ANAEROBIC DIGESTION IN A MULTI-STAGE PLUG FLOW BIOREACTOR: REVISITING AN AGE-OLD PROCESS WITH MODERN MOLECULAR TOOLS

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ABSTRACT:
To address knowledge gaps in the complex interacting microbial associations that underpin anaerobic digestion, a mesophilic (25°C) continuous-flow four-stage reactor was constructed to separate both spatially and temporally the component microbial groups. The reactor influent consisted of primary settled sewage sludge (PSSS) and the organic fraction of municipal solid waste (OFMSW). Chemical (volatile fatty acids, sulphate, sulphide, chemical oxygen demand, gas) and molecular analyses were made during an operation period of 15 months. Spatial separation of the microbial groups resulted in process instability where acidogenesis/acetogenesis produced an effluent with a pH between 2 and 4 that inhibited the subsequent catabolic steps. An organic loading rate of 6.5 g COD d^{-1} prevented reactor acidification but resulted in low biogas production (0.04-0.12 l biogas l^{-1} hydraulic load d^{-1}).
Fluctuations in chemical and molecular profiles/characteristics, which may have been due to the inherently heterogeneous PSSS and OFMSW, were recorded and these were countered by the development of a model medium. The medium was then used to: explore reactor efficacy; and study pertinent microbial diversity and functional interactions.

INTRODUCTION

The UK has become increasingly dependent on foreign energy imports [1]. In 2005, these accounted for 40% of oil, 14% of gas and 69% of coal but by 2008 these had risen to 46%, 33% and 71%, respectively [2]. To mitigate reliance on these imports the UK and EU governments have identified many renewable technologies of which anaerobic digestion is one.

The UK annually produces > 10 Mt of organic fraction of municipal solid waste (OFMSW) while 10 billion litres (3 500 t dry weight) of sewage are generated daily [3,4]. In 2008, the approximate direct cost to treat these was £4.2 billion, with further costs incurred for dewatering, landfilling or incinerating sewage sludge. To reduce these costs, anaerobic digestion could replace aerobic catabolism of sewage to minimise sludge production and generate methane for site use or sale [5].

Whilst evidence of anaerobic digestion can be traced back to the Babylonian ruins, the process is still not fully understood and in some respects retains a black box status [6]. As a consequence, anaerobic digesters are often run with low loadings to avoid souring.

Although the catabolic stages of anaerobic digestion are well documented: hydrolysis [7,8]; acidogenesis by fermentation and β-oxidation [9,10]; acetogenesis [11]; and methanogenesis [12], the underpinning microbial associations (multi-species gene pool) have still to be characterised fully.

Currently, anaerobic reactor efficacy is determined empirically with the process monitored chemically through variables such as volatile fatty acid (VFA) concentrations, pH, biogas composition and yield, volatile solids and chemical oxygen demand [13,14]. Of these, VFA concentrations are perhaps the best indicator of the catabolic balance [13,15]. Increasing our understanding of anaerobic processes will facilitate accurate digester biomonitoring with high throughput molecular tools detecting rapid metabolic changes [16] and so ensuring pre-emptive rather than reactive response to imbalances.

With the advent of molecular tools, analysis of mixed and unknown species, by polymerase chain reaction (PCR) based techniques such as reverse transcriptase (RT)-PCR [17], denaturing gradient gel electrophoresis (DGGE) [18,19], PCR-single strand conformation polymorphism (SSCP) [20,21], and terminal restriction fragment length polymorphism (T-RFLP) [22,23], has become routine. However, with the exception of metagenomic techniques such as 454-pyrosequencing [24,25], most molecular tools require the use of PCR or prior knowledge of target DNA sequences. According to Kanagawa (2003) [26], the complex microbiology of anaerobic digestion results in multi-template PCR increasing the risk of bias and artifact formation. Reducing the complexity of the template can be accomplished through spatial and temporal separation under continuous culture conditions compartmentalising the different phases of anaerobic degradation in separate vessels. Following hydrolysis, the acidogenic microorganisms are characterized by the highest specific growth rates followed by the acetogenic and then the methanogenic species [9,12]. Thus, specific growth rate manipulations, through reactor volume changes, can separate the component species. Coutts *et al* (1986) [27] used an increasing volume three-vessel configuration to study the anaerobic catabolism of hexanoic acid by a microbial association isolated from landfill but the work was limited to chemical analysis. In the work presented here, the same approach was used but molecular analysis enabled definitive study of the microbiology.

MATERIALS AND METHODS

Bioreactor and Operating Conditions

Phase One

The multi-stage continuous plug flow reactor (Figure 1) consisted of four vessels (A - D) linked in series with culture volumes of 1(A), 1.2(B), 1.5(C) and 1.8 *l* (D). With a constant influent flow rate of 10 m*l* h^{-1}, the dilution rates for Vessels A to D were 0.01, 0.0083, 0.0067 and 0.0056 h^{-1}, respectively while Vessel A was supplemented with 5 g OFMSW every second day for the duration of Phase One. The organic loading rate to avoid reactor souring, was determined by preliminary investigations (data not shown). The array was overgassed with oxygen-free nitrogen to maintain anoxic conditions with each vessel maintained at 25°C by thermostatically controlled water baths.

Phase Two

For model feed studies, four vessels were used with an influent flow rate of 30 m*l* h^{-1} and culture volumes and dilution rates of: I, 0.3 *l*, 0.1 h^{-1}; II, 0.6 *l*, 0.05 h^{-1}; III, 1.6 *l*, 0.019 h^{-1}; and IV, 2.0 *l*, 0.015 h^{-1}.

Inoculum

Phase One

Domestic wastewater primary settled sewage sludge, anaerobic sludge from an upflow anaerobic sludge bed reactor treating food processing wastewater and the organic fraction of municipal solid waste collected from a domestic household in the north east of England were mixed in a ratio of 105:31.5:100 (w/w), respectively to give a total carbon:nitrogen ratio of 25:1. Vessel A was inoculated with 500 m*l* of this preparation followed by 200 m*l* of sterile deionised water.

Phase Two

The organic fraction of municipal solid waste was removed from Vessel A and the culture volume adjusted to 300 m*l*.

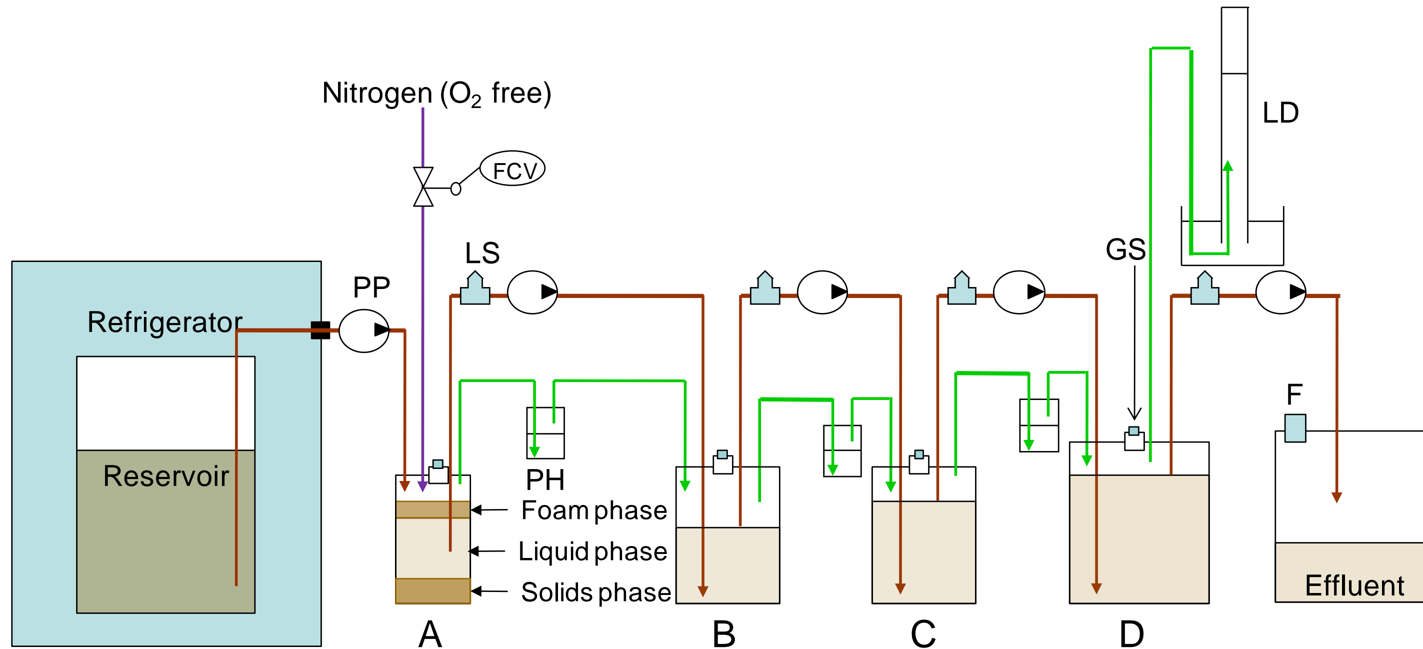


Figure 1. Multi-stage reactor consisting of four vessels A-D (Phase One) or I-IV (Phase Two) of increasing culture volume connected in series. PP, Peristaltic pump; FCV, Flow control valve; LS, Liquid sampling; GS, Gas sampling; PH, Pressure head (0.5% (w/v) citric acid acidified NaCl (20% w/v)); F, Filter (Nalgene, 0.2 µm); LD, Liquid displacement (0.5% (w/v) citric acid acidified NaCl (20% w/v)).

Influent

Phase One

The influent for Vessel A consisted of primary settle sewage sludge collected weekly and stored at 4°C and OFMSW collected over a two-week period and hand sorted to separate the food component with larger pieces cut by hand to increase their surface area prior to storage at 4°C.

Phase Two

The model feed consisted of a basic mineral salts medium [27] supplemented with final volatile fatty acid and sulphate concentrations based on data obtained from Vessel A during Phase One. The VFAs and sulphate were added as their sodium salts. Thus the medium contained the following (g l^{-1} deionised H_2O): K_2HPO_4, 1.5; NaH_2PO_4, 0.85; NH_4Cl, 0.9; $MgCl_2.6H_20$, 0.2; $NaHCO_3$, 0.5; Na_2CO_3, 0.2; $C_2H_3NaO_2.3H_2O$, 2.69; $C_3H_5NaO_2$, 1.38; $C_4H_7NaO_2$, 0.685; $C_5H_9NaO_2$ (1M), 5.1 m*l*; Na_2SO_4 (1M), 3.6 m*l*; vitamins solution, 1.0 m*l*; trace elements solution, 1.0 m*l*; trace minerals solution, 1.0 m*l*; nickel sulphate solution (1 mM), 1.0 m*l*; and resazurin (0.01% w/v), 1.0 m*l*. The vitamins solution contained (mg l^{-1}): L-biotin, 10; p-amino benzoic acid, 19; (±)α-lipoic acid, 20; folic acid, 10; pyridoxine HCl, 20; thiamine HCl, 20; riboflavin, 30; nicotinic acid, 50; pantothenate, 30; and cyanocobalamine, 20. The trace elements solution contained (mg l^{-1}): $FeCl_2.4H_20$, 1 500; NaCl, 9 000; $MnCl_2.4H_2O$, 197; $CaCl_2$, 900; $CoCl_2.6H_2O$, 238; $CuCl_2.2H_2O$, 17; $ZnSO_4$, 287; $AlCl_3$, 50; H_3BO_3, 62; and $NiCl_2.6H_2O$, 24. The trace minerals solution contained (mg l^{-1}): $NaMoO_4.2H_2O$, 48.4; $NaSeO_3$, 2.55; and $NaWO_4.2H_2O$, 3.3. The medium was filter sterilised (0.2 μm) and residual oxygen was removed by overgassing with oxygen-free nitrogen.

Sample Collection

Samples (5 m*l*) were collected from each vessel at regular intervals for volatile fatty acid and pH analyses. At each steady state, samples (25 m*l*) were collected for DNA extraction and sulphate, sulphide, COD and VFA analyses with 10 m*l* aliquots stored at -20°C.

Analyses

Chemical

For VFA analyses, effluent samples (0.9 m*l*) were acidified with formic acid (0.1 m*l*) and centrifuged at 12 000 x g (Eppendorf, 5810 R) for 5 min. The acids were identified and quantified with 20 μ*l* injections by gas chromatography (Shimadzu GC-2010). The GC was fitted with a BP21 capillary column (30 m x 0.56 mm i.d., 0.5 μm film, SGE Analytical Science) and a flame ionisation detector and was programmed as follows: the injector and detector temperatures were maintained at 230°C while the column temperature was held at 80°C for 6 min then increased at a ramp rate of 6°C min^{-1} to 150°C, followed by an increase to 230°C at a ramp rate of 40°C min^{-1} and a holding time of 2 min. Helium was used as the carrier gas at a rate of 10 m*l* min^{-1}. Calibration curves were created with a mixed standard of acetic, propionic, n-butyric, butyric, n-valeric, valeric, hexanoic and heptanoic acids (SUPELCO, VFA standard mix 46975-U). Chemical oxygen demand and sulphate and sulphide concentrations were quantified with a PalinTest 8100 photometer and the corresponding PalinTest kits. Total biogas production was determined after overgassing was discontinued for 16 h while analysis was by gas chromatography as above with 50 μ*l* samples and the following conditions: the injector and detector temperatures were maintained at 150°C while the column was held at 50°C. Calibration curves for methane were made with a standard (Sigma) and the concentrations were converted to molarity at standard temperature and pressure. pH values were determined with a PH213 pH meter (Hanna Instruments) fitted with a general use pH electrode (Fisher Scientific).

Nucleic Acid Extraction

Nucleic acids were extracted from culture samples using a protocol based on the method of Lemarchand *et al* (2005) [28]. Diethyl pyrocarbonate (30 μ*l*) was added to enable RNA recovery and the preparations were homogenised with a Precellys 24 (Bertin Technologies) for three cycles of 5 000 rpm for 30 seconds with a 30-second pause between each cycle. The final nucleic acid pellets were dissolved in 100 μ*l* TE buffer and stored at -80°C until required.

Polymerase Chain Reaction Conditions

Amplification was made with a Primus 96 Plus thermocycler (MWG-Biotech) and Promega master mix which contained initial concentrations of *Taq* DNA polymerase (50 U ml^{-1}), dATP (400 μM), dGTP (400 μM), dCTP (400 μM), dTTP (400 μM), $MgCl_2$ (3 mM) and BSA (10 mg ml^{-1}). The final reaction volumes were 50 μ*l* and included 2 μ*l* of template DNA. Three primer sets were used to amplify the 'universal' bacterial 16S rRNA genes (GC388F /530R) [29], sulphate-reducing *dsrB* gene (DSRp2060F / DSR4R) [30] and archaea (PRA46f / PREA1100r then PARCH304f / PARCH519r) [31]. Amplification conditions for each of the primer sets are shown in Table 1.

Table 1. Primer pairs and thermocycle programmes used for DNA amplification.

Primer Pairs	Final Primer Conc^{n} (μM)	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles
GC388F/ 530R	0.2	95°C 2 min	95°C 1 min	60°C 1 min	72°C 1.5 min	72°C 30 min	35
DSRp2060F/ DSR4R	1	94°C 4 min	94°C 1 min	55°C 1 min	72°C 1 min	72°C 10 min	35
PRA46f/ PREA110r	1	92°C 2 min	92°C 1 min	55°C 1 min	72°C 1 min	72°C 6 min	30
PARCH304f/ PARCH519r	1	92°C 2 min	92°C 1 min	55°C 1 min	72°C 1 min	72°C 6 min	30

Agarose Gel Analysis

Agarose gels for extracted nucleic acids (1% w/v) and PCR products (1.5% w/v) were prepared with 0.5X TBE and 30 μ*l* ethidium bromide (500 μg ml^{-1}). The gels were loaded with a mixture of 10 μ*l* of extracted nucleic acids/PCR products and 2 μ*l* of 6X loading buffer. Electrophoresis was run at 90 V for 90 min in 0.5X TBE running buffer. Visualisation of the extracted nucleic acids was then made with a UV transluminator (AlphaImager HP®, Alpha Innotech) at 302 nm and low intensity with Alphaview® software V 1.01.1.

Denaturing Gradient Gel Electrophoresis

The PCR products (20 μ*l*) from 'universal' bacterial primers were separated on a 10% (w/v) polyacrylamide gel with a 35% to 65% denaturing gradient. Sulphate-reducing bacteria and archaeal PCR products (20 μ*l*) were separated on an 8% (w/v) polyacrylamide gel with a 40% to 70% denaturing gradient. The gels were run on an Ingeny PhorU-2 DGGE at 110 V (2 gels) or 100 V (1 gel) for 18 h, stained with 1X SYBR Gold (Invitrogen) and visualised as above.

RESULTS AND DISCUSSION

Phase One

Figure 2 summarises changes in volatile fatty acid concentrations in the influent primary settled sewage sludge and Vessels A-D. As recorded in previous studies, the sludge showed variable VFA concentrations particularly for acetic (0.2 to 10.0 mM), propionic (0.2 to 6.9 mM), iso-butyric (< 0.1 to 3.1 mM), butyric (< 0.1 to 1.7 mM) and valeric acids (< 0.1 to 0.5 mM) [32,33]. Increases in the PSSS acetic (7.9 to 10.0 mM) and propionic (5.3 to 6.9 mM) acid concentrations were observed between days 45 and 65. In a study by Zhu *et al* (2008) [34] primary settled sewage sludge concentrations of these two acids were higher and varied from 27.5 to 10.4 mM and 11.6 to 4mM, respectively although a paucity of other comparable data in the published literature prevents further comparisons.

Throughout Phase One, Vessel A was characterised by increases in the concentrations of acetic, propionic, butytric and valeric acids (Figure 2). A study by Horiuchi *et al* (2002) [35] showed that butyric acid was the principal product of acidogenesis for a pH range similar to that recorded in Vessel A (pH 5 to 6), whilst Roy *et al* (2009) [36] used a plug flow reactor at 25°C and observed acetic acid to be the main product of hydrolysis and acidogenesis. The results of our study suggested that the β-oxidation of propionic acid to acetic acid was either absent or occurring at low rates, possibly due to a lack of a syntrophic partner [11]. For iso-valeric, hexanoic and heptanoic acids the concentrations were often below 1 mM (data not shown). Geraldi (2005) [12] reported that the anaerobic degradation of complex particulates similar to PSSS and OFMSW, via hydrolysis and acidogenesis, produced a range of products, particularly VFAs such as heptanoic and iso/n-hexanoic acids. Thus, the low concentrations of these acids in Vessel A could have indicated catabolism to acetic and propionic acids. In general, acetic and propionic acids were the principal products in Vessel A during Phase One, which suggested that hydrolytic, acidogenic and acetogenic microorganisms were established within this Vessel at a dilution rate of 0.01 h^{-1}.

In Vessels B and C, decreases in valeric and butyric acid concentrations to below detection and to > 0.251 mM in Vessel D suggested catabolism whilst concentration reductions in acetic (> 1 mM) and propionic (> 0.5 mM) acids in Vessels B-D were possibly indicative of sulphate-reducing bacteria or methanogenic archaea activity [37,38].

On day 162, the concentrations of acetic and propionic acids increased to 13.1 mM and 15.7 mM, respectively in Vessel C and, to a lesser degree, in Vessels B and D with concomitant pH decreases to 3.5, 3.76 and 5.25, respectively. The increases were not detected in previous samples and therefore did not originate from Vessel A. A possible explanation may have been the transfer of solid particulates down the array.

Throughout Phase One, progressive decreases in residual sulphate concentration of > 0.9 mM (Vessel A) and > 3.4 mM (Vessel B) were recorded with corresponding increases in sulphide concentrations of > 0.4 mM and > 1.3 mM (Figure 3). Sulphate was not detected in Vessels C or D, while sulphide concentrations between 0.3 and 0.02 mM were recorded for Vessel C but were below detection in Vessel D. This suggested the presence of an active sulphate-reducing community in Vessel A and/or Vessel B. The absence and/or low concentrations of sulphide observed in Vessels B-D could have resulted from wall growth sulphide precipitation as metallic sulphides [39,40]. The formation of metal sulphides and their accumulation could explain the lack of methane [39,40] and low biogas production (0.04 to 0.12 *l* biogas l^{-1} culture volume d^{-1}) compared to literature reports [33,41,42].

The DGGE profiles for the 'universal'16S rRNA (a), sulphate-reducing (b) and archaeal (data not shown) genes or communities exemplified microbial diversity in the influent PSSS and Vessels A–D for days 150, 154 and 166 (Figure 4). The highest richness of 52 operational taxonomical units (OTUs) was recorded for the 'universal' 16S RNA gene profiles, compared with the sulphate-reducing bacteria (SRB) (47 OTUs) and archaeal (28 OTUs) communities. So, although the SRB profiles were largely similar, suggesting an even strain distribution between the influent primary settled sewage sludge and Vessels A-D for all three sampling times, the 'universal' profiles were characterised by an additional five bands.

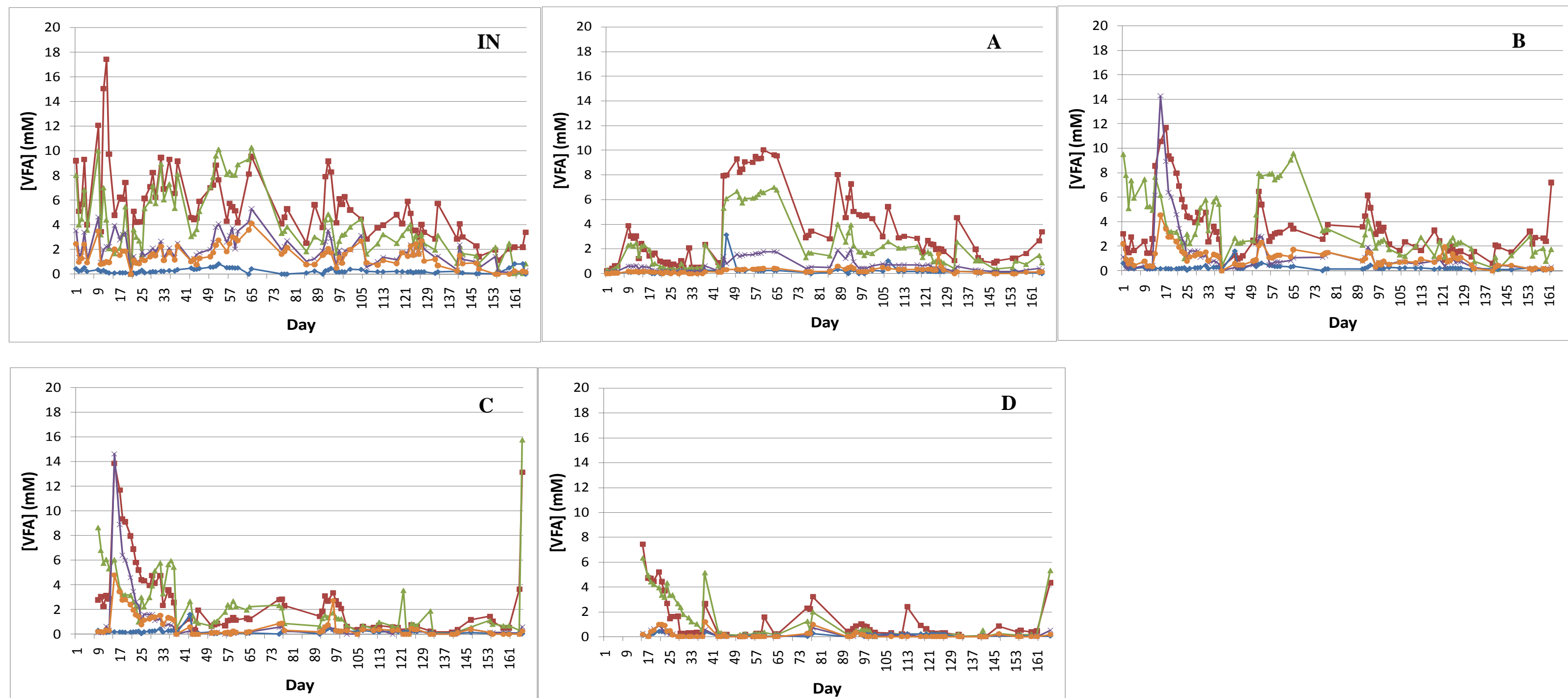
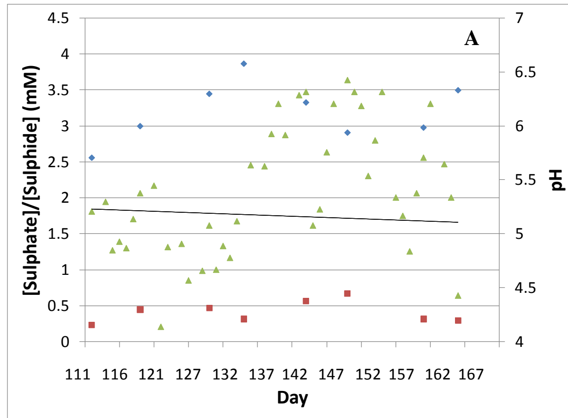


Figure 2. Changes in concentrations of acetic (■), propionic (▲), iso-butyric (♦), butyric (x) and valeric (●) acids, in the influent (IN) and during the anaerobic catabolism of primary settled sewage sludge and the organic fraction of municipal solid waste in Vessels A-D.



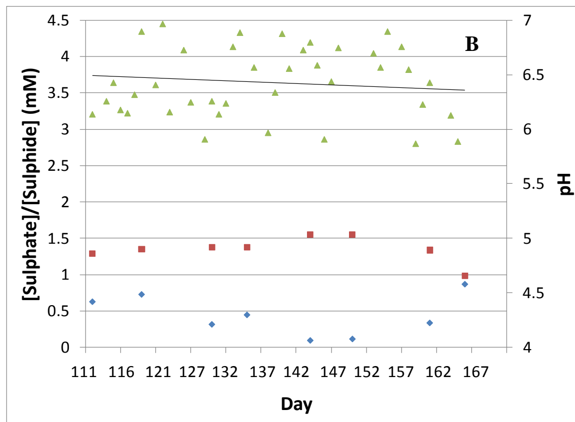


Figure 3. Changes in sulphate (♦) and sulphide (■) concentrations and pH (▲) in Vessels A and B during the anaerobic catabolism of primary settled sewage sludge and the organic fraction of municipal solid waste.

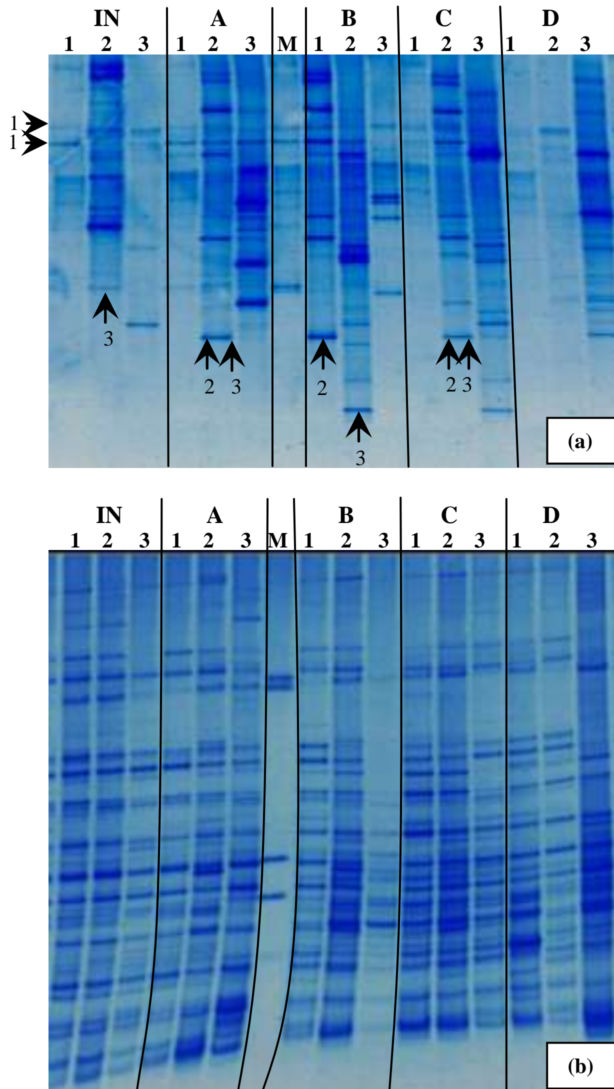


Figure 4. 'Universal' 16S rRNA gene (a) and sulphate-reducing bacteria (b) DGGE profiles of the influent (IN) and Vessels A-D on days 150 (1), 154 (2) and 166 (3). M designates the molecular weight marker Hyperladder I (Bioline).

Also, with the exception of two bands that were detected across most of the gel (Arrows 1) the 16S rDNA profiles showed recurrent shifts in the presence/absence and numerical dominances of most OTUs. There was, however, a common sub-pattern (Arrows 2) in Vessels A, B and C at days 154, 150 and 154, respectively.

Of the operational taxonomic units which originated from the primary settled sewage sludge influent, 15, 35 and 13 were 'universal', sulphate-reducing bacterial and archaeal, respectively. Throughout Phase One continuous re-inoculation from the PSSS may have affected the variability of the bacterial DGGE profiles although as Vessel A showed the greatest 16S rRNA gene diversity with 18 bands not present in the influent this is doubtful. The shift in bacterial diversity could have been indicative of the shift from an oxic to an anoxic environment. With the individual dilution rates of each vessel selected to ensure minimum species washout from the

total array, most species introduced via the influent were detected in all four vessels. This was most evident on day 154 when, with the exception of Vessel D, the high diversity of the influent was maintained in Vessels A-C (Arrows 3). However, on any given day it is invalid to make direct comparisons between individual vessels since steady states have not been reached in comparison with the preceding vessel. Thus, comparisons can only be made when each vessel has reached steady state.

It is generally accepted that the occurrence of archaea in anaerobic systems represents methanogenic communities [31,43,44]. Since these often occur in low copy numbers, and despite known limitations where the two-step analysis process possibly increases the potential for bias, nested PCR is used to facilitate their amplification [31,45]. Therefore, the shifts in the archaeal community profiles which were possibly due to fluctuations in both VFA concentrations (Figure 2) and pH, could also have reflected PCR bias. Nevertheless, the microbial profiles showed high population diversity and variability indicating that the community structure was complex and dynamic as reported previously [19,46-48].

Phase Two

The fluctuations in chemical and molecular profiles/characteristics discussed above, which, in part, may have been due to the inherently heterogeneous PSSS and OFMSW, were countered by the introduction of a model medium. The medium contained acetic, propionic, butyric and valeric acids in concentrations of 9.9, 7.2, 3.3 and 2.55 mM, respectively with 3.6 mM sulphate.

During this Phase, Vessel I was generally characterised by an increase in acetic acid concentration (9.9 to 13.7 mM) and decreases in propionic (7.2 to 1.9 mM), butyric (3.3 to 0.2 mM) and valeric (2.55 to 0.3 mM) acids (Figure 5). This suggested catabolism of propionic, butyric and valeric acids occurred in Vessel I at a dilution rate of 0.1 d^{-1}.
On days 13, 24, 25 and 30, Vessel I recorded decreased concentrations of acetic acid concomitant with increased propionic, butyric and valeric acid concentrations and stoichiometric analysis suggested a reduction in acetogenic activity. In contrast, on day 26, decreased butyric and valeric acid concentrations in conjunction with increased acetic acid possibly reflected higher acetogenic activity.

On day 30 Vessels II-IV were characterised by reduced concentrations of: acetic acid < 12.8 mM (II), < 8.7 mM (III), < 2.8 mM (IV); propionic acid < 2.5 mM (II), < 0.7 mM (III) and < 0.3 mM (IV); butyric acid < 0.4 mM (II), < 0.2 mM (III), < 0.1 mM (IV); and valeric acid < 0.5 mM (II), < 0.1 mM (III), < 0.1 mM (IV).

The lowered residual sulphate concentrations of < 1.6 mM (Vessel I) and < 1 mM (Vessel II), and the corresponding increased sulphide concentrations of < 1.2 mM (Vessel I) and < 1.4 mM (Vessel II) (Figure 6) recorded throughout Phase Two, suggested the consolidation of sulphate reduction in Vessel I. Sulphate-reducing bacteria are metabolically diverse and are capable of oxidising completely a wide range of substrates including propionic, butyric and valeric acids through multiple simultaneous pathways with and without sulphate [49,50]. Thus, catabolism of the volatile fatty acids in Vessel I was possibly due to SRB activity.

With the exception of the archaea, Phase Two DGGE banding patterns revealed simpler profiles than those observed for Phase One with 37, 39 and 28 OTUs recorded for the ‘universal’, sulphate-reducing bacterial and archaeal communities, respectively (Figure 7). Previous studies with synthetic and complex media have also reported simplified banding patterns [43,44,51] compared to reactors operating with wastewaters [52-54]. In the absence of continual re-inoculation from the primary settled sewage sludge and the organic fraction of municipal solid waste a simplified banding pattern resulted, which allowed for an increased focus on community dynamics during reactor operation. The ‘universal’ profiles showed several shared bands across the gel (a, Arrows 4) which varied only in numerical abundance. On day 28, Vessels I-III showed a replicated pattern with the numerical abundance of common bands (Arrows 5) either increased or decreased. In general, the variations in diversity and abundance observed in Vessels

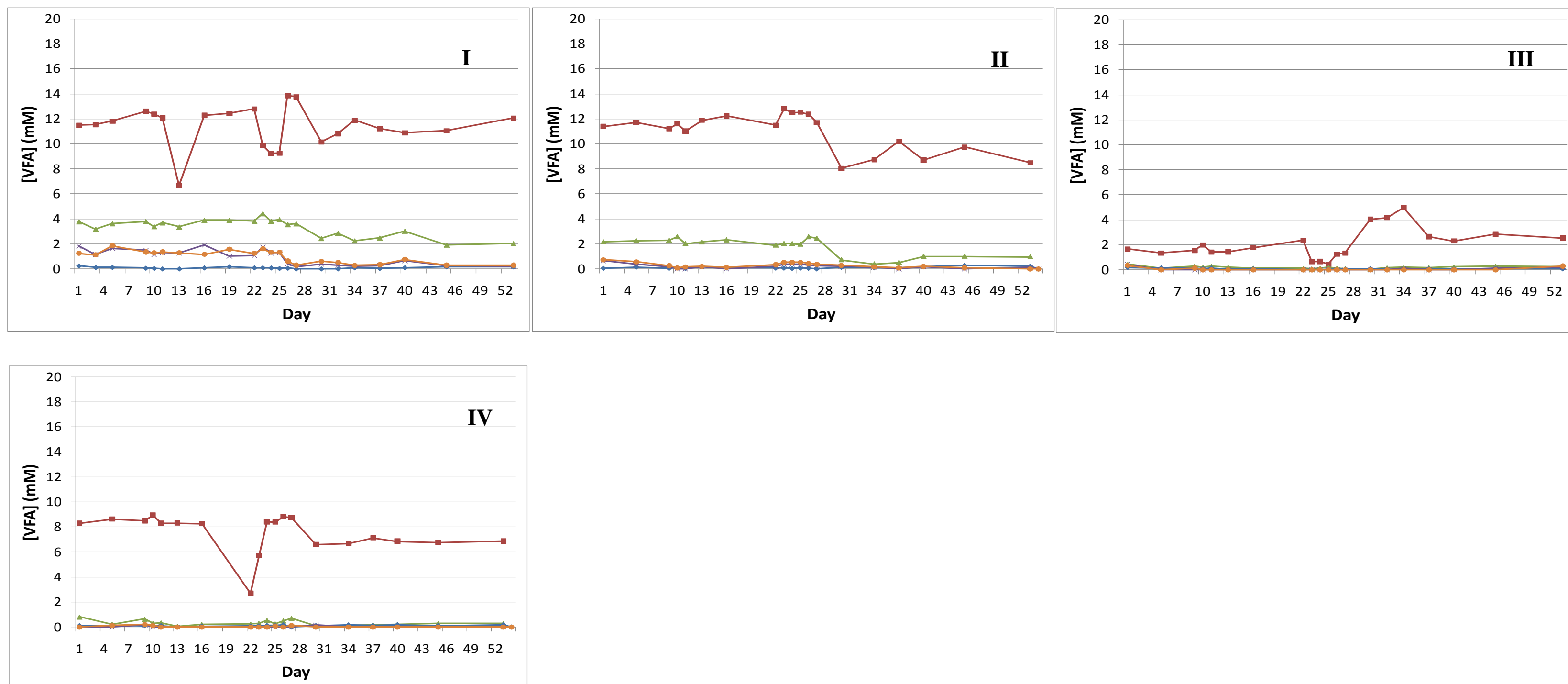


Figure 5. Changes in concentrations of acetic (■), propionic (▲), iso-butyric (♦), butyric (x) and valeric (●) acids during the anaerobic catabolism of a mixed volatile fatty acid feed in Vessels I-IV.

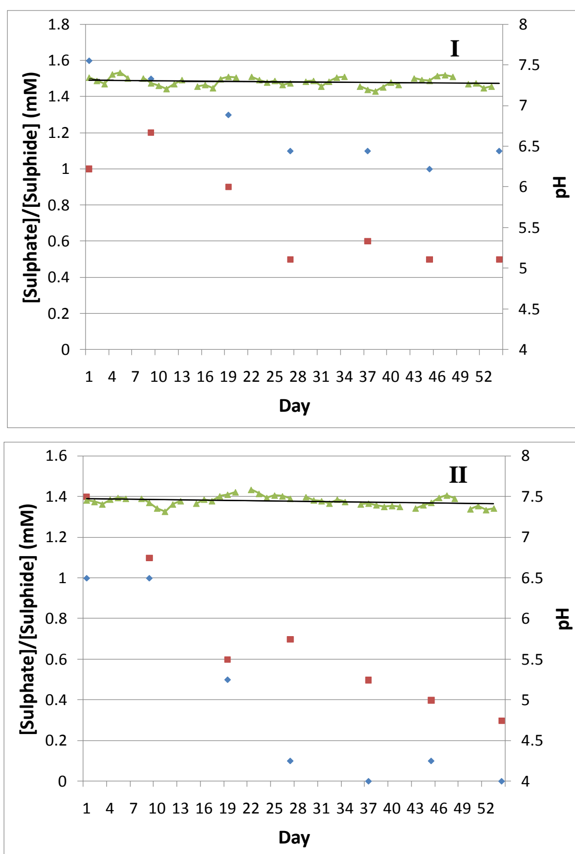


Figure 6. Changes in sulphate (♦) and sulphide (■) concentrations and pH in Vessel I and II during the anaerobic catabolism of a mixed volatile fatty acid influent.

I-IV for days 10, 18 and 28 were probably indicative of the species complement adjusting to the model feed.

As with the 'universal' bacteria, a decrease in diversity was recorded for the SRB communities (b). For all three sampling times, Vessel I recorded the most diverse and even profile with the highest number of numerically dominant bands that also had high-GC contents (Arrows 6). With the exception of Vessel III on day 10, Vessels II and III were each characterised by a replicated pattern with slight changes only in numerical abundance. For Vessel II, the changes in numerical dominance of some bands and the appearance of mostly non-numerically dominant bands (Arrows 7) between days 18 and 28, probably reflected the increased sulphate reduction. For Vessel IV, the increased diversity recorded from day 10 (1 OTU) to days 18 and 28 (22 and 24 OTUs) was possibly due also to species displacement down the array. A numerically abundant band that was recorded across all four Vessels (Arrows 8) highlighted a strain whose phylogenetic and functional significance should be determined.

The archaeal fingerprint during Phase Two (c) showed a slightly more complex community structure (32 OTUs) whilst operating with the model feed than for the waste feeds (28 OTUs). Generally, these profiles were characterised by 1-4 numerically dominant operational taxonomic units per vessel for the three sampling days. However, more low intensity bands were visible which suggested that the use of the model feed facilitated their detection. With the exception of

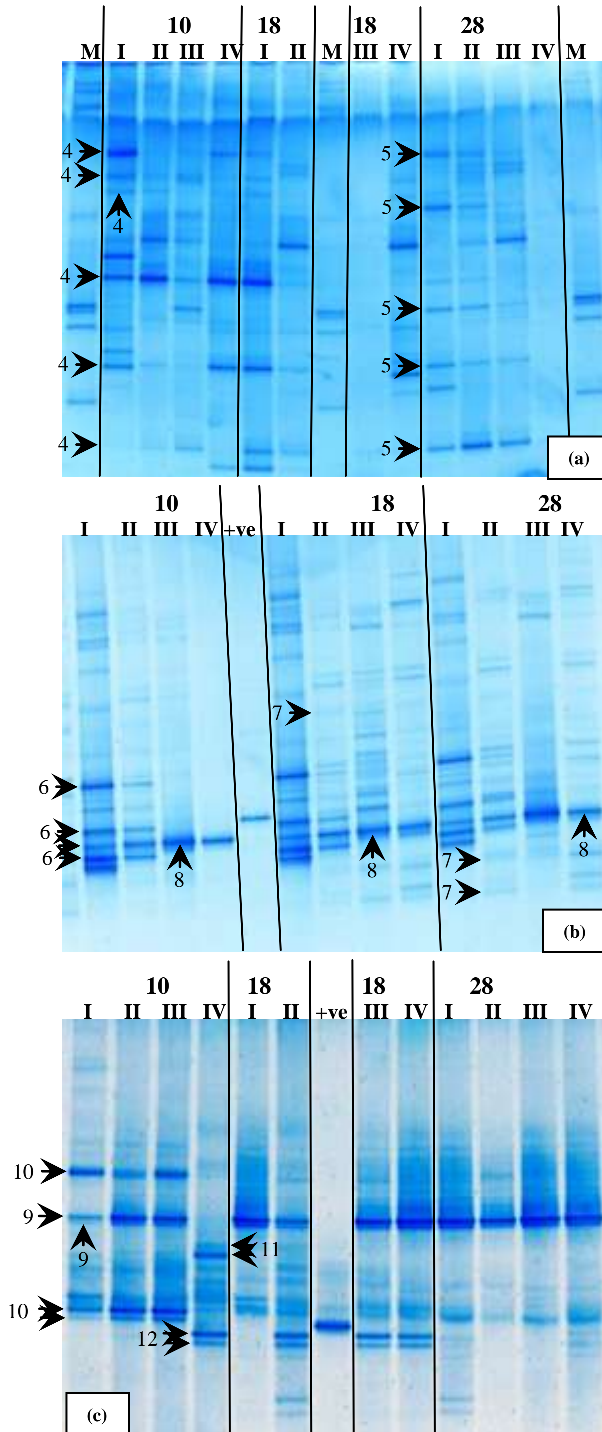


Figure 7. 'Universal' 16S rRNA gene (a), SRB (b) and archaea (c) DGGE profiles of Vessels I-IV on days 10, 18 and 28. The positive controls for the SRB (*Desulfovibrio ferrooxidans*, DSM642) and the archaeal (*Methanobacterium wolfei*, DSM2970) species are designated by +ve.

Vessel IV on day 10, two closely related species components with near constant numerical dominance occurred across the gel (Arrows 9). Also, Vessels I-III had very similar profiles with three high abundance bands (Arrows 10) that were little or non-detectable in Vessel IV. On day 10, Vessel IV was characterised by two unique bands (Arrows 11) and an additional two bands (Arrows 12) that were also detected on day 18 in Vessels II and III but were barely visible in Vessel I on day 28. The presence of archaeal bands in Vessels I and II operated at dilution rates of 0.1 and 0.05 h^{-1} may be explained by: not all the archaea were methanogens, hence they may have had higher specific growth rates; and biofilm formation on the vessel walls allowed growth independent of the dilution rate. The variability of the profiles suggested that the archaeal population was in a transient state following the change of feeds although further DGGE analysis would be required to confirm this.

CONCLUSION

Molecular analyses of the microbial associations underpinning anaerobic catabolism of primary settled sewage sludge and the organic fraction of municipal solid waste were hindered by the inherent microbiological load of the combined wastes as denaturing gradient gel electrophoresis is limited to the detection of only the numerically dominant members consisting of > 1% of the total community [52]. Therefore, the use of mRNA based techniques to investigate active community members [55] should allow a more focused analysis. The variability of the influent used for Phase One produced a range of volatile fatty acids in Vessel A, and the changes in the 'universal' and archaeal profiles may have reflected this. Thus, it is impossible to relate, with any degree of confidence, the changes in the chemical and molecular profiles.

The use of the model feed in Phase Two both produced simpler 'universal' and sulphate-reducing bacterial profiles and allowed the detection of less numerically dominant archaeal OTUs. Spatial separation was most apparent for the SRB communities but further analysis is required for the 'universal' and archaeal species complements. Archaeal operational taxonomic units in Vessels I and II suggested the presence of non-methanogenic species and/or biofilm methanogens. Therefore, further comparative analyses by DGGE, sequencing and microarray probing [55,56] of the planktonic and surface-attached communities should provide insights of the vessel species complements.

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REFERENCES

1. Department for Business and Regulatory Reform (2008) Digest of United Kingdom Energy Statistics 2008.
2. Department for Energy and Climate Change (2009) Digest of United Kingdom Energy Statistics 2009.
3. Department for Environment, Food and Rural Affairs (2007) UK Biomass Strategy.
4. Department for Environment, Food and Rural Affairs (2007) Waste Strategy for England 2007.
5. Nand K., Devi S.S., Viswanath P., Deepak S. and Sarada R. (1991) Anaerobic digestion of canteen wastes for biogas production: Process optimisation. Process Biochemistry, 26, 1-5.
6. Rivière D., Desvignes V., Pelletier E., Chaussonnerie S., Guermazi S., Weissenbach J., Li T., Camacho P. and Sghir A. (2009) Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. The ISME Journal, 3, 700-714.
7. Vavilin V.A., Rytov S.V. and Lokshina L.Y. (1996) A description of hydrolysis kinetics in anaerobic degradation of particulate organic matter. Bioresource Technology, 56, 229-237.

8. Batstone D.J., Keller J., Angelidaki I., Kalyuzhnyi S., Pavlostathis S.G., Rozzi A., Sanders W., Siegrist H. and Vavilin V. (IWA Task Group on Modelling of Anaerobic Digestion Processes) (2002) Anaerobic Digestion Model No. 1 (ADM1). IWA Publishing, London.
9. Stronach S.M., Rudd T. and Lester J.N. (1986) Anaerobic Digestion Processes in Industrial Wastewater Teatment. Springer-Verlag, Berlin.
10. Mata-Alvarez J., Macé S. and Llabrés P. (2000) Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Bioresource Technology, 74, 3-16.
11. Ragsdale S.W. and Pierce E. (2008) Acetogenesis and the Wood–Ljungdahl pathway of CO_2 fixation. Biochimica et Biophysica Acta - Proteins and Proteomics, 1784, 1873-1898.
12. Gerardi M.H. (2003) The Microbiology of Anaerobic Digesters. Wiley-IEEE. New Jersey, USA.
13. Ahring B.K., Sandberg M. and Angelidaki I. (1995) Volatile fatty acids as indicators of process imbalance in anaerobic digestors. Applied Microbiology and Biotechnology, 43, 559-565.
14. Liu J., Olsson G. and Mattiasson B. (2004) Short-term BOD (BODst) as a parameter for on-line monitoring of biological treatment process: Part I. A novel design of BOD biosensor for easy renewal of bio-receptor. Biosensors and Bioelectronics, 20, 562-570.
15. Ağdağ O.N. and Sponza D.T. (2005) Co-digestion of industrial sludge with municipal solid wastes in anaerobic simulated landfilling reactors. Process Biochemistry, 40, 1871-1879.
16. Talbot G., Topp E., Palin M.F. and Massé D.I. (2008) Evaluation of molecular methods used for establishing the interactions and functions of microorganisms in anaerobic bioreactors. Water Research, 42, 513-537.
17. Tatara M., Makiuchi T., Ueno Y., Goto M. and Sode K. (2008) Methanogenesis from acetate and propionate by thermophilic down-flow anaerobic packed-bed reactor. Bioresource Technology, 99, 4786-4795.
18. Cook K.L., Rothrock Jr. M.J., Lovanh N., Sorrell J.K. and Loughrin J.H. (2010) Spatial and temporal changes in the microbial community in an anaerobic swine waste treatment lagoon. Anaerobe, 16, 74-82.
19. O'Reilly J., Lee C., Chinalia F., Collins G., Mahony T. and O'Flaherty V. (2010) Microbial community dynamics associated with biomass granulation in low-temperature (15°C) anaerobic wastewater treatment bioreactors. Bioresource Technology, 101, 6336-6344.
20. Khelifi E., Bouallagui H., Touhami Y., Godon J. and Hamdi M. (2009) Bacterial monitoring by molecular tools of a continuous stirred tank reactor treating textile wastewater. Bioresource Technology, 100, 629-633.
21. Pobeheim H., Munk B., Müller H., Berg G. and Guebitz G.M. (2010) Characterization of an anaerobic population digesting a model substrate for maize in the presence of trace metals. Chemosphere, 80, 829-836.
22. Wang Z., Wang W., Zhang X. and Zhang G. (2009) Digestion of thermally hydrolyzed sewage sludge by anaerobic sequencing batch reactor. Journal of Hazardous Materials, 162, 799-803.
23. Xu K., Liu H. and Chen J. (2010) Effect of classic methanogenic inhibitors on the quantity and diversity of archaeal community and the reductive homoacetogenic activity during the process of anaerobic sludge digestion. Bioresource Technology, 101, 2600-2607.
24. Bibby K., Viau E. and Peccia J. (2010) Pyrosequencing of the 16S rRNA gene to reveal bacterial pathogen diversity in biosolids. Water Research, 44, 4252-4260.
25. Kröber M., Bekel T., Diaz N.N., Goesmann A., Jaenicke S., Krause L., Miller D., Runte K.J., Viehöver P., Pühler A. and Schlüter A. (2009) Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. Journal of Biotechnology, 142, 38-49.
26. Kanagawa T. (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). Journal of Bioscience and Bioengineering, 96, 317-323.

27. Coutts D.A.P., Senior E. and Balba M.T.M. (1987) Multi-stage chemostat investigation of interspecies interactions in a hexanoate-catabolizing microbial association isolated from anoxic landfill. Journal of Applied Bacteriology, 62, 251-260.
28. Lemarchand K., Berthiaume F., Maynard C., Harel J., Payment P., Bayardelle P., Masson L. and Brousseau R. (2005) Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. Journal of Microbiological Methods, 63, 115-126.
29. Manefield M., Whiteley A.S., Griffiths R.I. and Bailey M.J. (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. Applied and Environmental Microbiology, 68, 5367-5373.
30. Geets J., Borremans B., Diels L., Springael D., Vangronsveld J., van der Lelie D. and Vanbroekhoven K. (2006) *DsrB* gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria. Journal of Microbiological Methods, 66, 194-205.
31. Keyser M., Witthuhn R.C., Lamprecht C., Coetzee M.P.A. and Britz T.J. (2006) PCR-based DGGE fingerprinting and identification of methanogens detected in three different types of UASB granules. Systematic and Applied Microbiology, 29, 77-84.
32. Seghezzo L., Zeeman G., van Lier J.B., Hamelers H.V.M. and Lettinga G. (1998) A review: The anaerobic treatment of sewage in UASB and EGSB reactors. Bioresource Technology, 65, 175-190.
33. Carballa M., Omil F., Alder A.C. and Lema J.M. (2006) Comparison between the conventional anaerobic digestion of sewage sludge and its combination with a chemical or thermal pre-treatment concerning the removal of pharmaceuticals and personal care products. Water Science and Technology, 53, 109-117.
34. Zhu H., Parker W., Basnar R., Proracki A., Falletta P., Béland M. and Seto P. (2008) Biohydrogen production by anaerobic co-digestion of municipal food waste and sewage sludges. International Journal of Hydrogen Energy, 33, 3651-3659.
35. Horiuchi J.-I., Shimizu T., Tada K., Kanno T. and Kobayashi M. (2002) Selective production of organic acids in anaerobic acid reactor by pH control. Bioresource Technology, 82, 209-213.
36. Roy C.S., Talbot G., Topp E., Beaulieu C., Palin M. and Massé D.I. (2009) Bacterial community dynamics in an anaerobic plug-flow type bioreactor treating swine manure. Water Research, 43, 21-32.
37. Bitton G. (1994) Wastewater Microbiology. Wiley-Liss, Chichester, UK.
38. Boone D.R. and Castenholz R.W. (2001) Bergey's Manual of Systematic Bacteriology: The Archaea and the Deeply Branching and Phototrophic Bacteria Vol. 1. Springer, New York.
39. Isa Z., Grusenmeyer S. and Verstraete W. (1986) Sulfate reduction relative to methane production in high-rate anaerobic digestion: Microbiological aspects. Applied and Environmental Microbiology, 51, 580-587.
40. Chen Y., Cheng J.J. and Creamer K.S. (2008) Inhibition of anaerobic digestion process: A review. Bioresource Technology, 99, 4044-4064.
41. Habiba L., Hassib B. and Moktar H. (2009) Improvement of activated sludge stabilisation and filterability during anaerobic digestion by fruit and vegetable waste addition. Bioresource Technology, 100, 1555-1560.
42. Sosnowski P., Wieczorek A. and Ledakowicz S. (2003) Anaerobic co-digestion of sewage sludge and organic fraction of municipal solid wastes. Advances in Environmental Research, 7, 609-616.
43. O’Reilly J., Lee C., Collins G., Chinalia F., Mahony T. and O'Flaherty V. (2009) Quantitative and qualitative analysis of methanogenic communities in mesophilically and psychrophilically cultivated anaerobic granular biofilims. Water Research, 43, 3365-3374.
44. Hirasawa J.S., Sarti A., Del Aguila N.K.S. and Varesche M.B.A. (2008) Application of molecular techniques to evaluate the methanogenic archaea and anaerobic bacteria in the presence of oxygen with different COD:sulfate ratios in a UASB reactor. Anaerobe, 14, 209-218.

45. Liu X., Zhang Y., Yang M., Wang Z. and Lv W. (2007) Analysis of bacterial community structures in two sewage treatment plants with different sludge properties and treatment performance by nested PCR-DGGE method. Journal of Environmental Sciences, 19, 60-66.
46. Akarsubasi A.T., Ince O., Kirdar B., Oz N.A., Orhon D., Curtis,T.P., Head I.M. and Ince B.K. (2005) Effect of wastewater composition on archaeal population diversity. Water Research, 39, 1576-1584.
47. Palatsi J., Illa J., Prenafeta-Boldú F.X., Laureni M., Fernandez B., Angelidaki I. and Flotats X. (2010) Long-chain fatty acids inhibition and adaptation process in anaerobic thermophilic digestion: Batch tests, microbial community structure and mathematical modelling. Bioresource Technology, 101, 2243-2251.
48. Connaughton S., Collins G. and O'Flaherty V. (2006) Development of microbial community structure and actvity in a high-rate anaerobic bioreactor at 18°C. Water Research, 40, 1009-1017.
49. Visser A., Hulshoff P.L.W. and Lettinga G. (1996) Competition of methanogenic and sulfidogenic bacteria. Water Science and Technology, 33, 99-110.
50. Thauer R.K., Stackerbrandt E. and Hamilton H.A. (2007) Energy metabolism and phylogenetic diversity of sulphate-reducing bacteria. In: Sulphate-Reducing Bacteria: Environmental and Engineered Systems. 1st edn. (Ed. by L. Barton & W.A. Hamilton), pp 1-38. Cambridge University Press, UK.
51. van Leerdam R.C., de Bok F.A.M., Bonilla-Salinas M.,van Doesburg W., Lomans B.P., Lens P.N.L., Stams A.J.M. and Janssen A.J.H. (2008) Methanethiol degradation in anaerobic bioreactors at elevated pH (8): Reactor performance and microbial community analysis. Bioresource Technology, 99, 8967-8973.
52. Malin C. and Illmer P. (2008) Ability of DNA content and DGGE analysis to reflect the performance condition of an anaerobic biowaste fermenter. Microbiological Research, 163, 503-511.
53. LaPara T.M., Nakatsu C.H., Pantea L.M. and Alleman J.E. (2002) Stability of the bacterial communities supported by a seven-stage biological process treating pharmaceutical wastewater as revealed by PCR-DGGE. Water Research, 36, 638-646.
54. Liu C., Yuan X., Zeng G., Li W. and Li J. (2008) Prediction of methane yield at optimum pH for anaerobic digestion of organic fraction of municipal solid waste. Bioresource Technology, 99, 882-888.
55. Bodrossy L., Stralis-Pavese N., Konrad-Köszler M., Weilharter A., Reichenauer T.G., Schöfer D. and Sessitsch A. (2006) mRNA-based parallel detection of active methanotroph populations by use of a diagnostic microarray. Applied and Environmental Microbiology, 72, 1672-1676.
56. Gao H.,Yang Z.K., Gentry T.J., Wu L., Schadt C.W. and Zhou J. (2007) Microarray-based analysis of microbial community RNAs by whole-community RNA amplification. Applied and Environmental Microbiology, 73, 563-571.