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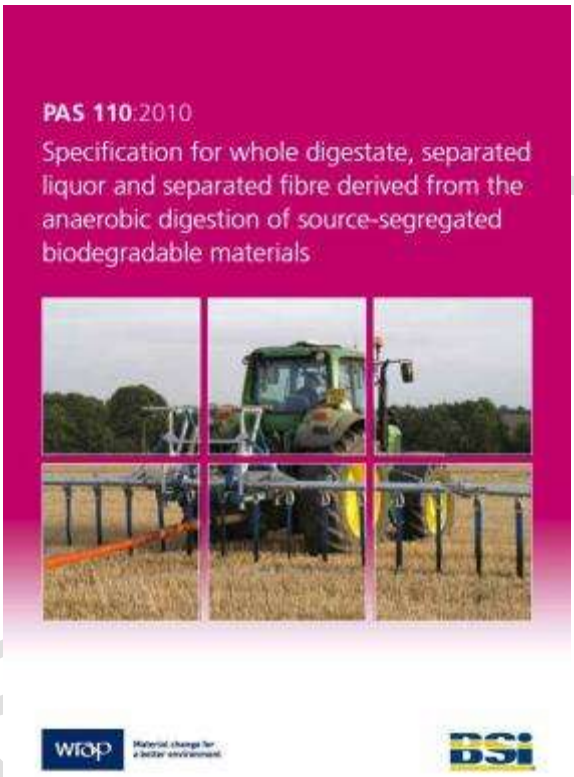
Final report

Review of the application of the Residual Biogas Potential test



A review of the application of the Residual Biogas Potential (RBP) test for PAS110 as used across the UK's Anaerobic Digestion industry, and a consideration of potential alternatives.

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Written by: Professor Charles J. Banks, Dr Sonia Heaven, Dr Yue Zhang, Dr Melanie Sapp and Dr Richard Thwaites.

UNIVERSITY OF Southampton

Front cover photography: PAS110 specification for anaerobic digestates

Executive summary

The Residual Biogas Potential (RBP) test was adopted as a component of the British Standards Institution's Publicly Available Specification 110 for anaerobic digestate with the specific purpose of providing evidence that an effective digestion process has taken place. Following feedback from UK AD operators, a review of the test was carried out to determine the test was fit for this purpose, and/or whether it could be replaced by a less time-consuming alternative.

The work carried out to achieve this included a survey to determine the RBP values for digestates from 24 currently operating UK anaerobic digestion (AD) plants treating waste from commercial and municipal sources. At the same time a range of digestate parameters was analysed to determine whether there was any useful correlation with the RBP value that could offer an alternative means of assessing the stability of the digestion process. The results were examined to assess the range of RBP values found, and to provide an insight into the reasons why specific digestates may pass or fail the current RBP limit.

The majority of samples (75%) provided by digester operators met the PAS 110 RBP limit of 0.25 l biogas g^{-1} VS. For samples that failed, in most cases there were clear indications of AD process instability or other issues that may have contributed directly to this failure and that could potentially be addressed by remedial measures at the plant. It therefore appears that the test is fulfilling its primary purpose of indicating whether an effective digestion process has taken place, and that the limit value is achievable in a plant that is operating well.

The AD plant operators were requested to supply samples taken directly from the outlet of the main digester, without post-digestion storage: in some cases further storage may have reduced the RBP value. The most appropriate sampling point may therefore be the last digester or post-digestion storage tank from which biogas is recovered, as this represents the end of the active process. On the same basis it is suggested that the RBP test should be applied to whole digestates only rather than to separated fractions, as separation is not itself a stabilisation process.

The work assessed the variability in the current RBP test by means of a multiple-replicated laboratory study using a single sample, multiple samples taken over a short period, and sequential samples taken over a longer period, from a commercial digester with a typical mid-range RBP value. The results indicated that the current test is repeatable and reliable. It is possible, however, that deterioration in process efficiency and thus digestate stability could occur over a short time period, and therefore the interval between testing may need to be reviewed. The current frequency of sampling does not allow consideration of rolling averages or percentile values to be used for assessment of compliance.

Preliminary investigations were carried out on potential 'rapid test' alternatives to the RBP protocol. Two approaches were used. The first was based on assessment of acid production after inhibition of methanogenesis, and the other on anaerobic digestion of the organic content of the digestate after separation of the microbial cell component: in both cases this gives a measure of the readily degradable material remaining in the digestate. Although the second approach showed some potential, both tests would require major development and any advantages were offset by the greater complexity of the test process. It was concluded that there are no other obvious candidates for an anaerobic biochemical assay that would be simpler and more rapid than the current RBP test. On analysis of the survey results, however, it appears that the duration of the RBP test could possibly be reduced to 10 days with a corresponding reduction in the limit value to 0.20 l biogas g^{-1} VS, without a major effect on the final outcome.

The RBP test does not appear to be a logical choice for assessing the performance of *aerobic* digesters, as the primary metabolic routes to stabilisation are different. There is, however, some potential for using aerobic respirometric techniques to assess both aerobic and anaerobic digestate stability as these techniques are based on determination of the biochemical conversion potential of the organic material present. A literature review showed that a reasonable correlation has been established between methane potential and aerobic respiration rates for anaerobic digestates. Another potential alternative could be the use of thermogravimetric analysis, but this approach would again require extensive verification to establish limit values. Self-assessment systems based on continuous monitoring of plant data including gas production and VS destruction could be used, but have the disadvantage that this type of data cannot be presented as a single limit value in a standard or specification.

An extensive review of English and German-language sources was carried out to establish the scientific rationale and basis for the adoption of a VFA standard under the proposed EU End-of-Waste criteria. The evidence found suggests that measurement of VFA is not an adequate means of assessing the degradation of input material, and this parameter is best used as an indicator of the stability of the process rather than of the final product. In the German RAL (German Institute for Quality Assurance and Certification) quality standard for digestate the VFA limit is also linked to requirement for a minimum retention time in the digester to ensure effective degradation.

Based on the findings of this work, as summarised above and presented in the main body of the report, the following conclusions were drawn:

- The RBP test is a satisfactory method for demonstrating that an effective digestion process has taken place, and the test procedure gave repeatable results.
- The current value of 0.25 l biogas g-1 VS appears appropriate and achievable.
- It is likely that the test duration could be reduced to 10 days, with a corresponding reduction in the limit value to 0.2 l biogas g-1 VS.
- It is recommended that the maximum period in which the net biogas production can remain negative is reduced from the current 5 days to 4.
- The sampling point should be specified as the outlet of the final tank from which biogas is collected for processing rather than simply vented.
- The RBP test should be applied to whole digestates only, rather than to separated fractions.
- The interval between testing may need to be reviewed.
- High VFA concentrations are known to occur in animal slurries and some aerobic composts that are commonly applied to land, and it is therefore considered inappropriate to consider setting an 'environmental outcome' VFA limit for digestates only.
- There are no grounds for using VFA concentration as a product stability criterion.
- The small number of comparative studies carried out has indicated good correlation between biogas potential tests and respirometric tests on digestates.
- The current RBP test protocol does not contain any instructions on the treatment of outlier results due to equipment failures (e.g. leakage) and it is suggested that a minor amendment is added to deal with this point.
- Preliminary work on a rapid anaerobic test showed some promising results but would require extensive test development.
- The RBP test does not appear to be a logical choice for assessing the performance of an aerobic digester, as the primary metabolic routes to stabilisation are aerobic in such systems.

Contents

Glossary

Abbreviations

AD	Anaerobic digestion
ADP1-24	Anaerobic digestion plant 1-24
ADOWG	Anaerobic Digestion Operators working Group
BAP	Biochemical acidogenic potential
BMP	Biochemical Methane Potential
COD	Chemical Oxygen Demand
IA	Intermediate Alkalinity
i/s	Inoculum to substrate ratio on a VS basis (g VS of inoculum per g VS substrate)
n/d	Not detected
PA	Partial Alkalinity
RBP	Residual Biogas Potential
RSD	Relative Standard Deviation
SOUR	Specific Oxygen Uptake Rate
TA	Total Alkalinity
TAN	Total Ammoniacal Nitrogen
TKN	Total Kjeldahl Nitrogen
TS	Total Solids (also known as Dry Matter, DM)
UoS	University of Southampton
VFA	Volatile Fatty Acids
VS	Volatile Solids (also known as Organic Dry Matter, ODM, and as Loss On Ignition, LOI)
WRAP	Waste and Resources Action Programme
WWTP	Wastewater Treatment Plant

Acknowledgements

We would like to acknowledge the help of the following: Dr Ying Jiang of the University of Southampton for carrying out the laboratory work; all those who contributed information to the literature review; the staff of the participating AD plants; and the EU FP7 VALORGAS project for support in additional sample analysis and data interpretation.

1.0 Introduction

The Residual Biogas Potential (RBP) test was adopted as a compulsory component of the British Standards Institution's Publicly Available Specification 110 for anaerobic digestate, often referred to as PAS110), with the specific purpose of providing evidence that an effective anaerobic digestion process has taken place. One indicator for this is a low degradable organic matter content in the output material: and the RBP test provides a practical method of demonstrating this, by quantifying the biogas produced through anaerobic biochemical conversion of any residual organic matter. This method is akin to the biochemical methods used in assessing the stability of aerobically treated material, where the residual organic matter is related to the oxygen demand. In both cases the tests do not quantify the degree of degradation that has already taken place, but are based on the assumption that a process which is performing well should be able to reduce the residual degradable material to a specified level.

Biogas potential can also be due to the presence of soluble intermediate metabolites such as volatile fatty acids (VFA), and the RBP protocol includes determination of VFA concentration as a pre-screening test, with a limit value based on the theoretical biogas yield from conversion of these intermediate products. In designing the original RBP protocol, however, it was considered that a low VFA concentration alone was not sufficient to indicate satisfactory degradation, as some untreated input materials may also have a low VFA content. This pre-screening was therefore not intended as a substitute for the RBP test, but was simply a means of avoiding a lengthy test procedure when a rapid method could demonstrate failure. A full discussion of the interpretation of VFA data is presented in the review section of this report.

After two years of test operation, feedback from the Biofertiliser Certification Scheme indicated that some AD plants were experiencing difficulty in achieving the required RBP limit. These included a number of plants which receive a high proportion of food wastes: at the same time, other plants accepting similar inputs were apparently meeting the standard with ease. It was therefore considered timely to check whether the RBP test was achieving its original purpose, and at the same time to investigate whether alternative and more cost-effective methods are available for demonstrating the recovery of input materials.

Although the RBP test was originally developed to show that an effective digestion process had been carried out, it has since been suggested that it could also provide an indication of the environmental impacts arising from the use of digestates, and thus could potentially be used to control these.

It was therefore proposed by the Waste and Resources Action Programme (WRAP) that a review of the RBP test should be carried out to consider the following points:

- The potential impact on digestate RBP values if samples were taken from the point of output from the digester, rather than from any post-digestion storage stage.
- Whether separate tests are needed to demonstrate that digestates will not cause unacceptable environmental harm when used according to Good Agricultural Practice: as the number of possible environmental indicators is large, it was specified that the study should focus on VFA and on any relationship between these and RBP values, to determine whether a single limit value could encompass both 'process' and 'product use' outcomes.
- Whether the RBP test should be applied to whole digestates and/or to subsequently separated fractions.
- Whether there are sufficient data to support statistical changes in the current pass/fail approach, such as the introduction of percentage compliance or rolling averages.
- Whether the current sampling intervals set by PAS110 are appropriate.

- Whether alternative methods for demonstrating recovery of input materials are available and might offer any advantages over the RBP test, including lower costs.
- Whether the RBP test could be applied to aerobic digestates, and if so, whether a different limit would be required.

To address these points the following programme of work was proposed and undertaken by the University of Southampton (UoS):

1. A survey was carried out to determine the RBP values for digestates from currently operating UK AD plants treating waste from commercial and municipal sources. At the same time a range of parameters were analysed for correlation with digestion process stability and process efficiency in terms of residual organic matter in the digestate. The results from this survey were then used to assess the range of RBP values currently found, and to provide an insight into the reasons why specific digestates may pass or fail the RBP criterion.

2. The statistical variability in the current RBP test was assessed by means of a multiple-replicated laboratory study using a single sample, multiple samples taken over a short period, and sequential samples taken over a longer period, from a commercial digester operating in the mid-range of RBP values.

3. A potential alternative to the RBP protocol in the form of a 'rapid' test was investigated: this was based on an adaptation of the specific methanogenic activity test in order to assay the organic content of digestate material separated from the microbial cell component of the digestate. The work also examined alternative methods of assessment of digestion efficiency, including continuous monitoring systems that could be implemented on a self-monitoring basis.

4. Advanced DNA sequencing was undertaken to reveal the microbial population structure in a range of commercial AD plants. The extent of any correlation of this with digestion performance and digestate stability (as assessed using the RBP test and conventional monitoring parameters as above) was examined.

5. An extensive review of English and German-language sources was carried out to establish the scientific rationale and basis for the adoption of a VFA standard under the proposed EU End-of-Waste criteria. This survey extended beyond the issue of digestate stability to include the use of VFA as an indicator of more general environmental impacts during product use, including soil quality.

This report presents the results of this work.

2.0 Comparative studies of RBP test

2.1 Survey of anaerobic digesters treating organic solid waste

A survey was carried out to determine the RBP values for digestates from currently operating UK AD plants treating source segregated and unsegregated wastes from commercial and municipal sources. A range of digestate parameters were also analysed for assessment of digestion process conditions and stability, and correlation with process efficiency in terms of residual organic matter in the digestate. The results were then used to review the range of RBP values found, and to provide an insight into the reasons why specific digestates may have passed or failed the RBP limit as current set in PAS110.

2.1.1 Sampling and test set-up

On 6 March 2012 discussions took place between UoS and WRAP to establish the best way of approaching AD plant operators about participation in the planned survey work. It was clear from the discussions that other surveys of AD plants were also being planned, and that coordination with these would be useful. These other initiatives were led by the trade associations, and in particular an on-going survey was being carried out by the Anaerobic Digestion Operators Working Group (ADOWG) to collect data for a response to the proposed EU End-of-Waste criteria for biowastes.

Following correspondence between WRAP and the other parties involved, it was agreed that a digestate sampling programme should be conducted as part of the current study. The trade associations would inform their members of this, and once this had been done UoS could go ahead and contact the plant operators directly. As soon as UoS was informed that these preliminary arrangements had been completed, a draft letter was prepared and sent to WRAP for approval (Appendix 1). This was sent out on 20 March 2012 to plant operators identified on the AD Portal (http://biogas-info.co.uk/maps/index2.htm) as treating waste feedstocks, as opposed to farm feedstocks or liquid effluents and sewage sludges. Address and contact details for the operators were initially obtained from the AD Portal and checked by web search and telephone enquiries. A follow-up reminder was sent to the listed organisations by e-mail on 3 April 2012.

Of the 33 organisations contacted, positive responses were received from 26 organisations responsible for 29 AD plants in total. Sample bottles and instructions accompanied by a short questionnaire were sent out to 25 of these respondents, asking them to send the samples back to Southampton in a pre-paid first class freepost package on Monday 28 or Tuesday 29 May 2012.

Samples from 22 AD plants, anonymised and coded ADP1-22, were received in time to be included in the first part of the comparative study which was set up on Saturday 2 June (within the 7-day period allowed by the current PAS110 RBP protocol). A further two samples (ADP23 and 24) were received late due to postal or other delays, and these were set up on Monday 11 June 2012. Because of the number of samples involved it was necessary to use four independent sets of RBP apparatus, with the samples in sets 1-3 allocated at random as shown in Table 1. The tests were carried out in accordance with the standard method as described in Walker et al. (2010), unless otherwise noted. Each sample was set up in triplicate using inoculum obtained from the anaerobic digester at Millbrook Wastewater Treatment Plant (WWTP), Southampton.

Table 1. Allocation of RBP samples in test sets

Set	Samples	No. of digesters
1	ADP8, 9, 19, 20, 22 plus 2 positive controls and 3 inoculum-only controls	20
2	ADP1, 3, 4, 10, 12, 13, 14, 17, plus 3 inoculum-only controls	27
3	ADP2, 5, 6, 7, 11, 15, 16, 18, 21 plus 2 inoculum-only controls	29
4	ADP23, 24 plus 3 positive controls and 3 inoculum-only controls	12

The inoculum for the first three sets (ADP1-22) was collected on Friday 1 June, sieved and left overnight before use. Inoculum for the fourth set was collected on Sunday 10 June 2012 and was also sieved and left overnight. Inoculum-only controls were included in each set, and positive controls in sets 1 and 4. It was decided that the tests should be set up within the shortest possible time and using the same batch of inoculum for as many of the samples as possible: therefore, in view of the large number of samples, only the total solids (TS) content of the digestate was measured before setting up the test. This was used to estimate the digestate volatile solids (VS) content and the required amount of sample, with the result that the inoculum/substrate (i/s) ratio sometimes differed from the range suggested in the standard test.

In two cases (ADP 10 and 13) where the digestate solids content was low, the amount of sample used in the test was reduced in order to ensure that there was sufficient material left for other analyses: the amount of inoculum was therefore increased slightly to maintain a constant volume in the test reactor, and this also affected the i/s ratio. In both cases the gas production curves were examined to ensure that these changes had no adverse effect on the RBP result.

Biogas volumes were recorded manually, and on each occasion when the gas collection cylinders were refilled a gas sample was taken for determination of the biogas composition (results not reported here).

Analytical methods

Digestate samples were also analysed for a number of other parameters:

- Total and volatile solids were determined according to Standard Method 2540 G (APHA, 2005).
- Total Kjeldahl Nitrogen (TKN) and total ammoniacal nitrogen (TAN) were determined using a Kjeltech digestion block and steam distillation unit, according to the manufacturer's instructions (Foss Ltd, Warrington, UK).
- Alkalinity was measured by titration with 0.25 N H_2SO_4 to endpoints of pH 5.75 and 4.3, in order to allow calculation of total (TA), partial (PA) and intermediate (IA) alkalinity (Ripley et al., 1986).
- VFA were quantified in a Shimadzu GC-2010 gas chromatograph (GC) with a flame ionisation detector (FID) and a capillary column type SGE BP-21. The carrier gas was helium at a flow of 190.8 ml min^{-1} and a split ratio of 100 to give a flow rate of 1.86 ml min^{-1} in the column and a 3.0 ml min^{-1} purge. The GC oven temperature was programmed to increase from 60 to 210°C in 15 minutes with a final hold time of 3 minutes. The temperatures of injector and detector were 200 and 250°C, respectively. Samples were prepared by centrifugation of digestates and then acidification of the supernatant in 10% formic acid. A standard solution containing acetic, propionic, iso-butyric, n-butyric, iso-

valeric, valeric, hexanoic and heptanoic acids, at three dilutions to give individual acid concentrations of 50, 250 and 500 mg l^{-1} respectively, was used for calibration.

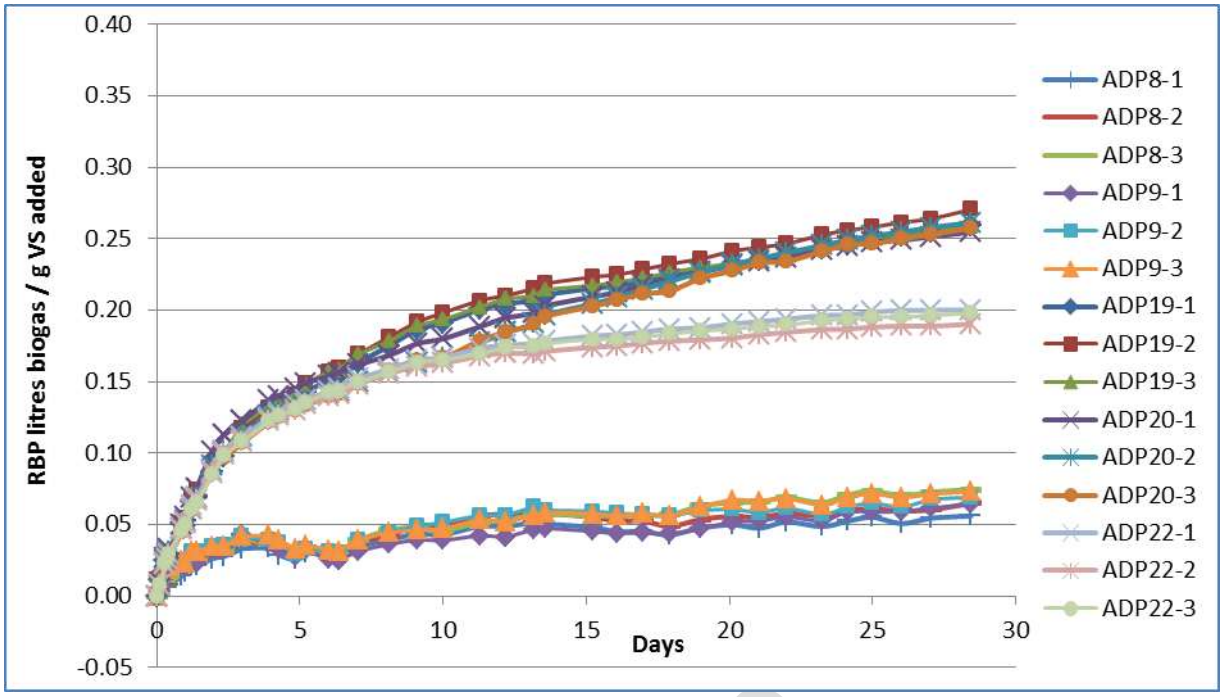
- Palmitic, stearic and oleic acids, representing the most common long chain fatty acids (LCFA) found in anaerobic digesters, were determined using a rapid non-derivatisation method (Jiang et al., 2012). Briefly, the LCFA were extracted from digestate samples using a hexane-methyl tertiary butyl ether (50:50, v/v) mixture. The LCFA were quantified using a Shimadzu GC-2010 GC, with the FID at 280°C, a capillary column type SGE BP-21; makeup flow: 30 ml min-1 (helium); column flow: 2.0 ml min-1 (helium); oven temperature: initial 160°C, ramp rate 10°C min-1, final 225°C, final hold 20 min; injection volume 1 µl. Standard solutions of palmitic, stearic and oleic acids in hexane-methyl tertiary butyl ether mixture, at three dilutions to give individual acid concentrations of 50, 100 and 250 mg l^{-1} respectively, were used for calibration.
- Total and soluble chemical oxygen demand (COD) was analysed by adapting the closed reflux titrimetric method (5220C, APHA 2005). Diluted samples (2 ml) were put into borosilicate culture tubes (16 x 100 mm), and then 0.1 ml of 500 g l^{-1} silver nitrate and 3.8 ml modified COD reagent (Ficodox Plus, Fisher Scientific UK Limited, Loughborough, UK) were added in sequence. The contents were thoroughly mixed and secured with PTFE-lined screw caps, then the tubes were heated at 150±2 ºC for 2 hours using a COD heating block. After cooling to room temperature, the tube contents were titrated using 0.025 M standard ferrous ammonium sulphate titrant with ferroin as indicator.
- Elemental analysis (C, H, N) was carried out according to the manufacturer's instructions (Flash EA-1112, Thermo Finnigan, UK), with L-Aspartic Acid, atropine and nicotinamide as standards.
- A CAL2k-ECO bomb calorimeter (CAL2k, South Africa) was used to measure calorific value (CV).
- Trace element concentrations (Co, Fe, Mo, Ni and Se) were determined using ICP–MS or ICP–OES at a commercial laboratory (Severn Trent Services, Coventry, UK) after in-house hydrochloric-nitric acid digestion (SCA, 1986).

2.1.2 Test results

The results for each set of RBP samples are shown in Figures 1-4. The final RBP values are given in Tables 2-5 together with the physico-chemical characteristics of each digestate. The individual RBP test results reported in accordance with the specification are given in Appendix 3 with the additional physico-chemical characterisation for each sample.

Set 1. Samples ADP8 and 9 showed a very low RBP of 0.065 litres g-1 VS added, with a similar gas production profile in all cases. There was no clear reason for the slight decrease in the rate of gas production relative to the control between days 4-7. The digestate was characterised by low VFA, relatively high concentrations of LCFA, high alkalinity and IA/PA ratio and a very low percentage of VS in the TS content. The questionnaire received with the sample indicated these two plants received similar input materials.

Figure 1. RBP results for samples in Set 1



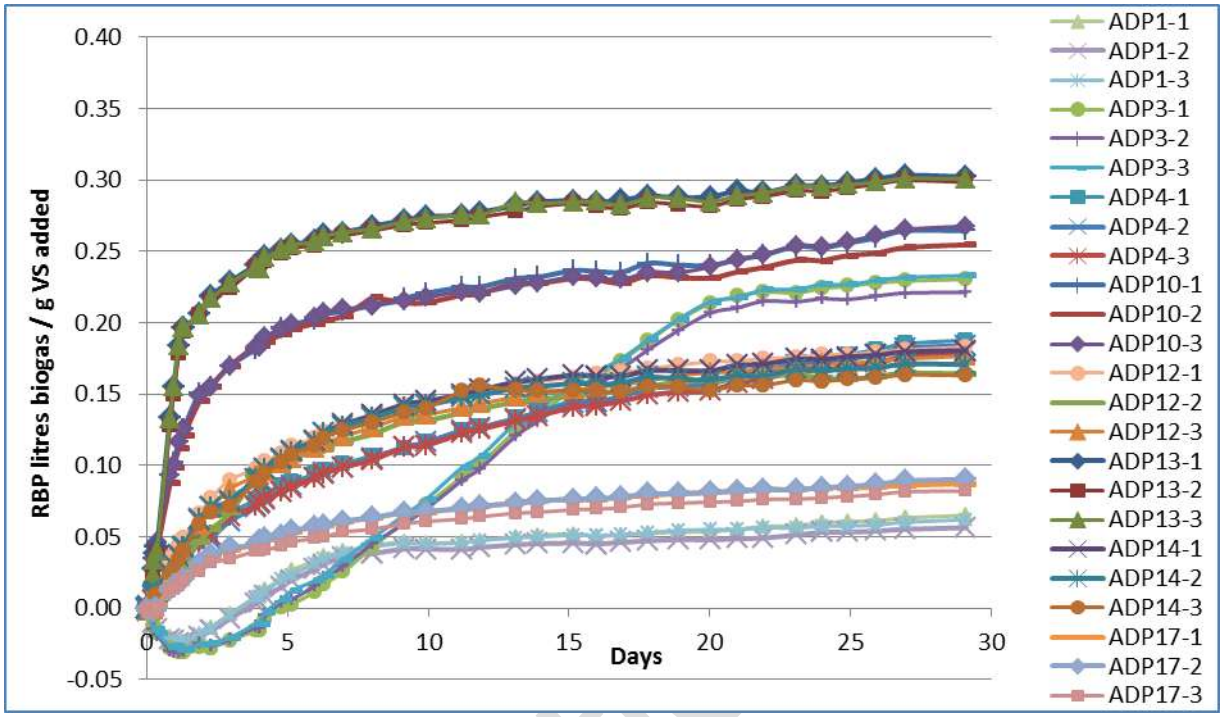
Sample ADP22 gave a classic RBP profile for a well-stabilised digestate, with about half of the total biogas production occurring in the first 2-3 days followed by a long 'tail' from less readily degradable material. The sample RBP of 0.196 l g-1 VS added was comfortably within the RBP limit of 0.25 l g-1 VS added, and triplicate values showed good agreement. The digestate had low VFA but some accumulation of palmitic acid. The TAN and TKN were relatively high, contributing to high total alkalinity but a low ratio of intermediate to partial alkalinity (IA/PA ratio). The digestate had a good balance of trace elements.

The RBP of ADP19 was marginally over the RBP limit at 0.263 l g-1 VS added. This digestate had very low VFA and no detectable LCFA. The solids content of the digestate was low, and this was reflected in low concentrations of TKN and TAN on a wet weight basis. The shape of the RBP curve indicates a high proportion of slowly degradable material present in the digestate, with the result that cumulative gas production had not plateaued by the end of the 28-day test. The concentration of trace elements in the digestate showed no deficiencies when considered in relation to the low nitrogen concentration.

ADP20 showed a similar profile to ADP19 but with more variation between the replicates in the early and middle stage of the test. The RBP value was again just marginally above the limit at 0.258 l g-1 VS added, with the cumulative total still increasing at the end of the 28-day period. The sample had a total VFA concentration of around 1.4 g l-1, composed mainly of acetic and propionic acids, and a high concentration of stearic acid. TKN and TAN concentrations were not particularly high, giving a moderate total alkalinity of 13 g CaCO3 l 1. A high proportion of the digestate solids were VS, again suggesting the presence of a sparingly degradable fraction. It is also possible that the microbial population of the plant could be inhibited by an excess of trace elements as the cobalt concentration was approximately 10 times the recommended value, with nickel around 30 times higher.

Set 2. Samples ADP4, 12 and 14 were all comfortably within the RBP limit. These digestates all had low or very low VFA and low or intermediate TAN concentrations. All three digesters showed a reasonable trace element balance.

Figure 2. RBP results for samples in Set 2



The RBP value for ADP13 was above the specified limit, at 0.301 l g-1 VS added: agreement between the triplicate sub-samples was good. This digestate had a total VFA concentration of 3963 mg l-1 (equivalent to 5200 mg COD l-1) but was below the RBP test cut-off value for VFA with a value of 0.41 g COD g-1 VS. The VFA profile showed that the highest proportion was acetic acid, but all acids up to C7 were present in detectable quantities. The digestate was very low in essential trace elements, in particular cobalt.

ADP10 also just exceeded the limit with an RBP value of 0.262 l g-1 VS added, despite a very low VFA content. The gas production curve for this sample indicated a substrate with a relatively large fraction of slowly degradable material. TAN concentrations in the digestate were also low. Again, this digestate had a very low concentration of the essential trace element cobalt.

ADP17 also had a low VFA concentration, but showed a small accumulation of palmitic acid. The TAN concentration was relatively high at 3.58 g N l-1, a value at which some inhibition of acetoclastic methanogens might occur. Apart from nickel, other trace elements were generally below recommended levels and the absence of acid accumulation together with the very low RBP indicates that this digester may be working at a low organic loading rate.

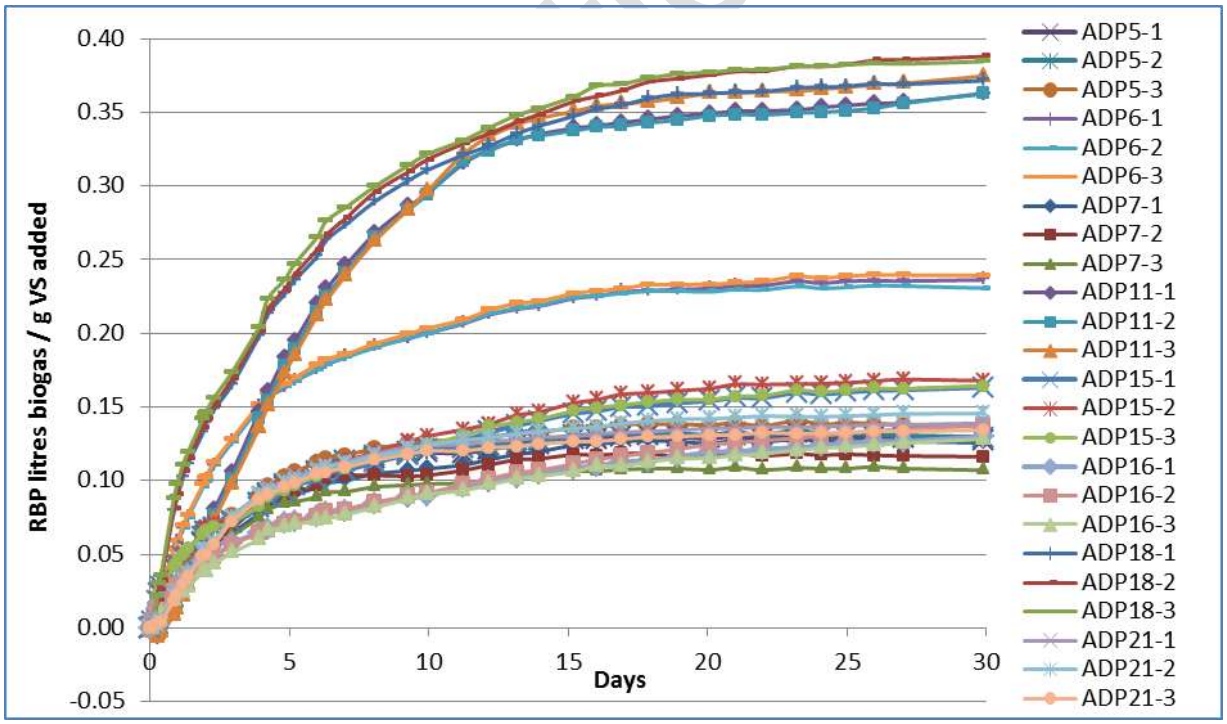
ADP1 and 3 both showed inhibition in the early stages of the RBP test: in the case of ADP3 the sample was very close to failure on the basis that net gas production was below zero for almost 5 days. The ADP3 digestate had a total VFA concentration of 4.9 g VFA l-1, and this may have been sufficient to account for the inhibition seen. On a COD basis the VFA content was 6.1 g COD l-1 or 0.29 g COD g-1 VS, below the limit value of 0.43 g COD g-1 VS. The VFA accumulation requires further investigation as the digester appears to have a well-balanced trace element composition, although the concentration of iron was surprisingly low and may be causing other essential elements to be preferentially complexed and therefore

unavailable. In the case of ADP1 the VFA content was low and there is no clear cause of inhibition in the parameters analysed: net gas production did recover to a positive value within the 5-day limit, however, and the sample passed with a low RBP value of 0.061 l g-1 VS added. Similar instances have been observed in tests carried out previously but the causes are currently unclear.

Set 3. ADP6 was below the RBP limit at 0.235 l g^{-1} VS added. The digestate had a low-to-moderate VFA concentration typical of plants that have a high TAN content but are receiving adequate trace element supplementation. The remaining samples in this set (ADP5, 7, 15, 16 and 21) were all comfortably below the RBP limit and as expected showed low VFA concentrations. Four of these samples showed high TAN and TKN values (Table 4) but all of the plants concerned reported that they were carrying out trace element supplementation, and this is supported by the digestate trace element concentrations.

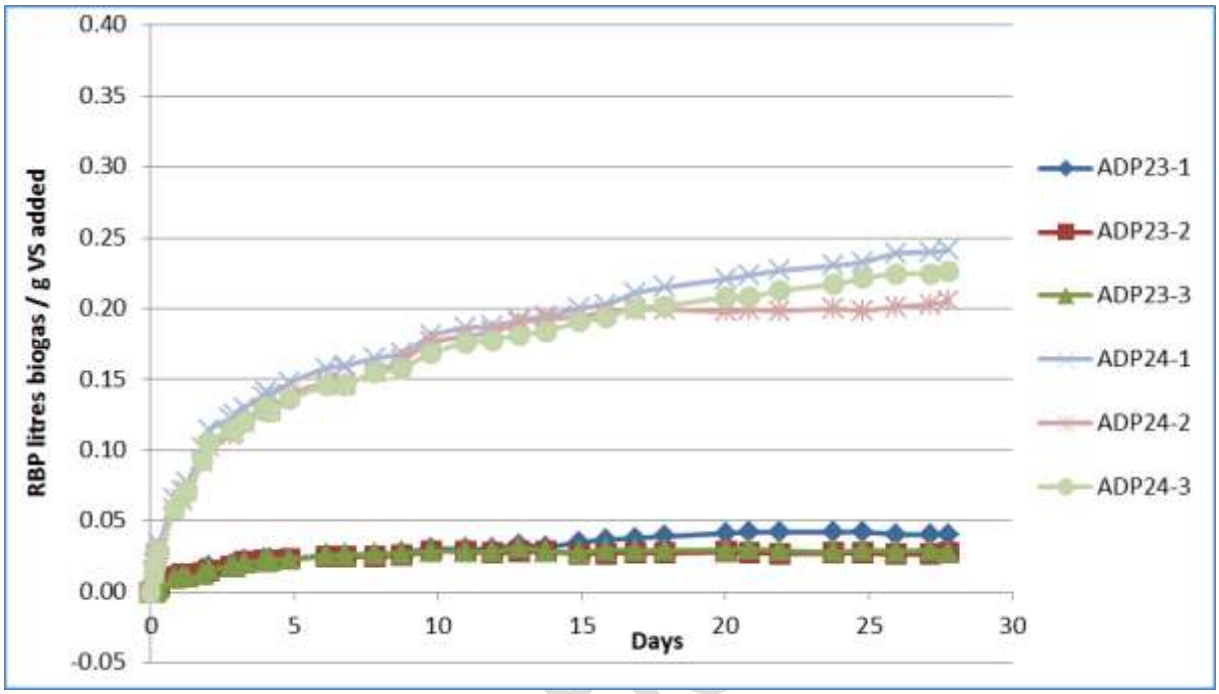
ADP11 and 18 both failed to meet the RBP limit. ADP11 passed the initial VFA screening test with a value of 0.38 g COD g^{-1} VS, despite having a total VFA concentration over 9 g VFA l^{-1}. The VFA concentration in ADP18 was 3.9 g VFA l^{-1} giving a screening test result well below the limit, at 0.17 g COD g^{-1} VS. ADP11 had very high TKN and TAN values of 9.23 and 6.54 g N l^{-1} respectively, and experience suggests that trace element concentrations in this digester may be insufficient to compensate for the failure of the acetoclastic population by supporting the alternative hydrogenotrophic methanogenic pathway. The TKN and TAN concentrations in ADP18 were lower at 4.87 and 3.00 g N l^{-1} respectively, but the trace element profile shown is probably sufficient to explain the accumulation of VFA in these conditions.

Figure 3. RBP results for samples in Set 3



Set 4. Both ADP23 and 24 were below the RBP limit, with ADP23 showing an extremely low value of 0.032 l g^{-1} VS added, similar to the values for ADP8 and 9: in each of these cases VS made up only a small proportion of the feedstock solids, suggesting a stable material. ADP23 also showed some accumulation of LCFA similar to that in ADP8 and 9.

Figure 4. RBP results for samples in Set 4



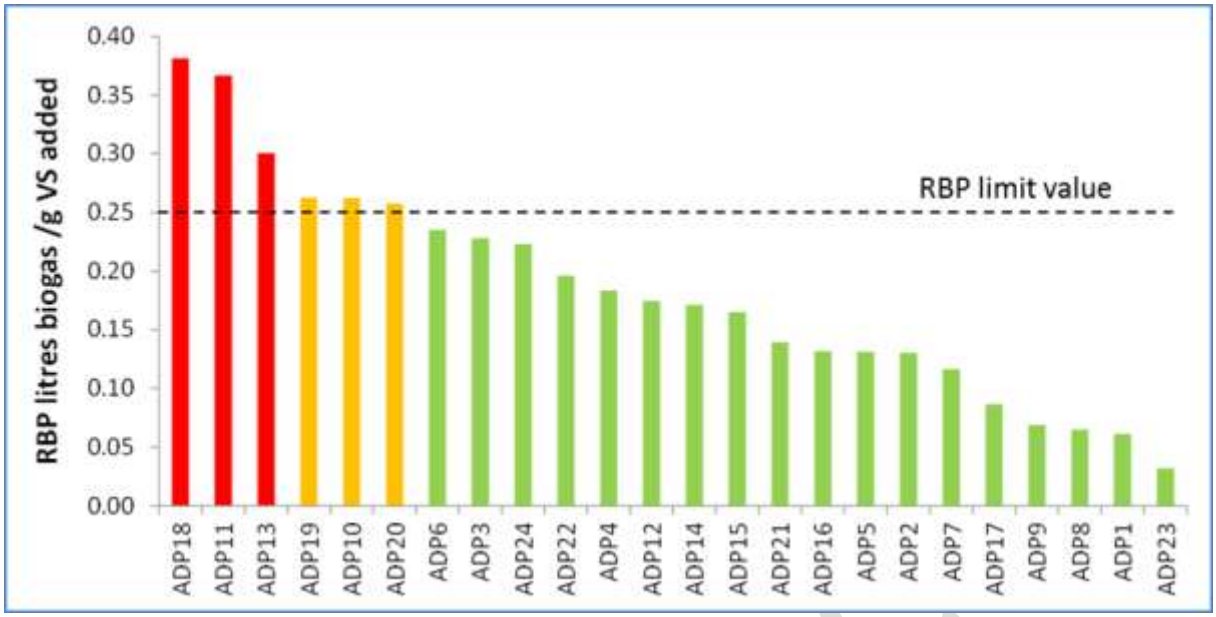
2.1.3 Discussion of results

Figure 5 summarises the RBP results for all samples tested. Of the 24 samples, three were clear failures and the probable reasons for this are discussed above: briefly, ADP11 and 18 both had high TAN and TKN values without trace element supplementation to support the hydrogenotrophic pathway, and correspondingly high VFA concentration. The TAN and TKN values in ADP13 were lower and probably insufficient to induce ammonia toxicity, but this digestate was also very deficient in cobalt which is essential for both acetoclastic and hydrogenotrophic methanogenesis.

Three other digestates were on the borderline. ADP10 was low in trace elements including iron, and appeared to contain a proportion of slow-degrading solids. In the case of ADP19 there is no single clear reason for the relatively high residual biogas potential, but again the gas production curve indicated a high proportion of slowly-degrading solids. ADP20 was similar in terms of the kinetics of gas production, but had slightly higher VFA; the trace element concentration was 10 times higher than required, possibly contributing to this. For this type of material some form of post-digestion storage including biogas collection (secondary digestion) is considered the best solution. Samples taken from this final point in the treatment process are likely to show a lower RBP value than those taken from the primary digester.

Taken together, the results therefore indicate that the original limit value of 0.25 l biogas g-1 VS was well chosen for its purpose, and failure to meet it indicates that there is potential to improve the performance of the plant.

Figure 5. RBP results - all samples



With a view to reducing the time and cost of the test, the 28-day RBP results were compared with the values after 10 days of the test (Figure 6). The results show good agreement with the exception of one point, corresponding to the sample for ADP3 which had a long period of negative net biogas production. This can also be seen in Figure 7a where the 10 and 28-day results for each digestate are compared. If the result for ADP3 is removed the correlation coefficient increases to 0.96 with a slope of around 0.82, suggesting that the current RBP value might correspond to a limit of around 0.20 l biogas g^{-1} VS added at 10 days. Figure 7b shows the 10-day values for the samples tested. It can be seen that the three clear failures are still well above the lower limit, and in fact still exceed the original value of 0.25 l biogas g^{-1} VS added, in part reflecting the high proportion of readily degradable material present in the form of VFA.

If the value of 0.25 l biogas g^{-1} VS were applied at 10 days the three marginal failures would pass, as the slower-degrading components have not yet been fully converted. If a value of 0.20 l biogas g^{-1} VS was substituted, one of the marginal failures remains a failure while the others are now just below the limit; one other sample would exceed the limit at 0.201 l biogas g^{-1} VS added (Figure 7). Both the multiply replicated test above and anonymised data (not shown) from RBP tests carried out at the Open University suggest a RSD% value of about 4-6% is achievable for a well-conducted test on a homogeneous substrate.

Figure 6. Relationship between 10-day and 28-day RBP values (litres biogas g^{-1} VS added)

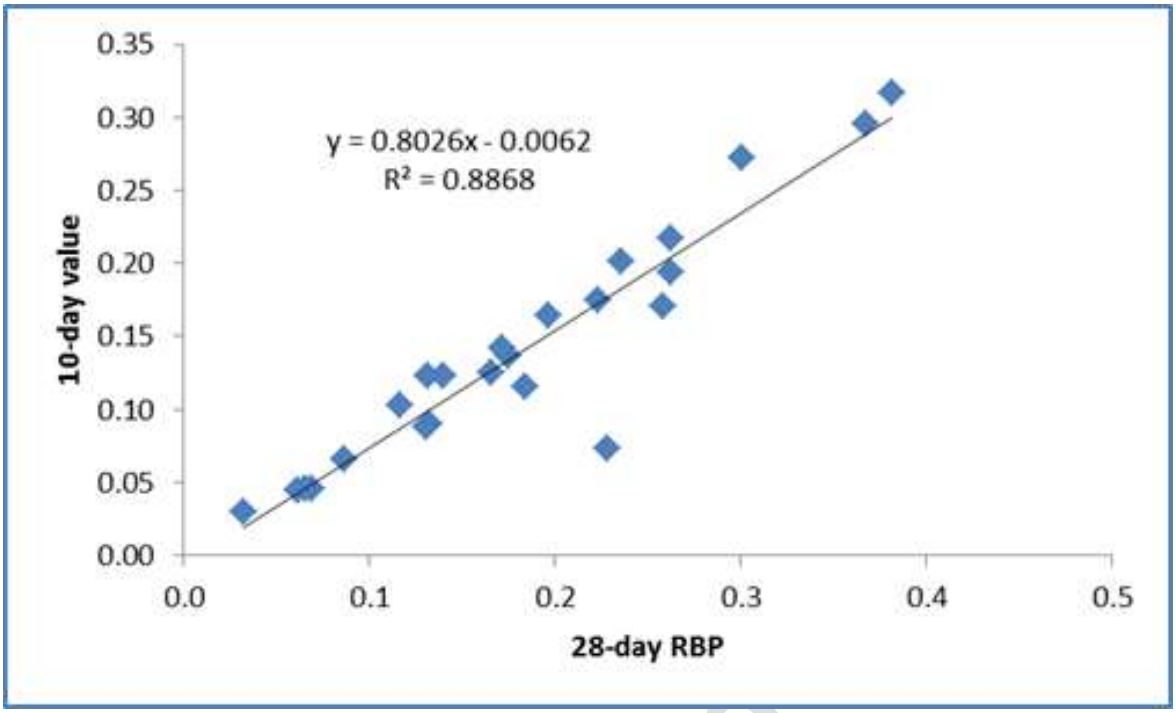
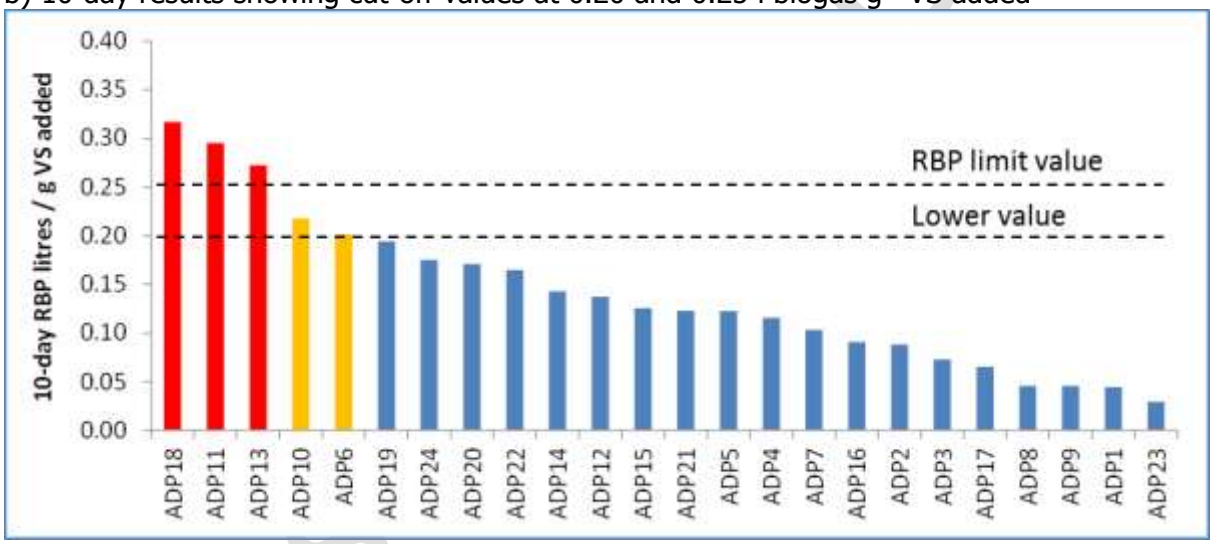


Figure 7. Comparison of 10-day and 28-day values from RBP test

a) Comparison of results for each digestate

b) 10-day results showing cut-off values at 0.20 and 0.25 l biogas g^{-1} VS added



2.1.4 Relationships between parameters

Correlation coefficients for RBP and the various parameters measured are shown in Tables 2-5 below. As expected no very strong relationships were found. Total VFA and acetic acid concentration account for about 40% of the variation in RBP value. Total and volatile solids content account for around 36% and 29% respectively. Other parameters showing some slight relationship include calorific value and elemental composition. If multiple factors are considered, the combination of total VFA and TS accounts for around 65% of variation and total VFA and VS for 61%: it is reasonable to consider these measurements as independent, as in the method used for TS and VS determination the VFA is likely to evaporate and not be included in the solids content. None of these parameters, however, appear sufficiently promising to warrant their use as a predictor for the RBP value.

The correlation between VS and biochemical methane potential (BMP) found by Schievano et al. (2008) (see Literature Review, Appendix 2) does not appear to hold true to the same extent for digested materials, and as noted in their work the inclusion of digestates reduced the correlation coefficients.

Table 2. RBP values and other digestate parameters - VFA

	RBP	VFA (mg l^{-1})									As COD	
		Acetic	Propionic	Iso-Butyric	n-Butyric	Iso-Valeric	n-Valeric	Hexanoic	Heptanoic	Total VFA	mg COD l^{-1}	mg COD g^{-1} VS
	l g^{-1} VS											
ADP1	0.061	106.1	n/d	n/d	n/d	n/d	n/d	n/d	n/d	106.1	113.1	4.8
ADP2	0.130	178.2	15.4	n/d	n/d	n/d	n/d	n/d	n/d	193.6	213.2	5.7
ADP3	0.228	3240.2	1236.5	118.8	40.3	220.6	21.6	n/d	n/d	4878.0	6104.6	293.6
ADP4	0.184	29.1	8.3	n/d	n/d	n/d	n/d	n/d	n/d	37.4	43.5	1.4
ADP5	0.131	846.8	128.4	15.1	4.7	21.5	n/d	n/d	n/d	1016.4	1176.1	17.4
ADP6	0.235	684.0	62.3	7.6	7.4	14.6	n/d	n/d	n/d	776.0	880.2	27.0
ADP7	0.116	247.4	80.4	n/d	n/d	4.8	n/d	n/d	n/d	332.6	395.0	10.5
ADP8	0.065	111.6	n/d	n/d	n/d	4.1	n/d	n/d	n/d	115.6	127.2	2.5
ADP9	0.069	183.6	n/d	n/d	n/d	3.8	n/d	n/d	n/d	187.4	203.4	5.0
ADP10	0.262	14.4	260.1	21.9	n/d	27.9	n/d	n/d	n/d	324.3	505.2	41.7
ADP11	0.367	2633.8	5909.6	117.5	20.1	526.8	54.9	n/d	n/d	9262.8	13175.8	381.8
ADP12	0.175	134.5	5.9	4.6	4.5	9.1	5.9	13.4	27.0	204.9	291.9	9.9
ADP13	0.301	2662.2	379.2	233.2	191.3	416.6	52.0	19.3	9.6	3963.3	5200.3	408.1
ADP14	0.171	23.3	6.1	n/d	n/d	7.3	n/d	n/d	n/d	36.7	48.9	1.8
ADP15	0.165	335.5	7.5	7.8	n/d	13.6	n/d	n/d	n/d	364.5	410.8	13.7
ADP16	0.132	19.2	n/d	n/d	n/d	n/d	n/d	n/d	n/d	19.2	20.5	1.0
ADP17	0.087	250.3	8.8	n/d	n/d	n/d	n/d	n/d	n/d	259.0	280.0	6.4
ADP18	0.381	2706.8	928.1	95.6	23.5	107.3	10.4	n/d	n/d	3871.6	4743.6	176.5
ADP19	0.263	36.7	8.8	n/d	n/d	4.8	n/d	n/d	n/d	50.3	62.2	3.8
ADP20	0.258	832.2	571.9	12.8	8.2	15.6	n/d	n/d	n/d	1440.6	1821.3	40.5
ADP21	0.140	39.8	10.8	n/d	n/d	3.3	n/d	n/d	n/d	53.9	65.5	1.7
ADP22	0.196	184.7	20.9	4.5	n/d	7.9	n/d	n/d	n/d	218.1	252.8	5.6
ADP23	0.032	89.69	29.22	11.40	7.66	19.69	13.28	29.79	59.43	260.1	445.9	4.2
ADP24	0.223	299.39	18.81	3.53	2.43	6.88	3.94	6.38	11.92	353.3	422.3	42.9
R^2 with RBP		0.440								0.449		
p value		0.000								0.000		

Table 3. RBP values and other digestate parameters - Solids, calorific value, elemental composition and COD

	RBP	Solids (g/kg)					Calorific Value	Elemental CNH			COD	
	l g^{-1} VS	TS g kg^{-1} WW		VS g kg^{-1} WW		VS/TS	MJ kg^{-1} TS	N (%)	C (%)	H (%)	Total mg O_2 l^{-1}	Dissolved
ADP1	0.061	36.1	±0.10	23.76	±0.26	65.8%	14.86	6.0	36.0	4.2	34622	11999
ADP2	0.130	49.8	±0.08	37.12	±0.22	74.5%	16.38	5.6	39.2	4.5	42772	6669
ADP3	0.228	32.9	±0.22	20.79	±0.17	63.3%	17.11	5.8	37.7	4.5	33019	16108
ADP4	0.184	49.1	±0.03	30.39	±0.04	61.9%	14.89	5.5	37.4	4.6	40350	2735
ADP5	0.131	93.3	±0.41	67.65	±0.48	72.5%	18.61	7.3	34.8	4.5	88635	21565
ADP6	0.235	46.4	±0.04	32.59	±0.03	70.2%	17.53	6.6	40.1	4.9	44041	12137
ADP7	0.116	56.6	±0.71	37.49	±0.73	66.3%	16.13	6.1	38.3	4.2	40694	15160
ADP8	0.065	178.3	±0.98	50.05	±0.90	28.1%	3.29	2.2	17.1	1.7	57292	11191
ADP9	0.069	137.4	±0.10	40.44	±0.10	29.4%	4.41	2.2	12.5	1.6	39315	8450
ADP10	0.262	17.2	±0.06	12.11	±0.02	70.6%	20.26	6.1	46.2	5.6	28619	3162
ADP11	0.367	47.8	±0.01	34.51	±0.05	72.2%	22.26	8.5	47.7	5.3	75387	35943
ADP12	0.175	46.5	±0.01	29.51	±0.08	63.4%	14.78	5.7	36.9	4.0	36119	7822
ADP13	0.301	20.4	±0.03	12.74	±0.06	62.5%	17.02	6.3	38.9	4.4	28689	9997
ADP14	0.171	36.9	±0.23	26.50	±0.11	71.8%	18.26	6.1	42.4	4.9	31682	10170
ADP15	0.165	43.6	±0.36	29.98	±0.38	68.8%	16.21	6.6	38.5	4.5	46880	9526
ADP16	0.132	35.0	±0.16	21.47	±0.08	61.3%	15.25	5.8	32.9	4.5	19631	4504
ADP17	0.087	58.0	±1.31	43.56	±1.18	75.1%	19.86	5.2	44.1	5.3	65628	11873
ADP18	0.381	36.6	±0.12	26.88	±0.05	73.4%	19.22	6.5	43.5	5.6	39180	18842
ADP19	0.263	29.8	±0.26	16.42	±0.21	55.0%	13.35	5.7	30.7	3.8	26505	6394
ADP20	0.258	59.0	±0.69	44.97	±0.84	76.2%	17.76	4.3	42.2	4.8	55736	16995
ADP21	0.140	52.9	±1.24	38.76	±1.34	73.2%	17.90	6.9	44.6	4.7	58576	14743
ADP22	0.196	64.6	±1.34	44.93	±1.16	69.5%	16.41	6.4	38.5	4.5	68516	15575
ADP23	0.032	211.5	±0.88	106.18	±1.04	50.2%	12.24	2.4	33.2	2.6	82916	12591
ADP24	0.223	20.9	±0.23	9.85	±0.64	47.2%	10.74	5.3	24.9	2.9	17765	9518
R^2 with RBP		0.356		0.264		0.153	0.277	0.324	0.228	0.319	0.047	0.124
p value		*0.002*		*0.010*		*0.059*	*0.008*	*0.004*	*0.018*	*0.004*		

Table 4. RBP values and other digestate parameters - pH, Alkalinity, TKN and TAN, LCFA

	RBP	TAN		TKN		TAN/TKN	Alkalinity				pH	LCFA (mg l^{-1})		
							TA	PA	IA	IA:PA		Palmitic	Stearic	Oleic
	l g^{-1} VS	g N kg^{-1}WW		g N kg^{-1}WW		%	mg $CaCO_3$ kg^{-1}							
ADP1	0.061	3.94	±0.02	5.96	±0.01	66.1%	19285	15576	3709	0.24	8.09	8.7	n/d	n/d
ADP2	0.130	1.70	±0.01	3.53	±0.01	48.2%	12521	9027	3494	0.39	8.30	n/d	n/d	n/d
ADP3	0.228	5.38	±0.02	6.75	±0.03	79.7%	21800	15159	6642	0.44	8.23	n/d	n/d	n/d
ADP4	0.184	0.62	±0.02	3.28	±0.02	18.9%	7972	6419	1553	0.24	7.41	5.3	n/d	n/d
ADP5	0.131	7.98	±0.01	12.37	±0.06	64.5%	33380	25871	7509	0.29	8.35	n/d	n/d	n/d
ADP6	0.235	4.04	±0.04	6.48	±0.07	62.4%	17523	12424	5099	0.41	8.17	n/d	n/d	n/d
ADP7	0.116	5.12	±0.00	7.48	±0.06	68.5%	24505	16622	7883	0.47	8.45	5.7	n/d	n/d
ADP8	0.065	2.80	±0.02	4.91	±0.01	56.9%	46426	28117	18309	0.65	8.14	18.1	42.5	10.0
ADP9	0.069	2.69	±0.02	4.44	±0.05	60.5%	58195	20924	37271	1.78	8.16	15.1	32.6	13.8
ADP10	0.262	0.40	±0.01	1.29	±0.02	31.2%	3306	2197	1109	0.50	7.33	5.2	n/d	n/d
ADP11	0.367	6.54	±0.02	9.23	±0.10	70.9%	23986	15622	8365	0.54	8.35	10.4	n/d	n/d
ADP12	0.175	3.32	±0.03	5.21	±0.03	63.8%	17643	13105	4538	0.35	8.04	n/d	n/d	n/d
ADP13	0.301	2.22	±0.03	3.14	±0.02	70.6%	9453	5222	4231	0.81	7.62	n/d	n/d	n/d
ADP14	0.171	2.71	±0.01	4.64	±0.01	58.4%	12224	8981	3243	0.36	8.10	n/d	n/d	n/d
ADP15	0.165	2.80	±0.00	5.29	±0.07	52.8%	15730	12140	3590	0.30	8.37	6.1	n/d	n/d
ADP16	0.132	0.44	±0.01	2.25	±0.01	19.6%	3965	1850	2115	1.14	7.50	n/d	n/d	n/d
ADP17	0.087	3.58	±0.01	6.12	±0.01	58.5%	17196	13658	3538	0.26	8.90	8.5	n/d	n/d
ADP18	0.381	3.00	±0.06	4.87	±0.04	61.5%	11906	7765	4141	0.53	7.92	9.4	n/d	n/d
ADP19	0.263	1.49	±0.01	2.85	±0.00	52.3%	7705	5794	1911	0.33	7.92	n/d	n/d	n/d
ADP20	0.258	2.42	±0.02	4.27	±0.03	56.7%	13698	9981	3717	0.37	8.16	n/d	41.0	n/d
ADP21	0.140	4.62	±0.03	6.85	±0.03	67.6%	21129	16311	4817	0.30	8.42	7.6	n/d	n/d
ADP22	0.196	5.76	±0.02	8.55	±0.01	67.4%	23562	18336	5227	0.29	8.15	10.4	n/d	n/d
ADP23	0.032	3.00	±0.02	5.61	±0.03	53.5%	24848	14704	10143	0.69	8.07	22.0	71.3	n/d
ADP24	0.223	2.25	±0.01	3.11	±0.02	72.2%	12783	10070	2714	0.27	8.32	n/d	n/d	n/d
R^2 with RBP		0.000		0.009		0.017	0.220	0.220	0.142	0.031	0.080	0.173	0.146	1.000
p value							0.021							

Table 5. RBP values and other digestate parameters - trace elements

	RBP	Trace element (mg l^{-1})				
	l g^{-1} VS	Cobalt	Iron	Molybdenum	Nickel	Selenium
ADP1	0.061	0.06	326	0.23	0.63	0.01
ADP2	0.130	0.85	131	0.29	1.20	0.07
ADP3	0.228	0.24	51	0.13	0.25	0.19
ADP4	0.184	0.08	1144	0.30	0.50	0.01
ADP5	0.131	0.24	1021	0.43	1.75	0.04
ADP6	0.235	0.28	2032	0.19	0.60	0.02
ADP7	0.116	0.21	91	0.18	0.39	0.06
ADP8	0.065	1.08	2327	0.94	7.74	<0.0101
ADP9	0.069	0.98	1923	0.69	5.06	<0.0084
ADP10	0.262	0.03	87	0.07	0.24	<0.0058
ADP11	0.367	0.09	539	0.14	0.50	0.03
ADP12	0.175	0.42	175	0.30	0.71	0.07
ADP13	0.301	0.04	211	0.08	0.19	0.01
ADP14	0.171	0.25	234	0.08	0.28	0.01
ADP15	0.165	1.04	111	0.30	1.02	0.13
ADP16	0.132	0.45	1166	1.46	1.67	<0.0059
ADP17	0.087	0.09	152	0.25	1.08	0.02
ADP18	0.381	0.09	64	0.18	0.26	0.02
ADP19	0.263	0.19	348	0.28	0.56	0.07
ADP20	0.258	9.06	227	5.68	30.37	0.58
ADP21	0.140	1.42	294	0.50	1.33	0.16
ADP22	0.196	1.21	1056	0.45	1.55	0.12
ADP23	0.032	2.58	4250	1.17	13.79	0.07
ADP24	0.223	0.89	37	0.26	0.82	0.12
R^2 with RBP			0.215			
p value			0.022			

2.2 Assessment of variability in RBP test results

In this part of the work tests were carried out on digestate from ADP7. This plant was selected as a previous RBP study had suggested it would be likely to have a typical mid-range RBP value. The following tests were carried out:

a) to determine the variability in RBP test results, a single sample was divided into 19 sub-samples which were tested at the same time under the same conditions.
b) to assess the effect of small-scale variability in sampling, multiple samples were tested that had been collected from the same sampling point within a short period of time on one day. Each sample was tested in triplicate.
c) to assess the effect of variability over time, samples of digestate were taken from a single digester over a two-month period. Each sample was tested according to the standard RBP protocol using inoculum from Millbrook WWTP, Southampton.

2.2.1 Variability in a single sample

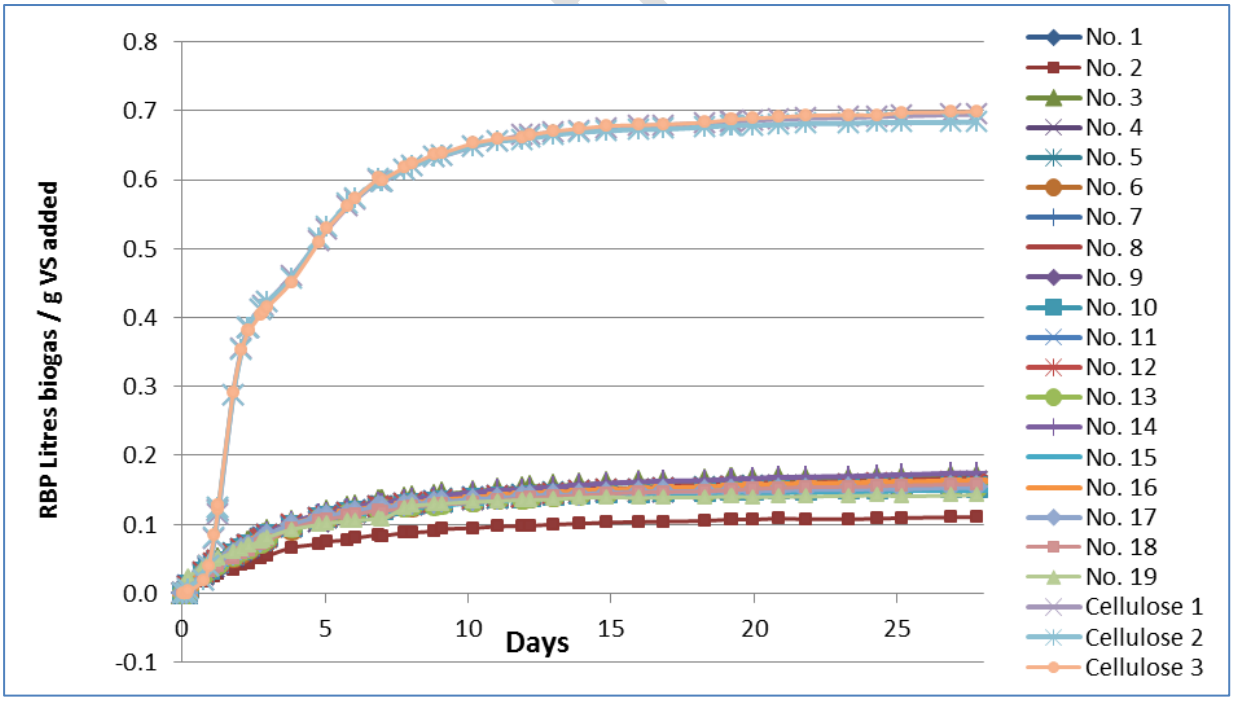
A single sample was taken directly from the digester at ADP7 and transported to the laboratory at UoS on the same day. An RBP test was carried out in accordance with the standard method (Walker et al., 2010) but with 19 replicates of the sample material and 5 of the inoculum-only control, rather than the standard triplicates. Triplicate cellulose positive controls were included as required in the test. Inoculum total and volatile solids were measured in duplicate rather than triplicate, but showed good agreement. Tables 6 and 7 show the reported data for the RBP test and Figures 8 and 9 show the graphical output. For clarity the RBP values for the inoculum are shown separately in Figure 10.

Table 6. Total and volatile solids contents for digestate, control sample (cellulose) and inoculum in single sample test

Parameter Unit	TS %WW	VS %WW	VS %TS	average TS g TS kg^{-1} WW	average VS %WW	average VS g VS kg^{-1} WW
Cellulose	96.01 95.98 95.90	95.95 95.91 95.85	99.9% 99.9% 99.9%	95.97	95.90	959.03
Digestate	5.73 5.82 5.77	3.87 3.94 3.84	67.4% 67.7% 66.6%	5.78	3.88	38.84
Inoculum	3.82 3.82	2.45 2.45	64.0% 64.0%	3.82	2.45	24.46

The quality control measures prescribed in the standard RBP test indicated that the test was satisfactory. Inoculum biogas production during the test was approximately 1.2 litres, corresponding to an RBP of 0.121 l biogas g^{-1} VS added. The RBPs of the reference material and the digestate samples were not negative at any point in the test. With the exception of sample 19, all of the plotted RBP values for inoculum, reference material and digestate sub-samples were smooth with no obvious spikes or inconsistencies, as can be seen from Figure 8. The RBP value for the reference material was 0.692 litres biogas g^{-1} VS added and was therefore greater than the stipulated minimum of 0.5 litres biogas g^{-1} VS added. The test results were therefore considered valid.

Figure 8. Digestate and reference material RBPs - single digestate sample (19 replicates)



Seventeen of the 19 replicates showed closely similar behaviour. Of the remaining two, one (sample 2) showed no distinctive behaviour but the cumulative gas production was noticeably lower, indicating a minor leak (Figure 8). This sample was rejected and the data were not included in calculation of the results. The other (sample 19) showed an unusual pattern of gas production from early on in the test, which may have been due to sample inhomogeneity, blockage or minor leakage.

Figure 9. Digestate RBP - single sample (19 replicates)

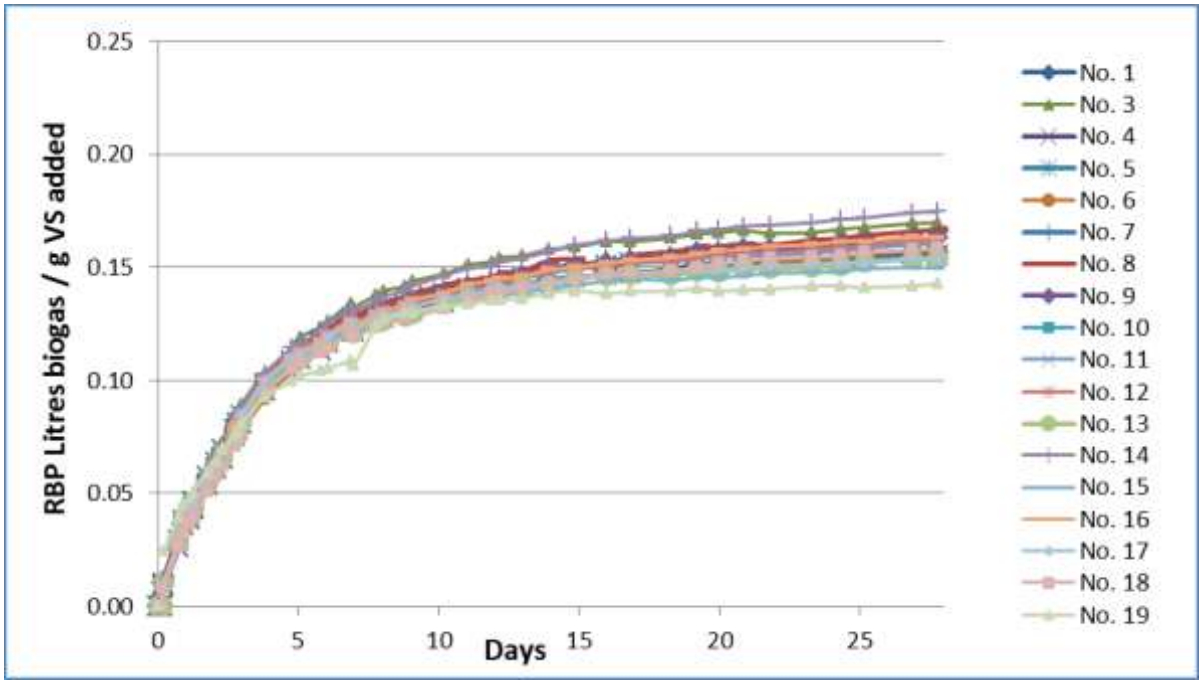
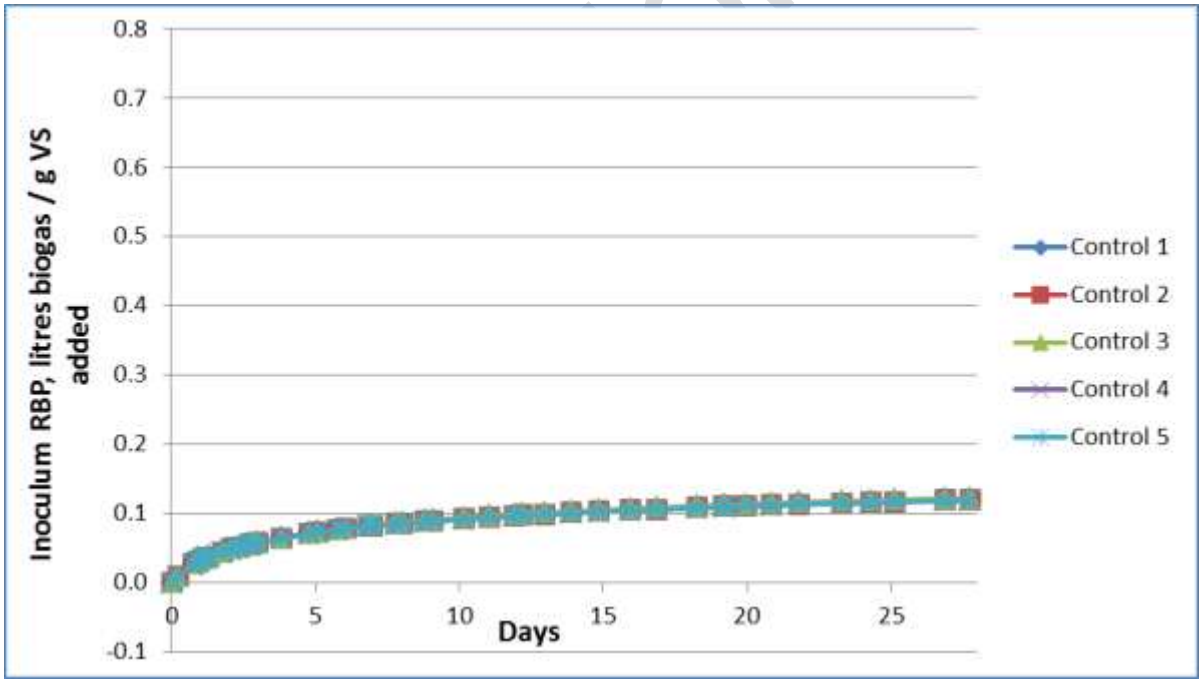


Figure 10. Inoculum RBP on same scale as main RBP results - single sample



The average RBP value for the 18 replicates was 0.157 l biogas g^{-1} VS added, with a relative standard deviation (RSD) of 4.66%. If the value for sample 19 is rejected, the RBP value becomes 0.160 litres biogas g^{-1} VS added and the RSD falls to 3.98%.

Table 7. Results for repeatability RBP

Sample	VS g kg^{-1} WW	weight of material added (g)	weight of inoculum added (g)	Gas Production (l)	Gas from inoculum (l)	VS ratio [a]	RBP value l g^{-1} VS	Ave. RBP l g^{-1} VS	SD	RSD%
1	38.84	58.5	346.5	1.379	1.023	3.73	0.157	0.159[b]	0.007[b]	4.66 [b]
2		58.5	342.0	1.260	1.010	3.68	0.110	0.160[c]	0.006[c]	3.98[c]
3		58.0	345.0	1.401	1.019	3.75	0.170			
4		61.5	348.0	1.398	1.027	3.56	0.155			
5		58.0	342.0	1.373	1.010	3.71	0.161			
6		66.5	348.0	1.433	1.027	3.30	0.157			
7		61.0	342.5	1.398	1.011	3.54	0.163			
8		58.0	346.5	1.398	1.023	3.76	0.167			
9		60.5	343.0	1.395	1.013	3.57	0.163			
10		58.5	342.0	1.362	1.010	3.68	0.155			
11		57.5	342.0	1.369	1.010	3.75	0.161			
12		59.5	345.0	1.393	1.019	3.65	0.162			
13		57.5	342.5	1.355	1.011	3.75	0.154			
14		58.5	344.5	1.414	1.017	3.71	0.175			
15		57.5	347.0	1.358	1.025	3.80	0.150			
16		58.5	342.0	1.380	1.010	3.68	0.163			
17		59.5	342.0	1.364	1.010	3.62	0.153			
18		62.5	342.5	1.396	1.011	3.45	0.159			
19		58.5	345.0	1.343	1.019	3.72	0.143			
Cellulose	959.03	1.54	401.5	2.211	1.185	6.65	0.694	0.692	0.008	1.09
		1.54	401.0	2.194	1.184	6.64	0.684			
		1.54	404.5	2.226	1.194	6.70	0.699			
Inoculum	24.46	-	401.5		1.196		0.122	0.121	0.001	1.13
		-	400.0		1.176		0.120			
		-	400.0		1.198		0.122			
		-	400.0		1.167		0.119			
		-	400.0		1.172		0.120			

[a]Inoculum/substrate on a VS basis for the RBP tests (should be around 4 for digestate and 6 for reference RBP tests); [b]Not including sample 2; [c]Not including sample 2 and 19.

2.2.2 Variability in samples taken over a short interval

Samples for this test were taken directly from ADP7 and transported to the laboratory at UoS. The samples were taken on the same day as for the single sample repeatability test described above at hourly intervals from 09:00 to 16:00. RBP tests were carried out in accordance with the standard method (Walker et al., 2010) with each sample tested in triplicate and five replicates for the inoculum-only control. The same inoculum was used as for the repeatability tests, but separate inoculum-only controls were provided to take into account any minor variations in equipment and set-up (e.g. temperature of waterbaths). Tables 8 and 9 show the reported data for the RBP test and Figure 11 shows the graphical output. For clarity the RBP values for the inoculum are shown separately in Figure 12.

Table 8. Total and volatile solids contents for digestate, control sample and inoculum in hourly sampling test

Parameter Unit	TS %WW	VS %WW	VS %TS	average TS g TS kg^{-1} WW	average VS %WW	average VS g VS kg^{-1} WW
Cellulose[a]	96.01	95.95	99.9%	95.99	95.90	959.03
	95.98	95.91	99.9%			
	95.98	95.85	99.9%			
09:00	5.95	4.02	67.6%	5.89	3.97	39.73
	5.83	3.92	67.3%			
10:00	5.79	3.92	67.8%	5.82	3.95	39.48
	5.86	3.97	67.8%			
11:00	5.72	3.82	66.7%	5.75	3.86	38.63
	5.78	3.91	67.6%			
12:00	5.82	3.94	67.7%	5.80	3.89	38.93
	5.77	3.84	66.6%			
13:00	5.76	3.86	67.1%	5.81	3.92	39.15
	5.86	3.97	67.8%			
14:00	5.72	3.85	67.3%	5.71	3.83	38.25
	5.70	3.80	66.7%			
15:00	5.79	3.93	67.8%	5.85	3.96	39.56
	5.90	3.98	67.5%			
16:00	5.76	3.84	66.8%	5.79	3.86	38.60
	5.81	3.88	66.7%			
Inoculum[a]	3.82	2.45	64.0%	3.82	2.45	24.46
	3.82	2.45	64.0%			

[a]As in repeatability test

As in the associated repeatability test, quality control measures indicated that the test was satisfactory. Inoculum biogas production during the test was approximately 1.2 litres, corresponding to an RBP of 0.120 l biogas g^{-1} VS added, very close to the value of 0.121 l biogas g^{-1} VS added found in the parallel test using the same inoculum. The RBPs of the reference material and the digestate samples were not negative at any point in the test. The plotted RBP values for inoculum, reference material and digestate sub-samples were smooth with no obvious spikes or inconsistencies, and the test was therefore considered to be valid.

All but one of the replicates showed closely similar behaviour. Replicate 3 on the sample taken at 14:00 showed signs of possible leakage from around day 5, and was therefore ignored in the calculations.

Figure 11. RBP test results for samples taken at hourly intervals in one day

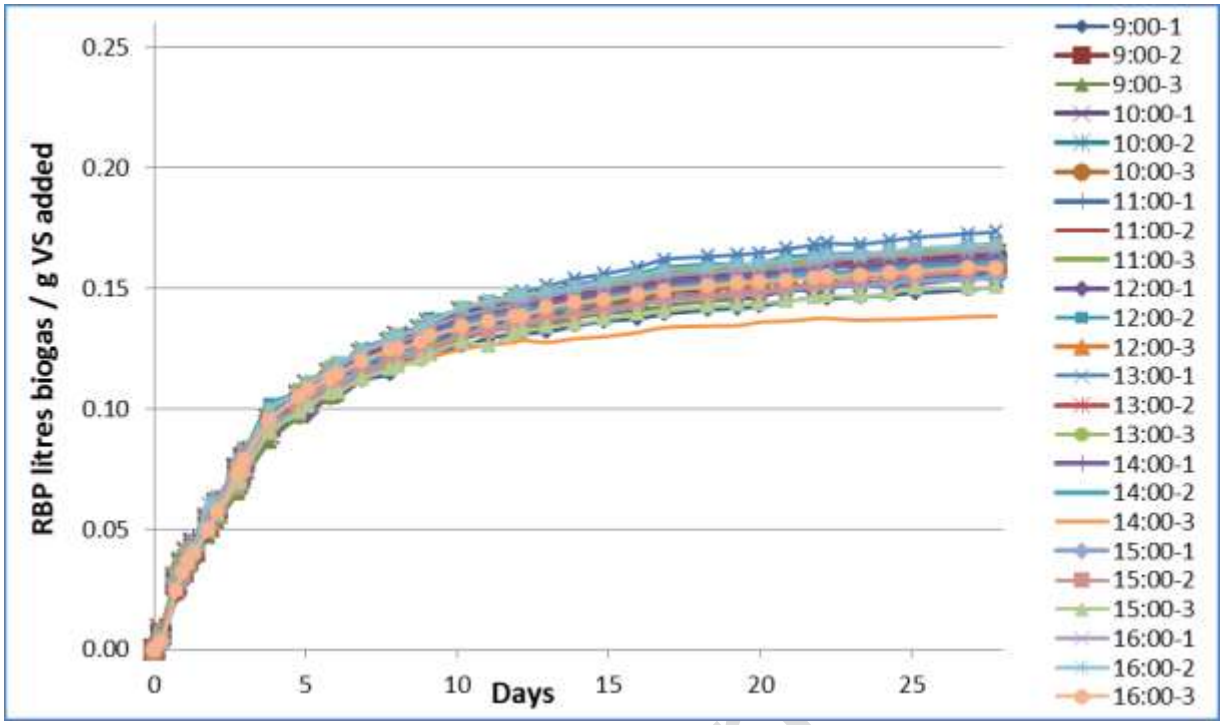
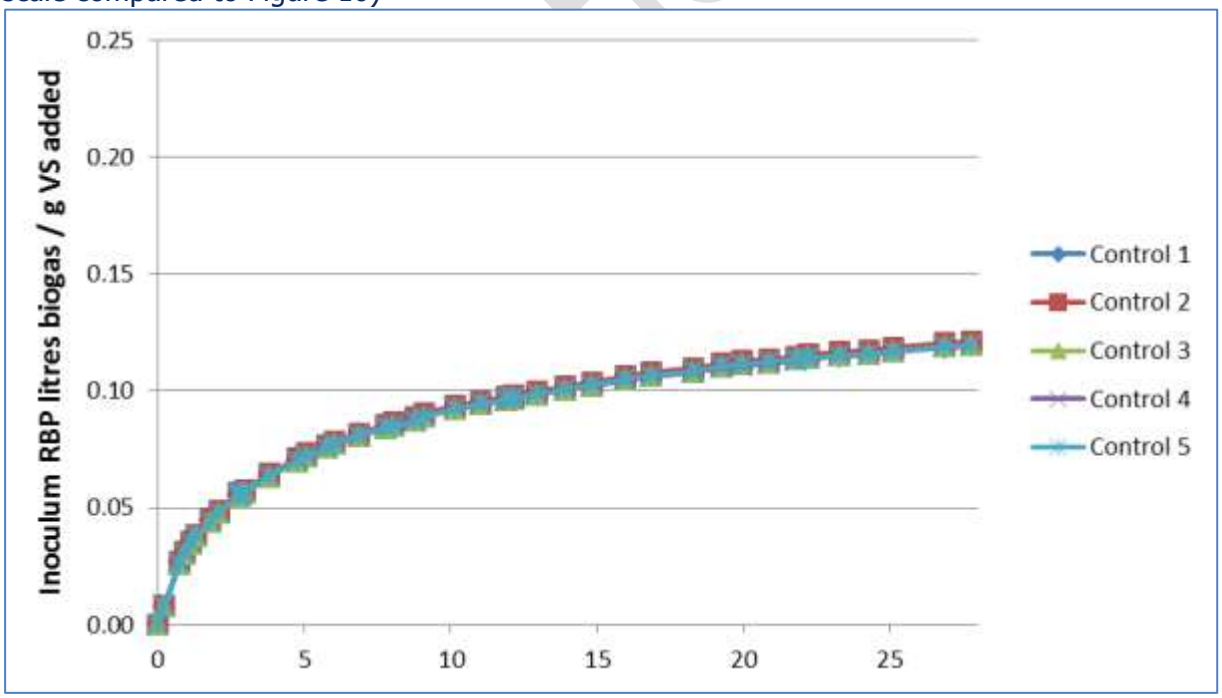


Figure 12. Inoculum RBP for samples taken at intervals in one day (note change in y axis scale compared to Figure 10)



The average RBP values for the 8 samples ranged from 0.154 to 0.167 litres biogas g^{-1} VS added, indicating little variation in digestate properties over the course of the day. The average for all samples was 0.161 l biogas g^{-1} VS added with a RSD of 3.81%. These results are closely similar to the value of 0.160 l biogas g^{-1} VS added (RSD 3.98%) for the single sample in the repeatability test, which was taken at 12:00 noon.

Table 9. Results from hourly sampling RBP

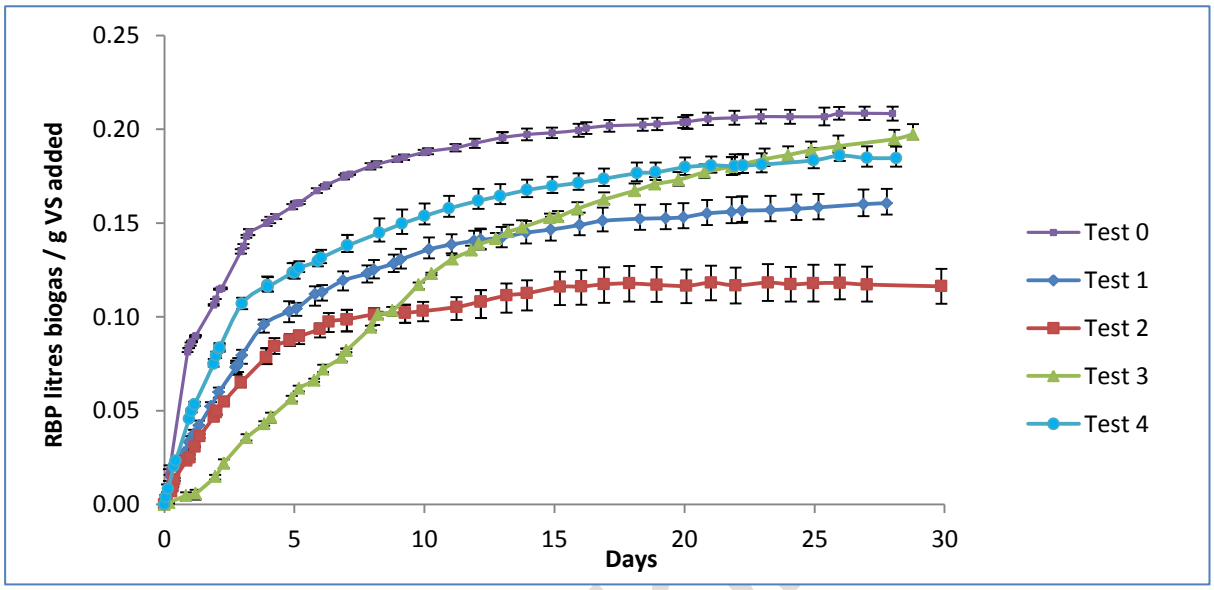
Sample	VS g kg^{-1} WW	weight of material added (g)	weight of inoculum added (g)	Gas Production (l)	Gas from inoculum (l)	VS ratio[a]	RBP value l g^{-1} VS	Ave. RBP	SD	RSD%
09:00-1	39.73	58.5	344.0	1.356	1.006	3.62	0.151	0.156	0.00	3.1%
09:00-2		58.0	343.5	1.374	1.004	3.65	0.160			
09:00-3		64.5	343.5	1.403	1.004	3.28	0.156			
10:00-1	39.48	62.0	343.5	1.406	1.004	3.43	0.164	0.162	0.00	1.5%
10:00-2		65.0	345.0	1.424	1.009	3.29	0.162			
10:00-3		60.5	342.0	1.380	1.000	3.50	0.159			
11:00-1	38.63	62.5	343.0	1.378	1.003	3.48	0.156	0.160	0.00	3.0%
11:00-2		60.5	342.0	1.386	1.000	3.58	0.165			
11:00-3		60.5	342.0	1.373	1.000	3.58	0.160			
12:00-1	38.84	59.5	342.0	1.356	1.000	3.62	0.154	0.161	0.01	4.5%
12:00-2		58.0	342.0	1.379	1.000	3.71	0.168			
12:00-3		58.5	342.5	1.363	1.001	3.69	0.159			
13:00-1	39.15	59.0	341.0	1.397	0.997	3.61	0.173	0.167	0.01	4.1%
13:00-2		61.0	341.5	1.380	0.998	3.50	0.160			
13:00-3		58.5	341.5	1.383	0.998	3.65	0.168			
14:00-1	38.25	58.0	341.5	1.359	0.998	3.77	0.163	0.162[1]	0.00[1]	0.6%[1]
14:00-2		58.0	343.5	1.362	1.004	3.79	0.161			
14:00-3		59.5	341.5	1.313	0.998	3.67	0.138			
15:00-1	39.56	58.5	342.5	1.359	1.001	3.62	0.155	0.154	0.00	2.5%
15:00-2		60.0	343.5	1.380	1.004	3.54	0.158			
15:00-3		60.5	342.0	1.360	1.000	3.50	0.150			
16:00-1	38.60	57.5	344.0	1.385	1.006	3.79	0.171	0.166	0.01	4.1%
16:00-2		58.5	344.5	1.387	1.007	3.73	0.168			
16:00-3		59.5	342.0	1.363	1.000	3.64	0.158			
All [b]								0.160	0.01	4.7%
Inoculum	24.46		400.0		1.166		0.119	0.120	0.00	0.7%
			400.5		1.185		0.121			
			400.5		1.168		0.119			
			400.5		1.167		0.119			
			401.0		1.169		0.119			

[a] Inoculum/substrate on a VS basis (should be around 4 for digestate and 6 for reference RBP tests); [b] Not including sample 14:00-3

2.2.3 Variability in samples taken over a more extended period

RBP tests were set up for samples from the selected digester on 18 March (Test 1), 2 June (Test 2), 7 July (Test 3) and 24 August 2012 (Test 4). Another RBP test on digestate from this source had also been carried out 29 February 2012, using the same equipment and source of inoculum, and results for this are included as Test 0.

Figure 13. Results for RBP tests on digestate from one source over an extended period



All five of the samples tested between 29 February and 24 August passed. The RBP values for Test 0, 1 and 2 show a steady fall in RBP (Figure 13): this may be attributable to implementation of a new trace element supplementation strategy. The result for Test 3 shows a different profile, with an increase in the final value: it is understood that substantial modifications to the site were in progress at this time which changed both the feeding regime and the digestate composition. The result from Test 4 show a return to a biogas production profile similar to those for the previous tests, with a final RBP value below that found in Test 3 and intermediate between those in Test 0 and 1. Taken together, these results again help to confirm the robustness of the RBP limit value as they indicate that a well-operated plant can comfortably meet the criterion even when undergoing significant changes in operating conditions. The improvements in RBP value seen during the implementation of process interventions further support the view that the test can provide a useful indicator of efficient process performance to the plant owner.

2.3 Alternative stability assessment methods

Two potential alternatives to the RBP protocol were investigated. One was based on the rate of acid production when methanogenesis was inhibited, and the other on an adaptation of the specific methanogenic activity test to assay the organic content of digestate material separated from the microbial cell component of the digestate. Also reported are some alternative methods of assessment of digestion efficiency, including continuous monitoring systems that could be implemented by industry on a self-monitoring basis.

2.3.1 Biochemical Acidogenic Potential test

The Biochemical Acidogenic Potential (BAP) test was first introduced in the 1990s (Lie and Welander, 1997; Ruel et al., 2002) for the wastewater treatment industry to predict the potential of wastewater to produce VFA from readily degradable organic material. As the test is relatively quick, it was considered to have some potential for adaptation and application to determining the readily degradable material present in anaerobic digestates.

In the BAP test the sample is fermented under anaerobic conditions until the VFA concentration reaches a stable maximum. Unlike the RBP test, however, methanogenesis during the BAP test is inhibited and the biochemical processes of anaerobic digestion terminate at acid production. This inhibition of methanogenesis requires pre-treatment using chemical methods such as addition of bromoethane sulfonate (BES) or pH adjustment (Mohan et al., 2006; Ruel et al., 2002). Physical methods such as freezing or heat treatment are also possible; the latter is known to eliminate or inhibit methanogens while allowing spores from the spore-forming bacteria to survive (Noike et al. 2002, Fang et al. 2006, Han and Shin 2004).

An increase in VFA concentration in digestate samples treated in this way is therefore likely to reflect the amount of biodegradable material in the sample. According to Lie and Welander (1997), in a BAP test the samples are allowed to ferment spontaneously without inoculation. This significantly simplifies the test procedure and eliminates variation in results caused by different inoculum and substrate/inoculum ratios. A further potential advantage of the BAP test is that the result can be related to RBP/COD test on the basis of an empirical formula.

A preliminary experiment was carried out to test whether heat pre-treatment could inhibit methanogenesis, but at the same time leave biological acidogenesis unaffected.

Method

The digestate sample was taken from a 75-litre mesophilic digester fed on food waste. The digester was in a stable condition and did not have any signs of process stress (i.e. no VFA accumulation).

1.5 l of the digestate was heated to 105oC in a 2-litre conical flask sealed with tin foil. 150 ml aliquots of the digestate were then measured into 200 ml Erlenmeyer flasks. To test for VFA production 1.5 g glucose (COD equivalent = 1.605 g), 1.354 g cellulose (COD equivalent = 1.605 g) and 5.3g fresh food waste (COD equivalent = 1.6 g, based on typical food waste COD of 0.3 g COD g-1 fresh matter) were added to separate flasks. The VFA production potential of these three substrates was compared to the VFA potential of the digestate itself. The controls used in the experiment consisted of digestate that had not received heat pre-treatment, and to these was added glucose (1.5 g) and cellulose (1.354 g). The headspace of all sample flasks (122 ml) was flushed with pure nitrogen (BOC, UK) and then sealed with rubber bungs fitted with 3-way valves to allow gas sampling. Each set of conditions was set up in triplicate as shown in Table 10.

Table 10. BAP experiment set-up

ID	Inoculum treated type	Substrate added	Substrate quantity (g)
1, 2 and 3	Heat pre-treated	Glucose	1.5
4, 5 and 6	Heat pre-treated	Cellulose	1.354
7, 8 and 9	Control (non-treated)	Glucose	1.5 (7), 1.354 (8 and 9)
10, 11 and 12	Control (non-treated)	Cellulose	1.354
13, 14 and 15	Heat pre-treated	Food waste	5.3
16, 17 and 18	Heat pre-treated	Digestate only	0

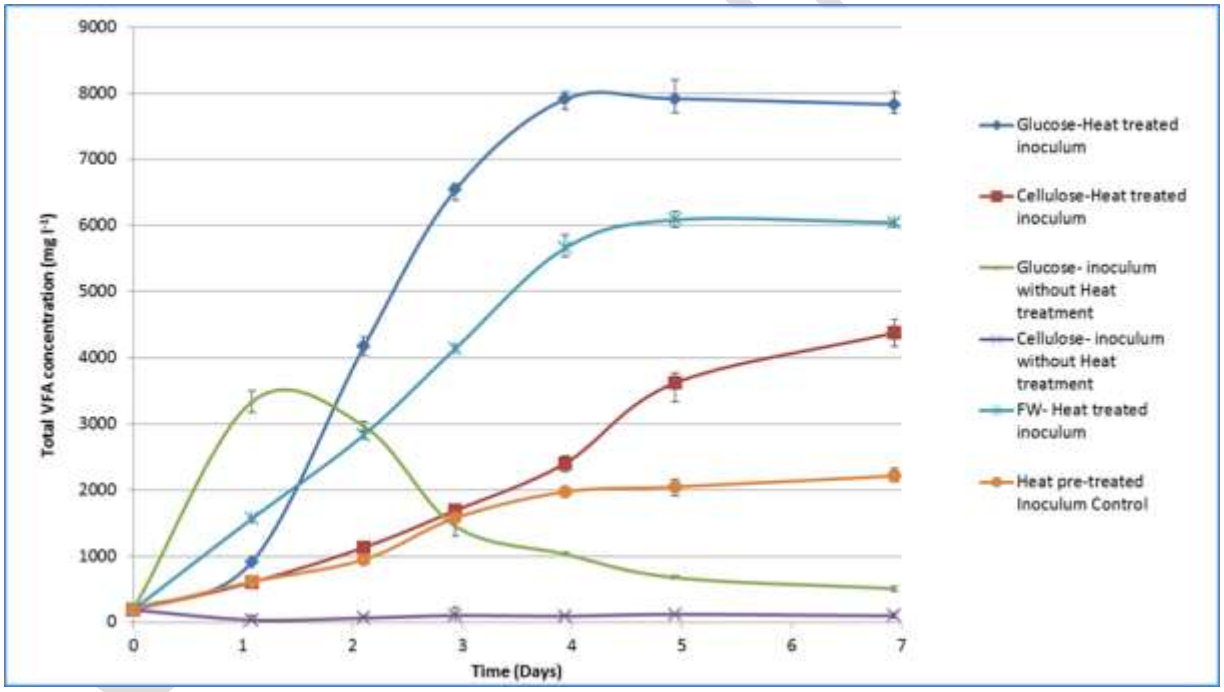
Methane production was monitored by taking 5 ml of headspace gas from each flask and analysing this by GC-TCD. Acid production was monitored by taking 1 ml of digestate in a hand pipette for analysis of VFA. This required removing the bung and on each occasion the headspace was flushed with nitrogen and then sealed again.

Results

The control flasks without heat treatment and to which glucose had been added showed a rapid initial production of VFA; this decreased after one day with a corresponding appearance of a high percentage of methane in the headspace. In the flasks to which cellulose had been added there was no increase in VFA, but detection of methane in the headspace indicated the cellulose was being broken down.

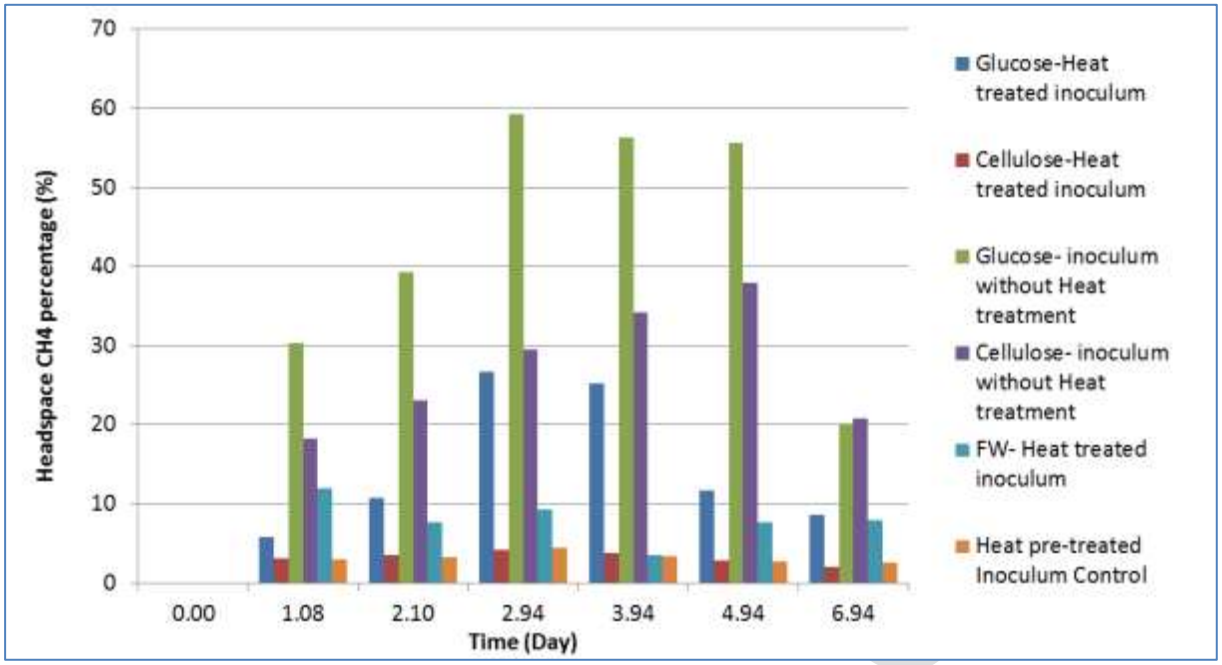
In the flasks where the digestate had been heat treated there was a mixed response, both in terms of the quantity and rate of acid production. In the flask with glucose supplementation there was a lag in acid production of almost one day and then a very rapid rate of production, accumulating 1.185 g of acid after 4 days. Where food waste was added there was no lag period and 0.89 g of acid were produced after 5 days' incubation. As expected, cellulose showed a much slower rate of acid production and this had not stabilised at the end of the 7-day incubation period; but by this time 0.59 g of acid had accumulated. The food waste digestate itself produced 0.288 g acid and appeared to stabilise after about 5 days. The rate of acid production in the cellulose and food waste digestate were almost identical in the early stages, suggesting that the food waste digestate contained very little readily degradable material but included a proportion of slowly degradable material with gas production characteristics similar to that of cellulose.

Figure 14. VFA production in methanogenesis-inhibited digestate samples with and without substrate, and in non-heat treated controls.



Methane production was observed in all of the flasks with heat treated digestate; this was, however, a small percentage of that found in the untreated controls (Figure 15). The result suggested the heat pre-treatment method needed further optimisation: it can be seen from the heat treated digestate with glucose added that methane production happened early in the fermentation and decreased later, probably as a result of acid accumulation. It is therefore possible that better inhibition of methanogenesis might be obtained by heat treatment followed by acidification of the digestate to a pH of around 6.5.

Figure 15. Headspace methane percentage



The BAP test has shown an interesting result and could be further researched to establish its sensitivity in differentiating between residual digestates, where the available substrate is likely to be very limited. The test may also pose difficulties where the digestion process has failed or is in the process of failing due to the accumulation of VFA in the digester, although in such cases a high starting value of VFA would also be recorded. Further work would be required to develop this as a test protocol, however, and even under the best circumstances the test is still likely to need a duration of several days.

2.3.2 Rapid serum bottle test

In the original research proposal it was suggested that it might be possible to develop a rapid residual biogas or methane potential test by rendering any residual substrate in the digestate more readily available, by first separating it from the microbial mass and then homogenising it to a very small particle size. The biogas potential would then be tested in a small-scale serum bottle test over a period of several hours to a few days. The concept relies upon the successful separation of undigested substrate from the digestate, and to do this a differential low speed centrifugation technique could be used. The resulting microbial cell-free extract could then be homogenised using a destruction technique e.g. by ultrasound or mechanical destruction, and the ensuing biogas production measured in the serum bottle using a gas pressure sensor. The biogas potential would be expressed on the basis of the quantity of extractable VS from the digestate being tested; and the rate of gas production might indicate how readily degradable this VS is.

Method

To assess the rate of biogas production, a preliminary test was set up using food waste as a surrogate for potentially extractable residual substrate from digestate. This preliminary experiment was also used to test the equipment, including the gas pressure measurement system. The surrogate used was source segregated domestic food waste which had been hand sorted to remove contaminants and then homogenised using a macerating grinder (S52/010 Waste Disposer, IMC Limited, UK). Two different concentrations of this food waste were prepared by diluting the homogenised material with deionised water in the ratio of 1:1 and 1:3 for high and low concentrations respectively. These prepared samples had a volatile solids content of 128.58 and 64.28 g kg-1 wet weight, respectively. After dilution the material

was further homogenised using a kitchen blender (Kenwood Limited, Havant, UK) for 5 minutes at maximum power (350 Watt).

Anaerobic digester sludge from Millbrook WWTP, Southampton was used as the inoculum. The test was carried out using crimp-top serum bottles with capacity of 119 ml.

1 g of each dilution of the food waste was added to separate serum bottles in triplicate with 19 g of inoculum. The headspace of the serum bottle was then flushed by N2¬/CO2 (80:20) (BOC, UK) before being sealed using a crimp cap with a PTFE coated septum. Three positive controls were prepared by adding 1 ml of 82 g l-1 (1 mol l-1) sodium acetate solution to each of 3 serum bottles which then received 19 g of inoculum. Inoculum-only controls were also prepared using 20 g of inoculum sludge in each of three serum bottles. The test and control bottles were incubated at 37oC with moderate agitation at ~50 rpm (Hybaid Maxi 14, Thermo Scientific, UK). The headspace pressure of each serum bottle was measured at regular time intervals using a digital absolute pressure meter (Digitron 2025P, Digitron Instrumentation Ltd, Cambridge, UK).

Pressure readings were then converted to gas volume and corrected to STP conditions using the ideal gas law: PV = nRT where P is the pressure of the gas, V is the gas volume (which in this case is the fixed headspace volume), n is the amount of gas (in moles), T is the gas temperature and R is the ideal gas constant (8.314 J•K−1•mol−1). The gas volume was also corrected to take into account any water vapour using the Goff-Gratch equation:

$$\text{Log}_{10}\, e_w = -7.90298\,(373.16/T-1) + 5.02808\,\text{Log}_{10}(373.16/T) - 1.3816\,10^{-7}\,(10^{11.344\,(1-T/373.16)} - 1) + 8.1328\,10^{-3}\,(10^{-3.49149\,(373.16/T-1)} -1) + \text{Log}_{10}(1013.246)$$

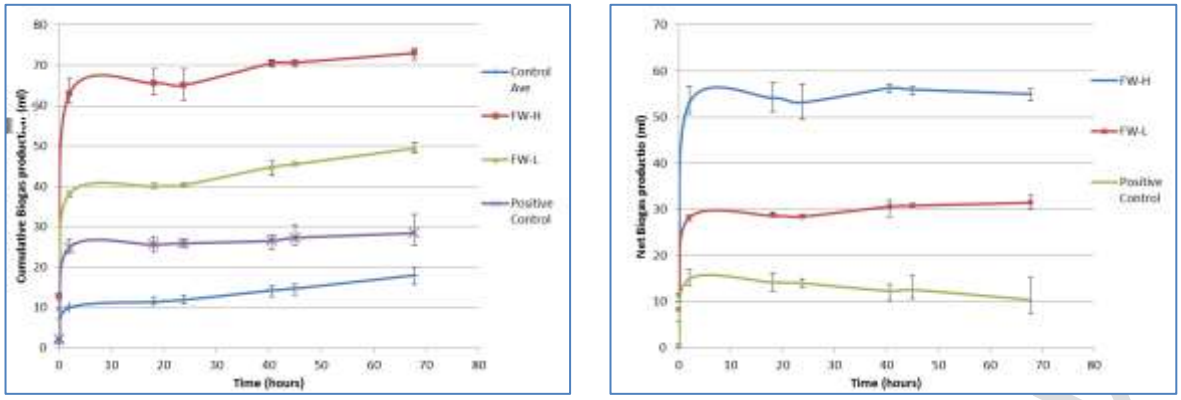
where e_w is the saturation water vapour pressure (in hPa) and T is the air temperature (in K).

Results

Both the food waste and the sodium acetate substrates showed a rapid degradation, with most of the net cumulative biogas production achieved within five hours. The weight of food waste added to each test series was of 0.128 and 0.064 g for the high and low concentration tests respectively. This difference in weights produced a differential biogas yield, with the food waste at high concentration giving 56.3 ml of gas compared to 30.6 ml for the low concentration. The gas production per g of VS added was almost equal at 0.440 and 0.478 l g 1 VS. The slightly lower specific biogas in the high concentration sample was possibly due to the higher pressure generated in the headspace altering the gas solubility, and to volume loss in the meter itself. The ratio of inoculum VS to food waste VS used in the tests was 3.5 and 7.0 for the high and low concentrations respectively.

The test results are shown in Figure 16a as the cumulative biogas production and in Figure 16b as the net cumulative biogas production

Figure 16. Biogas production kinetic of food waste and sodium acetate in serum bottle test



a) Cumulative biogas production b) Net biogas production

The trial showed the potential of the serum bottle test to differentiate between the quantity of degradable substrate in the sample. The surrogate substrate used and the positive control were, however, both readily degradable and the rate of response for extracted residual substrates could be considerably lower. For this reason a further trial was carried out using digestates of known high and low RBP value.

2.3.3 Comparative study of ADP11 digestate (High RBP value) and ADP17 digestate (Low RBP value) using the rapid serum bottle test

Samples

Digestates from ADP11 and ADP17 were selected as these had respectively the highest (0.367 l g-1 VS) and lowest (0.087 l g-1 VS) RBP values of those tested.

Residual substrate separation from digestate

A low speed centrifugation method (Lindahl and Bakken, 1995) was used to separate feedstock residue particles from microbial biomass, based on the differential rates of sedimentation of the two fractions (Fægri et al., 1977). To achieve this 25 ml samples were centrifuged at 1000 x g for 15 minutes in 50 ml centrifuge tubes using a refrigerated centrifuge at 4 oC (Thermo Scientific Heraeus X1R Refrigerated Centrifuge). The supernatant fraction which contained the microbial biomass and any soluble substrate was weighed then discarded. 20 g of pelleted material was then ultrasonically homogenised (W-225, Heat System-Ultrasonics Inc., Farmingdale, New York, USA) at an output frequency of 20 kHz using 20% power for 5 x 1 minute periods. The sample tube was maintained in an ice bath and 30-second rest intervals were given after each 1-minute sonication period. A sample of pelleted material was also tested without homogenisation.

Respirometric test setup

The test was carried out in 119 ml serum bottles and monitored in the same way as the preliminary test described above. The set-up of the experiment is shown in Table 11.

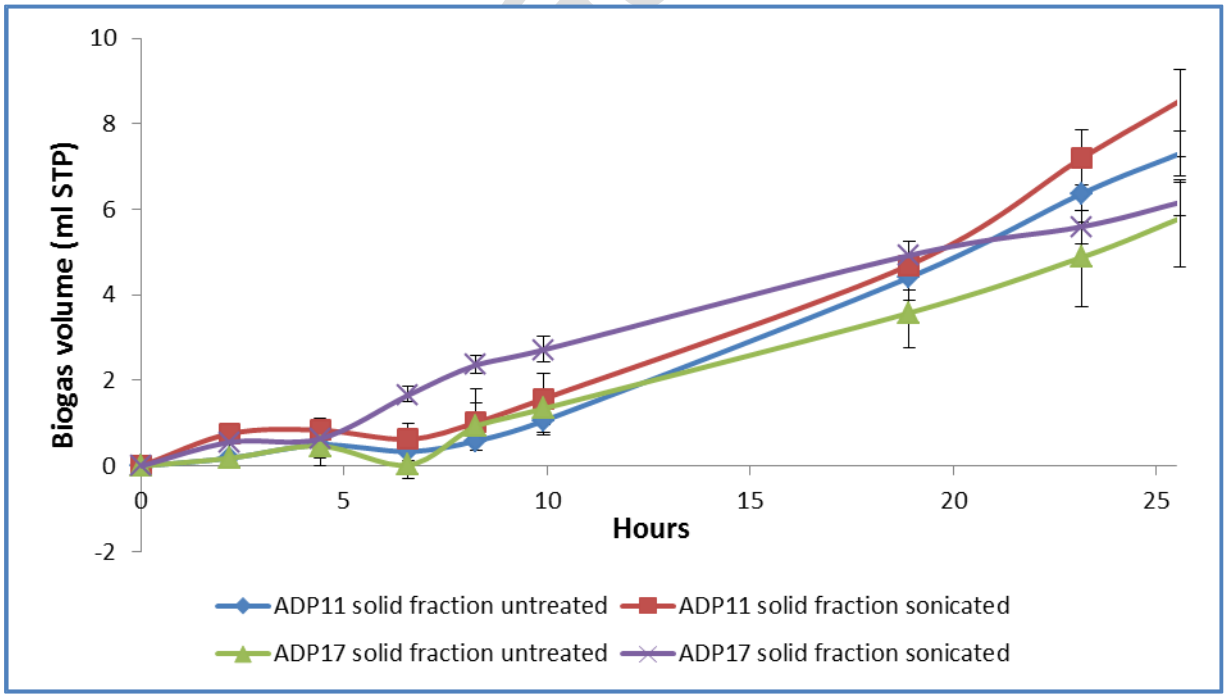
Table 11. Experiment set-up for the comparison study of ADP11 and ADP17 digestate

Sample ID	Sample weight (g)	Inoculum weight (g)	Inoculum VS (g)	Sample VS (g)
ADP11 solid fraction untreated control 1	4.027	16.015	0.379	0.612
ADP11 solid fraction untreated control 2	4.012	16.022	0.380	0.610
ADP11 solid fraction untreated control 3	4.005	16.102	0.382	0.609
ADP11 solid fraction sonicated 1	4.017	16.033	0.380	0.611
ADP11 solid fraction sonicated 2	4.021	16.041	0.380	0.611
ADP11 solid fraction sonicated 3	4.001	16.025	0.380	0.608
ADP17 solid fraction untreated control 1	4.024	16.018	0.380	0.415
ADP17 solid fraction untreated control 2	4.024	16.052	0.380	0.415
ADP17 solid fraction untreated control 3	4.017	16.027	0.380	0.414
ADP17 solid fraction sonicated 1	4.015	16.031	0.380	0.414
ADP17 solid fraction sonicated 2	4.022	16.11	0.382	0.415
ADP17 solid fraction sonicated 3	4.031	16.025	0.380	0.415
Inoculum control 1	0	20.014	0.474	
Inoculum control 2	0	20.005	0.474	
Inoculum control 3	0	20.201	0.479	

Results

The biogas production in ml (as determined from corrected pressure readings) was plotted against time after subtracting the inoculum-only control value to give a net cumulative value over a 24-hour period. The graph of the average values with error bars showing the range is given in Figure 17.

Figure 17. Cumulative biogas volume over 25 hour period for treated and untreated samples



Two approaches are commonly applied to the interpretation of respirometric data of this type: either using a cumulative value over a set period or using a maximum rate within a defined period. From the shape of the graph in Figure 17 it appears that there is a period of lag or acclimation for the first ~8 hours, after which there is a period when the rate of gas production is approximately uniform for each sample up to ~25 hours. Although the data are

not shown, gas production after 48 hours reduced in both samples. As the aim of the experiment was to see if a rapid test is possible, data were taken for the period between 10-25 hours. It might be assumed that a similar pattern will be seen for other digestates, as this profile is fairly typical of the RBP test, with the maximum rate of biogas production often seen in the period between 12-48 hours. The slope of the line represents the maximum gas production rate in response to the substrate added at the start of the test. This substrate was derived by centrifugation of whole digestate, and therefore the rate of gas production needs to be interpreted in terms of the VS content of the original sample. To achieve this, the following calculation is used:

- The maximum net biogas production rate (MNBPR) is calculated from the slope of the biogas production curve between 10-25 hours.
- The ratio of the amount of centrifuged pellet used in the test to the total amount of centrifuged pellet is calculated on a wet weight basis (the pellet ratio).
- The maximum net rate of substrate-related biogas production from the original digester VS is calculated from the MNBPR divided by the pellet ratio and by the quantity of VS (g) in the original digestate sample. This is referred to as the Residual Substrate-Induced Production Rate (RSPR). In the example given the average RSPR for ADP11 was 0.555 ml $hour^{-1}$ g^{-1} digestate VS for the untreated sample and 0.518 ml $hour^{-1}$ g^{-1} digestate VS for the sonicated sample. The equivalent values for the digestate from ADP17 were 0.341 and 0.336 ml $hour^{-1}$ g^{-1} digestate VS, respectively.

The procedure tested was a very preliminary attempt to see if it was possible to quantify how the initial (maximum) rates of gas production varied between samples of known high and low RBP value. The results obtained suggest that the method may have some potential, although refinement and extensive verification with a wide range of samples would be required. It is thought, however, that the test could be simplified by making use of typical automatic pressure monitoring equipment such as oxitop bottles (WTW, Weilheim, Germany), and that the rather complicated step of measuring the VS of the centrifuged pellet could be omitted as it is not essential for the calculation described.

Table 12. Results for rapid anaerobic respiration test

	Raw VS g VS kg^{-1} WW	Sample g WW	Sample VS g VS	Pellet g WW	Used g WW	Pellet ratio	MNBPR ml $hour^{-1}$		RSPR ml $hour^{-1}$ g^{-1} VS of digestate		
										Ave	RSD%
ADP11	34.5	25.4	0.88	5.2	4.03	0.769	0.44	0.57	0.648		
			0.88		4.01	0.767	0.37	0.48	0.551		
			0.88		4.01	0.765	0.31	0.41	0.466	0.555	16.4%
			0.88		4.02	0.767	0.41	0.53	0.607		
			0.88		4.02	0.768	0.28	0.37	0.422		
			0.88		4.00	0.764	0.35	0.46	0.524	0.518	17.9%
ADP17	43.6	25.5	1.11	6.1	4.02	0.657	0.23	0.34	0.310		
			1.11		4.02	0.657	0.26	0.39	0.349		
			1.11		4.02	0.656	0.26	0.40	0.363	0.341	8.1%
			1.11		4.02	0.655	0.23	0.35	0.311		
			1.11		4.02	0.656	0.25	0.37	0.336		
			1.11		4.03	0.658	0.26	0.40	0.362	0.336	7.6%

2.3.4 Continuous monitoring and other rapid methods

Alternative methods of assessment of digestion efficiency, including continuous monitoring systems that could be implemented on a self-monitoring basis, were also considered.

The tests carried out in the current work indicated that there was very little useful correlation between normal digestion plant static analytical tests and the RBP value, with the best correlation from a combination of VFA and total solids accounting for only around 65% of the RBP (see section 2.1.3). Approaches based on the use of these parameters were therefore rejected.

Continuous monitoring systems could also be used as a method of assessment of digestion efficiency. These could be based either on the consumption of substrate (input waste) or on the production of breakdown product (biogas or methane). The anaerobic digestion process is potentially able to convert a high proportion of the BMP, as demonstrated in long retention energy crop digesters and also in cases involving co-digestion e.g. of animal manures and energy crops. Where, for example, 99% of the BMP is removed from a typical waste with a methane potential of 100 m3 CH4 tonne-1 wet weight, the residual biogas potential would be around 2 m3 tonne-1 wet weight of digestate (assuming 50% CH4 : 50% CO2). According to the correlation graph produced by the Environment Agency (2005), for a digestate VS content of around 7% this is equivalent to a DR4 value of ~11000 mg O2 kg-1 VS, corresponding to a well-stabilised compost.

For a commercial anaerobic digester treating waste, a target for the proportion of BMP converted to methane in the heated mixed digestion tank itself might be around 85%, with the residual methane potentially collectable from post-digestion storage tanks. This value could be used as a minimum for the digester alone, with a higher value if anaerobic post-digestion storage is utilised. This approach involves knowing the BMP value of the typical input material, the tonnage applied and the amount of biogas or methane produced. A mass balance approach of this type can show the long-term performance of the plant and, if proper records are kept, should be able to account for over 90% of the mass on a wet weight basis (Banks et al., 2011).

The biogas produced can also be related to the amount of carbon converted, and if the carbon content and weight of the input materials are known this can be expressed as a percentage efficiency. A simpler method is to consider the weight reduction in volatile solids based on the typical VS content of the input materials and of the digestate; with high solids substrates it may be useful to assess this on a mass balance basis. VS destruction is very dependent on the type of input material, however, and it would therefore be necessary to relate this to the nature of the organic carbon using techniques such as fibre analysis or NIR spectroscopy.

It may also be worth re-considering the use of aerobic respiration measurements, as the literature review (see Appendix 2) confirmed that these can show a good correlation with the RBP or BMP of the digestate. The Specific Oxygen Uptake Rate (SOUR) test appears to offer most potential as it is rapid and easy to perform, and is carried out on liquid suspensions making it suitable for both 'wet' and 'dry' digestion processes as well as separated liquors and fibres. Further work would be needed to confirm the relationship, particularly in the low-level ranges for RBP and aerobic respiration rate.

2.4 Measurement of microbial communities in AD plants by DNA Sequencing

The efficiency of digestion is likely to be intrinsically linked to the structure of microbial communities within the digester: for example the stability of communities is known to be related to methanogenic potential (Werner et al., 2011), while populations of specific methanogens can emerge that are adapted to particular feedstocks (Banks and Thwaites,

unpublished data). Well-acclimatised, stable reactors processing food waste may contain populations of methanogens with higher tolerance to ammonia than those from poor-performing plants (Banks et al., 2012).

Detailed characterisation of complex microbial communities, such as those occurring in AD reactors, is now possible using next-generation DNA sequencing methods. This technology can very rapidly generate several hundred thousand DNA sequences which allow an in silico reconstruction of microbial communities in a sample. The results provide a characterisation of the digesters sampled in the current study, including those that do and do not meet current RBP standards, with statistical analysis of the relationships between microbial community structure and others digestate parameters. They also provide a baseline measurement of anaerobic communities in a wide range of AD processes, which could potentially be used in future to support the development of rapid test kits for monitoring changes in digester performance associated with community structure and biochemical functions. The results of the study carried out on this aspect of the work are presented in Appendix 3.

2.5 Literature review

An extensive review of English and German-language sources was carried out to establish the scientific rationale and basis for the adoption of a VFA standard under the proposed EU End-of-Waste criteria for biowastes. This survey extended beyond the issue of digestate stability to include the use of VFA as an indicator of more general environmental impacts including soil quality. The results of the review are presented in Appendix 2.

3.0 Discussion

3.1 RBP test analyses

From the comparative study of 24 AD plants in section 2.1 it is clear that the majority of samples taken directly from the end of the digestion process, without subsequent storage were able to comply with the current limit value of 0.25 l biogas g-1 VS added. The three samples which failed by a large margin all had high VFA concentrations on a wet weight basis, in one case close to the PAS110 screening value of 0.43 g COD g-1 VS. The indication is that the process at each of these three plants is suffering some instability, and complete and efficient conversion of methanogenic substrates is not occurring.

One sample showed strong inhibition in the early stages of the RBP test, but gave a measured RBP value below the test limit. This type of behaviour has been seen in previous tests (UoS unpublished data), and may indicate that during the period of inhibition substrate is converted to a mixed pool of metabolic intermediates, some of which may not have the potential for further conversion to biogas. After a period of time positive biogas production resumes as methanogenic substrates are gradually converted to a point where acid inhibition is removed. The non-metabolisable products remain, however, resulting in a loss of biogas production that is never recovered. For this reason it is important to keep both the VFA pre-screening and the 5-day limit on negative biogas production in the RBP test, although the latter might be better reduced to 4 days.

Three further samples were marginally over the 28-day RBP limit. None of these samples had a very high VFA concentration and all showed a biogas production profile indicating a relatively high proportion of slowly degradable material in the digestate, possibly reflecting the type of substrates received. One of the samples had a slightly raised VFA concentration of 1.4 g VFA l-1, and this could have contributed around 0.09 l biogas g-1 VS to the measured RBP value. This VFA concentration represents some process inefficiency and could potentially be reduced by appropriate interventions at the plant. If the results for all three samples were considered on the basis of a 10-day RBP test with a limit value of 0.2 l biogas g-1 VS two would have passed due to the proportion of biogas produced during the final stages of the test; the third sample would have failed. In all three cases it is likely that this type of digestate would benefit from post-digestion storage with secondary biogas collection to improve the overall process efficiency and biogas yield.

The results indicated that provided the quality assurance procedures in the RBP test protocol are followed, the agreement between triplicates is generally good and the kinetic behaviour of the sub-samples is also similar, providing confidence in the reproducibility of the test result. Both the multiple replicate test and the anonymised data from RBP tests carried out at the Open University suggest a RSD% value of about 4-6% is achievable for a well-conducted test on a typical digestate. The RBP test protocol, however, does not contain any instructions on the treatment of outlier results due to equipment failures (e.g. leakage) and it is suggested that a minor amendment is added to deal with this point. The original value of 0.25 l biogas g-1 VS appears to be realistic and achievable for the 28-day test. On the basis of the current data set this could be reduced to a limit value of 0.20 l biogas g-1 VS for a 10-day test period without a major effect on the outcome: one plant went from being a pass on the 28-day test to a marginal fail on the 10-day test, however, while two went from fail to pass. Whatever limit value is applied, some results may be only marginally above or below it. Where plants are operating above or close to the current limit value, however, it appears likely that there is potential for improvement of their performance.

The multiple replicate tests (section 2.2) confirmed the repeatability of the test results for multiple sub-samples and samples taken from the same digester over short periods. The results of sampling over a longer period were interesting: while all five of the samples tested

between 29 February and 24 August passed, they did show some changes in the RBP value: in this case an improvement with time for the first 3 samples followed by a change associated with site operating conditions, then a recovery to former performance. It appears that the RBP test can provide a useful indication of small changes in digestate properties that may reflect changes in operating status. It is also possible, however, that deterioration in digestate quality could occur over a similar or shorter time period, and therefore the interval between testing may need to be reviewed. The current frequency of sampling does not allow consideration of rolling averages or percentile values to be used for assessment of compliance.

As there was no opportunity to visit the participating plants, and therefore to make an assessment of the most suitable sampling point, all operators were asked to provide samples directly from the main digester to provide consistency in the survey. It is known, however, that some of these sites have post-digestion storage tanks, and the RBP of samples taken from the outlet of these may be lower than from the main digester, if biogas production continues during the storage period. The original PAS110 document does not specify the sampling point, but it may be sensible for this to be from the outlet of the main digester or from any post-digestion storage tank from which biogas is recovered, as this represents the end of the active process: since the RBP standard is intended to assess process performance, this is the most appropriate end point. On the same basis it is suggested that the RBP test should be applied to whole digestates only, rather than to separated fractions, as separation is not itself a stabilisation process. It is not appropriate to use the RBP test to determine the likely degree of environmental impact of the materials when applied to land, as soil processes are predominantly aerobic and the oxygen demand exerted by the material is likely to be more relevant: the respirometric tests currently used for assessing the stability of composts will provide this information. Similarly the RBP test does not appear to be a logical choice for assessing the performance of an aerobic digester, as the primary metabolic routes to stabilisation are aerobic in such systems.

3.2 Alternative assessment methods

From this initial survey it is clear that setting a stability value based on a VFA concentration of 0.43 g COD g-1 VS would not give the same result as the 28-day RBP test; and more importantly would not guarantee that digestates with a high content of degradable but unhydrolysed organic material would fail. It is probably for this reason that the German RAL recommendation for a VFA standard includes a minimum retention period within the digester, as in this case hydrolysis and acidification will have taken place even under unfavourable conditions, and the VFA content simply indicates ineffective conversion of these intermediates to biogas. Review of the available data from both the current work and in the literature review (Appendix 2) showed there was no useful correlation between RBP and VFA. The only firm conclusion that could be drawn was that digestates with a high VFA content will almost certainly fail an RBP test, but samples with a lower concentration will not necessarily pass. A VFA value used in conjunction with the requirement for a minimum retention time could, however, be an acceptable method of demonstrating that an effective digestion process has occurred.

In the work on investigation of a more rapid anaerobic testing procedure for biological stability of digestates two concepts were explored: the Biochemical Acidogenic Potential (BAP) test, and the Rapid Serum Bottle test. The disadvantages of the BAP test were that complete inhibition of the methanogenic population was difficult to achieve, and that reaction rates were still fairly slow. The BAP test is also slightly more difficult to interpret compared to biogas production in the RBP test, as the latter gives a direct measurement of the conversion of substrate originally present in the digestate by the simple technique of measuring gas volume, whereas the former relies on analysis of VFA. The problem of effective inhibition of methanogenesis could probably be resolved, but any interventions that alter the microbial community may potentially alter the test outcome. Because of the greater difficulty in

performing the test and the relatively long duration, the BAP test does not seem to offer major advantages over the RBP test, particularly if a 10-day version of the RBP is introduced. The Rapid Serum Bottle technique was able to distinguish between samples with high and low concentrations of undigested food waste within a period of hours rather than days, using more compact test equipment than the RBP or BAP tests. The test would require extensive development and cross-correlation, however, and does not give a definitive measurement of the actual residual biogas potential of the material, which is a useful parameter in its own right for determining the overall process efficiency. The Rapid Serum Bottle test is also more complex in terms of equipment and technique. One of the major advantages of the current RBP test was that it was specifically developed to allow technical personnel at AD plants to carry out their own in-house checks, using only simple low-cost equipment and facilities.

The tests carried out in the current work indicated that there was very little useful correlation between normal digestion plant static analytical tests and the RBP value. Other methods based on continuous monitoring could be used, as discussed in section 4, but have the disadvantage that this type of data cannot be presented as a single limit value in a standard or specification. They have the advantage, however, that they provide a continuous history of plant performance and a baseline against which any variations can be readily observed. They also ensure that proper records are kept of critical control parameters within the process, and are available for inspection and approval at whatever interval is required. Although VFA concentration is specifically mentioned in the German RAL quality standard for digestate it is clear from extensive examination of the related literature that the purpose of its inclusion in the standard is to demonstrate that the digestion process itself is stable: this is accompanied by a further condition of a minimum retention time in the digester. The use of VFA as an indicator of process stability was first recommended by McCarty et al. (1961); no literature could be found that specifically relates the VFA concentration to product stability. The current review is careful to distinguish between process stability as indicated by VFA concentration, and product stability which cannot be determined using this parameter alone. Further description and discussion of other methods for assessing digestates, including the role of VFA as a monitoring parameter, are given in Appendix 2.

4.0 Conclusions

The RBP test is a satisfactory method for demonstrating that an effective digestion process has taken place, and the test procedure gave reproducible and repeatable results. The results of the work carried out indicated that the current RBP test was repeatable and reliable. The current value of 0.25 l biogas g-1 VS appears appropriate and achievable. The majority of samples provided by digester operators were able to meet this standard. For those that failed, in most cases there were clear indications of process instability which almost certainly contributed directly to this failure, and which could potentially be addressed by remedial measures at the plant. Some digestates were close to the RBP limit because they appeared to contain a proportion of slow-degrading organic material: this could be effectively addressed by considering the sampling point and/or system design, including the provision of post-digestion secondary storage with gas collection. It therefore appears that the test is fulfilling its primary purpose, and that the sampling point should be specified as the outlet of the final tank from which biogas is collected for processing rather than simply vented.

There is no other obvious anaerobic biochemical assay that would be simpler and more rapid than the current RBP test, although the duration of the current test could probably be reduced to 10 days with a corresponding reduction in the limit value to 0.20 l biogas g-1 VS. The interval between testing may need to be reviewed, however, in view of the rapidity with which changes may occur in a digester's operational status. It is also recommended that the maximum period in which the net biogas production can remain negative is reduced from the current 5 days to 4.

An extensive literature review indicated that there may be some potential for aerobic respiration tests, as typically applied to compost materials. The small number of comparative studies carried out has indicated good correlation between biogas potential tests and respirometric tests on digestates. Thermogravimetric analysis and near-infrared spectroscopy could also be considered; but the work on these two techniques is limited and substantial verification would need to be carried out. Both techniques also involve quite complex and expensive apparatus and it is unlikely that they could routinely be carried out by the plant operators. Preliminary work on a new rapid anaerobic test showed some promising results but would also require extensive test development. The measurement of VFA is not an adequate means of assessing the degradation of input material, and the value of this parameter is in indicating the stability of the process rather than the product.

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Appendix 1 Text of letter to plant operators

Dear XXXX

PAS 110 Digestate Stability Test

As part of the PAS 110 review WRAP has asked us to review the digestate stability test and to resolve some of the questions raised about this at the WRAP workshops held earlier this year.

It is likely that you will have received some details of this work from the REA, AFOR or ADOWG (or even from all three), so we will not take your time repeating this information.

We would like to test as many digestates as possible from operating UK digesters. The work will be done anonymously, but we will give feedback to the sample providers on the RBP test results and on any other digestate parameters measured (solids content, volatile fatty acids etc).

If you are willing to supply a sample, please simply return this letter to us in the freepost envelope provided: if you need to change the address or other details just write them on this letter. We will then send you a sample bottle with a postage-paid jiffy bag, and ask you to send it back to us on a particular day. All the samples can then be tested together against the same controls and using the same inoculum.

We hope you will be able to support this, and in return we may be able to give you some additional insight into how your digester is performing.

Best regards

Charles Banks
Professor of Environmental Biotechnology
Tel 02380 594650 cjb@soton.ac.uk

Appendix 2 Literature Review

This review concerns approaches to the determination of digestate stability. It also considers the scientific basis and rationale behind the use of an organic acid standard, as currently specified in Germany.

A2.1 Biological stability

A definition was proposed by Lasaridi and Stentiford (1998), the basis of which is that biological stability is determined by the extent to which readily biodegradable organic matter has decomposed. As the degradation process occurs gradually, in order to establish stability it is necessary to identify a target point on a recognised scale of values. Stable biomass is characterised by being rich in recalcitrant organic molecules, resulting in a reduced level of metabolic activity as shown by oxygen uptake under aerobic conditions or methane production under anaerobic conditions. These parameters, together with a number of others, have been suggested as a means of quantifying the stability of organic waste. The inter-relationships between parameters and the scientific justification for their use are considered in the review, and, where appropriate, they are compared.

Stability is not considered from the viewpoint of the environmental impact that a bioprocessed product may have on the soil, except briefly in relation to the use of a volatile fatty acid (VFA) or 'organic acid' standard.

A2.2 Biogas and methane potential testing

A variety of biogas/methane potential tests have been developed over the years and these have generally been reported as being good at describing organic matter degradability.

The first tests in the 1960-70s were developed by McCarty and his team at Stanford University and used a modified Warburg respirometer (McCarty et al., 1963). By the mid-1970s this rather complicated approach had given way to the first dedicated biochemical methane potential (BMP) tests using serum bottle techniques (Owen et al., 1979). In these early small-scale procedures microbiological nutrient supplements were often included and special precautions taken to ensure complete exclusion of oxygen in setting up the tests. The serum bottle test approach continues to be used, but in many cases with further simplifications. Adani et al. (2001), Schievano et al. (2008) and Tambone (2010) all used serum bottle methods for residual biogas testing.

A typical test uses a serum bottle of small volume (100 or 250 ml) to which is added a pulverised dried sample (Ø < 1 mm) of the test material and a wet methanogenic inoculum, sometimes with deionized water added to balance the solids of the substrate to that of the inoculum. Control blanks are set up and bottles are flushed with N2, sealed with hermetic caps, and incubated at a specified temperature (often 37 or 55oC), until no further biogas production is detected. Gas is measured by pressure release into a syringe or by measuring the pressure inside the bottle before releasing it. The technique has some limitations because of the small biogas volumes produced and also concerns over the loss of volatile carbon during the sample drying stage (Banks and Zhang, 2010).

The same principle is used in the procedure developed by Hansen et al. (2004) except a much larger volume is used (2000 ml bottle), the sample is not dried, and positive cellulose controls are included as well as control blanks; all gas volumes are corrected to STP, and the flush gas contains 20% CO2 to buffer initial pH changes. Attention to such details has been followed through by the establishment of an International Working Group with the task of defining the nature and conditions of a methane potential test (Rozzi and Remigi, 2004; Angelidaki et al., 2008). This work is still in progress and at present no internationally agreed standard

procedure exists. It is there therefore likely that there will be variations in the results reported, even for apparently the same materials (Raposo et al., 2011a). A thorough review of the different methods being used has recently been reported by Raposo et al. (2011b).

The test procedure developed for the measurement of the residual biogas potential in the UK (Walker, 2010) took into account the recommendations of the international working group as far as possible, but within the remit of having a test that could be set up and run without specialist equipment or a high level of operator training.

A2.3 Residual methane or biogas potential (RBP and RMP) inter-relationships

General parameters

The relationships between biogas (or methane) potential, digestibility, and chemical parameters of both fresh substrates and digested materials have been explored by a number of authors. These are reviewed here only where part of the work specifically relates to the residual methane or residual biogas potential of digestate (RMP or RBP)

Schievano et al. (2008) analysed digestate samples taken over an eight-month period from 4 digesters and 1 post-digestion tank at a co-digestion plant in Italy receiving a mixed waste input. The samples were tested for biogas potential as well as a range of biological and chemical parameters which included: oxygen demand in a 20-hour respirometric test (OD_{20}), total solids (TS), volatile solids (VS), total organic carbon (TOC), total Kjeldahl nitrogen (TKN), ammonia, cell solubles (CS), acid detergent fibre (ADF), lignin (ADL), cellulose, and hemicellulose.

Results from a regression analysis showed that the closest relationship to biogas potential was with volatile solids (VS), with a regression coefficient of 0.806. This suggested to the authors that plant operators could easily obtain a quick assessment of the biogas potential of both substrate mixtures and digestate slurries by measuring the VS, and doing this would help them monitor the AD process by estimating degradation and biogas yields. It was also suggested, however, that there were risks to using such a simple relationship and for this reason a multiple linear regression was carried out on the data. This indicated that using VS and respiration rate (OD_{20}) together as independent variables gave a significant increase in the regression coefficient (see below: *respiration rate*).

Respiration rate

Respiration rate is commonly applied for the assessment of stability of compost material. A number of techniques have been used to determine this, based on the uptake of oxygen or the production of CO_2. Common methods include: the specific oxygen uptake rate (SOUR), the 20-hour oxygen demand (OD_{20}) and the more complicated 4-day dynamic respiration rate as used in the UK PAS100 specification. There are also variations on these based on the method of measurement, time of test, and test matrix. Some of these are mentioned below: for more detailed information on the tests referred to it is necessary to consult the original cited work. Only those studies in which respirometric tests have been compared to methane (or biogas) potential tests are reported. There is a much more extensive literature simply on the use of respiratory tests to assess stability, but this is beyond the scope of the current review.

The earliest comparison of the two techniques is that of Muller et al. (1998) in work carried out to establish landfill acceptance criteria and support the formulation of the German regulations that specify this. The study used a respiration activity test based on CO_2 production over a 96-hour period and an anaerobic digestion test carried out over 21 days at 35°C with dried ground samples and a methanogenic inoculum. Results from the two tests were compared to the biodegradable organic matter (OM_{bio}), with correlation coefficients (R^2) of 0.9 and 0.93 respectively. When the biogas and respirometric tests were compared directly the correlation coefficient was 0.77, suggesting that both methods could be used to assess the

stability of landfill input materials. The biogas potential test was thought to reflect reactions found in anaerobic landfills better than any of the other parameters which were evaluated for quantifying stability. The authors, however, in conjunction with the Bund der Ingenieure fir Wasserwirtschaft, Abfallwirtschaft und Kulturbau **(**BWK-l) working group on residual MSW finally considered respiration activity measurements as the most appropriate parameter for defining stability of mechanically biologically processed MSW. This led to the recommendation of 5 mg O_2 g^{-1} TS as a landfill acceptance criterion. Based on the correlation graph and equation presented by Muller et al. (1998), this is equivalent to a biogas potential of around 0.015 l g^{-1} TS. The correlations found were regarded as reliable as the study compared a large number of waste samples derived from a number of different trials.

Cossu and Raga (2008) compared the 4-day cumulative oxygen consumption (respiratory index, AT_4) with the 21-day biogas potential on excavated samples taken from three sanitary landfills. The correlation between the results (R^2) was 0.63, 0.89 and 0.82 for the individual sites and 0.8 when the results were considered collectively. The comparison for mechanically pre-treated waste gave a R^2 value of 0.6 indicating a less reliable correlation when used on non-stabilised waste organic matter.

Schievano et al. (2009) looked at the correlation of oxygen uptake to biogas potential for different substrates. These were first dried and then tested for a range of parameters including oxygen demand (20 hours) and biogas potential using a serum bottle method (Schievano et al., 2008). The results showed a significant linear regression between these two parameters (R^2 = 0.73). This was a better correlation than that found in earlier work (Schievano et al., 2008) which had also included digestates within the samples analysed. The correlation between biogas potential and other parameters was not as good and it was concluded that the only parameter able to predict biogas potential indirectly was the oxygen demand. The resolution of the correlation could be improved by using a linear regression approach in which volatile solids (VS) concentration was considered alongside oxygen demand (OD_{20}). This allowed more accurate prediction of the biogas potential (BP) which could be estimated using the equation BP = 26.15 $OD_{20}^{0.5}$ + 1.38 VS - 997.59. Using this relationship the regression coefficient was 0.88. Later, Scaglia et al. (2010), working in the same group, used the dynamic respiration index (DRI) to estimate the biogas production of biologically treated municipal solid waste and showed that this gave similar results to the cumulative oxygen uptake (AT_4); they also proposed a model allowing results from the two techniques to be mutually transformed.

Tambone et al. (2009) looked at the biological stability of samples from a co-digestion plant using the specific oxygen uptake rate (SOUR test) and the biogas potential using a serum bottle method (Schievano et al., 2008). The authors concluded that the measurement of the cumulative oxygen uptake rate provided results similar to the biogas potential test but in a much shorter time (2 days compared to 60 days) and could therefore be considered a fast and useful test to measure the biological stability of digestate. They also correlated the results to spectroscopic measurements using ^{13}C CPMAS NMR analysis which can distinguish 5 types of carbon in the NMR spectrum: this confirmed that there had been degradation of carbohydrate-like molecules and an increase in more recalcitrant long chain aliphatic-C. A further study (Orzi et al., 2010) used the same stability data sets and correlated these against the odour potential of the samples in which the volatile components were quantified by gas chromatography mass spectrometry (GC-MS) and an electronic nose (EN).

Barrena et al. (2009) looked at the stability of mechanical-biological recovered (MBR) materials. The correlation between biogas potential (both at 21 days and ultimate), the 24-hour specific respiration index (SRI_{24}), and the maximum and 24-hour dynamic respiration indices (DRImax and DRI_{24}) was significant. A higher correlation coefficient, however, was found between biogas potential and the 4-day cumulative oxygen consumption (AT_4). The

authors concluded that anaerobic tests could be used to predict the stage of biodegradation of organic matter in solid waste samples even if the process was aerobic. They also suggested this gave support to the view that the biodegradation of organic matter is not affected by the redox state, as both aerobic and anaerobic indices showed an identical evolution profile. Similar results were found by Ponsa et al. (2008) for other MBT plant configurations which included anaerobic digestion processes.

Trzcinski and Stuckey (2011) looked at the stability of digestate from a 2-stage laboratory-scale digestion process using standard respirometric techniques. When the solids retention time (SRT) in the 1st stage hydrolysis reactor was >70 days the respiration rate was low, and increased when the SRT was reduced to 20 days. It was suggested that the VFA concentration in the hydrolysis reactor contributed to the difference in respiration rate and could explain the differences between the different SRTs. The residual biogas potential was in the range 0.1-0.16 l g^{-1} VS when the SRT was greater than 70 days and increased past the threshold of 0.25 l g^{-1} VS established for the UK PAS110 (BSI, 2010) as the SRT was reduced to 20 days. The authors also compared the residual methane potential (RMP) with the ultimate biodegradability of the feedstock and found that this matched relatively well with the VS removal in the digester.

Wallace et al. (2011) found a good relationship between BOD and RBP values for 8 digestates: the R^2 correlation coefficient was 0.8 after removal of one very high BOD outlier from the data set. The authors stated, however, that the BOD test is an aerobic biological assay which uses diluted, but unfiltered, sample liquids and this tends to reflect all forms of carbon present in the original samples. It is thus possible for the test to produce very different results from the anaerobic RBP test, depending on the digestate composition. They stated that digestates containing high levels of ligno-cellulose material, which is resistant to anaerobic decomposition, may produce low RBP values but high BOD values. This conclusion led them to recommend further investigation before the BOD test could be adopted for testing the stability of anaerobic digestates.

A2.4 Alternative methods to assess stability of digestates and composts

Thermal analysis techniques

On the basis of the loss of energy undergone by materials during biological stabilisation, Otero et al. (2002) proposed thermal analysis (thermo-gravimetric analysis (TGA) and differential thermal analysis (DTA)) as a technique for evaluating the degree of stabilisation of biosolids. The use of these techniques was justified as there is mineralisation and conversion of organic matter into humic substances during biological stabilisation processes. As a result there is a reduction in the energy available for microbial metabolism as the stabilisation process takes place. Using thermal techniques Otero (2002) developed an ignition index which gives information on the combustible organic fraction and the energy released. Otero et al. (2002) then used the technique to assess the stability of wastewater biosolids, and Pietro and Paola (2004) proposed its use to monitor the composting process when applied to the organic fraction of municipal solid wastes (OFMSW) and vegetable wastes. In essence, interpretation of the results from the DTA technique looks at the decrease in weight loss at temperatures below 200°C in the treated sample (which indicates a reduction in biodegradable organic matter due to treatment) and the displacement of the weight loss region to a temperature above 500°C (which indicates complex 'humic like' organic matter typical of the residual materials remaining after biodegradation).

Gomez et al. (2005) compared the profiles of wastewater primary solids, the OFMSW and a mixture of these two substrates with digestate using thermal analysis techniques. They concluded that there were clear differences in the profiles of the individual substrates and also between them and their respective digestates. As a simple expression of stability the findings could be summarised by reference to two criteria. Firstly, for non-stabilised substrates 50% of

the original mass was lost below 400°C whereas for stabilised digestate less than 50% of the mass was lost at 450°C. Secondly, in all digestate samples the DTA signal showed energy release at high temperatures (around 500°C) which was not present in the original substrates. Gomez's group has gone on to use the technique to assess the stability of a number of digestates and to investigate the digestion process further by combining thermal analysis with mass spectrometry (Gomez et al., 2007a), Fourier transform infrared spectroscopy (FTIR) (Cuetos et al., 2010), and nuclear magnetic resonance (NMR) (Gomez et al. 2007b and Gomez et al., 2011). A recent study by Ali et al. (2012) also looked at DTA with FTIR to assess changes in compost stability.

As well as reporting weight loss differences in the DTA profiles they also considered that FTIR spectroscopy was a promising technique, as new peaks could be seen in composted material compared to the original substrate. The intensity of these new peaks in the FTIR spectra could indicate the stability and maturation time of composted material. Provenzano et al. (2010) tested a wide range of waste materials and showed that the FTIR spectra were related to their chemical composition, and were distinct from the spectra of the digestates derived from them, although these inherited the main spectroscopic features of the original waste. In their work, however, they did not demonstrate any way in which the spectra could be used to quantify stability.

Near-infrared spectroscopy

Lesteur et al (2011) carried out the first comprehensive study in which near infrared (NIR) spectroscopy has been used to predict the Biochemical Methane Potential value of municipal solid waste. NIR has been used successfully in the past for compositional analysis on a range of products and it has also been used in making estimates of the digestibility of forage feedstocks, it is therefore a good candidate method for the rapid screening of materials for their biogas or methane potential. To do this it is necessary to calibrate the technique and produce a predictive model through which the spectra can be interpreted in terms of BMP. To do this they used a calibration set of 51 samples with a BMP range from 23 - 400 ml CH_4 g^{-1} VS and then tested and validated the model with a further 51 samples; these ranged from MSW to individual components of paper, cards, foodstuffs and green waste.

The spectral analysis included data pre-treatment and the use of a truncated spectrum (1668–2500 nm) which helped improve the robustness of the model. The results obtained were very good with a correlation coefficient (R^2) of 0.76 over the range of BMP values. The technique may not, however, be sensitive enough to distinguish with accuracy the residual methane potential of digestates as the standard error of prediction was around 28 ml CH_4 $g^{-}1$ VS., this resolution may be better with a less heterogeneous range of material. Lesteur's study has shown the usefulness of this technique and developing a model more suited to the RBP range rather than the BMP range, and calibrating this with a representative number of samples may be worthwhile as once done the technique is rapid and reliable.

Changes in organic matter content (VS)

Muller et al. (1998) observed that biological activity diminished at OM_{bio} levels between 20 and 25% dry matter (TS). They concluded, however, that it would be wrong to deduce generally from this that an OM_{bio} content of 20-25% TS ensures that biological activity is minimised. This is only true where the organic matter content has been degraded by more than 60% and is subsequently stabilised. It was not possible to make any statement on the stability of the processed MSW based on its OM_{bio} content alone, without knowing the OM_{bio} content of the original material. The stability can be assessed if the OM_{bio} of the unprocessed MSW is known and used as reference: in this way the level of degradation is known and can be used in subsequent calculations.

An alternative review to the one presented here, in which rapid methods to determine biochemical methane potential are discussed is that of Lesteur (2010). The range of methods covered is larger as the focus is on BMP and not RMP, it is however relevant, well written and includes critical appraisal of the techniques considered. In addition to the techniques considered here they also include two destructive methods: pyrolysis and the Advanced Oxidation Process. Both of these can provide data that is used either to predict the chemical composition of the organic matter, which is subsequently used to predict the BMP, or to predict the BMP directly by regression.

A2.5 The use of organic acids or volatile fatty acids in assessing stability

One of the purposes of the current review is to look at the use of organic acids, and more specifically VFA, as an indicator of digestate stability. This arises from use of this criterion in the German RAL GZ 245 and 246, which are voluntary standards of the Bundesgütegemeinschaft Kompost (http://www.kompost.de) that monitors and controls most German composting sites and a large number of digesters (Personal Communication Arthur Wellinger, 27/07/12). Participating digestate producers undergo periodic testing according to these RAL standards. In 2010 the limiting value for organic acids in these standards was reduced from the previous value of 4500 mg l^{-1} to 1500 mg l^{-1}. At the time of the change the committee responsible for the RAL standard published the following statement: "The parameter 'organic acids' is a suitable indicator of degradability or stability of digestate products. The hitherto analysis method is kept".

Dr Andreas Kirsch (http://www.kompost.de/index.php?id=160&L=0%3Cbr%2F%3E) of Bundesgütegemeinschaft Kompost gave the following e-mail response (13/08/2012) to an enquiry from Dr Ludwig Gredmaier (Southampton University) when asked about the reasons for introducing the 1500 mg l^{-1} limit.

German	**English translation**
Sehr geehrter Herr die Festlegung von Vorgaben für die Gütesicherung erfolgt über unseren Bundesgüteausschuss, in dem Wissenschaftler, Behörden und Betreiber vertreten sind. Der Ausschuss hat sich bereits mehrfach mit den Vorgaben zum Vergärungsgrad befasst. Mit dem Vergärungsgrad soll die Abbaustabilität der abgegebenen Gärprodukte beschrieben werden. Dies ist hinsichtlich des Geruchs und von Klimagasemissionen bei der Aufbringung der Gärprodukte relevant.	Dear Sir Quality control is done through our Federal Quality Committee, which is made up of scientists, government bodies and plant operators. The Committee has discussed the degree of digestion on several occasions. Degree of digestion is meant to be an indicator of degradability of the digestate. This is important in terms of both odour and climate gas emissions.
Die Senkung des Grenzwertes für Organische Säuren auf 1.500 mg/l Essigsäureäquivalent erfolgte, da der bisherige Grenzwert von Bundesgüteausschuss als zu hoch angesehen wurde. Von Seiten der Betreiber im Güteausschuss wurde 1.000 mg/l als Maximalgehalt vorgeschlagen. Der Grenzwert wurde dann unter Berücksichtigung von Messtoleranzen auf 1.500 mg/l festgelegt. Die Einhaltung dieses Grenzwertes wurde als gute fachliche Praxis gesehen. Eine gesonderte wissenschaftlich erarbeitete Grundlage ist daher nicht vorhanden.	Lowering the limiting values for organic acids to 1500 mg/l propionic acid equivalent was introduced because the original value was considered too high. Plant operators proposed to have 1000 mg/l as the maximum value. Due to measuring tolerances the value chosen was 1500 mg/l. This is a value that has evolved from practice and it was not based on scientific research.

Uns ist bewusst, dass es bezüglich der Bewertung des Vergärungsgrades noch zahlreiche offene Fragen gibt. Zur nächsten Sitzung wird daher der Umgang mit dem Parameter "Vergärungsgrad" nochmals auf die Tagesordnung gesetzt. Gibt es Ihrerseits weitere Erkenntnisse hierzu??	We agree that there are a number of open questions concerning digestate stability (degree of digestion). In our next Committee meeting we will have this on the agenda again. Would you have any new scientific findings on this matter?
Für weitere Fragen stehe ich Ihnen gerne zur Verfügung. Grüße aus Köln Andreas Kirsch	Please feel free to contact me any time. Regards from Cologne Dr Andreas Kirsch

The view that VFA can be used as a measure of the 'degree of digestion' is still under debate, however, and has been disputed in Germany: In 2007, an expert meeting on dry digestion standards took place in eastern Germany (Gülzower Fachgespräche, 2007). A quote from the page 137 of the meeting notes is given below.

German	**English translation**
"Die Eignung des Faktors Essigsäureäquivalent zur Beurteilung der Restgasaktivität der vergorenen Substrate wird angezweifelt, da zwischen säureäquivalent von niedrigen Gehalten an flüchtigen Fettsäuren und dem unter x mg/l im Restgaspotenzial kein hinreichend enger Zusammenhang bestehen würde."	The suitability or fitness of VFA as an indicator of residual methane potential of digestate is to be disputed. No clear correlation exists between VFA content of x mg/l and residual methane gas potential.

An internet search in German of documents which relate to the stability of digestate or the role of VFA in the process produced the following relevant information:

A publication of the Bavarian State Research Center for Agriculture on 'Ensuring process stability in agricultural biogas plants' (LfL, 2007)

German	**English translation**
"Die am häufigsten gebildeten niederen Fettsäuren sind Essig-, Propion- und iso-Buttersäure.	The most common VFAs are acetic, propionic and iso-butyric acids.
Die Erfahrung zeigt, dass die Gesamtkonzentration an flüchtigen Fettsäuren im Fermenter unter einem Wert von 4000 mgeq.*L-1 liegen sollte. Die Konzentration der Essigsäure sollte unter 3000 mg*L-1 liegen, die der Propionsäure unter 1000 mg*L-1und die der iso-Buttersäure bei kleiner 500 mg*L-1. Das optimale Verhältnis von Essig- zu Propionsäure beträgt 2:1. Die Grenzwerte dieser und weiterer Parameter sind in Tabelle 3 zusammengestellt."	Practical experience has shown that the concentration of total VFAs in the fermenter must be below 4000 mg l^{-1}. The concentration of acetic acid should be below 3000 mg l^{-1}, propionic acid below 1000 mg l^{-1} and iso-butyric acid below 500 mg l^{-1}. The optimum ratio between acetic and propionic acid is 2:1. See Table 3 for a summary.

'Table 3' referred to in the German text above

	Main fermenter	Secondary stage fermenter
Acetic acid	<3000 mg l^{-1}	<1000 mg l^{-1}
Propionic acid	<1000 mg l^{-1}	<500 mg l^{-1}
Iso-butyric acid	<<500 mg l^{-1}	<<500 mg l^{-1}
Total VFA	<4000 mg total l^{-1}	<2000 mg total l^{-1}

A second publication from the same institute on 'Biogas plants - laboratory parameters and process control' (LfL, 2010) contains several sections on VFA content.

German	English translation
"Flüchtige Fettsäuren sind ein Zwischenprodukt im Biogasprozess. Sie wirken bei zu hoher Konzentration hemmend auf die Prozessbiologie. Durch die Analyse der einzelnen Säuren können Aussagen zum Zustand des Prozesses getroffen werden. Die Essigsäure sollte daher im niedrigen, einstelligen Grammbereich pro Liter liegen, die Konzentration der Propionsäure deutlich darunter, weitere Fettsäuren eine Zehnerpotenz niedriger. Für die Bestimmung der Fettsäuren werden sehr unterschiedliche Methoden verwendet. Es gibt Methoden, die einen Summenparameter für die Fettsäuren ermitteln ohne die einzelnen Komponenten zu bestimmen. Einzelne Fettsäuren können nur chromatographisch bestimmt werden. Die Praxis der Laboruntersuchungen zeigt jedoch, dass die chromatographisch bestimmten und hieraus berechneten Werte für ein Essigsäureäquivalent mit den Werten, die als Summenparameter direkt bestimmt werden, keine gute Übereinstimmung aufweisen. Die Bestimmung der Fettsäuren sollte daher einem guten Labor vorbehalten bleiben." (page 9)	Volatile fatty acids are an intermediate product in the biogas process. At high concentrations they inhibit the process biology. Analysis of individual acids can help in characterising the process conditions. Acetic acid content should be around 1 g per litre, and propionic acid should be much lower, other fatty acids should be lower by a factor of 10. Several methods exist for the determination of the fatty acids. One method determines the sum of the acids without individual acids. Individual acids can only be determined with gas chromatography. It has been shown in laboratory tests that the acetic acid equivalent sum does not correlate well with the sum of individual acids (from gas chromatography). The determination of volatile fatty acids should therefore only be conducted in a good laboratory. (Page 9).
"DIN 38414 beschreibt Abwasseruntersuchungen mit Distillation und Titration." (page 13)	DIN 38414 describes a method for VFA measurement by titration and distillation. (Page 13).
"Es gibt jedoch keine einheitliche Methodenvorschrift für die Probenvorbereitung und die Analyse." (page 14)	No harmonised, consistent method exists for sample preparation and analysis (of VFAs). (Page 14).

It became apparent from the internet search and by consultation with academic contacts in Germany and Austria that there is no underlying reason why organic acid or VFA should be considered as a stability parameter for digestate, other than that it is an indicator of the stability of the digestion process itself. The group working at the University of Vienna (BOKU; *www.ifa-tulln.ac.at*) responded to a request from Prof Charles Banks (e-mail of 08/03/12) for information on the reasons for the use of a VFA standard to assess digestate (odour, soil toxicity, process stability, product stability) as:

I talked to Günther Bochmann and Franz Kirchmeyr and figured out the two main reasons why the content of volatile acids should be controlled:

-) Odor emissions: In Austria it is sometimes a big problem to have biogas plants permitted. Often neighbours and people of the surrounding areas are afraid of being bothered by odor emissions (smells). This can become a major problem in permitting biogas plants.
-) Emission of green house gases: In the end the concentration of volatile fatty acids can be an indicator of the degree of degradation. The residual methane potential of digestate depends to some extent on the VFA in the digestate. If you have high residual VFA you can assume that you will also have a comparably high residual methane potential. At the moment we have an ongoing research project where we are establishing a measuring method based on "long path infrared" where emissions can be measured directly at the plant. However, direct emission measurements onsite are quite rare, however, I think there are already some available in English. If you need any special literature with this regard, please let me know. In Germany and Austria the emission of biogas plants is a point which will be important in future, if we sell biogas plants as very positive for climate change, we will have to guarantee processes where potential methane emissions are reduced/optimised. One very big issue in this regard are greenhouse gas emissions from digestate storage or land application.

Bernhard DROSG IFA-Tulln
BOKU - University of Natural Resources and Life Sciences, Vienna
Institute for Environmental Biotechnology
A-3430 Tulln, Konrad Lorenz Str. 20

The response from Prof. Bernd Linke from the Leibniz-Institut für Agrartechnik (http://www.atb-potsdam.de) was:

Dear Charles,
Please excuse that I can answer only now, but it took some time until I got some necessary information. I had contact with the Federal Association of compost quality in Germany (Dr. Reinhold). Please find attached some technical bulletins. Base of these bulletins was a monitoring of full scale biogas plants in Germany. With respect to the bulletin of VFA limit I have tried to translate it. In the last years there is no much scientific literature regarding the VFA as quality parameter. Please find attached a publication from the "Bayerische Landesanstalt für Landwirtschaft (LfL)" from 2010. The colleagues examined practice facilities to find out weaknesses for optimal operation. In chapter 5.2 (page 63) Figure 51-53 show biogas potential of digestate related to the biogas produced in the digester as function of the VFA concentration, measured in the last heated digester step. The authors stated, the VFA can be used as an indicator for the activity of degradation and hence the potential residual gas formation from the digestate. However, in my point of view the biogas potential from the digestate is influenced from other parameter, e.g. HRT or OLR in the digesters. On the base of the biogas-messprogramm (see below) I have plotted some data with respect of the VFA in the digestate, the HRT , OLR, VS and the proportion of crops in the mixture with animal slurry on VS basis (see excel sheet). However, the VFA was measured from the last step of the digester, not after 60 days storage of the digestate. It can be expected, that the VFA will decrease after some month and will comply the VFA limit according the Federal Association of compost quality in Germany.
http://mediathek.fnr.de/broschuren/bioenergie/biogas/biogas-messprogramm-ii-61-biogasanlagen-im-vergleich.html

Best regards
Bernd Linke Prof.Dr.agr.habil.Dipl.-Ing.
Leibniz-Institut für Agrartechnik Potsdam-Bornim e.V.
Abteilung Bioverfahrenstechnik

The translation referred to in Prof Linke's message is below:

New limit for Organic acids in fermented quality assured products

In the RAL quality backups fermentation product (RAL-GZ 245) and cultivated biomass fermentation product (RAL-GZ 246), the parameter "Organic acids" an integral part of control investigations of external monitoring. In the light of test results, the degree of degradation or the stability of quality assured fermentation products can be described. High levels indicate a low stability or incomplete digestion. At low levels can be expected from an extensive fermentation. In developing the quality and test 9 years ago now, at first, a limit of 4,000 mg / l fermentation product (acetic acid equivalent) was determined.

The comparatively high value was chosen because the time for this parameter still existing uncertainties with the intention to review it at a later date for its adequacy. For checking this value the Federal Quality Committee had used in the past year, a working group with representatives from the thematically relevant the field of research and practice. At its April 2009 meeting of the Federal Quality Committee has been discussing the results and decided to lower the value of 4.000 to 1.500 mg / l. The Committee has therefore followed the proposal of the working group.

The Federal Quality Committee has in this mater the following decision statements made:

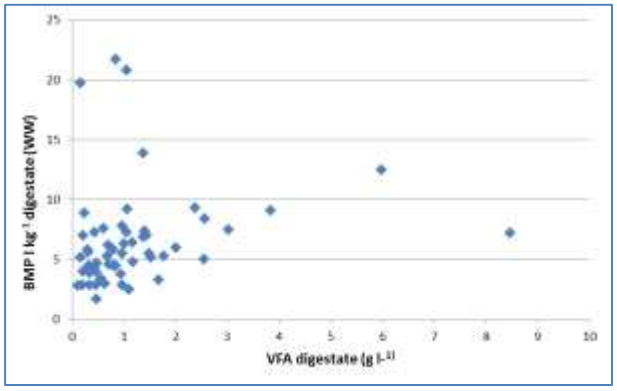
- The "Organic acids" is a useful measure to describe the degree of degradation and the stability of fermentation. The current methodology for the determination will be maintained.
- Digestate with quality label must be to a large extent digeste and stable against decomposition. This is appropriate when the organic acids in the digestate is less than 1,000 mg / l. In the RAL quality backups for fermentation product, taking into account possible fluctuations should be a limit of 1,500 mg / l .
- Operators of quality assured biogas plants, which exceed the lowered threshold should be allowed an appropriate transitional period.

The Federal Quality Association will take the necessary steps is the change in the quality and test the quality backups of fermentation product (RAL-GZ 245) and cultivated biomass fermentation product (RAL-GZ 246) and apply from 2010. Necessary adjustments should - if necessary - take place until early 2010. The operators of quality assured biogas plants have already been informed by post.

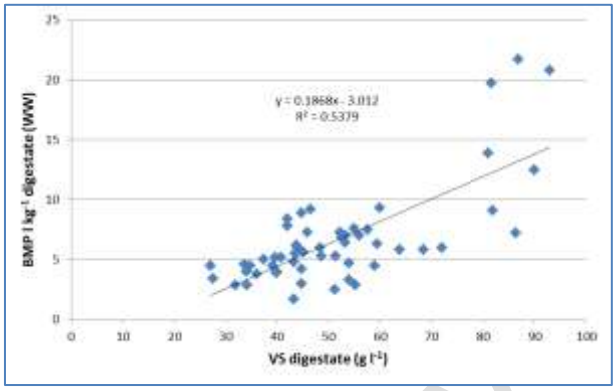
For more information: Federal Compost Quality Association, Von-der-bettors-Straße 25, 51149 Cologne, Germany Phone: 02203/35837-0, Fax: 02203/35837-12, e-mail: info@kompost.de, Internet: www.kompost.de
Source: H & K 01/09, page 14, Dr. Andreas Kirsch (BGK eV).

The data provided by Prof Linke shows the performance of 59 biogas plants in relation to VFA and biochemical methane potential (BMP) carried out at both 37°C and 22°C over a 60-day period. From the data it can be seen that 11 plants (19%) exceeded the current VFA standard, although this was without post-digestion storage, which could reduce VFA concentrations. The data showed no clear relationship between VFA and BMP (Figure A2.1a); there is a trend but not a good correlation between the BMP and VS of the digestate (Figure A2.1b). It is clear that the BMP increases with increasing load on the plant (Figure 1c), and as the increased load will lead to a decreased retention time the plants with short retention times (<50 days) tend to show a higher BMP value (Figure A2.1d).

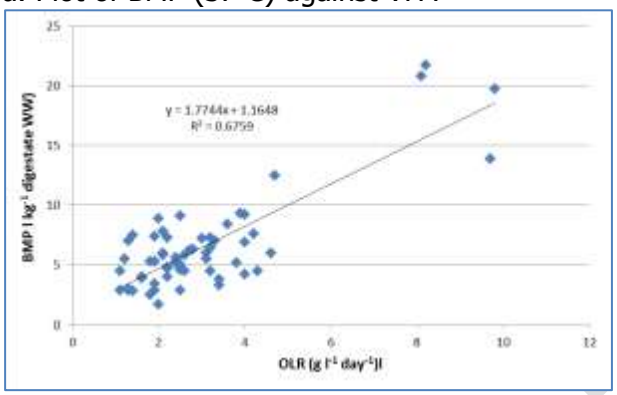
Figure A2.1. Data from the German biogas messprogramm



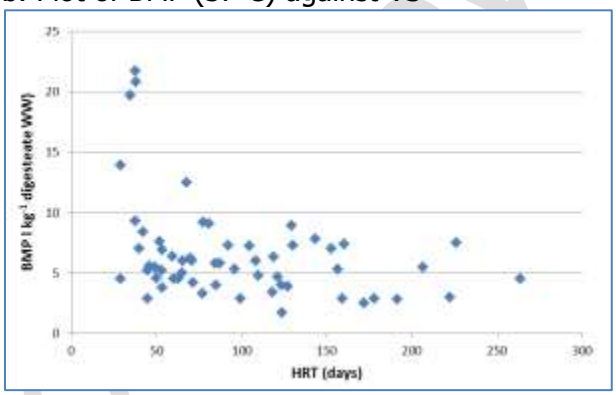
a. Plot of BMP (37°C) against VFA



b. Plot of BMP (37°C) against VS



c. Plot of BMP (3 °C) against OLR

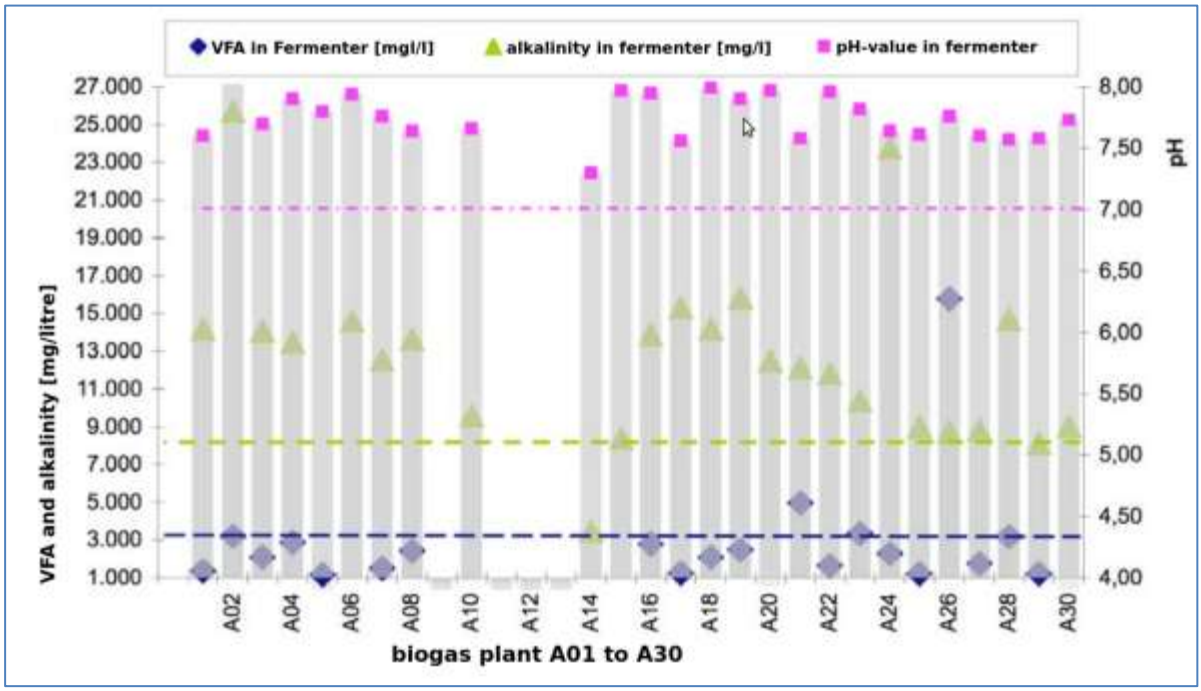


d. Plot of BMP (37°C) against HRT

Searching the German literature also produced a further dataset showing the VFA concentrations that might be expected in agricultural biogas plants. The data are taken from Biogasbericht Südtirol (2008).This study reported VFA concentrations at 30 biogas plants, with the associated text as follows:

German	English translation
Gemäß Abbildung 5 zeigt der Bestand durchwegs Werte im Normalbereich. 5 Anlagen liegen an der Schwelle der flüchtigen Fettsäuren (A02, A04, A16, A23, A28), A21 und A26 liegen deutliche darüber. Kritisch wird eine erhöhte Fettsäurekonzentration vor allem da, wo die Alkalinität unter 8.000 mg/l sinkt, da ein „Kippen" der Fermenterbiologie eintreten kann. In einem solchen Fall sinkt der ph-Wert unter 7. Keine der Anlagen war zum Zeitpunkt der Erhebung „gekippt". Gut ausgelastete Anlagen können durchaus hohe Fettsäurekonzentrationen aufweisen (FFS > 3.000mg/l), wobei jedoch eine Nachgärung sichergestellt sein sollte, um keine Energieverluste zu erleiden.	As seen in Figure 5 all VFA values are within normal range. Five biogas plants are close to the limit for VFA content (plant numbers A02, A04, A16, A23, A28), and plants A21 and A26 are clearly above it. VFA content becomes critical if alkalinity is below 8000 mg/l where the fermenter biology can 'topple over'. In these cases the pH value decreases to under 7. No plant was at risk at the time of the survey. If plants are run at high loading rate they can exhibit high VFA concentrations (> 3000 mg/l), but a 2nd stage fermenter should be installed to avoid energy loss

Figure A2.2. VFA across the 30 plants (taken from Figure 5 in Biogasbericht Südtirol (2008))



The above two datasets indicate that a VFA standard of 1500 mg l^{-1} may be difficult to achieve in practice, but the data do not take into account any post-digestion storage that may further reduce the VFA concentration. Also, the last set of data is taken from a 2008 publication and may be even older than that; since that time our knowledge of the process and its control has improved (Banks et al., 2012).

It is clear from the above literature review and correspondence that the VFA (or organic acid) concentration in the digester is not a measure of the stability of the digestate. It is, however, a long-established means of assessing the stability of the digestion process itself (McCarty et al., 1963; McCarty, 1964; Lettinga, 1995). If considered as part of a package of requirements (as in the German RAL) it might be assumed that satisfactory degradation is taking place if the process does not result in the accumulation of fermentation intermediate products and also has a stated minimum digestion period.

A2.6 Importance of VFA as a criterion, unrelated to digestate stability

The literature search revealed other more definitive reasons why VFA might be considered as a standard for digestate.

Odour. Wallace et al. (2011) considered VFA and odour in digestates but found no relationship between either the VFA or the RBP values and odour in the samples tested. Orzi et al. (2010) looked specifically at the relationship between odour emission from anaerobic digestion and other process stability parameters. Although VFA analysis was carried out VFAs were not detected in air emissions from the digestate samples used. VFA were not related to any other stability parameter in this work, nor was it suggested that the VFA concentration could be used as a measure of potential odour nuisance.

Phytotoxicity. A number of references relate organic acid concentrations in composts to plant phytotoxicity. In some cases the concentration of organic acids has also been used as a measure of the degree of 'maturity' or level of completeness of composting. The California Compost Quality Council (2001) states that some immature composts may contain high amounts of free ammonia, certain organic acids or other water-soluble compounds that can limit seed germination and root development. It is also stated that organic acids can increase

the apparent respiration rate. The document gives concentration values for VFA in mmoles g^{-1} dry weight for very mature (<200), mature (200-1000), and immature (>1000) composts. Results linking VFA to phytotoxicity have been known for many years, but the mechanism has been poorly understood. In a survey of 712 compost samples (Brinton, 1998) organic acid levels between 75 and 51,474 mg kg^{-1} TS were recorded with 15% exceeding 10,000 mg kg^{-1} TS and 2.5% exceeding 25,000 mg kg^{-1} TS, giving concern as to the potential for phytotoxicity and odour release. Further work assessed the maturity of these compost samples based on wheat and cress seedling growth in peat:compost blends (Brinton and Tränkner, 1999). Growth responses were compared to CO_2 evolution and volatile organic acid (VOA) content, and from this comparison the critical concentrations of volatile organic acids leading to phytotoxicity were estimated to be approximately 5000 mg kg^{-1} TS. Evidence of phytotoxicity from anaerobic digester residues was also found by Salminen et al. (2001) in studies using material from anaerobic digestion of slaughterhouse wastes, but critical concentrations are not given.

It is interesting to note that although the German RAL 245 and 246 specify a VFA standard for digestate, the same limit does not apply for compost: yet it is clear that high VFA concentrations can and do exist, particularly in immature composts. High VFA concentrations are also known to occur in animal slurries that are commonly applied to land and it is therefore inappropriate to consider this as an impact criterion for digestates only: the significance of VFA in digestate is simply that a low value indicates process stability.

Appendix 3 Analysis of microbial communities

A3.1 Executive Summary

During this project to evaluate the RBP test, UoS sampled digestate from anaerobic digesters across the UK treating municipal and commercial solid wastes. Availability of these samples and associated measurements provided an opportunity to assess the diversity of microflora in these digesters and assess any correlations between community structure and physico-chemical parameters. Samples were analysed at Fera using pyrosequencing to create *in silico* representations of bacterial and archaeal populations.

The efficiency of biogas plants is linked to the productivity of the microbial communities responsible for the conversion of waste to methane, a concept well-known to AD operators who acclimatise reactors (i.e. the microbial communities within them) to a waste feedstock and seek to maintain conditions conducive to the acclimatised community. There are no tools in common usage to monitor the composition of AD microbial communities, however, and despite being instrumental to a digester's efficiency, microbial data is not used to warn of failure or assist in maintaining an equilibrium. Chiefly this is due to the lack of knowledge on the composition of microbial communities: detailed data is scarce, particularly for the archaea responsible for producing methane. This study provided an opportunity to characterise microbial communities across a range of digesters and test how these correlate with physico-chemical parameters. The most striking finding was the high diversity and variation in community structure between different digesters.

Bacteria. The bacterial community consisted mainly of members belonging to the phyla Bacteroidetes, Firmicutes and Thermotogae with varying diversity levels. No correlation could be found between bacterial community and RBP values, but volatile solids were strongly correlated with bacterial phyla, favouring mainly members of the Firmicutes. Interestingly, the effect strength of certain factors was different depending on taxonomic level: ammonia was the significant factor affecting bacterial species, whilst volatile solids played a smaller role.

Methanogens. The distribution of methanogen taxa appeared very variable across the samples including the dominance of some taxa in most samples (63%) whilst a few harboured high methanogen diversity. Overall, the richness of methanogen communities ranged from 33 to 321 taxa. Of these, 19 taxa were identified as abundant with the majority of taxa related to Methanosarcinales, Methanomicrobiales and Methanobacteriales. The detection of a high number of relatives of *Methanosarcina* spp. e.g. *M. thermophila* suggests that the major methanogenesis pathway in these reactors is acetoclastic. No significant link could be detected between methanogen diversity and RBP values but there was a strong effect of alkalinity and volatile solids as well as micronutrients, in particular iron. The latter can possibly be attributed to the key role iron plays for essential enzymes in methanogenesis.

Overall, measured variables like RBP or volatile fatty acids did not explain the full variability and differences observed in community structure for either bacteria or methanogens. This finding supports the notion that too little information is available on microbial communities participating in anaerobic digestion processes to understand fully the relationships between regularly measured variables and microbial components responsible for the process itself; although it is also possible that measurements did not include physico-chemical parameters that are more closely linked to differences in microbial communities. Furthermore total communities were studied, including potentially dormant and inactive components. Future studies should also incorporate a differentiation between active and total communities to

identify if active components are more closely linked to variables like volatile solids and volatile fatty acids and thus could be better controlled.

A3.2 Methods

Samples from digesters around the UK were provided by Southampton University in June 2012 as part of the assessment of the Residual Biogas Potential (RBP) test performed on digesters intending to comply with the PAS110 digestate specification. The digestate samples were stored at -20°C upon arrival (see Table A3.1 for a full list).

DNA was extracted from 500 mg of digestate using the Power Soil extraction kit (MO BIO Laboratories, Carlsbad) according to the instructions of the manufacturer. This method included a bead-beating step, which was performed for 5 min. All DNA extracts were eluted with 60 mL of Tris buffer (10mM) and stored at -20°C until further analysis.

Microbial community analyses were performed using two different taxonomically-informative DNA sequence targets. To study bacterial communities PCR amplification of the V1-V3 region of the 16S rRNA gene (Hamady et al 2008) was carried out using the following primers (underlined) with Roche 454 pyrosequencing adaptors (in italics) and unique identifiers (NNNN, see Table A3.1):

16S_27F (5'-*CCATCTCATCCCTGCGTGTCTCCGACTCAG*NNNNAGAGTTTGATCMTGGCTCAG-3')
and 16S_519r (5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*GWATTACCGCGGCKGCTG-3').

For coverage of the methanogenic community a fragment of the methyl Co-A reductase gene (*mcrA*) common to all known methanogens (Luton *et al*., 2002) was applied using the primers (underlined):

mcrAf (5'-*CCATCTCATCCCTGCGTGTCTCCGACTCAG*NNNNGGTGGTGTMGGATTCACACARTAYG CWACAGC-3') and mcrAr (5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*TTCATTGCRTAGTTW GGRTAGTT-3')

with Roche 454 pyrosequencing adaptors (in italics) and unique identifiers (NNNN, see Table A3.1). The proof-reading polymerase Phusion (New England Biolabs) was used for the amplification of all targets. Next generation sequencing (NGS) of all amplicons was carried out using the GS FLX System (Roche). Sequence data were processed to remove low quality reads and short sequences (<250 bp). To assign taxonomy to the bacterial sequences, the Naïve Bayesian rRNA classifier of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) was used applying a bootstrap value of 80%. Sequences obtained from mcrA amplicons were clustered based on nucleotide identity of >97% similarity (Altschul et al. 2007) using uclust (Edgar 2010). Representative sequences of each cluster were aligned with *mcrA* sequences from the Genbank database and a phylogenetic tree was constructed using MEGA 4 (Tamura et al 2007) to locate each cluster in the taxonomic hierarchy.

Statistical analyses were carried out using the subroutines multidimensional scaling (MDS) and analysis of similarities (ANOSIM) of the PRIMER 5 software suite (PRIMER-E, Ltd., UK). For ordination and ANOSIM Bray–Curtis similarities based on square root transformed data were used. Additionally, hierarchical agglomerative clustering of Bray–Curtis similarities was performed using the complete linkage method of the PRIMER software. For understanding relationships between community dynamics and parameters measured like the Residual Biogas Potential, total volatile solids, alkalinity, volatile fatty acids, micronutrients and ammonium detrended correspondence analysis (DCA) was used to test whether weighted-averaging techniques or linear methods were appropriate. The CANOCO software package for Windows 4.53 (Biometris, the Netherlands) was used for this analysis. For the analysis of bacterial community profiles the longest gradients resulting from DCA were 0.998 for the analysis

based on RDP classified (phylum level) and 2.651 for the analysis of 3% distance clustered (species level) 16S rRNA gene data. The values indicated a linear relationship for the phylum level in contrast to the species level showing a tendency towards a unimodal relationship. Based on this result, bacterial community data at the phylum level were analysed using RDA (Redundancy Analysis) whereas bacterial community data at the species level were analysed using CCA (Canonical Correspondence Analysis) (Lepš & Šmilauer, 2003) to compare sample–environment correlations. Results for the methanogenic community based on mcrA sequences revealed a very long gradient in DCA indicating unimodal responses to the factors included so that CCA was used to analyse correlations in detail.

A3.3 Results

A3.3.1 Bacterial community

A total of 177,356 sequences with an average length of 380 bp were obtained from sequencing of the V1-V3 region of the 16S rRNA gene. The sequence number obtained was highly variable across the analysed samples ranging from over 19,000 to as little as 402 sequences (Table A3.1).

Table A3.1. Overview of sequences obtained from sequencing of the V1-V3 region of the 16S rRNA gene

Sample	Sequences	Sample	Sequences
ADP1	8,483	ADP13	19,432
ADP2	17,624	ADP14	9,479
ADP3	8,129	ADP15	402
ADP4	9,705	ADP16	4,680
ADP5	1,327	ADP17	8,191
ADP6	4,599	ADP18	7,333
ADP7	4,472	ADP19	12,405
ADP8	8,516	ADP20	886
ADP9	4,670	ADP21	9,249
ADP10	5,776	ADP22	8,909
ADP11	408	ADP23	5,166
ADP12	4,285	ADP24	13,230

Overall, the bacterial community consisted of members belonging mainly to the phyla Bacteroidetes (24%), Firmicutes (21%) and Thermotogae (11%) (Figure A3.1). Smaller fractions were observed for members of the phyla Actinobacteria (2%), Proteobacteria (0.8%), Synergistetes (0.56%), Chloroflexi (0.3%), Spirochaetes (0.06%), Acidobacteria (0.03%), Armatimonadetes (0.02%) and SR1 (0.02%). All samples contained members of the Bacteroidetes, Firmicutes and Thermotogae, but respective contributions were highly variable. Furthermore, a high contribution by Actinobacteria could be found in sample ADP 4, whereas most other samples contained lower levels. Ordination and clustering were used to illustrate differences between bacterial communities (classified sequences only) on the phylum level (Figure A3.2). Based on 70% similarity the following samples formed four separate groups: group 1: ADP 4 and 16; group 2: ADP 19, 18, 3, 24 and 1; group 3: ADP 10, 17, 6, 12, 22, 8, 14, 2, 9, 13; group 4: ADP 7, 21, 23, 5, 15, 20, 11.

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Figure A3.1. Overview of the bacterial community structure, phylum level

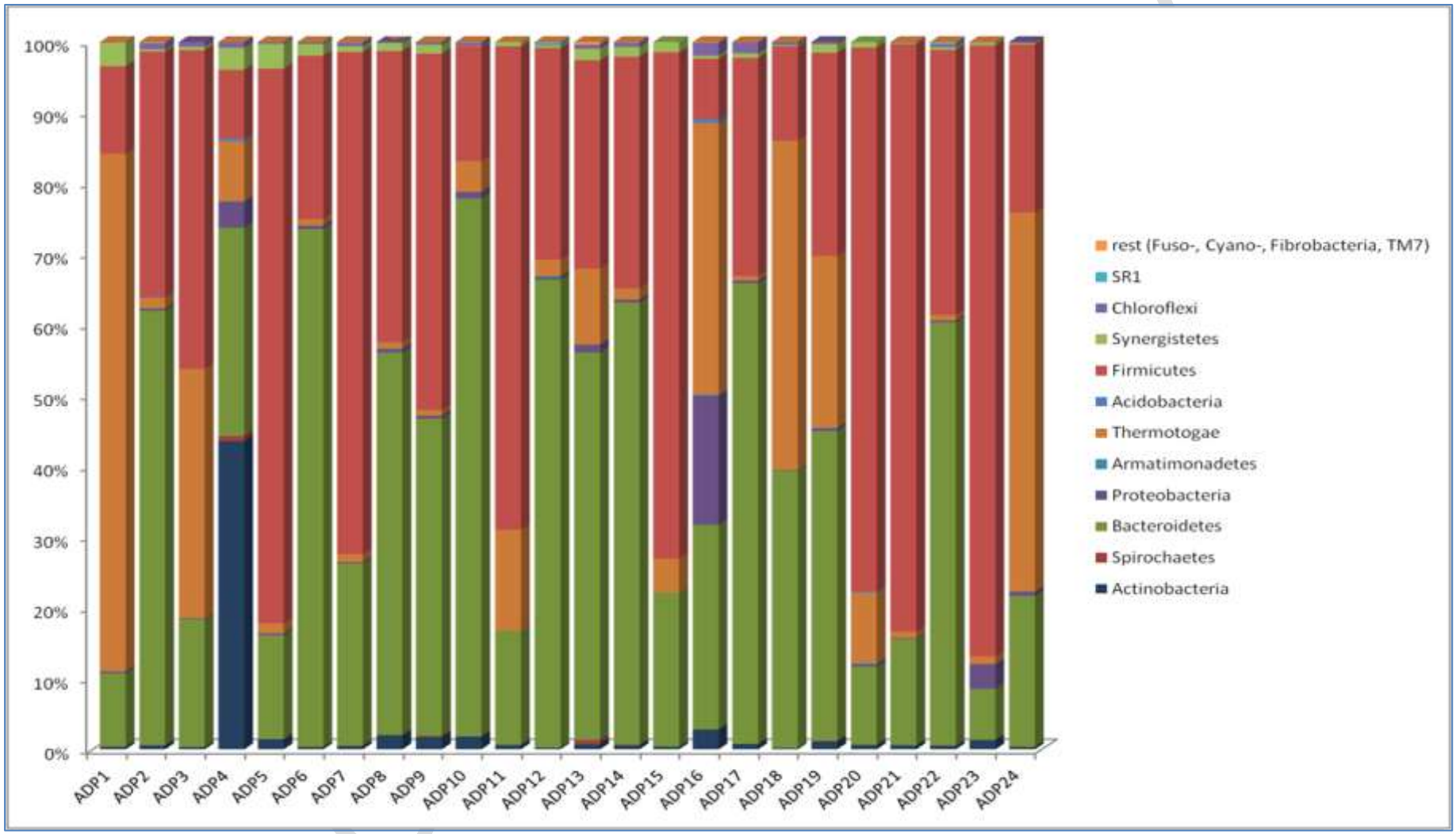
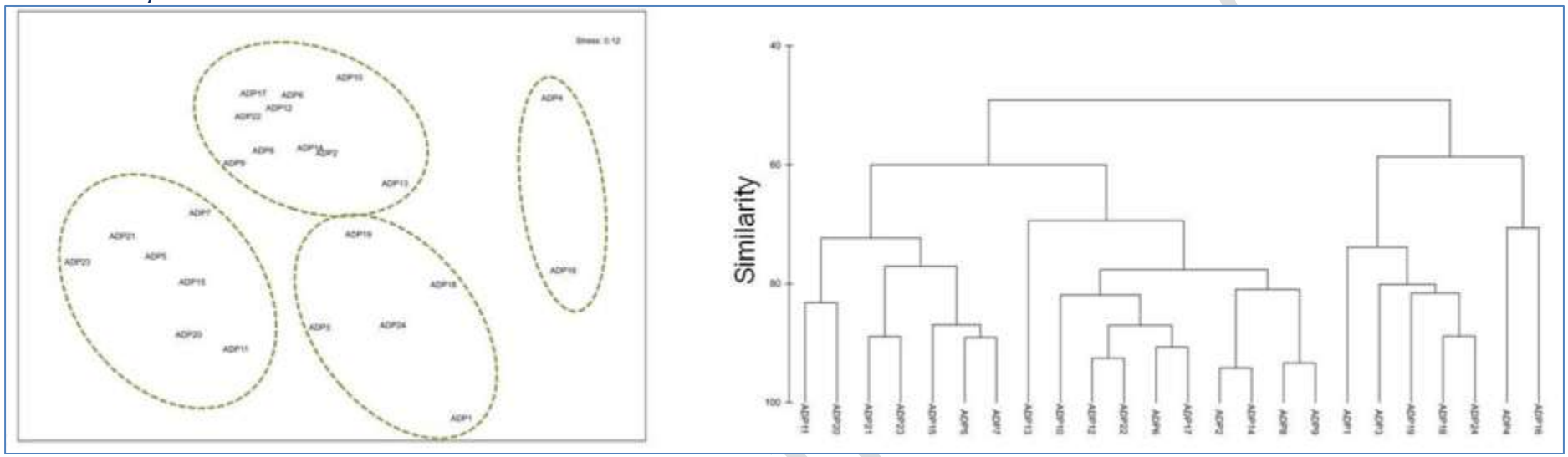
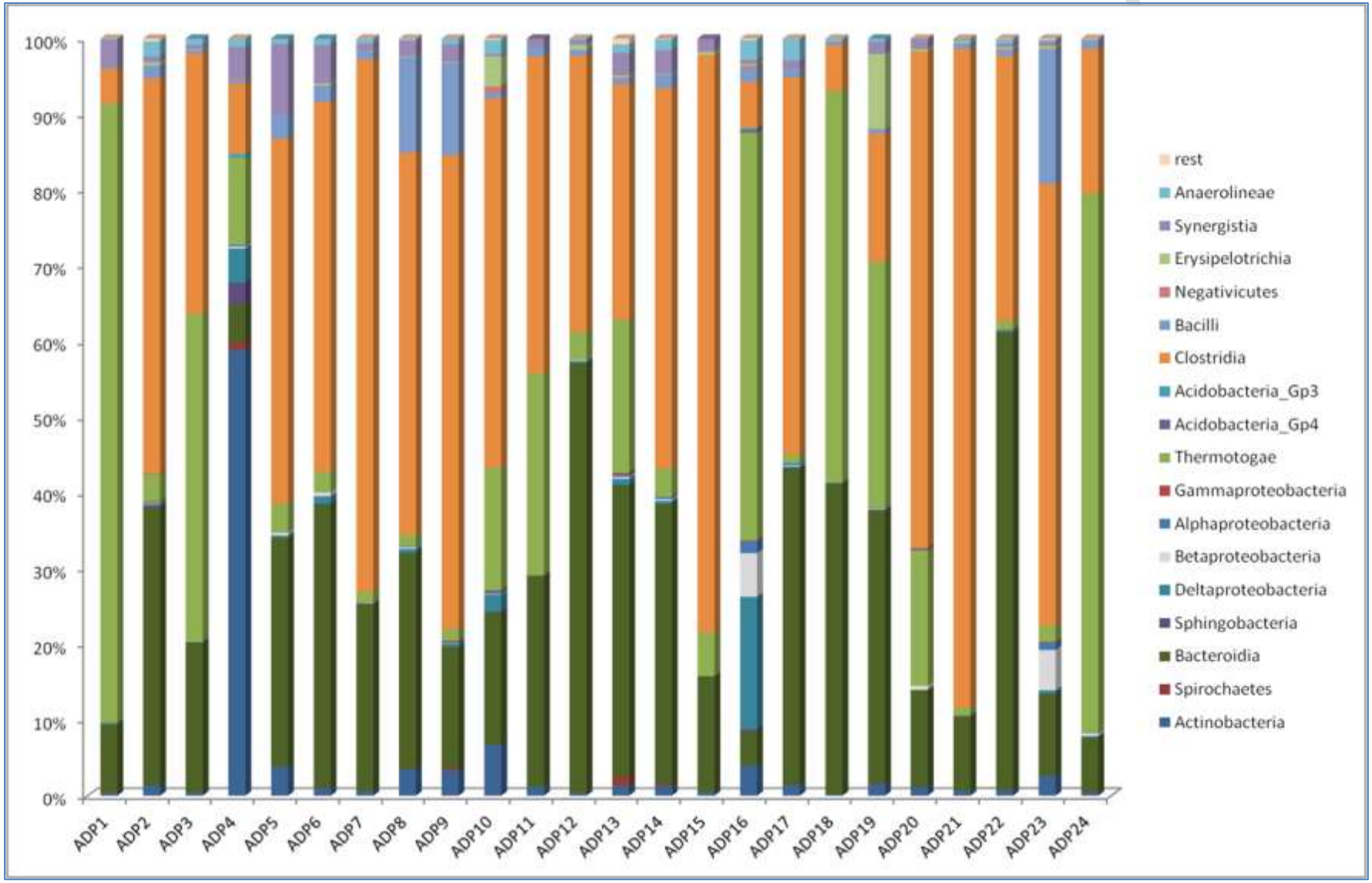


Figure A3.2. nMDS biplot (A) and cluster analysis (B) based on bacterial community profiles, phylum level. A: Circles indicate groupings based on 70% similarity



Looking at the community structure on the level of class revealed that the majority of sequences belonged to members of Clostridia (13.8%), Thermotogae (11%) and the Bacteroidia (9.8%) (Figure A3.3). Smaller fractions of sequences were related to the following classes: Actinobacteria (2%), Spirochaetes (0.06%), Sphingobacteria (0.08%), Deltaproteobacteria (0.4%), Betaproteobacteria (0.19%), Alphaproteobacteria (0.07%), Gammaproteobacteria (0.016%), Acidobacteria Gp4 and Gp3 (0.006 and 0.017% respectively), Bacilli (0.7%), Negativicutes (0.02%), Erysipelotrichia (0.35%), Synergistia (0.56%) and Anaerolineae (0.3%). Among the most prevalent classes the genera *Lactobacillus* sp. (Bacilli), *Clostridium* sp. XVIII, *Tissierella* sp. and *Sedimentibacter* sp. (Clostridia), *Petrotoga* sp. and *Kosmotoga* sp. (both Thermotogae) as well as *Anaerorhabdus* sp. (Bacteroidia) were found to be highly abundant and present in most samples.

Figure A3.2. Overview of the bacterial community structure, class level



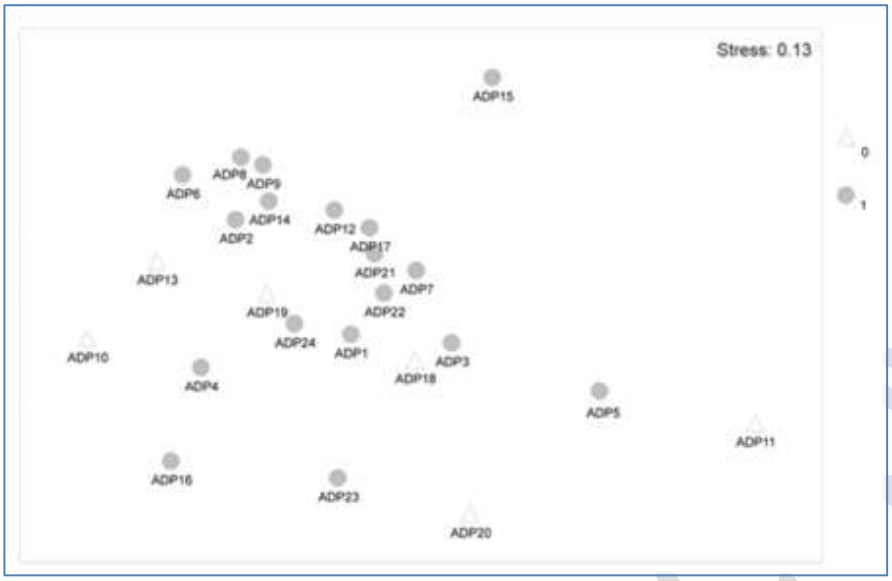
Since a high level of unclassified sequences was observed, comparison of bacterial communities was further carried out based on clustering of OTUs at species level (3% sequence distance) using RDPs complete linkage clustering tool. The numbers of clusters obtained are provided in Table A3.2. When compared with the chao 1 richness estimator coverage of 47-73% of the present diversity was covered on the species level. The lowest coverage was observed for the bacterial community of APD11 covered with 47%; however, the diversity of most samples was covered by more than 60%. Lowest richness was found in samples ADP 5, 11, 15 and 20, which could be linked to low sequence coverage. The diversity of the bacterial community was assessed using the Shannon-Weaver index (RDP) which ranged from 4.949-2.567 with ADP9 showing the highest diversity (Table A3.2).

Table A3.2. Species diversity and richness at 3% sequence distance

	Bacteria		
Sample	*Clusters*	*Shannon (Diversity)*	*chao 1 (Richness)*
ADP1	626	2.602	891.152
ADP2	1517	3.601	2,404.447
ADP3	1023	4.211	1,563.507
ADP4	1150	4.256	1,866.809
ADP5	303	3.951	460.067
ADP6	668	4.050	1,031.073
ADP7	589	3.789	842.022
ADP8	1074	4.011	1,807.647
ADP9	907	4.949	1,283.043
ADP10	620	3.224	936.700
ADP11	93	2.933	197.545
ADP12	637	3.973	923.033
ADP13	1314	2.884	2,036.573
ADP14	1095	3.703	1,766.192
ADP15	119	3.243	176.125
ADP16	913	4.942	1,656.864
ADP17	882	3.684	1,359.783
ADP18	543	2.567	781.750
ADP19	1210	4.288	1,659.044
ADP20	260	4.221	437.364
ADP21	867	3.152	1,313.841
ADP22	986	4.023	1,355.879
ADP23	826	4.397	1,257.937
ADP24	1204	3.794	1,670.221

Prior to ordination, it was tested via ANOSIM whether the Residual Biogas Potential (RBP) as measure of efficiency can be linked to bacterial community structure. For this, a nominal variable of pass/fail was introduced based on the assumption that failing the RBP test applies if RBP> 0.25 l/g VS. ANOSIM revealed a small effect (R=0.264, p=0.048) of RBP on the bacterial community structure; however, failed or passed samples did not show strong similarities in ordination (Figure A3.4). Furthermore, no link between pass/fail and richness or diversity in the samples could be observed.

Figure A3.4. nMDS biplot based on species level community structure. 1: RBP passed samples, 0: RBP failed samples



To understand bacterial community-environment relationships, RDA was performed for classified 16S rRNA gene data (phylum level) using the factors RBP, total volatile solids (VS), alkalinity, ammonia and total VFA. No collinearity was observed for these explanatory variables. RDA revealed that the environmental variables included were able to explain 35.3% of variation in the dataset (Table A3.3). Volatile solids were identified as significant factor shaping the bacterial community (Table A3.4) and was strongly linked to samples ADP 5, 7 and 23 (Figure A3.5), whereas ammonia and VFA displayed a lower and not significant correlation with the dataset. Correlation with RBP test results showed the lowest conditional effects. When the effect of RBP was tested as only variable included, a small and non-significant result was obtained.

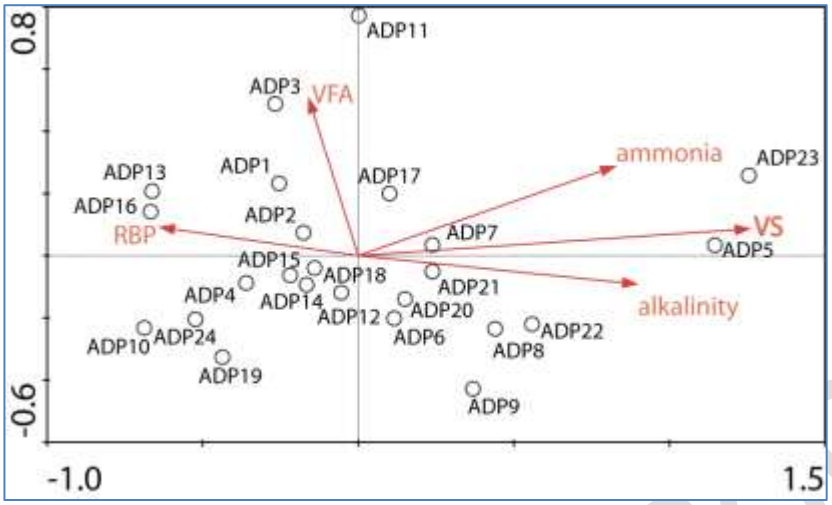
Table A3.3. Bacterial community – RDA results based on phylum level

Axes	Eigenvalues	Cumulative percentage variance of species data	Cumulative percentage variance of species–environment correlation
Axis 1	0.246	24.6	69.8
Axis 2	0.069	31.5	89.3
Axis 3	0.034	35.0	99.1
Axis 4	0.002	35.2	99.7

Table A3.4. Marginal and conditional effects of forwardly selected environmental variables produced by RDA using phylum level community structure

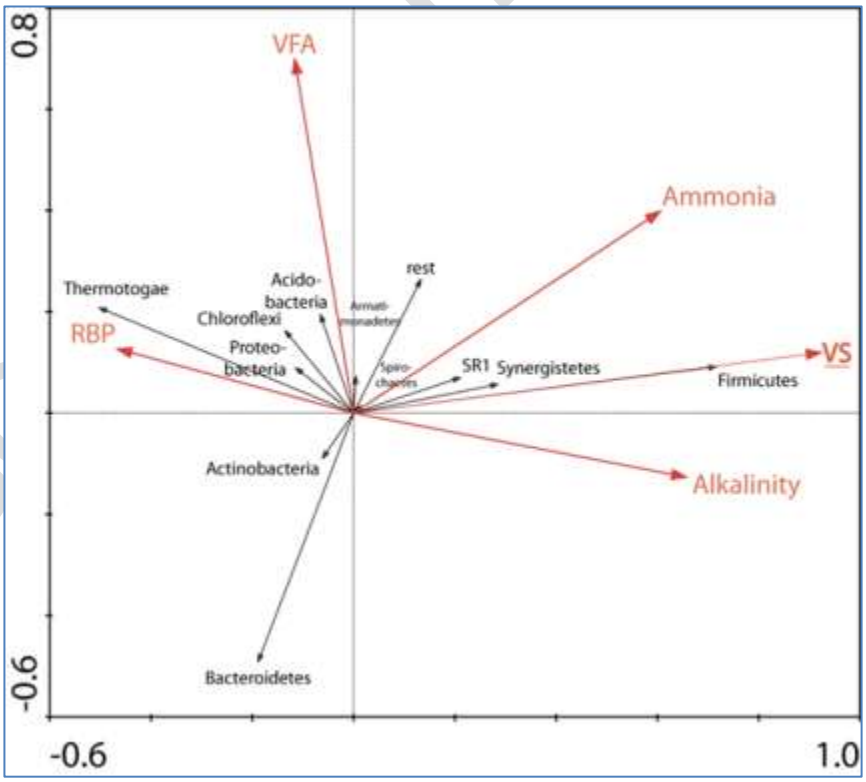
	Marginal Effects	**Conditional Effects**		
Variable	Lambda1	Increase in variation	P	F
VS	**0.21**	**0.21**	**0.001**	**6.01**
ammonia	0.12	0.06	0.185	1.64
alkalinity	0.11	0.03	0.466	0.89
VFA	0.04	0.03	0.42	0.94
RBP	0.06	0.02	0.751	0.46

Figure A3.5. RDA biplot illustrating correlations between bacterial communities from digesters and parameters ammonia, volatile solids (VS), alkalinity, VFA and RBP; phylum level



RBP displayed low contribution to explaining the variation in the dataset. Only 35.3% of variation on phylum level could be explained by the factors included, however, potentially indicating a gap within in the suite of factors measured to reflect AD bacterial community performance. To understand which phyla are linked to measured factors, RDA was used to illustrate those relationships (Figure A3.6).

Figure A3.6. RDA biplot illustrating correlations between bacterial phyla from digesters and parameters ammonia, volatile solids (VS), VFA, alkalinity and RBP



Volatile solids were strongly correlated with Firmicutes, whilst values for increasing RBP were correlated with Thermotogae and Proteobacteria. Additionally, VFA was correlated with Acidobacteria, but Actinobacteria and Bacteroidetes did not show any positive correlation to

parameters included in the analysis. Instead, a negative correlation could be observed with ammonia. In a second step, correlations between reactor parameters and bacterial communities based on species level were calculated using CCA. The results showed that the factors included namely volatile solids, ammonia, VFA, RBP and alkalinity could only explain a small fraction of variation in the dataset. The first two axes of CCA were able to illustrate 51.7 % of the covered species-environment correlations (Table A3.5). Using automated forward selection the variable ammonia showed a significant correlation with the samples (Table A3.6). Also volatile solids and alkalinity displayed contributions to conditional effects, but could not be shown to be significant.

Table A3.5. CCA results correlating environmental variables ammonia, volatile solids, alkalinity, VFA and RBP and the bacterial community based on species level

Axes	Eigenvalues	Cumulative percentage variance of species data	Cumulative percentage variance of species–environment correlation
Axis 1	0.279	8.3	30.1
Axis 2	0.200	14.2	51.7
Axis 3	0.187	19.7	71.8
Axis 4	0.139	23.8	86.8

Table A3.6. Marginal and conditional effects of forwardly selected environmental variables produced by CCA using species level community structure

	Marginal Effects	**Conditional Effects**		
Variable	Lambda1	Increase in variation	P	F
Ammonia	**0.27**	**0.27**	**0.001**	**1.90**
Volatile solids	0.21	0.19	0.056	1.39
Alkalinity	0.19	0.19	0.097	1.38
VFA	0.19	0.15	0.264	1.14
RBP	0.17	0.13	0.703	0.91

Linking bacterial communities to parameters measured revealed strong correlations between ammonia and samples ADP 5, 3 and 22, whereas samples ADP 17 and 18 were correlated with volatile solids (Figure A3.7). Alkalinity could be linked to samples ADP 6, 7, 1, 12 and 21. Furthermore, a negative relationship could be assumed for samples ADP 13, 14, 15, 16, 4 and 2 with ammonia as well as for samples ADP 12, 14, 15, 8 and 24 with volatile solids. A negative correlation was observed between samples ADP 13 and 2 and the factor ammonia.

To gain an understanding of the bacterial species affected by measured factors, CCA was illustrated using OTUs (Operational Taxonomic Units) limited to those contributing >1% across all samples (Figure A3.8). Based on the resulting biplot, certain bacterial species could be correlated with chosen parameters like ammonia displaying strong links between OTUs U00022, U00004, U00114 and U00046 and this factor (Figure A3.8). OTUs U00004 and U00114 belong to the Firmicutes whereas OTU U00046 belongs to the Bacteroidetes and is closely related to the genus *Proteiniphilum* sp.

Figure A3.7. CCA biplot displaying correlations between bacterial communities based on species level data from digesters and parameters ammonia, volatile solids (VS), VFA, alkalinity and RBP

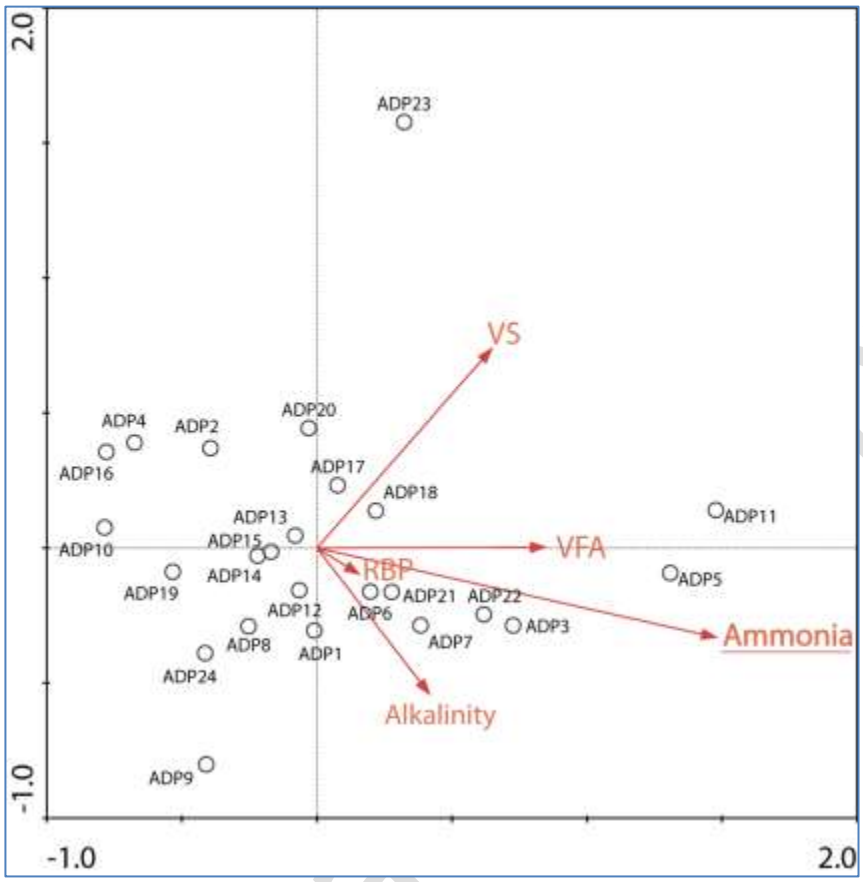
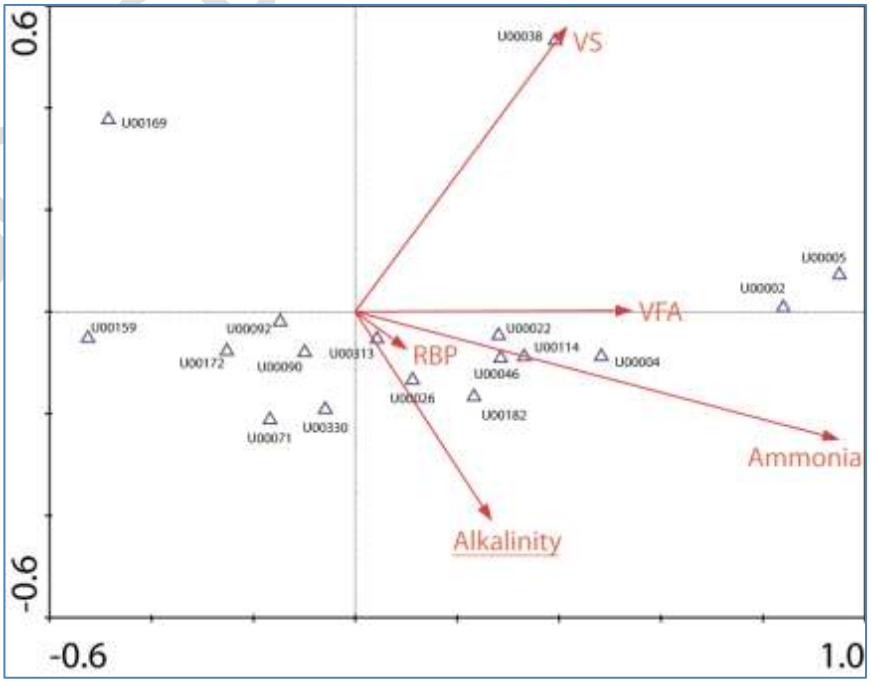


Figure A3.8. CCA biplot displaying correlations between selected bacterial species from digesters and parameters ammonia, volatile solids (VS), VFA, alkalinity and RBP



It is thought that alkalinity can be used as an indicator for the buffer capacity of the substrate counteracting the pH decreasing effect of raising VFA levels (Weiland 2010). Therefore, those samples and bacterial species linked to ammonium and alkalinity increase are assumed to indicate a well-balanced acetogenesis. An observable alkalinity reduction is indicative of acetogens producing volatile acids at a faster rate than methanogens are able to convert into methane, leaving acids available to consume alkalinity. Also ammonium is produced during acid fermentation and reflects the efficiency of this process. Based on this understanding, samples from reactors ADP 1, 3, 5, 6, 7, 12, 21 and 22 appear to contain a well-functioning bacterial community. In these reactors, both, the phylum and OTU approach indicated that RBP measurements were not correlated with overall community structure. Furthermore, Thermotogae seemed to be positively correlated with rising RBP but negatively correlated with alkalinity giving rise to the assumption that an increase in Thermotogae might indicate suboptimal conditions. Overall, Bacteroidetes and Firmicutes were described as part of a core group of bacteria in anaerobic digestion of wastewater sludge (Rivière et al. 2009) and as major components during thermophilic digestion of rye silage and winter barley straw (Rademacher et al. 2012). Firmicutes were also found to be prevalent in the anaerobic digestion of plant biomass and pig manure (Wirth et al. 2012), whereas Thermotogae were a less important component in the digestion of wastewater sludge (Rivière et al. 2009). Members of this phylum were found to be present with a high proportion in thermophilic reactors (Sasaki et al. 2011); however at this point their presence cannot be related directly to temperature conditions. Furthermore, it was assessed if micronutrients and specifically the volatile fatty acid acetic acid could be correlated with certain bacterial communities.

Table A3.7. CCA results based on species level including micronutrients iron, molybdenum and iron as well as acetic acid

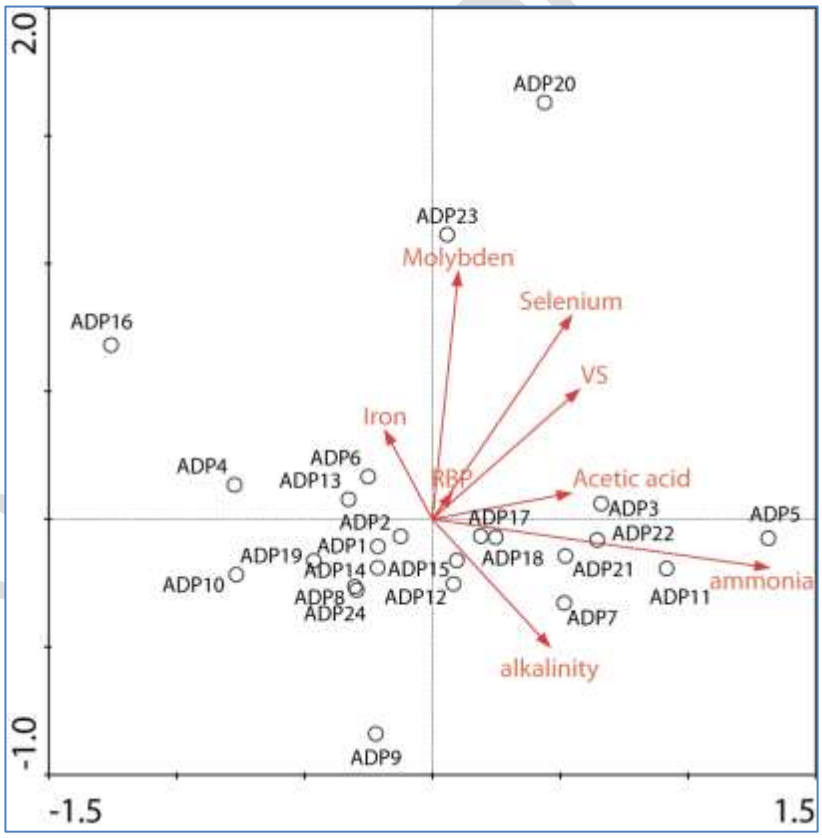
Axes	Eigenvalues	Cumulative percentage variance of species data	Cumulative percentage variance of species–environment correlation
xis 1	0.293	8.7	20.7
Axis 2	0.236	15.7	37.4
Axis 3	0.198	21.5	51.4
Axis 4	0.175	26.7	63.8

In this analysis, a larger fraction of variation in the community could be explained by the chosen variables, of which automated forward selection revealed ammonium to be highly significant (Table A3.8). Although molybdenum and selenium appeared strongly correlated with samples ADP 23 and 20 (Figure A3.9), their effect could not be shown to be significant.

Table A3.8. Marginal and conditional effects of forwardly selected environmental variables produced by CCA using species level bacterial community structure including micronutrients and acetic acid

	Marginal Effects	**Conditional Effects**		
Variable	Lambda1	Increase in variation	P	F
Ammonia	**0.27**	**0.27**	**0.001**	**1.90**
Molybdenum	0.21	0.21	0.078	1.51
Alkalinity	0.19	0.18	0.11	1.38
Iron	0.18	0.19	0.055	1.43
Selenium	0.21	0.17	0.124	1.23
Volatile solids	0.21	0.15	0.231	1.18
RBP	0.17	0.12	0.57	0.97
Acetic Acid	0.16	0.12	0.659	0.91

Figure A3.9. CCA biplot displaying correlations between bacterial communities, species level from digesters and parameters ammonia, volatile solids (VS), alkalinity, RBP, the micronutrients selenium, molybdenum and iron as well as acetic acid



A3.3.2 Methanogen community

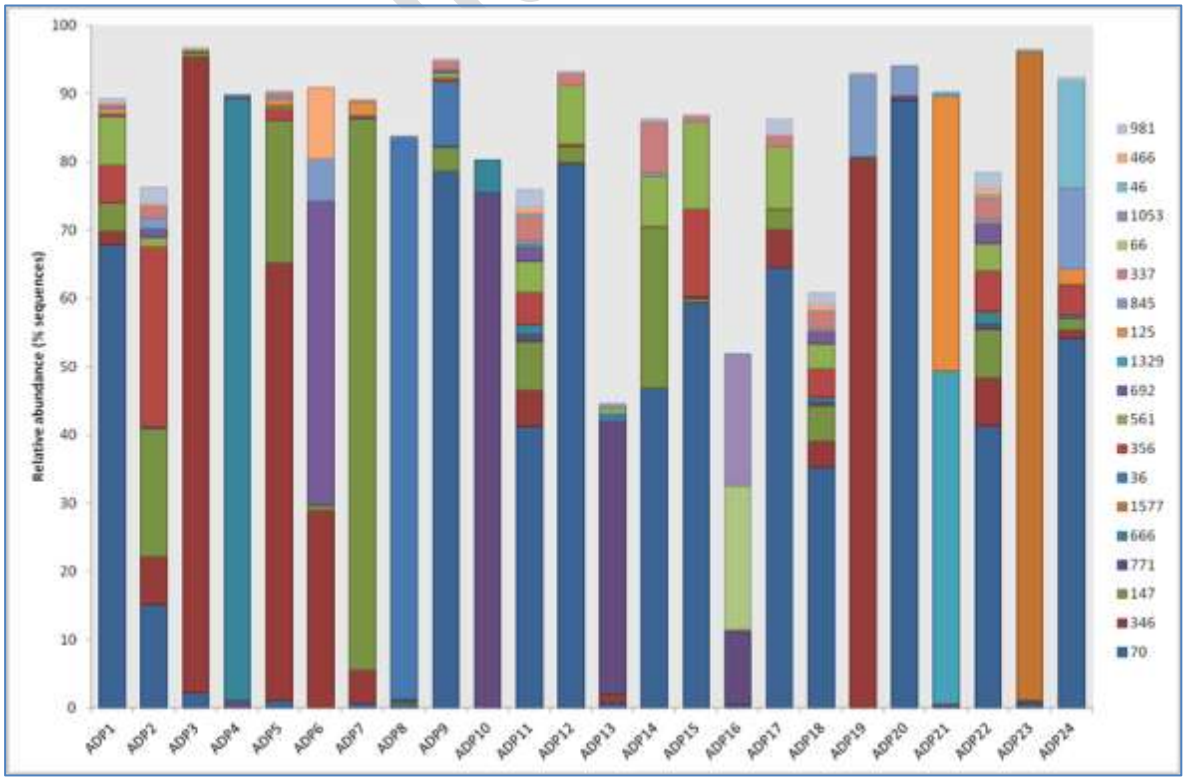
Overall, a total of 128,533 sequences were obtained using pyrosequencing of the amplified *mcr*A gene. Highest sequence numbers were obtained from samples ADP 1, 2, 12, 17 and 19 with highly variable numbers as low as 587 for ADP 21 (Table A3.9).

Table A3.9. Overview of sequences obtained from sequencing of the *mcr*A gene

Sample	Sequences	Clusters 3% distance	Sample	Sequences	Clusters 3% distance
ADP1	8696	212	ADP13	5510	320
ADP2	9463	260	ADP14	5334	145
ADP3	2292	53	ADP15	7528	136
ADP4	2483	72	ADP16	3196	213
ADP5	1127	59	ADP17	8664	186
ADP6	5819	88	ADP18	2429	157
ADP7	3332	73	ADP19	10153	130
ADP8	1198	37	ADP20	3049	77
ADP9	2172	69	ADP21	587	33
ADP10	1569	63	ADP22	6637	289
ADP11	7660	321	ADP23	5057	60
ADP12	21329	222	ADP24	3249	112

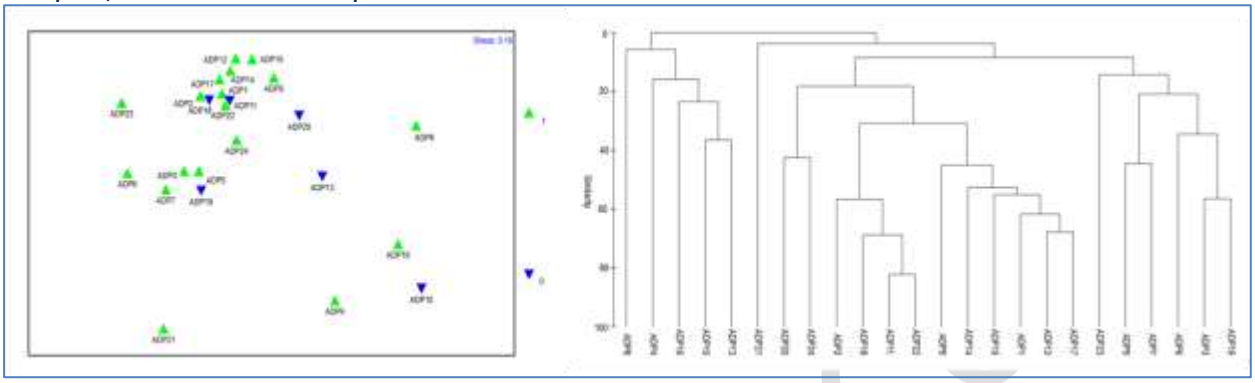
After removing single hits from the dataset, the distribution of methanogen taxa appeared very variable highlighting the dominance of some taxa in most samples (63%) and high diversity in others (Figure A3.10). The number of clusters at 3% genetic distance resembling species level varied from 33 to 321 clusters (Table A3.9) illustrating high richness in samples ADP1, 2, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22 and 24 containing over 100 methanogen OTUs each. Of these, 19 methanogen taxa were identified with a relative abundance >0.5% across all digesters. Interestingly, samples ADP 13 and 16 displayed high diversity including many taxa with low proportions across the dataset (Figure A3.10). It was tested, if there was a significant link between RBP and the methanogen community using ANOSIM (global R: -0.004, p=0.461), revealing no relationship.

Figure A3.10. Community structure of methanogens based on OTUs present >0.5 across the dataset



To illustrate similarities between digesters, ordination and clustering were used both illustrating a high degree of dissimilarities between samples (Figure 11). The following groups displayed 40% similarity in community structure: group 1: ADP 9, 14, 15, 1, 12, 17, group 2: ADP 20, 24, group 3: ADP 2, 18, 11, 22, group 4: ADP 3, 19, group 5: ADP 5, 7 (Figure A3.11).

Figure A3.11. nMDS biplot based on methanogen community structure. 1: RBP passed samples, 0: RBP failed samples



To link OTUs to relatives of known methanogens a neighbour-joining tree was constructed. This revealed that the majority of taxa were related to Methanosarcinales but also members of the Methanomicrobiales and Methanobacteriales (Figure A3.12). Furthermore, some clusters could not be related to known methanogens leaving their phylogeny unknown. Major taxa within the Methanosarcinales could be found widely distributed across samples, although some occurred as main taxon in certain reactors like OTU 1577 solely found in sample ADP 23 contributing to 95 % of the community (Figure A3.13).

Figure A3.12. Neighbour joining tree using p-distance including 356 methanogen taxa. Clusters highlighted in red indicate taxa with a relative abundance of more tha 0.5% across the dataset. Phylogenetic analyses were conducted in MEGA4

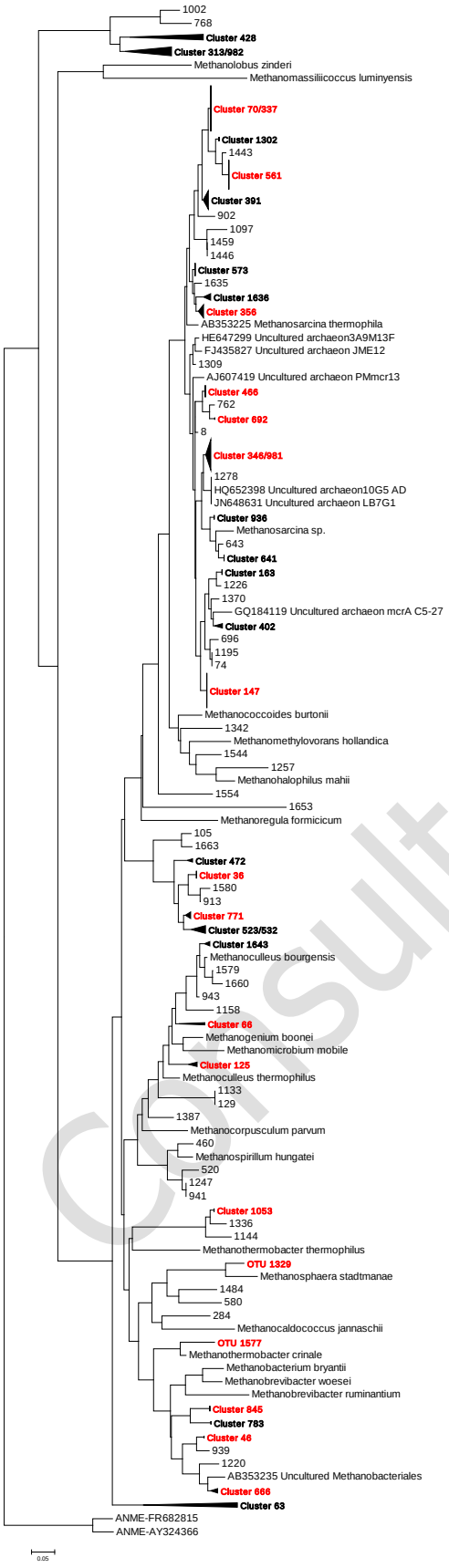
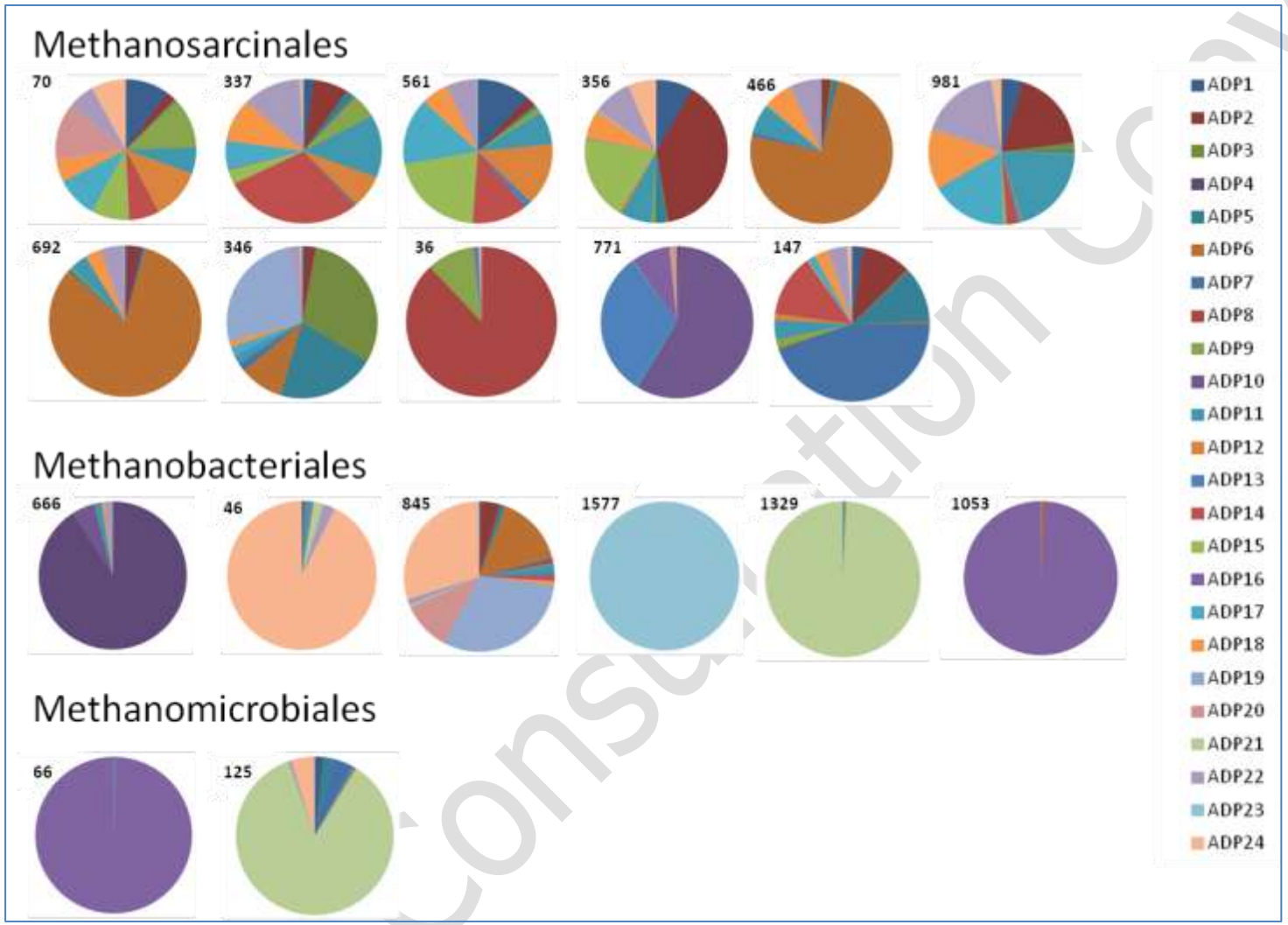


Figure A3.13. Distribution of methanogen taxa >0.5% across samples digesters



To analyse the relationship between the methanogen community and factors like ammonia, volatile solids and alkalinity, CCA was applied. In a first step it was tested whether the factors alkalinity, RBP, ammonia, total VFA and volatile solids were correlated with the dataset and if specific factors measured would be able to explain the observed variation in the methanogen community. Overall, a low fraction of variation in the community could be explained by the chosen variables, of which 60.4% could be illustrated by the first two axes (Table A3.10), including alkalinity and volatile solids indicating the strongest influence (Figure A3.14). Samples ADP 9 and 8 displayed a strong correlation with alkalinity whereas correlation of volatile solids with samples ADP 2, 4, 5, 17 and 20 was weaker. The other samples could be linked with ammonia and RBP of which the latter appeared negatively correlated with alkalinity. Interestingly, none of the variables included in the analysis revealed a significant effect using automated forward selection (Table A3.11). When the effect of RBP values were tested separately, a low and non-significant results was obtained.

Figure A3.14. CCA biplot displaying correlations between methanogen communities based on species level data from digesters and parameters ammonia, volatile solids (VS), VFA, alkalinity and RBP

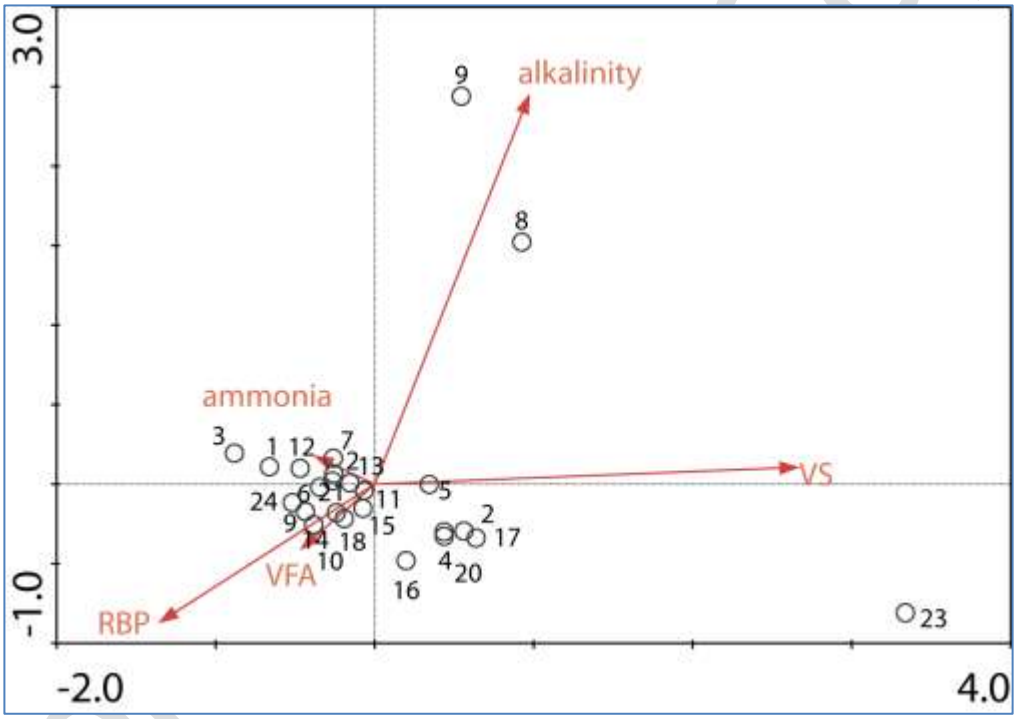


Table A3.10. CCA results based on the methanogen community including the variables ammonia, alkalinity, RBP, total VFA and volatile solids

Axes	Eigenvalues	Cumulative percentage variance of species data	Cumulative percentage variance of species–environment correlation
Axis 1	0.38	7.9	33.6
Axis 2	0.304	14.2	60.4
Axis 3	0.282	20	85.2
Axis 4	0.107	22.2	94.6

Table A3.11. Marginal and conditional effects of forwardly selected environmental variables produced by CCA using methanogen community structure and the variables ammonia, alkalinity, RBP, total VFA and volatile solids

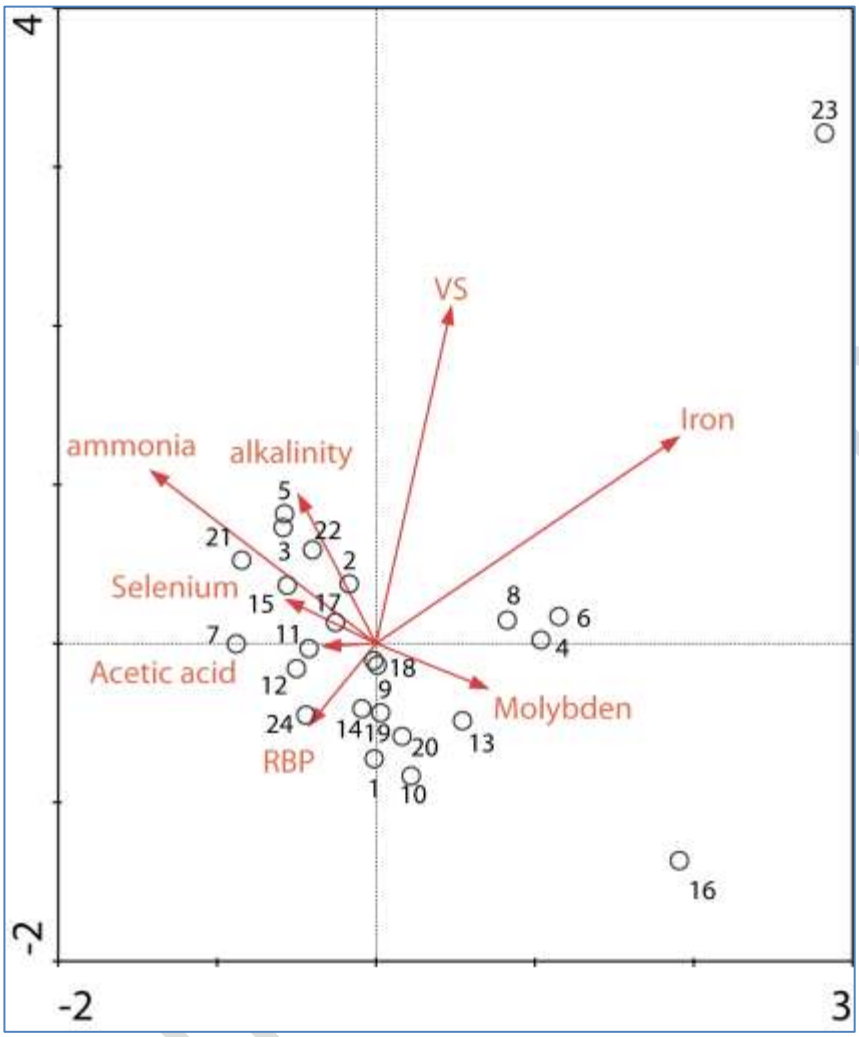
	Marginal Effects	**Conditional Effects**		
Variable	Lambda1	Increase in variation	P	F
Volatile solids	0.34	0.34	0.058	1.69
Alkalinity	0.3	0.29	0.134	1.45
Ammonia	0.25	0.28	0.101	1.39
RBP	0.16	0.11	0.948	0.53
VFA	0.08	0.11	0.948	0.59

It is well known that the lack of micronutrients can hamper methanogenesis leading to the assumption that the levels of specific nutrients can be potentially linked to methanogen community composition. Furthermore, it was tested how acetic acid in particular can be linked with community structure. CCA revealed that the variables included were able to explain a larger proportion of the variation observed compared with the analysis not including micronutrients. Iron, volatile solids, ammonia and alkalinity displayed the strongest effect on community structure (Figure A3.15). The first two axes of CCA could illustrate 51% of the correlation between variables and community variation (Table A3.12).

Table A3.12. CCA results based on methanogen community structure including micronutrients iron, molybdenum and selenium as well as acetic acid, RBP, volatile solids (VS), alkalinity and ammonia

Axes	**Eigenvalues**	**Cumulative percentage variance of species data**	**Cumulative percentage variance of species–environment correlation**
Axis 1	0.507	10.5	27.5
Axis 2	0.433	19.5	51
Axis 3	0.313	26	67.9
Axis 4	0.166	29.4	76.9

Figure A3.15. CCA biplot displaying correlations between methanogen communities based on species level data from digesters and parameters ammonia, volatile solids (VS), acetic acid, alkalinity, RBP, and the micronutrient iron, molybdenum and selenium



Using automated forward selection revealed that both iron and alkalinity showed significant effects with iron as the strongest factor (Table A3.13). It appeared that samples ADP 4, 6 and 8 were strongly linked with iron and that samples positively correlated with RBP showed negative correlation with iron namely ADP 24, 12 and 11. Similarly, samples positively correlated with alkalinity were negatively correlated with molybdenum including ADP 2, 3, 5, 15, 17, 21 and 22 (Figure A3.15).

Since iron is involved in all electron transport processes including enzymes like pyruvate:ferredoxin oxidoreductase shown to be present in cultivated methanogens like *Methanosarcina barkeri* (Bock et al 1997), its importance in shaping methanogen community structure is not surprising. It is also essential for methanogens i.e. for key enzymes like the formylmethanofuran dehydrogenase catalysing the reduction of CO_2 (Blaut 1994).

Table A3.13. Marginal and conditional effects of forwardly selected environmental variables produced by CCA using species level community structure including micronutrients and acetic acid

	Marginal Effects	**Conditional Effects**		
Variable	Lambda1	Increase in variation	P	F
Iron	**0.39**	**0.39**	**0.02**	**1.94**
Alkalinity	**0.3**	**0.36**	**0.02**	**1.87**
Volatile solids	0.34	0.29	0.061	1.49
Molybdenum	0.17	0.18	0.357	1
Selenium	0.16	0.24	0.201	1.26
Ammonia	0.25	0.13	0.764	0.7
Acetic Acid	0.13	0.14	0.777	0.71
RBP	0.16	0.11	0.896	0.56

Interestingly, acetic acid did not display a strong effect on the community when analysed within this set of variables. Increasing levels of ammonia were negatively correlated with molybdenum as was alkalinity. A direct link between methanogen diversity and correlation to specific factors could not be found. Overall, the analysed digesters were dominated by members of Methanosarcinales, whose diversity was very high. This included a high number of relatives of *Methanosarcina* spp. e.g. *M. thermophila* assumed to choose the aceticlastic pathway for methanogensis. Methanobacteriales played also an important role especially for those reactors that were dominated by members of this order like ADP 4, 21 and 23. Major factors explaining variations in methanogen community structure were the micronutrient iron and alkalinity.

A.3.4 References

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A3.5 Samples and unique identifiers (MIDs)

Sample	mcrA MID-sequence	Bacterial 16S MID-sequence
ADP1	1-ACGAGTGCGT	21-CGTAGACTAG
ADP2	2-ACGCTCGACA	22-TACGAGTATG
ADP3	3-AGACGCACTC	23-TACTCTCGTG
ADP4	4-AGCACTGTAG	24-TAGAGACGAG
ADP5	5-ATCAGACACG	25-TCGTCGCTCG
ADP6	6-ATATCGCGAG	26-ACATACGCGT
ADP7	7-CGTGTCTCTA	27-ACGCGAGTAT
ADP8	8-CTCGCGTGTC	28-ACTACTATGT
ADP9	9-TAGTATCAGC	29-ACTGTACAGT
ADP10	10-TCTCTATGCG	30-AGACTATACT
ADP11	11-TGATACGTCT	31-AGCGTCGTCT
ADP12	12-TACTGAGCTA	32-AGTACGCTAT
ADP13	1-ACGAGTGCGT	33-ATAGAGTACT
ADP14	2-ACGCTCGACA	34-CACGCTACGT
ADP15	3-AGACGCACTC	35-CAGTAGACGT
ADP16	4-AGCACTGTAG	36-CGACGTGACT
ADP17	5-ATCAGACACG	37-TACACACACT
ADP18	6-ATATCGCGAG	38-TACACGTGAT
ADP19	7-CGTGTCTCTA	39-TACAGATCGT
ADP20	8-CTCGCGTGTC	40-TACGCTGTCT
ADP21	9-TAGTATCAGC	41-TAGTGTAGAT
ADP22	10-TCTCTATGCG	42-TCGATCACGT
ADP23	3-AGACGCACTC	47-TGTGAGTAGT
ADP24	11-TGATACGTCT	43-TCGCACTAGT

Waste & Resources Action Programme

The Old Academy
21 Horse Fair
Banbury, Oxon
OX16 0AH

Tel: 01295 819 900
Fax: 01295 819 911
E-mail: info@wrap.org.uk

Helpline freephone
0808 100 2040

www.wrap.org.uk

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