic micro-organisms with all elements needed for their metabolism, is reported in Section F.1; the characteristics and the calibration procedure of the gas-chromatograph that has been employed for the determination of the gas composition are discussed in Section F.2; the two-point titration method to measure alkalinity and volatile fatty acids concentration is outlined in Section F.3; the organic carbon content of a test compound and the composition of the biogas resulting from its anaerobic biodegradation can be theoretically calculated, according to the procedures outlined in Section F.4.

F.1 NUTRIENTS AND MINERAL SALTS MEDIUM

Micro-organisms need **nutrients** and **trace elements** (minerals and vitamins) for their metabolism.

Nitrogen, phosphorous, sulphur, calcium, magnesium, iron, cobalt and nickel have proven to be essential to sustain high acetate utilisation rates: e.g. Takashima and Speece (1989) indicate that to prevent a reduction in activity of the biomass, the NH_4-N concentration in the reactor must be maintained in excess of 40 to 70 mg/ℓ. Several nickel or cobalt ion-containing enzymes, such as coenzyme F_{420}, are involved in methanogenesis and therefore it is not surprising that trace metals such as nickel, cobalt and iron are found to be stimulatory to anaerobic processes. Thus an obligate requirement of iron (0.020 mg), cobalt (0.004 mg) and nickel (0.003 mg) per gram of acetate is reported by Speece (1996). The empirical cell formula $C_5H_7O_2NP_{0.06}S_{0.1}$ indicates that there is an obligate requirement for sulphide: Zehnder and co-workers (1980) reported an optimal growth to occur at 0.001 to 1 mg S/ℓ (as sulphide).

There is no general consensus on whether these *growth factors* are readily available and at a sufficient concentration in a sludge; when low concentration of biomass (e.g. 1 g TS/ℓ) are used, it may be necessary to supplement the culture with nutrient and trace elements. Besides, the bioavailability of trace metals may be hindered in the presence of sulphide, carbonate and phosphate ions due to the low solubility of their precipitates (Speece, 1996).

The theoretical minimum COD:N:P ratio is 350:7:1 for highly loaded systems and 1000:7:1 for lightly loaded systems (Speece, 1996).

The presence of an additional carbon source can also enhance degradation, in co-metabolic processes.

The nutrients and minerals solution (NMS) that was used for the experiments presented in this report, contained trace elements, minerals and vitamins, according to Owen et al. (1979) with some modifications. The stock solutions for the preparation of the medium are presented in Table F.1: each one is to be prepared in deionised water and stored in a cold room (at 4 °C). We have not rigorously quantified their *shelf-life*: however, a safe approach is to prepare relatively small volumes which can be used up within 2 to 3 months after preparation.

Table F.1 – Stock solutions for the preparation of the minerals and nutrients medium.

Stock solution	Composition	Concentration (g/ℓ)
S2	Resazurin	5755
S3	$(NH_4)_2HPO_4$	26.7
S4	$CaCl_2 \cdot 2H_2O$	16.7
	NH_4Cl	26.6
	$MgCl_2 \cdot 6H_2O$	120
	KCl	86.7
	$MnCl_2 \cdot 4H_2O$	1.33
	$CoCl_2 \cdot 6H_2O$	2
	H_3BO_3	0.38
	$CuCl_2 \cdot 2H_2O$	0.18
	$Na_2MoO_4 \cdot 2H_2O$	0.17
	$ZnCl_2$	0.14
S5	$FeCl_2 \cdot 4H_2O$	370
S6	$Na_2S \cdot 9H_2O$	500
S7	Biotin	0.002
	Folic acid	0.002
	Pyridoxine hydrochloride	0.01
	Riboflavin	0.005
	Thiamin	0.005
	Nicotinic acid	0.005
	Panthotenic acid	0.005
	p-aminobenzoic acid	0.005
	Thioctic acid	0.005

The vitamins *p*-aminobenzoic acid and thioctic acid were unavailable, therefore were omitted from stock solution S7.

The method for the preparation of the medium is illustrated hereafter.

4. Add 1 ℓ of deionised water to a 2 ℓ Pyrex beaker.
5. Add the following stock solutions: S2 (1.8 mℓ), S3 (5.4 mℓ) and S4 (27.0 mℓ).
6. Add deionised water up to a volume of 1.8 ℓ.
7. Boil for 15 min whilst flushing with oxygen-free nitrogen (1 ℓ/min).
8. Cool to room temperature.
9. Add the following stock solutions: S7 (18.0 mℓ), S5 (1.8 mℓ) and S6 (1.8 mℓ).

10. Add sodium bicarbonate ($NaHCO_3$: 8.4 g) in powder form.
11. Flush with oxygen-free nitrogen until pH stabilises around 7.1.
12. Autoclave at 121 °C, for 30 min.
13. Store at 4 °C until use.

F.2 GAS-CHROMATOGRAPHIC DETERMINATION OF GAS COMPOSITION

The biogas composition was analysed with a GOW MAC 350 gas chromatograph, equipped with a thermal conductivity detector (TCD) which can detect nitrogen, methane and carbon dioxide. A Haysep D stainless steel column (L 4 m, ID 2.2 mm, OD 3.2 mm) was used for the separation, according to the following conditions:

- Column oven: 25 °C
- Detector: 25 °C, 100 mA
- Filaments: 40 °C
- Injection port: 40 °C

The carrier gas was helium at a flow rate of 24.5 mℓ/min. The retention times of nitrogen, methane and carbon dioxide were approximately 1.05, 1.39 and 2.30 min respectively. Gas samples were withdrawn from the serum bottles or laboratory-scale reactors by inserting the needle of a gas- or pressure-lock syringe (100 μℓ) through the butyl rubber septum (GR-2 9.5 mm, Supelco) and withdrawing 40 to 60 μℓ of headspace gas. The peak area was recorder with a Varian 4270 integrator with the attenuation set at 1.

To set-up the integrator, the following procedure is to be used.

1. Switch the power on; then press the DIALOG key.
2. Enter a FILE NAME if desired, then press ENTER.
3. The batch program is ready to acquire the set of parameters which are needed to interpret the chromatogram and automatically integrate the peak areas. A matrix of (5 x 3) parameters is to be entered. The three parameters indicate the times (TT and TV) and the codes (TF) which identify each one of five distinct events. E.g. should the detector be receiving a signal stronger than the background after 5 min (TT: 5), this would be treated as an error (TF: ER) and the detection period would be terminated (Line 5 in the sequence corresponds to the 'safety' termination).

   Press ENTER after each entry, to prompt the next parameter in the sequence.

	TT	**TF**	**TV**
Line 1	0.01	AZ	1
Line 2	0.01	CS	0.5
Line 3	0.01	PM	1
Line 4	0.01	AT	1
Line 5	5	ER	5

4. At the next prompt, press ENTER to exit.
5. Press ENTER, to END DIALOG.
6. Press PRINT FILE to display program code

The calibration of the gas chromatograph was performed using pure gases (UHP, Afrox Scientific). Gas samples of given volumes were withdrawn by inserting the gas-lock syringe into a butyl rubber septum positioned on the outlet of the gas bottle. The flow rate on the regulator was set to 16 ℓ/min and 0.35 ℓ/min for carbon dioxide and methane respectively. Sets of five to six volumes were injected (ranging from 10 to 60 $\mu\ell$), in 3 to 5 replicates. The detailed calibration procedure was as follows.

1. Set up the sampling line. Make sure that the integrator and GC settings are correct.
2. Check the septum seal on the sampling line: as a precaution, the septum should be changed every 30 to 35 injections, to prevent gas leaks through the injection port.
3. Check the reading on the pressure gauge and ensure that there is no fluctuation.
4. Record the ambient temperature and the gauge pressure.
5. Withdraw a sample of the gas i ($i = N_2$, CH_4 and CO_2) of defined volume ($v_{i,j}$, $j = 10$, …, 60 $\mu\ell$): lock the syringe until the point of injection:
6. Inject the gas sample in the GC and wait for integrator analysis.
7. Record the retention time (RT) and Area ($A_{i,j}$) of the gas with the highest area.
8. Repeat steps 5 to 7, for all the j volumes of the first gas, from the lowest to the highest volume.
9. Repeat steps 5 to 7, a number of times sufficient to generate a statistically significant set of data for the first gas (five replicates should be sufficient).
10. Repeat steps 5 to 9, for the other gases.

The raw data for the GC calibration on methane and on carbon dioxide are reported in Tables F.2 and F.3. The respective calibration curves are plotted in Figures F.1 and F.2: the data points (diamonds) are the average values of each set of replicates (column *Ave*, in the Tables); the solid lines represent the 95 % confidence intervals for the calibration curve (the slopes and intercepts of the regression lines are indicated below the Figures, as *calibration factor* and *detection limit*). As one can see, the determination coefficient of the regression is very high ($R^2 \geq 99.9\%$) and the detection limits very low ($v_0 \leq 5$ $\mu\ell$).

Table F.2 – Raw data for the calibration curve of METHANE.

Volume	Area					Ave	StDev	VC
($\mu\ell$)	1st	2nd	3rd	4th	5th			(%)
10	4 183	4 150	4 495	4 269	4 343	4 288	138	3
20	10 557	11 244	11 047	11 380	11 320	11 110	333	3
30	15 299	16 226	15 939	17 284	17 316	16 413	877	5
40	22 471	22 565	22 609	23 659	23 800	23 021	651	3
50	29 938	29 481	29 579	30 229	30 209	29 887	347	1
60	36 069	35 810	36 004	36 624	36 724	36 246	404	1

Table F.3 – Raw data for the calibration curve of CARBON DIOXIDE.

Volume	Area					Ave	StDev	VC
(μℓ)	1st	2nd	3rd	4th	5th			(%)
10	6 196	5 755	5 763	5 269	5 421	5 681	359	6
20	15 015	14 192	13 920	13 866	14 088	14 216	465	3
30	21 677	23 511	22 056	21 035	21 687	21 993	925	4
40	29 753	31 234	31 949	29 617	29 354	30 381	1 142	4
50	41 226	40 265	38 024	37 575	37 632	38 944	1 688	4
60	47 785	45 063	n.p.	n.p.	n.p.	46 424	1 925	4

n.p., not performed; Ave, average; StDev, standard deviation; VC, variation coefficient

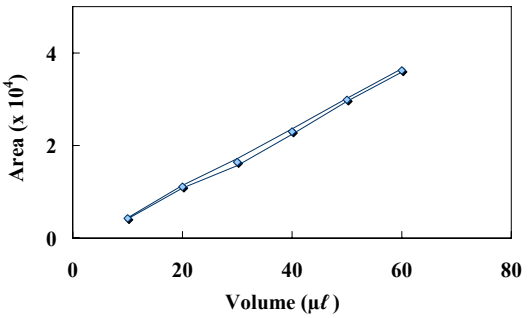


Figure F.1 – Calibration curve for methane.

Calibration factor: **636** Area/μℓ (R^2 99.90 %)
Detection limit: **3** μℓ

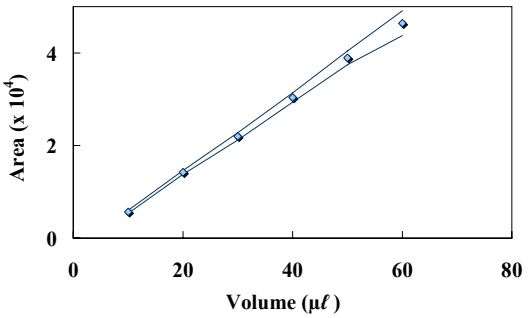


Figure F.2 – Calibration curve for carbon dioxide.

Calibration factor: **818** Area/μℓ (R^2 99.97 %)
Detection limit: **3** μℓ

For mass balances purposes, the number of moles of each component (n_i), in a sample, can be calculated from the gas law:

$$P_i \cdot V = n_i \cdot R \cdot T \qquad (F.1)$$

where P_i is the partial pressure (bar) of each constituent, V is the total volume (ℓ), R is the gas coefficient: 0.0821 ℓ·bar/(mol·K), T is the temperature (K).

The partial pressure of a component is calculated as:

$$P_i = (P_{atm} - P_w) \cdot \chi_i \qquad (F.2)$$

where P_{atm} is the atmospheric pressure: 1.013 bar, P_w is the water vapour pressure (bar), χ_i is the molar fraction of each constituent (mol/mol).

The water vapour pressure was calculated as (Batstone et al., 2002):

$$P_w = 3.225 \cdot 10^9 \cdot e^{0.05393 \cdot T} \qquad (F.3)$$

F.3 TWO-POINT TITRATION METHOD

A spreadsheet based on the two-point titration method developed by Anderson and Yang (1992) to determine the alkalinity and VFA concentration was implemented to analyse titration data and is described hereafter.

Input data	*Symbol*	*Units*
▪ Volume of the sample of mixed liquor	V	mℓ
▪ Concentration of the titrating solution	c	mol/ℓ
▪ Initial pH	pH_0	-
▪ Initial volume of titrating solution (*)	v_0	mℓ
▪ Volume of titrating solution dosed to reach pH 5.1	v_1	mℓ
▪ Volume of titrating solution dosed to reach pH 3.5	v_2	mℓ

(*) It denotes the volume of acid added to the sample prior to starting the titration, e.g. to inactivate the biological activity.

The following constants will be used:

$H_0 = 10^{-pH_0}$; $H_1 = 10^{-5.1}$; $H_2 = 10^{-3.5}$

$K_1 = 6.6 \cdot 10^{-7}$ $K_2 = 2.4 \cdot 10^{-5}$

Calculate the following:

$$A_i = \frac{H_i - H_0}{K_2 + H_i}; \quad B_i = \frac{H_i - H_0}{K_1 + H_i}; \quad C_i = \frac{v_i \cdot c}{V} \cdot 1000 \quad (i = 1, 2)$$

Output	*Formula*	*Symbol*	*Units*
▪ Bicarbonate alkalinity	$\frac{C_1 \cdot A_2 - C_2 \cdot A_1}{B_1 \cdot A_2 - B_2 \cdot A_1}$	BAlk	mol/ℓ
▪ Dissociated VFA	$\frac{C_2 \cdot B_1 - C_1 \cdot B_2}{B_1 \cdot A_2 - B_2 \cdot A_1}$	dVFA	mol/ℓ
▪ Total VFA	$dVFA \cdot \frac{H_0 + K_2}{K_2}$	tVFA	mol/ℓ

F.3.1 The accuracy of alkalinity and VFA determination through titration

The equilibrium of the inorganic carbon is the key factor that one must take into consideration when measuring pH; since we used a titration method to determine alkalinity and volatile acids concentration, these estimates too can be affected, if the concentration of carbon dioxide in the liquid varies significantly.

The problem obviously arises when a sludge sample is withdrawn from the digester and taken to the laboratory to be analysed. Factors that influence the equilibrium are primarily the carbon dioxide partial pressure, the temperature and the mixing conditions and, to a smaller degree, the microbial activity, the turbidity of the suspension and the surface area exposed to air.

- The sudden drop in the partial pressure of carbon dioxide from the reactor (typically, 0.2 to 0.8 bar) to the environment (0.03 bar) favours the stripping of CO_2 hence the pH raises.
- The drop in temperature from the operating temperature of 35 °C to ambient temperature opposes the stripping of CO_2 hence the pH decreases.
- Turbulent conditions favour the stripping of CO_2.
- Microbial activity generates CO_2 within the suspension but this effect is partially counterbalanced by the simultaneous alkalinity generation (aceticlastic methanogenesis).
- A high turbidity, i.e. a high solid content of the mixed liquor may oppose the stripping of CO_2.
- A wide surface area at the liquid/atmosphere interface favours the stripping of CO_2.

The first two factors could be quantified with precision, using the laws of aquatic chemistry, whilst the other may be more difficult to estimate. One would rather minimise these potential interferences by:

- minimising the exposure of the sludge sample to the air, e.g. by capping the container until the measurement is completed (if possible);
- performing all determinations which involve pH, at the operating temperature;
- limiting or avoiding mixing the sample (if possible); and
- using a narrow and deep container, e.g. a cylinder.

It is beyond the scope of this discussion to analyse this topic in details; we rather approached this issue from a pragmatic point of view.

In a few occasions, we took a sludge sample, split it into two sub-samples (F, NF), immediately filtered one of the two, and then titrated both. Mixing is required for an accurate titration, so we distinguished between a ‘quick’ and a ‘slow’ titration: in the former case, as soon as the sample had reached the titration points (i.e. 5.1 and 3.5), the volume titrated was recorded; in the latter case, after the titration points had been reached, the sample was left and the carbon dioxide enabled to re-equilibrate (the pH response is much faster than the kinetics of gas/liquid exchange) and additional titrant was carefully added until the titration points were passed.

The change in temperature was unavoidable, as the titration apparatus could not be housed inside the temperature controlled room; so temperature was recorded during the procedure.

The results (Figure F.3) seem to indicate that the factors mentioned above do not significantly affect the determination, at least when the entire procedure is completed within 1 h from sampling, the sludge is contained in a cylinder and magnetically stirred at low speed.

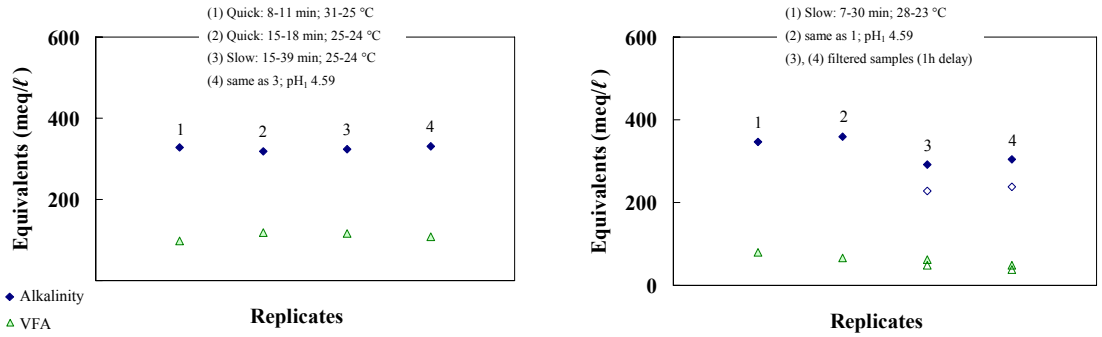


*Figure F.3 – Evaluation of the impact of mixing and filtration on the determination of alkalinity and VFA concentration in a sample of sludge. (**A**) All replicates are not filtered. (**B**) Filtered (full symbols) vs. unfiltered replicates (empty symbols): the values for the unfiltered samples are re-scaled to take in account the dilution effect. Samples 2 and 4 in both batches were titrated until pH 4.59 instead of 3.5. Quick and slow titration are explained in the text.*

F.4 STOICHIOMETRIC CALCULATIONS

F.4.1 Organic carbon content

The theoretical COD of a compound of known chemical composition can be calculated as follows.

$$Cr_2O_7^{2-} + 8H^+ \rightarrow 2Cr^{3+} + 4H_2O + 3O \tag{F.4}$$

$$C_cH_hO_oN_n + \alpha \cdot Cr_2O_7^{2-} + (8\alpha + n) \cdot H^+ \rightarrow c \cdot CO_2 + \frac{h + 8\alpha - 3n}{2} \cdot H_2O + n \cdot NH_4^+ + 2\alpha \cdot Cr^{3+} \tag{F.5}$$

where:

$$\alpha = \frac{2}{3}c + \frac{1}{6}h - \frac{1}{3}o - \frac{1}{2}n \tag{F.5a}$$

$$COD = \frac{3}{2}\alpha \text{ mole } O_2 \tag{F.5b}$$

This calculation can be used to determine the theoretical COD of the mass balance components.

An example of the calculation is presented and few additional examples summarised in (Table F.4).

An empirical formula for **biomass** is: $C_5H_7O_2N$.

Substituting the values $c = 5$; $h = 7$; $o = 2$; $n = 1$ in equation F.5a, we obtain:

$$\alpha = \frac{10}{3} \text{ mole } O_2$$

And equation F.5b gives the COD equivalent of one mole of biomass:

$$1 \text{ mole } C_5H_7O_2N = \frac{3}{2}\alpha = 5 \text{ moles of } O_2$$

Considering the formula weight of the biomass (113 g/mol), this translates into 1.416 g COD/g of biomass.

Table F.4 – Stoichiometric calculation of the chemical oxygen demand.

Compound	Formula	Molecular weight (g/mol)	COD equivalent (g COD/g)
Biomass	$C_5H_7O_2N$	113	1.416
Nutrient stock solution	$C_{12}H_{14}O_3N_2$	234	1.709
Acetic acid	CH_3COOH	60	1.067
Ethanol	C_2H_5OH	46	2.087

F.4.2 Biogas composition

For monitoring anaerobic digestion, the methane-to-carbon dioxide ratio represents a rapid and sensitive parameter: any sudden noticeable change in this ratio is indicative of unbalanced conditions. The volume of biogas produced per unit mass of volatile solids destroyed is an important and sensitive process control indicator to assess the activity of the micro-organisms and the progress of digestion.

The earliest definition of the stoichiometry of anaerobic digestion was that of Buswell and Müller (1952), which provides a good approximation of the biogas composition from a waste of known chemical composition:

$$C_cH_hO_o + \left(c - \frac{h}{4} - \frac{o}{2}\right)\cdot H_2O \rightarrow \left(\frac{c}{2} - \frac{h}{8} + \frac{o}{4}\right)\cdot CO_2 + \left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4}\right)\cdot CH_4 \qquad (F.6)$$

The equation reflects algebraically that the higher the oxidation state of the carbon in the organic substrate, the lower the proportion of methane in the biogas (Figure F.4).

An example of the calculation is presented hereafter.

The formula for **starch** is $C_6H_{10}O_5$, which gives the following values of the constants in equation F.6:

$$c = 6;\ h = 10;\ o = 5$$

which in turns results in the theoretical gas composition of:

$$2\cdot CO_2 : 2\cdot CH_4$$

This implies that the biogas produced from the anaerobic degradation of starch will be a 1:1 mixture of methane and carbon dioxide.

McCarty (1964) advanced this equation more comprehensively, as follows:

$$C_cH_hO_oN_n + \left(2c + n - o - \frac{9sd}{20} - \frac{ed}{4}\right)\cdot H_2O \rightarrow$$

$$\left(c - n - \frac{sd}{5} - \frac{de}{8}\right)\cdot CO_2 + \frac{de}{8}\cdot CH_4 + \frac{sd}{20}\cdot C_5H_7O_2N + \left(n - \frac{sd}{20}\right)\cdot\left(NH_4^+ + HCO_3^-\right) \qquad (F.7)$$

where:

$$d = 4c + h - 2on - 3n$$

s and e indicate the fraction of COD synthesized and converted to methane respectively.

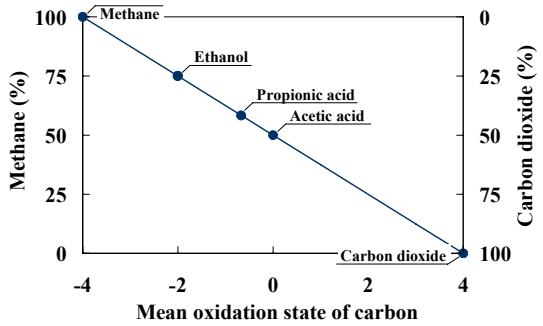


Figure F.4 – The theoretical composition of the biogas is a function of the mean oxidation state of carbon in the substrate (adapted from: Gujer and Zehnder, 1983)

ANNEXURE G

Results and Discussion

This section contains the details of the experiments described in Chapter 4.

G.1 SERUM BOTTLE TESTS

The following tests are included in this Section.

- Conventional toxicity (ATA) and biodegradability tests (BMP) on landfill leachates (Section G.1.1).
- Conventional toxicity and biodegradability tests on sizing textile effluent (Section G.1.2).
- An improved toxicity and biodegradability test was conducted on a synthetic dye effluent prepared in the laboratory (Section G.1.3); the operating conditions for this test differed from the conditions prescribed for a serum bottle activity test, but yet represented an improvement of the conventional toxicity or biodegradability protocols.

  The results are particularly interesting in that they clearly indicate that the biomass was eventually able to adapt to the initially inhibiting substances present in the effluent.
- Anaerobic activity tests (AAT) on industrial effluents, namely size, distillery, scour and dye effluents: three tests (Sections G.1.4 to G.1.6) were aimed at assessing the biodegradability and toxicity of the effluents in isolation; two tests (Sections G.1.7 and G.1.8) looked at the possible beneficial effects, in terms of biodegradability and toxicity of digesting two effluents in combination.
- Furthermore, the outcomes of the control units in the five AAT is compared in Section G.1.9 to assess the reproducibility of an activity assay conducted using the serum bottle method: in fact, the source of the seed inoculum had been consistent throughout the five tests, the only differences being in the way the sludge samples were handled.
- Lastly, a method for the accurate calculation of the mass balance in a serum bottle activity test is discussed in Section G.1.10.

Note on the calculation of the activity of the biomass

When the composition of the off-gas is not measured, the mass balance of equation B.4 cannot be used to calculate the specific methanogenic activity of the seed inoculum.

This occurred in the conventional toxicity and biodegradability (Sections G.1.1 and G.1.2) tests, where the gas composition was not determined at all or discontinuously.

Under these circumstances, one can only report the activity of the biomass, in terms of activity of the sludge as a whole, i.e. as the rate of biogas production (mℓ of biogas/d or mℓ of biogas/g VS/d). This information does not univocally translate into a corresponding *methanogenic* activity, which is the quantity of interest, because the knowledge of the methane fraction of the biogas is missing.

This has a further consequence: the comparison of the performance of the seed inocula between different tests is virtually impossible.

Assuming that the gas composition evolves in the same way for the same test material, or in other words, assuming that the gas composition only depends on the type of test material and not or to a negligible extent on its concentration, one could consider that the estimation of the activity of the sludge, as the rate of total gas production, is sufficient to characterise the effect of the test material, hence to assess its toxicity. This assumption is questionable in many instances, as shown in the case of the distillery effluent and is certainly not acceptable when different test materials are considered.

This in principle would make the comparison of the effect of different test materials virtually impossible: one could only compare the *relative* effects, as reduction of the reference activity of the sludge, but not the *absolute* activity displayed by the sludge in presence of one or the other test material.

G.1.1 Conventional toxicity and biodegradability tests on landfill leachates

This Section presents the detailed results of the conventional ATA and BMP conducted on the three landfill leachates (i.e. Holsfontein, Aloes and Shongweni). Refer to Sections 4.1.1.1 and 4.1.1.2 for an extensive discussion.

A consistent template has been used to report the outcomes of these assays.

Firstly, the composition of the serum bottles, for each test is reported in form of table, by listing all *constituents* and a few *parameters* of interest that characterise each group of replicates. Each column in these tables indicates a group of three replicates.

In the following pages, the outcomes of the control units and of each level of concentration of the test materials are reported separately in graphical form. Two sets of plots are presented.

- For the toxicity tests (Section G.1.1.1), the biogas production curves and the sludge activity curves, as the rate of total biogas production (mℓ/d), as explained above. The composition of the biogas was only measured at the end of the test. This single determination, together with the short duration of the conventional ATA tests, is not sufficient to perform meaningful calculations of the Specific Methanogenic Activity (SMA), which in fact is the key-parameter when toxicity is to be assessed. Therefore, plots of the biogas composition and of the volume of methane produced can not be reported (as it is recommended in the protocol proposed in Annexure B).
- For the biodegradability tests (Section G.1.1.2), the biogas production and the biogas composition curves. The composition of the biogas was measured discontinuously, on a weekly basis. This is sufficient to calculate a COD mass balance, thus to estimate the extent of biodegradation achieved by the test materials, following the methodology discussed in Chapter 4.

Unlike in Chapter 4, where average values are plotted, these plots show each replicate individually, to visually evaluate the reproducibility of the results.

The rationale of maintaining the same scale for the series of plots is to facilitate the comparison between groups, despite the fact that in some instances details might disappear.

Table G.2 – Composition of the serum bottles for the toxicity tests on the landfill leachates.

Constituents		**Control**	**0.04 %**	**0.4 %**	**4 %**	**10 %**	**20 %**	**40 %**
Substrate [(a)]	mℓ	2	2	2	2	2	2	2
NMS	mℓ	28	28	28	28	28	28	28
Sludge	mℓ	30	30	30	30	30	30	30
Effluent	mℓ	0	0.04	0.4	4	10	20	40
Total volume [(b)]	mℓ	100	100	100	100	100	100	100
Ratio v/v	%	-	**0.04**	**0.4**	**4**	**10**	**20**	**40**
Test No 1: Holsfontein leachate								
Organic m.	g COD/ℓ	0.86	0.88	1.1	3.6	7.7	14.5	28.1
Solids	g VS/ℓ	5	5	5	5	5	5	5
S-to-X	-	0.16	0.17	0.21	0.7	1.4	2.7	5.2
Test No 2: Aloes leachate								
Organic m.	g COD/ℓ	0.86	0.86	0.93	1.6	2.7	4.5	8.2
Solids	g VS/ℓ	5	5	5	5	5	5	5
S-to-X	-	0.16	0.16	0.17	0.3	0.5	0.8	1.5
Test No 3: Shongweni leachate								
Organic m.	g COD/ℓ	0.86	0.86	0.87	0.97	1.1	1.4	2.0
Solids	g VS/ℓ	5	5	5	5	5	5	5
S-to-X	-	0.16	0.16	0.16	0.18	0.2	0.3	0.4

[(a)] Acetate/propionate mixture: 75 mg of sodium acetate + 25.6 mg of sodium propionate per 100 mℓ.
[(b)] The remaining volume was filled with distilled water.

Table G.3 – Composition of the serum bottles for the biodegradability tests on the landfill leachates.

Constituents		**Control**	**Holsfontein**	**Aloes**	**Shongweni**
Substrate	mℓ	0	0	0	0
NMS	mℓ	70	66	66	60
Sludge	mℓ	30	30	30	30
Effluent	mℓ	0	4	4	10
Total volume	mℓ	100	100	100	100
Ratio v/v	%	-	**4**	**4**	**10**
Organic m.	g COD/ℓ	-	2.7	0.7	0.28
Solids	g VS/ℓ	5	5	5	5
S-to-X	-	-	0.54	0.15	0.06

G.1.1.1 Toxicity assays

Holsfontein leachate

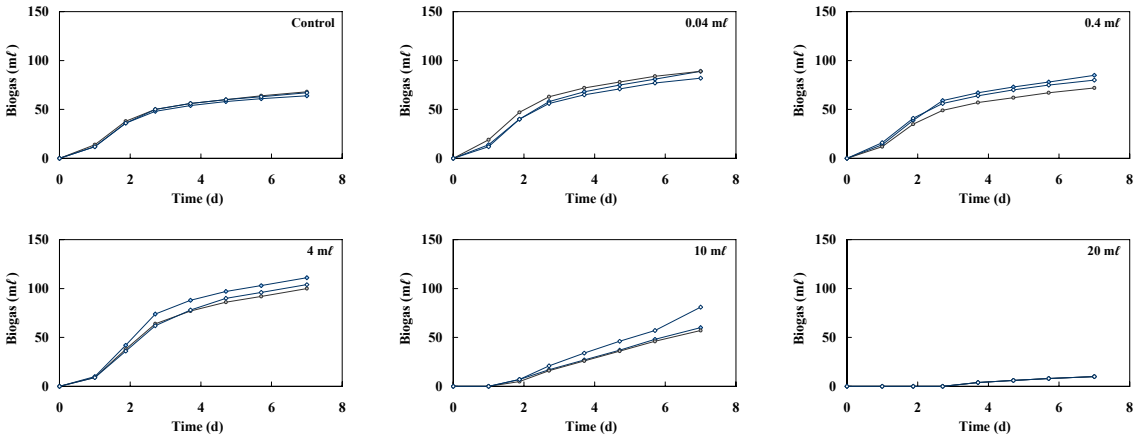


Figure G.1 – Biogas production.

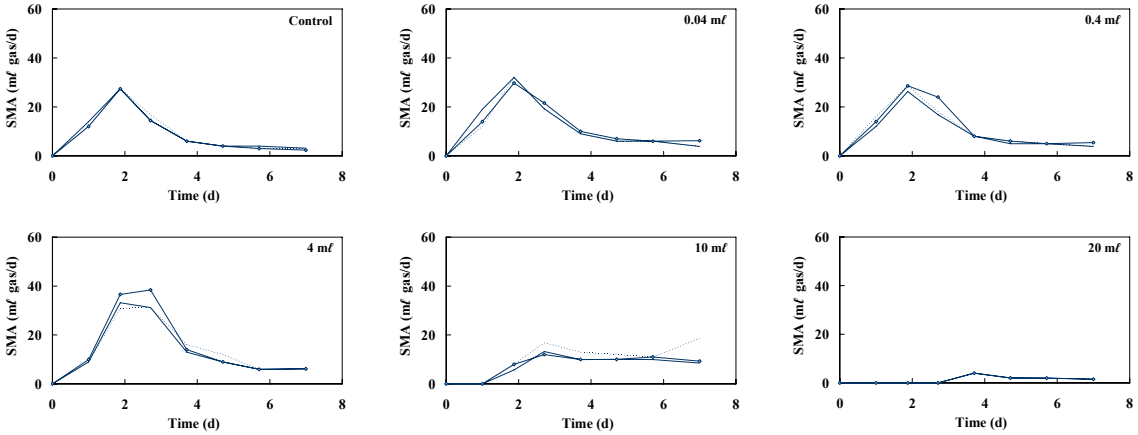


Figure G.2 – Activity of the sludge.

Plots for the 40 % v/v concentration are not included, because the gas production was negligible throughout the test period.

Aloes leachate

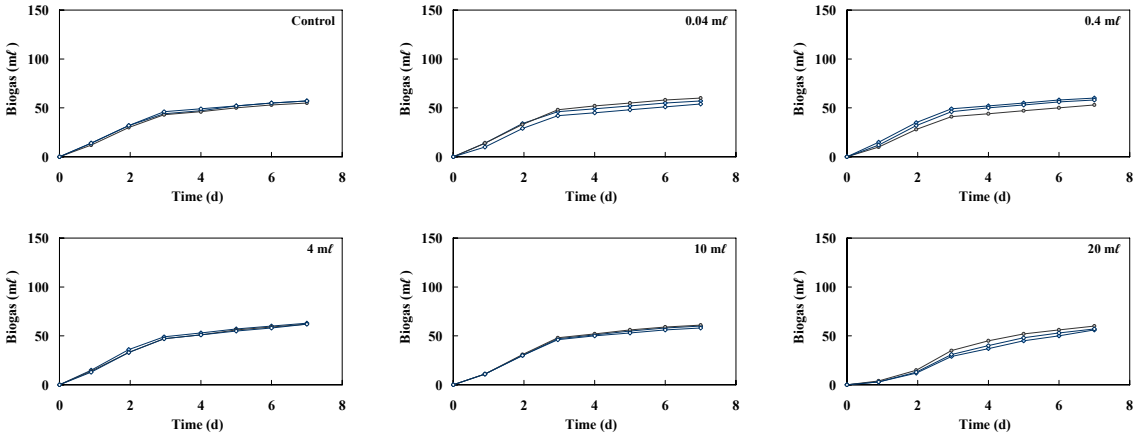


Figure G.3 – Biogas production.

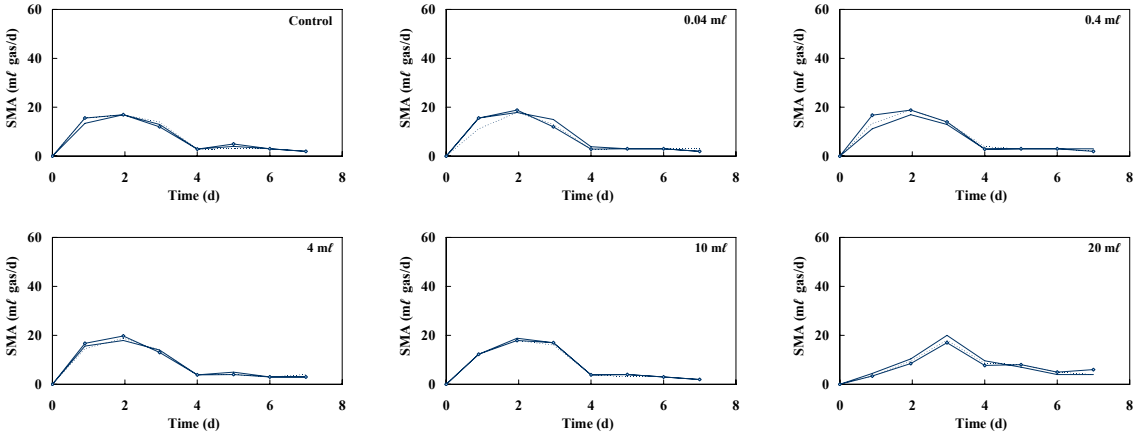


Figure G.4 – Activity of the sludge.

Plots for the 40 % v/v concentration are not included, because the gas production was negligible throughout the test period.

Shongweni leachate

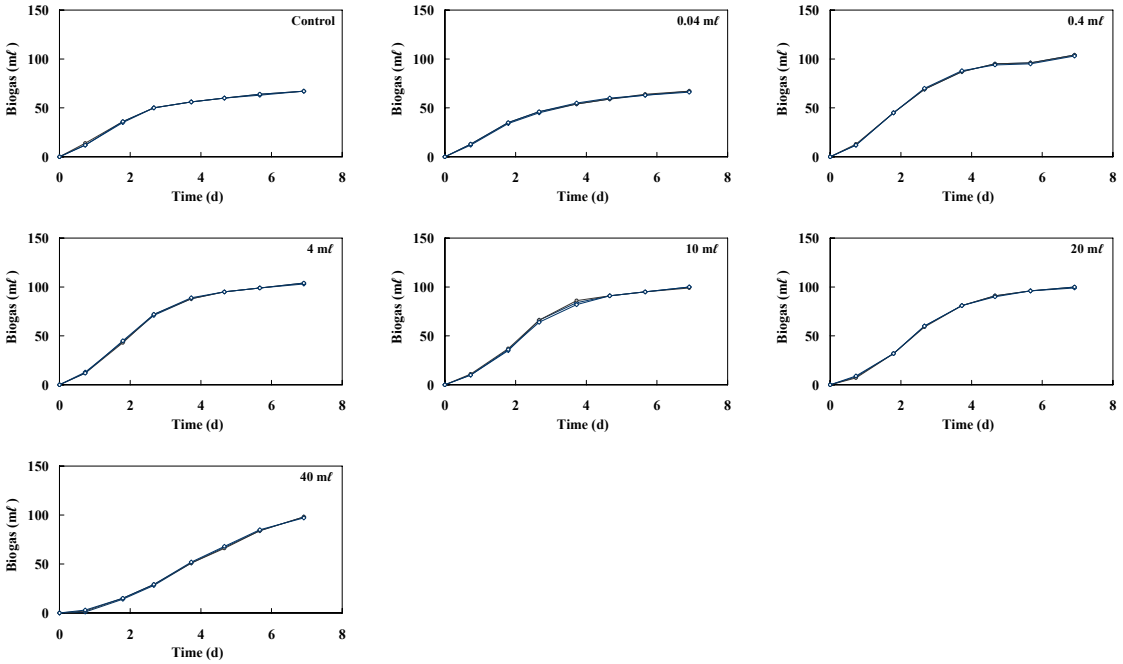


Figure G.5 – Biogas production.

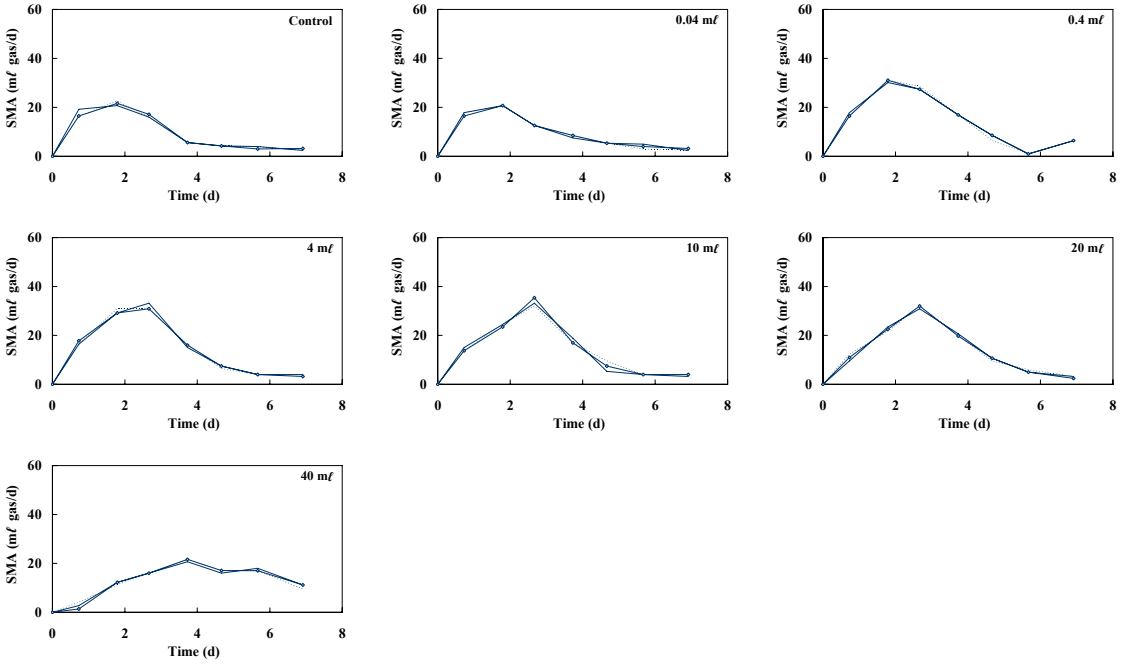
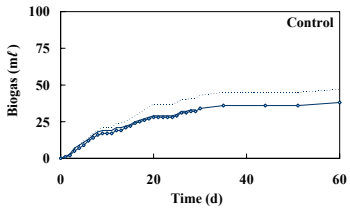


Figure G.6 – Activity of the sludge.

G.1.1.2 *Biodegradability assays*

Holsfontein leachate



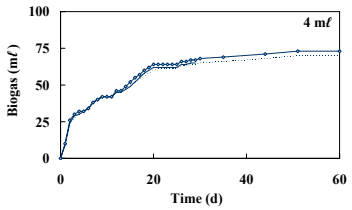
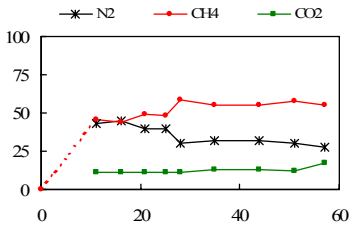


Figure G.7 – Biogas production.



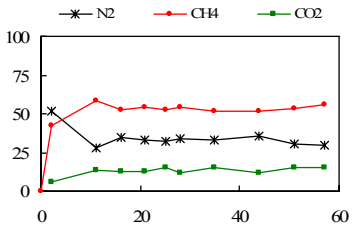
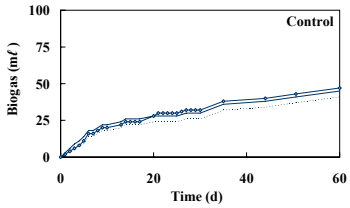


Figure G.8 – Gas composition.

Aloes leachate



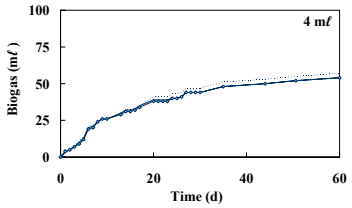
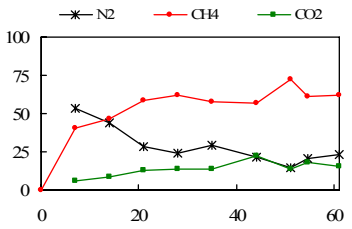


Figure G.9 – Biogas production.



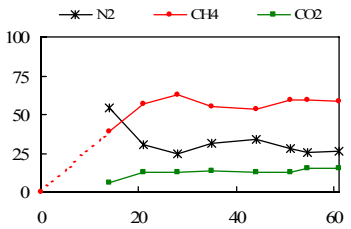


Figure G.10 – Gas composition.

Shongweni leachate

Figure G.11 – Biogas production.

Figure G.12 – Gas composition.

G.1.2 Conventional toxicity and biodegradability tests on textile effluents

This Section presents the detailed results of the conventional ATA and BMP conducted on the sizing textile effluent labelled 'Recipe No 2' and on its individual components, i.e. wax, PVA and starch. Refer to Sections 4.1.1.3 and 4.1.1.4 for an extensive discussion.

The composition of the serum bottles for each test is reported in form of table; the outcome of each group of serum bottles for the controls and for the test materials is reported separately, in graphical form. Two sets of plots are presented, i.e. the biogas production curves and the biomass activity (for the ATA tests); the biogas production curves and the gas composition (for the BMP tests): the gas composition was determined on one unit only per group of replicates and therefore may show unexpected profiles. The activity of the sludge was not calculated for the BMP test, because the gas composition was measured discontinuously and the frequency of one value per week does not allow a meaningful calculation of a rate of methane production.

Table G.4 – Composition of the serum bottles for the toxicity test on the sizing effluent 'Recipe No 2'.

Constituents		**Control**	**0.4 %**	**4 %**	**10 %**	**20 %**	**40 %**
Substrate [(a)]	mℓ	2	2	2	2	2	2
NMS	mℓ	28	28	28	28	28	28
Sludge	mℓ	30	30	30	30	30	30
Effluent	mℓ	0	0.4	4	10	20	40
Total volume [(b)]	mℓ	100	100	100	100	100	100
Ratio v/v	%	-	**0.4**	**4**	**10**	**20**	**40**
Sizing effluent (mixture)							
Organic m.	g COD/ℓ	0.86	1.2	4.1	9.0	17.1	33.3
Solids	g VS/ℓ	5	5	5	5	5	5
S-to-X	-	0.16	0.22	0.8	1.7	3.2	6.2
Starch							
Organic m.	g COD/ℓ	0.86	1.0	2.4	4.8	8.7	16.5
Solids	g VS/ℓ	5	5	5	5	5	5
S-to-X	-	0.16	0.19	0.45	0.9	1.6	3.1
Wax							
Organic m.	g COD/ℓ	0.86	0.88	1.0	1.3	1.7	2.6
Solids	g VS/ℓ	5	5	5	5	5	5
S-to-X	-	0.16	0.16	0.19	0.24	0.32	0.49
PVA							
Organic m.	g COD/ℓ	0.86	1.1	2.9	6.0	11.1	21.3
Solids	g VS/ℓ	5	5	5	5	5	5
S-to-X	-	0.16	0.20	0.54	1.1	2.1	4.0

[(a)] Acetate/propionate mixture: 75 mg of sodium acetate + 25.6 mg of sodium propionate per 100 mℓ.

Table G.5 – Composition of the serum bottles for the biodegradability test on the sizing effluent 'Recipe No 2'.

Constituents		**Control**	**Reference**	**Mixture**	**Wax**	**PVA**	**Starch**
Substrate [(a)]	mℓ	0	2	0	0	0	0
NMS	mℓ	30	30	30	30	30	30
Sludge	mℓ	70	68	66	66	60	60
Effluent	mℓ	0	0	4	4	10	10
Parameters							
Total volume [(b)]	mℓ	100	100	100	100	100	100
Ratio v/v	%	-	-	**4**	**4**	**10**	**10**
Organic m.	g COD/ℓ	-	0.86	4.1	1.0	6.0	4.7
Solids	g VS/ℓ	5	5	5	5	5	5
S-to-X	-	-	0.16	0.8	0.21	1.2	0.9

G.1.2.1 Toxicity assays

Sizing effluent No 2: mixture

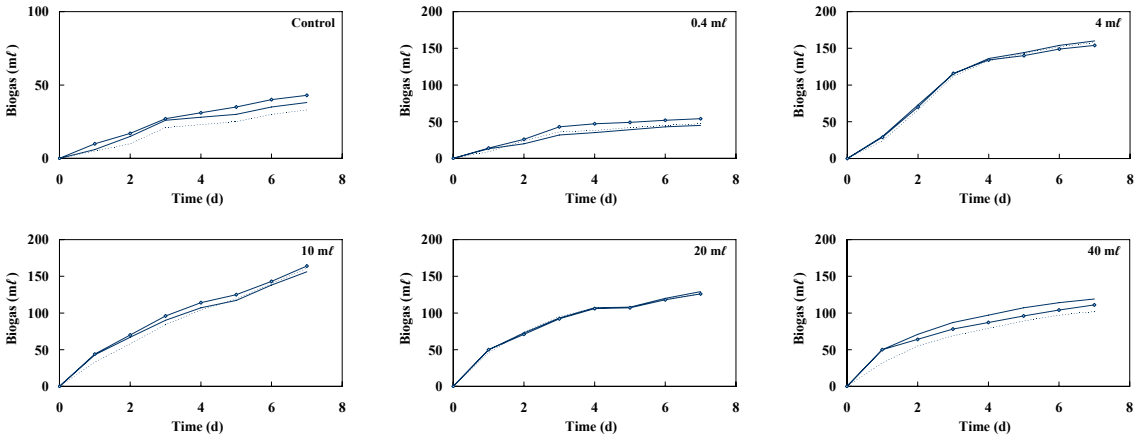


Figure G.13 – Biogas production.

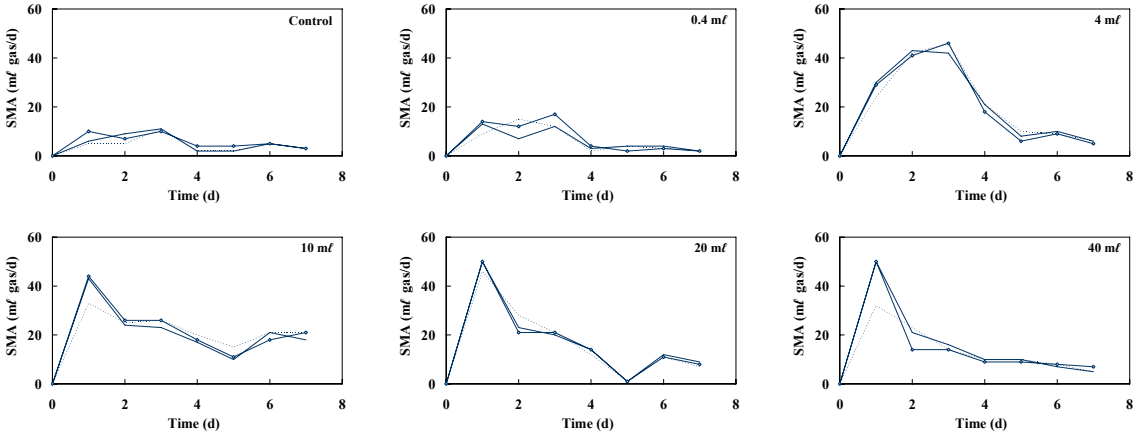


Figure G.14 – Activity of the sludge.

Sizing effluent No 2: starch

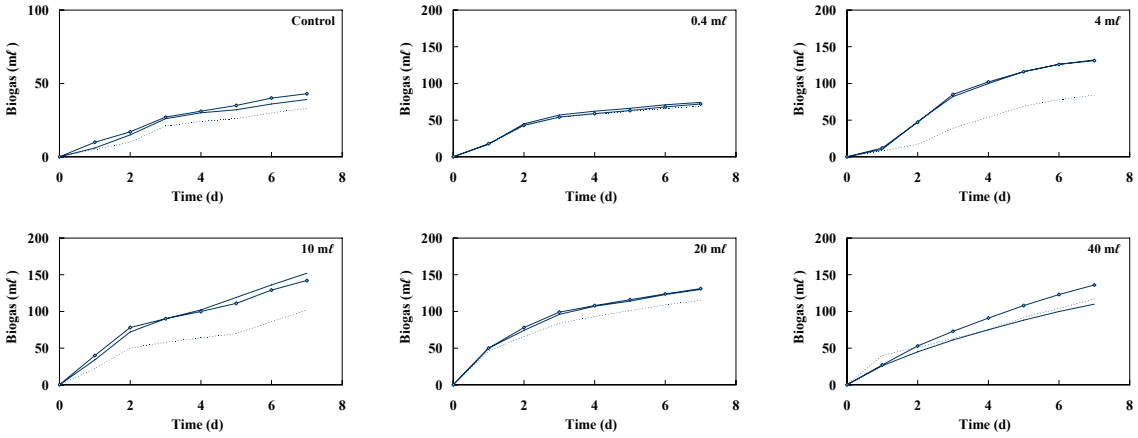


Figure G.15 – Biogas production.

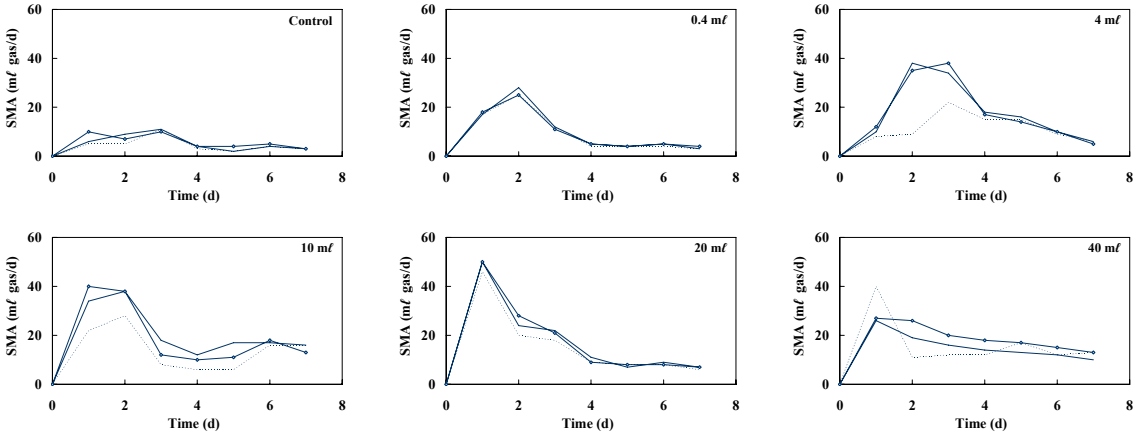


Figure G.16 – Activity of the sludge.

Sizing effluent No 2: wax

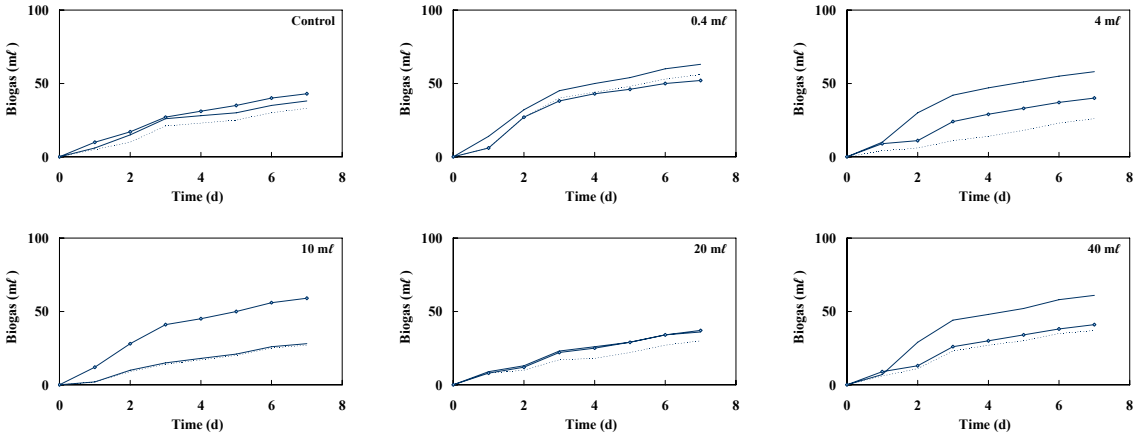


Figure G.17 – Biogas production.

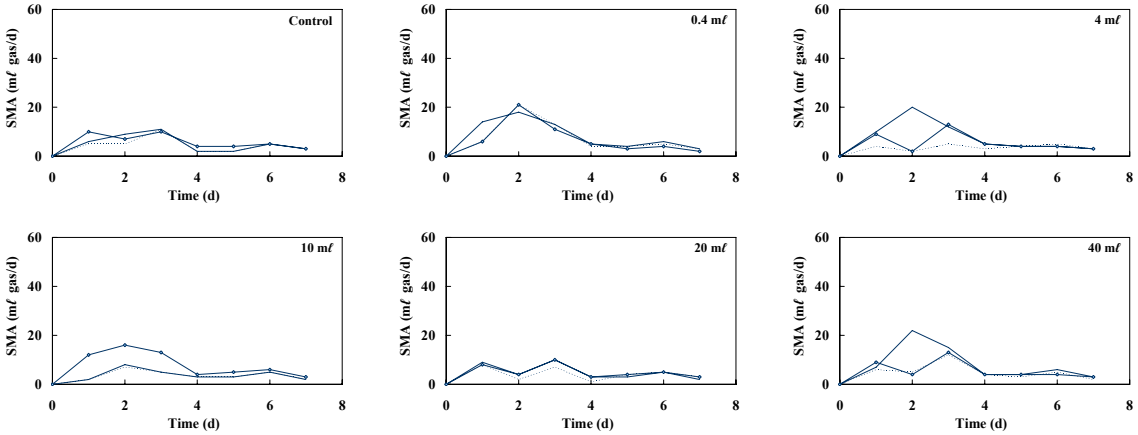


Figure G.18 – Activity of the sludge.

Sizing effluent No 2: polyvinyl alcohol (PVA)

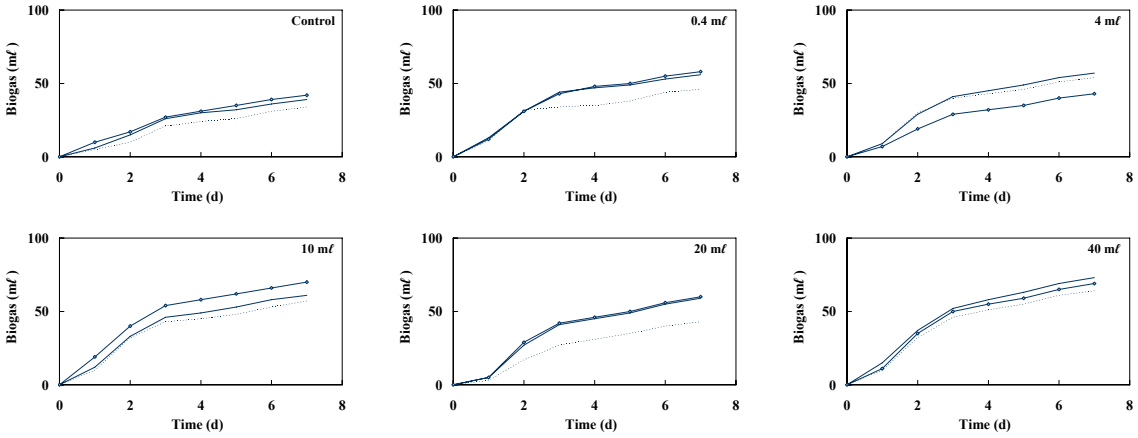


Figure G.19 – Biogas production.

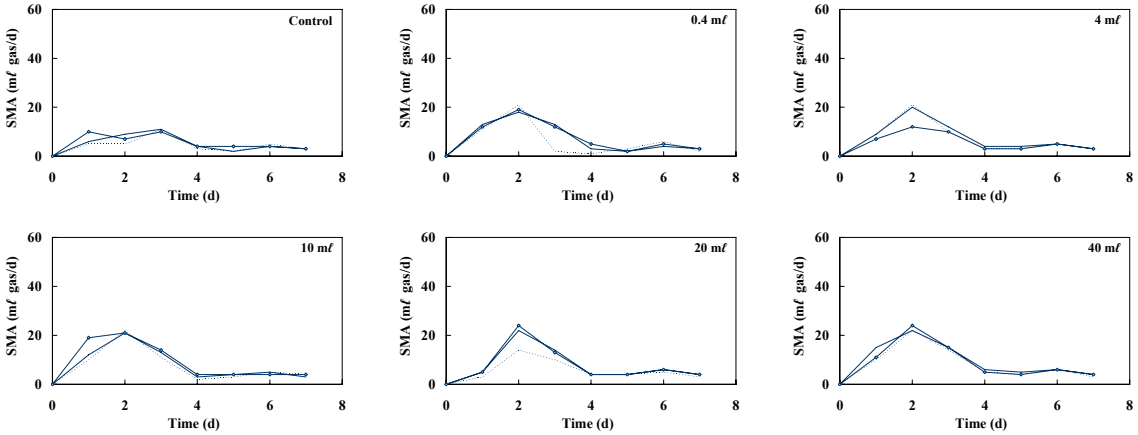


Figure G.20 – Activity of the sludge.

G.1.2.2 *Biodegradability assays*

Sizing effluent No 2

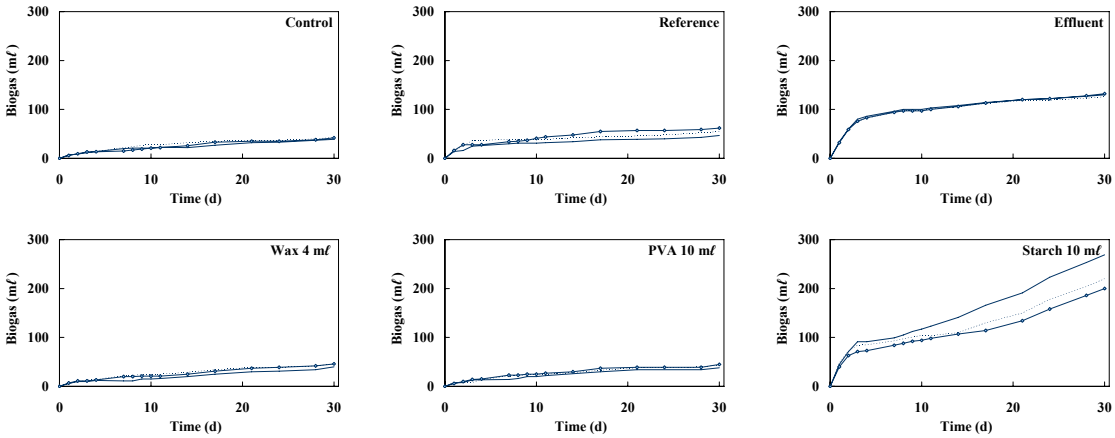


Figure G.21 – Biogas production.

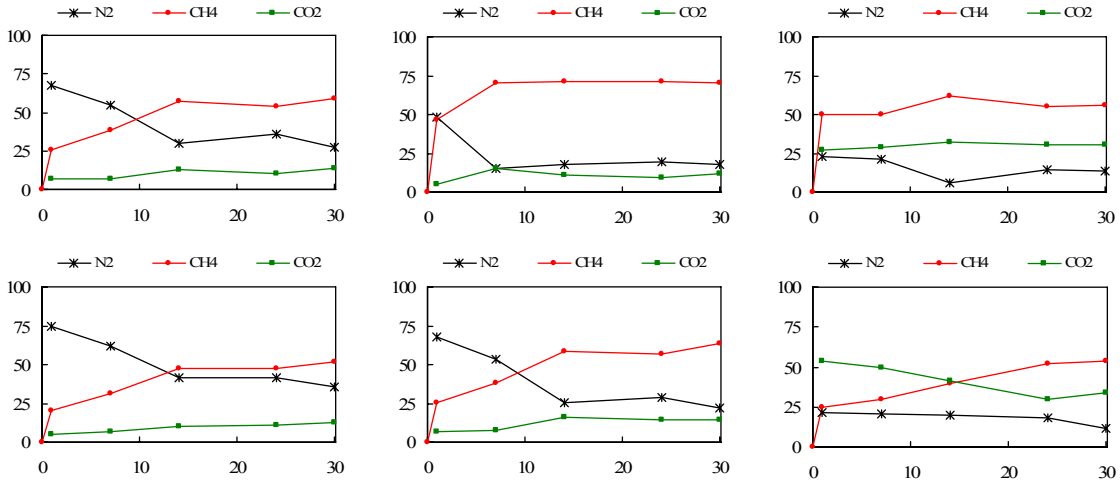


Figure G.22 – Gas composition.

G.1.3 Improved activity test: synthetic dye effluent

This section present the detailed output of the activity test conducted on a synthetic dyeing effluent (Section 4.1.2). Four sets of plots are shown, for each group of serum bottles, i.e. controls, 10 % and 25 % v/v: the cumulative biogas production; the net cumulative methane production; the gas composition; and the specific methanogenic activity.

The following table reports the composition of each group of serum bottle, along with a few summary indicators which can be compared to the guidelines for the set-up of an activity test, discussed in Section B.2.

Table G.6 – Composition of the serum bottles for the toxicity test on synthetic dye effluent.

Constituents		**Controls**	**10 %**	**25 %**
Acetate	g	1.00	1.00	1.00
NMS	mℓ	0	0	0
Sludge	mℓ	100	90	75
Effluent	mℓ	0	10	25
Parameters				
Total volume	mℓ	100	100	100
Organic m.	g COD/ℓ	4.7	5.1	5.7
Solids	g VS/ℓ	7.5	6.8	5.6
S-to-X	-	0.6	0.7	0.8
Ratio v/v	%	-	**10**	**25**

Control units	Dye: 10 %	Dye: 25 %

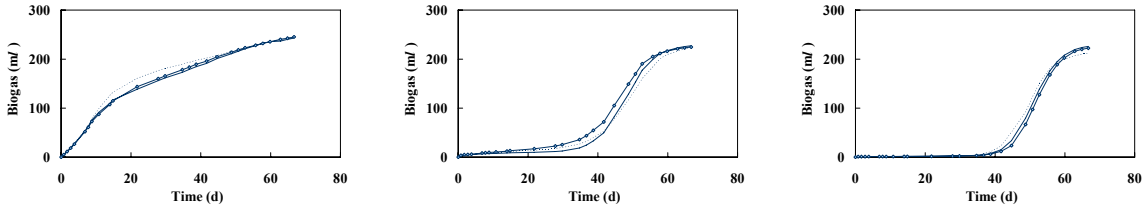


Figure G.23 – Biogas production.

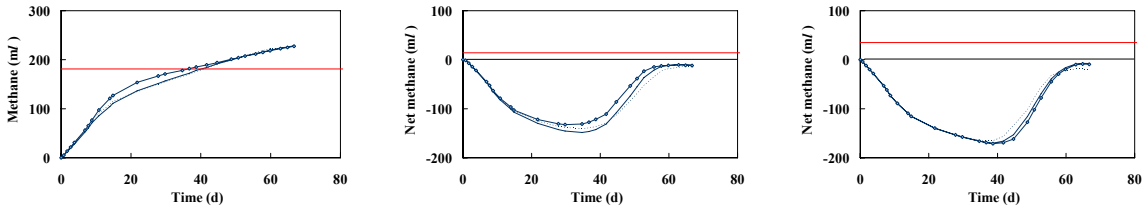


Figure G.24 – Methane production.

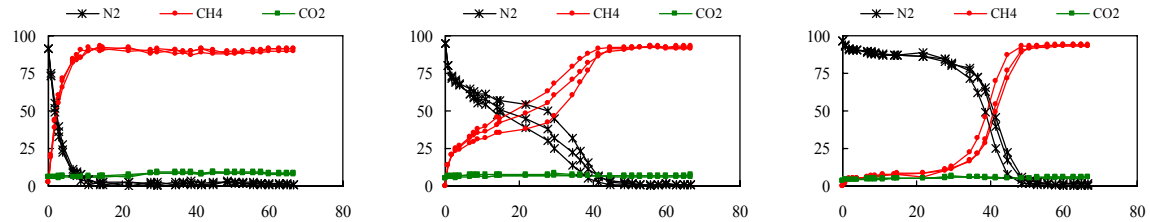


Figure G.25 – Gas composition.

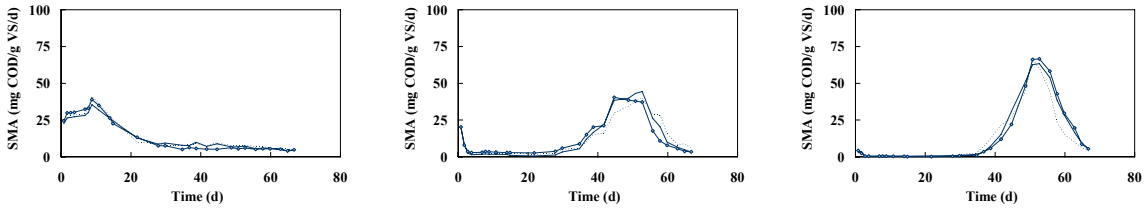


Figure G.26 – Specific methanogenic activity.

G.1.4 Modified anaerobic activity tests: test No 1

This Section presents the detailed results of the first test conducted on the industrial effluents, i.e. size, distillery and scour. Refer to Section 4.1.3.1 for an extensive discussion.

Table G.7 reports the quantities of each components that were added to the vials, in the section *constituents*; and some *parameters* of interest which characterise each group of replicates. Each column in the Table indicates a group of three replicates.

In the following pages, the outcomes of the three control groups and of each test material are reported separately. Four sets of plots are presented, i.e. the biogas production curves; the methane production curves; the gas composition curves and the methanogenic activity curves. The cumulative volume of methane for the test units is calculated as *net* volume.

As discussed in Chapter 4, the AAT was initially designed to evaluate the inhibitory effect of the test materials on the aceticlastic biomass. Therefore, two 'functionally' different types of organic substrate are present in each unit, i.e. acetate as reference and the respective test materials. The biodegradability values reported in Chapter 4 are therefore calculated by comparing the net methane produced to the methane expected based on the test material only. Whereas, the specific methanogenic activity is calculated on each test unit individually (and the inhibition effect can be calculated by comparing this value to the SMA in the control unit).

Table G.7 – Composition of the serum bottles for the toxicity test on industrial effluents (No 1).

Constituents		**Controls**			**Size effluent**			**Distillery effluent**			**Scour effluent**		
Acetate	g	0.35	0	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
NMS	mℓ	10	0	0	10	10	10	10	10	10	10	10	10
Sludge	mℓ	40	40	40	40	40	40	40	40	40	40	40	40
Effluent	mℓ	0	0	0	5	15	35	5	15	35	5	15	35
Parameters													
Total volume	mℓ	50	40	40	55	65	85	55	65	85	55	65	85
Organic m. [(1)]	g/ℓ	5.5	0	6.8	11.9	21.7	34.5	16.1	32.4	53.5	5.3	5.1	4.9
Solids [(2)]	g/ℓ	24	30	30	22	18	14	22	18	14	22	18	14
S-to-X	-	0.2	0	0.2	0.6	1.2	2.5	0.7	1.8	3.8	0.2	0.3	0.3
Ratio v/v	%	-	-	-	**9**	**23**	**41**	**9**	**23**	**41**	**9**	**23**	**41**

[(1)] in COD units;
[(2)] expressed as Volatile Solids (VS).

Control units		
Sludge, acetate and NMS	Sludge only	Sludge and acetate

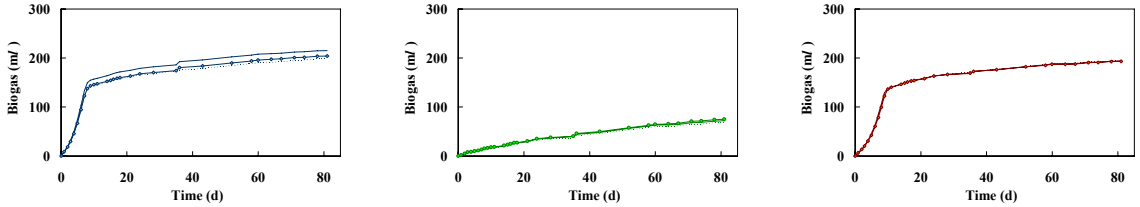


Figure G.27 – Biogas production.

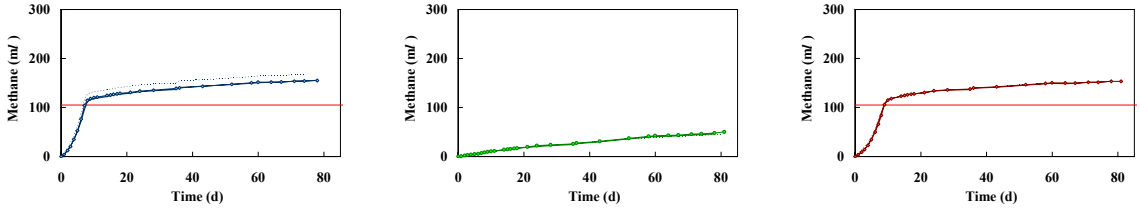


Figure G.28 – Methane production.

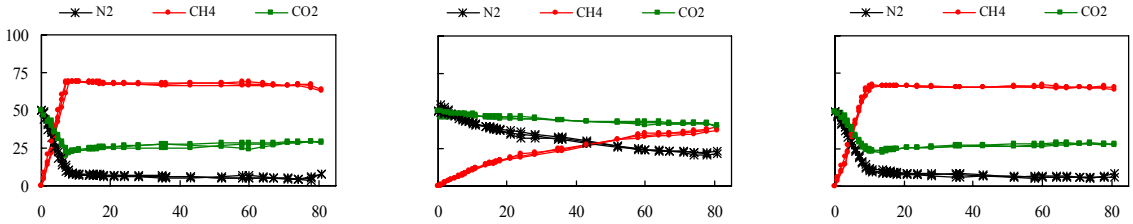


Figure G.29 – Gas composition.

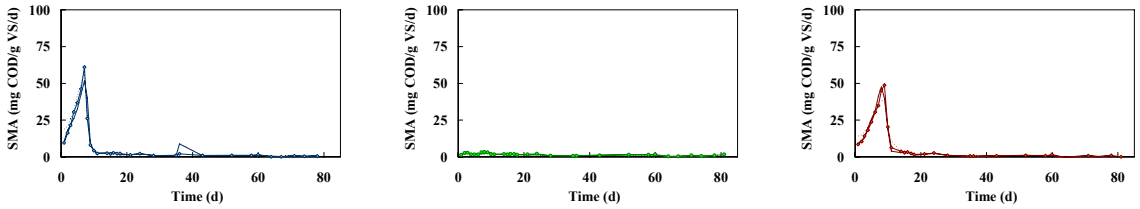


Figure G.30 – Specific methanogenic activity.

Test compound: Size effluent		
Low	Medium	High

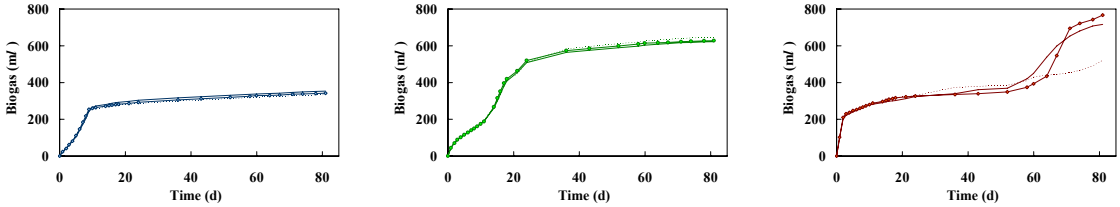


Figure G.31 – Biogas production.

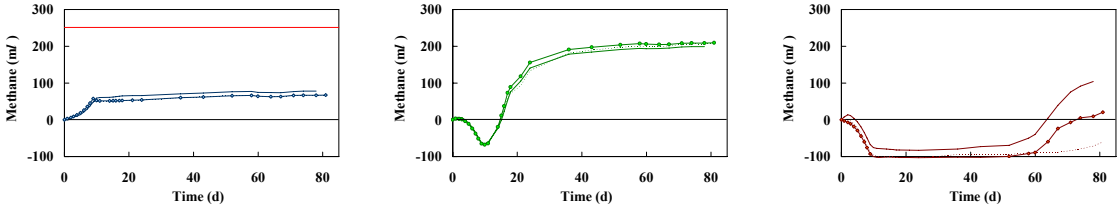


Figure G.32 – Net methane production.

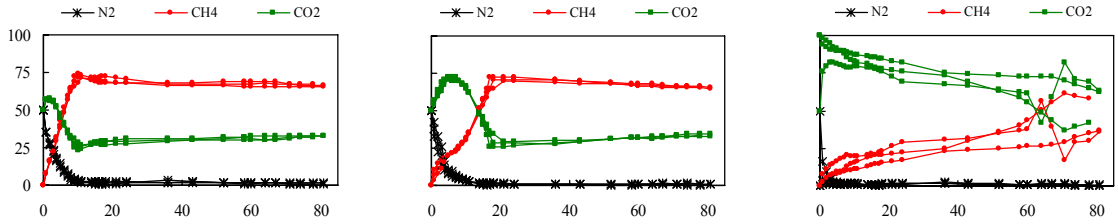


Figure G.33 – Gas composition.

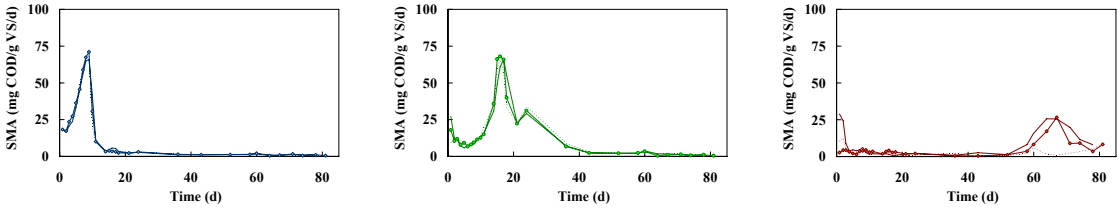


Figure G.34 – Specific methanogenic activity.

Test compound: Distillery effluent		
Low	Medium	High

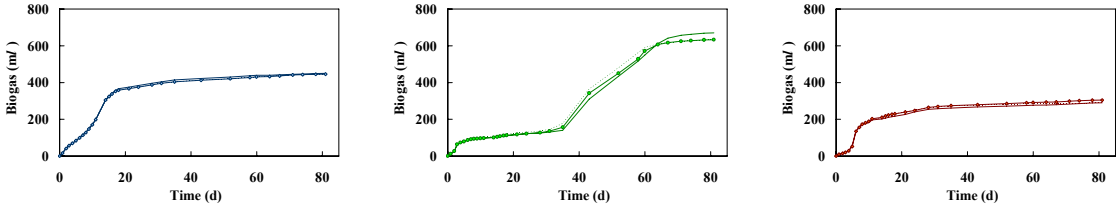


Figure G.35 – Biogas production.

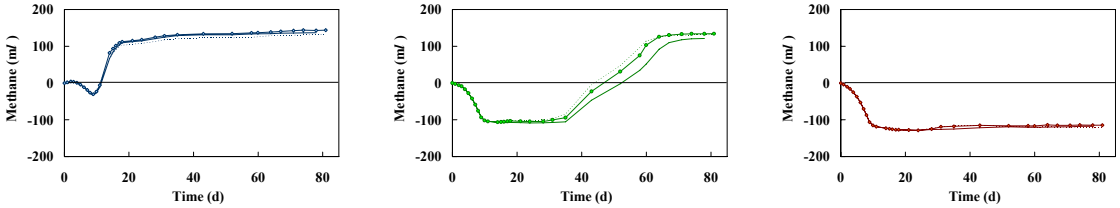


Figure G.36 – Net methane production.

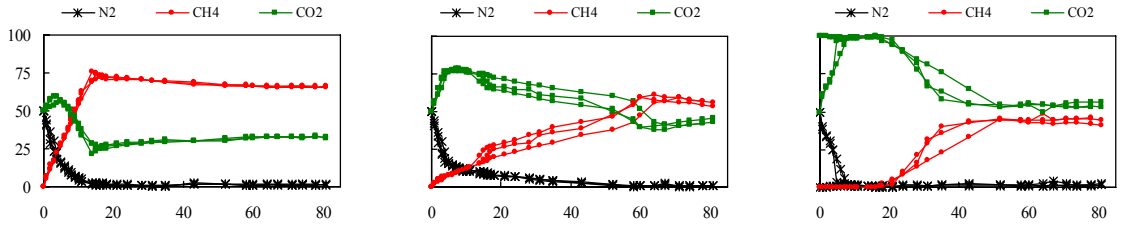


Figure G.37 – Gas composition.

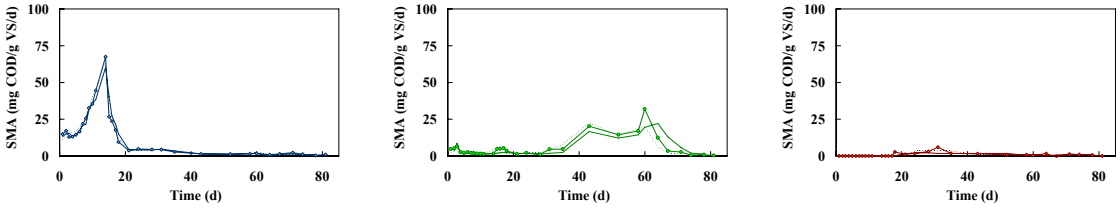


Figure G.38 – Specific methanogenic activity.

Test compound: Scour effluent		
Low	Medium	High

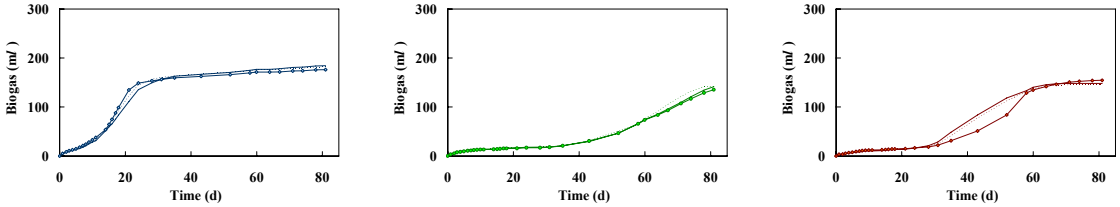


Figure G.39 – Biogas production.

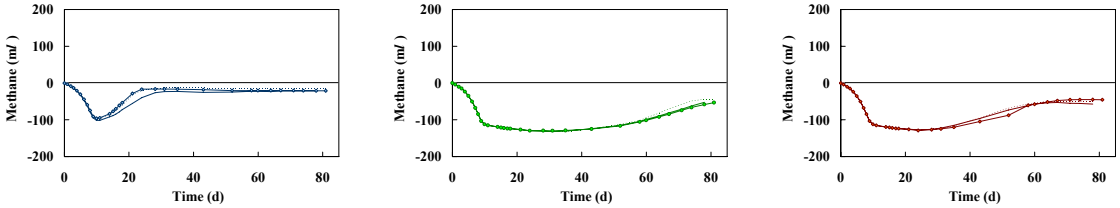


Figure G.40 – Net methane production.

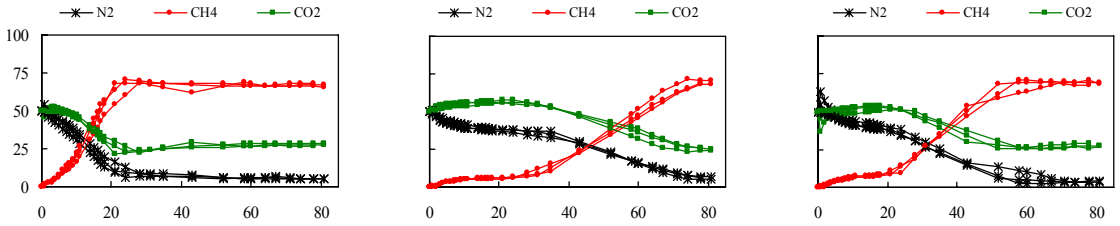


Figure G.41 – Gas composition.

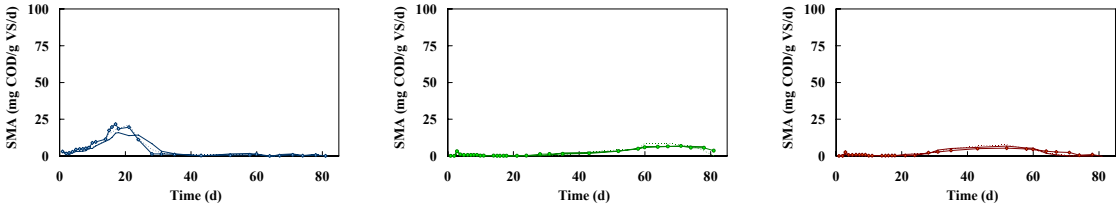


Figure G.42 – Specific methanogenic activity.

G.1.5 Modified anaerobic activity tests: test No 2

The same template described in the previous Section has been adopted, i.e. a Table which summarises the set-up of the vials and the resulting operating conditions, followed by the specific results for each experimental group. Each individual column of the table denotes a set of three identical replicates. The three columns under each heading indicate the three levels of concentration tested, which are termed low, intermediate and high in the following pages.

Table G.8 – Composition of the serum bottles for the toxicity test on industrial effluents (No 2).

Constituents		**Size effluent**			**Distillery effluent**			**Scour effluent**			**Synth. dye effluent**		
Acetate	g	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
NMS	mℓ	10	10	10	10	10	10	10	10	10	10	10	10
Sludge	mℓ	40	40	40	40	40	40	40	40	40	40	40	40
Effluent	mℓ	0.5	2.5	5	0.5	2.5	5	0.5	2.5	5	0.1	0.25	0.5
Parameters													
Total volume	mℓ	50.5	52.5	55.0	50.5	52.5	55.0	50.5	52.5	55.0	50	50	51
Organic m. [(1)]	g/ℓ	6.2	8.8	11.9	6.6	11.0	16.1	5.4	5.4	5.3	5.5	5.5	5.5
Solids [(2)]	g/ℓ	19	19	18	19	19	18	19	19	18	19	19	19
S-to-X	-	0.3	0.5	0.7	0.3	0.6	0.9	0.3	0.3	0.3	0.3	0.3	0.3
Ratio v/v	%	**1.0**	**4.8**	**9.1**	**1.0**	**4.8**	**9.1**	**1.0**	**4.8**	**9.1**	**0.2**	**0.5**	**1.0**

(note: the composition of the control units was identical to the one reported in Table G.1)
(1) in COD units;
(2) expressed as Volatile Solids (VS).

Control units		
Sludge, acetate and NMS	Sludge only	Sludge and acetate

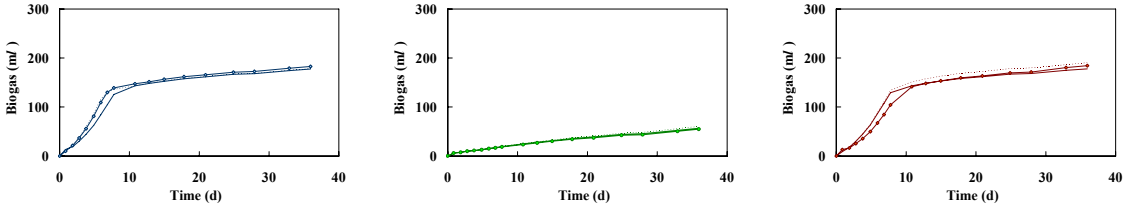


Figure G.43 – Biogas production.

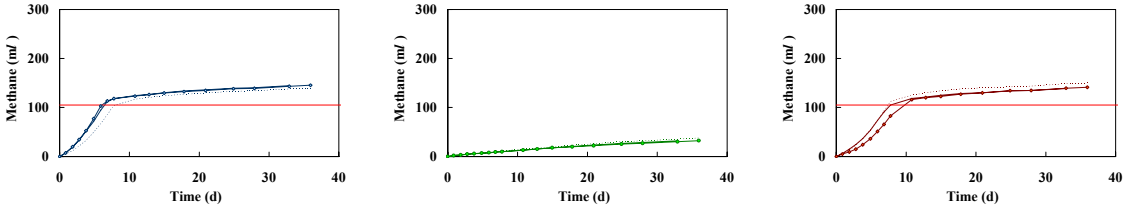


Figure G.44 – Methane production.

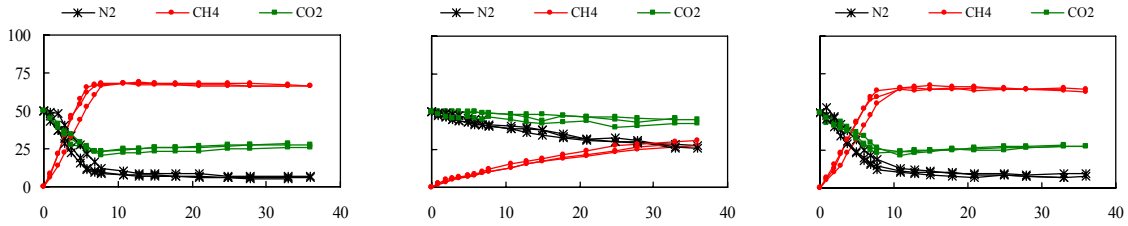


Figure G.45 – Gas composition.

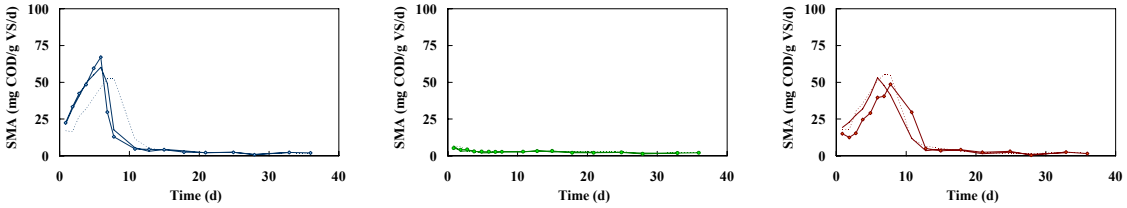


Figure G.46 – Specific methanogenic activity.

Test compound: Size effluent		
Low	Medium	High

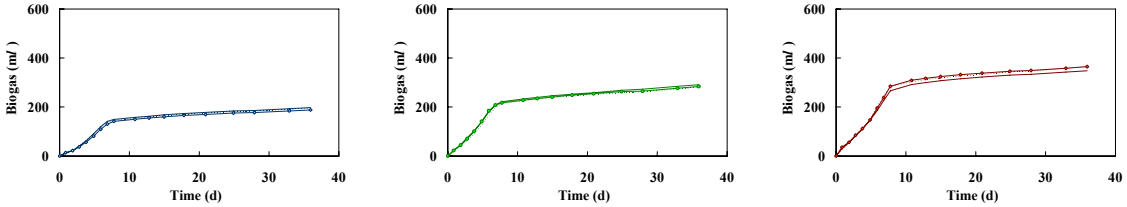


Figure G.47 – Biogas production.

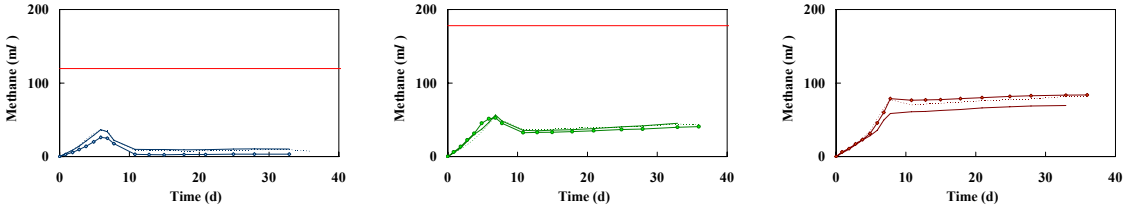


Figure G.48 – Net methane production.

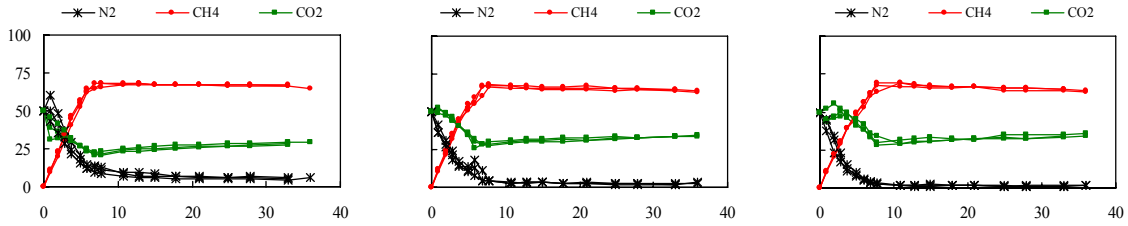


Figure G.49 – Gas composition.

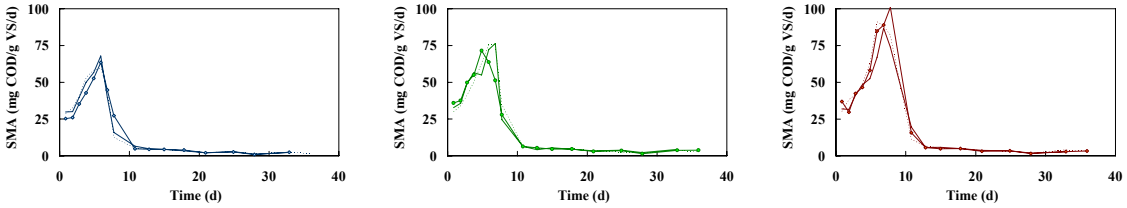


Figure G.50 – Specific methanogenic activity.

Test compound: Distillery effluent		
Low	Medium	High

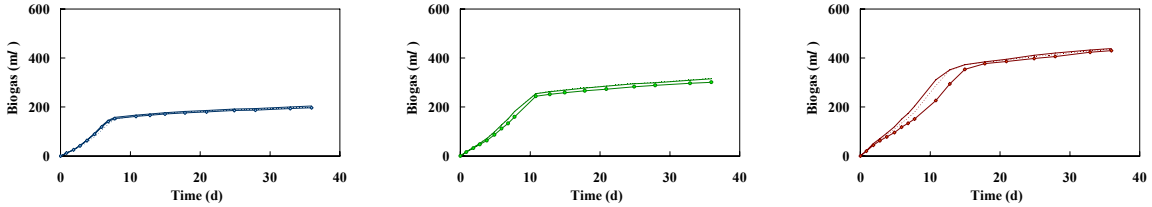


Figure G.51 – Biogas production.

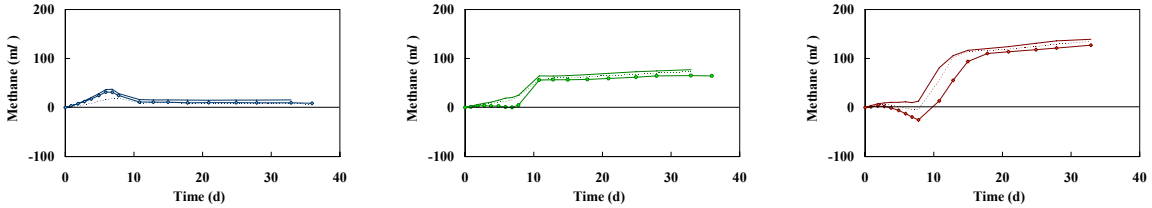


Figure G.52 – Net methane production.

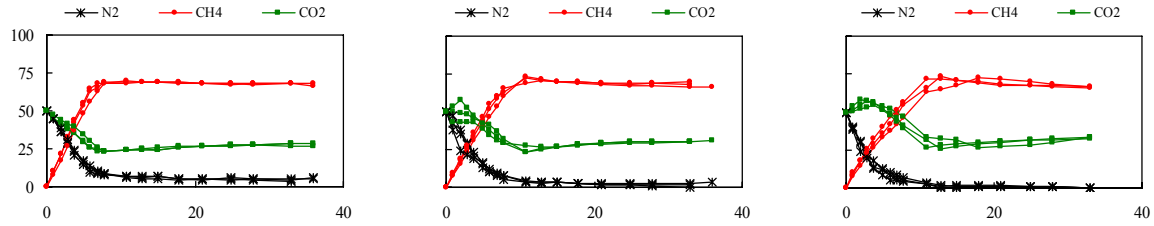


Figure G.53 – Gas composition.

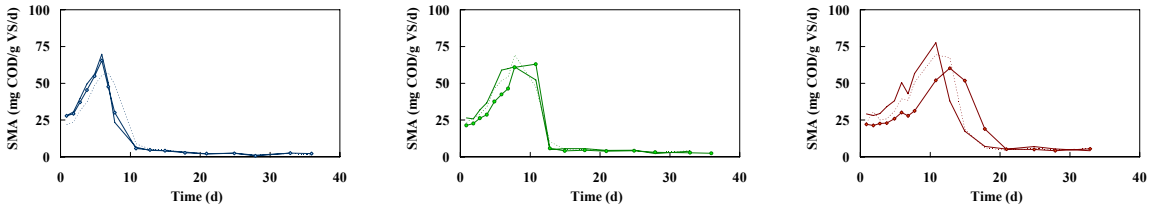


Figure G.54 – Specific methanogenic activity.

Test compound: Scour effluent		
Low	Medium	High

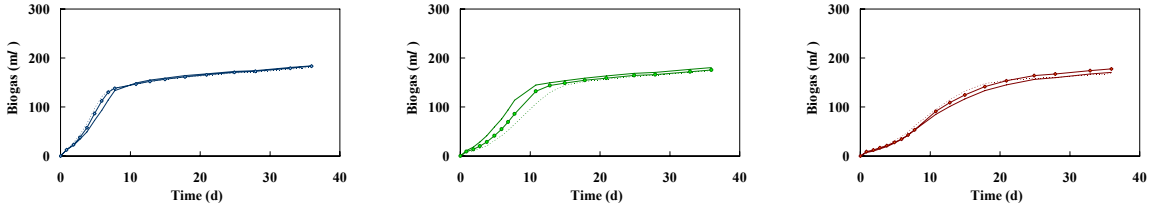


Figure G.55 – Biogas production.

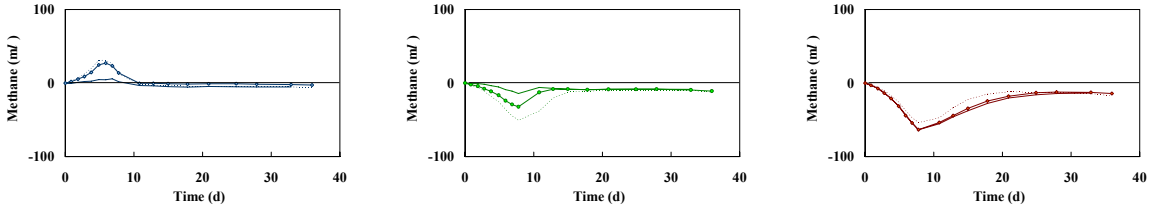


Figure G.56 – Net methane production.

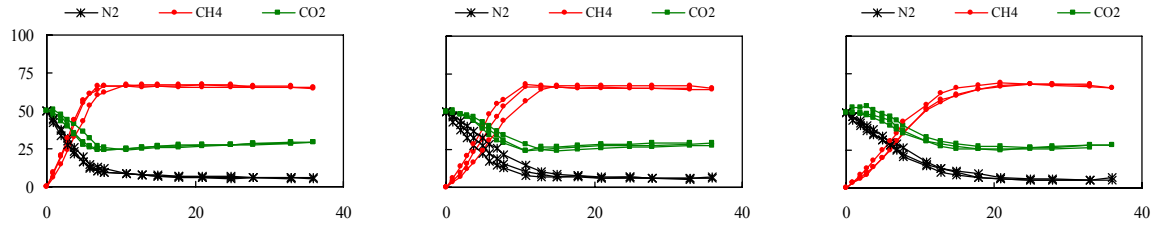


Figure G.57 – Gas composition.

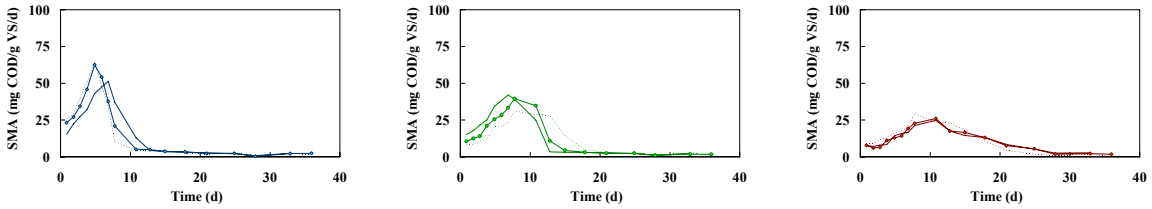


Figure G.58 – Specific methanogenic activity.

Test compound: Synthetic dye effluent		
Low	Medium	High

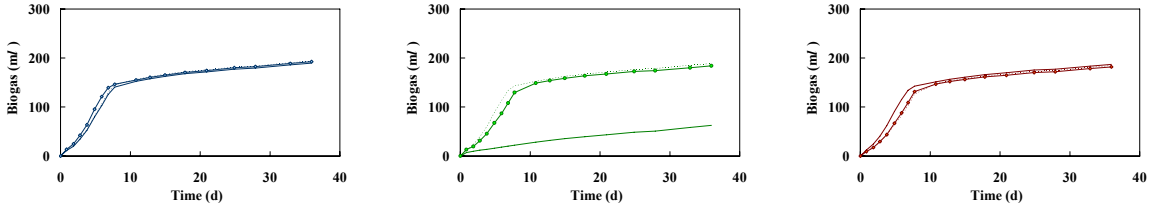


Figure G.59 – Biogas production.

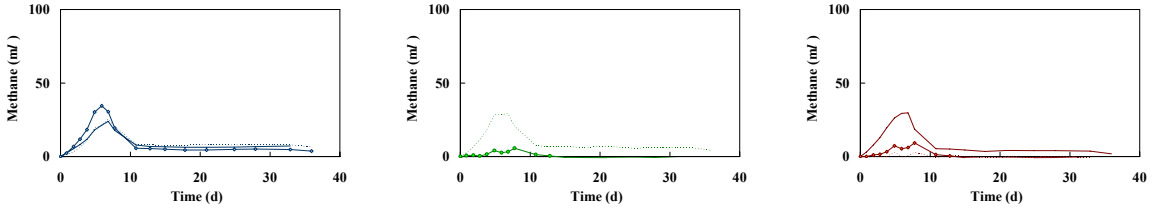


Figure G.60 – Net methane production.

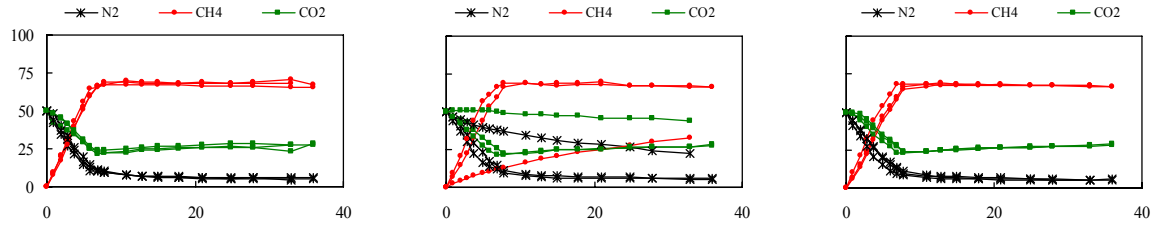


Figure G.61 – Gas composition.

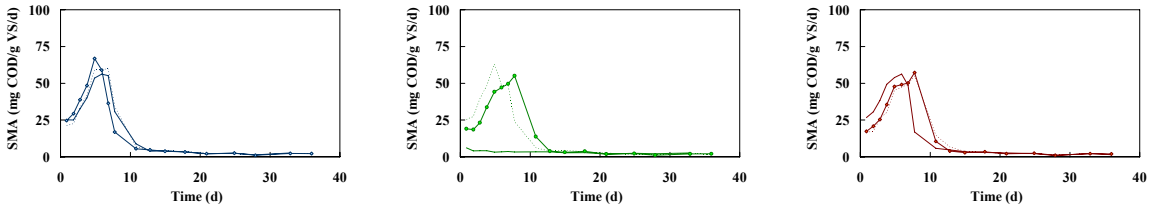


Figure G.62 – Specific methanogenic activity.

G.1.6 Modified anaerobic activity tests: test No 3

The usual template has been adopted and the same rationale is used to organise the Table, which summarises the set-up of the vials and the resulting operating conditions.

The sludge sample used to seed the vials was withdrawn from the Umbilo Wastewater Treatment Plant at a different time, therefore the outcome of this assay is not immediately homogeneous with that of tests No 1 and No 2. In Section G.1.9, the reproducibility of the activity assessment will be evaluated.

Table G.9 – Composition of the serum bottles for the toxicity test on industrial effluents (No 3).

Constituents		**Ctrl**	**Size effluent**			**Distillery effluent**			**1:1 mixture** [a]		
Acetate	g	0	0	0	0	0	0	0	0	0	0
NMS	mℓ	10	10	10	10	10	10	10	10	10	10
Sludge	mℓ	40	40	40	40	40	40	40	40	40	40
Effluent	mℓ	0	0.5	2.5	5.0	0.32	1.6	3.6	0.41	2.0	4.1
Parameters											
Total volume	mℓ	50	50.5	52.5	55.0	50.3	51.6	53.6	50.4	52.0	54.1
Organic m. [1]	g/ℓ	0	0.75	3.6	6.9	0.78	3.8	8.2	0.77	3.7	7.1
Solids [2]	g/ℓ	13.6	13.5	13.0	12.4	13.5	13.2	12.7	13.5	13.1	12.6
S-to-X	-	-	0.06	0.28	0.56	0.06	0.29	0.65	0.06	0.28	0.57
Ratio v/v	%	**-**	**1**	**4.8**	**9.1**	**0.6**	**3.1**	**6.7**	**0.8**	**3.9**	**7.6**

[1] in COD units;
[2] expressed as Volatile Solids (VS);
[a] the volumes of effluent (mℓ) reported indicates the sum of the contributions of the size and distillery effluents, which is calculated as 1:1 on a COD basis. The individual contributions are therefore: (0.25+0.16); (1.25+0.8) and (2.5+1.6) for the size and distillery effluents respectively.

Control units

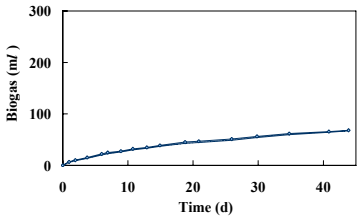


Figure G.63 – Biogas production.

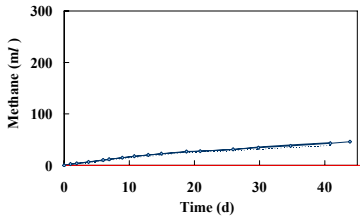


Figure G.64 – Methane production.

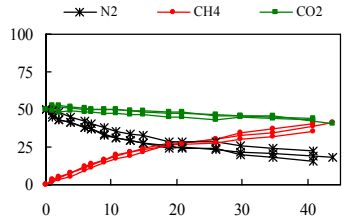


Figure G.65 – Gas composition.

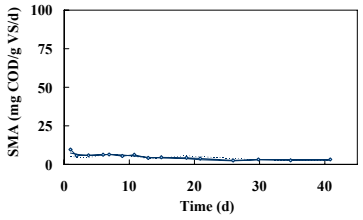


Figure G.66 – Specific methanogenic activity.

Test compound: Size effluent		
Low	Medium	High

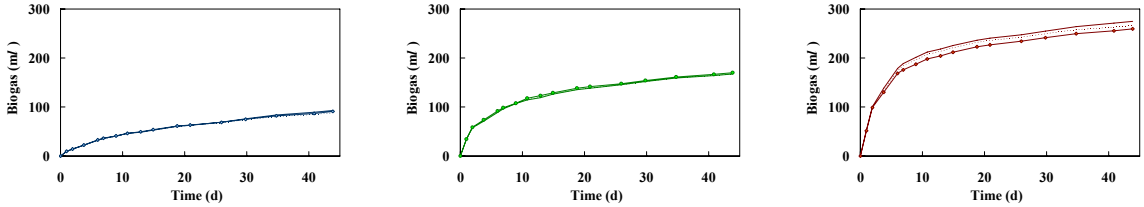


Figure G.67 – Biogas production.

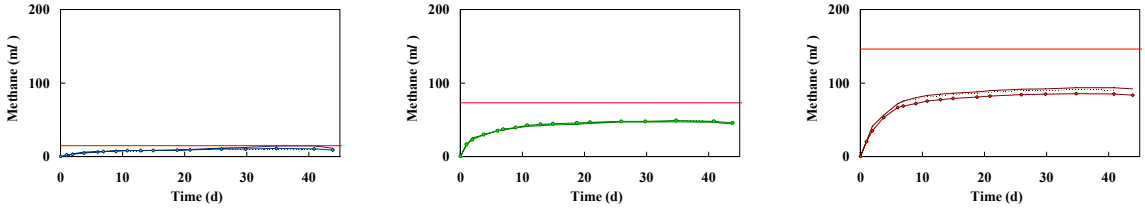


Figure G.68 – Net methane production.

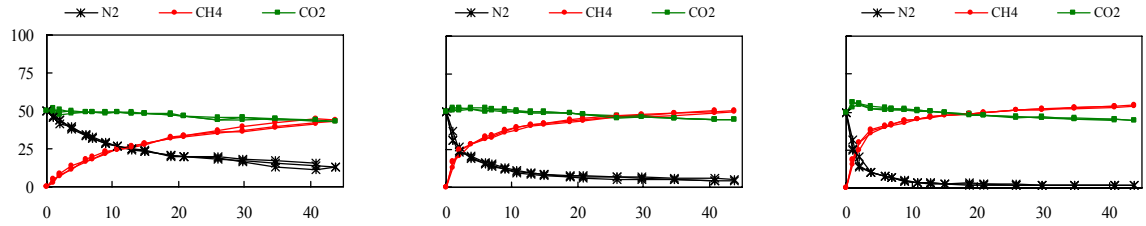


Figure G.69 – Gas composition.

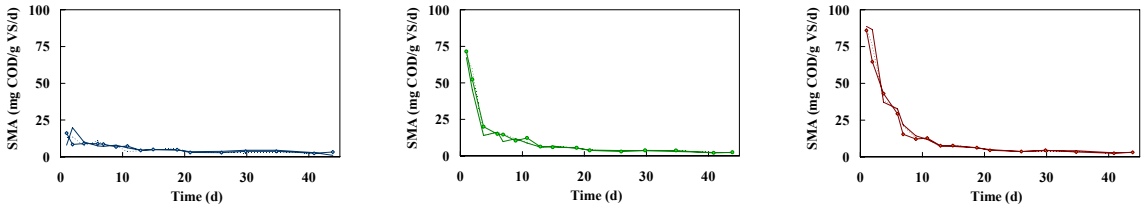


Figure G.70 – Specific methanogenic activity.

Test compound: Distillery effluent		
Low	Medium	High

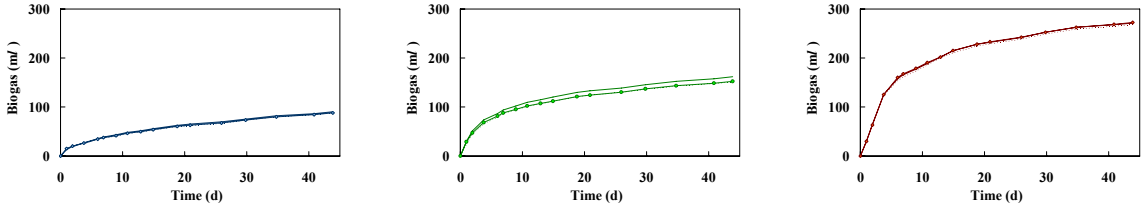


Figure G.71 – Biogas production.

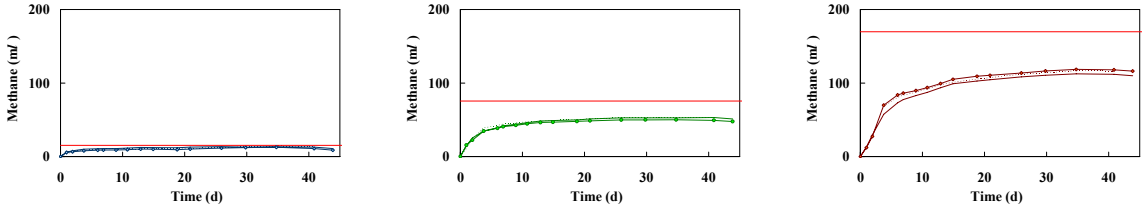


Figure G.72 – Net methane production.

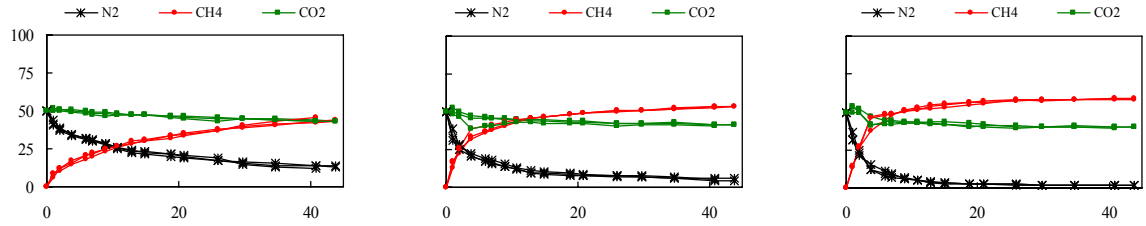


Figure G.73 – Gas composition.

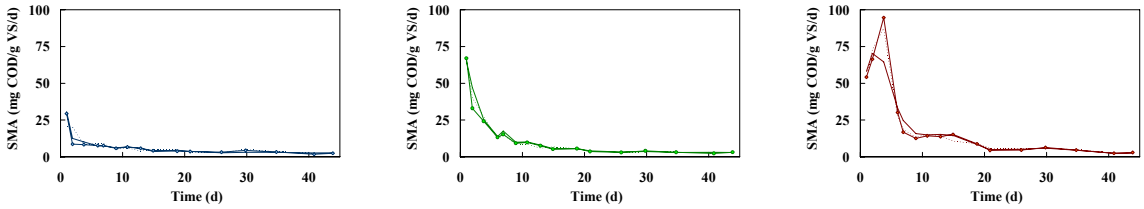


Figure G.74 – Specific methanogenic activity.

Test compound: Mixture (1:1)		
Low	Medium	High

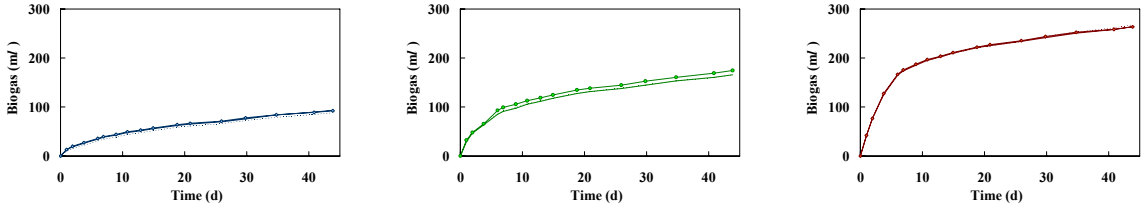


Figure G.75 – Biogas production.

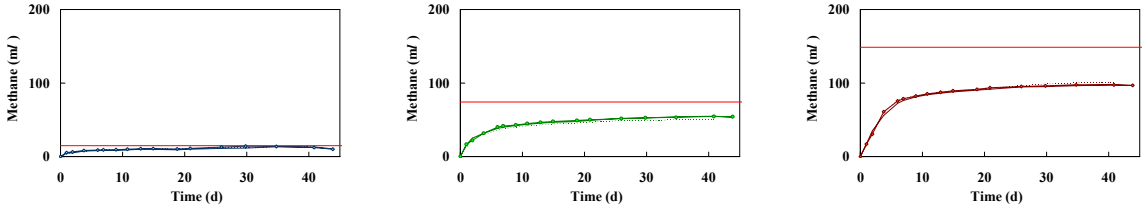


Figure G.76 – Net methane production.

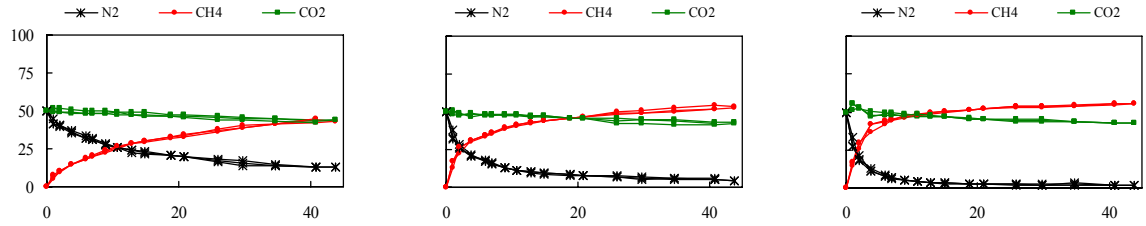


Figure G.77 – Gas composition.

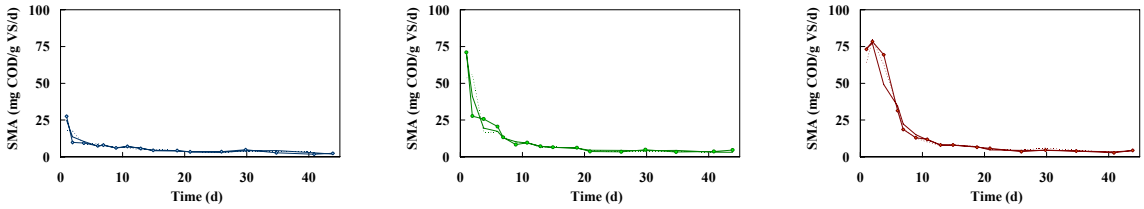


Figure G.78 – Specific methanogenic activity.

G.1.7 Modified anaerobic activity tests: test No 4

The same template described in the previous Section has been adopted, i.e. a Table which summarises the set-up of the vials and the resulting operating conditions, followed by the specific results for each experimental group.

Table G.10 – Composition of the serum bottles for the toxicity test on industrial effluents (No 4).

Constituents		**Ctrl**	**Size effluent**			**Distillery effluent**			**1:2 mixture** [(a)]		
Acetate	g	0	0	0	0	0	0	0	0	0	0
NMS	mℓ	10	10	10	10	10	10	10	10	10	10
Sludge	mℓ	40	40	40	40	40	40	40	40	40	40
Effluent	mℓ	0	2.5	5.0	10	1.5	3.0	6.0	1.9	3.8	7.3
Parameters											
Total volume	mℓ	50	52.5	55	60	51.5	53	56	51.9	53.8	57.3
Organic m. [(1)]	g/ℓ	0	3.6	6.9	12.7	3.6	6.9	13.1	3.6	7.1	13.0
Solids [(2)]	g/ℓ	11.7	11.2	10.7	9.8	11.4	11.1	10.5	11.3	10.9	10.2
S-to-X	-	-	0.32	0.65	1.3	0.31	0.63	1.25	0.32	0.65	1.27
Ratio v/v	%	**-**	**4.8**	**9.1**	**16.7**	**2.9**	**5.7**	**10.7**	**3.6**	**7.0**	**12.8**

Constituents		**2:1 mixture** [(a)]		
Acetate	g	0	0	0
NMS	mℓ	10	10	10
Sludge	mℓ	40	40	40
Effluent	mℓ	2.2	4.4	8.7
Parameters				
Total volume	mℓ	52.2	54.4	58.7
Organic m. [(1)]	g/ℓ	3.7	7.0	12.8
Solids [(2)]	g/ℓ	11.2	10.8	10.0
S-to-X	-	0.33	0.65	1.28
Ratio v/v	%	**4.2**	**8.1**	**14.8**

[(1)] in COD units;
[(2)] expressed as Volatile Suspended Solids (VSS);
[(a)] the individual contributions of the size and distillery effluents (mℓ) are: (1.2+2.4), (2.4+4.7) and (4.4+8.6), for the 1:2 mixture; (2.5+1.2), (4.7+2.3) and (8.6+4.2), for the 2:1 mixture.

Control units

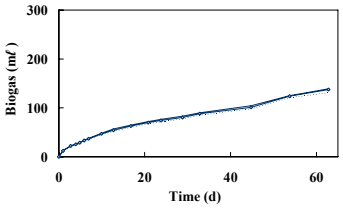


Figure G.79 – Biogas production.

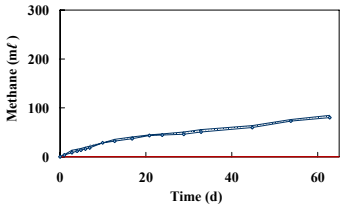


Figure G.80 – Methane production.

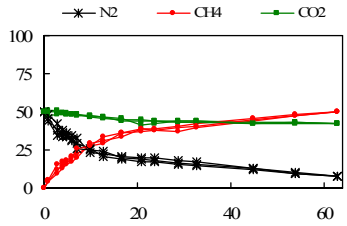


Figure G.81 – Gas composition.

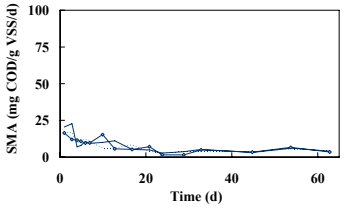


Figure G.82 – Specific methanogenic activity.

Test compound: Size effluent		
Low	Medium	High

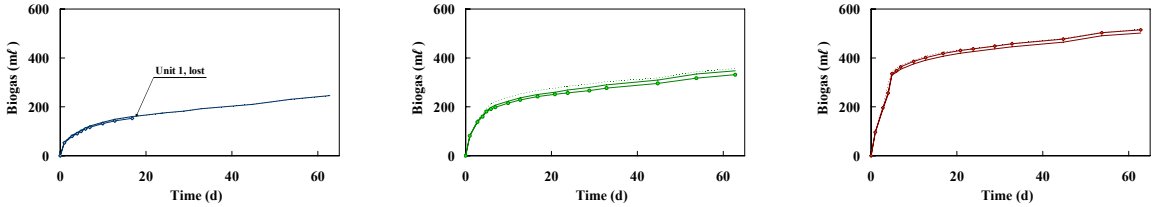


Figure G.83 – Biogas production.

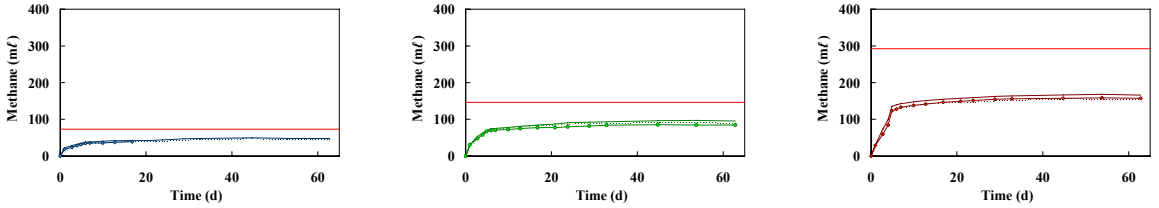


Figure G.84 – Net methane production.

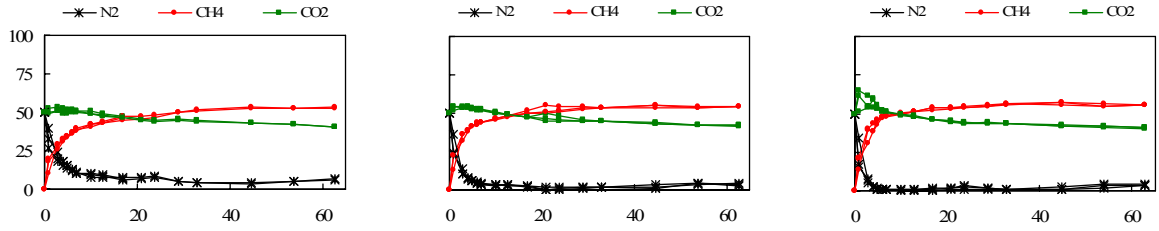


Figure G.85 – Gas composition.

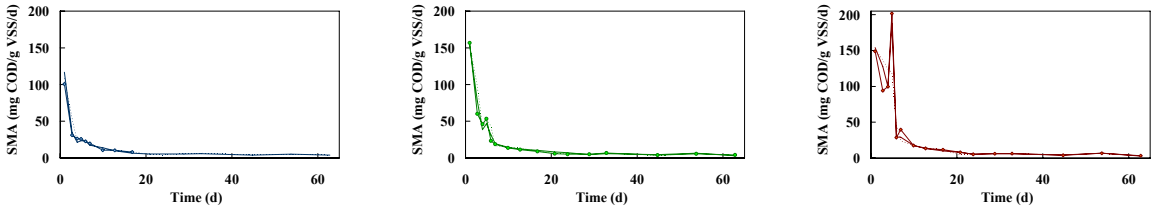


Figure G.86 – Specific methanogenic activity.

Test compound: Distillery effluent		
Low	Medium	High

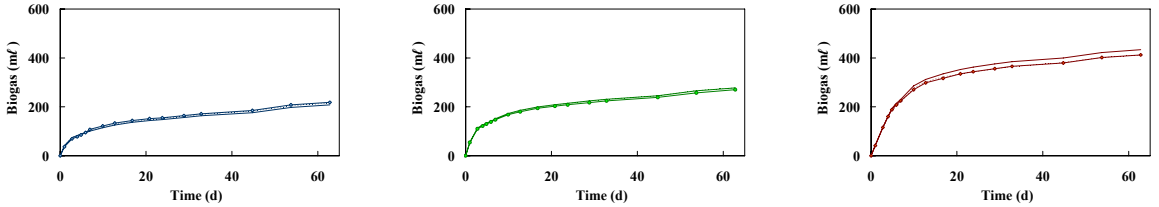


Figure G.87 – Biogas production.

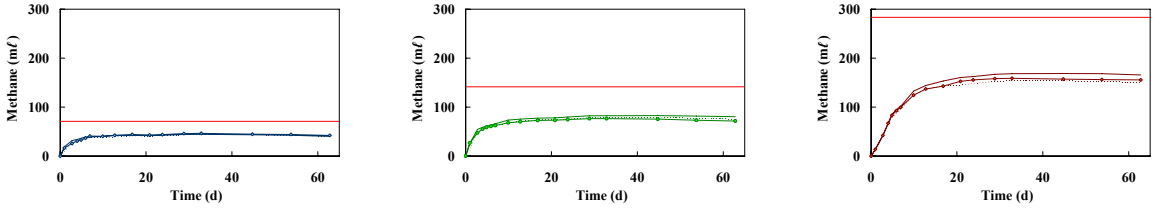


Figure G.88 – Net methane production.

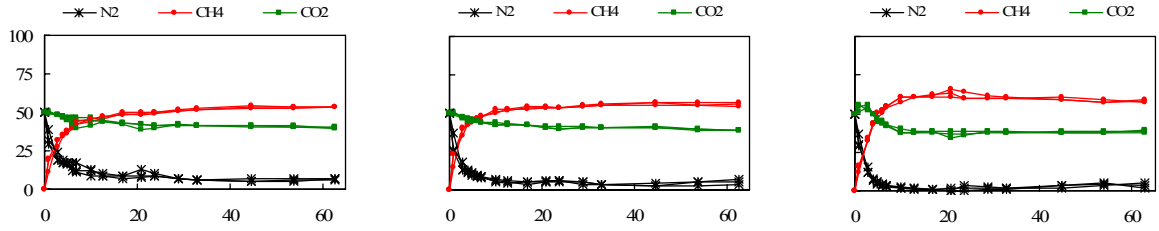


Figure G.89 – Gas composition.

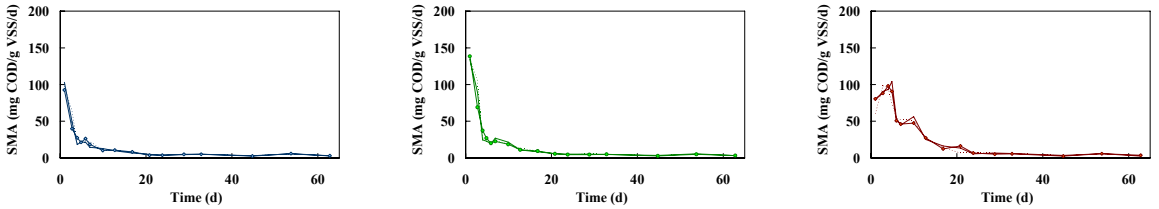


Figure G.90 – Specific methanogenic activity.

Test compound: Mixture (1:2)		
Low	Medium	High

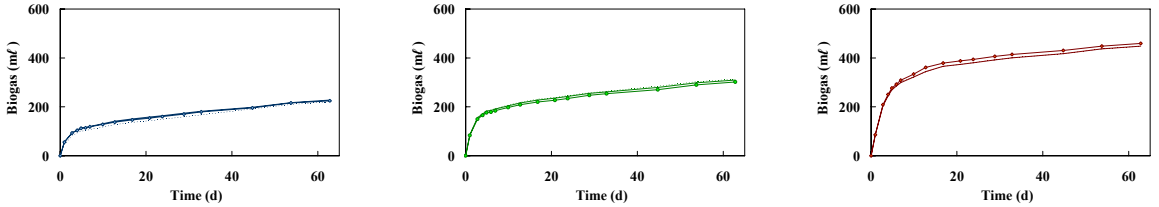


Figure G.91 – Biogas production.

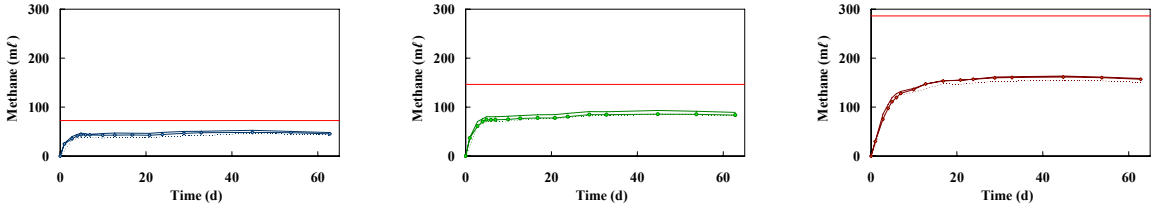


Figure G.92 – Net methane production.

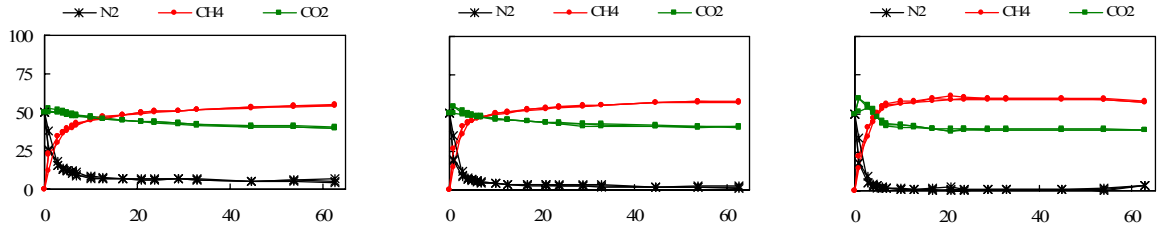


Figure G.93 – Gas composition.

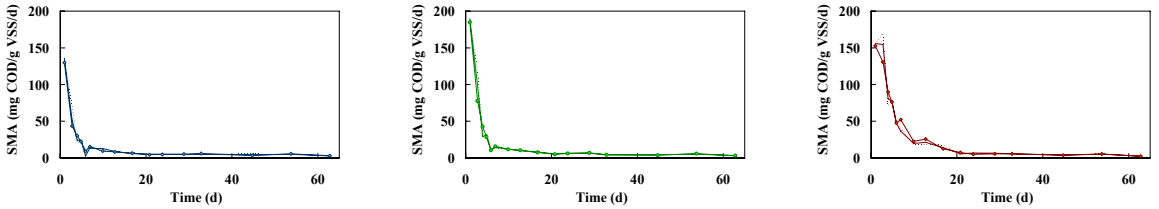


Figure G.94 – Specific methanogenic activity.

Test compound: Mixture (2:1)		
Low	Medium	High

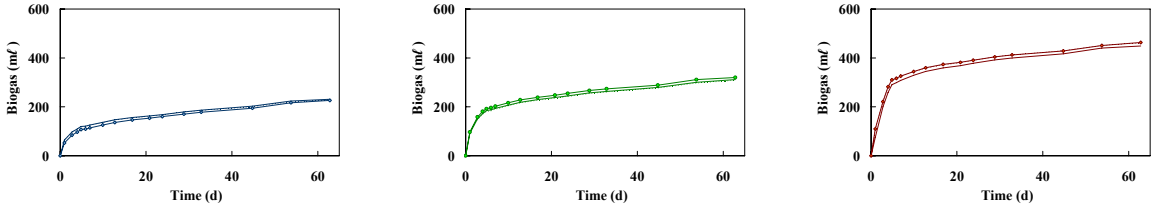


Figure G.95 – Biogas production.

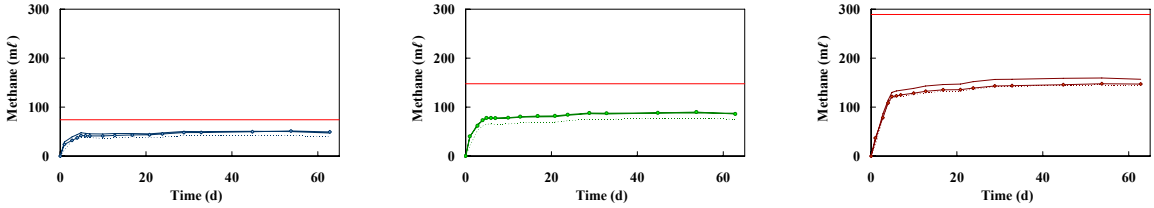


Figure G.96 – Net methane production.

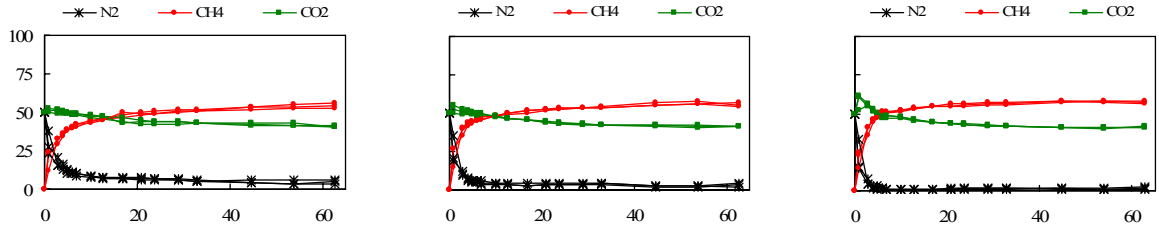


Figure G.97 – Gas composition.

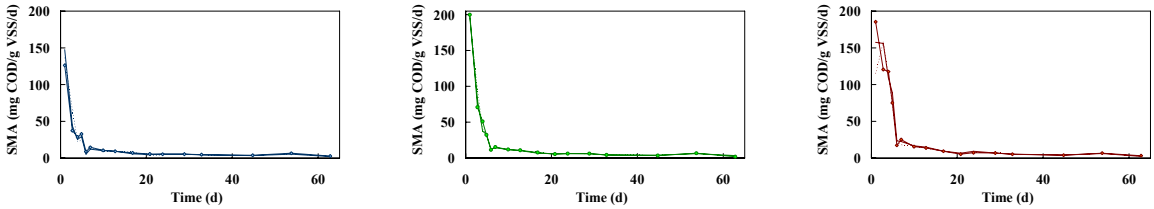


Figure G.98 – Specific methanogenic activity.

G.1.8 Modified anaerobic activity tests: test No 5

The same template described in the previous Section has been adopted, i.e. a Table which summarises the set-up of the vials and the resulting operating conditions, followed by the specific results for each experimental group.

In this test, two distinct co-digestion options were studied which involved the combinations of size and scour effluents and of distillery and synthetic dye effluents; the scour and the synthetic dye effluents act as the recalcitrant components of the mixtures. Three volumetric ratios were considered, i.e. same proportion of the two effluents (1:1), greater proportion of the recalcitrant effluent (1:2) and greater proportion of the labile effluent (2:1).

Table G.11 – Composition of the serum bottles for the toxicity test on industrial effluents (No 5) (cont.).

Constituents		**Ctrl**	**Size effluent**		**Scour effluent**		**1:2 mixture** [a]		**2:1 mixture** [a]	
Acetate	g	0	0	0	0	0	0	0	0	0
NMS	mℓ	10	10	10	10	10	10	10	10	10
Sludge	mℓ	40	40	40	40	40	40	40	40	40
Effluent	mℓ	0	1.0	2.0	20	40	13.7	27.2	7.3	14.6
Parameters										
Total volume	mℓ	50	51.0	52.0	70	90	63.7	77.2	57.3	64.6
Organic m. [1]	g/ℓ	0	1.5	2.9	1.1	1.8	1.2	2.0	1.4	2.4
Solids [2]	g/ℓ	11.7	11.5	11.3	8.4	6.5	9.2	7.6	10.2	9.1
S-to-X	-	-	0.13	0.26	0.14	0.27	0.14	0.27	0.13	0.27
Ratio v/v	%	**-**	**2.0**	**3.8**	**29**	**44**	**21**	**35**	**13**	**23**

Constituents		**1:1 mixture** [a]		**Distillery effl.**		**Synth. dye effl.**		**1:2 mixture** [b]	
Acetate	g	0	0	0	0	0	0	0	0
NMS	mℓ	10	10	10	10	10	10	10	10
Sludge	mℓ	40	40	40	40	40	40	40	40
Effluent	mℓ	10.5	20.5	1.0	2.0	10	20	7.0	14.0
Parameters									
Total volume	mℓ	60.5	70.5	51.0	52.0	60	70	57.0	64.0
Organic m. [1]	g/ℓ	1.3	2.2	2.4	4.7	2.0	3.4	2.1	3.7
Solids [2]	g/ℓ	9.7	8.3	11.5	11.3	9.8	8.4	10.3	9.2
S-to-X	-	0.13	0.26	0.21	0.42	0.20	0.41	0.20	0.41
Ratio v/v	%	**17**	**29**	**2.0**	**3.8**	**17**	**29**	**12**	**22**

[1] in COD units;
[2] expressed as Volatile Suspended Solids (VSS);
[a] the volumes of effluent (mℓ) reported indicates the sum of the contributions of the size and scour effluents, which is calculated on a COD basis. The individual contributions are therefore (for the size and scour effluents respectively): (0.3+13.3) and (0.7+26.5), for the 1:2 mixture; (0.7+6.7) and (1.4+13.2), for the 2:1 mixture; and (0.5+10.0) and (1.0+19.5), for the 1:1 mixture.
[b] the individual contributions of the distillery and synthetic dye effluents (mℓ) are: (0.3+6.7) and (0.6+13.3), for the 1:2 mixture; (0.6+3.3) and (1.3+6.6), for the 2:1 mixture; and (0.5+5.0) and (1.0+10.0), for the 1:1 mixture.

Table G.8 – (continued) Composition of the serum bottles for the toxicity test on industrial effluents (V).

Constituents		**2:1 mixture** [b]		**1:1 mixture** [b]	
Acetate	g	0	0	0	0
NMS	mℓ	10	10	10	10
Sludge	mℓ	40	40	40	40
Effluent	mℓ	3.9	7.9	5.5	11.0
Parameters					
Total volume	mℓ	53.9	57.9	55.5	61.0
Organic m. [1]	g/ℓ	2.2	4.1	2.1	3.9
Solids [2]	g/ℓ	10.9	10.1	10.6	9.6
S-to-X	-	0.20	0.40	0.20	0.41
Ratio v/v	%	**7**	**14**	**10**	**18**

[1] in COD units;
[2] expressed as Volatile Suspended Solids (VSS);
[b] the individual contributions of the distillery and synthetic dye effluents (mℓ) are: (0.3+6.7) and (0.6+13.3), for the 1:2 mixture; (0.6+3.3) and (1.3+6.6), for the 2:1 mixture; and (0.5+5.0) and (1.0+10.0), for the 1:1 mixture.

Control units

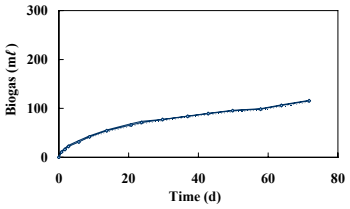


Figure G.99 – Biogas production.

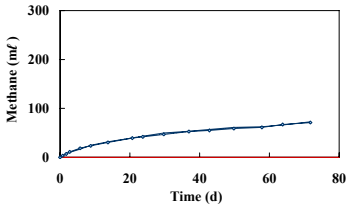


Figure G.100 – Methane production.

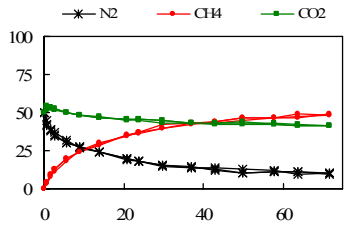


Figure G.101 – Gas composition.

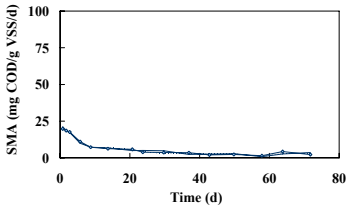


Figure G.102 – Specific methanogenic activity.

Test compound: Size effluent	
Low	High

Figure G.103 – Biogas production.

Figure G.104 – Net methane production.

Figure G.105 – Gas composition.

Figure G.106 – Specific methanogenic activity.

Test compound: Scour effluent	
Low	High

Figure G.107 – Biogas production.

Figure G.108 – Net methane production.

Figure G.109 – Gas composition.

Figure G.110 – Specific methanogenic activity.

Test compound: Mixture (1:2)	
Low	High

Figure G.111 – Biogas production.

Figure G.112 – Net methane production.

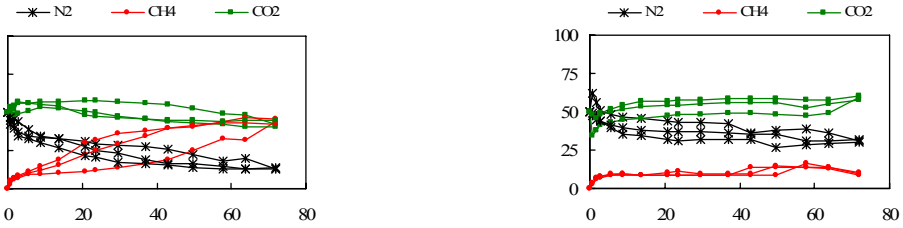


Figure G.113 – Gas composition.

Figure G.114 – Specific methanogenic activity.

Test compound: Mixture (2:1)	
Low	High

Figure G.115 – Biogas production.

Figure G.116 – Net methane production.

Figure G.117 – Gas composition.

Figure G.118 – Specific methanogenic activity.

Test compound: Mixture (1:1)	
Low	High

Figure G.119 – Biogas production.

Figure G.120 – Net methane production.

Figure G.121 – Gas composition.

Figure G.122 – Specific methanogenic activity.

Test compound: Distillery effluent	
Low	High

Figure G.123 – Biogas production.

Figure G.124 – Net methane production.

Figure G.125 – Gas composition.

Figure G.126 – Specific methanogenic activity.

Test compound: Synthetic dye effluent	
Low	High

Figure G.127 – Biogas production.

Figure G.128 – Net methane production.

Figure G.129 – Gas composition.

Figure G.130 – Specific methanogenic activity.

Test compound: Mixture (1:2)	
Low	High

Figure G.131 – Biogas production.

Figure G.132 – Net methane production.

Figure G.133 – Gas composition.

Figure G.134 – Specific methanogenic activity.

Test compound: Mixture (2:1)	
Low	High

Figure G.135 – Biogas production.

Figure G.136 – Net methane production.

Figure G.137 – Gas composition.

Figure G.138 – Specific methanogenic activity.

Test compound: Mixture (1:1)	
Low	High

Figure G.139 – Biogas production.

Figure G.140 – Net methane production.

Figure G.141 – Gas composition.

Figure G.142 – Specific methanogenic activity.

G.1.9 Evaluating the reproducibility of an anaerobic activity test

The five AAT on industrial effluents were conducted independently using three batches of sludge collected from the anaerobic digester at the Umbilo Wastewater Treatment Plant: the first, third and fourth tests were conducted immediately after the sludge was sampled; whereas the second and the fifth tests were conducted using sludge from the first and third batches respectively that had been stored at 4 °C until use.

The control sets of the AAT No 1 and No 2 (Sections G.1.4 and G.1.5) were identical and comprised:

- three vials (Reference), containing 40 mℓ of the seed inoculum, 10 mℓ of NMS and sodium acetate;
- three vials (Control), containing 40 mℓ of the seed inoculum only; and
- three vials (labelled: no NMS), containing 40 mℓ of the seed inoculum and sodium acetate.

The control sets of the AAT No 3, 4 and 5 (Sections G.1.6, G.1.7 and G.1.8) comprised only the Control group supplemented with the NMS, i.e. containing 40 mℓ of the seed inoculum and 10 mℓ of NMS, without external substrate.

The conditions at which the five tests were carried out are therefore not entirely homogeneous, both in terms of sludge handling and of set-up; however, a comparison can be attempted, based on the concentration of solids.

Table G.12 – Volume of gas and methane fraction after 35 days of incubation (N=3).

	Solids	Biogas volume (mℓ)			Methane fraction (%)		
	(g/ℓ)	*Reference*	*Control*	*no NMS*	*Reference*	*Control*	*no NMS*
Test 1	23-30 [(a)]	178±7	39±2	170±1	67±1	24±1	66±0
Test 2	19-24 [(a)]	180±3	57±3	184±6	67±1	28±2	65±1
Test 3	14 [(a)]	-	61±1	-	-	35±2	-
Test 4	12 [(b)]	-	88±2	-	-	41±2	-
Test 5	12 [(b)]	-	73±2	-	-	43±1	-

[(a)] as volatile solids
[(b)] as volatile suspended solids

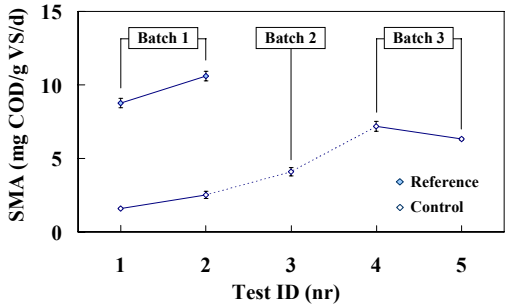


Figure G.143 – Overview of the average specific methanogenic activity (with reference to the volume of methane produced after 35 d of incubation), in the reference and control units in the five activity tests.

- Biogas production and composition: Figure G.144 compares the curves of biogas volume obtained in the five tests separately. The first test lasted over 80 d since one of the objectives was to verify the possibility of a long-term adaptation of the biomass to some of the test materials. The other tests were generally interrupted when all the units displayed a negligible methanogenic activity.

  One can observe a clear similarity between the two sets of curves of tests No 1 and No 2 and between the curves of tests No 4 and No 5; whereas, the curve of test No 3 stands on its own. This grouping matches the three different batches of sludge, used to seed the serum bottles, as explained above.

  However, looking at tests No 1 and No 2 it is also apparent that although both the volume and the composition of biogas after 35 d of incubation (Table G.12) are almost identical in the two sets that were given acetate as external substrate, a great difference emerges between the control sets (that cannot be explained through a difference in the concentration of solids, which was in fact lower in test No 2). This may be tentatively explained by observing that if the substrate concentration in the sets supplemented with acetate (5-7 g COD/ℓ) reached an inhibitory level (which is likely to be the case), then the microbial activity in those units would only attain a *limited* level. This conclusion is supported by the outcome of the subsequent tests, as discussed later.

  On the other hand, the two control sets which were not given acetate seem to suggest that a period of starvation is in fact beneficial to the performance of the sludge as inoculum.

  The better outcome of the control units in test No 3 as compared to the corresponding units in tests No 1 and No 2, both in terms of volume of gas produced and of fraction of methane in the gas (Table G.12), can be attributed to the addition of the NMS. However, two different batches of sludge were used to inoculate the serum bottles, therefore this cannot be taken as conclusive evidence of the beneficial effect of the use of the NMS.

  Finally, the set-up for the control units in tests No 4 and No 5 was identical, in that they received sludge originating from the same batch and were supplemented with the NMS. Although the solids were quantified as volatile suspended solids and therefore the outcome of these tests is not directly comparable to that of the previous tests, the volume of gas and the fraction of methane measured in tests No 4 and No 5 match very well.

  This latter observation and the generally high reproducibility of each independent set of serum bottles (as indicated by the small standard deviations reported in Table G.12) show that the serum bottle method can certainly provide accurate and reliable outputs.

- Activity: Figure G.145 presents the curves of the specific methanogenic activity calculated for the reference and control units in the five tests separately: the objective is the comparison of the *endogenous* SMA, i.e. the activity in the absence of substrate, across the five tests.

  For tests No 1 and No 2, the curves obtained on the reference units are reported, to clearly show the remarkable decrease in the value of SMA, when the external substrate (acetate) and the residual

organic matter in the sludge are exhausted, i.e. after approximately 20 d. The same trend is not so clearly visible in the other tests, since no external substrate was supplemented to 'magnify' the SMA in a first period, with the exception of test No 5, in which the activity decrease almost linearly for the first 10 d, thus indicating the progressive consumption of the residual organic matter.

Accordingly, the values of *endogenous* SMA reported in the last plot (Figure G.145 F) are calculated after day 30 in each test. Despite the differences in the set-up and sludge characteristics discussed so far, one can assume a 95 % confidence interval ranging of 2.3-2.6 mg COD/g VS/d (N=44) (or 3.3-4.2 mg COD/g VSS/d, N=32) for the endogenous activity of the sludge.

This discussion is summarised in Figure G.143, in which the *average* specific methanogenic activity in the reference (tests No 1 and No 2) and in the control units (all tests) is depicted. The *average* SMA is calculated by dividing the flow rate of methane, averaged on the 35 day-period (mℓ/d), by the amount of biomass present in each unit (g VS) and by converting this value in COD units. The result however is not homogeneous, because the solids in tests No 4 and 5 were measured as volatile suspended solids.

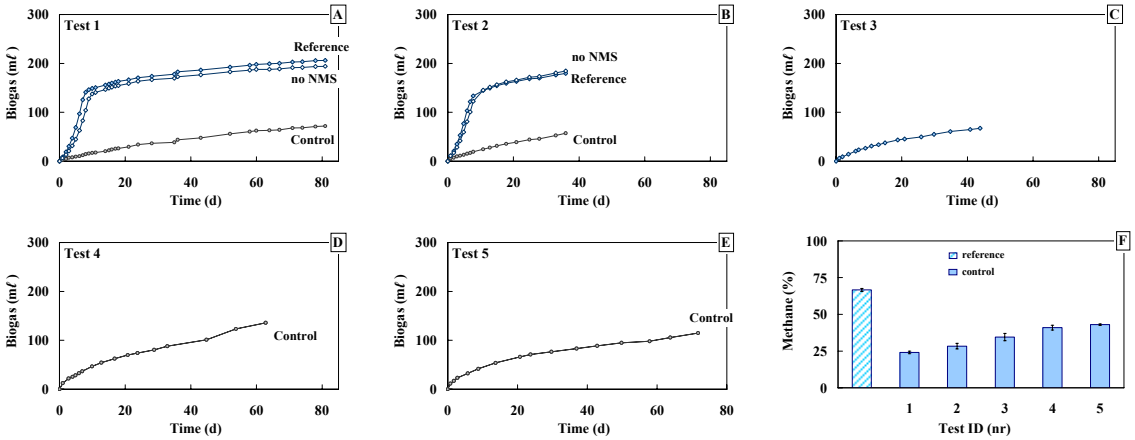


Figure G.144 – Volume of biogas produced and overview of the methane fraction in the biogas (after 35 d), in the control units in the five activity tests.

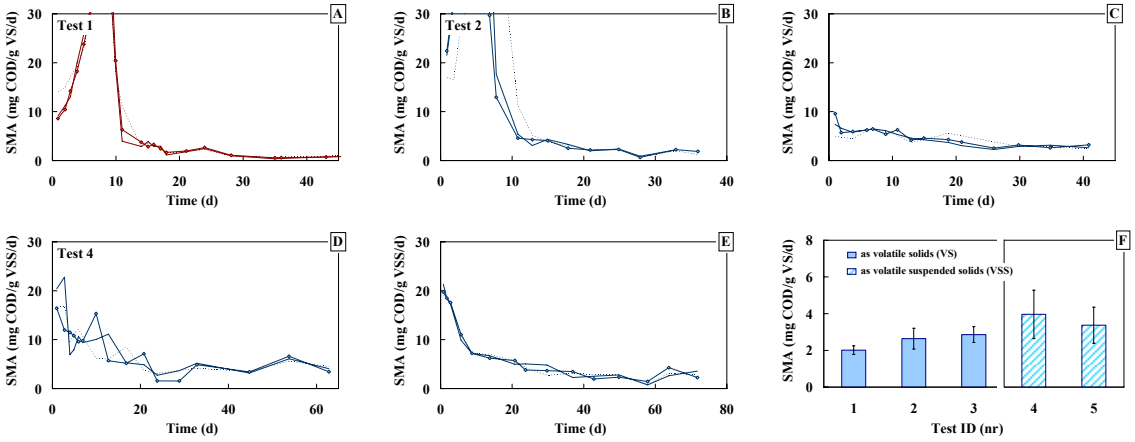


Figure G.145 – Specific methanogenic activity and overview of the endogenous *SMA, in the control units in the five activity tests.*

G.1.10 The accurate determination of the gas volume for an activity test

It might happen (in fact, it is almost always the case) that the volume of gas produced between two subsequent re-equilibrations exceeds the capacity of the syringe. Besides avoiding this circumstance by selecting an appropriate concentration of organic substrate and biomass and/or by releasing the excess pressure more frequently, the only way to proceed is to carry out a rigorous mass balance.

When multiple insertions of the needle are required, the determination of the volume of gas is inevitably inaccurate, because only the last determination is carried out at the atmospheric pressure: all other individual determinations are done while a certain volume of gas is left inside the vial and generates a residual overpressure. Ultimately, the volume of gas measured underestimates the actual volume of gas produced. This error can be compensated through a step-by-step mass balance described elsewhere (Remigi and Foxon, 2005).

This section is aimed at using the data of the activity test No 4 (Section 4.1.3.4) to demonstrate that by not taking into account the residual overpressure between subsequent insertions of the needle, the response of a test can be misleading.

Ideally, the accurate mass balance should be carried out straight after re-equilibration and the correct volume of gas recorded; alternatively, the operator must log the volume of every single partial re-equilibration. Since this information was not available for test No 4, a rigorous mass balance would be impossible. However a tentative post-processing of the data can be done by making a few reasonable assumptions.

Bearing in mind that three glass syringes were used in the laboratory at that time, i.e. a 2 mℓ, a 10 mℓ and a 50 mℓ syringe (this latter was damaged though and could only be filled up to the 30-40 mℓ mark with great care), we isolated the individual gas volume determinations larger than 30 mℓ.

For a headspace volume of 60-75 mℓ, volumes smaller than 20 mℓ (which would require up to two insertions of the needle) result in a negligible error: therefore a cut-off of 30 mℓ seemed reasonable.

The following criterion was also adopted: we assumed that volumes smaller than 60 mℓ were quantified using a hypothetical 20 mℓ syringe and volumes larger than 60 mℓ using the 50 mℓ (40 mℓ) syringe. Although in the practice a different succession of measurements was done, this assumption seemed to be a good compromise to ensure a qualitatively correct revision.

The results for the size and distillery effluents in isolation are presented in Figure G.146 in terms of biodegradability only. The specific methanogenic activity being calculated using the individual gas determinations would be adversely affected by the considerable degree of approximation of the data processing.

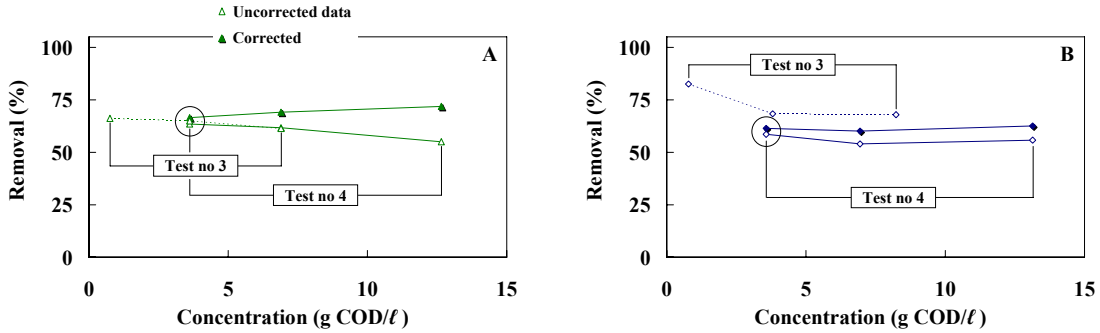


*Figure G.146 – Correction of the biodegradability calculated for the size (**A**) and for the distillery effluents (**B**), to take into account the residual overpressure for repeated insertions of the needle.*

It is evident in both instances that the potential error due to the unaccounted residual overpressure can be relevant, particularly at relatively high concentrations of the test material. This is obviously due to the progressive reduction of the volume of the headspace which in turn is due to the larger volume of test material needed; this is clearly more pronounced in the case of the size effluent which has a much lower COD than that of the distillery effluent.

At ca. 3.6 g COD/ℓ, the estimation of the biodegradability of the two test materials is relatively consistent, so that a 95 % confidence interval can be tentatively reported as 63-67 % and 57-63 % for the size and the distillery effluents respectively (circles in Figure G.146).

G.2 LABORATORY-SCALE TESTS: FINAL EXPERIMENT

This section reports a few experimental results and details that have not been included in Chapter 4.2 to facilitate the understanding of the general picture.

G.2.1 Batch phase

The outcome of the serum bottle AAT indicated that the distillery effluent was not particularly harmful to methanogenesis and sufficiently biodegradable. Nonetheless the use of it as feed for the laboratory-scale was closely monitored, after addition, in order to evaluate the effect on the biomass: frequent readings of the gas counter were taken as well as gas samples analysed on the GC in the 6 to 8 h immediately after feeding. It was therefore possible to monitor the biogas flow rate, thus estimating the biological activity (Figure G.147 A) and the evolution of the gas composition (Figure G.147 B).

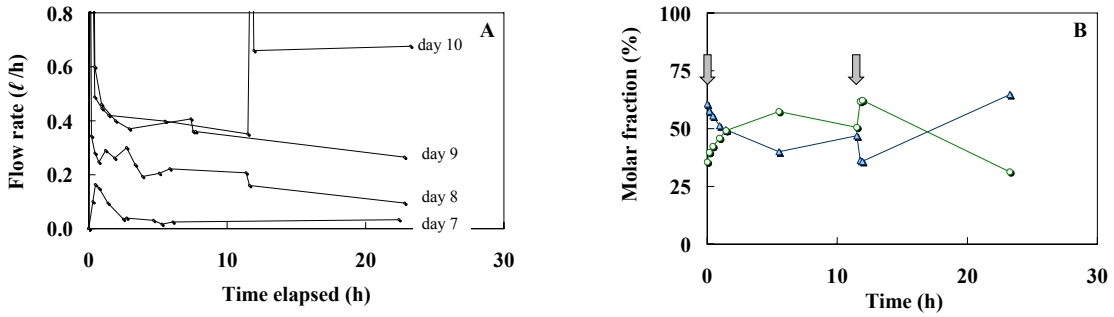


*Figure G.147 – Progression of the biogas flow rate from day 7 to day 10 of the batch experiment (**A**) and gas composition on the day 10, when two individual doses (arrows) were given 12 h apart (**B**).*

G.2.2 Accuracy of the liquid displacement gas measuring system

The performance of the digester can be assessed using two conceptually equivalent approaches, i.e. by comparing the amount of organic matter that is being fed (M_{fed}), either to the amount degraded (M_{deg}), or to the amount converted to methane (M_{CH}) –this latter being the ultimate sink of organic matter in anaerobic processes, if one neglects the fraction converted to new biomass. In fact, no significant growth of biomass can be reported throughout the experimental period (Figure 52 D).

The two approaches are based on the obvious mass balance on the organic matter:

$$M_{fed} = M_{in-R} + M_{deg} = M_{in-R} + (M_{CH} + M_{bio})$$

where M_{in-R} and M_{bio} indicate the amount of organic matter that remains undegraded in the suspension and the amount converted to biomass respectively.

However, the two corresponding estimations of the COD removal efficiency (η_{II} and η_{III}; equations D.5 and D.6, respectively) are not matching well (Figure 52 G).

We believe that the only possible reason is that the determination of the biogas flow rate conducted with the volumetric gas measuring system was not correct. This in turn may be due to an inaccurate calibration; an inherently faulty system (e.g. a portion of the gas generated in the digester could have been lost unaccounted, during the equilibration periods); or a progressive deterioration of the sensor.

A tentative evaluation of this issue can be done by examining the evolution of the difference between the two estimates of the COD degraded, i.e. one analytically measured by determining the COD of the effluent (which we consider highly reliable); the other one instrumentally measured by combining the information on the gas composition through gas chromatographic analysis to the volume of gas measured by liquid displacement.

From day 19, an acidified barrier solution was used. Ideally, no carbon dioxide should dissolve into an acidic solution, so that the volume of liquid displaced is equivalent to the total volume of gas generated. However, this is only true when the carbon dioxide in the solution and in the gas phase are in equilibrium (Rozzi and Remigi, 2004).

Under the experimental conditions though, the fraction of carbon dioxide in the biogas had not reached a steady-state (Figure 52 F), so that a *variable* portion of carbon dioxide may have been exchanged with the barrier solution throughout the experiment, thus introducing a source of error which is difficult if not impossible to account for.

Figure G.148 depicts the ratio between the methane generated (M_{CH}) and the COD degraded (M_{deg}). The following aspects can be observed.

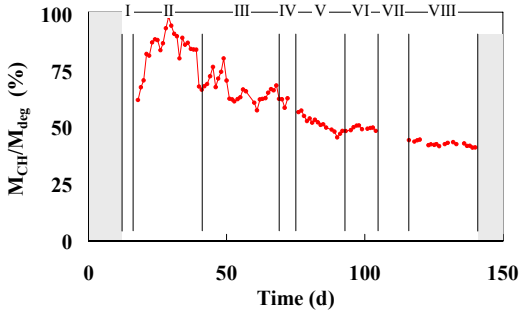


Figure G.148 – Mass balance: comparison of the estimates of the amount of COD degraded. M_{CH} and M_{deg} are the amounts of COD recovered as methane in the off-gas and disappeared from the liquid phase, respectively.

▪ The curve lies entirely below 100 %.

This indicates that the amount of methane measured *underestimates* the amount of organic matter which has actually been degraded.

▪ During an initial transient, the curve shows a positive trend.

This suggests that of the two terms of the ratio, the amount of COD degraded may be the most inaccurate. In fact, if some organic substrate was present in the seed sludge and had been

progressively degraded, it would have remained unaccounted for in the feed, whereas the amount of methane generated would have increased. This trend would have continued until the residual organic matter had been consumed. This seems to have occurred after about 35 days. Interestingly, a very similar trend is displayed by the concentration of solids (Figure 52 D), which decreased from 16 to 10 g VS/ℓ during the same period.

▪ Thereafter, the curve tends to level off at around 40-42 %.

A failure of the measuring device would have occurred at a particular moment in time and therefore resulted in a discontinuity of the curve. A progressively deteriorating signal (e.g. due to a slow loss of the barrier solution) would have resulted in a steadily decreasing curve.

Of the three possible origins of the pattern of Figure G.148 listed above, the inaccurate calibration seems to be the only one compatible.

In conclusion, the following recommendations are proposed:

i) that an alkaline barrier solution be used in the liquid displacement gas measuring device, as it ensures that all carbon dioxide gets consistently dissolved throughout the experimental period, thus the sole methane fraction of the biogas is measured;

ii) that the calibration of the two-level electric sensor be accurately performed, prior to starting the measurements and possibly at regular intervals, to monitor the conditions of the device; and

iii) that the initial organic matter present in the sludge be quantified.

G.2.3 Off-line activity tests

Off-line activity tests (in duplicate) were performed once a week on the sludge extracted from the digester.

The tests lasted 21-22 days and were staged into two experimental periods:

- first period (days 1 to 10): it was aimed at evaluating the SMA, at the time of sludge withdrawal and whether a significant portion of the residual COD had the potential of being biodegraded would the biomass have been given a longer time;
- second period (days 11 to 21): it was aimed at evaluating the *optimal* methanogenic activity, i.e. on acetate. Doses of 30 µℓ of acetic acid (glacial, 35.4 %) were spiked once a day.

In some cases, the test was extended to a third period, aimed at evaluating the methanogenic activity on the distillery effluent, in a fed-batch mode: 1 mℓ of a 1:1 stock solution of a concentrated (10 x) NMS and distillery effluent was spiked once a day.

The main results of the activity test are summarised in Table G.13 and discussed in Section 4.2.2.3.

Table G.13 – Summary of the results of the off-line activity tests.

Units are (g/ℓ) for COD and VSS; (mg COD/g VSS/d) for SMA and (%) for the recovery η.

Test [a]	Initial conditions		1st period		2nd period		3rd period	
	COD	VSS	SMA	η	SMA	η	SMA	H
day 15	8.6±0.2	16±1	24±2	≈ 30 %	52±8 [b]	> 97 %	87±5	> 70 %
day 22	13.1±0.4	14±0	17±2	≈ 18 %	26±2	40-50	n.p.	n.p.
day 36	29.0±1.2	10±0	13±1	≈ 10 %	93±12	> 115 %	18±2	≈ 19 %
day 43	29.4±0.0	12±0	29±4	13-20 %	68±9	100 %	17±3	14-22 %
day 50	29.5±1.2	9±0	68±0	≈ 19 %	79±4	67-72 %	n.p.	n.p.
day 84	30.2±0.9	≈ 8	22±1	≈ 7 %	n.p.	n.p.	n.p.	n.p.

[a] It indicates the day the AAT was started, with reference to the 'mother' digester.
[b] Activity reached a first level at 61±4 and few days later it dropped to 45±1. The value reported in the Table is an overall average.

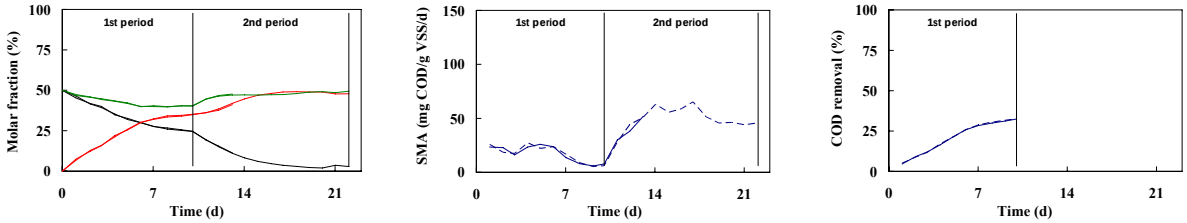


Figure G.149 – Off-line AAT on day 15: gas composition, methanogenic activity and COD removal.

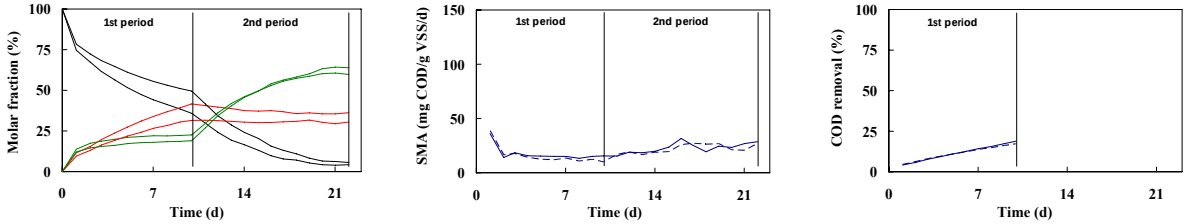


Figure G.150 – Off-line AAT on day 22: gas composition, methanogenic activity and COD removal.

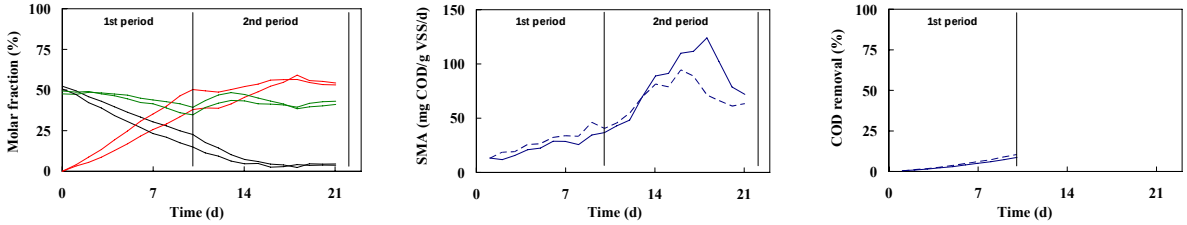


Figure G.151 – Off-line AAT on day 36: gas composition, methanogenic activity and COD removal.

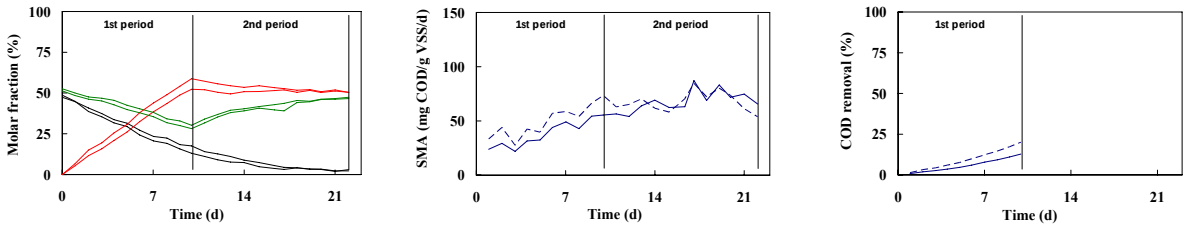


Figure G.152 – Off-line AAT on day 43: gas composition, methanogenic activity and COD removal.

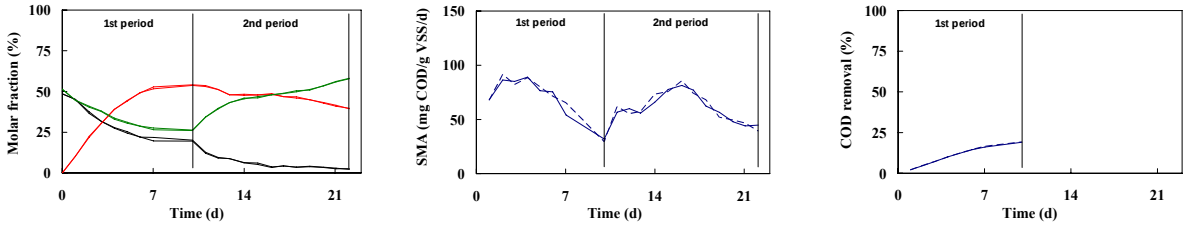


Figure G.153 – Off-line AAT on day 50: gas composition, methanogenic activity and COD removal.

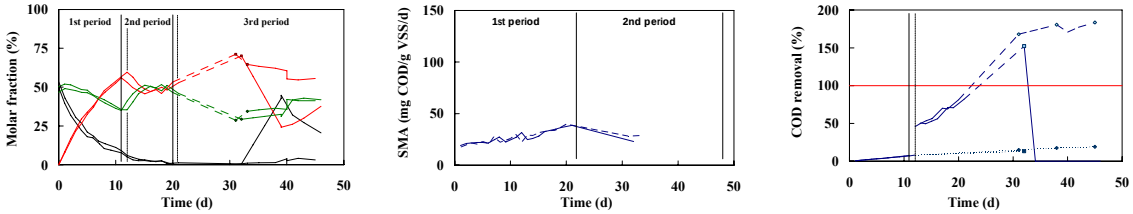


Figure G.154 – Off-line AAT on day 84: gas composition, methanogenic activity and COD removal.

ANNEXURE H

List of publications

IN PREPARATION

1. Remigi E and Foxon KM (2005). The serum bottle technique to assess microbial activity: a discussion of principles. Submitted to Water SA.

REFEREED PUBLICATIONS

1. Rozzi A and Remigi E (2004). Methods of assessing microbial activity and inhibition under anaerobic conditions: a literature review. *Rev. Environ. Sci. Biotechnol.*, **3**(**2**): 93-115.

CONFERENCE PROCEEDINGS

1. Remigi E and Ficara E (2005). pH-stat titration for the assessment of anaerobic biodegradability and activity. Submitted for oral presentation at the "VIII Taller y Simposio sobre Digestion Anaerobia", 2-5 October Punta del Este (Uruguay).

2. Remigi E and Buckley CA (2005). Protocol for the assessment of the disposal of organic concentrates by codigestion. Oral presentation at the "WISA/IWA conference: Management of residues emanating from water and wastewater treatment", 9-12 August, Johannesburg (South Africa).

3. Remigi E and Foxon KM (2004). Modelling of anaerobic digestion processes: a review. Oral presentation at the seminar "Ricerca e innovazione nella depurazione delle acque reflue. Giornate di studio in memoria del Prof. Alberto Rozzi" ("Research and advances in wastewater treatment. In memory of Prof. Alberto Rozzi"), 25-26 November, Milano (Italy).

4. Remigi E, Naidoo D and Buckley CA (2004). Mathematical modelling of a pH-stat bioassay. Poster at the "WISA 2004 Biennial Conference and Exhibition", 2-6 May, Cape Town (South Africa).

Rozzi and Remigi (2004). Rev. Environ. Sci. Biotechnol. **3**(**2**): 93-115.

METHODS OF ASSESSING MICROBIAL ACTIVITY AND INHIBITION UNDER ANAEROBIC CONDITIONS: A LITERATURE REVIEW

Alberto Rozzi [†] and Enrico Remigi [*]

DIIAR, Sezione Ambientale, Politecnico di Milano, 20133 Milano (Italy)

[(*)] Pollution Research Group - University of KwaZulu-Natal, 4041 Durban (South Africa)

[†] Passed away on April 7th 2003

Keywords: anaerobic, activity, inhibition, methods

ABSTRACT

This work reviews the existing methodologies for assessing microbial activity and inhibition under anaerobic conditions. The anaerobic digestion process consists of several metabolic steps: the Anaerobic Digestion Model No. 1 (ADM1) has attempted to describe these steps in the form of a mathematical model with the intention of providing a reference base for all further efforts in the modelling of anaerobic processes. The existence of a reference point for modelling has highlighted the fact that there is a lack of coherence between the many different methodologies for experimentally assessing anaerobic activity and inhibition.

A working group of the International Water Association was recently founded to harmonise existing methodologies with the ultimate intention of developing a unified reference procedure: a primary objective of the group will be the establishment of standard terminology in the field of anaerobic digestion, activity and inhibition assessment. Secondly, it will compare existing methodologies and develop standard protocols for assessing characteristics of anaerobic processes that may be entered directly into ADM1 and its successors.

This paper revises and enlarges a contribution presented by the authors at the workshop "Harmonisation of anaerobic biodegradation, activity and inhibition assays" (Ligthart and Nieman, 2002) and aims to promote a clear understanding of the currently established methodology.

Numerous methods have been developed over the past 30 years, since Van den Berg et al. (1974) measured methanogenic activity, by using a manometric device equipped with a photoelectric sensor to quantify the gas production. Methanogenesis is often the rate limiting step of the entire process and since the quantification of gas flow rate is relatively easy to perform, most of the methods reported in literature monitor the production of biogas. These methods can be termed volumetric or manometric methods, as the volume of biogas produced or the pressure increase due to gas production inside a close vessel are assessed, respectively. However, this same concept can be employed to assess activity

or inhibition of individual metabolic steps preceding the methanogenic one, providing that they are rate limiting for the whole process. The reliability of activity assessment through gas measurement has been proven to be strongly dependent on the equilibrium between liquid and gas phase in a closed vessel. This can be influenced by the amount and characteristics of the test substrate, the concentration of the biomass, the gas-to-liquid ratio, etc.: all these aspects will need to be addressed in the standard procedure.

Other direct or indirect methods, targeting physico-chemical or microbiological parameter exist and have been investigated by various authors.

Besides the interest for research purposes, the definition of reference methods to assess activity and inhibition can be of great interest for engineers, in both designing a plant and in process-control: the challenge in this field is to conceive a reliable, easy-to-perform, automated method that can monitor the state of an anaerobic digester online.

pH-STAT TITRATION FOR THE ASSESSMENT OF ANAEROBIC BIODEGRADABILITY AND ACTIVITY

Enrico Remigi [1] and Elena Ficara [2]

(1) Pollution Research Group - University of KwaZulu-Natal, 4041 Durban (South Africa)

(2) DIIAR, Sezione Ambientale, Politecnico di Milano, 20133 Milano (Italy)

INTRODUCTION

Numerous methods for measuring microbial activity (and related concepts, such as inhibition and biodegradability) under anaerobic conditions, have been developed since the early seventies. Methods which are specifically devised for anaerobic processes rely on the detection of biogas production, by measuring the volume of gas produced or the pressure generated inside a sealed vessel (Rozzi and Remigi, 2004). Recently, the scientific community has acknowledged the lack of reference methods to measure the activity of anaerobic biomasses as well as the lack of standardised conditions which would ensure the repeatability and the accuracy of the determination (Müller et al., 2004).

The principle of pH-stat titration applied to microbial processes enables the maintenance of pH at a constant value and the measurement of the biological activity by a controlled addition of an acidic (or alkaline) solution into a bioreactor (Ramadori et al., 1980). If protons are consumed (or produced) during the process, the pH of the solution will increase (or decrease) in a stoichiometric ratio to the reaction substrates and products; and the titration rate will provide a direct measurement of the reaction rate.

Due to the simplicity of both the measuring principle and of the instrumentation, pH-stat titration has been successfully applied to a variety of biological processes; other applications have been proposed which combine the concepts of pH- and substrate-stat to achieve more accurate control strategies (Rozzi et al., 2003).

The limit of the pH-stat technique is that it cannot be accurate in monitoring a single process which occurs in combination with other pH-affecting processes, because the change in pH observed is the cumulative response of the system to all proton-producing and consuming processes.

For instance, anaerobic digestion is a combination of several sub-processes which have different pH-effects and result in a pH profile, which starts in the acidic range, when the breakdown of the substrate to volatile acids is predominant, and gradually shifts to the alkaline range, when the production of methane becomes predominant. Under such circumstances, a conventional pH-stat assay would not be accurate, because the two phases of acidification and methanisation, occur simultaneously: therefore, a fraction of the volatile fatty acids is *internally* compensated for by an equivalent fraction of alkalinity, produced by the downstream methanogens. Ultimately, the bioassay would not sense a fraction of the reagents and of the products and the microbial activity would be incorrectly estimated.

RATIONALE OF THE METHOD

Ideally, by *uncoupling* the acidogenic and methanogenic phases, a pH-stat bioassay will enable the accurate determination of microbial activities. This can be done by modifying the experimental procedure and the set-up of the anaerobic set-point titration tests (Rozzi et al., 2001), as follows.

- An aliquot of the anaerobic sludge to be analysed is equally distributed in two identical reaction flasks. The first unit (A) is flushed with a mixture of nitrogen and carbon dioxide and the pH adjusted around neutrality. The second unit (B) is put on hold and stored at low temperature to minimise microbial activity.
- Flask A is immediately connected to the titration unit and the test started. If a substrate other than the one present in the sludge is to be tested, it must be supplemented at this point. The outcome of this test is a titration plot (i.e. the cumulative volumes of titrants dosed) consisting of two curves, which correspond to the acidogenic and methanogenic phases, respectively.
- Subsequently, flask B is connected to the titrimetric assay. Prior to starting the second test, a selective inhibitor of methanogenesis (e.g. bromo-ethane sulfonate or chloroform; Scholten et al., 2000) is spiked into the flask, in order to suppress the conversion of acids into methane. The outcome of this second test is the pH-stat titration response which relates to the acidogenic phase alone.

This procedure enables the assessment of the pH effect of the acidogenic phase in terms of alkaline titration in unit B and, *by difference*, the pH effect of the methanogenic phase from the overall titration curve obtained in unit A, as described later on.

A simplified mathematical model of anaerobic digestion has been implemented using AQUASIM to simulate the pH-stat titration test. It consists of three microbial steps, i.e. fermentation (in which an organic substrate is converted into volatile acids, described as acetate equivalents), acetotrophic and hydrogenotrophic methanogenesis. The ADM1 approach (Batstone et al., 2002) has been adopted in which *i*) the substrate uptake processes are described using Michaelis-Menten kinetics and *ii*) the growth of biomass is incorporated. The decay of the three microbial groups has been implemented with a first-order kinetics. An on-off pH-control, which simulates the flow of alkaline and acidic titrants, enables the generation of titration plots that can be used to fit experimental data.

INTERPRETATION OF THE TITRATION PLOTS

The mathematical model of the pH-stat titration assay has been used to simulate the outcome of a test conducted on a generic substrate: a typical titration plot is presented in Figure 1 and can be interpreted as follows.

In the methanogenesis non-inhibited unit (plot **a**), the amount of alkaline titrant dosed equals the *unbalanced* acidity produced, that is the portion of volatile acids exceeding the simultaneous alkalising

effect due to the acetate/hydrogen conversion to methane. Once the methanogenic process becomes predominant ($t > t_A$), a net alkalising effect, thus an acidic titrant addition, is evidenced, which corresponds to the *residual* portion of volatile acids present. In the methanogenic-inhibited unit (plot **b**), the alkali dosed tracks the *actual* acidity produced.

By comparing the alkaline titration curves obtained in unit A and in unit B, the 'missing' fraction of acids which remains undetected by the sensor can be calculated; therefore, the 'true' titration volume for the methanogenic step can be obtained by adding this fraction to the actual acid titration curve in unit A (Figure 2 a). The resulting 'true' acidification and alkalising effects are shown in Figure 2 b. During the interval (tA, tB) no titration takes place and the pH slightly increases, hence a fraction of acetate remains undetected: this can be regarded as an inherent inaccuracy of the method, which is a function of the pH tolerance.

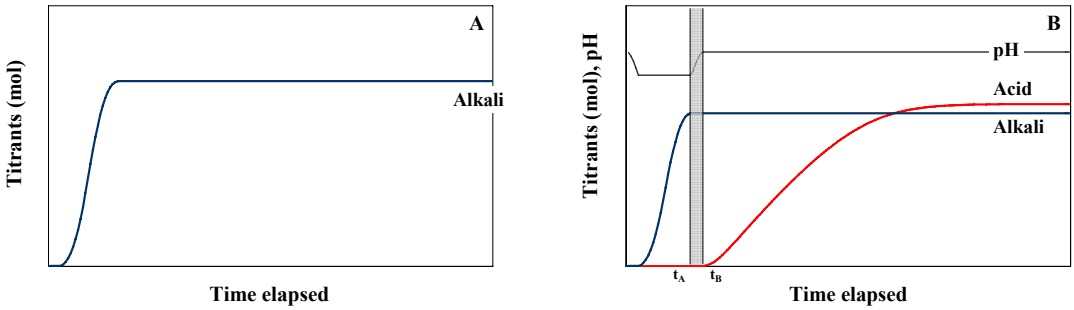


*Figure 1 – Simulated titration plots for a methanogenesis-inhibited (**A**) vs. non-inhibited (**B**) process.*

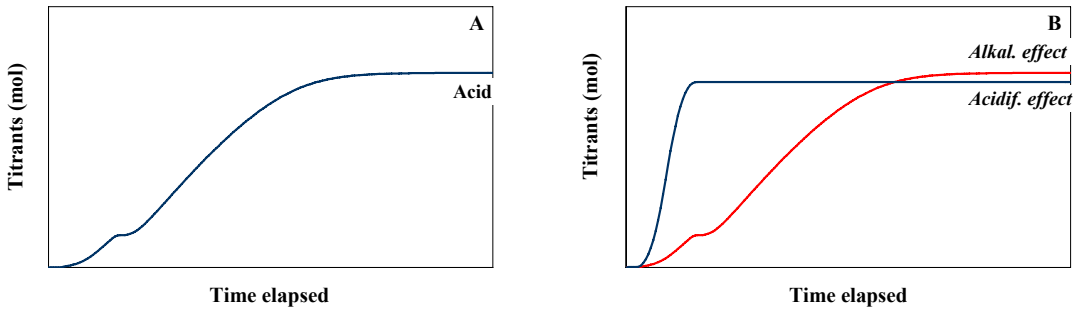


*Figure 2 – 'True' acid titration, during methanogenesis (**A**); acidification and alkalising effects in the methanogenesis non-inhibited unit (**B**).*

The following information can be obtained, by analysing the titration plots.

- The sludge methanogenic activity and the biodegradability of the substrate are calculated on the 'true' acidic titration curve (Figure 2 b): the former is proportional to the slope of the curve; the latter is the ratio between the cumulative equivalents of titrant dosed and the equivalents of substrate initially present or supplemented.
- Similarly, the fermentation rate is proportional to the slope of the 'true' alkaline titration curve (Figure 1 b), under the simplification that all volatile acids are measured as acetate equivalents.

- A positive difference between the amount of volatile acids and of methane will be found when a portion of the acids is not fully converted to methane.

The overall pH-effect of the simplified two-step digestion process is r_H (Pratt et al., 2003):

$$\frac{d}{dt}[H^+] = f_1 \cdot \frac{d}{dt}[HAc] + f_2 \cdot \frac{d}{dt}[CO_2] = r_H \quad (1)$$

$$\frac{d}{dt}[HAc] = f_{Ac} \cdot (1 - Y_{Su}) \cdot r_F - r_{AM} \quad (2)$$

$$\frac{d}{dt}[CO_2] = [C_{Glu} - f_{Ac} C_{Ac} (1 - Y_{Su}) - C_{Bio} Y_{Su}] \cdot r_F + [C_{Ac} - C_{CH_4} (1 - Y_{Ac}) - C_{Bio} Y_{Ac}] \cdot r_{AM} + [- C_{CH_4} (1 - Y_{H_2}) - C_{Bio} Y_{H_2}] \cdot r_{HM} \quad (3)$$

where $f_1 = \frac{[Ac^-]}{[Ac^-] + [HAc]} = \frac{10^{-pK_{ac}}}{10^{-pH} + 10^{-pK_{ac}}}$ and $f_2 = \frac{10^{-pK_1}}{10^{-pH} + 10^{-pK_1}}$; r_F, r_{AM} and r_{HM} denote the rates of fermentation, aceticlastic methanogenesis and hydrogenotrophic methanogenesis respectively.

Since the system works at constant pH, the titration rate equals the proton production rate ($r_t = r_H$).

In the methanogenesis-inhibited unit, the term r_{AM} in eqns. (2) and (3) equals zero and the titration rate becomes:

$$r_t(B) = \{f_1 \cdot f_{Ac} (1 - Y_{Su}) + f_2 [C_{Glu} - f_{Ac} C_{Ac} (1 - Y_{Su}) - C_{Bio} \cdot Y_{Su}]\} \cdot r_F \quad (4)$$

which shows that the fermentation rate is proportional to the titration rate through the stoichiometric parameters of the model. In the non-inhibited unit, r_t is a combination of both the hydrogenotrophic (r_{HM}) and aceticlastic (r_{AM}) rates. The former has generally a smaller effect on r_t and can be neglected:

$$r_t(A) = r_t(B) + \{f_2 [C_{Ac} - C_{CH_4} (1 - Y_{Ac}) - C_{Bio} \cdot Y_{Ac}] - f_{Ac}\} \cdot r_{AM} \quad (5)$$

However, the contribution of hydrogenotrophic methanogenesis to the production of methane is significant: therefore, if this latter is measured, this would provide a second equation in which the effect of r_{HM} is greater, thus allowing the estimation of both methanogenic rates separately.

CONCLUSIONS

We propose an experimental procedure in which a pH-stat titration sensor is used to assess biodegradability and microbial activity under anaerobic conditions. Although the pH-stat titration technique is inherently not suitable for monitoring simultaneous pH-affecting processes, by sequentially monitoring the undisturbed sludge and the sludge in which methanogenesis is inhibited, one is able to accurately calculate the parameters of interest.

A mathematical model of a simplified two-stage anaerobic process has been used to simulate the outcome of the titration assay. Experiments will be carried out to validate the model.

Should this technique prove to be reliable and accurate, it could become a valuable alternative to other common techniques used to assess anaerobic biodegradability and activity.

SYMBOLS

Y_i Biomass yield (g VS/g COD). The subscripts *Su*, *Ac* and H_2 denote the fermentative, the aceticlastic and the hydrogenotrophic biomass, respectively.

C_i Carbon content (mol C/g COD). The subscripts *Glu*, *Ac*, CH_4 and *Bio* denote Glucose, Acetate, Methane and the Biomass, respectively.

f_{Ac} Acetate yield (-) from the fermentation of the organic substrate.

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PROTOCOL FOR THE ASSESSMENT OF THE DISPOSAL OF ORGANIC CONCENTRATES BY CO-DIGESTION

Enrico Remigi and Chris Buckley

Pollution Research Group - University of KwaZulu-Natal, 4041 Durban (South Africa)

Keywords: co-digestion, industrial effluents, management

ABSTRACT

The province of KwaZulu-Natal is relatively well endowed with water resources and has thus attracted water intensive industries such as the textile industry. Increasing population and development has increased the pressure on the resource which together with proposed waste discharge charges have lead to increased costs for water supply and wastewater treatment. This has lead to the increasing adoption of various management tools, such as waste minimisation and water conservation.

These methods have proved effective in reducing both the water consumption and the discharge of large volumes of wastewaters. As a consequence, the residues have generally become more concentrated in organic pollutants which are not ideally suited for conventional aerobic treatment systems.

The existence of underutilised anaerobic digesters in the region has suggested the option of anaerobically treating municipal sludge (which is considered a *labile* substrate) in combination with those *high strength* industrial effluents which are potentially toxic for the microbial consortia if digested in isolation.

The advantages of *co-digestion* are that *i*) it is generally less expensive than the separate treatments; *ii*) due to the dilution, it can improve the digestibility of a highly concentrated effluent as well as counteract the presence of inhibitors or the lack of nutrients; and *iii*) it can also favour the rise of *co-metabolism*, which is often the only way to achieve detoxification of specific organic compounds.

This paper will present a protocol whereby the suitability of an organic effluent to be co digested can be assessed.

The protocol consists of assessing the anaerobic biodegradability and toxicity of an effluent and in defining the operating conditions that could be safely applied to the process.

A preliminary screening of the targeted effluent stream is carried out at the laboratory-scale, using a rapid titrimetric bioassay followed by a serum bottles assay. These tests enable the accurate quantification of the biodegradability and the toxicity and define a volumetric co-digestion ratio

between municipal sludge such that the effluent does not negatively affect the performance of the digestion process. If the sample passes these tests then, a co-digestion trial on a small-scale unit will simulate the full-scale process and is aimed at optimising the process and selecting the best operating conditions for the full-scale implementation.

The protocol includes a monitoring phase whereby the characteristics of the effluent source (which often vary as a consequence of changes in the production line) and the performance of the co-digestion process are verified. A sampling campaign at the co-digestion works monitors the activity of the sludge and the methanogenic toxicity of the effluent, so that action can be taken to ensure the continuous economical viability and environmental safety of the process.

MODELLING OF ANAEROBIC DIGESTION PROCESSES: A REVIEW

Enrico Remigi and Katherine Foxon

Pollution Research Group - University of KwaZulu-Natal, 4041 Durban (South Africa)

Keywords: anaerobic digestion, modelling

ABSTRACT

This paper gives an introduction to the chemistry and microbiology of anaerobic digestion and presents a review of the major developments that have taken place in the modelling of anaerobic digestion systems from the first recognised attempts in the sixties to the detailed mechanistic models that are being attempted today. A similar approach may be found in Rozzi and Passino (1985) and more recently in the Anaerobic Digestion Model No. 1 (ADM1) (Batstone et al., 2002). In presenting the evolution of the understanding of the processes of anaerobic digestion, a comprehensive picture of the individual constituents of current complex anaerobic digestion models evolves, and some clarity on the means by which they may be reasonably simulated may be obtained.

Anaerobic digestion has been used for more than a century for the stabilisation of waste sludges and degradation of organic wastes (McCarty, 1971). It is a multi-step process of parallel and sequential reactions in which complex substrates are degraded by different micro-organisms within the anaerobic microbial consortia to simple monomers that are finally converted to carbon dioxide and methane gas.

The anaerobic conversion of organic waste to methane and carbon dioxide occurs in the absence of oxygen, and consequently requires no direct energy input other than organic substrate to effect the biochemical conversions. Anaerobic digestion has significant advantages over other methods of waste treatment (Lettinga, 1995), including a low production of waste sludge (due to the slow growth rates of micro-organisms), low power requirements (unlike activated sludge processes which require oxygen) and formation of a useful product, methane, which is a source of energy. Moreover, the digested sludge is a good soil conditioner. However, low growth rates of the microbial populations mean that high biomass detention times are required to achieve practical treatment rates. Furthermore, anaerobic digestion has historically not enjoyed a good reputation, because of its poor record with respect to process stability.

Anaerobic digestion is commonly visualised as a biochemical chain reaction, where the product of one process is the reactant of the next; however, in reality, it is a complex system of *microbiologically mediated* and *physico-chemical* sub-processes, with feedback loops of substrate and product regulation and inhibition. In fact, there is a relatively narrow range of environmental or operating conditions in which all the sub-processes of the digestion can function simultaneously.

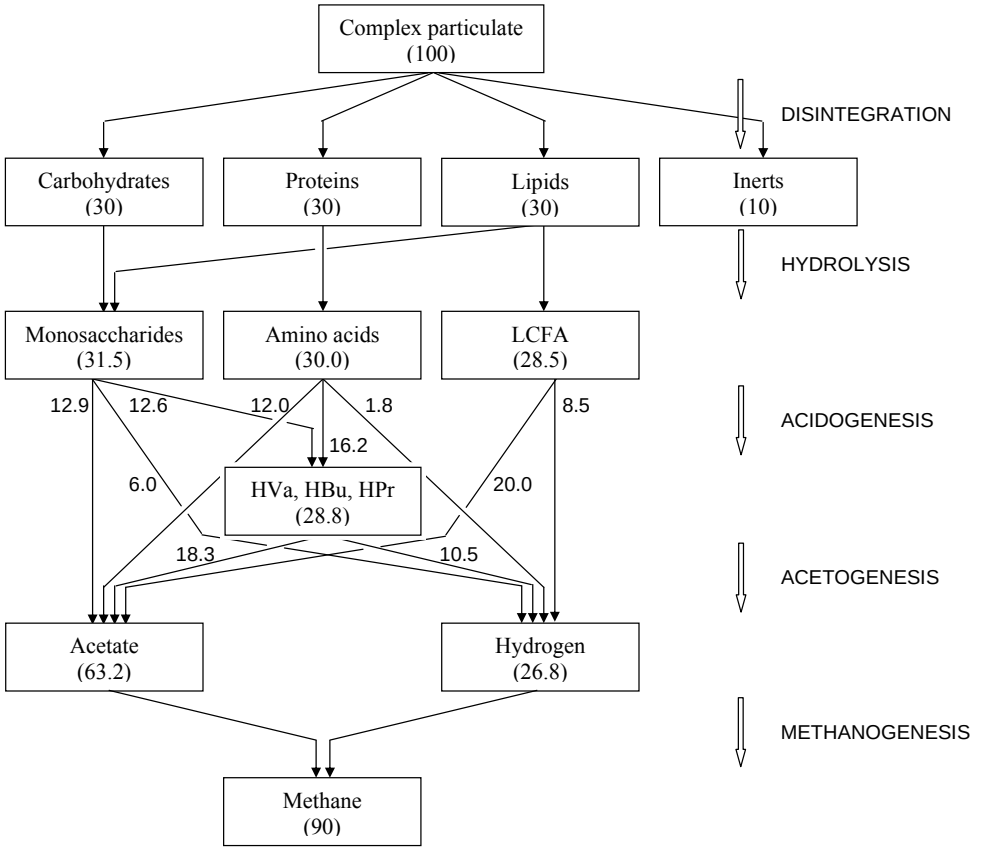

Figure 1 – Flow-diagram for the anaerobic degradation of a composite particulate material, as implemented in ADM1 (from Batstone et al 2002). Valerate (HVa), Butyrate (HBu) and Propionate (HPr) are grouped for simplicity. Figures in brackets indicate COD fractions.

BIOCHEMISTRY AND MICROBIOLOGY

Biochemical processes may be subdivided into extracellular processes, effected by enzymes (e.g., disintegration, hydrolysis) and intracellular processes.

Disintegration and hydrolysis: Complex materials such large molecular weight proteins, carbohydrates and lipids are hydrolysed extracellularly by enzymes excreted by hydrolytic bacteria, with some release of energy. This process can be divided into the *disintegration* of composite particulate material into smaller carbohydrate, protein and lipid fractions and the *hydrolysis* of large molecular weight compounds to *long chain fatty acids* and the monomers of sugar and protein compounds; to simple sugars; and to *amino acids*.

Anaerobic oxidation: *Long chain fatty acids* are oxidised to simple organic acids in a process called *anaerobic oxidation*, in which the carbon chain is sequentially shortened by two carbon atoms at a time. The final product of fatty acids with an even number of carbon atoms is *acetate* only; when the fatty acid has an odd number of carbon atoms, one mole of *propionate* is produced per mole of substrate. Relatively large amounts of dissolved hydrogen are released in this process.

Acidogenesis: *Amino acids* and *simple sugars* are fermented by the next category of bacteria, termed *acidogenic* or "acid formers" that produce simple organic acids such as acetic, propionic, butyric and lactic acids. The end product of acidogenesis is determined by the environmental conditions, as will be presented later (Mosey, 1983). Different amounts of di-hydrogen are produced depending on the acidogenesis end product.

Acetogenesis: A further category of bacteria (*acetogenic*) ferment *propionic, butyric and lactic acids* to *acetic acid*. In most cases, each group of acetogens can only ferment one type of organic acid.

Aceticlastic methanogenesis: The final stage in anaerobic digestion is the conversion of *acetic acid* to *methane* and *carbon dioxide* by a group of *Archaea* known as *aceticlastic methanogens*. The conversion to methane is the only strictly anaerobic step which results in the removal of chemical oxygen demand to the gas phase.

Hydrogenotrophic methanogenesis: *Hydrogenotrophic methanogenesis* is the production of methane from *dissolved hydrogen* and *carbon dioxide* by a select group of slow-growing methanogens. This last process can account for up to 30 % of the methane produced by anaerobic digestion of an organic waste.

Homoacetogenesis: *Homoacetogenesis* is the generation of acetic acid from *dissolved hydrogen* and *carbon dioxide*. Homoacetogens are one of the most versatile physiological group among the anaerobic bacteria. They utilise and transform one-carbon compounds and can carry out incomplete oxidation of reduced fermentation products released by other fermenting bacteria. Homoacetogens can use various substrates sequentially or simultaneously and may constitute an energy link from hydrogen, via acetate to heterotrophic methanogens.

Sulphate reduction: The presence of any sulphate in the waste will result in *sulphidogenesis*, the generation of sulphide from sulphate. As with methanogenesis, this process can be either aceticlastic or hydrogenotrophic. Organisms reducing sulphur can obtain the electrons directly by oxidising organic acids, or by oxidising the hydrogen produced by acetogens. Additionally, organic acids are used as a carbon source, and as a result, organisms reducing sulphur compete with the majority of groups in anaerobic digestion (Kalyuzhnyi and Fedorovich, 1998). Further complicating the effect on anaerobic systems, the reduced product, sulphide, is inhibitory to different extent for almost all microbial groups. Reduced sulphide has a similar acid-base system to the inorganic carbon system. Hydrogen sulphide is also a gas phase component, with a relatively high solubility.

Denitrification: Dissimilatory nitrate reduction, a form of anaerobic respiration, is the reduction of nitrate (NO_3^-) to nitrogen oxides – such as NO_2^-, NO, N_2O – and N_2. Denitrification has a higher yield per unit of substrate consumed than methanogenesis and competes for the same substrate. Denitrification intermediates have also been found to inhibit methanogenesis. This results in a decrease in methane production and an increase in alkalinity. In an overall methanogenic system, nitrate reduction can have significant impact on both the carbon and electron flow, on microbial competition and inhibition, and on gas composition.

PHYSICO-CHEMICAL PROCESSES

The physicochemical system can be defined as non-biologically mediated processes that commonly occur in anaerobic reactors. There are three broad types, listed below:

- Liquid-liquid processes: this mainly refers to ion association and dissociation with hydrogen and hydroxide ions. There are a number of important compounds, which have dissociation constants close to the operating pH of anaerobic systems.
- Gas-liquid processes (i.e., gas liquid transfer of carbon dioxide, methane, hydrogen, hydrogen sulphide and nitrogen gases).
- Liquid-solid processes (i.e., precipitation/solubilisation of ions).

Important physico-chemical subsystems include inorganic carbon, organic acids dissociation, sulphate, sulphite, sulphide, ammonia/ammonium, oxidised nitrogen, phosphate, and the gas liquid interaction hydrogen and methane gases.

THERMODYNAMICS AND SELF-REGULATION

From a thermodynamic point of view, the energy change per unit (mole) of substrate fermented in anaerobic digestion is very small; moreover, many organic compounds are fermented to methane in a step-wise process by different groups of micro-organisms, therefore the little energy yield from the overall conversion must be divided into even smaller packets. The extent to which bacterial growth occurs is a function of the energy released by the electron transfer and the efficiency of the energy utilisation by the micro-organism mediating the transfer. The low amount of energy released in anaerobic transformations results in a low growth yield for all types of anaerobic micro-organism, although the individual growth yields for each type of micro-organism depend on the energy release by the reaction that they mediate and the conditions under which they operate. McCarty (1971) uses a thermodynamic approach to understand which of the many possible reactions are likely. The dominant micro-organisms in a consortium will be those that can harvest the available energy in any substrate most rapidly and efficiently in a given environment. These species will outcompete other micro-organisms that utilise the same substrate.

This has consequences for process stability since upsets in environmental conditions can reduce the Gibbs free energy of a certain sub-process to the point where it is no longer thermodynamically feasible, or only at dramatically reduced rates. This in turn interrupts the supply of substrate and therefore energy to all subsequent processes that occur later in the metabolic chain. For example, the harmony between propionate oxidation, acetate decarboxylation and hydrogen oxidation is critical for a stable anaerobic digestion process. The optimal range for all three reactions (i.e., they must all be exergonic) is very narrow, and is mainly controlled by free propionate, hydrogen and acetate concentrations. (Gujer and Zehnder, 1983).

The peculiarity of the anaerobic digestion process is that most of the control is undertaken directly by the micro-organisms themselves: i.e., pH regulation by the formation and removal of short chain fatty acids and regulation of redox potential by formation and removal of trace concentrations of dissolved

hydrogen (Mosey, 1983). Andrews (1969) suggested that instability and failure of such an ecosystem might be caused by substrate inhibition: high concentrations of volatile fatty acid produced by the fast growing acidogenic bacteria inhibit the metabolism of the slowly growing methanogens. In fact, the specifics of pH and hydrogen regulation have been shown to be very complicated, and are not fully understood: product and substrate inhibition of several of the anaerobic sub-processes have been observed, resulting in feedback loops which assist in maintaining pH and redox conditions in ranges which allow anaerobic digestion to proceed. These issues are presented in greater detail later. It is however possible to increase the range of operating conditions and stability to a certain extent by a physical separation of acidogenic and methanogenic bacteria in serial reactors since they are sufficiently different with respect to their physiology and nutritional requirements (Van den Heuvel and Zoetemeyer, 1982).

From the preceding discussion, it is clear that the biochemistry of anaerobic digestion is extremely complicated, and sensitive to changes in environmental factors. The application of anaerobic digestion is limited by the ability to predict digestion performance and control anaerobic processes; Andrews and Graef (1971) pointed out that in the sixties, operation practice consisted only of a set of empirical rules, with no clear (and quantitative) understanding of the process and claimed that a dynamic model would ultimately enable the setting up of control procedures, thus allowing the prevention of failure. There is thus a three-fold need for the development of anaerobic digestion models in (1) understanding the interactions of the sub-processes (2) optimising design and operation for improved steady-state performance and stability and (3) controlling dynamic processes.

MODELLING

Modelling is the systematic application of kinetics to carefully defined processes within a carefully defined system. Process kinetics plays a central role in the development and operation of anaerobic treatment systems. Based on the biochemistry and microbiology of the anaerobic process, kinetics provide a rational basis for process analysis, control and design. A knowledge of kinetics allows for the optimisation of performance, a more stable operation, as well as better control of the process.

Biological growth kinetics are based on two fundamental relationships, i.e., substrate utilization rate (equation 1) and growth rate (equation 4). The growth rate is related to the substrate utilisation rate by a growth yield, the portion of substrate consumed which is converted into biosolids. In anaerobic digestion, the majority of substrate is converted into gaseous end-products with the concomitant release of energy, which is mostly required for the continuation of non-growth related cell functions. Endogenous respiration, cell maintenance, predation, cell death and lysis are processes leading to a decrease in cell mass. To account for the effect of these processes, a micro-organism decay rate is usually used for the modification of the growth rate. Microbiological process kinetics are therefore a function of three factors: cell growth, energy generation (the combination of which may be measured as substrate utilisation) and cell reduction (see: Appendix).

Each of the microbiologically mediated reactions described above is governed by a kinetic expression of the form of equation 1. These reactions are strongly interdependent since most processes consume products of other processes, and may be inhibited by actual concentrations of reactants or products, or by operating conditions affected by substrate utilisation or product generation. A complete mathematical description of the state-of-the-art of current understanding in a general anaerobic digestion system would necessarily be extremely complicated.

Therefore, despite improvements in understanding of anaerobic digestion, mathematical modelling is nevertheless faced with a compromise between detail and robustness: very detailed mechanistic models of many sub processes may provide an accurate theoretical representation of the processes, but are unwieldy and difficult to calibrate. A common problem with biological models is poor structural identifiability (Dochain et al., 1995): it is often not possible to identify independent parameter values for highly parameterised models given the possible experimental measurements available. Thus a calibration that appears to reproduce experimental data accurately may only be a local solution for parameter values, when the true parameter value set (or parameter estimation objective function global minimum) provides similar simulation results under the conditions tested, but cannot correctly simulate performance under different conditions.

It is often therefore necessary to select a less complicated model structure which leaves out highly specific sub processes but is able to describe the general picture sufficiently well and is therefore ideally applicable to process control (Batstone et al. 2002). A whole range of more robust options are available, from mechanistic models that are constructed from the rate limiting processes only, to fuzzy logic models, and control models that are constructed without considering the underlying mechanisms that cause process dynamics at all. Models in these categories may be able to predict events, but in general cannot explain why they occur.

There are three broad categories of mathematical models:

- Mechanistic models attempt to describe the interactions of the sub-processes as they are understood; in this category it is possible to differentiate between static and dynamic models.
- Fuzzy logic models are semi-mechanistic in that they predict process response to variations in inputs and conditions according to a set of carefully defined rules; however the relationships are based on the magnitude of the observed response, as opposed to an understanding of the biochemistry thereof.
- Neural networks provide an accurate nonlinear mapping between input-output pairs of data without knowing their functional relationship.

Much research has been directed towards the development of mechanistic models. This category is presented in the section that follows. As the two last categories are system specific, both in terms of calibration and application, their use is limited to monitoring and control of real systems. They are reviewed in the context of on-line and control applications.

MECHANISTIC MODELS

Most early attempts to kinetically describe anaerobic digestion relied upon the so-called rate-limiting step approach. Generally speaking, when a process is composed of a sequence of reactions, one step is usually very much slower than the other steps. The last slow step in a sequence of reactions has been called the rate-limiting or rate-determining step; that step that will cause process failure to occur under conditions of imposed kinetic stress. In the context of a continuous culture, kinetic stress refers to the imposition of a continually reducing value of the solids retention time until it is lower than its limiting value and results in washout of the micro-organisms. In anaerobic treatment, washout-type failure is associated with the slowest growing micro-organisms, usually methanogens, leading to near cessation of methane production, decreasing substrate consumption and build-up in the concentration of long- and short-chain fatty acids. Process failure in anaerobic digestion can also result from a disturbance in the redox potential or pH (or both) caused by a non-steady state accumulation of a reactant or product to a certain process, without necessarily involving wash-out. Souring incidents are of this type, where the Gibbs free energy of an acid removal or buffering process that is inhibited by low pH or high VFA concentration decreases, preventing a steady-state rate of acid removal that will maintain the pH or VFA concentration in sufficiently non-inhibitory ranges.

In anaerobic digestion, the rate-limiting step is related to the nature of the substrate, process configuration, temperature and loading rate (Speece, 1983). Although the rate-limiting approach leads to relatively simple mathematical descriptions of the process, a more universal and fundamental approach is required for a better understanding, which can only be satisfied by examining each major step of the anaerobic process separately.

The publication of the Anaerobic Digestion Model no. 1 (Batstone et al., 2002) is the first attempt to provide a generalised and comprehensive structure from which to begin mechanistic modelling of anaerobic processes. In the previous three decades, a variety of not necessarily homogeneous modelling tools have been developed for specific applications, which have not had a wide application in industrial practice by engineers, process technology providers and operators. The *Anaerobic Digestion Modelling Task Group* described ADM1 as the "first generalised model" of anaerobic digestion, and to have the advantages of being:

- a reference mathematical tool for full-scale plant design, operation and optimisation;
- a reference mathematical tool for further research on process optimisation and control, aimed at direct implementation in full-scale plants;
- a common basis for further model development and validation studies;
- a tool to assist in technology transfer from research to industry.

This model was developed as a result of a thorough review of the developments in anaerobic digestion modelling and the relative successes and failures of the different approaches that had been adopted. In this section, a review of the research that has lead to the current understanding of the mechanics of anaerobic digestion, as is embodied in ADM1, is presented.

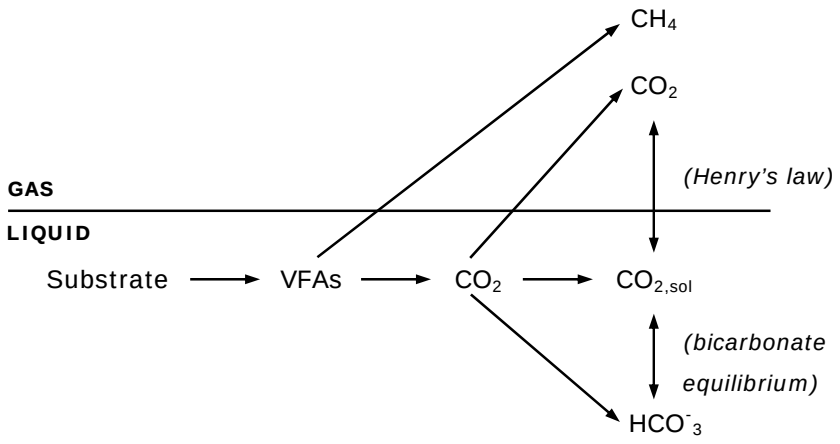

Figure 2 – McCarty's verbal model of the anaerobic process (from Rozzi et al., 1985 b).

DEVELOPMENT OF BIOCHEMICAL STRUCTURES OF ANAEROBIC MODELS

The first verbal model was presented by McCarty (1964), consisting of two stages characterised by the conversion of the organic matter into fatty acids and the conversion of these latter into methane. No differentiation of biological populations was included. This model used the approach of the "rate-limiting" step, i.e., the entire process is kinetically governed by the slowest step.

Lawrence and McCarty (1969) described anaerobic digestion as a three-step process (hydrolysis, acid production and methane production), and indicated that the methanogenic step was generally rate-limiting. In this work, studies on the kinetics of methanogenesis precursors acetic, propionic and butyric acids were reported, and Monod-kinetics were proposed for the rate-limiting step. Variations of Monod parameters with temperature were observed.

McCarty (1971) then described a steady-state model in which kinetics were described by a simple Monod relationship, cellular yield was expressed as a function of reaction energetics, and the rate of electron transfer in energy yielding reactions was dependent on temperature only. Therefore, carbohydrate, protein and hydrogen fermentation produce the highest relative yields as a result of the greater energy released per electron equivalent fermented. This model is most sensitive to variations in the cell retention time, and chiefly contributed to understanding the performance of a system to different cell retention times. A thermodynamic approach was presented to explain why certain biochemical routes and reactions are favourable under differing reaction conditions.

Andrews and Graef (1971) developed a one-population model in which the key features were the use of an inhibition function to relate volatile acids concentration and specific growth rate of methanogenic bacteria and the consideration of unionised volatile acids as both the growth-limiting substrate and inhibiting agent. The former allowed prediction of process failure due to high concentration of volatile acids at biomass residence times exceeding the wash-out residence time. The latter resolved the conflict as to whether low pH or high concentration of volatile acids are inhibitory,

as the concentration of unionised acids is function of both pH and total volatile acids concentration. The model removed the limitation of operation under constant pH (Andrews, 1969), by incorporating the interaction in and between liquid, gas and biological phases, since by modelling gas exchange, it is possible to obtain an indication of variations in pH value. The authors recognised the complexity of the series process, in which for instance, an increasing concentration of volatile acids can be caused by an increased rate of acid production by the acidogenic bacteria or by a decreased rate of consumption by the methanogenic bacteria. However, their model was limited by the assumption that the conversion of volatile acids to methane and carbon dioxide is rate-limiting (plus other limitations, minor in this context) and considered a reactor feed of acetic acid only.

Andrews and Graef (1971) also defined *stability indicators* and *control variables*: the former are those variables that display large variations in the shortest possible time after a potential process disturbance has affected the system; the latter are those that can be accurately determined and greatly affect the system. Control variables should be sensitive to process changes but not exhibit large oscillations as a result of them. For this reason, they are often associated with the gas phase, due to its smaller mass inertia. (Gujer and Zehnder, 1983).

Van den Heuvel and Zoetemeyer (1982) described a two-phase process with cell recycle in which the methanogenic step is rate limiting and unionised fatty acids are the rate limiting and inhibitory substrate. A set of dimensionless equations (for substrate and biomass dynamics) were presented and analysed for steady-state and dynamic conditions. The steady-state conditions show that there exists a domain of residence times with three steady-state solutions: a high conversion, a low conversion and a wash-out state. The high conversion state is stable. A numerical method (i.e., phase planes, where trajectories possess a time direction that converge at the steady-state) to study dynamic behaviour of the system was used to show the effects of different operating conditions on the performance of the process to prevent wash-out, e.g., inoculum size, hydraulic retention times, influent substrate concentration.

Rozzi (1984) presented a two population model, namely acidogenic and methanogenic micro-organisms, in which carbohydrates are converted to acetic acid and subsequently to methane and carbon dioxide. Both acidogenesis and methanogenesis were described using a Monod function. pH inhibition was accounted for in the functional description of maximum growth rate. The physico-chemical subsystem considered the inorganic carbon balance HCO_3^-/CO_2, the dissociation of acetate, Henry's law and mass and ionic balances. It assumed that gas and liquid phases are in equilibrium. The model was used to establish the most sensitive stability indicators and control variables (such as pH and bicarbonate alkalinity) on which to base process control strategies to cope with organic overloads.

Eastman and Ferguson (1981) first modelled hydrolysis of particulate organics, developing a two-stage model for the acid phase of anaerobic digestion (hydrolysis, followed by formation of fatty acids. The authors proposed first order kinetics for the former process, in which the rate constant can be a function of, amongst other factors, pH, temperature, microbial mass (the source of extracellular

enzymes), the particle size, etc. The formation of fatty acids is assumed to follow Monod kinetics. They showed that in the conversion of solids to fermentation products, the kinetics of the overall process is determined primarily by the rate of hydrolysis. The first-order function reflects the cumulative effect of the hydrolysis processes occurring and implies that not all the particulate material is degraded with equal facility: it is therefore most appropriate to complex, heterogeneous substrates. De Baere et al. (1984) developed a similar mathematical model for the degradation/hydrolysis of complex particulate organic matter using zero-order kinetics, in a complex function of the initial substrate and active biomass concentration. The model was applied to an anaerobic contact process, showing that hydrolysis is the rate-limiting step (and therefore that a form of pre-treatment of the particulate fraction or the addition of soluble wastes as auxiliary substrates can improve the hydrolysis rates).

A more detailed understanding of the microbiology and biochemistry of acidogenesis was developed in the early eighties (Zeikus, 1980), that showed that the overall picture of a two step digestion in which substrate was degraded first to organic acids, and then directly to methane was in fact much more complicated, in that: (i) volatile fatty acids other than acetic and formic are not directly degraded by methanogens; these must first to be metabolised by obligate hydrogen producing acetogens; (ii) the kinetics of such reactions strongly depend on the hydrogen partial pressure; (iii) for most substrates there are two different routes for methanogenesis, i.e., from acetic acid and from hydrogen.

Mosey (1983) proposed a "cornerstone" model in which the formation and removal of trace concentrations of dissolved hydrogen gas regulate redox potential. Glucose was selected as a representative organic substrate: under optimal reaction conditions, the preferred organic acid product of acid-forming bacteria from glucose is acetic acid, because it provides the highest energy yield for growth. Formation of butyric and propionic acids are the bacteria's response to accumulations of hydrogen: the first one reduces the output of hydrogen and the acid load on the system; the latter puts hydrogen production into reverse. The main route for glucose conversion to organic acid involves the transfer of hydrogen first to the carrier molecule nicotinamide adenine dinucleotide (NAD^+), converting it to $NADH$ and H^+ and is subsequently released into solution as dissolved hydrogen gas (see: e.g., Bailey and Ollis, 1986).

The Author described the rate equations for glucose uptake (i.e. acid formation), in terms of $NADH/NAD^+$ relative availability; $NADH$ and NAD^+ concentrations cannot be easily determined, but a more useful indicator can be estimated from the oxidation state of the NAD carrier molecule, which is related to the concentration of H_2 in the digester gas.

The same principles were applied to the conversion of propionic and butyric acids into acetic acid (acetogenesis) and to methane formation (methanogenesis) from either acetic acid or carbon dioxide and hydrogen. In conclusion, the model included four microbial populations, (i.e., two acetogenic populations; propionic and butyric acid utilisers, aceticlastic and hydrogenotrophic methanogens) and calculated the relative rates of formation of different acids in terms hydrogen partial pressure.

Gujer and Zehnder (1983) presented a widely used model with six processes and five microbial groups that could be used for steady-state evaluation. Importantly, this paper described the balance between propionate oxidation, acetate decarboxylation and hydrogen oxidation, and indicated the sensitivity of these processes to environmental conditions. The authors suggested that the partial pressure of hydrogen, and acetate concentration are suitable stability indicators. Partial pressure of hydrogen is an indicator of process upset as it is unlikely that a digester will remain stable when propionate oxidation has shifted to a thermodynamically unfavourable condition.

Rozzi et al. (1985, b) presented a four population model (Figure 3), which incorporated Mosey's kinetic equations. The acidogenic population were as obligate hydrogen producing bacteria, and hydrogen regulation was an important feature of the model. It also considered the nitrogen requirement for biomass synthesis and the pH influence on biomass dynamics (on both growth and decay); it neglected methane solubility in the liquid phase and the effect of ionic strength on physico-chemical equilibria. The main differences of this model to that of Mosey (1983) are associated with the calculation of gas production rates, and the treatment of hydrogen in the gas space: no fixed ratio between methane and carbon dioxide flow rates is assumed, allowing hydrogen gas to form part of the gas flow. A mathematical difficulty arises since the flow of gaseous hydrogen is the balance between the rates of production and consumption, and may therefore lead to instability in the solution. Mosey (1983) simulated a reactor using an artificially large overhead volume to damp the oscillations of hydrogen concentration. Rozzi adopted two approaches to calculate the hydrogen partial pressure, i.e., assuming that hydrogen is insoluble or considering the solubility of hydrogen: the two approaches prove to be practically equivalent. Rozzi et al. (1985, a) described a possible method of process control based on monitoring bicarbonate through the measurement of carbon dioxide gas evolution in an acid titration.

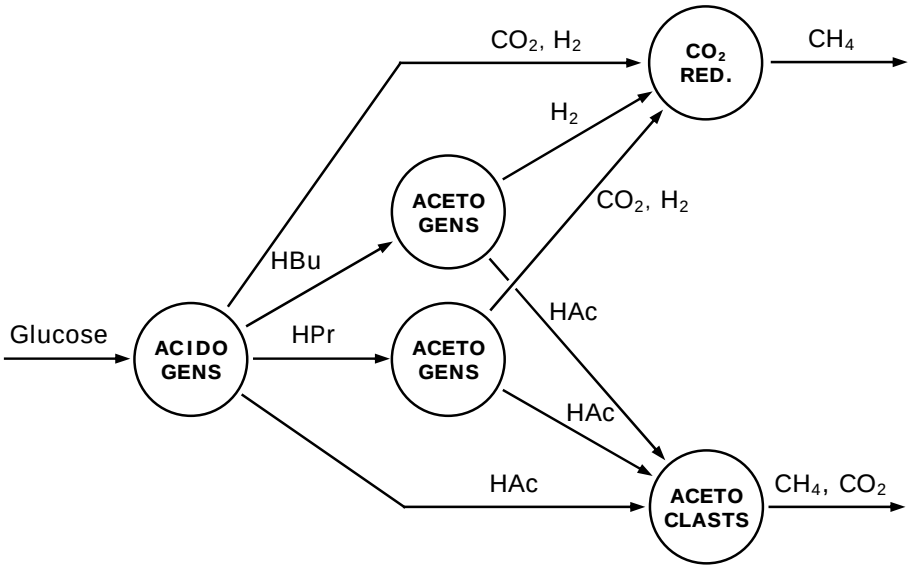

Figure 3 – Flow diagram of the 4-population model (from Rozzi et al., 1985 b).

Siegrist et al. (1993) attempted to develop a comprehensive mathematical model for (mesophilic) anaerobic digestion processes, based on Gujer and Zehnder (1983). It consisted of six biological processes and five microbial populations (amino acid and sugar fermentation, anaerobic oxidation of long chain fatty acids, acetogenesis, aceticlastic and hydrogenotrophic methanogenesis), the respective decay processes, stripping of biogas, the and dissociation of bicarbonate. The concept of a multi-component particulate substrate was introduced, although the model considered a single particulate substrate with a fixed composition of the components protein, carbohydrate and lipid that were hydrolysed in constant ratios to two intermediates, a lumped amino acid and sugar component, and a lipid hydrolysis product termed "fatty acids". All microbial processes were modelled with Monod kinetics, modified to incorporate pH dependence and inhibition due to increasing hydrogen partial pressure and acetate concentration. The model was able to simulate variations in load on a full-scale digester, but exclusion of acid-base dynamics at low pH values (dissociation of volatile fatty acids) limited its ability to describe reactor failure.

Vavilin et al. (1994) developed a seven population model consisting of one enzymatic step, i.e., hydrolysis, two acidogenic steps, one acetogenic step, two methanogenic steps, two sulphate-reducing steps, lysis and hydrolysis of cellular material after bacterial death. This model extended the concept of multiple hydrolysis products (viz. proteins, lipids and carbohydrates). All processes were described with an optimal rate (i.e., Monod equation) that is corrected using a number of factors such as temperature, substrate limitation and various sources of inhibition (pH, hydrogen partial pressure and concentration of unionised molecules, i.e. ammonia, sulphide and propionate; see: Appendix). The same structures of the hydrolysis process and rate expressions were later applied in ADM1. This model could be considered to be the natural predecessor of ADM1, although more complicated in that, unlike ADM1, it included sulphate-reducing processes.

Valentini et al. (1997) quantitatively assessed the influence of biomass concentration in a first order model (equation 6), with an exponent between 0 and 1 affecting the biomass concentration. The "qualitative" meaning of the exponent may be related to the relative availability of substrate: the higher the biomass concentration, the higher the fraction of biomass that does not have full access to substrate. In essence, the apparent biomass concentration is reduced as a result of substrate limitation to a portion of the biomass due to substrate diffusion constraints.

At low temperature and high hydrogen partial pressure, homoacetogenesis (generation of acetic acid from hydrogen and carbon dioxide) has been observed to dominate over hydrogenotrophic methanogenesis, and consequently, a greater proportion of methanogenesis occurs via the aceticlastic route than the hydrogenotrophic route than at higher temperatures. Vavilin et al. (1997) modified the earlier model (Vavilin et al., 1994) to include homoacetogenesis.

Angelidaki et al. (1999) published a comprehensive model of co-digestion of complex wastes with different characteristics and composition. The substrate is divided into carbohydrate, lipid and protein components. It involved two enzymatic processes, i.e., hydrolysis of particulate carbohydrates and proteins and eight biological processes, i.e., glucose-, lipid- and amino acid-degrading bacteria, four groups of acetogens and aceticlastic methanogens. It considered various sources of inhibition, i.e., free ammonia on methanogenesis, fatty acids on hydrolysis, acetate on acetogenesis (product inhibition), and inhibition of all bacterial groups by long chained fatty acids. Hydrogen was not explicitly modelled. For all microbial steps, Monod type kinetics were used with respect to the primary substrate; a secondary substrate (i.e., nitrogen) was also included in the same form; and inhibition was modelled as non-competitive or Haldane-type.

The current "state-of-the-art" in anaerobic digestion modelling is contained in the Anaerobic Digestion Model No. 1. This model is presented based on an extensive review of anaerobic digestion modelling and current understanding of the microbiology and biochemistry of the anaerobic digestion process. However it necessarily applies several simplifications to the existing knowledge in an effort to achieve an acceptable level of usability and mathematical robustness. Probably the main contribution of ADM1 is that it introduces generic nomenclature, units and definitions.

The model includes three overall biological (cellular) steps, i.e., acidogenesis (or fermentation), acetogenesis (anaerobic oxidation of organic acids) and methanogenesis. Extracellular processes are divided into a (partly non-biological) disintegration step and a hydrolysis step. Three of the processes (hydrolysis, acidogenesis, and acetogenesis) have a number of parallel reactions. The disintegration step is intended to include an array of steps such as lysis, non-enzymatic decay, phase separation, and physical breakdown. All extracellular steps were assumed first order, which is an empirical function reflecting the cumulative effect of a multi-step process (Eastman and Ferguson, 1981). Cellular kinetics are described by three expressions (uptake, growth and decay). The key rate equation is substrate uptake, which is based on substrate level Monod-type kinetics. Substrate uptake related kinetics (rather than growth related kinetics) are used to decouple growth from uptake. Biomass growth is implicit in substrate uptake, as it is linked to growth rate. First order biomass decay (to complex organics) is assumed and is an independent set of expressions.

KINETICS

This section presents a summary of the major developments and current understanding of the kinetics of the biochemical sub-processes of anaerobic digestion. Kinetics of inhibition and the effects of mass transfer considerations on the condition of anaerobic digestion are also presented.

In general, for biologically mediated processes, reaction rates follow Monod kinetics (equation 1). However, the range of environmental conditions under which these kinetics may be applied

unmodified is limited by inhibition of some or all of dissolved hydrogen concentration, unionised volatile fatty acids, pH value, ammonia and other external toxic or inhibitory substances. Many studies have been undertaken to determine the basic structure of the rate equations, and the mechanism of inhibition by various factors.

Kinetics of inhibition

Early models demonstrated that simple inhibition patterns could create complex model behaviour, although the specific types of inhibition were not fully clarified. Since then, inhibition patterns have been subject of many investigations and models of increasing complexity have been formulated along with the recognition of several important types of inhibitions and interaction among various bacterial groups. For a review on the description of substrate and product inhibition, see Mösche and Jördening (1999).

Inhibition kinetics considered are shown in equations 2 and 3 in the Appendix.

The most commonly used models of inhibition are Haldane kinetics (equation 2) and a non-competitive inhibition factor applied to Monod kinetics (equation 3.2). The latter has been used extensively because of the ease with which it may be applied to existing Monod kinetics without requiring the re-evaluation of kinetic constants. Other common types of inhibition are pH inhibition, usually modelled using empirical functions (equation 3.4) and competitive inhibition (equation 3.1). Secondary substrate Monod kinetics are included (equation 3.3) although they do not strictly represent a form of inhibition, but rather indicate secondary substrate limitation, which is necessary for example to describe decrease in growth rate when nitrogen is limited.

In general, the exact biochemical nature of inhibition mechanisms is not known, and authors are required to make a choice between inhibition models that most closely describe their own data, and ones which are easily comparable to the work of other researchers. For this reason, non-competitive inhibition functions are often selected because of the ease with which they may be multiplied to kinetic terms, and to facilitate comparison between researches (Batstone et al., 2002).

pH inhibition

pH inhibition is a combination of disruption of homeostasis and increased weak acids concentration at low pH, or weak bases inhibition and transport limitations at high pH, and affects all organisms to some degree. In the case of hydrolysis, inhibition at low or high pH is probably caused by partial denaturation of enzymes. Although not often used, functions for pH inhibition of hydrolysis are given in Sanders (2001). pH inhibition functions are often substituted by inhibition by high concentrations of unionised organic acids.

Inhibition by hydrogen

Hydrogen is an important product and reactant in most of the sub-processes of anaerobic digestion. Although only slightly soluble in aqueous systems, the partial pressure of hydrogen has a significant impact on the redox potential of the solution, and as previously presented, Mosey (1983) showed that there is a strong regulatory effect by hydrogen on the selection of biochemical pathway in fermentation of higher organic acids to acetic, propionic and butyric acids. Inhibition of anaerobic

processes by hydrogen is commonly modelled using non-competitive inhibition kinetics (Batstone et al., 2002), although other formulations of inhibition have been used (e.g., Hoh and Cord-Ruwisch, 1996).

Inhibition by unionised form of weak acids and bases

Free acid and base inhibition is the disruption of cell homeostasis by change in pH, caused by passive transport of the free acid or base across the cell membrane and subsequent dissociation. Because the relative amount of free acids or bases (compared to the ionic counterpart) is strongly pH dependent, the inhibition is also pH dependent, and empirical pH inhibition functions may include the cumulative effect of free acid or base inhibition. Free organic acids with pKa values from 4.7 to 4.9 at 25°C including acetic, propionic, butyric and valeric acids have been shown to exert this kind of inhibition. Ammonia is the main free base in anaerobic digesters, with a pK_a value of 9.3, and has been found to inhibit aceticlastic methanogenesis (Siegrist and Batstone, 2001). The effects of free organic acid inhibition are largely implicitly included in empirical pH function inhibition functions, although other specific functions have been proposed (Mösche and Jordening 1999), while the free ammonia inhibition is either implicitly included in the upper and lower empirical pH inhibition, or explicitly included in the free ammonia inhibition function.

Inhibition by external agents

Luong (1987) studied the inhibitory effect of butanol on yeasts and compared several mathematical functions that describe inhibition using the *F*-test for equality of variances. The author proposes a non-linear model, which incorporates a maximum substrate concentration (S_m) above which microbial growth ceases and which accounts for both substrate availability and inhibition.

Non-inhibition terms that are treated as inhibition terms include ammonia limitation as a secondary substrate for all organisms (necessary to describe halt in growth at very low ammonia concentrations, and to prevent negative ammonia concentrations; equation 3.3), and competitive uptake of butyrate and valerate by C^{4+} oxidising organisms (equation 3.1).

Kinetics of specific processes

In general, anaerobic processes are modelled with Monod kinetics, with modification by various inhibition terms as described in the previous section. It is necessary that a researcher thoroughly understands the implications of any selection of kinetic expression, both from a biochemical perspective, and the perspective of mathematical properties of the model.

Batstone et al. (2002) provides a well-balanced reference point in this regard. Below is a brief description of kinetic considerations for specific processes where not explicitly covered elsewhere.

Hydrolysis

Two conceptual models have been proposed for hydrolysis, i.e. the organisms secrete enzymes to the bulk liquid where they adsorb onto a particle or react with a soluble substrate; and the organisms attach to a particle, produce enzymes in the vicinity of the particle and benefit from soluble products released by the enzymatic reaction. In anaerobic mixed culture systems, the latter mechanism was found to be dominant (Sanders et al. 2000).

This process has generally been modelled with first-order kinetics (equation 5).

Pavlostathis and Gossett (1986) presented a conceptual model for the anaerobic digestion of biological solids, in which the sludge cells first undergo death, followed by hydrolysis; thus biosolids become available substrate for the anaerobic biomass in the digester: both processes were found to follow first-order kinetics. Similar concepts were presented by Gujer and Zehnder (1983).

Fermentation

The process by which sugars and amino acids and similar smaller organic molecules are converted to low molecular weight organic acids can occur without an external electron acceptor, by a finely balanced transfer of small packages of energy, and consequently is very sensitive to thermodynamic conditions which dictate the overall Gibbs free energy of a specific biochemical pathway. Mosey (1983) showed that both the kinetics of the fermentation reaction, and the specific biochemical pathway were dictated by the ratio of the internal biochemical carrier ion NAD^+ and its unionised form $NADH$, which in turn is related to the partial pressure of hydrogen gas. Other authors have proposed Monod kinetics modified with non-competitive terms for inhibition by hydrogen partial pressure. Anaerobic oxidation of long chain fatty acids however requires an external electron acceptor, and is consequently less sensitive.

Homoacetogenesis and hydrogenotrophic methanogenesis

For the description of the rate of homoacetogenesis and hydrogenotrophic methanogenesis, Vavilin et al. (1998) used the lesser of the values of the Monod functions for hydrogen and for carbon dioxide as limiting substrates. An affinity for hydrogen determines the competition ability of the various hydrogenotrophs. At mesophilic conditions, homoacetogens have much higher H_2 threshold levels (520-950 ppm) than sulphate-reducers and methanogens. Therefore, they are usually considered to be non-dominant in anaerobic digesters. However, below 20 °C, homoacetogens can play a significant part in hydrogen oxidation because of the low activity of methanogenic organisms at low temperatures (Conrad et al., 1989).

Kinetics of physico-chemical systems

A detailed discussion of the kinetics of physico-chemical processes is not included as this is commonly available in texts on aquatic chemistry (see: e.g., Stumm and Morgan, 1996). However, all anaerobic systems produce biogas, and the effect of mass transfer characteristics across the gas-liquid interface, as well as the diffusion of dissolved gases and mixing effects of gas bubbles may need to be taken into account to accurately describe an anaerobic application. A further complication arises from the disparity between physico-chemical and biological process rates, between which there may be several orders of magnitude.

Liquid-liquid interactions such as acid-base association/dissociation reactions in organic acid, inorganic carbon, phosphate, sulphate and ammonia systems, have relatively high rates, with equilibrium being achieved in milliseconds. Liquid-gas interactions (such as evolution of hydrogen, carbon dioxide and methane gases) are often described as equilibrium processes with equilibrium achieved in the order of seconds. When the liquid phase is relatively dilute, Henry's law can be used

to describe the equilibrium relationship (equation 8). The overall gas-liquid mass transfer rate is described by equation 9. Solid-liquid processes such as precipitation and dissolution may have rate constants an order of magnitude bigger. In comparison, anaerobic growth processes may have doubling times in the order of several days or longer.

These differences are well-known to cause stiffness problems in numerical integration when performing simulations, although they can be dealt with by the use of stiff solvers, or by using a combined algebraic/differential approach.

The physicochemical system is very important when modelling anaerobic systems because (1) a number of biological inhibition factors depend on correct speciation within the physico-chemical (e.g., pH, degree of ionisation of acid-base pairs, partial pressure of gases); (2) major performance variables such as gas flow and carbonate alkalinity are dependent on correct estimation of physicochemical processes and (3) the major operating cost is often associated with pH control with a strong acid or base. In this case the control set-point (pH) and inputs are calculated from physicochemical estimation.

Accurate estimation of the physico-chemical system may also improve interpretation of observations: Pauss et al. (1990) showed that gases in anaerobic digesters may be supersaturated to a significant degree in relation to effluent organics and total COD balance. Therefore, dynamic gas transfer equations should be used to describe gas-liquid transfer. The most common equations follow the two-film theory; derivation is described by Stumm and Morgan (1996) and the mass flux is a combination of the driving force and the rate equation.

Effect of mass transfer on kinetics

The previous descriptions of kinetic considerations apply to dispersed, well-mixed systems; however, anaerobic sludges often have physical properties which differ considerably from this situation. In many applications, aggregation of microbial consortia in dense conglomerates or pellets is observed, and has been shown to improve sludge retention by improving sludge settlability and enhance anaerobic digestion by optimally locating micro-organisms in micro-environments best suited to their activity. Non-dispersed sludges may be observed as free pellets inside the reactor, or biofilms attached to reactor walls or media within the reactor. The formation of biofilms and pelletisation of sludges leads to an uncoupling of the hydraulic and microbial (solids) retention times. Importantly, a range of mass transfer considerations are introduced by the fact that not all micro-organisms are equally exposed to bulk liquid conditions (Pavlostathis and Giraldo-Gomez, 1991).

External mass transfer considerations

During the transport of substrate from the bulk liquid to the surface of the aggregate of the methanogenic consortium, different transport mechanisms need to be considered depending of the size of the substrate. Due to the complex hydrodynamic situations involved, semi-empirical correlations are used to obtain the rate of external mass transfer.

Particle deposition in biofilms can interfere with the uptake of soluble substrates and can reduce the volumetric activity of the biofilm. An additional complication is the interaction between the physical

transport of the particle to the surface of the biofilm and the chemical attachment of the particle to the biofilm.

Internal mass transfer considerations

Mass transport inside the biological matrix is usually considered to be by diffusion only and may be modelled using Fick's law. The available information suggests that the diffusion coefficients for solutes inside the methanogenic aggregates are between 10 and 30% of those found in clean water. Outward diffusion of gases in the form of bubbles can create enough disturbance to alter the whole diffusional pattern in the film (Henze and Harremoes, 1983). If the outward transport of gases is slower than their biological production, gas will accumulate and lead to super saturation in the biofilm matrix. In this case, a bubble would form and the aggregate would burst or the biofilm would slough off.

Effect of temperature on kinetics

Most commercial anaerobic applications are operated in the mesophilic temperature range, around 35 °C, and much of the associated experimental work, including determination of kinetic constants has been performed at these temperatures. It is important to note that temperature has a significant effect on most biological kinetic parameters in anaerobic digestion, as well as selection of biochemical pathways. It also affects the value of the equilibrium constants of the physico-chemical system, including pK_a and solubility constants.

The effect of temperature on process rates is usually modelled with an Arrhenius type relationship while the Van't Hoff equation describes the temperature dependence of equilibrium constants (equation 7). Batstone et al. (2002) may be consulted for a more thorough examination of temperature dependence in anaerobic digestion.

ON-LINE MODELLING AND CONTROL

A major objective of modelling is to obtain a basis from which process response can be predicted in order to develop an appropriate control strategy. The development of complex mechanistic models has both assisted in the interpretation of experimental observations, and been a driving force in designing experiments to better understand the mechanisms and microbiology of anaerobic digestion. However, the level of detail that is found in the current generation of mechanistic models is too great for direct application in on-line monitoring and control for two reasons: (1) the availability of on-line measurements for calibration and identification is considerably less then the requirements of the models and (2) the models in question are highly non-linear both in the variables and the parameters, and therefore linearization and discretisation of models for implementation in control algorithms requires considerable simplification.

As in the development of kinetic models, control algorithms may be based on either a simplification of the understood process centred around a measurable variable with fairly well understood response to system changes, or a semi-mechanistic, or non-mechanistic approach which predicts process response from empirical relationships while ignoring the reason for the observed response.

Two categories of models are presented, fuzzy logic and artificial neural networks (ANN). The theory of the methodology of developing and implementing these models is not presented here.

The literature contains many references to the application of models to control of real applications, a selection of which is presented below.

Andrews and Graef (1971) acknowledged that no single variable will always indicate the onset of digester failure, therefore several variables should be monitored for good control, e.g., pH, alkalinity, etc.

Jones et al. (1989) presented an interesting application of stochastic analysis to anaerobic digestion. From a mechanistic point of view, this approach is a significant simplification from the developments that preceded, to facilitate linearization and discretisation. An extended Kalman filter was used in the state estimation algorithm, which combined online measurements with a two step mechanistic model, and allowed the estimation of unmeasured process states. This was implemented on-line with the measurement of biogas flow-rate and composition and pH, and off-line with soluble organics measurements.

An excellent example of the application of a simple dynamic model to control strategies was presented by Beteau et al. (1999). A kinetic model of a two-stage process was employed; conversion of the effluent into acetic acid and further into methane using two microbial populations, acid-base equilibria for acetate and bicarbonate and gas-liquid equilibrium were modelled. No inhibition of acidogenesis was modelled since the focus was on methanogenesis as the most common source of instability. Four common measurements (i.e., bicarbonate alkalinity, total acetic acid, undissociated acetic acid and pH), and three control action (i.e., dilution rate, addition of bicarbonate alkalinity in the feed and addition of a base) were used to define the control algorithm. The performance of the process was described using a "phase portrait", in which the ability of the system to adapt to causes of instability or perturbations was described in terms of its capacity to find a different equilibrium point that ensures the degradation of the organic load. Considerable simplification was required, in order to (i) define a suitable control strategy and (ii) define a "distance" of the current state of the process from the desired equilibrium state.

Possibly more appropriate to control applications are a range of semi-mechanistic and non-mechanistic modelling options that do not attempt to simulate the intricacies of the microbiological and physico-chemical processes but predict the response of the anaerobic system to changes in conditions (e.g., temperature, pH, bulk concentration of reactants) and inputs (flow rates, concentrations) including fuzzy logic and artificial neural networks.

FUZZY LOGIC

Fuzzy sets theory accommodates uncertainty in the recognition process facilitates the elaboration of complex decision rules from a range of cause and effect training cases. This is achieved by defining categories of model inputs (e.g., concentration: low, medium, high) and calculating model outputs by relating different inputs with a series of qualitative calculations and conditional decisions.

Marsili-Libelli and Muller (1996) successfully applied the fuzzy theory to anaerobic digestion.

NEURAL NETWORKS

Neural networks are powerful tools for learning complex mappings from a set of examples, deliberately constructed to make use of some organisational principles resembling those of the human brain. An Artificial Neural Network generally has three layers, i.e., input, hidden and output layers. Connection weights can change in response to the inputs and states of the network, which provides the ANN with the ability to adapt, learn and to model non linear systems. The neurons are connected using various topologies, e.g., random, feed forward, etc.

Guwy et al. (1997) applied ANN to control bicarbonate alkalinity in a fluidised-bed anaerobic digester.

Steyer et al. (1997) combined fuzzy logic and ANN, with a qualitative knowledge-based model for the diagnosis of an anaerobic digestion process. Fuzzy logic "qualifies" a measurement into a fuzzy value, to define whether the variable is in a faulty condition. Since the operating conditions vary, i.e., the "faulty" condition during start-up is different from the "faulty" condition during normal operation (i.e., thresholds need to be adapted), artificial neural networks are very efficient in mapping the measurement changes to the evolving faulty situation and ideal to handle multivariable non linear relationships, as in anaerobic digestion processes. The last step in the development of the control algorithm is the application of a simple knowledge basis, to manage cross-relationships between variables.

The neural fuzzy system is a fuzzy system that uses a learning algorithm derived from ANN theory to determine its parameters by processing data. Tay and Zhang (1999) successfully applied this concept to an upflow anaerobic sludge bed reactor (UASB) and to an anaerobic fluidised bed reactor (AFBR): the same model calibrated (or instructed) on a laboratory scale UASB was successfully applied to a larger unit, while the model of an AFBR was able to accurately interpret experimental data.

CONCLUSIONS

This paper has presented an overview of the developments in anaerobic digestion modelling and briefly discussed its application to on-line monitoring and control. In the space of thirty five years, the understanding of anaerobic digestion has evolved dramatically; the implementation of current understanding into kinetic models has similarly seen a change from simple one- and two-population models in the sixties, to models with eight or more biological populations and integrated biochemical and physico-chemical processes today.

The biggest conceptual developments have been in the appreciation of the subtleties of the thermodynamic control of the sub-processes of anaerobic digestion, as observed in the dynamics of the redox potential and pH value, and their effect on free energy of reaction. Consequently, rate expressions have been modified to include kinetic terms that have the effect of "selecting" biochemical pathways. As the complexity of the models have increased, so the sophistication of the

tools and methods for implementing and integrating them have developed; however, the current level of complexity in state-of-the-art mechanistic models is too high for on-line and control applications. This has lead to the development of alternative methods of predicting process response, such as the implementation of fuzzy logic and artificial neural networks.

There are still considerable gaps in current understanding of anaerobic digestion; an opportunity exists for researchers to use modelling to interpret experimentally observed phenomena and to drive experimental analysis to better understand the processes.

Anaerobic digestion has the potential to be a powerful waste treatment option with associated benefits of harvestable energy generation. Lack of process stability due to the small range of thermodynamically feasible operating conditions has historically limited its popularity, however development of on-line monitoring and control tools based on a range of modelling techniques will considerably enhance its application on a commercial level.

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APPENDIX

The following table summarises is a collection of the main formulae referred to in the text.

$\frac{dS}{dt} = -k_m \cdot \frac{S}{K_S + S} \cdot X$	Monod (or: Michaelis-Menten) kinetics [(a)] It describes the rate of uptake of the soluble substrates (S). ▪ S substrate concentration, $[M \cdot L^{-3}]$ ▪ k_m maximum uptake rate, $[T^{-1}]$ ▪ K_S half-saturation constant, $[M \cdot L^{-3}]$ ▪ X biomass concentration, $[M \cdot L^{-3}]$	(1)
$\frac{dS}{dt} = -k_m \cdot \frac{S}{K_S + S + \frac{S^2}{K_I}} \cdot X$	Haldane kinetics [(b)] It describes the inhibitory effect of the soluble substrates (S), at high concentrations. ▪ K_I inhibition coefficient, $[M \cdot L^{-3}]$	(2)
$\frac{dS}{dt} = -k_m \cdot \frac{S}{K_S + S} \cdot X \cdot I_1 \cdot (...) \cdot I_n$	Generalised Monod kinetics It is proposed by Batstone et al. (2002) as a convenient mathematical expression, to account for several forms of inhibition. ▪ $I_1, \ldots, I_n$ inhibition factors, [-]	(3)
$I = \frac{1}{1 + \frac{S_I}{S}}$	Competitive uptake [(c)] ▪ S_I competitive substrate concentration, $[M \cdot L^{-3}]$	(3.1)
$I = \frac{1}{1 + \frac{S_I}{K_I}}$	Non-competitive inhibition [(c)] ▪ S_I concentration of the competitive species, $[M \cdot L^{-3}]$ ▪ K_I inhibition coefficient, $[M \cdot L^{-3}]$	(3.2)
$I = \frac{1}{1 + \frac{K_I}{S_I}}$	Secondary substrate [(c)] ▪ S_I secondary substrate concentration, $[M \cdot L^{-3}]$ ▪ K_I half-saturation constant for the secondary substrate, $[M \cdot L^{-3}]$	(3.3)
$I = \frac{1 + 2 \cdot 10^{0.5(pH_{LL} - pH_{UL})}}{1 + 10^{(pH - pH_{UL})} + 10^{(pH_{LL} - pH)}}$	Empirical (two-sided) pH inhibition function [(c)] ▪ pH_{UL}, pH_{LL} upper and lower limits for pH respectively, [-]	(3.4)

[(a)] The Michaelis-Menten rate equation was developed for homogenous enzymatic reactions. It *strictly* applies to a single strain of bacteria, growing on a single rate-limiting substrate.
It assumes that all other substrates and nutrients are present in excess and that the products of the reaction do not accumulate sufficiently to inhibit the fermentation.
Note: in the Monod kinetic model, the predicted steady-state concentration of the substrate in the effluent of a completely stirred tank reactor (CSTR) is *independent* from the influent substrate concentration. Other kinetic models, e.g. Contois, explicitly account for the organic loading which in fact may affect the digester performance. For an extensive review, see Pavlostathis and Giraldo-Gomez (1991).
[(b)] As K_I approached infinity, the Haldane relationship approaches equation (1).
This model has some complications in that it implies that cells can grow indefinitely, which is clearly not true: in general, there is a definite substrate concentration above which growth ceases. See Luong (1986).
[(c)] From Batstone et al., 2002.

(continued)

$\frac{dX}{dt} = Y \cdot \frac{dS}{dt} - k_d \cdot X$	Monod yield kinetics It describes the net growth of the biomass (X). ▪ Y biomass yield, [-] ($\mu_m = Y \cdot k_m$) ▪ μ_m maximum growth rate, [T^{-1}] ▪ k_d decay rate, [T^{-1}] The term of decay may equally translate the concept of "maintenance energy", whereby only the surplus substrate is available for growth; or the concept of death of the micro-organisms.	(4)
$\frac{dS_X}{dt} = -k_H \cdot S_X$	First-order kinetics It is generally used to describe the hydrolysis of particulate organic matter (S_X). ▪ k_H rate constant, [T^{-1}]	(5)
$\frac{dS_X}{dt} = -k_H \cdot S_X \cdot X^{\alpha}$	Generalised first-order kinetics Rate equation for hydrolysis, proposed by Valentini et al. (1997), to account for the *indirect* effect of biomass on enzymes concentration. ▪ k_H rate constant, [dimensions are function of α] ▪ α empirical factor, [-]	(6)
$\ln \frac{K_2}{K_1} = \frac{\Delta H_0}{R} \cdot \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$	Van't Hoff equation It relates the variation of the equilibrium constants with temperature, to the thermodynamics of the reaction. ▪ ΔH_0 standard enthalpy, [$M \cdot L^2 \cdot T^{-2} \cdot mole^{-1}$] ▪ R gas law constant ($8.314 \cdot 10^{-2}$ bar·$Mol^{-1} \cdot K^{-1}$) Lawrence and McCarty (1969) used the same type of function to interpret the variation of the half-saturation constant K_S (which is the *driving force* of a biochemical reaction) and used an empirical factor ϑ instead of $\frac{\Delta H_0}{R}$.	(7)
$K_H \cdot p_{gas} - S_{liq} = 0$	Henry's law It describes the equilibrium between liquid and gas phase (at a given temperature). ▪ K_H dimensionless Henry's law constant [-] ▪ p_{gas} steady-state gas phase partial pressure, [$M \cdot L^{-1} T^{-2}$] ▪ S_{liq} steady-state liquid phase concentration, [$M \cdot L^{-3}$]	(8)
$\frac{dS_{liq}}{dt} = k_{L,a} \cdot \left(K_H \cdot p_{gas} - S_{liq} \right)$	Liquid-gas mass transfer It describes the dynamics of the liquid-gas interaction (at a given temperature). ▪ $k_{L,a}$ overall transfer coefficient [T^{-1}]; it depends on mixing and temperature conditions and liquid properties	(9)

MATHEMATICAL MODELLING OF A pH-STAT BIOASSAY

Enrico Remigi, Dinesh Naidoo and Chris Buckley
Pollution Research Group - University of KwaZulu-Natal, 4041 Durban (South Africa)

ABSTRACT

Growing general concern about environmental protection and increasingly stringent regulations mean there is a need for an improved efficiency of process control in municipal and industrial wastewater treatment plants to ensure that the quality of the final effluents is consistently good. Instruments used in preliminary screening surveys should be simple, give rapid responses and require a limited chemical load.

Titration (or pH-stat) bioassays, have recently been developed to measure the activity of bacterial populations and adequately fulfil these needs. They are presently used to monitor anaerobic digesters and to test the potential inhibition of industrial effluents to anaerobic treatment.

However, when many processes which can influence the pH occur within the system, the accuracy of a titrimetric measurement can be questioned. This is the case of anaerobic digestion applications. In order to clarify this issue, a mathematical model of a pH-stat bioassay has been derived from the ADM1 which is the state-of-the-art for modelling of anaerobic digestion.

This new model, when calibrated using experimental data, would be a valuable tool for data analysis of biodegradability or pre-screening activity tests.

INTRODUCTION

A titration (or pH-stat) bioassay is an instrument whereby the activity of a microbial population is evaluated on-line by monitoring the flow rate of a titrating solution of a strong acid or base which compensates for the production or consumption of alkalinity (or acidity), thus maintaining the pH of the environment at a constant level (2). In principle, the titration technique can be applied to any biological or physico-chemical reaction which causes a pH change. In such a system, it is *assumed* that the titration rate is proportional to the reaction rate (i.e.: the substrate uptake rate, in case of a biological reaction; 9).

Recent applications to complex pH-affecting processes (6) showed that, although the technique was promising due to the relatively short duration of a test, the titrimetric method cannot be considered as a stand-alone technique to obtain an accurate quantitative estimate of microbial activity, when many simultaneous processes influence the pH. The suitability of a pH-stat bioassay for a rapid screening of anaerobic biodegradability of industrial effluents prior to co-digestion has been evaluated (5): sodium hydroxide and hydrochloric acid were used to compensate for fatty acids production and acetate

consumption, respectively. This application demonstrated the need for a better insight, in an otherwise apparently straight-forward process.

In this endeavour, a mathematical model of anaerobic digestion has been adapted from the Anaerobic Digestion Model No.1 (ADM1; 1) for simulating an activity test carried out with a titration bioassay: this latter in fact is limited to detecting the production of excess acidity or alkalinity as the overall outcome of the different processes, whereas the ADM1 describes seven biochemical processes and microbial groups.

The simplified model consists of two sequential steps, performed by three microbial populations, i.e.: fermentative, aceticlastic methanogenic and hydrogenotrophic methanogenic micro-organisms, according to the following schematic (units: COD):

$$S \rightarrow S_{ac} + S_{h2} + X_f$$

$$S_{h2} \rightarrow S_{ch4} + X_{h2}$$

$$S_{ac} \rightarrow S_{ch4} + X_{ac}$$

where S and X denote the soluble and particulate components, i.e.: the substrate and the biomass, respectively. The first process (i.e.: the fermentation of the organic substrate S to acetate S_{ac} and hydrogen S_{h2}) results in a net acidity production, whereas the conversion of acetate and hydrogen to methane (S_{ch4}) results in a net alkalinity production.

MATERIALS AND METHODS

The titration bioassay - Principle of operation

A titrimetric procedure in which the targeted microbial step was aceticlastic methanogenesis was originally designed by Lee et al. (3) and further improved by Rozzi et al. (9). The MAIA (Methanogenic Activity and Inhibition Analyser) pH-stat bioassay consists of a 1 ℓ thermostated conical flask; a pH electrode, connected to a titration unit; and a PC for control, data logging and data processing. The bioreactor is equipped with two inlet tubes for titrant addition and with other openings to flush gas through the system, add reagents and discharge the evolved biogas. The operating principle is that as the methanogenic activity starts, acetate is converted into methane and bicarbonate and the pH value of the mixed liquor tends to increase; to maintain a constant pH value, acetic acid (or another acid) is added by the titration unit. If the working pH is close to 7, the pH of the mixed liquor almost exclusively depends on the concentration of bicarbonate ions: under this assumption, the dissociation equilibrium of carbon dioxide in the liquid phase and the liquid-gas equilibrium are the main interfering processes. However, the contribution of inorganic carbon (i.e.: carbon dioxide and bicarbonate ions) to the change in pH can be controlled by keeping the concentration of carbon dioxide in the liquid phase (and its fraction in the gas phase) constant throughout the activity test (9): this is done prior to the addition of the substrate, by flushing the liquor with a N_2:CO_2 gas mixture

having the same carbon dioxide partial pressure as the expected biogas (which in a function of the mean oxidation state of carbon in the substrate).

The mathematical model

A Differential Algebraic Equation (DAE) approach has been adopted, where dynamic and equilibrium processes are defined. The first set of processes is fully determined by specifying their stoichiometric and kinetic component, corresponding to a system of differential equations. The latter set of processes is assumed to have a much faster therefore negligible dynamics (time constant) and is described by a system of algebraic equations. The model was implemented using the Aquasim® (7) software platform.

The model includes the following processes:

- Three biological uptake processes, i.e.: fermentation of the soluble substrate to acetate and hydrogen; hydrogenotrophic methanogenesis; and aceticlastic methanogenesis. All processes are described using a Michaelis-Menten kinetics. The decay of the three microbial populations has been set to follow a first-order kinetics.
- Four physico-chemical processes, i.e.: the dissociation equilibria of water, acetate and inorganic carbon (IC) and the charge balance. The liquid-phase equilibrium of carbon dioxide and bicarbonate has been described as a dynamic process, based on the K_{La} and the saturation concentration of carbon dioxide in the liquid phase.

Other characteristics of the model are:

- Two proportional controllers, which simulate the titration system:

  $\Phi = q \cdot c$ (mol/h)

  where q and c are the titrant flow-rate in (ℓ/h) and concentration in (mol/ℓ), respectively. Both are "switch" functions that are activated when the pH level falls out of the allowed range (± 0.02 units); q is proportional to the pH deviation from the set-point.
- In order to incorporate the effect of temperature on the physico-chemical equilibria and therefore to assess the interfering effect on the titration process, the equilibrium of inorganic carbon and liquid-gas exchange of carbon dioxide have been implemented as temperature dependent, as follows (temperature T in K):

  $K_{H,co2} = 10^{2.6747 - 0.0139 \cdot T}$

  $pK_{co2} = 17052 \cdot T^{-1} + 215.21 \cdot \log T - 0.12675 \cdot T - 545.56$

  $pK_{h2o} = - 0.0361 \cdot T + 14.9046$

  where $K_{H,co2}$, K_{co2} and K_{h2o} are the Henry's law constant ($mol \cdot \ell^{-1} \cdot atm^{-1}$) and the dissociation constants for carbon dioxide and for water, respectively.
- The influence of temperature on the metabolism of the microbial populations has been modelled by making the uptake rates vary as follows:

  $k_{max}(T) = k_{max}(20) \cdot \exp [\theta \cdot (T-293)]$

where: $k_{max}(20)$ and θ are the maximum uptake rate at 20 °C and an empirical factor for temperature correction (ranging from 0.01 to 0.1).

- Generally, an anaerobic sludge requires a certain period of time to reactivate, or to adapt to a new substrate. This lag-phase is simulated by defining the growth rate as a function of time, as:

  $t<t_{lag}$: $k_{max}(T) = k_{max}(T)\cdot[1 - \exp(-\gamma\cdot t / (t_{lag} - t))$

  where γ is an empirical factor (ranging from 0.01 to 0.1).

- No inhibition has been accounted for at this stage, although it is known that an increase of the hydrogen partial pressure may significantly affect acidogenesis (1).

The basic structure (and a first approximation for the parameters and for the constants) is derived from the ADM1 (Tables 1 and 2) and consists of two compartments, i.e.: a liquid and a gas phase. An additional "virtual" compartment (that mirrors the liquid phase) has been defined to calculate cumulative quantities, such as the titration curves.

Table 1 – Values of the parameters used for the simulations.

Description	Symbol	Units	Value
Fraction of methanogens	α	---	0.1
Stoichiometric fraction of acetate from solubles	β	---	0.75
Time constant for activation of slow fermenters	γ	---	0.1
Decay rate for fermentative micro-organisms	$k_{d,f}$	d^{-1}	0.072
Decay rate for aceticlastic methanogens	$k_{d,ac}$	d^{-1}	0.144
Decay rate for hydrogenotrophic methanogens	$k_{d,h2}$	d^{-1}	0.009
Max uptake rate (at 20 °C) for fermentative micro-organisms	$k_{m,f,20}$	g COD·g $VS^{-1}\cdot d^{-1}$	3.89
Max uptake rate (at 20 °C) for aceticlastic methanogens	$k_{m,ac,20}$	g COD·g $VS^{-1}\cdot d^{-1}$	1.87
Max uptake rate (at 20 °C) for hydrogenotrophic methanogens	$k_{m,h2,20}$	g COD·g $VS^{-1}\cdot d^{-1}$	9.07
Fermentation half-saturation constant	$K_{S,f}$	g COD·ℓ^{-1}	0.5
Aceticlastic methanogenesis half-saturation constant	$K_{S,ac}$	g COD·ℓ^{-1}	0.2
Hydrogenotrophic methanogenesis half-saturation constant	$K_{S,h2}$	g COD·ℓ^{-1}	$5\cdot10^{-5}$
Coefficient for temperature dependency of uptake rates	θ	---	0.09
Lag-time for activation of fermentative biomass	t_lag	D	0.125
Fermentative biomass yield	Y_f	g VS·g COD^{-1}	0.272
Aceticlastic biomass yield	Y_{ac}	g VS·g COD^{-1}	0.005
Hydrogenotrophic biomass yield	Y_{h2}	g VS·g COD^{-1}	0.024

Table 2 – Values of the physical constants used for the simulations.

Description	Symbol	Units	Value
Non dimensional Henry's law constant for methane	H_{CH4}	---	0.0315
Non dimensional Henry's law constant for nitrogen	H_{N2}	---	0.0162
Liquid/gas exchange coefficient for methane	$K_{La,CH4}$	d^{-1}	$2 \cdot 10^5$
Liquid/gas exchange coefficient for carbon dioxide	$K_{La,CO2}$	d^{-1}	10
Association/dissociation kinetic constant for CO_2/HCO_3	$k_{kin,CO2}$	d^{-1}	$2 \cdot 10^3$
Dissociation constant for acetate ($-\log_{10}$)	pK_{ac}	---	4.76
Atmospheric pressure	p_{atm}	bar	1.013
Universal gas constant	R	$\ell \cdot bar \cdot (K \cdot mmol)^{-1}$	$8.2 \cdot 10^{-5}$

RESULTS AND DISCUSSION

The primary objective of this mathematical model is to interpret the outcome of an anaerobic biodegradability assay carried out using a titrimetric sensor. However, the authors (5; 6) have raised some concerns on the accuracy of such a simple way of monitoring the process, which detects pH as the only variable.

The following aspects are therefore addressed hereafter:

1 - the dilution effect of titration. This issue was initially neglected by Rozzi et al. (9): they stated that the titration volume was generally very small compared to the working volume. This is actually not true as reported by Remigi (6). Other researchers (4) showed that this also makes the assumption of constant substrate concentration in a conventional activity test unrealistic.

2 - the effect of temperature. A variation in the working temperature would not result in a bias of the titrimetric assessment, because it would be reflected in a similar variation in biological activities and therefore in a variation of the titration rates. However, temperature also affects the dissociation and the solubility of carbon dioxide: this would constitute an interfering effect not related to the biological processes.

3 - the accuracy of the titration method, when two concomitant pH-affecting processes are to be separately monitored. As the two processes do not occur separately in time (typically, as soon as a certain amount of acids is produced by fermentation, a fraction of it is converted to methane), a fraction of the acidity produced cannot be sensed by the controlling unit but is internally compensated by an equivalent generation of alkalinity.

Dilution effect

The fundamental assumption of the titrimetric detection of microbial activity is that pH (and therefore alkalinity, or the concentration of bicarbonate ions) is maintained constant throughout the test. As an example, during an aceticlastic activity assay, this is achieved by compensating the biologically produced alkalinity through the addition of an *equivalent* amount of acid: if this assumption was true,

it could be correctly assumed that the titration rate is a direct measure of the microbial activity. However, if a constant methanogenic activity generates a_0 moles of alkalinity (in the unit time, e.g.: moles per day), the control unit will tend to neutralise this surplus by adding a volume v (litres per day) of titrating solution (having a concentration $c = v/a_0$) As the titration takes place, the excess bicarbonate ions get diluted in an increasing volume, thus the true excess alkalinity (moles per litre) sensed by the control unit gets smaller. The overall result is that a smaller amount of acidity is released by the sensor to compensate for the alkalinity generated, therefore a lower activity $a < a_0$ will be measured. This circumstance is depicted in Figure 1: as one should expect, the more concentrated the titrating solution, the smaller the error in the activity estimation (but the lower is also the sensitivity of the detection; 9).

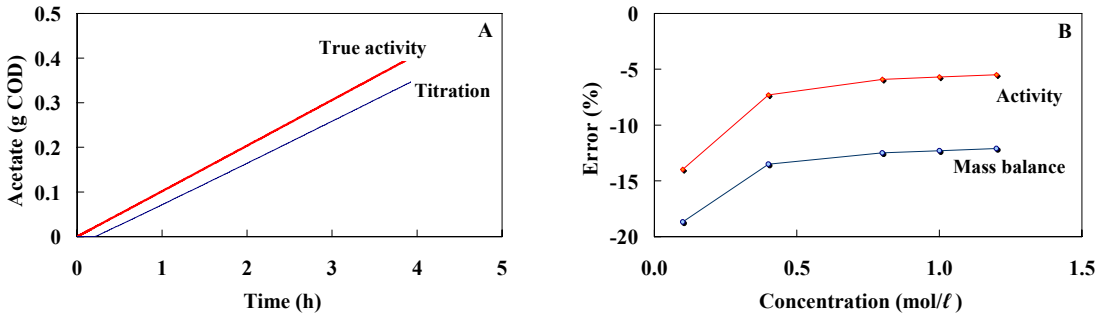

*Figure 1 - Simulated aceticlastic activity test: the estimated activity is lower than the true microbial activity (**A**), due to the titration volume. This effect in a function of the strength of the titrating solution (**B**) and results in a systematic error ranging from 5 to 10 % on the activity and from 12 to 15 % on the mass balance.*

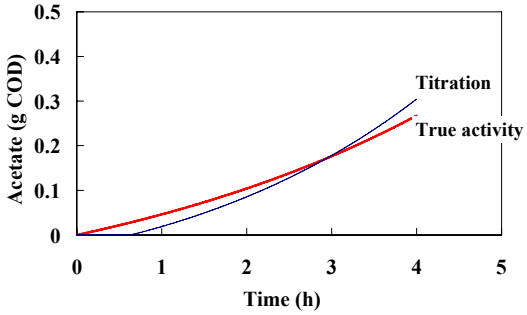

Figure 2 - Simulated aceticlastic activity test, at increasing temperature (ranging from 25 to 35 °C): the estimated activity is higher than the true microbial activity, due to CO_2 stripping from the liquid phase.

Temperature effect

In an anaerobic environment, purely physico-chemical processes related to the presence of carbon dioxide (i.e.: the CO_2/HCO_3 dissociation equilibrium and the gas exchange between the liquid and the gas phase) interfere with the determination of microbial activity through pH (9). This interference must be controlled by maintaining the carbon dioxide fraction in the gas phase constant: this requires that the working temperature does not vary during the test. Figure 2 shows the effect of an increase in

temperature: as both the bacterial metabolism and the CO_2 concentration change, the titration cannot correctly monitor the variation of microbial activity; over time, the alkalising effect of CO_2 stripping becomes predominant and results in an overestimation of activity. In order to measure microbial activity at different temperatures, subsequent activity tests must be carried out by keeping the temperature constant and readjusting the physico-chemical system after each change.

Monitoring two sequential pH-affecting processes

Notwithstanding these factors which influence the accuracy of the estimates, the suitability of a titrimetric assay to measure the activity of a *single* microbial population (specifically aceticlastic methanogens) has been proven elsewhere (9): this application can outcompete the generally labour-intensive conventional methods. More advanced applications have been attempted, such as the assessment of anaerobic biodegradability of an organic substrate (5) and the monitoring of the Anammox process (8). Both applications are characterised by the coexistence of two processes which have an opposite influence on the pH: in the former, the conversion of the substrate to fatty acids requires an alkali titration and the conversion of the acids to methane (through acetate and hydrogen) requires an acidic titration. One should expect that a fraction of the excess alkalinity generated during the methanogenic phase is masked by the simultaneous production of an equivalent amount of excess acidity by the fermentative micro-organisms. Therefore, the accuracy of both the activity and biodegradation (i.e.: mass balance) assessment is affected by the extent of this internal neutralisation: this is a function of many factors, such as the characteristics of the sludge used as inoculum (i.e.: the relative abundance or activity of the two microbial groups).

Simulation of experimental data

The mathematical model was used to simulate a biodegradation assay on glucose.

The monitored variables, i.e.: the two titration curves and the pH were interpolated to the respective simulated variables and the model parameters adjusted. The results (Figure 3) show that experimental and modelled outputs fit very well, thus indicating that the *simplified* model is sufficiently accurate to interpret a titrimetric activity test.

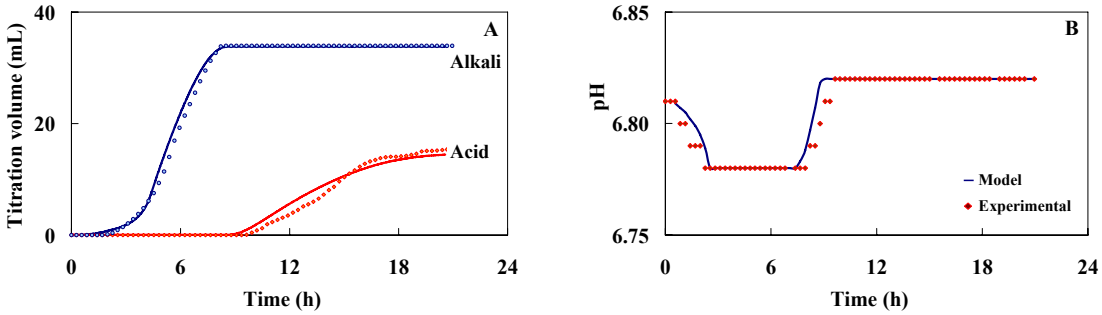

*Figure 3 - Simulation of a biodegradation test (substrate: glucose): titration curves (**A**) and pH (**B**). Generally, the model fits well to the experimental data; however, it can be noted that the acidification stage (alkali titration) is much better simulated than the methanisation one.*

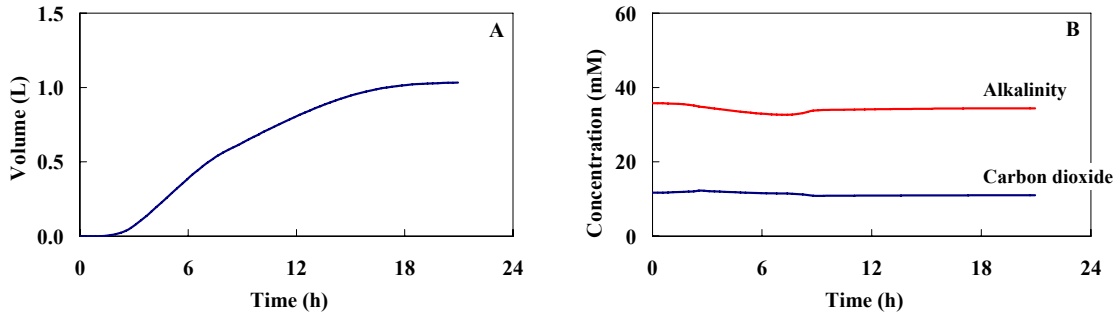

*Figure 4 - Simulation of a biodegradation test (substrate: glucose): biogas volume (**A**) and carbon dioxide and bicarbonate concentration in the liquid phase (**B**). Experimental data not available.*

One should note that the output of the model can very close trace the acidification stage, almost perfectly overlapping the titration and pH curves, whereas a larger deviation occurs on the methanisation stage: the simulated acidic titration starts earlier and levels off more gradually than the experimental one. This might indicate that an intermediate step (which mimics the conversion of fatty acids to acetate) is necessary in the mathematical model, to delay the availability of acetate. The expected biogas production and inorganic carbon concentration in the liquid phase are also reported (Figure 4), although not measured during the test: as one should expect, the two metabolic stages are partially simultaneous, as a considerable fraction of the ultimate gas production is generated while acidic titration is active (Figure 4 a). Moreover, the physico-chemical system is not perfectly equilibrated throughout the test, as the carbon dioxide fraction in the gas phase changes: this is mirrored by the slight change in alkalinity between 0 and 10 hours (Figure 4 b).

CONCLUSIONS

A pH-stat bioassay has been successfully applied to the assessment of microbial activity in presence of a single pH-affecting process. The use of a mathematical model allowed to investigate issues such as dilution and temperature effects on the accuracy of the estimates (aspects which have been overlooked until now), thus enabling to determine the systematic error of this technique.

When two or more processes occur within the system under study, the response of a titration sensor becomes even less accurate. The implementation of a simple two-step anaerobic digestion model, incorporating a fine pH control function, enables to quantify the influence of various disturbances and possibly the optimal experimental conditions which enhance the sensitivity of the instrument.

ACKNOWLEDGEMENTS

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ANNEXURE I

Capacity building and transfer of knowledge

I.1 THESES

1. Rakgotho T (2005). Toxicity and biodegradability assays for hazardous landfill leachate and textile size effluents. *MTech*, Durban Institute of Technology (South Africa).

2. Naidoo D (2003). A pre-screening tool for the anaerobic treatment of complex industrial effluents and wastewaters. *MSc Eng*, University of Natal (South Africa).

3. Petlane M (2003). The feasibility of treating dye or coloured textile effluent in an anaerobic digester. *BSc (Hon)* in Environmental Science, Department of Geography and Environmental Science.

4. Ndimande S (2000). Evaluation of an industrial effluent for anaerobic digestion. *BTech*, ML Sultan Technikon (South Africa).

5. D'Ambrosio D (2000). Estimation of kinetics constants for anaerobic sludges in steady-state and transient conditions. *MSc Eng*, Politecnico di Milano (Italy).

I.2 COURSES

1. Buckley C (2005). Biological effluent treatment processes. *Master post graduate course*. University of KwaZulu-Natal.

2. Remigi E (2004). WEST® Software for simulation in environmental engineering. *Doctoral course in "Theory and practice of pilot-plants"*. Politecnico di Milano (Italy), March-April.

3. Remigi E (2003). The use of mathematical modelling for biochemical processes: the software Aquasim®. *Undergraduate course in "Applied Biochemical Engineering"*, 4th year Chemical Engineering, University of KwaZulu-Natal.

4. Remigi E (2003). Introduction to anaerobic digestion modelling of wastewater. *Seminar held at SASOL*, April 9th.

I.3 CO-OPERATIONS

The primary capacity building aspect that was included in this contract was to involve ML Sultan Technikon researchers and students to conduct and facilitate research aimed at achieving the objectives set out in this project. This project has also sought to encourage chemical engineering students into pursuing postgraduate studies by providing vacation work and student assistantships. The PRG has collaborated with Prof Rozzi and his group at the Department of Hydraulic, Environment and Survey Engineering, Politecnico di Milano (Italy), in order to improve the PRG's knowledge on anaerobic bioassays.

1. EU-COST. Following the EU workshop on "Harmonisation of anaerobic biodegradation, activity and inhibition assays" in Italy in 2002, a reciprocal exchange of experiences developed between the Project Team and Dr W-R Müller of the Institut für Siedlungswasserbau, Wassergüte- und Abfallwirtschaft (University of Stuttgart, Germany). Dr Müller has been working on anaerobic testing for the last five years, using two types of equipments (i.e. Eudiometer and Methanomat®) for biodegradability assessment. The Methanomat® seems very promising, because it incorporates a titration system and an automated system for gas measurement and carbon dioxide detection. He was also the external examiner of Dr Remigi's PhD thesis.
2. NRF/Flanders
3. ML Sultan/DIT. The Pollution Research Group has formed a research relationship with Dr B. Odhav of the Department of Biological Sciences.
4. Politecnico di Milano (Italy)

I.4 LINKAGE WITH OTHER WRC PROJECTS

1. WRC Project K5/1538: Anaerobic Digestion for the Treatment of Toxic and High Strength Organic Wastes : a case study in eThekwini Municipality.
2. WRC Project K5/1456 : Biotechnological co-treatment of saline and sewage wastewaters with integrated recovery and reuse of water and organic and inorganic components for sustainable development".

3 WRC Project K5/1248: Evaluation of the Anaerobic Baffled Reactor for Sanitation in Dense Peri-urban Settlements

4 WRC Project K5/1629 : Research into urine diversion toilets in eThekwini

5 WRC Project K5/1630: Scientific support for the design and operation of Ventilated Improved Pit-latrines (VIPs)

ANNEXURE J

Content of the CD

Water Research Commission Project No K5/1074	
🗁 **Data**	
🗁 01 – Serum bottle	
🗁 01 – Leachates	01 - ATA-Holsfontein.xls
	01 - BMP-Holsfontein.xls
	02 - ATA-Aloes.xls
	02 - BMP-Aloes.xls
	03 - ATA-Shongweni.xls
	03 - BMP-Shongweni.xls
🗁 02 – Textiles	01 - ATA-Recipe 2.xls
	01 - BMP-Recipe 2.xls
🗁 03 – Industrials	01 - AAT-Dyestuff.xls
	02 - AAT-test 1.xls
	03 - AAT-test 2.xls
	04 - Codig test 3.xls
	05 - Codig test 4.xls
	06 - Codig test 5.xls
	07 - Codig test 4-corrected.xls
	08 – AAT summary.xls
🗁 02 – Lab-scale	01 - Distillery.xls
🗁 **Report**	WRC K5-1074.pdf
	Annex A-Protocol.pdf
	Annex B-Serum bottle.pdf
	Annex C-pH-stat titration.pdf
	Annex D-Reference guide.pdf
	Annex E-Database.pdf
	Annex F-Materials.pdf
	Annex G-Results.pdf
	Annex H-Publications.pdf
	Annex I-Capacity.pdf
	Annex J-CD.pdf