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THEME ENERGY.2009.3.2.2
Biowaste as feedstock for 2nd generation

VALORGAS

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D4.4: Experimental data on mesophilic and thermophilic anaerobic microbial consortia as a basis for design of process interventions to achieve stable food waste digestion

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Lead contractor for this deliverable
University of Southampton (Soton)

Revision [1]

D4.4: Experimental data on mesophilic and thermophilic anaerobic microbial consortia as a basis for design of process interventions to achieve stable food waste digestion

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Revision

Minor changes to text including revision and harmonisation of dates and day numbers in figures.

D4.4: Experimental data on mesophilic and thermophilic anaerobic microbial consortia as a basis for design of process interventions to achieve stable food waste digestion

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D4.4: Experimental data on mesophilic and thermophilic anaerobic microbial consortia as a basis for design of process interventions to achieve stable food waste digestion

1 Introduction

Anaerobic digestion is conducted by three main groups of microorganisms: hydrolytic-fermentative bacteria, syntrophic oxidising bacteria, and methanogens. Methanogens mediate the final stage of anaerobic conversion and are a highly specialised group of microorganisms with a restricted range of feedstocks (Thauer, 2008). There are two major methanogenic pathways in anaerobic digestion of organic materials, i.e. acetoclastic and hydrogenotrophic methanogenesis. The former pathway cleaves acetic acid to CH_4 and CO_2, and the latter reduces CO_2 to CH_4 using the intermediate product H_2. It was originally believed that in a 'normal' anaerobic digestion process 70% of CH_4 is formed via acetoclastic methanogenesis (Jeris and McCarty, 1965; Gujer and Zehnder, 1983). Recent studies, however, have demonstrated that the environmental parameters in digesters greatly influence the methanogenic community structure and therefore the methanogenic pathway: for example when the ammonia concentration is sufficient to inhibit the acetoclastic methanogens (Angelidaki and Ahring, 1993; Karakashev et al., 2005; Schnurer and Nordberg, 2008; Banks et al., 2012). Under such circumstances, the conversion of acetic acid to methane may rely entirely on syntrophic acetate oxidisation and hydrogenotrophic methanogenesis. Acetate oxidisation and hydrogenotrophic methanogenesis uses different enzymatic systems from acetoclastic methanogenesis, resulting in an additional requirement for certain trace elements such as selenium (Müller, 2003; Thauer et al., 2008; Stock and Rother, 2009; Zhu and Tan, 2009). Information on the methanogenic community structure may therefore indicate the dominant methanogenic pathway and provide a basis for the design and assessment of appropriate interventions to ensure process stability.

Earlier results from the VALORGAS project (Banks et al., 2012) clearly showed that under mesophilic conditions the acetoclastic methanogenic population in food waste digesters is lost, leaving the conversion of substrate to methane entirely via hydrogenotrophic methanogens. It is known that thermophilic digesters are also sensitive to ammonia toxicity (Angelidaki and Ahring, 1993) and because of the higher temperatures used it is likely that the toxicity threshold is at a lower total ammonia concentration than found in mesophilic digesters. Experimental work was therefore carried out to assess changes in the methanogenic population structure in relation to increasing ammonia concentrations under both mesophilic and thermophilic conditions.

In this report, microbial community structure were analysed under different digestion scenarios, including

1) digestion of source segregated domestic food waste at mesophilic and thermophilic temperature. Microbial community structures were compared with those for low-nitrogen synthetic food waste in thermophilic conditions, and with high-nitrogen food waste, which was made of real-world food waste spiked with urea, in mesophilic conditions. This work was based in Soton, and used fluorescence in-situ hybridisation (FISH), ^{14}C radioisotope labelling, and gene sequencing techniques.

2) autoclaved food waste digestion in mesophilic conditions and comparison with a parallel digestion trial with untreated food waste. This work was conducted by MTT, Finland, and terminal restriction fragment length polymorphism (T-RFLP) analysis was employed; and

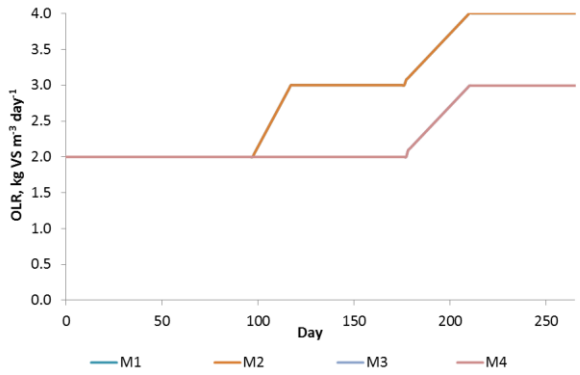
3) two-stage thermophilic food waste digestion by UNIVR, Italy using FISH.

The results of each set of investigations are presented in the following sections.

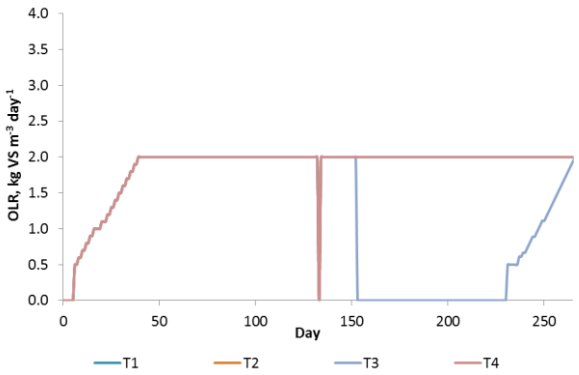
2 Anaerobic microbial community analysis at Soton

2.1 Digester operation

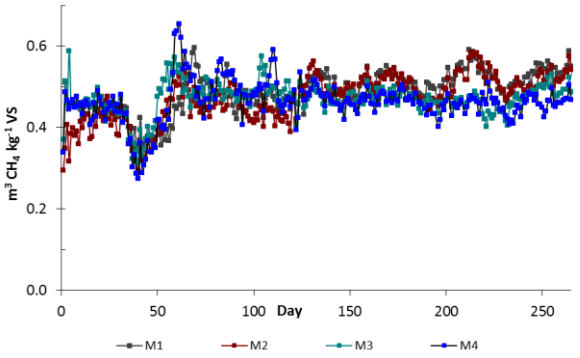
Eight laboratory-scale digesters with a working volume of 4 litres were used in the study. These were inoculated from a digester treating municipal wastewater biosolids which was known to have a predominantly acetoclastic methanogenic population. Two pairs of digesters were operated at mesophilic temperatures (35 ^{o}C) and fed on source segregated domestic food waste at final organic loading rates (OLR) of 3 and 4 kg volatile solids (VS) m^{-3} day^{-1}. Two pairs of digesters were acclimated to thermophilic conditions (55 ^{o}C) as described in Yirong et al. (2013a). One pair was fed on the same source segregated domestic food waste as used in the mesophilic study. The second pair was fed on a low-nitrogen food waste composed of food materials known to have a low nitrogen content, e.g. certain types of fruit and vegetable. Because of the high moisture content of these materials the volatile solids content of the low-N food waste was adjusted to a concentration similar to that of the source segregated food waste by the addition of alpha-cellulose powder. The waste prepared was designed to have a TKN of around 2000 mg N kg^{-1} wet weight. The performance of the digesters and their digestate characteristics were continuously monitored for key parameters as described in Yirong et al (2013a and b). The parameters on digestion operation are shown in Figure 1: M1, 2 and M3, 4 were two pairs of digesters running in mesophilic conditions with final OLR of 4 and 3 kg VS m^{-3} day^{-1}; T3, 4 were the pairs of digester fed with identical food waste but operated in thermophilic conditions; T1, 2 were digesters running in thermophilic conditions with low-nitrogen food waste.



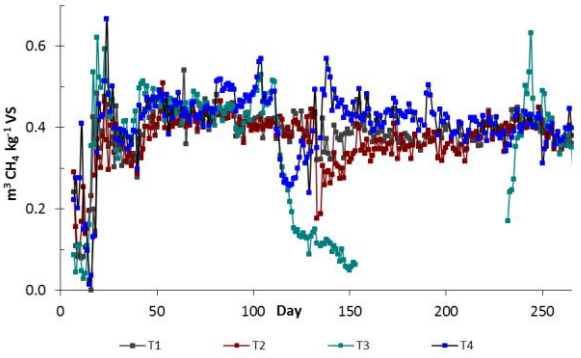
(i) OLR mesophilic digesters



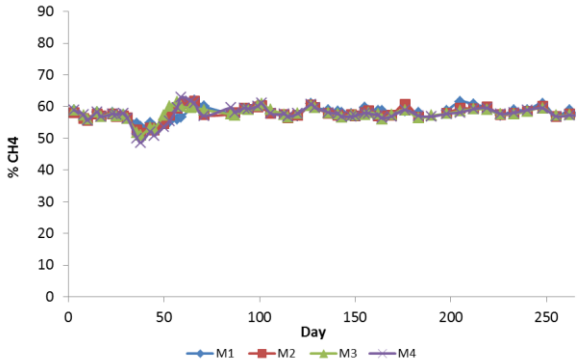
(ii) OLR thermophilic digesters



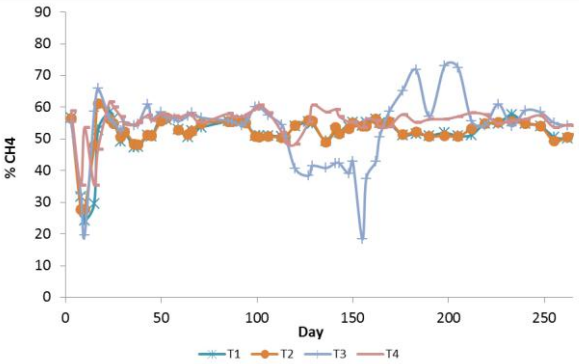
(iii) Specific methane production (mesophilic)



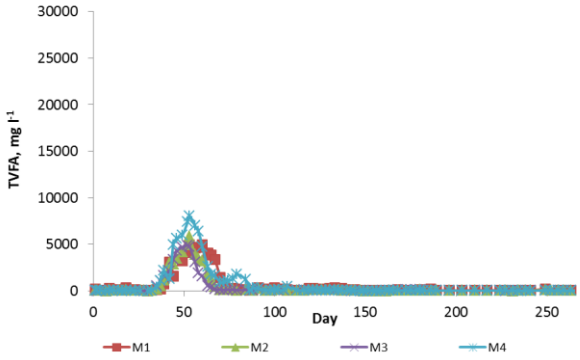
(iv) Specific methane production (thermophilic)



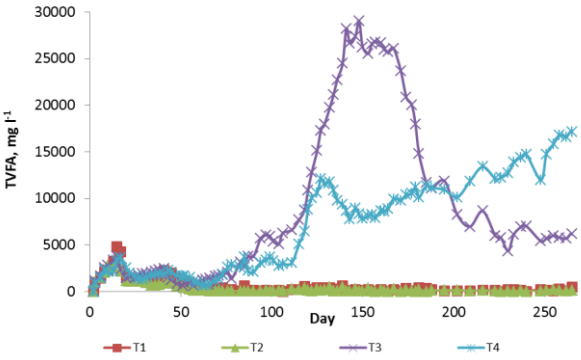
(v) Biogas methane content (mesophilic)



(vi) Biogas methane content (thermophilic)

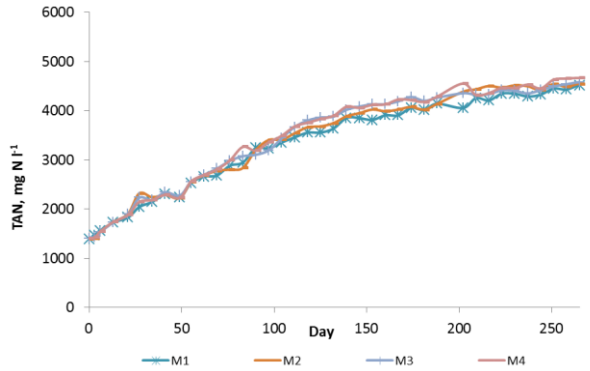


(vii) Total VFA concentration (mesophilic)

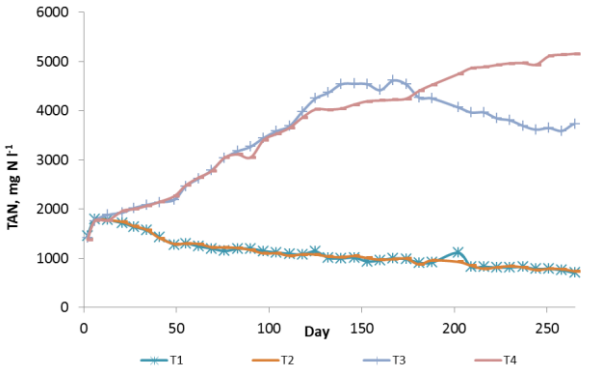


(viii) Total VFA concentration (thermophilic)

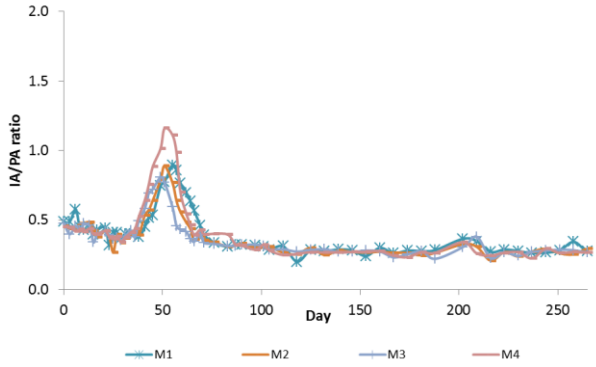
Figure 1a. Organic loading rate, specific methane yield biogas methane composition and total VFA concentration in digesters M1-4 and T1-4



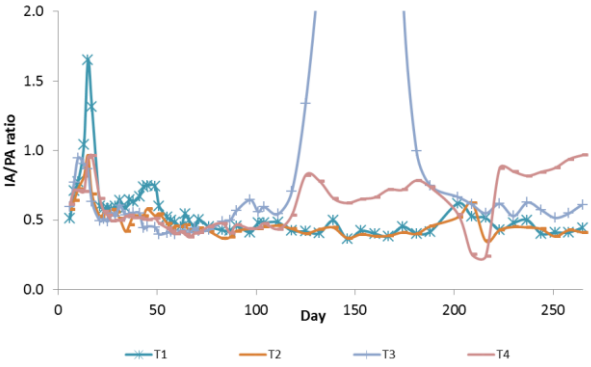
(i) TAN concentration (mesophilic)



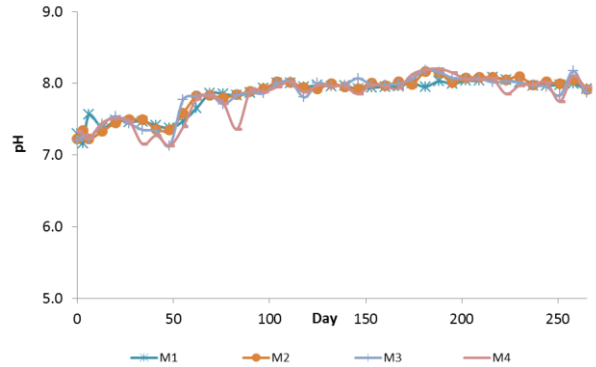
(ii) TAN concentration (thermophilic)



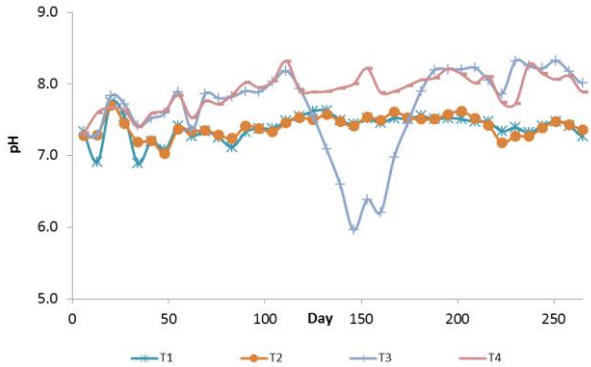
(iii) IA/PA ratio (mesophilic)



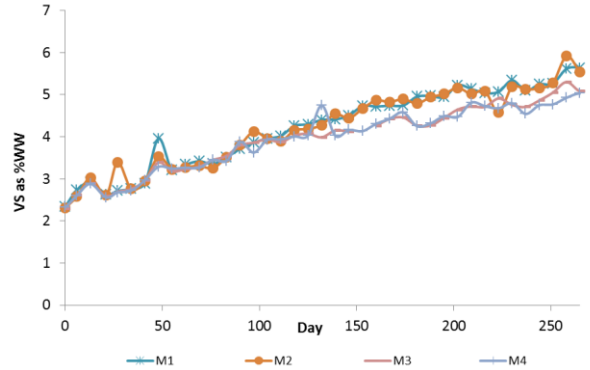
(iv) IA/PA ratio (mesophilic)



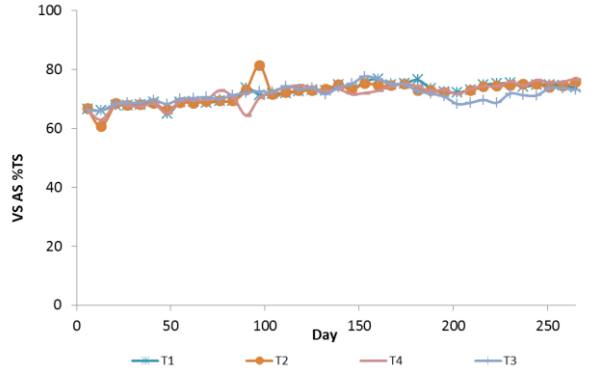
(v) pH (mesophilic)



(vi) pH (mesophilic)



(vii) VS concentration



(viii) VS concentration

Figure 1b. Total ammonia nitrogen, pH, IA/PA ratio and VS concentration in digesters M1-4 and T1-4

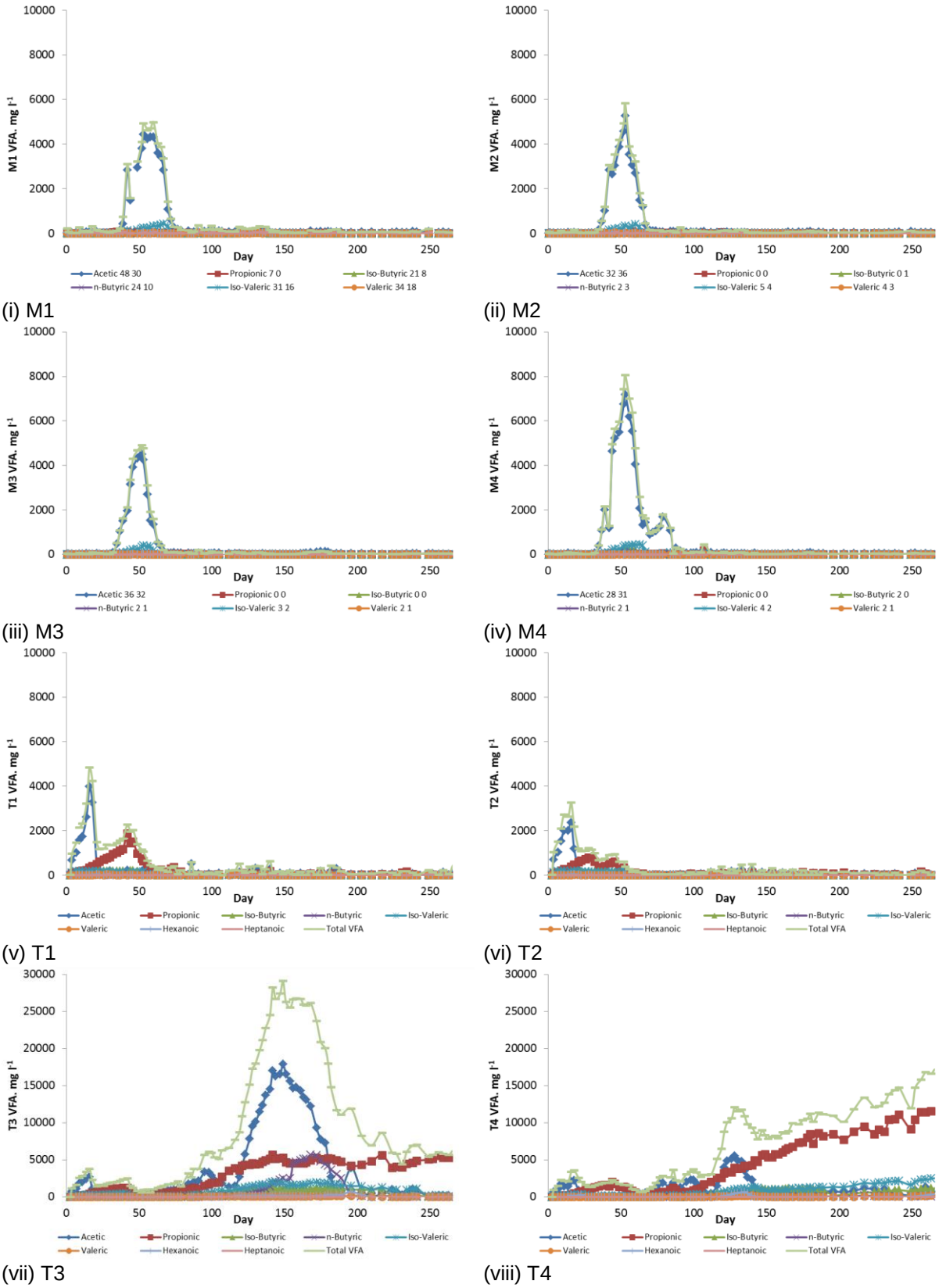


Figure 1c. VFA profiles in digesters M1-4 and T1-4 Note change of y-axis scale for T3 and T4

Samples were taken and fixed for FISH analysis on a weekly basis. Samples for radioisotope labelling test were taken from M1-4 after 10 months of operation. Samples for gene sequencing were taken from the mesophilic digester M1 at approximately one-month intervals from the start date on days corresponding to the FISH analysis samples. A further three samples (symbol NN, HN and FW) were also included in gene sequencing and related statistical analysis. NN and HN were mesophilic digesters at OLR 3 kg VS m^{-3} day^{-1} after 5 months' operation on source segregated food waste (NN) and on source segregated food waste with added urea (HN): the inoculum for these digesters was taken from a food waste digester operated over an extended period (Serna Maza et al., 2013). FW was a mesophilic digester operated at OLR 6 kg VS m^{-3} day^{-1} with full TE supplementation for a period of 3.8 years: the inoculum for this digester was sewage sludge digestate and the organic loading rate was gradually increased from 2 to 6 kg VS m^{-3} day^{-1} until the time of sampling. Digestate parameters at the time of sampling for gene sequencing are shown in Table 1 below.

Table 1. Digestate parameters at time of sampling for gene sequencing

Sample name		M1_0	M1_34	M1_55	M1_76	M1_97	M1_118	M1_174	M1_209	M1_265	FW_875	NN_140	HN_140
Digester		M1	M1	M1	M1	M1	M1	M1	M1	M1	FW	NN	HN
Day of operation		0	34	55	76	97	118	174	209	265	875	140	140
pH		7.3	7.47	7.47	7.85	7.93	7.95	8	8.03	7.92	7.55	8.03	8.21
ammonia	mg as N /l	1386	2144	2530	2884	3235	3556	4060	4257	4519	2416	4339	8603
Intermediate alkalinity	mg as CaCO3/l	2314	3029	5609	3666	3773	2914	4336	5109	4593	2979	4563	7539
Partial Alkalinity	mg as CaCO3/l	4755	7513	6281	10918	11818	14570	15448	14700	16635	8631	18049	29546
Total Alkalinity	mg as CaCO3/l	7069	10542	11890	14584	15591	17484	19784	19809	21228	11610	22613	37085
TS	%	3.49	3.98	4.49	4.53	5.54	5.77	6.46	6.87	7.51	7.73	6.66	6.25
VS (wet weight basis)	%	2.34	2.76	3.21	3.35	3.86	4.26	4.74	5.14	5.63	6.31	4.94	4.64
VS (dry weight basis)	%	67.12	69.36	71.45	73.97	69.76	73.82	73.42	74.8	74.91	81.69	74.19	74.34
Specific methane production	m3 CH4/kg VS	0.341	0.401	0.367	0.472	0.466	0.444	0.505	0.543	0.532	0.396	0.438	0.436
VFA													
Acetic	mg/l	39	40	4244	173	114	88	81	78	55	108	33	145
Propionic	mg/l	3	58	61	23	34	104	10	0	3	18	0	642
Iso-Butyric	mg/l	14	5	37	4	8	25	0	0	0	0	0	0
n-Butyric	mg/l	17	0	14	4	3	5	0	0	0	0	0	0
Iso-Valeric	mg/l	24	10	250	8	25	42	0	0	3	0	0	0
Valeric	mg/l	26	0	0	9	3	9	0	0	0	0	0	0
Hexanoic	mg/l	40	0	1	17	2	15	0	0	0	0	0	0
Heptanoic	mg/l	52	0	2	27	2	14	0	0	1	0	0	0
Total VFA	mg/l	216	114	4608	265	189	301	92	78	62	126	33	787

2.2 Methods for microbial community analysis

2.2.1 Fluorescence in-situ hybridisation

Both mesophilic and thermophilic digesters were sampled for methanogenic microbial community structure analysis using the Fluorescent In-Situ Hybridisation (FISH) technique. Density gradient centrifugation with Nycodenz (Sigma-Aldrich, UK) was used before performing the FISH fixation to separate the microbial biomass in the digestate from other components including both partially digested input material and the non-digestable components of the food waste (Banks et al., 2012). The separated microbial biomass was then fixed with 4% of paraformaldehyde (Sigma-Aldrich, UK) solution and used for FISH analysis (Daims, 2005). The oligonucleotide probes (Thermo Electron Biopolymers, Ulm, Germany), as detailed in Table 2 below, and the hybridisation stringency were chosen based on a previous study (Karakashev et al., 2006). Hybridised samples were viewed using a Nikon Eclipse E400 epi-fluorescence microscopy. 10 different microscrope fields were randomly selected for each hybridisation treatment. Typical images showing the effect of density gradient centrifugation and of FISH are given in Figure 2 and 3.

Table 2. Oligonucleotide probes used with target groups and optimised formamide concentrations

Probe name	Target group	Probe sequence (5'-3')	Fluoro-chrome
EUB338	*Bacteria (most)*	GCTGCCTCCCGTAGGAGT	Cy3
EUB338+	*Bacteria (remaining)*	GCWGCCACCCGTAGGTGT	Cy3
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	6-Fam
MX825	*Methanosaetaceae*	TCGCACCGTGGCCGACACCTAGC	Cy3
MS1414	*Methanosarcinaceae*	CTCACCCATACCTCACTCGGG	Cy3
hMS1395	MS1414-helper	GGTTTGACGGGCGGTGTG	-
hMS1480	MS1414-helper	CGACTTAACCCCCCTTGC	-
MG1200	*Methanomicrobiales*	CGGATAATTCGGGGCATGCTG	Cy3
MB1174	*Methanobacteriales*	TACCGTCGTCCACTCCTTCCTC	Cy3
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	Cy3

Note: W, A+T mixed base.

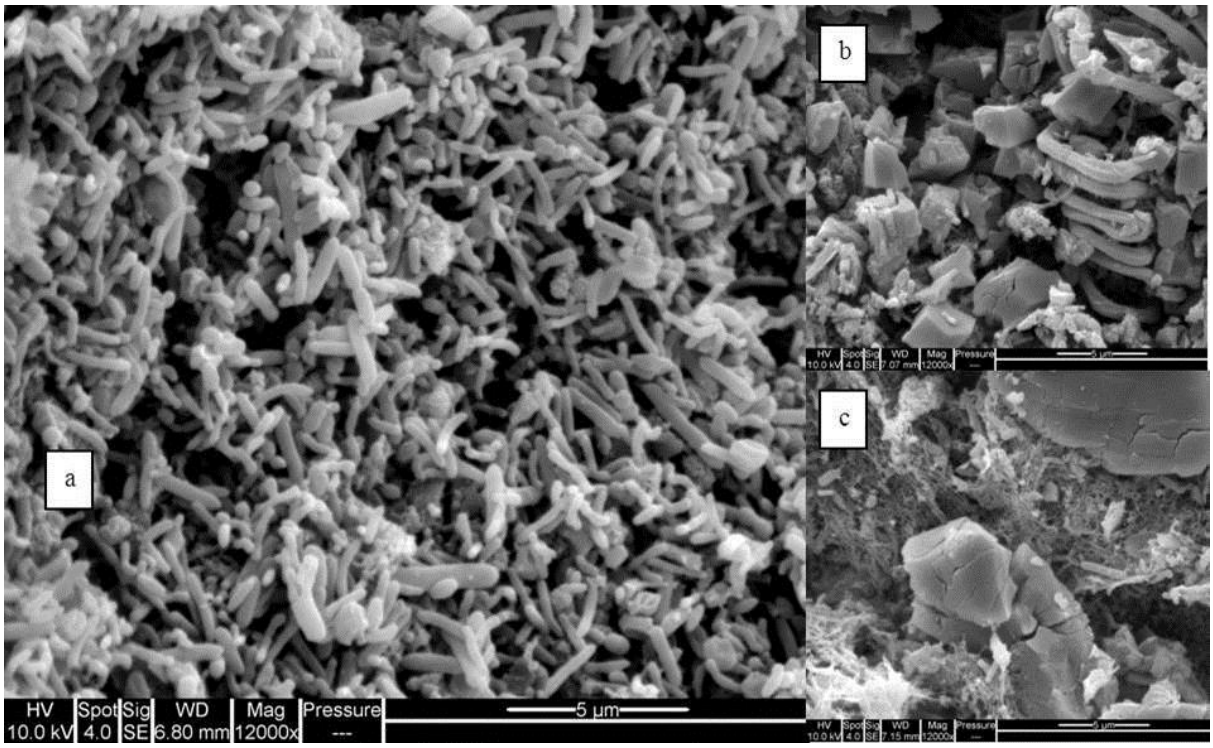
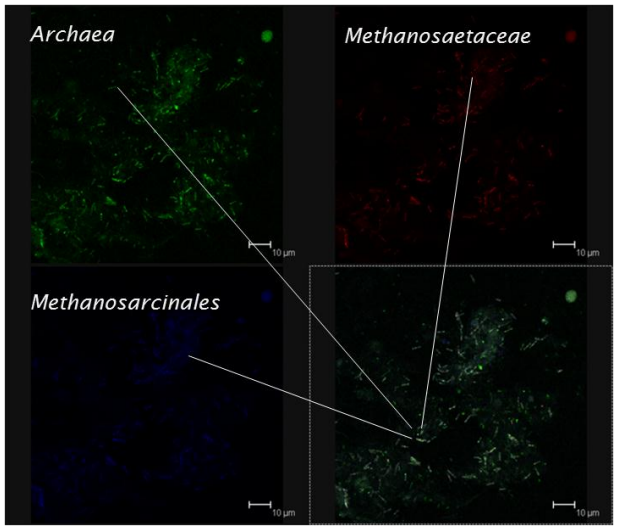
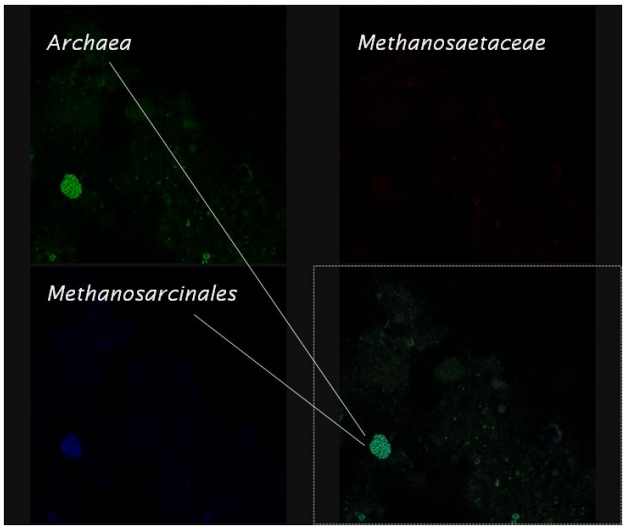


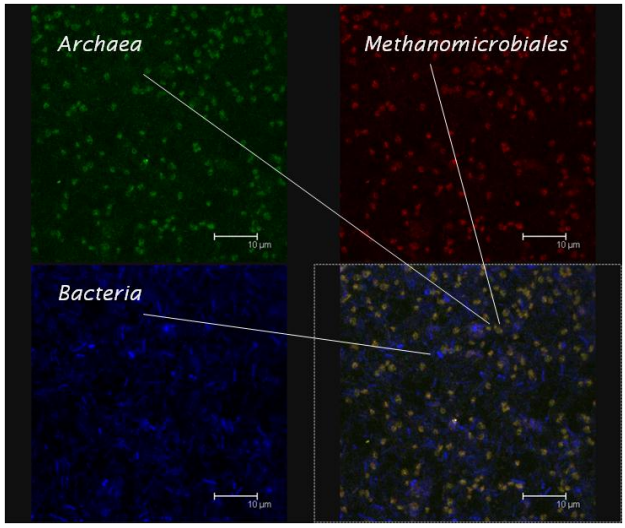
Figure 2. a) Food waste digestate sample after density gradient centrifugation b) and c) Residual material after density gradient separation of the microbial cells



Inoculum – a wide range of methanogens



After 3 months – development of family of *Methanosarcinacaea*



After 1.5 years – development of order of *Methanomicrobiales*

Figure 3. Methanogenic community structure profile with time in a mesophilic food waste digester.

2.2.2 Radioisotope labelling test on methanogenic pathway

The metabolic pathway for methanogenesis in mesophilic digesters M1-4 was determined by labelled [2-^{14}C] sodium acetate analysis on duplicate samples (Jiang, 2012). Each 15 g sample of digestate was mixed with anaerobic medium in the ratio of 1:2 and 0.15 ml of $^{14}CH_3COONa$ solution with a specific activity of 10 kBq ml^{-1} was added (MP biomedical, Solon, OH, USA). The mixture was incubated in 119 ml crimp top serum bottles at 37 ^{o}C for 48 hours. At the end of the incubation process the sample/medium mixture was acidified with 2 ml of 1mM H_2SO_4 and sparged using N_2 and O_2 gas mix (9:1 on a volume basis). As shown in Figure 4, the CO_2 and CH_4 produced were firstly passed through 20 ml 5M NaOH before CH_4 was oxidised to CO_2 in a tube furnace consisting of a heating block within which was embeded a quartz tube (6.2mm OD, 4mm ID, 180mm in length, H. Baumbach & Co Ltd,

Suffolk, UK) packed with copper (II) oxide. The operating temperature was regulated at 800±5°C using a temperature controller (Omega DP7004, Manchester, UK). The sparge gas then carried the CO_2 generated from CH_4 to a second CO_2 trap filled with 20 ml 1M NaOH. After absorption, 1 ml of each alkali trap and 1 ml of the centrifuged sample/medium mixture were added into 15 ml Gold Star multi-purpose liquid scintillation cocktail (Meridian Biotechnologies Ltd, Surry, UK) and counted in a Beckman Coulter LS6500 scintillation counter. The ratio of $^{14}CO_2$ and $^{14}CH_4$ can be used to indicate the methanogenic pathway: $^{14}CO_2/^{14}CH_4 > 1$ is regarded as having high-level of acetate oxidisation and hydrogenotrophic methanogenesis activity (Karakashev et al., 2006).

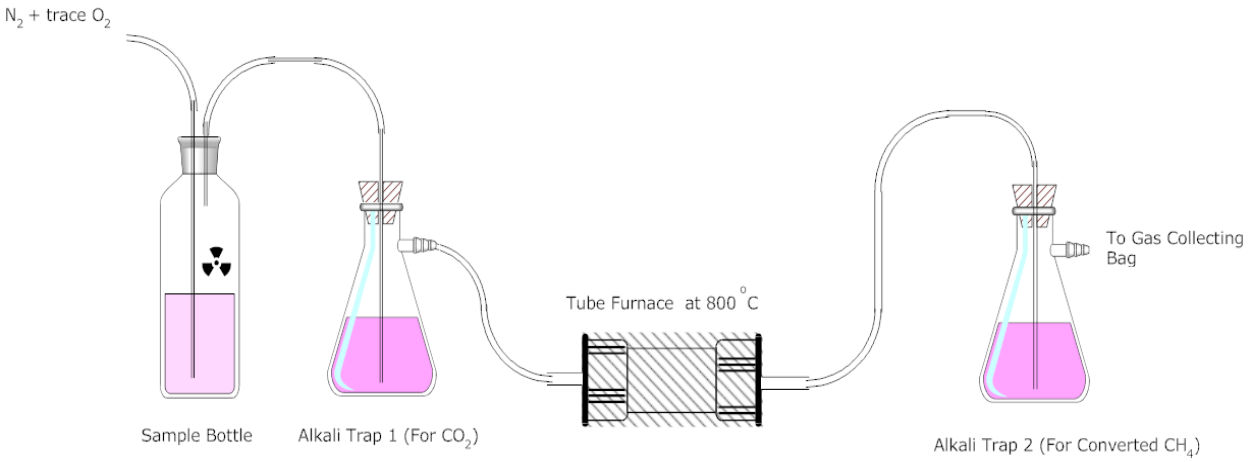


Figure 4. Schematic illustration of radioisotope ^{14}C labelling experiment.

2.2.3 Gene sequencing and statistical analysis

DNA was extracted from 250 mg of digestate using the Power Soil extraction kit (MO BIO Laboratories, Carlsbad) according to the instructions of the manufacturer. This method included a bead-beating step, which was performed for 5 min. All DNA extracts were eluted with 60 mL of Tris buffer (10mM) and stored at -20°C until further analysis.

Microbial community analyses were performed based on two different targets. To study bacterial communities PCR amplification of the V1-V3 region of the 16S rRNA gene was carried out using the following primers (underlined) with Roche 454 pyrosequencing adaptors (in italics) and unique identifiers (NNNN):
16S_27F (5'-*CCATCTCATCCCTGCGTGTCTCCGACTCAG*NNNNAGAGTTTGATCMTGGCTCAG-3') and 16S_519r (5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*GWATTACCGCGGCKGCTG-3').

For coverage of the methanogenic community a fragment of the methyl Co-A reductase gene (*mcrA*) common to all known methanogens (Luton *et al*., 2002) was used using the primers (underlined) with Roche 454 pyrosequencing adaptors (in italics) and unique identifiers (NNNN):
mcrAf (5'-*CCATCTCATCCCTGCGTGTCTCCGACTCAG*NNNNGGTGGTGTMGGATTCACACARTAYGCWACAG C-3') and mcrAr (5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG*TTCATTGCRTAGTTWGGRTAGTT-3')

Unique microbial identifiers (MIDs) were used to barcode individual amplicons to allow their identification after pyrosequencing. The proof-reading polymerase Phusion (New England

Biolabs) was used for the amplification of all targets. Next generation sequencing (NGS) of all amplicons was completed using the GS FLX+ System (Roche). Emulsion PCR was carried out according to the manufacturer's instructions (Roche).

The obtained sequence data were processed using the Galaxy platform to remove low quality reads and short sequences (<100 bp). To assign taxonomy to the bacterial sequences, the Naïve Bayesian rRNA classifier of the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) was used applying a bootstrap value of 50%. Sequences obtained from mcrA amplicons were clustered based on nucleotide identity using the BLASTclust algorithm (Altschul *et al*., 1997) with parameters set to cluster sequences of >97% similarity over 90% of their length. Representative sequences of each cluster were aligned with *mcrA* sequences from the Genbank database and a phylogenetic tree was constructed to indicate the taxonomic identity of each cluster.

Statistical analyses were carried out using the subroutines multidimensional scaling (MDS) and analysis of similarities (ANOSIM) of the PRIMER 5 software suite (PRIMER-E, Ltd., UK). For ordination and ANOSIM Bray–Curtis similarities were calculated. MDS was calculated using 10 random starting configurations of sample points. It was assumed that the final configuration was optimal unless other configurations displayed lower stress levels. Additionally, hierarchical agglomerative clustering of Bray–Curtis similarities was performed using the complete linkage method of the PRIMER software. For understanding relationships between community dynamics and abiotic factors like ammonia, pH, volatile fatty acids and total solids, it was tested whether weighted-averaging techniques or linear methods were appropriate, detrended correspondence analysis (DCA) was performed using CANOCO for Windows 4.53 (Biometris, the Netherlands). The longest gradients resulting from DCA were 3.452 for the analysis based on the 16S rRNA gene and 2.316 for the analysis based on the mcrA gene. Since the values indicate a unimodal relationship for the 16S rRNA gene based analysis and a tendency towards unimodal relationships for the mcrA gene based data (Lepš and Šmilauer, 2003), CCA (Canonical Correspondence Analysis) was performed to compare species–environment correlations. Explanatory variables included pH, ammonia, Intermediate alkalinity, Partial Alkalinity, total Alkalinity, total solids (TS), VS on a fresh matter basis, VS on a TS basis, specific methane production, total volatile fatty acids (VFA) and individual VFA such as acetic acid, propionic acid, iso-butyric acid, n-butyric acids, iso-valeric acid, valeric acid, hexanoic acid and heptanoic acid. Generally, CCA was performed as described by Lepš and Šmilauer (2003). An automated forward selection was used to analyse intersample distances for both communities. First, the variance inflation factor (VIF) of environmental variables was calculated. Variables displaying a value greater than 20 of this factor were excluded from CCA analyses, assuming collinearity of the respective variable with other variables included in the examined dataset. The null hypothesis that species composition is independent of the measured variables was tested using constrained ordination with automated forward selection and a permutation test. The analysis was performed without transformation of data, applying a partial Monte Carlo permutation test (999 permutations) including unrestricted permutation. The partial Monte Carlo permutation test provided the conditional effect of each variable. For all community ordination analyses, biplot scaling was used.

2.3 FISH and radioisotope labelling results for mesophilic and thermophilic trials

2.3.1 Methanogen community structure profile in mesophilic digesters

FISH analysis was carried out on a series of samples taken from these two pairs of mesophilic digesters. It should be mentioned that although a range of pretreatment methods were trialled, e.g. mechanical and ultrasonic pretreatment, it was still very difficult to distribute the microbial biomass evenly on the microscope slides due to the robust microbial floc formed in the digesters. Therefore methanogens were present on the microbial slides either as planktonic single cells or aggregates of various sizes. This caused great difficulty in counting the percentage of different groups of methanogens in the entire methanogen category; the approximate results in the following Tables 3-6 reflected this problem.

In addition, it is possible that the specific FISH probes sometimes did not hybridise with the designed targets (false negative error) under standard hybridisation condition, which caused the sum of the percentage coverage of individual methanogen groups to be less than 100%. A number of measures were taken to tackle this problem, such as overnight hybridisation and lowered hybridisation stringency. For the samples from digesters at a final organic loading rate of 4 kg VS m^{-3} day^{-1}, these measures had only very limited effect, however, and therefore the methanogenic community structure analysis as shown in Table 3 does not cover the whole digester operation period.

Despite all the above-mentioned problems, the overall trend of the methanogenic community structure showed certain patterns in these two pairs of mesophilic food waste digesters. As shown in Table 4, the dominant methanogens in the inoculum belonged to the family of *Methanosaetaceae*, which are known to utilise the acetoclastic methanogenesis metabolic pathway. The predominant methanogenic group gradually shifted from *Methanosaetaceae* to *Methanosarcinaceae*, and finally stabilised as *Methanomicrobiales* in the digester running at an OLR of 3 kg VS m^{-3} day^{-1}. This observation was similar to previous FISH results for another batch of long-term operated mesophilic food waste digesters (Banks et al., 2012). The high proportion of *Methanoimicrobiales* at a high concentration of total ammonia nitrogen (TAN) indicated that the dominant acetic acid degradation pathway in food waste digestion was that of syntrophic acetate oxidising acetogenesis and hydrogenotropic methanogenesis. Blasco et al. (2013) also stated that *Methanomicrobiales* and *Methanococcales*, as well as *Methanosarcina* were present in a 11-litre mesophilic food waste digester when TAN concentration was around 4000 mg N l^{-1}. In that study, however, only *Methanosarcina* was detected at TAN concentrations of less than 2000 mg l^{-1}.

Table 3. Methanogen community structure profile in mesophilic digester at final OLR of 4 kg VS m^{-3} day^{-1}

Operation time (days)	*Methanosaetaceae*	*Methanosarcinaceae*	*Methanomicrobiales*	*Methanobacteriales*	*Methanococcales*	TAN (mg N l^{-1})
0	> 60 %	< 10 %	< 10 %	< 20 %	< 20 %	1400
34	> 60 %	< 10 %	< 10 %	< 20 %	< 20 %	2140
55	> 60 %	< 10 %	< 10 %	< 20 %	< 20 %	2530
97	< 20 %	> 70 %	~ 20 %	< 10 %	< 10 %	3240
118	~ 20 %	> 70 %	< 20 %	< 10 %	< 10 %	3560
174	~ 50 %	< 30 %	< 30 %	< 10 %	< 10 %	4060
209	> 30 %	< 50 %	< 30 %	< 10 %	< 10 %	4260
237~601	Very limited positive response so that fluorescence emission was not detectable					

Table 4. Methanogen community structure profile mesophilic digester at final OLR of 3 kg VS m^{-3} day^{-1}

Operation time (days)	*Methanosaetaceae*	*Methanosarcinaceae*	*Methanomicrobiales*	*Methanobacteriales*	*Methanococcales*	TAN (mg N l^{-1})
0	> 60 %	< 10 %	< 10 %	< 20 %	< 20 %	1400
34	> 60 %	< 10 %	< 10 %	< 20 %	< 20 %	2180
55	> 60 %	< 10 %	< 10 %	< 20 %	< 20 %	2560
73	< 20 %	> 70 %	~ 10 %	< 10 %	< 10 %	2900
90	< 20 %	> 70 %	~ 20 %	< 10 %	< 10 %	3100
111	< 20 %	> 70 %	< 10 %	< 10 %	< 10 %	3670
146	~ 30 %	< 30 %	> 50 %	< 10 %	< 10 %	4090
174	~ 20 %	< 30 %	> 70 %	< 10 %	< 10 %	4280
209	~ 10 %	< 30 %	> 80 %	< 10 %	< 10 %	4330
237	< 10 %	< 30 %	> 80 %	~ 10 %	< 10 %	4350
265	< 10 %	< 30 %	> 80 %	~ 10 %	< 10 %	4580
335	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	5100
363	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	5420
384	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	5650
419	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	5550
447	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	5600
475	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	5750
510	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	6200
545	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	6020
573	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	6290
601	< 10 %	< 10 %	> 80 %	< 10 %	< 10 %	5860

Radioisotope labelling tests using [2-^{14}C] sodium acetate were carried out on the mesophilic digestate around day 280 when the TAN concentration increased to above 4500 mg N l^{-1}. The ratio of $^{14}CO_2$ / $^{14}CH_4$ was 2.0 and 1.4 for digesters at OLR of 3 and 4 kg VS m^{-3} day^{-1}, respectively. This indicated the dominant methanogenic pathway was hydrogenotrophic methanogenesis, and thus supported the results of the FISH analysis.

2.3.2 Methanogenic community structure profile in thermophilic digesters

In Table 5 it can be seen that the dominant methanogen group in the low nitrogen thermophilic digester shifted from *Methanosaetaceae* to *Methanosarcinaceae*. The dominant methanogenic pathway in this case is unclear, because the family *Methanosarcinaceae* can use both acetoclastic and hydrogenotrophic pathways.

The methanogen community structure in the thermophilic digester fed with normal food waste also changed over time (Table 6). After around 120 days of operation, however, when the TAN concentration rose to above 4000 mg N l^{-1} and total volatile fatty acid concentrations reached 15000 mg l^{-1}, it became difficult to detect fluorescence emission during microscopic observation of samples from these digesters. This may indicate that the activity of methanogens as a whole was very low at that stage and the density of ribosomal RNA did not reach the threshold level to show strong fluorescence emission for light microscopic observation. FISH analysis therefore failed as a technique to detect the presence or absence of specific groups of methanogens under these circumstances.

Table 5. Methanogen community structure profile in thermophilic digester fed with low nitrogen food waste

Operation time (days)	*Methanosaetaceae*	*Methanosarcinaceae*	*Methanomicrobiales*	*Methanobacteriales*	*Methanococcales*	TAN (mg N l^{-1})
0	> 60 %	< 10 %	< 10 %	< 20 %	< 20 %	1400
6	> 70 %	< 10%	< 10 %	< 10 %	< 10 %	1790
62	~ 70 %	< 10%	< 10 %	< 10 %	< 10 %	1240
90	< 10 %	> 70 %	< 10 %	< 10 %	< 10 %	1200
125	< 10 %	> 50 %	~ 20%	~ 10 %	< 10 %	1140
153	< 10 %	> 70 %	< 10 %	< 10 %	< 10 %	930
181	< 10 %	> 50 %	< 10 %	~ 20 %	< 10 %	910
216	< 10 %	> 80 %	< 10 %	~ 10 %	< 10 %	830
251	< 10 %	> 80 %	< 10 %	< 10 %	< 10 %	790
279	< 10 %	> 80 %	< 10 %	< 10 %	< 10 %	650

Table 6. Methanogen community structure profile in thermophilic digester fed with normal food waste

Operation time (days)	*Methanosaetaceae*	*Methanosarcinaceae*	*Methanomicrobiales*	*Methanobacteriales*	*Methanococcales*	TAN (mg N l^{-1})
0	> 60 %	< 10 %	< 10 %	< 20 %	< 20 %	1400
6	~ 30 %	~ 20 %	~ 40 %	< 10 %	< 10 %	1760
20	~ 60 %	~ 20 %	~ 20 %	< 10 %	< 10 %	1930
62	~ 40 %	~ 20 %	~ 40 %	< 10 %	< 10 %	2620
90	< 10 %	~ 50 %	~ 30 %	< 10 %	< 10 %	3270

2.4 Gene sequencing results and statistical analysis

2.4.1 Bacterial dataset

61,560 high quality sequences distributed among the different samples were obtained from 16S amplicon sequencing. The bacterial community consisted mainly of sequences related to the phyla Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria with other phyla being present at lower levels (Figure 5).

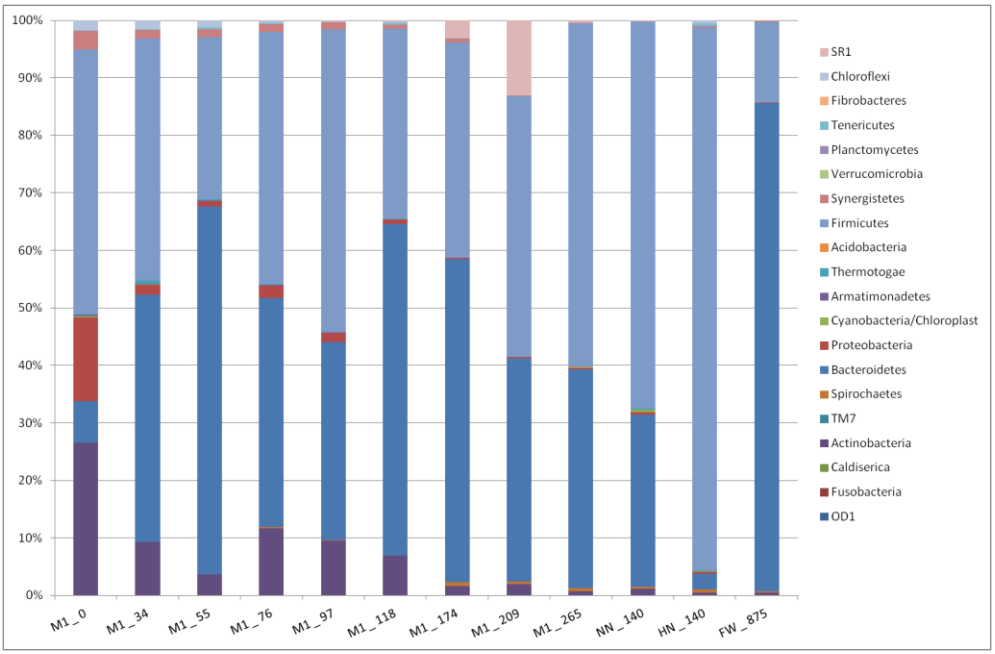


Figure 5. Bacterial community structure on phylum level

When looking at the family level of the bacterial communities (Figure 6), large proportions of sequences belonged to Porphyromonadaceae, Cryomorphaceae, Clostridiales_Incertae Sedis XI, Peptostreptococcaceae, Intrasporangiaceae, Micrococcaceae, Microbacteriaceae, Clostridiaceae 1, Synergistaceae and the Ruminococcaceae.

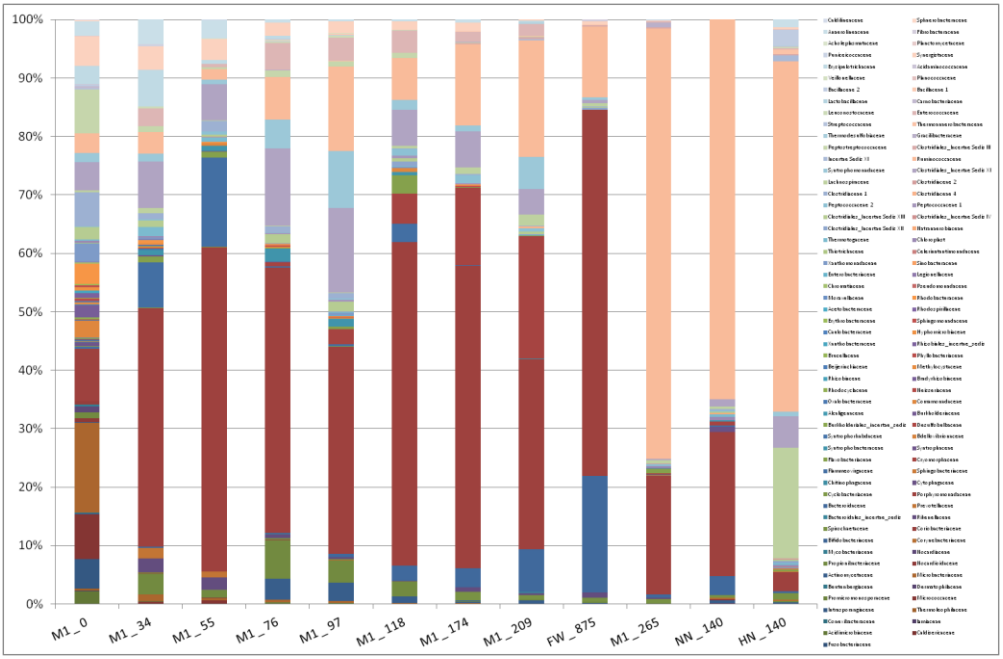


Figure 6. Bacterial community structure on family level

Ordination was used to illustrate differences between bacterial communities that were considered fairly similar on the phylum level (Figure 7) except for sample M1_0. On the genus level, samples M1_0 and HN_140 appeared different from the other samples (Figure 8). This result was confirmed by clustering as shown in Figure 9.

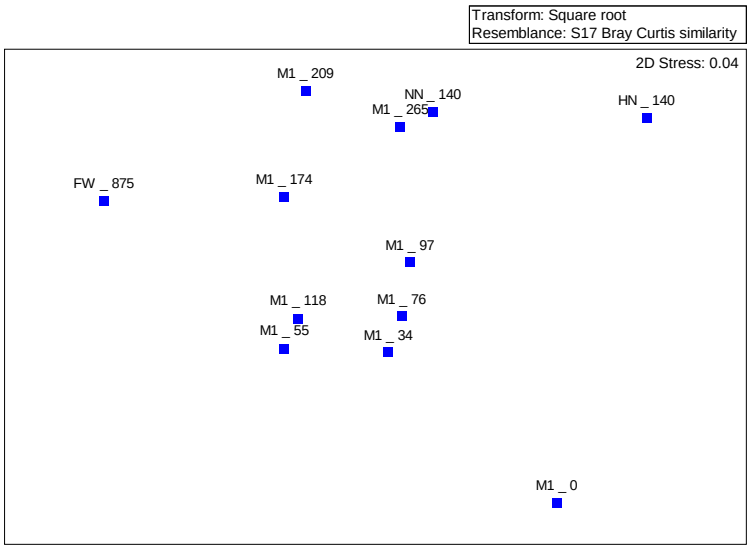


Figure 7. nMDS based on bacterial community profiles, phylum level

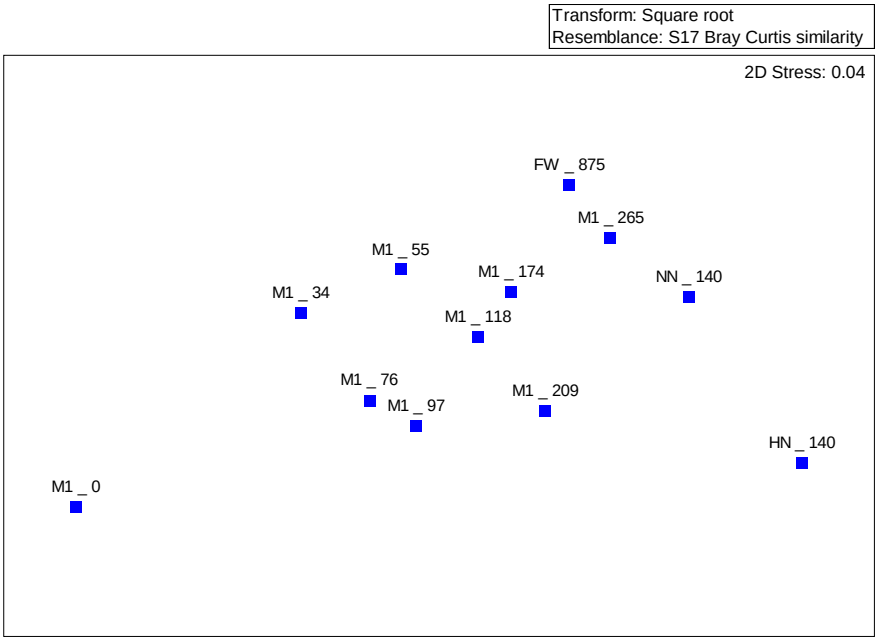


Figure 8. nMDS based on bacterial community profiles, genus level

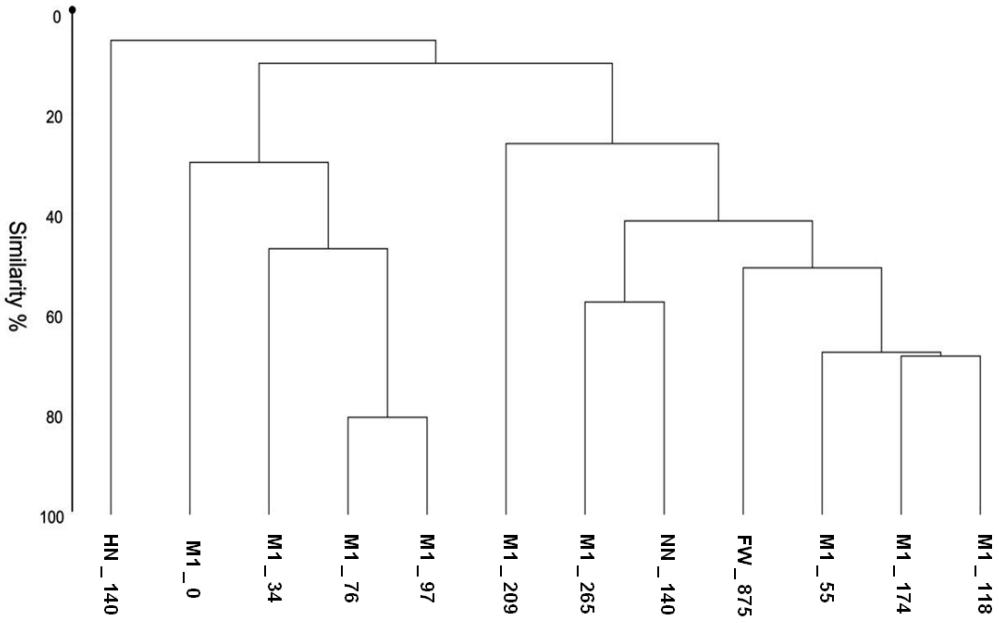


Figure 9. Cluster analysis based on bacterial community profiles, genus level

To understand community-environment relationships, CCA was performed. Due to co-linearity, some explanatory variables had to be removed from the dataset so that the factors pH, ammonia, total solids (TS), total VFA and the VFAs acetic, propionic, iso-butyric, n-butyric and heptanoic acid remained. Overall, CCA revealed that the environmental variables included were able to explain 99.8% of variation in the dataset.

It can be seen from CCA that specific samples were mainly correlated with VFAs whereas other samples were strongly correlated with pH, total solids and ammonia (Figure 10). Especially the correlation with iso-butyric acid for samples M1_76 and M1_97 and to a smaller extent the correlation of samples M1_34, M1_55 and M1_118 with acetic acid indicates an effect on communities by these VFAs. Similarly, some samples appeared to be negatively correlated with propionic acid (M1_34, M1_55, M1_76 and M1_97) or heptanoic acid (FW_875 and M1_174). Monte Carlo permutation revealed statistical significance for the parameters total solids and ammonia.

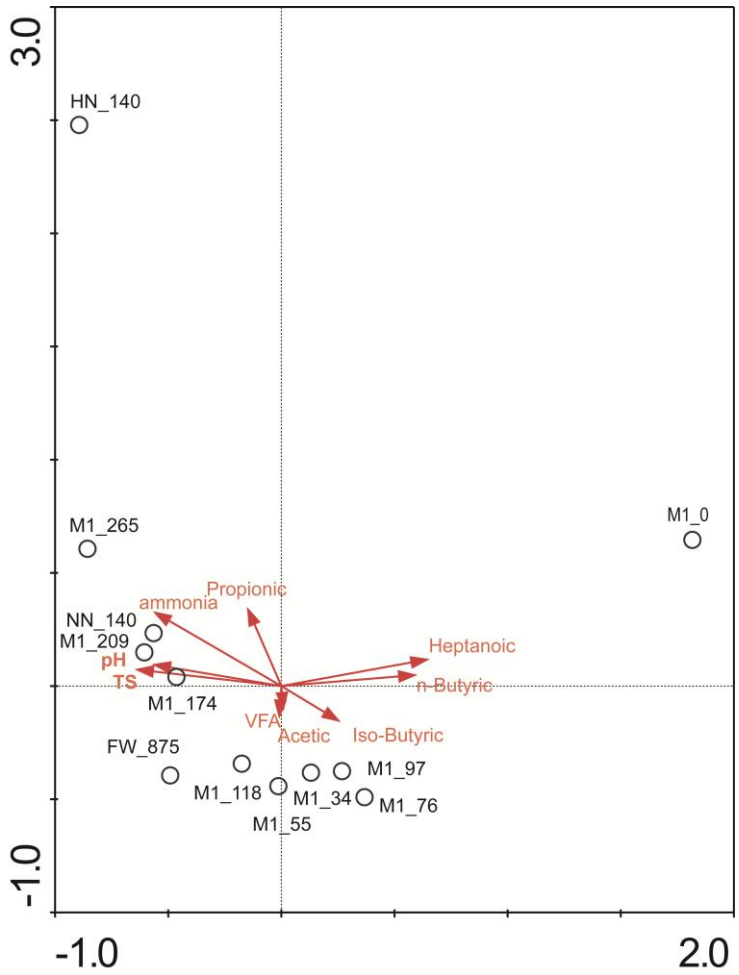


Figure 10. CCA based on bacterial community profiles

2.4.2 Methanogen community

In total, 52,916 sequences were obtained from mcrA amplicon sequencing. For analyses of community structure and its relationship with environmental variables as well as taxonomy, a minimum of 2 sequences within a cluster was chosen.

The main clusters present in the studied samples belonged to members of the Methanosarcinales (clusters 1, 3, 4, 7, 9 and 11), the Methanobacteriales (clusters 2, 8 and 10), a currently unknown clade related to previously extracted sequences from food digesters (cluster 6) and the Methanomicrobiales (cluster 5). Furthermore, clusters could be related to *Methanosphaera stadtmanae*, *Methanothermobacter crinale*, *Methanoculleus bourgensis*,

Methanoculleus thermophilus, *Methanoregula formicicum*, *Methanospirillum hungatei*, *Methanosaeta concilii*, *Methanosaeta* sp., *Methanosarcina* sp., and sequences obtained from a food digester namely F-G878RAR07H0OA4 (Figure 11 and Table 7).

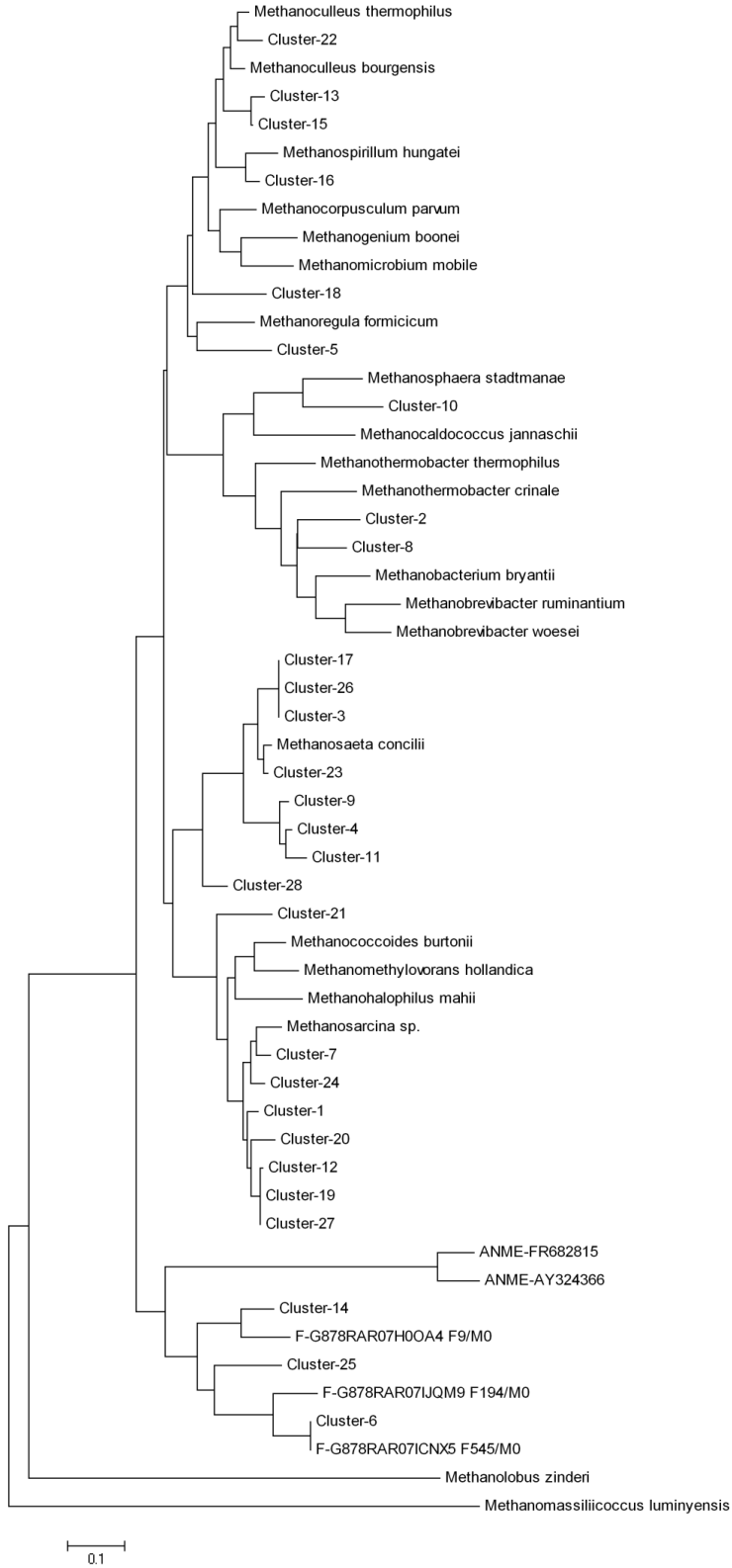


Figure 11. Phylogenetic tree based on mcrA sequence analysis

Table 7. Summary of present mcrA clusters per sample

Cluster	Order	Family	Relative	M1_0	M1_34	M1_55	M1_76	M1_97	M1_118	M1_174	M1_209	M1_265	FW_875	NN_140	HN_140
10	Methanobacteriales	Methanobacteriaceae	Methanosphaera stadtmanae	■	■	■	■		■	■	■			■	■
2	Methanobacteriales	Methanobacteriaceae	Methanothermobacter crinale	■	■	■	■	■	■	■	■	■	■		■
8	Methanobacteriales	Methanobacteriaceae	Methanothermobacter crinale	■	■	■	■	■	■	■	■		■		
13	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus bourgensis							■			■		
15	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus bourgensis							■			■		■
22	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus thermophilus	■	■					■		■			
5	Methanomicrobiales	Methanoregulaceae	Methanoregula formicicum	■	■	■	■	■	■				■		■
16	Methanomicrobiales	Methanospirillaceae	Methanospirillum hungatei	■	■	■		■		■					
18	Methanomicrobiales	unknown	na	■	■					■	■				
3	Methanosarcinales	Methanosaetaceae	Methanosaeta concilii	■	■	■	■	■	■	■	■	■	■	■	■
17	Methanosarcinales	Methanosaetaceae	Methanosaeta concilii				■	■		■	■				
23	Methanosarcinales	Methanosaetaceae	Methanosaeta concilii	■			■	■							
26	Methanosarcinales	Methanosaetaceae	Methanosaeta concilii	■								■	■		
4	Methanosarcinales	Methanosaetaceae	Methanosaeta sp.	■	■	■	■	■		■	■				■
9	Methanosarcinales	Methanosaetaceae	Methanosaeta sp.	■	■	■	■	■			■				
11	Methanosarcinales	Methanosaetaceae	Methanosaeta sp.	■	■	■				■					■
28	Methanosarcinales	Methanosaetaceae	na	■							■	■			
1	Methanosarcinales	Methanosarcinaceae	Methanosarcina sp.	■	■	■	■	■	■	■	■	■	■	■	■
7	Methanosarcinales	Methanosarcinaceae	Methanosarcina sp.	■	■	■	■	■	■	■	■	■			■
12	Methanosarcinales	Methanosarcinaceae	Methanosarcina sp.				■	■	■	■	■	■		■	■
19	Methanosarcinales	Methanosarcinaceae	Methanosarcina sp.				■	■		■	■		■		
20	Methanosarcinales	Methanosarcinaceae	Methanosarcina sp.				■	■	■	■					
27	Methanosarcinales	Methanosarcinaceae	Methanosarcina sp.	■							■		■		
21	Methanosarcinales	unknown	na					■	■	■	■				
14	unknown	unknown	F-G878RAR07H0OA4	■	■	■	■			■					
6	unknown	unknown	F-G878RAR07ICNX5	■						■	■	■	■		
25	unknown	unknown	na	■					■						

A comparison of methanogen community structure in digesters fed with food waste over several months revealed a strong shift between samples M1_55 and M1_75 (Figure 12). This shift appeared to be linked to a reduction in diversity and an increase of a member of the Methanosarcinales (cluster 1) and decline of a member of the Methanobacteriales (cluster 2). In comparison, the sample derived from a digester fed on food waste for around 4 years (FW_875) consisted mainly of sequences belonging to an unknown clade.

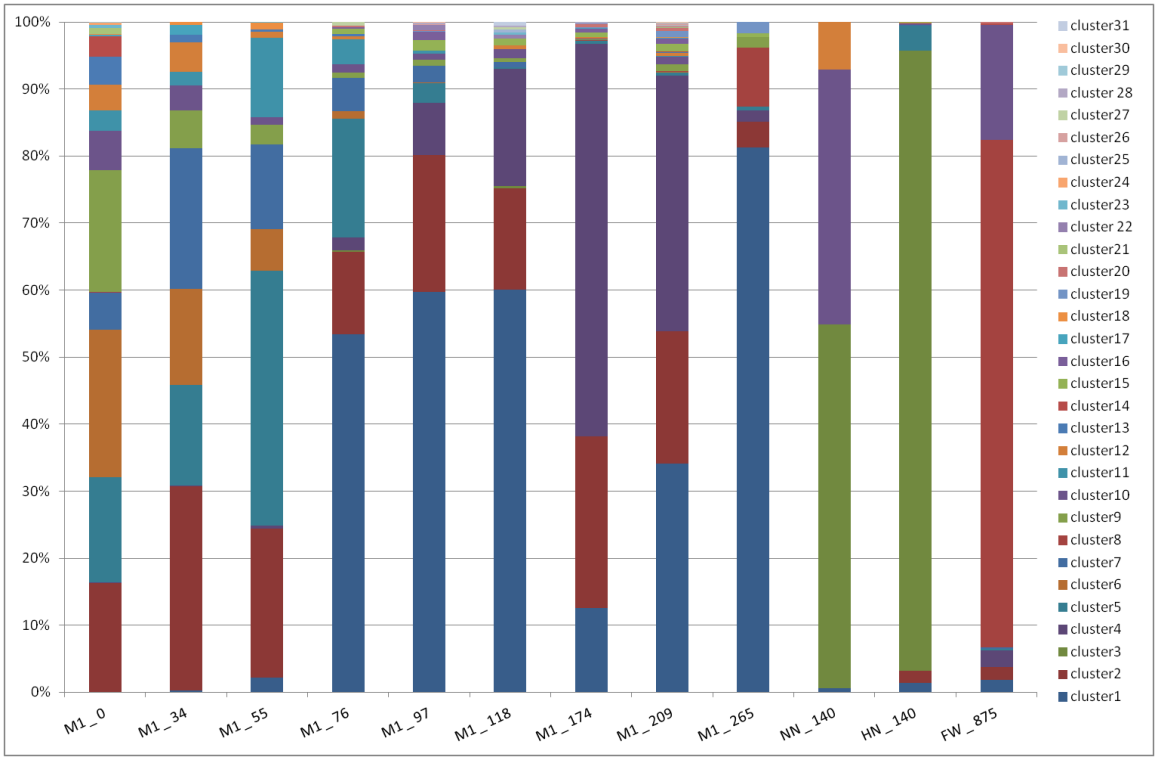


Figure 12. Community structure of methanogens based on mcrA sequencing

The differences between communities was validated by clustering and ordination (Figures 13 and 14) confirming a strong shift in community structure in digester M1 between day 55 and 76. Methanogen community structure in reactor FW_875 appeared completely different from the other samples. Also sample HN_40 and NN_140 were more similar to each other than the other samples in the datasets.

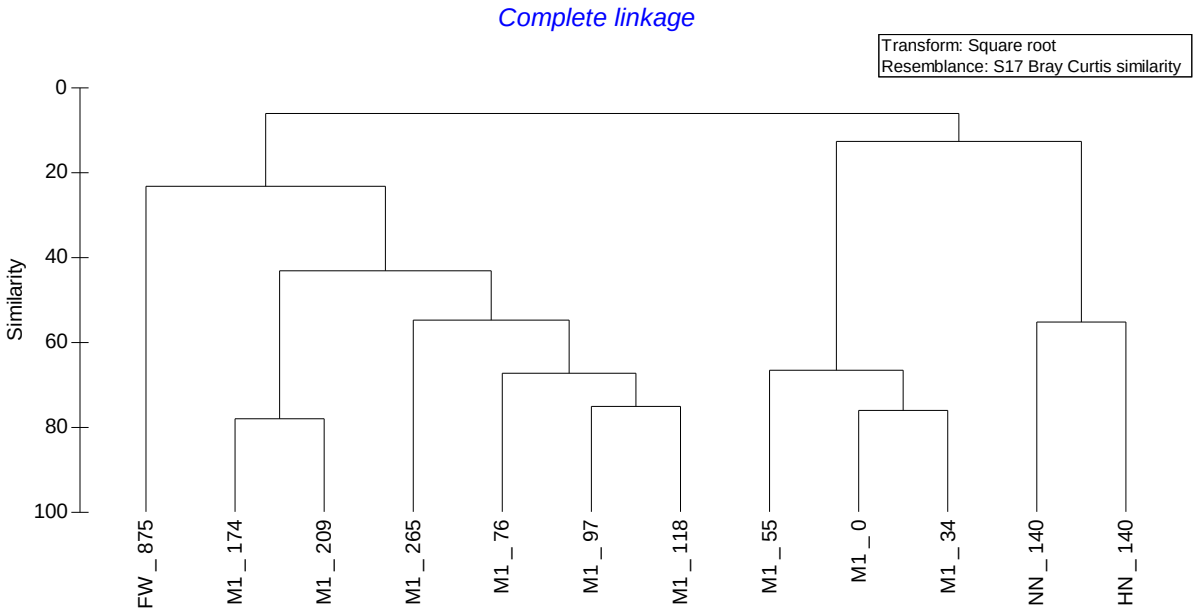


Figure 13. Cluster analysis based on mcrA sequences

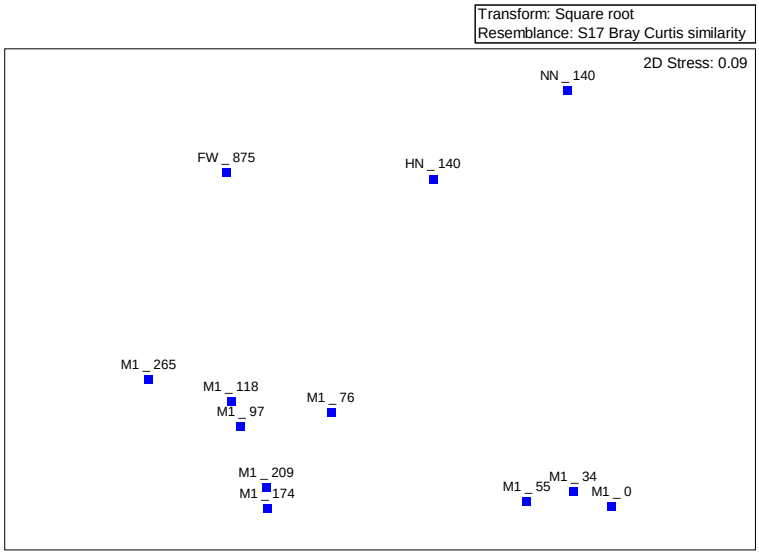
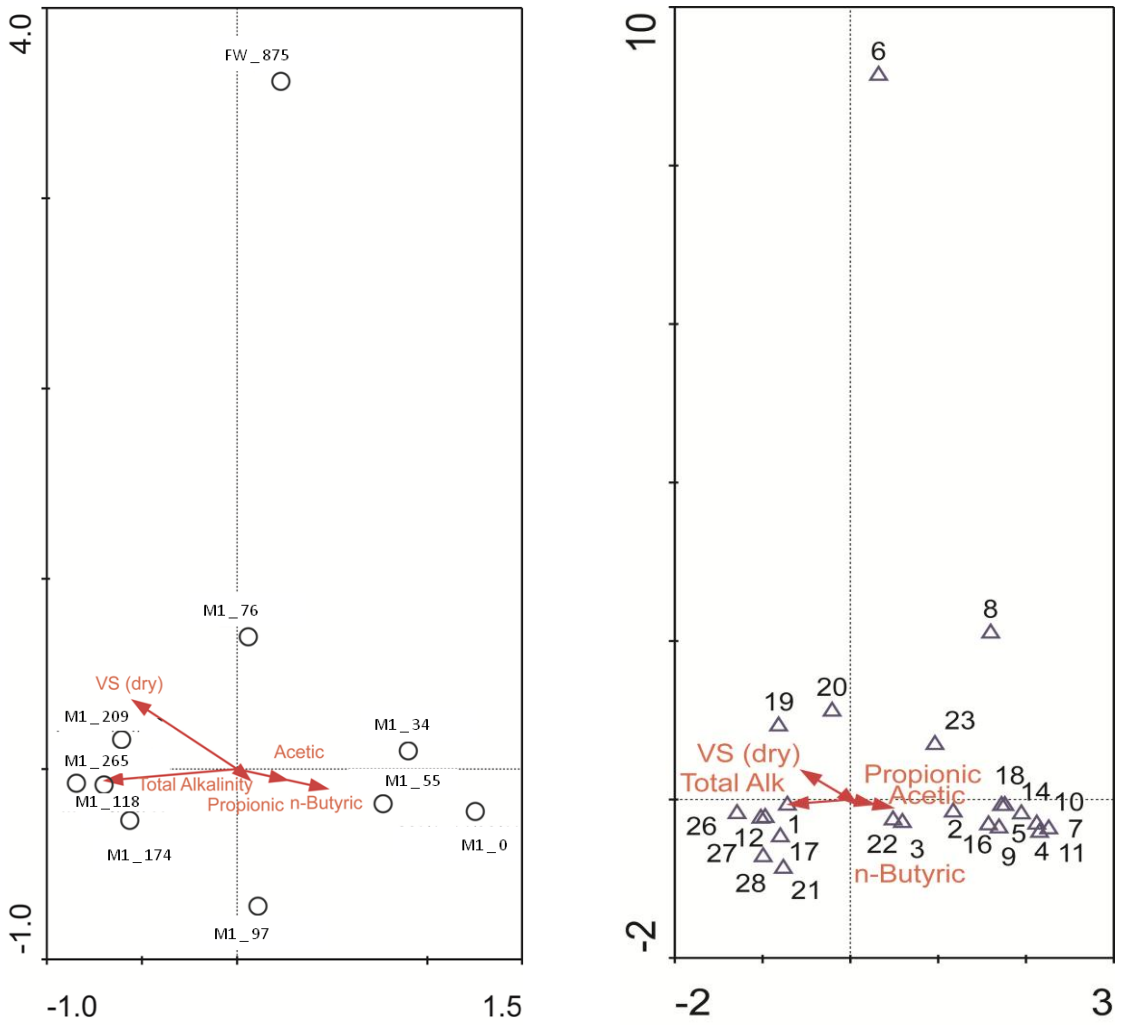


Figure 14. nMDS based on mcrA sequences

For the analysis of environment-species relationships CCA was conducted including the factors total alkalinity, VS (dry weight basis) as well as the VFAs acetic, propionic and n-butyric acid. Overall, the abiotic factors explained 87.8% of the variation in the community.

CCA showed a strong correlation of total alkalinity with the samples M1_174, M1_118 and M1_265, whereas volatile solids were correlated with sample M1_209 (Figure 15). The strongest correlation with the different VFA species could be found for n-butyric acid being linked with M1_55 and M1_0, but also acetic and propionic acid could be correlated with those samples. Similarly, certain methanogens could be linked with abiotic factors. Especially certain clusters related to *Methanoculleus thermophilus*, *Methanosaeta concilii*, *Methanothermobacter crinale*, *Methanospirillum hungatei*, *Methanosaeta* sp, *Methanoregula formicicum*, *Methanosarcina* sp., *Methanosphaera stadtmanae*, unclassified sequence F-G878RAR07H0OA4 and an unknown member of Methanomicrobiales (clusters 22, 3, 2, 16, 9, 5, 4, 11, 7, 10, 14 and 18) were correlated with an increase in respective VFAs. Two members of Methanosarcinales were positively correlated with an increase in volatile solids (clusters 19 and 29), whereas some methanogens were linked to total alkalinity (clusters 26, 27, 28, 12, 21, 1, 17). The latter clusters all belong to Methanosarcinales. Factors correlating positively with clusters 23 and 8 (relatives of *Methanosaeta concilii* and *Methanothermobacter crinale*) were not included in the dataset, but it could be seen from Figure 15 that these phylotypes were negatively correlated with total alkalinity.



a) sample-environment correlation b) species-environment correlation

Figure 15. CCA based on mcrA sequence data

The methanogenic structure analysis results by FISH and by gene sequencing on M1 showed reasonably good agreement. Due to the demonstrated limitation of FISH analysis, also due to the high resolution of gene sequencing analysis, more samples on M1-4 and T1-4 digesters have been sent out for gene sequencing. It is noted that the detailed statistical analysis on gene sequencing results also provided insight information on the relation between microbial community structure and digester operating parameters. Appendix A is a paper draft as an example on how to link the information on microorganisms with digester physicochemical characteristics.

3 Anaerobic microbial community analysis at MTT

3.1 Digester operation

Two pairs of 11-litre working volume anaerobic digesters were used in this study at mesophilic condition (37 °C). The batch of source segregated domestic food waste used in this study was collected in Ludlow, UK. Half of the food waste was treated with a novel double-auger autoclave (AeroThermal Group Ltd, UK) in 160 °C and 6.2 bars (autoclaved FW), and the rest was left untreated (untreated FW). Both portions were then passed through a macerating grinder (S52/010 Waste Disposer, IMC Limited, UK) to reduce particle size prior to use. One pair of digesters was fed with autoclaved FW, and the other pair with untreated FW.

At the beginning of the experimental run, both digesters used for T-RFLP analysis (R1M untreated and R3A autoclaved) were inoculated with sewage sludge digestate from the Biovakka Suomi Ltd Turku plant. Digesters were started at an OLR of 2 kg VS m^{-3} day^{-1} and hydraulic retention time (HRT) of 117 and 94 days for untreated R1M and autoclaved R3A, respectively. On day 151 the OLR was raised to 3 kg VS m^{-3} day^{-1} and on day 256 to 4 kg VS m^{-3} day^{-1}, shortening HRTs from 78 to 58 days in R1M and from 63 to 47 days in R3A. Microbial sampling was done in OLRs of 3 and 4 kg VS m^{-3} day^{-1}. Reactors were supplemented with trace element solutions containing cationic elements Al (0.1 mg l^{-1}), B (0.1 mg l^{-1}), Co (1.0 mg l^{-1}), Cu (0.1 mg l^{-1}), Fe (5.0 mg l^{-1}), Mn (1.0 mg l^{-1}), Ni (1.0 mg l^{-1}), Zn (0.2 mg l^{-1}) and oxyanions Mo (0.2 mg l^{-1}), Se (0.2 mg l^{-1}) and W (0.2 mg l^{-1}).

Digestate samples for chemical analyses and microbial samples during the sampling period (days 231-328, OLRs 3 and 4 kg kg VS m^{-3} day^{-1}) were collected at regular intervals. More detailed information on digester operation can be found in VALORGAS deliverable D3.3 and Tampio et al. (2013).

3.2 T-RFLP analyses

3.2.1. Methods for T-RFLP analysis

Total community DNA was extracted from selected samples based on CH_4, pH and TAN profiles. Approximately 0.25g of sample was used for extraction using FastDNA® SPIN Kit for Soil (MP Biomedicals, US) according to manufacturer protocol. DNA extractions were visualised by ethidium bromide staining after gel electrophoresis in 1% (w/v) agarose and 1x

TBE buffer. Quantifications of genomic DNA were performed using a NanoDrop ND1000 (NanoDrop Technologies, Wilmington, DE, U.S.).

16S rRNA genes were amplified in duplicate for each sample using the bacterial primers, pA and pH (Edwards et al, 1989) and the archaeal primers, 2AF and 915R (Stahl et al., 1991, DeLong et al., 1992). Both forward primers were labeled at the 5'-end with the phosphoramidite dye 6-FAM and the reverse primers with VIC (Applied Biosystems). One microliter of DNA extracts was applied in the PCR mix for bacteria and two for archaea. The cycle profiles used were: denaturation at 95 °C for 1 min, annealing at 55 °C (archaea) or 52 °C (bacteria) for 1 min extension at 72 °C for 3 min; the number of cycles was 35, and a final extension of 20 min at 72 °C. The amplicons were purified using a PCR purification kit (Qiagen, Venlo, Netherlands) and quantified using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, U.S.). The purified PCR product (100 ng) was digested with the *Hha*I and *HpyA*V restriction enzymes for bacteria and *Hha*I and *Taq*I for archaea (Fermentas, St. Leon-Rot, Germany). DNA fragments were precipitated with 95% ethanol and washed with 70% ethanol, then vacuum dried and resuspended in 15 µL of distilled water. 1 µL was mixed with 9 µL of formamide containing GeneScan 1200 LIZ Size Standard (Applied Biosystems, Halle, Belgium) and separated on a 3500xL Genetic Analyzer (Applied Biosystems, Halle, Belgium).

3.2.2 Statistical analysis of the T-RFLP data

The T-RFLP electropherograms were analysed using Peak Scanner Software v.1.0 (Applied Biosystems, Halle, Belgium). The relative abundances of T-RFs were determined by calculating the ratio between the height of each peak and the total peak height of all peaks within one sample. A cut off point for fragment sizes included in further analysis was >20 bp. Terminal restriction fragment length polymorphism (T-RFLP) analysis was done in triplicate for each sample. The restriction endonucleases selected for further analyses were those producing the highest numbers and best size distribution of T-RFs *in silico* using the online program MiCA ISPaR (Shyu et al., 2007). After testing them on the extracted DNA one enzyme showed the most suitable, thus *Hha*I using reverse and forward primers was chosen for further analyses of archaea and bacteria, respectively. MiCA APLAUS (Shyu et al., 2007) was used to infer the plausible community structure based on our data.

Only peaks with more than 2% abundance were considered. The range-weighted richness was determined as the number of peaks in each electropherogram. Interpretation of T-RFLP profile was made using Principal Coordinates Analysis (PCoA) with Bray Curtis similarity index. The software used for this part of the study was PAST v.2.15 (PAleontological Statistic, Hammer et al., 2001) and Qiime (Caporaso et al., 2010). Shannon's diversity index (*H*) was calculated on T-RFLP data using PAST software as $H = \Sigma\ \rho_i \ln \rho_i$ (Magurran, 1998). To statistically test whether microbial assemblages varied between bioreactor treatments, a one-way analysis of variance (ANOVA) was performed (SAS® software package, Version 9.2).

3.3 T-RFLP analysis results

T-RFLP fingerprints of bacterial 16S rRNA gene fragments revealed a total of 33 terminal restriction fragments (T-RFs) on the forward fragment and 20 T-RFs on the reverse fragment of the enzyme *Hha*I for bacterial population (Figure 16). For archaea 17 T-RFs on the forward fragment and 18 T-RFs on the reverse fragment were generated with the same enzyme (Figure 17). This implies lower diversity of the archaeal community than the bacterial population in both digesters (the tested enzymes *Hpy*AV for bacteria and *Taq*I for archaea showed a lower number of T-RFs). Some T-RFs were detected with high abundance; thereby, on bacteria the T-RFs 377, 555, 560 and 1084 accounted for an average of 70% of the total population and the reverse fragments 431, 435 and 436 an average of 61%. Meanwhile the archaea *Hha*I forward fragments T-RFs 321 and 326 accounted for a range of abundance from 34-94% (Figure 17).

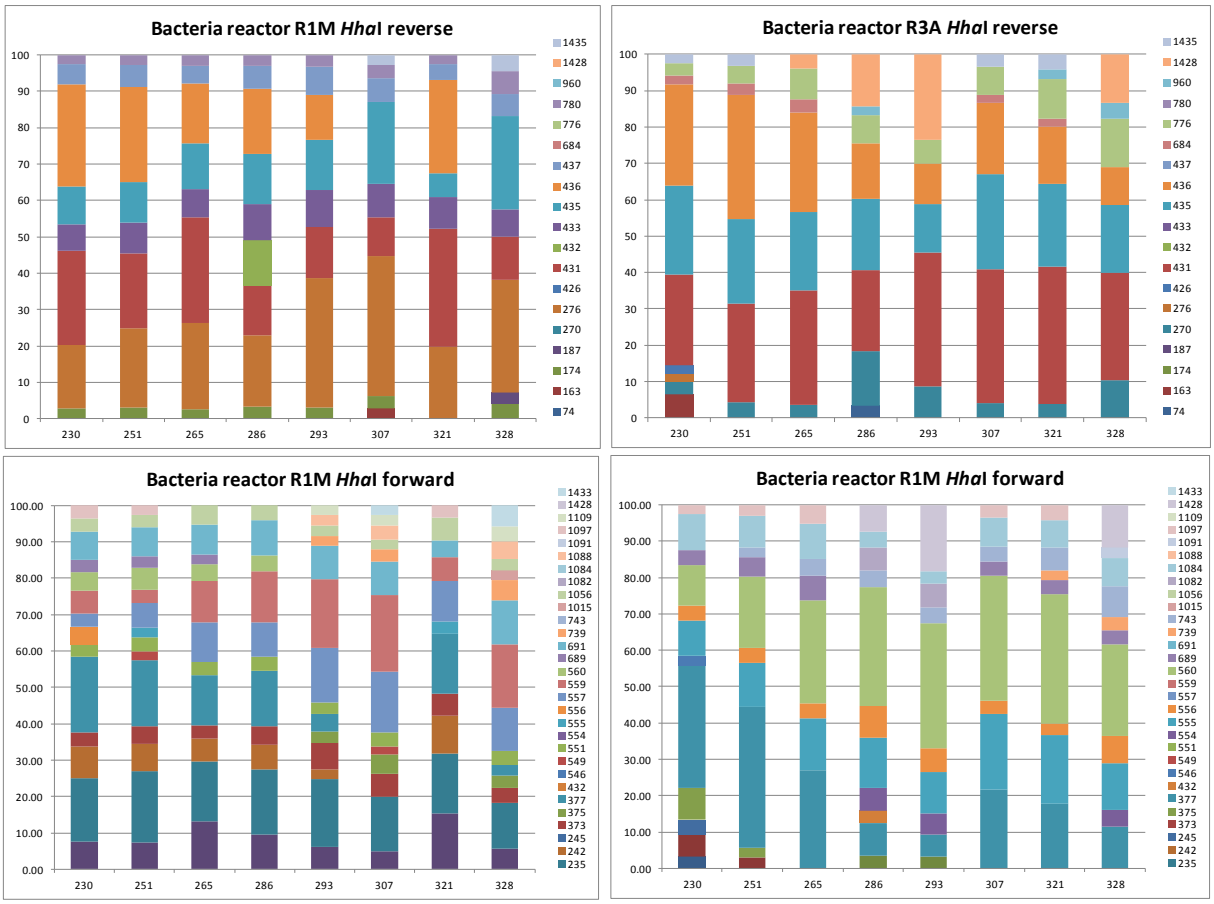


Figure 16. Relative abundance of 16S rRNA gene fragments retrieved from the anaerobic digesters R1M and R3A during the sampling period based on T-RFLP analyses with *Hha*I enzyme forward and reverse labeled primers for bacterial populations. The length of T-RFs in base pairs (bp) was indicated.

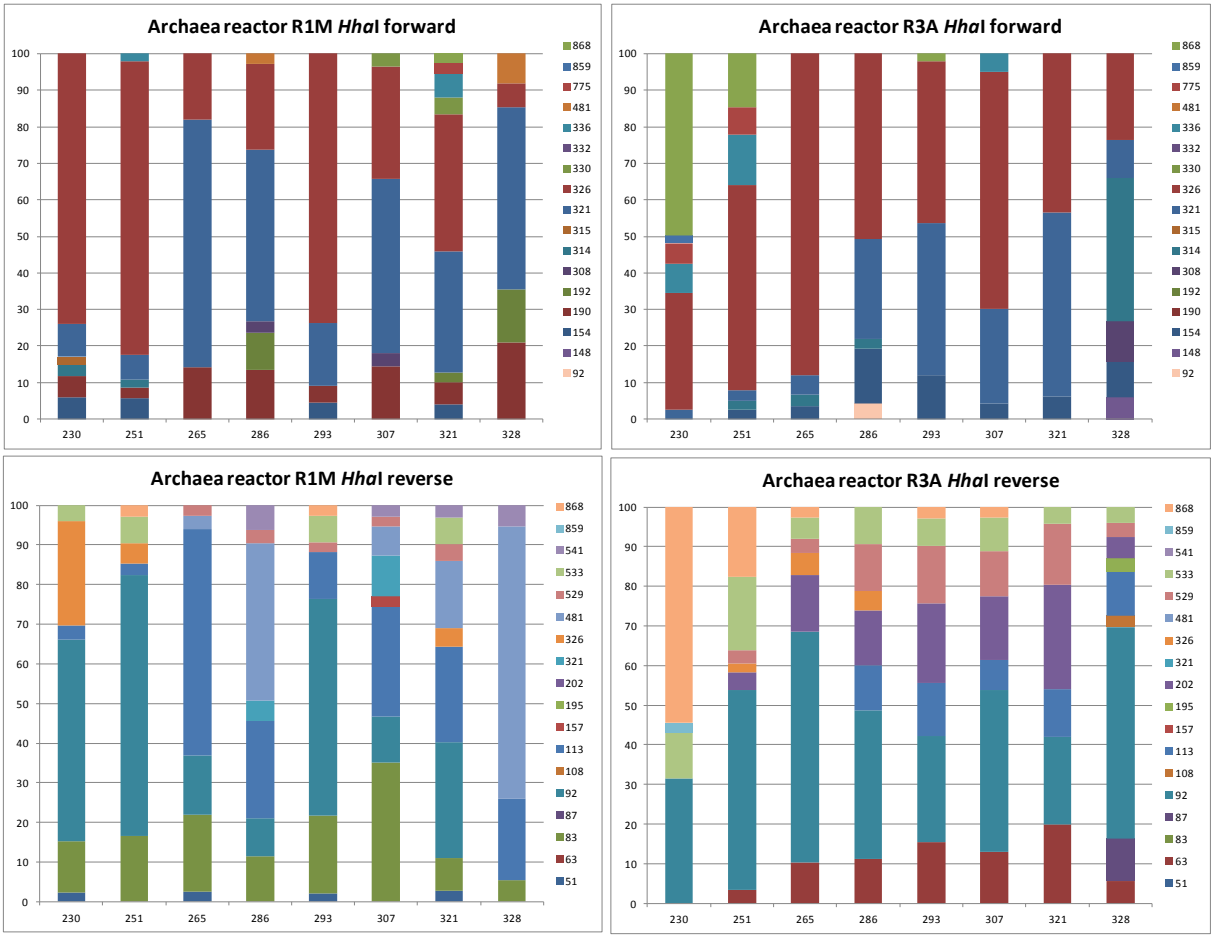


Figure 17. Relative abundance of 16S rRNA gene fragments retrieved from the anaerobic digesters R1M and R3A during the sampling period based on T-RFLP analyses with *Hha*I enzyme forward and reverse labeled primers for archaeal populations. The length of T-RFs in base pairs (bp) was indicated.

It was found that for fragments 182 and 376 *Taq*I enzyme accounted for 91% of total abundance of population. The change of OLR did not influence much in the appearance or disappearance of specific T-RFs but a clear change of the abundance of some T-RFs on bacterial and archaeal populations was revealed. The presence of T-RFs within STRs was very similar during sampling period showing mainly difference in abundances between the digesters. The methane production was notably higher (2-17%) in the untreated digester (R1M) compared with the autoclaved digester (R3A) (Figure 18).When comparing Shannon-Wiener diversity indexes the digester R1M showed a higher diversity than the R3A for bacterial communities during the course of the process whereas the correlation of indexes on archaea populations seemed to be more or less random (Figure 19). PCoA with Bray Curtis similarity index was used to visualise relationships among bacterial and archaeal communities. PCoA of 16S rDNA T-RFLP data revealed clustering related to digester (for bacterial communities variance between reactors was up to 74%) but not so clearly related to date (13%) although there was a segregation based on the change of OLR on day 259 (see Figure 20a). Association between the community structure and pretreatments or change of OLR was observed but not so clearly for the archaea population compared to bacteria (Figure 20b).

When archaeal T-RFLP data was analysed using APLAUS application it was detected that the genus *Methanosarcina* was present during all the period on both digesters, meanwhile the genera *Methanocalculus* and *Methanoculleus* (both belonging to *Methanomicrobiales* order) and genus (family *Methanococcaceae*) were present in the R1M during all the period but were undetectable in R3A after the day 251. Moreover, a large fraction of 16S-rDNA T-RFs could only be assigned to uncultured archaeon, demonstrating that numerous microorganisms are still unclassified or unknown. When analyzing bacterial population it was found that the genus *Clostridia* as well as *Bacillus* was found in both digesters allocated in several sizes of T-RFs. Regarding to the community structure only 7.14% of the forward fragments and 27.7% of the reverse for the enzyme *Hha*I showed significant differences ($p<0.05$) between reactors (autoclaved *vs* untreated). Meanwhile for bacterial population the significance was 76% and 69% of the T-RFs for the forward and reverse fragment of the same enzyme, respectively.

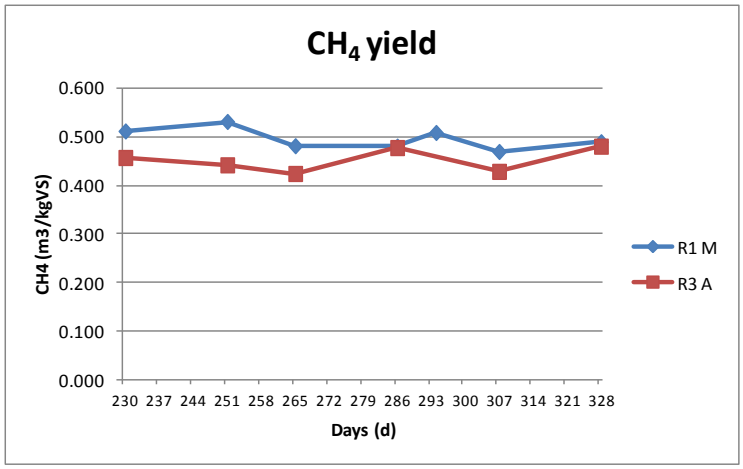


Figure 18. Methane production during the studied period on both digesters.

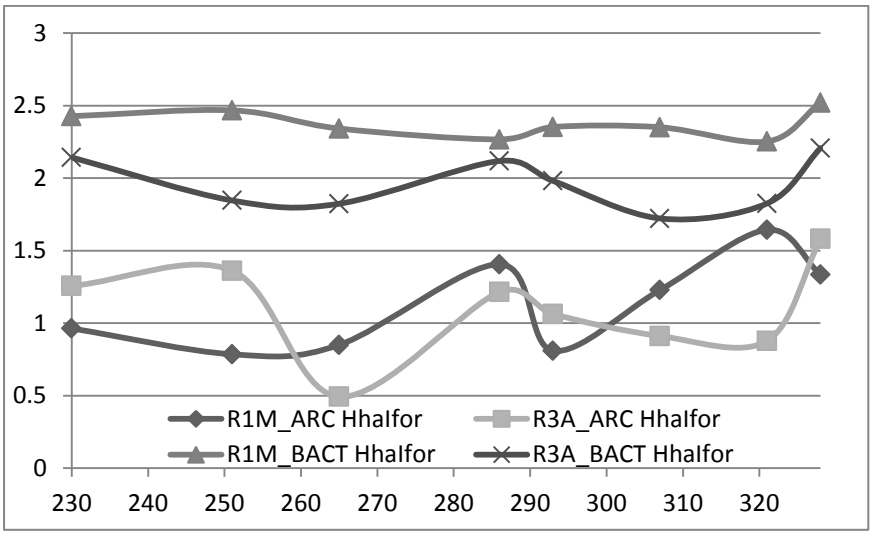


Figure 19. Shannon-Wiener diversity indexes of archaea and bacteria

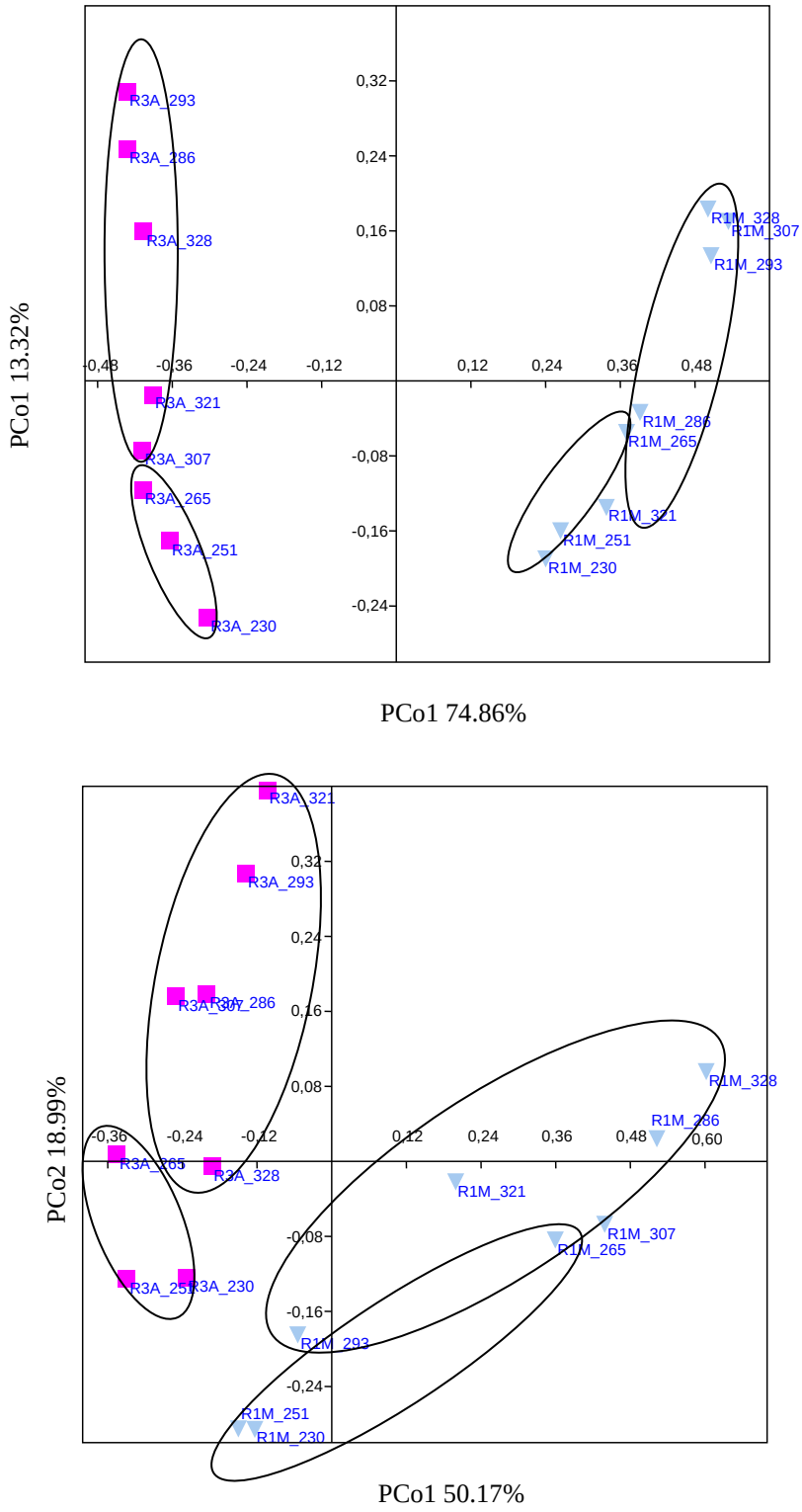
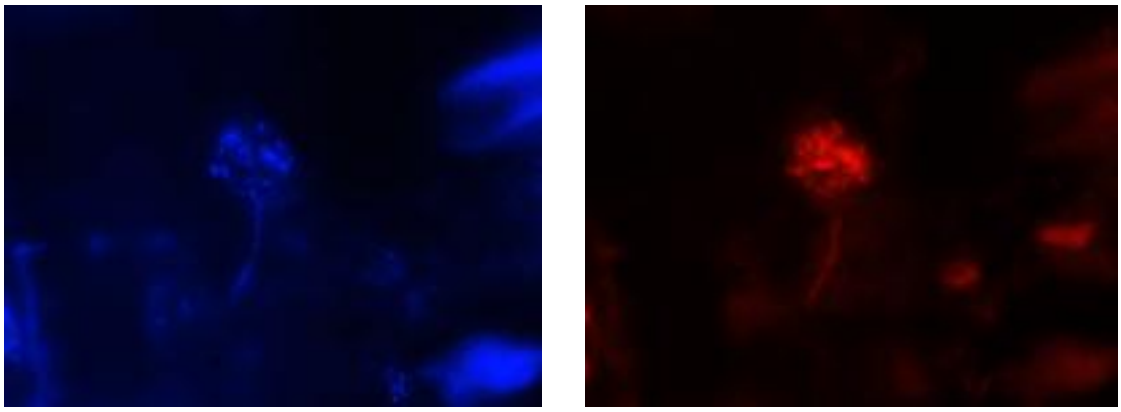


Figure 20. Differences in microbial community structure based on principle coordinates analysis of T-RFLP peak patterns in bacteria with the forward fragment obtained with the enzyme *Hha*I (a) and archaea with the reverse fragment obtained with the enzyme *HhaI* (b). The circles denote the communities before and after the change of OLR.

4 Anaerobic microbial community analysis at UNIVR

FISH analysis was also conducted for a two-phase thermophilic anaerobic digestion process on the effluent of dark fermentation reactor and methanogenic reactor (Cavinato et al., 2011).

The microbiological analysis was able to identify *Methanosarcinae* was acclimated in the dark fermentation reactor (Figure 21), and hydrogenotrophic methanogenic microorganisms *Methanomicrobiacea* and *Methanospirillacea* (Figure 22) and hetero-trophic *Methanosarcinae* in the methanogenic reactor.



DAPI FISH probe MS1414

Figure 21. Cluster of *Methanosarcinaceae* in dark fermentation reactor

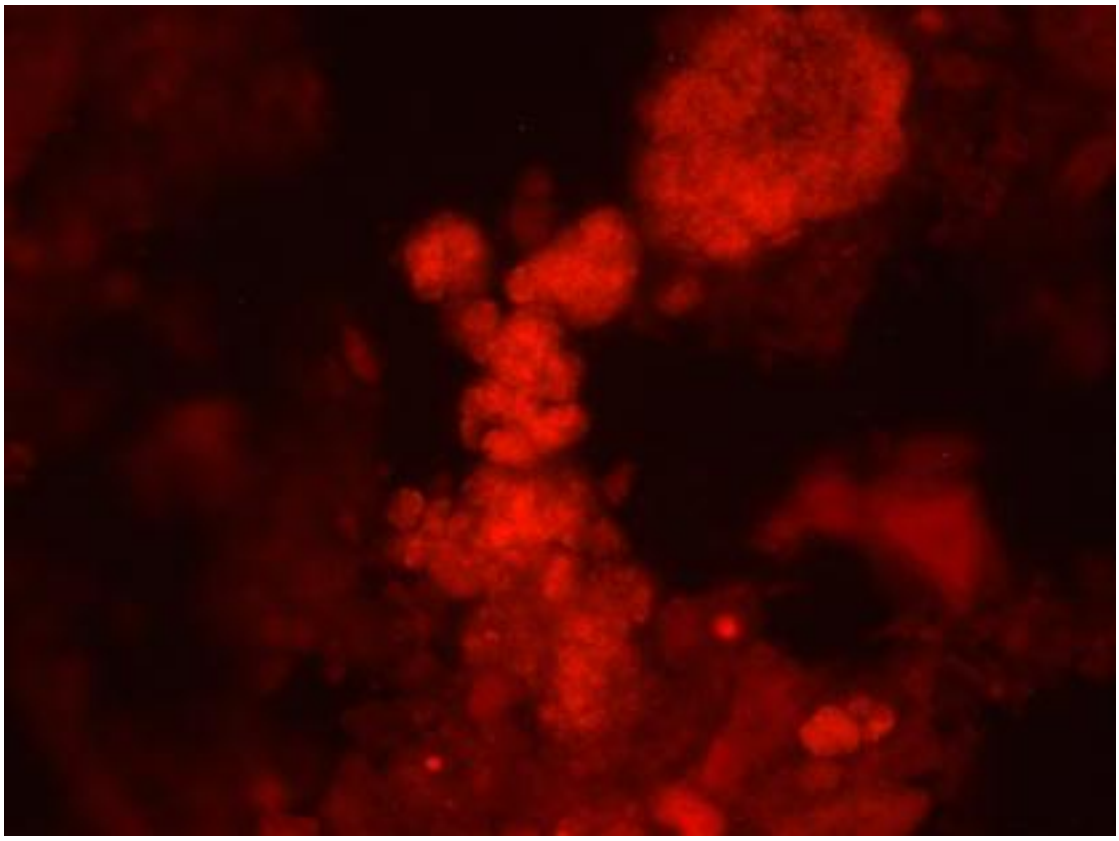


Figure 22. FISH image using probe MG1200 (*Methanomicrobiacea* & *Metahnospirillacea*)

5 Conclusions

It can be seen from the above molecular analyses that the microbial community structure changed after feeding with food waste materials. During acclimation to food waste digestion at mesophilic and thermophilic conditions, the methanogenic communities became dominated by distinct hydrogenotrophic methanogens and/or hetero-trophic *Methanosarcinaceae*. In the case of *Methanosarcinaceae*-dominated digesters, the main methanogenic pathway could only be detected by radioisotope labelling tests which were not conducted for each set of trials. However, these analyses showed the higher contribution of the hydrogenotrophic methanogenesis pathway which indicated the elevated requirement for certain essential trace elements. The trace element supplementation strategy should therefore reflect this demand. FISH techniques demonstrated limitations when used on digestate analysis; gene-sequencing analysis was then arranged for better microbial community structure investigation.

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Note: Papers marked * are outputs from the FP7 VALORGAS project

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