Performance of a three-stage membrane bioprocess treating the Organic Fraction of Municipal Solid Waste and evolution of its archaeal and bacterial ecology

Antoine P. Trzcinski [a], Michael J. Ray [b], David C. Stuckey [a]*

[a] Department of Chemical Engineering, Imperial College of Science and Technology and Medicine, Prince Consort Road, London SW7 2AZ, UK.

[b] Department of Biology, Imperial College of Science and Technology and Medicine, Prince Consort Road, London SW7 2AZ, UK.

E-mail: *a.trzcinski05@ic.ac.uk*. Tel.: +44 (0) 7826814053

* Corresponding author. E-mail: *d.stuckey@ic.ac.uk*. Tel.: +44 (0)20 7594 5591

Fax: +44 (0)20 7594 5629

[b] E-mail: michael.ray@imperial.ac.uk. Tel.: +44 (0)20 7589 5111

ABSTRACT

A novel three-stage bioprocess achieved 75% Volatile Solids (VS) removal at an Organic Loading Rate (OLR) of 4g VS/L.day, a Solids Retention Time (SRT) of 66 days, a Hydraulic Retention Time (HRT) of 20 days, at a temperature of 35°C. The bioprocess consisted of an anaerobic hydrolytic reactor (HR) where the solids and liquid fractions of the Organic Fraction of the Municipal Solid Waste (OFMSW) were separated with a mesh. The leachate was pumped to a Submerged Anaerobic Membrane Bioreactor (SAMBR) and the treated permeate was polished in an Aerobic Membrane Bioreactor (AMBR). Denaturing Gradient Gel Electrophoresis (DGGE) and DNA sequencing analyses indicated that the increase in methane content in the HR caused by the excess sludge recycle from the SAMBR was associated with an increase in the number of hydrogenotrophic species, mainly *Methanobrevibacter sp.*, *Methanobacterium formicicum* and *Methanosarcina sp*. At 20ºC VS removal dropped to 50% in the HR and some DGGE bands disappeared when compared to 35°C samples, while some bands such as the one corresponding to *Ruminococcus flavefaciens* were reduced in intensity. The species associated with the COD-polishing properties of the AMBR correspond to the genera Pseudomonas, Hyphomonas and Hyphomicrobiaceae. These results highlight the positive effect of recycling the excess sludge from the SAMBR to re-inoculate the HR with hydrogenotrophic species.

Keywords: Anaerobic Digestion; Organic Fraction of Municipal Solid Waste; Denaturing Gradient Gel Electrophoresis; Membrane Bioreactor; nitrification.

List of abbreviations

AMBR	Aerobic Membrane Bioreactor
AOB	Ammonia-Oxidizing Bacteria
COV	Coefficient of Variation (%)
DGGE	Denaturing Gradient Gel Electrophoresis

GPR	Gas Production Rate (L STP.day^{-1})
HR	Hydrolytic Reactor
HRT	Hydraulic Retention Time (days)
LMH	Flux (L.m^{-2}.h^{-1})
MSW	Municipal Solid Waste
MLTSS	Mixed Liquor Total Suspended Solids (g.L^{-1})
MLVSS	Mixed Liquor Volatile Suspended Solids (g.L^{-1})
NLR	Nitrogen Loading Rate (g N.L^{-1}.day^{-1})
NOB	Nitrite-Oxidizing Bacteria
OFMSW	Organic Fraction of Municipal Solid Waste
OLR	Organic Loading Rate (g COD or VS.L^{-1}.day^{-1})
PCR	Polymerase Chain Reaction
SAMBR	Submerged Anaerobic Membrane Bioreactor
SCOD	Soluble Chemical Oxygen Demand (mg.L^{-1})
SRT	Solid Retention Time (days)
STP	Standard Temperature and Pressure
TCOD	Total Chemical Oxygen Demand (mg.L^{-1})
UASB	Upflow Anaerobic Sludge Blanket
UF	Ultrafiltration
VFA	Volatile Fatty Acids (mg.L^{-1})
VS	Volatile Solids

1. Introduction

Municipal Solid Waste (MSW) is a growing concern in the world, and European authorities discourage municipalities landfilling as their waste management strategy. In this respect, anaerobic digestion offers many advantages such as the destruction of organic components without the addition of oxygen, the production of useful by-products such as a gaseous fuel and stabilized solid residue that can be sold as a soil fertilizer (Speece, 1996).

Anaerobic digestion at lower temperatures is interesting because it allows the operational costs of the digester to be reduced. It is known that lower temperatures can affect gas production rates because of the lower substrate affinity of mesophilic bacteria under these conditions. Many researchers are engaged in studies on anaerobic digestion, but relatively little work has been done regarding the production of biogas at sub-mesophilic temperatures. Table 1 shows different processes treating organic solid waste with their performances at low temperatures.

Anaerobic digestion of organic solids is also characterized by particulate COD which generates wastewater or leachate with a very high organic content. Some authors have treated wastewater with high suspended solids concentrations (Harada et al., 1995; Hogetsu et al., 1992; Kimura, 1991; Norddahl and Rohold, 2000; Robinson, 2005). OLRs of 3-5 kg COD.m^{-3}.d^{-1} were achieved with COD removals of 80% or higher. Particulate cellulose has been treated in an UASB fitted with a cross-flow UF membrane, and there was no tendency for cellulose to accumulate in the reactor at 2 days HRT and an OLR of 2.5 kg COD. m^{-3}.d^{-1},of which 50% was particulate COD (Harada et al., 1995). A COD removal of 98% could be maintained and the flux decreased significantly but remained in the range 21-42 LMH due to frequent washings and membrane replacement every 40-50 days of operation. The flux for a SAMBR treating leachate has been reported to be normally in the range 5-10 LMH (Robinson, 2005). The use of a membrane allows the methanogens to be kept within the second reactor, which enables it to reach HRTs as low as 0.4 days and high organic loading rates of 20 g COD.L^{-1}.d^{-1} (Trzcinski and Stuckey, 2009). However, the lignocellulosic suspended solids in the leachate built up in the SAMBR because of the complete retention of solids, therefore, excess sludge should be removed from the SAMBR to keep the MLTSS below 20 g/L, as this was found to be a threshold above which the flux dropped considerably (Trzcinski and Stuckey, 2009). Ideally, a workable permeate flux of 3-5 LMH should be obtained to reach minimum HRTs of 0.25-0.42 days.

However, little is known about the effect of recycling excess sludge on the microbial populations and the performance of the first and second stage of a two-stage process treating the OFMSW with liquid recycle. Therefore, the objective of this paper was to find a trade off between the SRT and the COD removal at the lowest HRT in the SAMBR. Another objective was to investigate the effect of sludge recycle to the HR on the archaeal and bacterial populations. In addition to detecting species normally found by traditional culturing methods,

DGGE offers the prospect of detecting species that may be present in the habitat in a viable but non-culturable state. The objective of this study was to apply DGGE to this novel three-stage membrane process to understand in a more fundamental manner changes in the microbial ecology and physiology of the system in relation to process operating conditions.

2. Materials and methods

2.1 Feedstock

The simulated OFMSW mixture used in this study consisted of 41% Kitchen Waste (KW), 11% Garden Waste (GW) and 48% Paper Waste (PW) on a wet basis. The collection, storage and preparation of the OFMSW feedstock can be found elsewhere (Trzcinski and Stuckey, 2009). The properties of the feedstock can be found in Table 2.

2.2 Reactors

The HR had a working volume of 10 litres and was mixed intermittently (15 min ON-15 min OFF). The leachate was treated in a SAMBR, while the permeate of the SAMBR was post-treated aerobically in an AMBR. The permeate of the AMBR was recycled to the HR in order to maintain the moisture and alkalinity of the system, and also to dilute the incoming COD and organic acids. The SAMBR and the AMBR were 3-L reactors maintained at 35ºC and fitted with new Kubota polyethylene flat sheet membranes with a total surface of 0.1 m^2 and a pore size of 0.4 μm. Details of the HR, SAMBR and AMBR can be found elsewhere (Trzcinski and Stuckey, 2009). The HR was operated at 35ºC until day 100, and then at 20ºC until the end of the experiment. Fresh tap water was added to the HR with the feedstock on days 5, 6, 7, 12, 17, 20, 25, 27, 30, 34, 36, 38, 42, 44, 46, 47, 50, 57, 62, 68, 70, 73, 80, 85, 92, 99, 106, 114, 117 and 120 in order to keep a constant working volume of 10 litres.

2.3 Inoculation and Start-up

The HR was inoculated with 9 L of anaerobic sludge from a conventional full scale digester treating waste activated sludge (Mogden, UK) (MLTSS = 36.5 and MLVSS = 20.4 g/L). The final volume was achieved with 1 L tap water containing 200 g of OFMSW feedstock to reactivate the inoculum. The HR was fed daily at a constant OLR of 4g VS/L.day throughout the experiment. The SAMBR was started up on day 2 with 300 mL sludge from a SAMBR treating leachate (Trzcinski and Stuckey, 2009), and the final volume was adjusted to 3L with the anaerobic biomedium defined in Owen et al. (1979). The initial MLTSS and MLVSS were 6.6 and 3 g/L, respectively. The AMBR was inoculated with the aerobic sludge fed on the permeate of a SAMBR treating leachate (Trzcinski and Stuckey, 2009). The initial MLTSS and MLVSS were 3.6 and 2 g/L, respectively.

2.4 Analytical Methods

The measurement of pH (Jenway) was accurate to within ±0.02 units. The Mixed Liquor Total Suspended Solids (MLTSS), Volatile Suspended Solids (MLVSS), Fixed Suspended Solids (FSS), Soluble Chemical Oxygen Demand (SCOD) and Total Chemical Oxygen Demand (TCOD) were measured as described in Standard Methods (APHA, 1999). Their coefficient of variation (COV) for ten identical samples was 4%, 3.1%, 7.1%, 2.6% and 9.9%, respectively. Volatile fatty acids (VFAs) were measured using a Shimadzu Gas Chromatograph with a flame-ionized detector and a SGE capillary column (12mx0.53mm ID-BP21 0.5µm). The COV was 3% for ten identical samples. The gas production rate (GPR) was measured using a Tedlar bag connected to the top of the HR, while the water (2% H_2SO_4, 10% NaCl) displacement method was used to measure the GPR in the SAMBR. The composition of biogas was determined using a Shimadzu GC-TCD fitted with a Porapak N column (1500×6.35 mm). The COV for 10 identical samples was 2%. Ammonia-Nitrogen

was measured using the Nesslerization method by reading absorbance at 425 nm. The COV was equal to 6.6% for 10 identical samples. Nitrite and nitrate were analyzed by Dionex Ion Chromatography. The COV for 5 identical samples was 1.8%.

For the DGGE analysis, the DNA from the mixed culture was extracted using the FastDNA SPin for soil kit from MP Biomedicals. Samples were taken from the HR on days 35, 99 and 137, from the SAMBR on days 66, 94 and 125, and from the AMBR on days 99 and 125. Archaeal DNA was amplified using a nested PCR reaction in a G-Storm thermocycler. The first PCR used the primers 46F and 1017R (Akarsubasi et al., 2005; Gray et al., 2002), then 1 µL of this PCR was used as the template for the second PCR reaction using the primers 344F-GC and Univ522R. Bacterial DNA was amplified using the primers 341F-GC and 907R (Fernandez et al., 2008; Liu et al., 2008). The DNA specific to ammonia-oxidizers was amplified using the primers CTO189FGC and CTO654R (Kowalchuk et al., 1998; Stephen et al., 1998). The presence of PCR products was confirmed by electrophoresis on 1% agarose gels stained with ethidium bromide. DGGE of the 16Sr DNA PCR products was carried out using the D CODE system (Bio-Rad Laboratories Ltd) according to the manufacturer's instructions and protocols. A 6% and a 8% polyacrylamide gel was used for the electrophoresis of PCR products generated with bacterial and archaeal primers, respectively. The PCR products were electrophorised at 60V for 16 hours at 65ºC with a denaturing gradient ranging from 40 to 60% for bacterial primers (Liu et al., 2008), 45-65% for archaeal primers and 38-50% for ammonia-oxidizing bacteria (Kowalchuk et al., 1998; Stephen et al., 1998). The gel was stained with SYBR green before visualizing on a UV transilluminator and imaged. The DGGE technique cannot differentiate between dead and living cells; band intensity is not always correlated with population density, as DNA extraction efficiencies vary between microorganisms (Prakitchaiwattana et al., 2004), and also because some primer pairs can show bias in amplification of some species over others resulting in misleading band

intensities on the DGGE gel (Wittebolle et al., 2008). The DGGE bands of greatest intensity were excised, eluted in DNA-free water overnight at 4ºC and then re-amplified. The purified PCR product was then cloned with the pCR 2.1-TOPO cloning kit (Invitrogen) according to the manual instructions. Sequencing was performed by MWG (London, UK). DNA sequences analyses were performed using the BLAST server of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Sequences from the DGGE analysis described above were deposited in the GenBank database under accession numbers GQ369766 to GQ369774 for Archaea and GQ369775 to GQ369787 for bacteria. All of the dendrograms were constructed using the neighbour-joining method (with bootstrapping) of the ClustalX2 programme.

3. Results and discussion

3.1 Performance of the Hydrolytic Reactor

3.1.1 Start-up

The HR was operated at a SRT of 66 days based on the daily mass of VS withdrawn from the HR. The HRT was maintained circa 20 days in the HR. The SRT and HRT were different because of the solid-liquid separation in the HR by means of the cylindrical 1 mm stainless steel mesh. Although the HR was fed at a constant OLR of 4g VS/L.day a constant strength was not observed in the leachate. Figure 1A shows that during start up the TCOD, SCOD and VFAs gradually increased. The undulating look of all the curves in Figure 1 is due to the addition of large amounts of fresh water on a single day rather than a small addition every day. The addition of a large quantity of fresh water in one feed to keep the working volume constant at 10 L may have resulted in the dilution of TCOD, SCOD, VFAs, alkalinity and a change in pH. In fact, the drops of SCOD and VFAs on days 28, 34 and 49 can all be

attributed to fresh water addition on the same day or a few days before sampling. The average fresh water addition was 150 mL/day, which is equivalent to 320 L/ton of feedstock treated. However, our process did not produce wastewater because the permeate of the AMBR was recycled to the HR. Therefore, the only outputs of the process were water by evaporation, biogas and moist compost. This is to be compared with actual full scale plants that produce 305 kg of wastewater per ton of biowaste treated (Van Zanten, 2005).

During start up a rapid pH drop in the HR was observed (Figure 1C). This indicates that fermentable sugars were converted to VFAs resulting in a pH drop due to the insufficient alkalinity (3800 mg equivalent $CaCO_3$/L on day 14). The pH even reached 5.5 on day 17, and to avoid a further drop, small doses of alkalinity (20-30 g of $NaHCO_3$) were added on days 17, 18, 19, 21 and 23. The alkalinity then increased to 5300 on day 21 and the pH to 5.8. After start-up, the leachate reached very high values in terms of SCOD and VFAs (as COD); a peak of 28 and 18.7 g/L on day 49 was observed for SCOD and VFAs, respectively. VFAs consisted mainly of acetic and propionic acids throughout the whole run (Figure 1B). N-butyric acid was also observed at concentrations up to 2200 mg/L around day 50, but it then decreased to zero. Propionic acid became the dominant acid after day 40, thus after the period considered as the start up. Acetate concentration, however, dropped significantly after day 50, and its production is only possible if the hydrogen partial pressure is sufficiently low (Harper and Pohland, 1986). As the partial pressure increases, the NADH is used to produce more reduced products such as propionic and butyric acids as the conversion of acetate become energetically unfavourable. With elevated hydrogen levels the production of propionic acid predominates at neutral pH values, but as the pH level becomes acidic then the production of butyric acid will begin to predominate (McCarty and Mosey, 1991).

3.1.2 Methane production rate

Figure 1C shows the evolution of percentage methane in the headspace. The percentage reached 55% in the HR on day 53, after which it decreased slowly. The biogas production rate was measured on days 58, 59 and 60 and the average for the HR was 13.47 L STP (±1.2) of biogas per day of which 5.96 L STP (±0.7) was methane. This corresponds to 148 (±17) ml CH_4 STP/g VS in the HR only. If the gas production in the SAMBR is also taken into account, it was found that 2.1 L of biogas at 62% methane was produced in 12 hours on day 57. This is equivalent to 2.42 L CH_4 STP in 24 hours. Thus the contribution of the SAMBR was 60 mL CH_4 STP/g VS. Thus the total methane yield was 208 mL CH_4 STP/g VS, of which 71% was obtained in the HR. It is noteworthy that this yield was relatively close to the ultimate biodegradability obtained after 242 days (216 mL CH_4 STP/g VS in Table 2).

3.1.3 Effect of Temperature on Performance

The HR was operated at 35ºC at a constant OLR of 4g VS/l.day and a SRT of 66 days until day 100. The VS removal was calculated from day 28 to day 99 to ignore the effect of the start up. The VS destruction was found to be 75% at a relatively high solids retention time of 66 days and a HRT of 20 days. On day 101, the recirculation of hot water in the copper coil placed around the HR was stopped so that the HR was operating at room temperature (20ºC) until the end of the experiment. As a result, the VS destruction dropped to 50%. A significant and immediate effect of the temperature drop was the decrease of VFAs (Figure 1B). This drop in VFAs compared to the SCOD concentration is in line with Bolzonella et al. (2005) who noticed that VFA concentrations dropped to 15% of the SCOD when the temperature was in the range 14-22ºC. In this study the VFA/SCOD ratio was in the range 14-27% at 20ºC compared to acidification yields in the range of 50-90% at 35ºC. Similarly, the SCOD decreased from 15.6 g/l on day 98 to 10 g/l on day 101. This indicates that the hydrolysis rate was strongly affected by the decrease in temperature. This was correlated with a build up of

TCOD and thus a decrease in the liquefied fraction of the leachate (SCOD/TCOD ratio) which is consistent with Uemura and Harada (2000) who observed a drop in the SCOD/TCOD ratio in the range 37-56% at 19-22ºC. In this study, the SCOD/TCOD ratio dropped from 35% on day 98 to only 16% at the end of the experiment.

3.1.4 Effect of SRT on Performance

On day 115, the SRT was lowered to 38 days, while the HRT was kept around 20 days. The effect of lowering the SRT in the HR was a drop in the VS removal to 45%. This drop was expected as at lower SRTs a given mass spends less time in the HR compared to at higher SRTs. Although the SRT dropped by 42%, the VS removal dropped by 5% only due to the uncoupling of the SRT and HRT by the mesh. Another consequence was the build up of recalcitrant TCOD after day 100 (Figure 1A) because of the hydrolysis was slowed due to the HR being at ambient temperature. This was linked to a dropping GPR as the average methane production decreased to only 1.74 L CH_4 STP/day. Moreover, there was virtually no gas produced in the SAMBR due to an excessively low SRT which caused the build up of SCOD in the bulk.

3.2 Performance of the SAMBR

3.2.1 Effect of SRT on Performance

In this section the effect of SRT was investigated and Figure 2B shows the evolution of SRT versus HRT. The HRT was determined by the available flux, but because it dropped rapidly low HRTs could not be maintained for very long, and the HRT had to be re-increased on many occasions. Previous results (Trzcinski and Stuckey, 2009) have shown that a successful start up in the SAMBR can take place at a HRT of 5.5 days. Thus in this experiment start up was attempted at a low HRT of 1.3 days in order to investigate the performance of the

SAMBR during start up using an acclimatised inoculum. The SRT was initially set at 95 days by removing sludge daily from the SAMBR.

From Figure 2A it can be seen that the leachate was not stabilized because the SCOD was between 1 and 2 g/l and VFAs were up to 1 g/l in the bulk. This indicated that methanogens were not as active or in sufficient concentrations as hydrolytic and acidogenic bacteria during start up. The dominance of propionic acid over acetic acid in the SAMBR (Figure 2C) indicates, however, that it is more likely that there was an insufficient amount of propionate oxidizers in the inoculum or a lack of syntrophic associations. The first twenty days have shown that an efficient start up can not take place at a HRT of 1.3 even with an inoculum acclimatized to the leachate medium. However, when the HRT was increased to 3.9 days on day 17, and after 20 days, the VFA concentrations dropped to below 30 mg/L and the SCOD in the permeate was below 600 mg/L, thus meeting the criteria for a stabilized leachate. Despite 20 days as start up period, it can be said that the SAMBR can start up more rapidly than the HR due to the complete retention of bacteria by the membrane which helps to create syntrophic associations in the reactor as demonstrated by low VFAs level after 20 days. This is a very interesting feature of the SAMBR when considering that UASB reactors typically need long start up periods such as 2-4 months (Najafpour et al., 2006). Shorter start-up renders SAMBRs more attractive through saving of time and money.

The SCOD and VFA concentrations remained stable until day 50 showing that operation at a SRT of 95 days and HRT of 9.6 days was possible with a SCOD removal greater than 95%. On day 57, the SRT was halved to 45 days while the HRT remained at 9.6 days. This drop in SRT had an immediate impact on VFA removal as their concentration went over 1 g/L, and on SCOD removal as its concentration increased to 3 g/L. Interestingly, due to cake formation on the membrane the effluent was relatively unaffected by the shock and its SCOD remained around 600 mg/L. The value of SCOD and VFAs on day 70 was lower which indicated that

the SAMBR could recover from the SRT shock to 45 days. On day 72, a simultaneous shock of SRT to 25 days and HRT to 2 days was disastrous because the SCOD increased rapidly to 10 g/L, while the VFA concentration reached 1.5 g/L. This time the permeate COD was affected by the shock because it reached 6.6 g/L, and as a result the SCOD removal decreased to between 50 and 60%.

On day 77, the HRT was set back to 9.6 days because the flux in the SAMBR was not sufficient to sustain that HRT. Indeed, the high suspended solids (≥40 g/L) levels were linked to a rapid drop in the flux from 6.6 to 1 LMH in only 35 days of operation. The HRT of 9.6 days allowed the system to return to normal levels in terms of SCOD and VFAs. On day 85, the HRT was set at 3.7 to determine whether the system could cope with that HRT and the same SRT of 25 days. Although the VFA concentrations remained low (≤ 500 mg/L), the bulk SCOD increased to 5-6 g/L meaning that the system could not cope with 3.7 days HRT because of SCOD build up. The same observation was made at a SRT of 12.5 days after day 104.

3.3 Performance of the AMBR

The AMBR (SRT = 300 days, HRT = HRT of the SAMBR) acted as a COD polishing step as well as an ammonia removal unit throughout the whole experiment. During the start up (0 to 20 days), the SAMBR permeate contained up to 2 g COD/L but this residual COD was eliminated in the AMBR (Figure 3A), showing that the AMBR can assist the SAMBR during start up, and thereby achieve high COD removals from the very first days and decrease the start up period required to treat the leachate from the HR. Based on the SCOD content of the leachate, the SCOD removal was always greater than 90% due to the AMBR.

On day 72, the AMBR remained relatively stable during a simultaneous SRT and HRT shock in the SAMBR that led to a permeate containing 6-7 g COD/L (1 to 2 g VFAs/L). As a result,

the COD removal in the AMBR temporarily increased to 90% between days 72 and 77, and this indicates that the AMBR can also assist the SAMBR in cases where the anaerobic step can not cope with an organic shock which is in line with other researchers (Xu et al., 2008). Moreover, the ammonia removal was not affected by the COD load during these days. The ammonia nitrogen coming out of the SAMBR was typically in the range 200 - 500 mg N/L, which resulted in a Nitrogen loading rate (NLR) to the AMBR equal to or lower than 0.07 g N/L.day, except between days 72 and 77 when it reached 0.24 g N/L.day; the ammonia removal remained above 95% throughout the run, and thus the AMBR bulk and permeate consisted of nitrite and nitrate. The sum of NO_2^--N and NO_3^--N correlated with the total amount of ammonia-nitrogen fed to the AMBR (within the range of accuracy), indicating that stripping of NH_3 was negligible although the pH was above 9 (Figure 3B). Moreover, the residual nitrite (maximum 25% of the total inorganic nitrogen) indicated that the nitrification was not complete in the AMBR, possibly due to a lack of syntrophic associations between ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) because of the high shear in the AMBR (Wittebolle et al., 2008). Another reason may have been the high temperature and pH of the AMBR which shifted the ammonia equilibrium towards the most toxic form which is NH_3. In our case free ammonia was in the range 1.6-7 mg/L which is greater than the range of 0.1-1 mg/L reported to be inhibitory for NOB by Anthonisen et al. (1976). Unfortunately, after day 80 the AMBR remained practically unfed due to flux problems as well as a high HRT and low SRT in the SAMBR. Also because the AMBR was operated at 35ºC, its liquid level was low due to water evaporation which translated to an increase in COD concentration above 1 g/L. After day 80, the COD removal was not calculated for that reason.

3.4 Microbial community structure and population dynamics

PCR-DGGE analysis of the microbial community composition in the bioreactors sludge indicated major differences in the archaeal and bacterial populations between day 0 and day 137. Figure 4 shows the evolution of the archaeal population in the HR. Nine bands could be identified, and these are listed in the dendrogram included in Figure 4 (bands a to i). On day 35 the DNA sequence of the two main bands in the HR was similar to *Methanobacterium formicicum* and *Methanosarcina sp.*, corresponding to bands d and e respectively, and this was associated with a low methane percentage (≤10%), and these species could cope with the concentrations of acids encountered during the start up which were not greater than 3000 mg/L. Later, concentrations of up to 7000 mg/L of propionic acid were observed (Figure 1B) which indicates that the hydrogen conversion rate was not fast enough, or that the distance between hydrogen-producing and hydrogen-consuming species (interspecies distance) was not short enough, or that the hydrogenotrophs were not present in sufficient number. These syntrophic associations are essential in anaerobic reactors as they are associated with small distances between H_2 producers and H_2 consumers which allows them to keep low hydrogen partial pressures in the reactor. The response of the system can be seen on day 99 in Figure 4 where the number of bands almost doubled from 10 bands on day 35 to 18 bands on day 99, and several new bands appeared the DNA sequences of which were similar to members of the genera *Methanosarcina and Methanobrevibacter* (corresponding to bands b, c and g). This increase in the number of bands was associated with an increase in methane to 30-55%. The recirculation of sludge would have caused the appearance of hydrogenotrophs as highlighted by Jarvis et al. (1995).

Three species were found to belong to the genus *Methanosarcina* (representing bands b, e and g, respectively), and it is known that members of that genus can grow by obligate CO_2 reduction with H_2, methyl reduction with H_2, aceticlastic fermentation of acetate, or methylotrophic catabolism of methanol, methylated amines, and dimethylsulfide (Maeder et

al., 2006). It is also known that it dominates at acetate concentrations greater than 350 mg/L (Ehlinger et al., 1987), so it was not surprising to find it in the HR.

Methanobacterium formicicum (similar DNA sequence to band d) on the other hand appeared as a strong band with Archaea specific primers in the HR as soon as day 35 when the recirculation rate from the SAMBR was only 30 mL/day (SRT of SAMBR = 90-100 days). It can also produce methane from H_2 and CO_2. Methanobacteriales, mainly *M. formicicum*, represent the majority of the hydrogenophilic methanogens in anaerobic bioreactors (Leclerc et al., 2004; McHugh et al., 2003). In this study it was found that *M. formicicum* (band d) and *Methanosarcina sp.* (band e) were present throughout the experiment, and were not affected by the changes in SRT or temperature; thus it can be inferred that these can withstand thermal fluctuations. The presence of these hydrogenotrophic Archaea confirms the presence of hydrogen in the HR headspace which was responsible for the high concentrations of propionic and butyric acids observed in Figure 1. The shear and agitation existing in the HR for hydrolysis purposes may cause aggregate disruption and loss of syntrophic associations. Good methane yields in the HR at pHs below 6.5 were possible at a recirculation rate of 30 ml of SAMBR sludge per day. The closer syntrophic distances in the flocs formed in the SAMBR may have sheltered the methanogens from the lower pH in the HR.

On day 137 bands b and c appeared with a DNA sequence similar to members of the genus *Methanosarcina* and *Methanobrevibacter*, respectively. These two bands increased in intensity only when the HR was operated at 20ºC; this strongly indicates that these Archaea grew better under sub-mesophilic temperatures. However, it should be kept in mind that using a nested PCR with primers having specificity issues may have resulted in non-specific amplification that can influence the intensity of specific bands (Wittebolle et al., 2008).

The species *Methanospirillum sp.* and *Methanoculleus sp.* showed good similarity to the DNA sequences from bands h and i, respectively, these appeared as faint bands at the end of

the experiment (on day 137), although they seem to be present in the first lane (day 35) as well. These are other hydrogen scavengers (Muller et al., 2008), and their predominance at lower temperatures is consistent with Connaughton et al. (2006) who observed that anaerobic digestion in the sub-mesophilic range favours hydrogenotrophic methanogenesis as one of its main routes of methane production.

DGGE analysis of the bacterial community resulted in the identification of different species (Figure 5). The exact number of bands was difficult to determine due to the "smearing" of DNA on the gel.The bacterial populations in the HR and SAMBR were different although at the same time some bands appeared in common in both reactors. This suggests that the population in the SAMBR was strongly influenced by the population of the HR due to the presence of the bacteria in the leachate. However, some bands had an increased intensity in the SAMBR (bands j, k and l) which could be a result of more suitable growth conditions in the SAMBR such as constant neutral pH and the complete retention by the membrane.

The DNA sequence from band j in Figure 5 showed good similarity to the anaerobic cellulolytic bacterium *Ruminococcus flavefaciens* which is one of the critically important inhabitants of the rumen (Sadanari et al., 2008). It is known that members of this genus can degrade cellulose by producing an extracellular cellulase, whereas members of the genus *Fibrobacter* contain a periplasmic cellulase to break down cellulose. Because of this, cells of *Fibrobacter* must remain attached to the cellulose fibril while digesting it (Madigan and Martinko, 1984). The presence of *R. flavefaciens* in our reactors contradicts the literature that states that *F. succinogenes, R. albus* and *R. flavefaciens* species are among the most important cellulose degrading bacteria in the rumen, while in landfills and anaerobic digesters other species of *Firmicutes*, primarily *Clostridia*, tend to be dominant (O'Sullivan et al., 2007). Two very close relatives of *R. flavefaciens* (bands i and j) were found in common in the SAMBR

and the HR regardless of the HRT and SRT. Interestingly, their corresponding bands eventually disappeared on day 137 when the HR was operated at room temperature, and this coincided with the drop in VS removal, suggesting inadequate conditions for *R. flavefaciens*. This could explain why less carbohydrate was fermented and less acetate was produced after day 100, and this coincided with a drop in SCOD and VFA concentrations in Figure 1A.

Band m was excised and the DNA sequence showed good similarity to *Spirochaeta sp. buddy*. Previous *Spirochaeta* related sequences have also been reported from anaerobic digesters treating wine distillation waste (Godon et al., 1997) and mesophilic and thermophilic methanogenic sludges (Sekiguchi et al., 1998). Fernandez et al. (1999) found that the bacterial and archaeal community were dominated by *Spirochaeta* and *M. formicicum*, respectively, in a stable reactor fed with glucose. Members of this genus ferment carbohydrates and produce acetate, succinate and lactate (Garrity, 2005), but also hydrogen and carbon dioxide (Fernandez et al., 1999). Figure 5 shows that band m was found in the SAMBR at all sampling times, whereas it was present in the HR only on day 35.

Bands o, p, q and r in Figure 5 were found exclusively in the AMBR indicating its bacterial population was very different to the one in the SAMBR. Band o DNA sequence showed good similarity to the bacterium *Nitrosomonas halophila*. *N. halophila* was, however, not identified from an excised and sequenced band using the CTO primer specific for ammonia-oxidizers DGGE analysis. However, this does not rule out its absence from the AMBR, just that the band was not of a significant intensity for sequencing. Band r was identified as a member of the genus *Hyphomicrobiaceae* that are known to be chemoorganotrophic on one-carbon compounds such as methanol, methylamine, formaldehyde and formate (Madigan and Martinko, 1984). All that is needed for that microorganism to grow is a mineral salts medium lacking organic carbon, and it can use nitrate as an electron acceptor under anoxic conditions.

However, denitrification was not observed in the AMBR which suggests that they grew solely on recalcitrant COD.

Figure 6 shows the evolution of the population of AOB in the HR and the AMBR. Bands s, t, u and v were excised from the DGGE gel, and their respective DNA sequence was similar to that of members of the genus *Nitrosomonas*, except for band v whose corresponding sequence was more similar to those from the genus *Nitrosospira* (Figure 6). It is clear from Figure 6 that AOB species were present in the AMBR which correlates with the conversion of ammonia to nitrite and nitrate. Interestingly, a band was found in the HR but only on day 137. This band v was excised and the DNA sequence was similar to the corresponding sequence from *Nitrosospira sp. Nsp40*. It has been reported that at low DO levels, this AOB can use nitrite or nitrate as an artificial electron acceptor and generate nitrous oxide gas (Ritchie and Nicholas, 1972). Moreover, the HR effluent contained no nitrite and less than 25 mg/L nitrate which shows that nitrate reduction took place in the HR because many anaerobic bacteria and Archaea can use nitrate as a source of cellular nitrogen (Madigan and Martinko, 1984).

5. Conclusions

At an OLR of 4 g VS/l.day a high VS removal was obtained in the HR where solids and liquid retention times were uncoupled. The SAMBR achieved 95% COD removal at SRTs down to 45 days and at a minimum HRT of 3.9 days. The emergence of hydrogenotrophic species was observed in the HR due to the excess sludge recycled from the SAMBR which contributed to re-inoculate the HR and maintain high methane yields in the HR even at pH below 6.5.

Acknowledgements

This research was supported by a grant from the Department of Environment, Food and Rural Affairs (DEFRA) in the UK.

References

Akarsubasi, A.T., Ince, O., Kirdar, B., Oz, N.A., Orhon, D., Curtis, T.P., Head, I.M., Ince, B.K., 2005. Effect of wastewater composition on archaeal population diversity. Water Research, 39, 1576-1584.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G., 1976. Inhibition of Nitrification by Ammonia and Nitrous-Acid. Journal Water Pollution Control Federation, 48, 835-852.

APHA, 1999. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington D.C.

Bolzonella, D., Fatone, F., Pavan, P., Cecchi, F., 2005. Anaerobic fermentation of organic municipal solid wastes for the production of soluble organic compounds. Industrial and Engineering Chemistry Research, 44, 3412-3418.

Chandler, J.A., Hermes, S.K., Smith, K.D., 1983. A low cost 75 kW covered lagoon biogas system Energy from Biomass and Wastes VII, Lake Buena Vista, FL, Institute of Gas technology, Chicago, pp. 23.

Connaughton, S., Collins, G., O'Flaherty, V., 2006. Development of microbial community structure and activity in a high-rate anaerobic bioreactor at 18ºC. Water Research, 40, 1009-1017.

Ehlinger, F., Audic, J.M., Faup, G.M., 1987. Relationship between the Concentration of Acetate in the Feed and the Composition of a Biofilm in an Anaerobic Filter. Environmental Technology Letters, 8, 197-207.

Fernandez, A., Huang, S.Y., Seston, S., Xing, J., Hickey, R., Criddle, C., Tiedje, J., 1999. How stable is stable? Function versus community composition. Applied and Environmental Microbiology, 65, 3697-3704.

Fernandez, N., Diaz, E.E., Amils, R., Sanz, J.L., 2008. Analysis of microbial community during biofilm development in an anaerobic wastewater treatment reactor. Microbial Ecology, 56, 121-132.

Garrity, G.M., 2005. Bergey's Manual of Systematic Bacteriology. 2nd ed. Springer, New York.

Godon, J.J., Zumstein, E., Dabart, P., Habouzit, F., Moletta, R., 1997. Molecular microbial diversity of an anaerobic digester as determined by small-subunit rDNA sequence analysis. Applied and Environmental Microbiology, 63, 2802-2813.

Gray, N.D., Miskin, I.P., Kornilova, O., Curtis, T.P., Head, I.M., 2002. Occurrence and activity of Archaea in aerated activated sludge wastewater treatment plants. Environ. Microbiol., 4, 158-168.

Harada, H., Momonoi, K., Yamazaki, S., Takizawa, S., 1995. Application of anaerobic-UF membrane reactor for treatment of a wastewater containing high strength particulate organics. Water Science and Technology, 30, 307-319.

Harper, S.R., Pohland, F.G., 1986. Recent Developments in Hydrogen Management during Anaerobic Biological Waste-water Treatment. Biotechnology and bioengineering, 28, 585-602.

Hogetsu, A., Ishikawa, T., Yoshikawa, M., Tanabe, T., Yudate, S., Sawada, J., 1992. High-Rate Anaerobic-Digestion of Wool Scouring Waste-Water in a Digester Combined with Membrane-Filter. Water Science and Technology, 25, 341-350.

Jarvis, A., Nordberg, A., Mathisen, B., Svensson, B.H., 1995. Stimulation of conversion rates and bacterial activity in a silage-fed two-phase biogas process by initiating liquid

recirculation. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 68, 317-327.

Kanwar, S.S., Guleri, R.L., 1994. Performance evaluation of a family-size, rubber-balloon biogas plant under hilly conditions. Bioresource Technology, 50, 119-121.

Kimura, S., 1991. Japan's aqua renaissance '90 project. Water Science and Technology, 23, 7-9.

Kowalchuk, G.A., Bodelier, P.L.E., Heilig, G.H.J., Stephen, J.R., Laanbroek, H.J., 1998. Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. Fems Microbiology Ecology, 27, 339-350.

Leclerc, M., Delgenes, J.P., Godon, J.J., 2004. Diversity of the archaeal community in 44 anaerobic digesters as determined by single strand conformation polymorphism analysis and 16S rDNA sequencing. Environ. Microbiol., 6, 809-819.

Liu, S.T., Yang, F.L., Gong, Z., Meng, F.G., Chen, H.H., Xue, Y., Furukawa, K.J., 2008. Application of anaerobic ammonium-oxidizing consortium to achieve completely autotrophic ammonium and sulfate removal. Bioresource Technology, 99, 6817-6825.

Madigan, M.T., Martinko, J.M., 1984. Biology of microorganisms. Brock ed. Pearson Benjamin Cummings, USA.

Maeder, D.L., Anderson, I., Brettin, T.S., Bruce, D.C., Gilna, P., Han, C.S., Lapidus, A., Metcalf, W.W., Saunders, E., Tapia, R., Sowers, K.R., 2006. The *Methanosarcina barkeri* genome: comparative analysis with *Methanosarcina acetivorans* and *Methanosarcina mazei* reveals extensive rearrangement within Methanosarcinal genomes. Journal of Bacteriology, 188, 7922-7931.

McCarty, P.L., Mosey, F.E., 1991. Modelling of anaerobic digestion processes (A discussion of concepts). Water Science and Technology, 24, 17-33.

McHugh, S., Carton, M., Mahony, T., O'Flaherty, V., 2003. Methanogenic population structure in a variety of anaerobic bioreactors. FEMS Microbiology, 219, 297-304.

Meher, K.K., Murthy, M.V.S., Gollakota, K.G., 1994. Psychrophilic anaerobic digestion of human waste. Bioresource Technology, 50, 103-106.

Muller, N., Griffin, B.M., Stingl, U., Schink, B., 2008. Dominant sugar utilizers in sediment of Lake Constance depend on syntrophic cooperation with methanogenic partner organisms. Environ. Microbiol., 10, 1501-1511.

Najafpour, G.D., Zinatizadeh, A.A.L., Mohamed, A.R., Hasnain Isa, M., Nasrollahzadeh, H., 2006. High-rate anaerobic digestion of palm oil mill effluent in an upflow anaerobic sludge-fixed film bioreactor. Process Biochemistry, 41, 370-379.

Norddahl, B., Rohold, L., 2000. The Biorek concept for the conversion of organic effluent to enrgy, concentrated fertilizer and potable water. [Online]. Available from: http://www.bioscan.dk/The_BIOREK_Concept_May_2000.pdf.

O'Sullivan, C.A., Burrell, P.C., Clarke, W.P., Blackall, L.L., 2007. The effect of biomass density on cellulose solubilisation rates. Bioresource Technology, 99, 4723-4731.

Owen, W.F., Stuckey, D.C., Healy, J., J. B., Young, L.Y., McCarty, P.L., 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Research, 13, 485-492.

Prakitchaiwattana, C.J., Fleet, G.H., Heard, G.M., 2004. Application and evaluation of denaturing gradient gel electrophoresis to analyse the yeast ecology of wine grapes. FEMS Yeast Research, 4, 865-877.

Ritchie, G.A.F., Nicholas, D.J., 1972. Identification of sources of nitrous-oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*. Biochemical Journal, 126, 1181-1191.

Robinson, A.H., 2005. Landfill leachate treatment 5th International Conference on Membrane Bioreactors, Cranfield University, UK.

Sadanari, J., Brulc, J.M., Levy-Assaraf, M., Rincon, M.T., Flint, H.J., Berg, M.E., Wilson, M.K., White, B.A., Bayer, E.A., Lamed, R., Borovok, I., 2008. Cellulosome gene cluster analysis for gauging the diversity of the ruminal cellulolytic bacterium *Ruminococcus flavefaciens*. FEMS Microbiology Letters, 285, 188-194.

Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H., Nakamura, K., 1998. Phylogenetic diversity of mesophilic and thermophilic granular sludges determined by 16S rRNA gene analysis. Microbiology-Sgm, 144, 2655-2665.

Singh, L., Maurya, M.S., Ram, M.S., Alam, S.I., 1993. Biogas production from night soil - Effects of loading and temperature. Bioresource Technology, 45, 59-61.

Speece, R.E., 1996. Anaerobic Biotechnology for Industrial Wastewaters. Archae Press, Nashville, Tennessee, USA.

Stephen, J.R., Kowalchuk, G.A., Bruns, M.A.V., McCaig, A.E., Phillips, C.J., Embley, T.M., Prosser, J.I., 1998. Analysis of beta-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. Applied and Environmental Microbiology, 64, 2958-2965.

Trzcinski, A.P., Stuckey, D.C., 2009. Continuous treatment of the Organic Fraction of Municipal Solid Waste in an anaerobic two-stage membrane process with liquid recycle. Water Research, 43, 2449-2462.

Uemura, S., Harada, H., 2000. Treatment of sewage by a UASB reactor under moderate to low temperature conditions. Bioresource Technology, 72, 275-282.

Van Zanten, W., 2005. Caddet Dutch National Team Composting versus Digestion of Biowaste. [Online]. Available from: www.caddet.org/public/uploads/pdfs/newsletter/3-98art3.pdf.

Wittebolle, L., Vervaeren, H., Verstraete, W., Boon, N., 2008. Quantifying community dynamics of nitrifiers in functionally stable reactors. Applied and Environmental Microbiology, 74, 286-293.

Xu, Y., Zhou, Y., Wang, D., Chen, S., Liu, J., Wang, Z., 2008. Occurrence and removal of organic micropollutants in the treatment of landfill leachate by combined anaerobic-membrane bioreactor technology. Journal of Environmental Sciences, 20, 1281-1287.

Zeeman, G., Sutter, K., Vens, T., Koster, M., Wellinger, A., 1988. Psychrophilic digestion of dairy cattle and pig manure: Start-up procedures of batch, fed-batch and CSTR-type digesters. Biological Wastes, 26, 15-31.

List of Figures

List of Tables