

**Denaturing Gradient Gel Electrophoresis (DGGE) analysis of
Archaeal and Bacterial populations in a Submerged Anaerobic
Membrane Bioreactor (SAMBR) treating landfill leachate at low
temperatures.**

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Abstract

This study investigated the evolution of archaeal and bacterial populations of two Submerged Anaerobic Membrane Bioreactors (SAMBRs) operating at a mean Solids Residence Time (SRT) of 30 (SAMBR30) and 300 days (SAMBR300) at mesophilic and psychrophilic temperatures. The SAMBRs were fed with leachate produced in a hydrolytic reactor (HR) treating the Organic Fraction of Municipal Solid Waste (OFMSW). The archaeal fingerprint using Denaturing Gradient Gel Electrophoresis (DGGE) showed different populations in the first and second stage of the two-stage anaerobic process. A build up of Volatile Fatty acids (VFAs) was observed at 20°C in SAMBR30, while in SAMBR300 the VFAs only built up at 10°C. The dominant bacterial species in the HR belonged to *Prevotella* and *Thauera*, while the dominant ones in SAMBR300 belonged to *Sphingobacteriales*, *Anaerovorax*, *Spirochaetaceae*, *Hydrogenophaga*, *Ralstonia*, *Prevotella* and *Smithella*. The low bacterial diversity in SAMBR30 compared to SAMBR300 resulted in a persistently high Soluble Chemical Oxygen Demand (SCOD) (>2 g/L) in the bulk reactor due to an insufficient residence time for bacteria to carry out the degradation of recalcitrant COD found in the leachate.

Keywords: Anaerobic digestion; submerged anaerobic membrane bioreactor; denaturing gradient gel electrophoresis.

List of abbreviations

COD	Chemical Oxygen Demand (mg.L ⁻¹)
COV	Coefficient of Variation (%)
DGGE	Denaturing Gradient Gel Electrophoresis
GPR	Gas Production Rate (L.L ⁻¹ .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)

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)
VFA	Volatile Fatty Acids (mg.L ⁻¹)

Introduction

Many researchers have reported significant effects of temperature on anaerobic microorganisms, and lowering the operational temperature is known to lead to a decrease in the maximum specific growth and substrate utilisation rates. Under psychrophilic conditions (10-15°C), chemical and biological reactions proceed much slower than under mesophilic conditions (30-40°C) (Lettinga *et al.*, 2001). Low temperatures were reported to affect biogas production, methanogenic activity and microbial community composition, and can also result in an exhaustion of cell energy, a leakage of intracellular substances or complete lysis (Kashyap *et al.*, 2003). COD removal efficiencies are typically in the range 93-99% at mesophilic temperatures, but when the temperature is decreased to ambient, a few studies have reported lower COD removals: 65-75% COD removal was achieved in an Upflow Anaerobic Sludge Blanket treating landfill leachate (Kettunen and Rintala, 1998). At 24°C, a COD removal of up to 75% was achieved with a 10 hours hydraulic retention time (HRT), and the highest organic loading rate (OLR) applied was 10 kg COD.m⁻³.d⁻¹ (Kettunen *et al.*, 1996).

There is a lack of information regarding the microbial populations in anaerobic digestion, especially at lower temperatures. *Methanosaeta* and *Methanosarcina* were reported to be dominant at low and elevated concentrations of acetate, respectively, while *Methanobacteriaceae* were the most abundant hydrogenotrophic methanogens in mesophilic digesters fed with simulated municipal solid waste (Griffin *et al.*, 1998). Connaughton *et al.* (2006) noted the emergence of hydrogenotrophic *Methanocorpusculum*-like, and *Methanococcales* and *Methanobacteriales*

organisms when treating synthetic VFA-based wastewater at 18°C using a mesophilic inoculum. Moreover, they noticed an apparent reduction in the abundance of acetoclastic methanogens from the *Methanosaeta* and *Methanosarcina* groups. In addition to methanogenic *Archaea*, *Crenarchaeota* were also detected. Regarding bacterial species, McHugh et al. (2004) have observed variations in population structure, but no clear pattern could be discerned when gradually decreasing the temperature from 37 to 16°C in a reactor treating a synthetic wastewater consisting of acetate, ethanol, butyrate and propionate. At 16°C they detected gram-positive bacteria such as members of the groups *Bacillus*, *Lactobacillus* and *Streptococcus*, while the remaining clones showed strong similarities with *Bacteroides* and *Spirochaeta*. Their studies focused on synthetic wastewaters, however, very little is known about bacterial communities in anaerobic membranes bioreactors treating complex wastewater such as landfill leachate. Moreover, the identification and selection of such microorganisms capable of treating leachate at low temperatures would be important when monitoring the stability of a psychrophilic digester and optimising its performance.

Studying the response of anaerobic reactors to low temperatures is vital, especially when the temperature change affects the rate-limiting step by altering the growth rate of individual species, and hence changes the predominant group of microorganisms in the long run. In addition, heating of anaerobic reactors requires energy and capital expenditure, and hence efficient digestion of wastewater at low temperatures (5-10°C) would be highly desirable to lower costs and increase energy output. Hence, the identification and selection of such microorganisms capable of treating leachate at low temperatures would be important when monitoring the health of a psychrophilic digester and optimising its performance. The development of high rate anaerobic reactor designs such as the SAMBR containing high biomass concentrations should enable the biomass to operate at lower temperatures where the specific rate per unit of biomass is lower. However, the microbial population in suspension in the SAMBR and membrane biofilm operating at low temperatures is

not well documented. Thus the purpose of this study was to investigate the evolution of archaeal and bacterial species in suspension and on the membrane biofilm of the SAMBR treating municipal solid waste leachate at ambient and psychrophilic temperatures.

Experimental section

Wet mesophilic two-stage lab-scale anaerobic process

The hydrolytic reactor (HR, 10L working volume) producing the leachate was an acrylic cylinder with a stainless steel mesh which followed a concentric arrangement inside the cylinder, and had a grid of 1 mm holes (Fig. 1). A stirrer moved inside the mesh allowing two pieces of rubber to rub against the perforated mesh: the speed of the stirrer was 40 rpm (Heidolph) and was operated intermittently (15 min ON-15 min OFF). The HR was inoculated with 10 L of anaerobic sludge and was maintained at 37°C throughout the study and was fed daily with a simulated feedstock of the OFMSW at an organic loading rate of 4-5 g volatile solids.L⁻¹.day⁻¹ until day 46, and 10 g volatile solids.L⁻¹.day⁻¹ from day 47 until the end of the experiment. Details of the HR, composition and properties of the municipal solid waste feedstock can be found elsewhere (Trzcinski and Stuckey, 2009). In addition to screening in the HR, the leachate pumped from the HR was also centrifuged (1500 rpm for 20 minutes) daily to avoid a build-up of recalcitrant suspended solids in the laboratory scale SAMBR, and the supernatant was poured into an intermediate tank from which the SAMBRs were fed. The leachate was treated in two SAMBRs, SAMBR30 and SAMBR300, operating at mean SRTs of 30 days and 300 days, respectively. The SRT was controlled by removing a known volume from the SAMBR daily. The SAMBRs were 3 litre reactors fitted with a Kubota polyethylene flat sheet membrane (0.1 m² surface area - pore size of 0.4 micron) (Trzcinski and Stuckey, 2009). The SAMBR contained a standing baffle designed to direct the fluid to the upcomer and downcomer regimes. The biomass was continuously mixed using headspace biogas

that was pumped through a stainless steel tube diffuser to generate coarse bubbles. The bubbles pushed the sludge flow upward between the membrane module and the reactor wall in the upper section. The biogas sparging rate was set at 3 L/m².hr to minimize cake formation on the membrane. SAMBR300 was inoculated with an acclimatised anaerobic sludge treating leachate, and its initial Mixed Liquor Total Suspended Solids (MLTSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) were 5.2 and 3.9 g/L, respectively, whereas in SAMBR30 it was 4.4 and 3.2 g/L, respectively. The SAMBRs were operated at 35°C until day 95, at 20°C from day 95 to day 100 and at 10°C after day 100 using a chiller (Model RC400, Grant). Microbial communities from the bioreactors were sampled by taking a small volume with a syringe whereas the sample from the biofilm formed on the membrane was taken at the end of the experiment after opening the bioreactor. The permeates of SAMBR30 and SAMBR300 were recycled to the HR or used to moisten the fresh feedstock in order to minimise fresh water consumption (Fig. 1).

Analytical Methods

The measurement of pH (Jenway) was accurate to within ± 0.02 units. Mixed Liquor Total Suspended Solids (MLTSS), Mixed Liquor Volatile Suspended Solids (MLVSS), sulfate concentration and Soluble Chemical Oxygen Demand (SCOD is COD after filtration through a 0.2 micron filter) were measured as described in Standard Methods (APHA, 1999). Their coefficient of variation (COV) for ten identical samples was 4, 3.1, 5 and 2.6 %, respectively. Volatile fatty acids (VFAs: acetic, propionic, iso- and n-butyric, iso- and n-valeric and n-caproic acids) were measured using a Shimadzu Gas Chromatograph with a flame-ionization detector and a SGE capillary column (12m x 0.53mm ID-BP21 0.5 μ m). The mobile phase was helium, and the injector, column and detector temperature were 200, 80 and 250°C, respectively. The COV was 3 % for ten identical samples. The water (2 % H₂SO₄, 10 % NaCl) displacement method was used to measure the gas production rate (GPR: $L_{\text{biogas}} \cdot L_{\text{reactor}}^{-1} \cdot \text{day}^{-1}$) 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 $\pm 2\%$.

For the DGGE analysis, the microbial community structure was evaluated when the reactor reached equilibrium state and after several alterations in the operational conditions. Samples were collected from the SAMBRs sludge, and the DNA from the mixed culture was extracted using the FastDNA SPin for soil kit from MP Biomedicals. The PCR mixture (50 μL) contained 2 μL of each primer, 0.2 μL of the Taq Polymerase (Promega), 2 μL of dNTPs stock solution (10 μM), 5 μL of Taq buffer solution containing MgCl_2 and between 1 and 20 μL of DNA template. The final volume was adjusted to 50 μL using DNA-free water. Archaeal DNA was amplified using a nested PCR reaction in a G-Storm thermocycler. First, the DNA template was amplified using the primers 46F (5'-YTA AGC CAT GCR AGT-3') and 1017R (5'-GGC CAT GCA CCW CCT CTC-3') in 25 μL . The temperature program was: initial denaturation 95°C for 3 minutes, then 35 cycles of denaturation at 95°C for 1 minute, annealing at 40°C for 1 minute, elongation at 72°C for 1 minute and the final elongation took place at 72°C for 7 minutes (Akarsubasi et al., 2005; Gray et al., 2002). Then, 1 microliter of the PCR product was used for the second PCR reaction in 50 μL using the primers 344F-GC (5'-*CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC GGG GHG CAG CAG GCG CGA*-3') and Univ522R (5'-GWA TTA CCG CGG CKG CTG-3'). The temperature program was: initial denaturation 95°C for 3 minutes, then 35 cycles of 95°C for 1 minute, 53°C for 1 minute, 72°C for 1 minute and the final elongation took place at 72°C for 7 minutes (Akarsubasi et al., 2005; Gray et al., 2002). Bacterial DNA was amplified using the primers (with GC clamp in italics) 341F-GC (5'-*CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCT ACG GGA GGC AGC AG*-3') and 907R (5'-CCG TCA ATT CCT TTR AGT TT-3') (Muyzer et al., 1993) in 50 μL PCR volume. The temperature program was: initial denaturation 94°C for 5 minutes, then 30 cycles of 94°C for 1 minute, 52°C for 1 minute,

72°C for 1 minute and the final elongation took place at 72°C for 10 minutes. Presence of PCR products was confirmed by electrophoresis on 1 % agarose gels stained with ethidium bromide. DGGE of the 16S rDNA PCR products was carried out using the D CODE system (Bio-Rad Laboratories Ltd) according to the manufacturer's instructions and protocols. A 6 % polyacrylamide gel was made up if bacterial primers were used, while 8 % was used for archaeal primers. The PCR products were electrophorised at 60 V for 16 hours at 65°C with a denaturing gradient ranging from 40 to 60 % for bacterial primer and 45-65 % for archaeal primers. A 100 % solution contains 7 mol/L urea and 40 % formamide. The gel was stained in 1 X TAE buffer containing SYBR green before visualizing on a UV transilluminator and photographed. The brightest DGGE bands were cut out to determine which species were dominant in each reactor. The bands were then eluted in DNA-free water overnight at 4°C and re-amplified using the same set of primers as for the first PCR. The purified PCR product was then cloned with the pCR 2.1-TOPO cloning kit (Invitrogen) according to the manual instructions. Briefly, 1 µL of vector was mixed carefully with 1 µL of salt solution (Invitrogen) and 4 µL of purified PCR product. Meanwhile chemically competent *Escherichia coli* were placed on ice and 2 µL of the cloning reaction was mixed with the competent cells. The cells were transformed using a heat shock protocol (30 seconds at 42°C), then incubated for 1 hour at 37°C in S.O.C. medium and eventually spread onto Luria-Bertani agar plates containing 50 µg/mL of kanamycin to incubate overnight at 37°C. Three well-separated colonies were picked from the plate using a pipette tip and were grown between 12 and 16 hours in LB medium (without agar) at 37°C. The plasmid DNA from cloned colonies was then purified using the purification kit from Qiagen (QIAprep Spin Miniprep Kit) and the resulting DNA was sent off for sequencing (Cogenics, 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 were deposited in the GenBank database under the accession numbers HQ113101 for the Archaea and HQ111174 to HQ111193 for bacteria.

Results and Discussion

Submerged Anaerobic Membrane Bioreactor (SAMBR) performance

The SCOD and VFAs concentrations were monitored in the bulk and the permeates of SAMBR30 and SAMBR300 in order to control the stability at various hydraulic retention times (HRTs) and operating temperature. Generally, the VFA concentration in the leachate from the HR was in the range 1-3 g/L, but peaked on some occasions up to 7 g/L. Acetic and propionic acids were the main acids produced, so the other VFAs were omitted from Fig. 2. Figure 2 (top) shows that SAMBR30 could start up at 3.7 days HRT and 35°C, and that it could achieve more than 90 % SCOD removal at 1.5 days HRT. When the temperature dropped to 20°C, SAMBR30 did not cope and the SCOD removal dropped to below 50 %, while VFAs started to build up. SAMBR300 was fed with centrifuged leachate from the HR from day 34 onwards because results obtained from SAMBR30 showed a high SCOD in the bulk (>2 g/L) presumably due to the low sludge retention times of 30 days. SAMBR300 was therefore run at 300 days solid retention time to investigate if lower bulk SCODs could be attained. The initial HRT was set at 19 days to check that the inoculum was active, and was then reduced gradually to low HRTs and temperatures. SAMBR300 achieved 95 % SCOD removal at 1.1 days HRT and 20°C (Fig. 2 bottom). When the temperature dropped to 10°C SCOD and VFAs concentrations started to build up in the bulk presumably due to lower degradation rates at psychrophilic temperatures.

The methane content and the biogas production rate are shown in Table 1 where it can be seen that a methane percentage of 70 % was obtained in SAMBR300, even at 20°C. However, a drop in the biogas production rate was observed in both SAMBRs when the temperature was set at 20°C. At

10°C the methane percentage dropped to 55 % in the SAMBR headspace, while the gas production rate was only 1 and 1.7 L.L⁻¹.day⁻¹ in SAMBR30 and SAMBR300, respectively.

Archaeal population in HR, SAMBR30 and SAMBR300

The archaeal populations from the HR, SAMBR30 and SAMBR300 were analyzed using the DGGE technique as described in Material and Methods, and the gel picture is shown in Fig. 3. The brightest bands were cut and sequenced, but no match with known Archaea was found. Only one sequence from a band in the gel was found to have a high similarity (100 %) to the sequence of *Methanosarcina mazeii*. It was found in SAMBR30, but its presence in the other reactors can be ruled out because of bands at similar migration distances in the gel. Furthermore, the DGGE picture shows that the SAMBR can start up within a week using an acclimatised inoculum since methanogens were detected in SAMBR30 after only 2 days and this was confirmed by the absence of VFAs at a HRT of 3.7 days (Fig. 2, top).

In SAMBR30, the detected archaeal diversity was lower than in SAMBR300 for the samples taken on days 64 and 85. There were a maximum of five or six bands detected in the former, while the latter had up to 12 bands. The lower SRT in SAMBR30 appears to be the main reason, nevertheless, the lower archaeal diversity did not impede VFA degradation as their concentrations remained low until the shock to low temperatures on day 95. Eventually on day 115, the SAMBR was opened and a sample from the biofilm on the membrane was taken. The archaeal population on the biofilm formed on the membrane was very diverse and this shows evidence that Archaea remain attached to the membrane of a SAMBR even at very low HRTs, which may not necessarily be the case in a more conventional anaerobic reactor in which the hydraulic flowrate is suddenly increased. However, it should be born in mind that differences between lanes in the gel may be the result of

different amounts of DNA loaded into each well. On day 113 a lower archaeal activity was observed at 10°C as indicated with a build up of acetate and propionate to 760 and 340 mg/L, respectively. This build up started even when the temperature was set at 20°C on day 95 which indicates that the number of methanogens was not sufficient to cope with the load at 20°C because the specific reaction rate of the methanogens decreased. It can be seen from the bands on lane 115 that the biofilm formed on the membrane in the SAMBR contained methanogenic species even at 10°C.

Two main species were detected on day 49 in SAMBR300 at an HRT of 14 days, and this was sufficient to keep low VFA concentrations; however, with time the number of bands increased to more than 12 as the HRT was reduced on day 87 to 1.1 days at 35°C. The sample taken on days 85 and 92 were the samples containing the most bands, but only one dominant species was sufficient to guarantee complete VFA degradation as it was the case on day 49. Interestingly, the population changed markedly as the temperature was set at 20°C on day 95 which resulted in a decrease in the number of bands to five. This showed that different species responded differently to the shock from 35 to 20°C. This observation was supported by the fact that on day 113 a build up of VFAs was observed at 10°C due to lower growth rate and metabolic activity of Archaea at such low temperatures. In contrast with SAMBR30, SAMBR300 could maintain complete VFA degradation at 20°C due to the high SRT that enabled a greater number of methanogens to be present in the bulk, but not at 10°C where the acetate and propionate concentrations increased to 2330 and 670 mg/L, respectively. The last lane (day 115) in Fig. 3 shows the archaeal population present on the biofilm formed on the Kubota membrane. Based on our DGGE picture it was found that in both SAMBRs the archaeal fingerprint of the biofilm was very similar to the one in the bulk, and this may be because there were psychrotolerant mesophiles present.

Bacterial population in HR, SAMBR30 and SAMBR300

Fig. 4 shows the bacterial population in the HR, SAMBR30 and SAMBR300, and the closest match associated with the DNA extracted from bands 1 to 21 can be found in Table 2. The lane noted MS on the right hand side of Fig. 4 is a sample of the anaerobic sludge from Mogden wastewater treatment plant (UK) that was used as inoculum in the experiment. The bacteria identified were an unclassified *Bacteroidetes* and a *Propionibacteriaceae* bacterium. By comparing this lane with other lanes it can be seen that an important change in bacterial population occurred during this study.

From Fig. 4 and Table 2, it can be seen that the dominant bacterial species in the HR belonged to *Prevotella* and *Thauera*, while the dominant ones in SAMBR300 belonged to *Sphingobacteriales*, *Anaerovorax*, *Spirochaetaceae*, *Hydrogenophaga*, *Ralstonia*, *Prevotella* and *Smithella* although the absence of the species in the HR cannot be totally ruled out. This suggests that the membrane in the SAMBR allows different genus to appear in the second stage reactor compared to a single stage process. Madigan and Martinko (1984) reported that members of the *Prevotella* genus are starch decomposer found in the rumen, and their fermentation products are formate, acetate and succinate. As far as SAMBR30 was concerned, the bacterium *Brachymonas denitrificans* was found in band 16 on day 2. It is an aerobic chemoorganotrophic bacterium able to denitrify and can also remove phosphorus (Shi and Lee, 2007). The last band in Table 1 was found to be *Thauera phenylacetica* and it was detected in the HR on day 113. This bacterium is a nitrate-reducing bacterium capable of anaerobically degrading methoxylated aromatic compounds with a bacterium from the genus *Clostridium* (Mechichi et al., 2005). The *Thauera* strain was found to use metabolic intermediates such as protocatechuate, acetate and butyrate as a carbon source anaerobically or aerobically in the presence of nitrate. In SAMBR30 no bands were seen on day 85 because of a failed PCR reaction. On day 113, DNA was detected on the agarose gel but only as a faint band, and no band could be seen on the DGGE gel, probably due to insufficient amounts of DNA. Nonetheless, bands were

observed on the biofilm developed on the membrane of SAMBR30 (Lane day 115) which suggests that some of the degradation occurring in SAMBR30 towards the end of the experiment was probably due to the bacteria attached to the biofilm. However, it should be born in mind that differences between lanes in the gel may be the result of different amounts of DNA loaded in each well.

The most diverse population was observed on day 64 in SAMBR300 with more than 20 bands which contrasts with SAMBR30 where the low SRT did not allow the bacteria to grow in sufficient numbers. Indeed, this was associated in SAMBR30 with a persistently high SCOD (> 2 g/L) in the bulk compared to values in the range of 400-1000 mg/L for SAMBR300 (Fig. 2). Nonetheless, both SAMBRs achieved about 95 % SCOD removal at 35°C and a HRT of 1.1-1.5 days because of the retention of SCOD by the cake/gel layer on the membrane that provided a similar permeate SCOD for both SAMBRs. The population changed dramatically when SAMBR300 was operated at 20°C on day 100. Band 4 was found to have a very similar sequence to the one of a member of the genus *Anaerovorax* which is known to be important for the digestion of polysaccharides and oligosaccharides (Krause *et al.*, 2008).

Band 11 was found to have a very similar sequence to a member of the *Syntrophaceae* group which includes propionate-utilizing bacteria (Ariesyady *et al.*, 2007). Propionate was found to build up frequently in the HR in previous experiments (Trzcinski and Stuckey, 2009). Therefore, the presence of this bacterium was associated with stability regarding propionate degradation in this experiment.

Band 5 was found to have a very similar sequence to a member of the phylum *Spirochaetes* also detected by McHugh *et al.* (2004). The sequence from bands 7 and 8 was found to be highly similar to the sequence of *Ralstonia* species that are reported to be hydrogen-oxidising bacteria (Song *et al.*, 2002). Amongst the other species detected in SAMBR300, it is worth highlighting the presence of a

sulphate-reducing bacterium in band 2 on day 64 only, but no attempt was made in this study to measure H₂S in order to confirm sulphate reduction. The temporary appearance of this bacterium strongly suggests that sulphate reduction was not important in the SAMBR, and there was no competition with methanogens for the carbon source. Moreover, its presence can be related to the inoculum used in the HR (from Mogden wastewater treatment plant, UK) which also contained a band at that position and is referred to as MS in Fig. 4. In the HR, band 17 was identified as Uncultured *bacterium clone* G-25 that was detected in a study where lactate and sulphate were degraded in a continuously stirred tank reactor in which acidogenic and sulphate-reducing bacteria were detected (Zhao et al., 2008). The presence of this bacterium in our system suggests that sulphate-reducing bacteria could have also been present in the HR as they were in SAMBR300 on day 64 (band 2 in Table 2). However, the concentration of sulphate remained in the range 30-40 mg/L in the HR and SAMBRs effluents showing that no significant sulphate reduction was taking place in the process.

A bacterium of the genus *Hydrogenophaga* was also found to have a very similar sequence to the one extracted from band 6 of SAMBR300. Some *Hydrogenophaga* species are chemolithotrophic bacteria at the expense of hydrogen or carbon dioxide (Willems, 1991). Normally, methanogens would consume hydrogen and CO₂ to produce methane according to the following reaction (de Bok et al., 2004; Mosey, 1983):



Alternatively, hydrogen could be utilized by hydrogen-consuming acetogenic bacteria such as *Clostridium aceticum* according to the following reaction:



However, in this study it was found that other bacteria (99 % similarity with *Hydrogenophaga* and a sulphate-reducing bacterium) might have used hydrogen as a source of energy (electron donor). Morvan and co-workers (1994) have shown that the rumen ecosystem can harbour other hydrogenotrophic populations such as H₂-utilizing sulphate reducers before the apparition of methanogens in the newborn lamb, and this underlines the competition that exists between different H₂-utilizing species. Methanogens and sulphate-reducing bacteria have a similar affinity for hydrogen (33 for some methanogens and 10 ppm, respectively) as pointed out by Morvan et al. (1994). However, in our study it is likely that methanogens outcompeted sulphate-reducing bacteria because of the residual 30-40 mg/L sulphate detected in the SAMBR. Uchida *et al.* (2008) also reported their presence in landfill leachate as they are known to play significant roles in the biodegradation of aromatic compounds, in denitrification, and in the bioremediation of contaminated environments.

Conclusions

An experiment with a wet two-stage lab-scale membrane bioprocess was performed in order to investigate its microbial population using the DGGE technique and it was found that archaeal populations were different in both stages of the two-stage anaerobic process with *Methanosarcina* as dominant species. The SRT in the SAMBR significantly affected the archaeal diversity in the bulk, but a low diversity did not impede VFA degradation. An increase in VFAs concentration was observed in SAMBR30 at 20°C, whereas in SAMBR300 VFAs build up only appeared at 10°C. The dominant bacterial species in the HR belonged to *Prevotella* and *Thauera*, while the dominant ones in SAMBR300 belonged to *Sphingobacteriales*, *Anaerovorax*, *Spirochaetaceae*, *Hydrogenophaga*, *Ralstonia*, *Prevotella* and *Smithella*. The low bacterial diversity in SAMBR30 compared to SAMBR300 resulted in a persistently high SCOD (>2 g/L) in the bulk reactor phase due to

insufficient residence time for the bacteria to degrade recalcitrant COD. The presence of a member of the genus *Hydrogenophaga* in SAMBR300 suggests that hydrogen was found in the SAMBR.

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Authors Disclosure statement

The authors declare that no competing financial conflicts exist.

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