Energy and Environment Research; Vol. x, No. x; 2014
ISSN 1927-0569 E-ISSN 1927-0577
Published by Canadian Center of Science and Education

454 pyrosequencing assessment of biodegradative bacteria from thermal hydrolysis processes

Fabrizio Colosimo[1], Richard Lord[1], Russell Thomas[2], Jonathan R Lloyd[3], Christopher Boothman[3] and Robert M Kalin[1]

[1]Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow, United Kingdom

[2]WSP Parsons Brinckerhoff Ltd., Bristol, United Kingdom

[3]Williamson Research Centre for Molecular Environmental Science, School of Earth, Atmospheric and Environmental Sciences, The University of Manchester, Manchester, united Kingdom

Correspondence: Fabrizio Colosimo, Department of Civil and Environmental Engineering, University of Strathclyde, 75 Montrose Street, Glasgow G1 1XJ, United Kingdom. Tel: +34(0)620206662. E-mail: fabrizio.colosimo@strath.ac.uk

Received: October XX, 201X Accepted: November XX, 201X Online Published: November XX, 201X

doi:10.5539/ URL: http://dx.doi.org/

The research is financed by WSP Parsons Brinckerhoff and Scottish Government Joint Research Partnership in Engineering

Abstract

Anaerobic treatment process is a cost-effective method for treating organic wastes, since the biogas formed can be used for heat/electricity production and the digester residues can be recycled for other applications. An innovative use of the digestate could be as biodegradative and methanogenic inoculum for the stimulation of methane production in gas-producing or depleted wells. The microbial communities involved in the biodegradation of petrochemical waste are similar to the indigenous microorganisms typically found in unconventional basins. These communities also follow the same cascade of reactions: from the initial breakdown of complex molecules to the production of intermediate compounds used by methanogens. This study carried out a culture-independent assessment of the bacterial community composition of a digestate from the Bran Sands Advanced Digestion Facility (Middleborough, UK) and compared the results with the microbial populations found in unconventional gas basins. The 454 pyrosequencing analyses revealed a bacterial community dominated by *Thermotogae*, *Bacteroidia*, *Clostridia* and *Synergistia*, which are typically found in unconventional gas systems. The classification of nucleotide sequence reads and assembled contigs revealed a genetic profile characteristic for an anaerobic microbial consortium running fermentative metabolic pathways. The assignment of numerous sequences was related to hydrocarbon decomposition and digestion of cellulosic material, which indicates that the bacterial community is engaged in hydrolysis of plant-derived material. The bacterial community composition suggest that the effluent of the digester can be used as a biodegradative inoculum for the stimulation of methane generation in unconventional wells, where events of microbial methanogenesis have been previously observed.

Keywords: Pyrosequencing, anaerobic degradation, petrochemical waste.

1. Introduction

Organic residues accumulate in large amounts as left-overs of industrial activities, food processing and as household refuse; these waste products are therefore available as a substrate for biogas production (methane (CH_4)) in anaerobic reactors (Wiesenthal et al., 2007). Petrochemical waste has a considerable advantage compared with renewable primary products (maize silage, fodder beat, green rye, etc.) as there is no competition between the use of the substrate for the production of biogas and the use of the substrate as food.

In general, the biogas-forming process is characterized by three phases which proceed simultaneously in a continually fed reactor. Initially, bacteria attack and utilize polymers (complex hydrocarbons such as carbohydrates, proteins, and lipids) by excreting hydrolytic enzymes (e.g. cellulases, cellobiases, xylanases, amylases, lipases, and proteases) (Weiland, 2010). This process is called (*i*) hydrolysis or primary fermentation (Schink, 1997). The major products released by these bacteria are carbon dioxide (CO_2), hydrogen (H_2), volatile fatty acids such as acetate, and a wide range of intermediates. Those intermediates are then further transformed to H_2, CO_2, acetate, and other acids during a process called (*ii*) acidogenesis/acetogenesis or secondary fermentation (Schink, 1997). H_2 and CO_2 or acetate is used by methanogenic archaea for the production of methane, in a process called (*iii*) methanogenesis or methanation) (Weiland, 2010). Finally, the methane is converted to electricity by non-bacterial processes. To close the cycle, in most cases, the digestate of the biogas-forming process can be recycled as fertilizer or soil amendments (Bogner et al., 2008).

The influence of the composition and diversity of the microbial community on the stability of the biogas-forming process and on biogas yield is of great interest (Weiland, 2010). So far, several studies have focused on the microbial diversity in biogas plants supplied with renewable primary products and liquid manure as substrates (Schnürer et al., 1999; Cirne et al., 2007; Schlüter et al., 2008; Weiss et al., 2008; Kröber et al., 2009; Liu et al., 2009; Nettmann et al., 2010). Whilst most of these studies focused on the *Archaea* community structure, more research efforts should address the complex interplay within the bacterial community that ferment complex organic material.

Research into anaerobic processes is currently undergoing a reawakening due to the development of techniques suitable for mechanistic linking of whole community function and phylogeny (Vanwonterghem et al., 2014), and as a platform to investigate phenomena such as direct interspecies microbial electron transfer (Morita et al., 2011). At the same time as these discoveries are enhancing our understanding of the process, the scope of applications as biotechnological processes is also expanding. As a waste valorisation process, anaerobic digestion is important, as it allows almost complete recovery of inherent chemical energy during relatively low cost conversion to methane, a transportable, vehicle and natural gas network compatible energy source (McCarty et al., 2011). Anaerobic digestion has been traditionally applied to either slurries (2-6% solids) in mixed reactors (Batstone and Jensen, 2011), or to low solids, concentrated industrial or domestic waste-waters in high-rate processes such as the Upflow Anaerobic Sludge Blanket (UASB) (Smith et al., 2012). Anaerobic processes have not been widely studied and applied to treat petrochemical residues or for treatment of industrial waste-products with high concentration of organics, largely due to treatment quality or process sensitivity.

This work analyses the composition and the diversity of the bacterial community of digestate sludge from the Bran Sands Advanced Digestion Facility. The anaerobic digester operates using a range of biowaste, such as sewage, sludge cake (Class A biosolid) as well as petrochemical waste. The recent development of 'next-generation sequencing' such as 454 pyrosequencing has made it possible to deep-sequence microbial communities in complex biological samples without the time-consuming cloning procedure. The technique has so far been used for the sequencing of metagenomes from a number of biogas reactors (Schluter et al., 2008;). Schluter et al., (2008)

described the bacterial community from a full-scale, completely stirred tank reactor (CSTR) digesting maize silage (63%) and green rye (35%) together with small amounts of chicken manure. Bacterial members of the taxonomic classes *Clostridia* and *Bacteroidetes* were most abundant. Among the *Archaea*, the hydrogenotrophic *Methanoculleus* sp. dominated, but the acetoclastic *Methanosarcina* sp. were also detected. Lee et al., (2012) used 454 pyrosequencing of the V1, V2, and V3 regions of the 16S rRNA gene to assess the microbial community in seven full-scale reactors over time. Six of the reactors treated waste-activated sludge (one of these in combination with smaller amounts of food waste), and one reactor treated night soil. Sequences belonging to *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Chloroflexi* were found to be the most abundant, and the bacterial population was influenced by the digestion temperature. Werner et al. (2011) characterized bacterial communities in nine full-scale granulated sludge reactors treating brewery waste water by targeting a part of the rRNA gene. These bacterial communities were dominated by *Syntrophobacterales*, *Desulfuromonales*, *Bacteroidetes*, *Spirochetes*, *Clostridia*, *Chloroflexi*, and *Synergistia*. The present study carried out a characterisation of the bacterial communities in the effluent of a full-scale digester to identify bacteria related to hydrolysis and acetogenesis processes. High-throughput 16S rRNA gene sequencing of the V1-V5 hypervariable region was carried out using 454 technologies to investigate the bacterial communities, and the set of sequences obtained were further analysed using bioinformatics tools. These bacteria could potentially be used for application in unconventional gas systems to achieve a continuous generation of biogenic CH_4 from existing producing wells or depleted wells. In unconventional gas systems, such inoculum could help to achieve a continuous generation of biogenic CH_4, since the bacterial and archaeal consortium are already adapted to high temperatures and high concentration of organic compounds.

2. Material and Methods

2.1. Samples

The samples used for this study were collected fresh from the effluent of the Bran Sands Advanced Digestion Facility, which employs thermal hydrolysis processes for the conversion of liquid, solid sewage waste and petrochemical waste into biogas. The samples were collected in plastic PTFE containers and stored at -80°C until analysed.

2.2. Plant description

The Bran Sands Advanced Digestion Facility was commissioned in 2009 and designed to treat up to 40,000 tds/y of sewage sludge comprising:

- 14,500 tds/y of indigenous sludge from the adjacent effluent treatment works;
- 1,500 tds/y of liquid imports;
- 24,000 tds/y of cake imports from satellite works.

Liquid imports and indigenous sludges are pumped into a storage facility before being screened and transferred to a pre-dewatering plant. Cake transfer pumps move the dewatered cake to reception silos before being pumped to the thermal hydrolysis buffer tanks and two parallel thermal hydrolysis streams (provided by Cambi, www.cambi.com, CAMBI GROUP AS Skysstasjon 11A, 1383 Asker). Each stream consists of a pulper, four

reactors and a flash tank. The header is supplied with steam from a combination of waste heat recovery modules, which recover high grade energy from the gas engine exhaust streams and triple fuelled boiler plant. The output from the second thermal hydrolysis process streams is combined before being split into three separate digesters. The three digesters provide 18 days retention time, based on 90% Effective Digester Volume (EDV) at design throughput. The mesophilic temperature within each digester is controlled by air blast sludge coolers installed on each feed line. Digested sludge is dewatered using centrifuges and transferred to cake storage. This cake store has been provided with separate bays to enable loading and unloading of cake by front loaders whilst also allowing cake to be stored for longer periods before transfer off-site (to increase de-waterability). Odourous air which is generated within the facility is ducted to the existing odour control plants, which make use of biofilter technology. Liquors generated by the pre and post-digestion plant operation are transferred to the main effluent treatment works for processing. Northumbrian Water's existing advanced digestion plant at Bran Sands on Teesside is shown in Fig. 1.

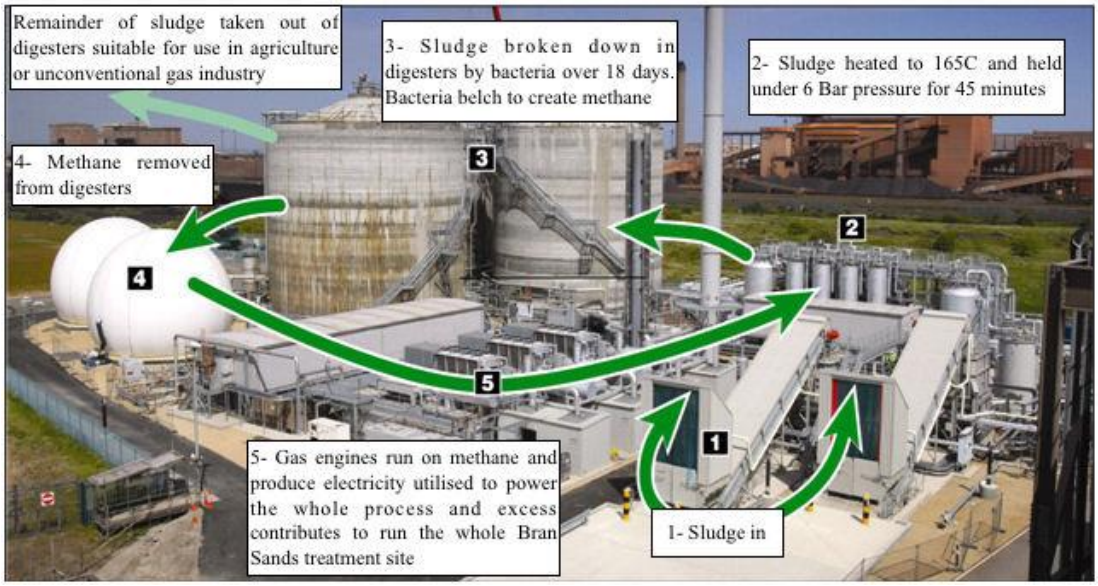

Figure 1. Schematic diagram of advanced digestion plant. Modified from North East Bioresources & Renewables (NEBR) (http://www.nebr.co.uk/).

2.3. 16S rRNA gene extraction

Prior to DNA extraction, all samples were shaken by hand before transferring 0.25 g to the bead-beating tube (PowerSoil DNA Kit PowerBead Tubes, MOBIO Laboratories, Carlsbad, CA, USA). The extraction was performed with few modifications of the manufacturer's protocol, and the DNA obtained was stored at -20°C until further analysis. Concentrations of double stranded DNA in the extracts were determined using the NanoDrop ND-1000 (Thermo Scientific).

2.4. 16S rRNA gene amplification and pyrosequencing

PCR of the V1-V5 hypervariable region of the bacterial 16S rRNA gene was performed using amplicon fusion universal bacterial primers 27F (Lane, 1991) and 907R, (Lane, 1995) synthesised by IDTdna (Integrated DNA Technologies, BVBA, Leuven, Belgium). The forward primer (5′ CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGAGAGTTTGATCMTGGCTCAG 3 ′)

consisted of a fusion containing the 454 Life Sciences 'Lib-L Primer A', a four-base 'key' sequence (TCAG), a unique ten-base barcode 'MID' (MID 5, Multiplex Identifier Adaptors, Roche. ATCAGACACG) sequence, and bacterial primer 27F.

The reverse fusion primer (5′ CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCGTCAATTCMTTTRAGTTT 3′) contained the 454 Life Sciences 'Lib-L Primer B', a 4 base 'key' sequence (TCAG), and bacterial primer 907R. The PCR amplification was performed in 75 µL volume reactions using 47.1 µl Roche PCR Grade Water, 7.5 µl Reaction Buffer (without $MgCl_2$), 9 µl 25 mM $MgCl_2$, 1.5 µl Nucleotide Mix, 3.75 µl DMSO, 1.2 µl 25M 907R Primer, 0.75 µl High Fidelity Enzyme Blend, 3 µl of sample DNA and 1.2 µl 25M The MID5 (Roche) was used to label the Forward Primer 27F. A negative and a positive control were also prepared for the PCR reaction, containing respectively 2 µl sterile H_2O and DNA extracted from *Geobacter sulfurreducens* (from the collection of The University of Manchester, School of Earth, Atmospheric and Environmental Sciences). The PCR conditions included an initial denaturing step at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, and a final elongation step at 72°C for 5 min. At the end of the run, the whole PCR product was mixed with 12.5 µl of 5x gel-loading dye, and 35 µl of the mixture was loaded on a 2% Tris-Acetate-EDTA/agarose gel. A 2000-100 bp ladder was also loaded on the gel that was run at 80 mV for ~ 2 h. At the end of the run, the DNA bands were observed on a Gel Doc 2000 Gel Imaging System (Bio-Rad Laboratories). Following gel electrophoresis, bands of the correct fragment size (~ 410 bp) were excised, purified using a QIAquick Gel Extraction Kit (Quiagen, Limburg, Netherlands) according to the manufacturer's protocol, and eluted in 30 µL of DNAse free H_2O. The purified PCR products were quantified using the NanoDrop ND-1000 (Thermo Scientific). The DNA products were then stored at 4°C until it was sequenced. The emulsion PCR was performed at The University of Manchester, School of Earth, Atmospheric and Environmental Sciences, the pyrosequencing run was performed at the sequencing facilities of Faculty of Life Science of The University of Manchester using a Roche 454 Life Sciences GS Junior.

2.5. Pyrosequencing data analysis

The 454 pyrosequencing reads were analysed using the Quantitative Insights Into Microbial Ecology pipeline (Qiime 1.8.0 release) (Caporaso et al., 2010b). Raw sequences were first assigned to the different samples by using the barcode sequences provided: sequences outside the 300–500 bp range were removed along with the reverse primer sequence, using the *split_libraries.py* script. The usearch 6.1 programme (Edgar, 2010) was used to perform filtering of noisy sequences and chimera checking. Operational taxonomic units (OTUs) were picked from and compared at 97% similarity with the May 2013 release (13_5) of greengenes OTU reference using the usearch 6.1 programme through the *pick_otus.py* script. The most abundant OTU sequence was chosen as a representative using the *pick_rep_set.py* script. Taxonomy assignment was based on the greengenes reference database (McDonald et al., 2012) using the Ribosomal Database Project Naive Bayes (RDP) classifier v 2.2 (Wang et al., 2007), with the confidence level set at 80% through the *assign_taxonomy.py* script. An OTU table was built using the *make_otu.py* script and a biological observation matrix file (BIOM) was built using *convert_biom.py* script. A OTU heatmap file was generated with the *make_otu_heatmap.py* script. The sequences were then aligned to the greengenes core reference alignment (De Santis et al., 2006) using PyNAST (Caporaso et al., 2010a) through the *align_seqs.py* script. Aligned sequences were then filtered using the *filter_alignement.py* script, and a phylogenetic tree was built through the *make_phylogeny.py* script (Price et al., 2009). Alpha diversity, alpha rarefraction and beta diversity were calculated using respectively the *alpha_diversity.py*, *alpha_rarefraction.py* and

beta_diversity_through_plots.py scripts. Jackknifing was performed with the *jackkinfed_betadiversity.py* script to directly measure the robustness of individual Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clusters and build jackknifed 2D and 3D PCoA plots. Sequences (average length of 400 bp) were analysed against the NCBI (USA) database using BLASTn program packages and matched to known 16S rRNA gene sequences to retrieve the closest relatives.

3. Results and Discussion

3.1. Phylogenetic analysis

The total number of sequences obtained was 3090, with an average length of ~ 400 bp. In total, 8 phyla, 9 classes, and 15 genera were found among Bacteria. The Chao1 index of the sample, at 3% distance and with 96% confidence intervals together with the α-diversity (Fig. 2), indicates a trend of higher microbial richness.

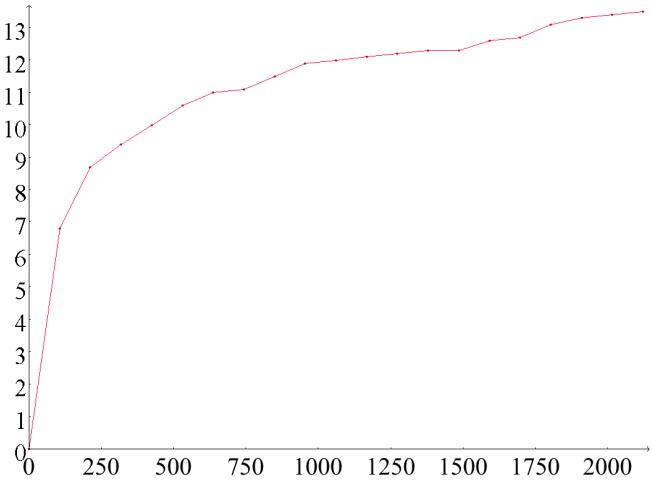

Figure 2. α-Diversity plot, showing the number of leaves in taxonomy.

The phylogenetic analysis showed a dominance of *Thermotogae* at the class level (Fig. 3), that accounted for the 60.71% of the sequence reads analysed. Microorganisms of this class have the genetic potential to oxidize butyrate to CO_2/H_2 and acetate. The second most abundant class is the *Bacteroidia* (15.28%), followed by *Clostridia* (10.42%) and *Synergistia* (7.18%). These observations suggest that the degrading consortium in the bioreactor consists of additional syntrophic interactions beyond the standard H_2-producing syntroph–methanogen partnership that may serve to improve community stability. Members of the *Thermotogae* in the bioreactor community may participate in the syntrophic interactions by producing acetate from an intermediate molecule. This intermediate molecule may be the butyrate produced by the *Clostridia* population. Lastly, a small presence of *Cloacamonae* belonging to the phylum WWE1 was also detected (3.33%), microorganisms in the bacterial phylum WWE1 have been implicated in cellulose degradation in anaerobic sludge digesters (Limam et al., 2014) and were also found in the coal-bearing strata of the Cherokee Basin, USA (Kirk et al., 2015). The presence of this of these bacterial classes suggest a putative role in the degradation of complex macromolecules.

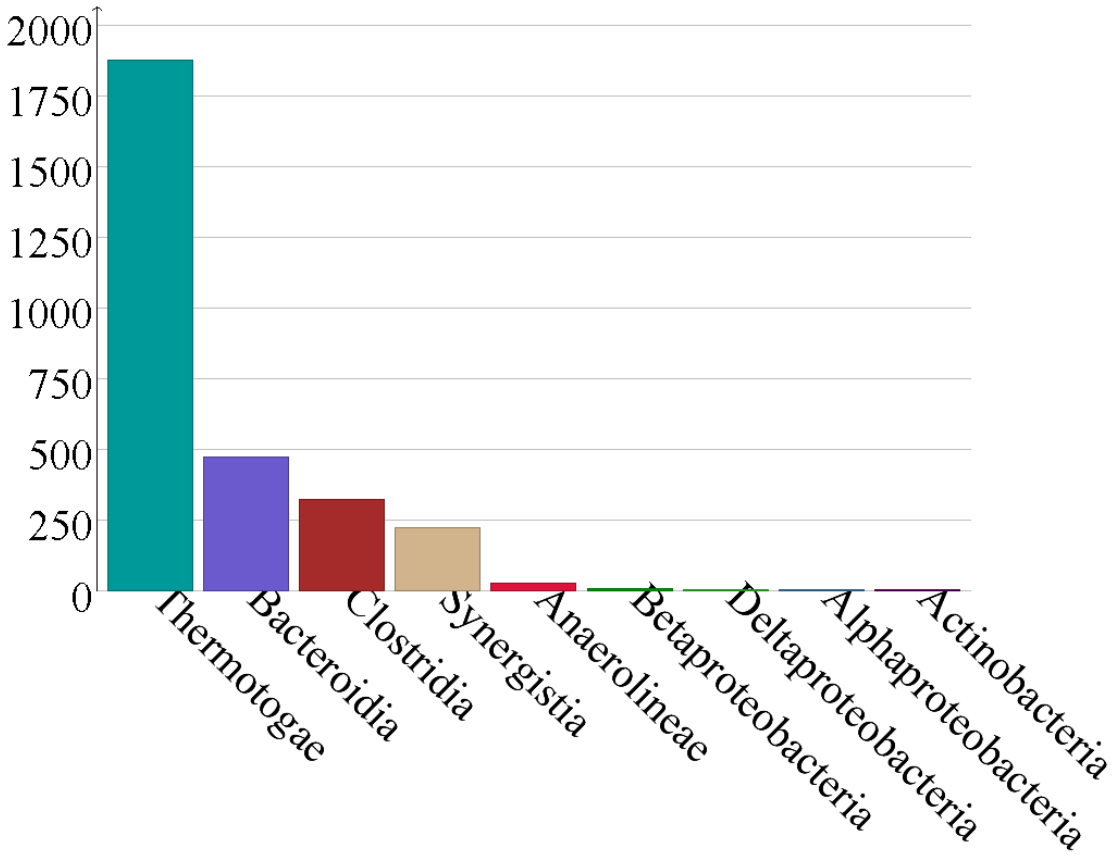

Figure 3. Bar chart showing the bacterial class in each sample that were tested.

At the genus level, the digestate sample was characterized by a significant dominance of unclassified members of the *Thermotogaceae* family (60.71%). Members of *Thermotogaceae* are moderately thermophilic to hyperthermophilic, fermentative bacteria, typically isolated from hydrothermal systems (Dahle et al., 2011). The cells of *Themotogaceae* members are wrapped in an outer membrane (toga), which confer resistance to high temperature. They typically metabolize carbohydrates and have varying amounts of salt and oxygen tolerance. (Huber et al., 1986) Members of these family were isolated from a number of subsurface environments: *Thermotoga subterranea* strain SL1 was found in a 70°C deep continental oil reservoir in the East Paris Basin, France (Li et al., 2007), *Petrotoga halophile* was isolated from an oil well in Congo and can grow in the presence of 5-9% of NaCl (Miranda-Tello et al., 2009). The genus *Thermotoga* is anaerobic and reduces cysteine and thiosulphate to hydrogen sulphide, while the genus *Petrotoga* has moderate halophilic species. Other genera of the *Thermotogaceae* include *Kosmotoga* (Dipippo et al., 2009), *Marinitoga* (Wery et al., 2001) and *Thermosipho* (Huber et al., 1989). The second most abundant genera revealed by the taxonomy analyses were unclassified members of the Porphyromonadaceae family, which is composed of two genera of environmental bacteria, *Porphyromonas* and *Dysgonomonas.* Within the classified genera (Fig. 3), the taxonomy classification analyses performed by 454 pyrosequencing identified as the most abundant genera, bacteria such as *Paludibacter*, Clostridium, Thermoacetogenium and Syntrophomonas (Respectively 12.62%, 6.08%, 3.33% and 3.01% abundance) (Fig. 4).

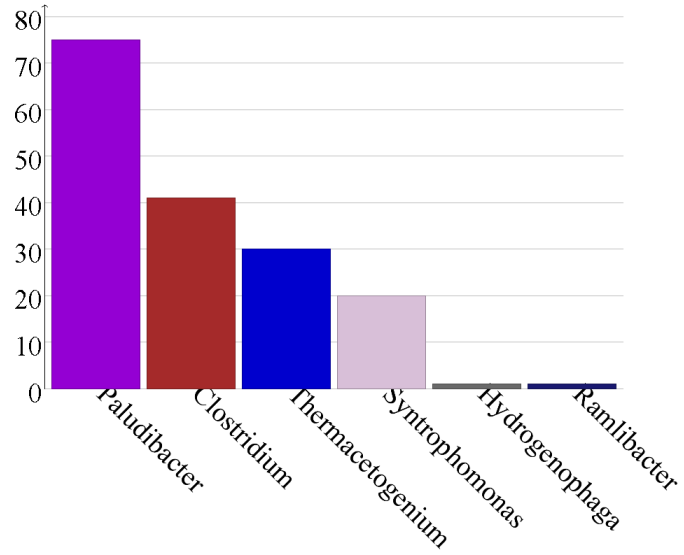

Figure 4. Bar charts showing the bacterial genera in each sample that were tested.

Further analyses were carried out using the BLAST programme package that matched the pyrosequencing data to known 16S rRNA gene sequences to find the closest known relatives to the OTUs identified in the sample (Table 1). This analysis showed that the dominant OTU was an uncultured *Thermotogae bacterium*, with 58.11% abundance and 97% identity. Other important bacteria retrieved using the BLAST search platform are two uncultured bacteria belonging to *Bacteroidia* (6.96% and 3.84% abundance respectively), both at 99% identity and *Aminobacterium colombiense* (3.57 % abundance and 99% identity), belonging to the *Synergistia* phylogenetic class. Also significant is the presence of the Uncultured WWE1 bacterium clone (3.53% abundance, 99% identity) belonging to *Cloacamonae, isolated from a mesophilic anaerobic digester* and of the *Defluviitoga tunisiensis* strain SulfLac1 (3.46% abundance, 99% identity), isolated from a thermophilic anaerobic digester.

Table 1. OTU's Sequence Reads Analysed Filtered by Counts.

OTU	Abundance (%)	Closest Match	Accession	Match (Identities)	Phylogenetic Class	Isolation source
1	58.11	*Uncultured Thermotogae bacterium clone QEEA3DA10*	CU918794	97%	*Thermotogae*	Mesophilic anaerobic digester
11	6.96	*Uncultured bacterium clone B045*	HG530299	99%	*Bacteroidia*	Mesophilic agricultural biogas reactor
1080	3.84	*Uncultured bacterium clone: 3MP-B-1HY-65*	AB731261	99%	*Bacteroidia*	Methane Production Process. Food waste bioreactor
7	3.57	*Aminobacterium colombiense strain DSM 12261*	CP001997	99%	*Synergistia*	Anaerobic lagoon of dairy wastewater treatment plant in Colombia
10	3.53	*Uncultured WWE1 bacterium from clone QEDN11CH09*	CU925933	99%	*Cloacamonae*	Mesophilic anaerobic digester
1000	3.46	*Defluviitoga tunisiensis strain SulfLac1*	NR_122085	99%	*Thermotogae*	Thermophilic and anaerobic whey digester
16	2.50	*Uncultured Firmicutes bacterium from clone QEDV1BH08*	CU919563	99%	*Clorstridia*	Mesophilic anaerobic digester
14	1.99	*Uncultured Bacteroidetes bacterium from clone QEDP2BA06*	CU924061	99%	*Bacteroidia*	Mesophilic anaerobic digester
20	1.85	*Uncultured bacterium clone:BSA2B-03*	AB175375	97%	*Synergistia*	Mesophilic anaerobic digester
25	1.47	*Tepidanaerobacter syntrophicus strain JL*	NR_040966	99%	*Clostridia*	Anaerobic sludge
403	1.30	*Uncultured bacterium clone ATB_CM_534_02*	KP151393	99%	*Thermotogae*	Thermophilic chicken dung-cow slurry fermentation

67	1.13	Bacterium enrichment culture clone 4-44	KF460362	99%	*Clostridia*	Paddy soil
1135	1.03	*Uncultured bacterium clone S1_3_440*	JQ106154	99%	*Thermotogae*	Anaerobic sludge digester
36	0.99	*Tepidanaerobacter acetatoxydans Re1*	NC_019954	99%	*Clostridia*	Unkn.
40	0.93	*Uncultured Chloroflexi bacterium clone QEEA1DG07*	CU918614	99%	*Anaeroloneae*	Mesophilic anaerobic digester

4. Conclusions

In this work, the biodegrading bacterial community of the Bran Sands Teesside anaerobic digester was investigated using 454 pyrosequencing technology followed by sequence data interpretation using bioinformatics tools. The main goal of this study was to investigate the bacterial community composition in an anaerobic digester that include petrochemical waste as a substrate for biogas production. The bacteria population found in the digestate samples is comparable in term of composition with the bacteria found in unconventional gas systems. DNA-based assessment of the microbial community structure in unconventional gas basins have shown that bacterial diversity is higher than archaeal diversity (Barnhart et al., 2013; Penner et al., 2010; Green et al., 2008). Bacteria related to Proteobacteria (mostly Beta, Delta and Gamma-proteobacteria), Actinobacteria, Bacteroidetes and Firmicutes seem to be widespread in unconventional systems such as coal-bed methane (Green et al., 2008; Jones et al., 2008; Jones et al., 2010; Li et al., 2008; Strąpoć and Picardal, 2008; Warwick et al., 2008) and shale gas (Meslé et al., 2015; M Meslé et al., 2013; Struchtemeyer and Elshahed, 2012). These taxonomic groups are known for their versatile metabolic activity and hydrocarbon degrading capabilities. The classification of nucleotide sequence reads and assembled contigs carried out in this study revealed a genetic profile characteristic for an anaerobic microbial consortium running fermentative metabolic pathways. Moreover, the assignment of numerous sequences was related to hydrocarbon decomposition and digestion of cellulosic material, which indicates that many species in the samples are engaged in hydrolysis of plant-derived material. Since hydrolysis is the rate limiting step in degradation of plant biomass (Noike et al., 1985) and within the biogeochemistry of coal-bed methane (Strąpoć et al., 2011) it would be worthwhile to learn more about microorganisms and their metabolic features involved in this process. Some genetic traces of organisms dominating the hydrolysis step were already identified in the metagenome data set. Future work will concentrate on isolation of corresponding bacteria and analysis of their genomic properties with the objective to optimise initial steps in the decomposition of substrates for biogas production. Putative key organisms involved in intermediate steps of methanogenesis were also identified. As a general conclusion, the bacterial community is comparable with results obtained from other mesophilic and slightly thermophilic digesters. Leven et al., (2007) studied the effect of process temperature on the anaerobic

digestion of organic household waste and reported a dominance of *Thermotogae* and *Clostridia* in their slightly thermophilic reactors, while *Bacteroidetes* and *Chloroflexi* were the main phyla in the mesophilic reactors. Their results are in agreement with our findings for the mesophilic reactor in Bran Sands, but their mesophilic community seems less diverse than in our digestate samples. In a study investigating the microbial community of seven anaerobic sludge digesters, Riviere et al., (2009) identified organisms from the *Betaproteobacteria* (class level), *Anaerolineales* (order level), *Bacteroidetes* (phylum level), and *Synergistetes* (phylum level) as 'core' organisms. These results are also in accordance, with the results obtained for the bioreactor investigated in the present study. However, while sequences belonging to *Anaerolineae* (*Chloroflexi*) and sequences from classes of *Proteobacteria* (Alpha, Beta, Delta, and Gamma) are characteristic of the reactor, sequences of the class *Bacteroidetes* were less present in our samples. Moreover, our results are comparable, to some extent, with the bacterial profile characterized in unconventional gas systems. More than 10 different bacterial phyla, among which Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria dominate, have been identified in coal and oil.

The bacterial diversity in low temperature unconventional reservoirs is essentially dominated by Proteobacteria from the alpha, beta, gamma and delta classes. Alpha-proteobacteria dominate in the mesophilic Enermark oil field in Canada where they represent around 50% of the bacterial diversity (Kryachko et al., 2012). a-proteobacteria represent circa 29% in sandstone and coal samples of the Eastern Ordos Basin in China. Gammaproteobacteria dominate in the Bokor oil field in Malaysia (Guo et al., 2012), Beta- and Gamma-proteobacteria are also present in the Alberta coal beds in Canada (Penner et al., 2010). In these settings, Proteobacteria represent over 70% of the total diversity. Members of the Alpha-proteobacteria are known degraders of water-insoluble compounds such as PAHs, and Gamma-proteobacteria include primary hydrocarbon degraders (Guo et al., 2012; Kryachko et al., 2012; Penner et al., 2010). A large number of Delta-proteobacteria are syntrophic bacteria, acetogens or secondary fermenters known to be associated with methanogenic Archaea, or sulphate-reducing bacteria, which can degrade hydrocarbons and organic acids with sulphate as the terminal electron acceptor (Zengler et al., 1999). Besides proteobacteria, Firmicutes are frequently observed, but sometimes in numbers close or below the detection levels of the studies, e.g. they can only be detected after cultivation. Firmicutes, which include fermenters and syntrophs able to hydrolyse water-soluble macromolecular compounds, homoacetogens, fatty acids- oxidizers, and acetogens are associated with oil formation waters (Yamane et al., 2011) and coal production waters (Guo et al., 2012; Wawrik et al., 2012). Conversely, Actinobacteria, which include cellulolytic organisms degrading poorly water-soluble organic compounds (Deng and Fong, 2011), are associated with bulk oil, coal, and sandstones. It is unclear why these organisms, many of which require oxygen to degrade cellulose, are present. Further elucidation is also required to establish whether these microorganisms are able to degrade the cellulose-like organic compounds in oil and coal. Nevertheless, there is a strong metabolic convergence towards the microorganisms typically found in anaerobic digesters and in unconventional systems.

Acknowledgements

The authors thank WSP Parsons Brinckerhoff for supporting and funding this work. We also thank the Northumbrian Water for providing samples from the Bran Sands Teesside Anaerobic digester and Scottish Government Joint Research Partnership in Engineering for providing funding for this work.

References

Barnhart, E. P., De Leon, K. B., Ramsay, B. D., Cunningham, A. B., Fields, M. W., De León, K. B., Ramsay, B. D., Cunningham, A. B., Fields, M. W. (2013). Investigation of coal-associated bacterial and archaeal populations from a diffusive microbial sampler (DMS). *Int. J. Coal Geol*. 115, 64–70. http://dx.doi.org/10.1016/j.coal.2013.03.006

Bogner, J., Pipatti, R., Hashimoto, S., Diaz, C., Mareckova, K., Diaz, L., Kjeldsen, P., Monni, S., Faaij, A., Gao, Q., Zhang, T., Ahmed, M. A., Sutamihardja, R. T. M., Gregory, R. (2008). Mitigation of global greenhouse gas emissions from waste: conclusions and strategies from the Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report. Working Group III (Mitigation). *Waste Manag. Res*. 26, 11–32. https://doi.org/10.1177/0734242X07088433

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., Sevinsky, J. R., Turnbaugh, P. J., Walters, W. A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. https://doi.org/10.1038/nmeth.f.303

Cirne, D.G., Paloumet, X., Björnsson, L., Alves, M. M., Mattiasson, B.(2007). Anaerobic digestion of lipid-rich waste. Effects of lipid concentration. *Renew. Energy* 32, 965–975. https://doi.org/10.1016/j.renene.2006.04.003

Dahle, H., Hannisdal, B., Steinsbu, B. O., Ommedal, H., Einen, J., Jensen, S., Larsen, Ø., Øvreås, L., Norland, S. (2011). Evolution of temperature optimum in Thermotogaceae and the prediction of trait values of uncultured organisms. *Extremophiles* 15, 509–516. https://doi.org/10.1007/s00792-011-0381-4

Deng, Y., Fong, S. S. (2011). Metabolic engineering of Thermobifida fusca for direct aerobic bioconversion of untreated lignocellulosic biomass to 1-propanol. *Metab. Eng*. 13, 570–577. https://doi.org/10.1016/j.ymben.2011.06.007

DiPippo, J. L., Nesbø, C. L., Dahle, H., Doolittle, W. F., Birkland, N. K., Noll, K. M.(2009). *Kosmotoga olearia* gen. nov., sp. nov., a thermophilic, anaerobic heterotroph isolated from an oil production fluid. *Int. J. Syst. Evol. Microbiol*. 59, 2991–3000. https://doi.org/10.1099/ijs.0.008045-0

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Green, M. S., Flanegan, K. C., Gilcrease, P. C. (2008). Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, U.S.A. *Int. J. Coal Geol*. 76, 34–45. https://doi.org/10.1016/j.coal.2008.05.001

Guo, H., Liu, R., Yu, Z., Zhang, H., Yun, J., Li, Y., Liu, X., Pan, J. (2012). Pyrosequencing reveals the dominance of methylotrophic methanogenesis in a coal bed methane reservoir associated with Eastern Ordos Basin in China. *Int. J. Coal Geol*. 93, 56–61. https://doi.org/10.1016/j.coal.2012.01.014

Huber, R., Langworthy, T. A., König, H., Thomm, M., Woese, C. R., Sleytr, U. B., Stetter, K. O. (1986). *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. *Arch. Microbiol*. 144, 324–333. https://doi.org/10.1007/BF00409880

Huber, R., Woese, C. R., Langworthy, T. A., Fricke, H., Stetter, K. O. (1989). *Thermosipho africanus* gen. nov., represents a new genus of thermophilic eubacteria within the "*Thermotogales*." *Syst. Appl. Microbiol.* 12, 32–37. https://doi.org/10.1016/S0723-2020(89)80037-2

Jensen, P. D., Ge, H., Batstone, D. J. (2011). Assessing the role of biochemical methane potential tests in determining anaerobic degradability rate and extent. *Water Sci. Technol*. 64, 880–886. https://doi.org/10.2166/wst.2011.662

Jones, E. J. P., Voytek, M. a, Corum, M. D., Orem, W. H. (2010). Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. *Appl. Environ. Microbiol*. 76, 7013–22. https://doi.org/10.1128/AEM.00728-10

Kirk, M. F., Wilson, B. H., Marquart, K. A., Zeglin, L. H., Vinson, D. S., Flynn, T. M. (2015). Solute concentrations influence microbial methanogenesis in coal-bearing strata of the Cherokee basin, USA. *Front. Microbiol*. 6, 1–14. https://doi.org/10.3389/fmicb.2015.01287

Kröber, M., Bekel, T., Diaz, N. N., Goesmann, A., Jaenicke, S., Krause, L., Miller, D., Runte, K. J., Viehöver, P., Pühler, A., Schlüter, A. (2009). Phylogenetic characterization of a biogas plant microbial community integrating clone library 16S-rDNA sequences and metagenome sequence data obtained by 454-pyrosequencing. *J. Biotechnol*. 142, 38–49. https://doi.org/10.1016/j.jbiotec.2009.02.010

Kryachko, Y., Dong, X., Sensen, C. W., Voordouw, G. (2012). Compositions of microbial communities associated with oil and water in a mesothermic oil field. *Antonie Van Leeuwenhoek* 101, 493–506. https://doi.org/10.1007/s10482-011-9658-y

Lane, D. (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic Acid Techniques in bacterial systematics*. Wiley, New York 115 – 147.

Lane, D. J., Shah, J., Buharin, A., & Weisburg, W. G. (1995). Universal eubacteria nucleic acid probes and assay methods. *Biotechnol. Adv*. 13-3, 595.

Lee, S. H., Kang, H. J., Lee, Y. H., Lee, T. J., Han, K., Choi, Y., Park, H. D. (2012). Monitoring bacterial community structure and variability in time scale in full-scale anaerobic digesters. *J. Environ. Monit*. 14, 1893–905. https://doi.org/10.1039/c2em10958a

Levén, L., Eriksson, A. R. B., Schnürer, A. (2007). Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. *FEMS Microbiol. Ecol*. 59, 683–693. https://doi.org/10.1111/j.1574-6941.2006.00263.x

Li, D., Hendry, P., Faiz, M. (2008). A survey of the microbial populations in some Australian coalbed methane reservoirs. *Int. J. Coal Geol*. 76, 14–24. https://doi.org/10.1016/j.coal.2008.04.007

Li, H., Yang, S. Z., Mu, B. Z., Rong, Z. F., Zhang, J. (2007). Molecular phylogenetic diversity of the microbial community associated with a high-temperature petroleum reservoir at an offshore oilfield. *FEMS Microbiol. Ecol*. 60, 74–84. https://doi.org/10.1111/j.1574-6941.2006.00266.x

Limam, R. D., Chouari, R., Mazéas, L., Wu, T. D., Li, T., Grossin-Debattista, J., Guerquin-Kern, J. L., Saidi, M., Landoulsi, A., Sghir, A., Bouchez, T. (2014). Members of the uncultured bacterial candidate division WWE1 are implicated in anaerobic digestion of cellulose. *MicrobiologyOpen* 3, 157–167. https://doi.org/10.1002/mbo3.144

Liu, G., Zhang, R., El-Mashad, H. M., Dong, R. (2009). Effect of feed to inoculum ratios on biogas yields of food and green wastes. *Bioresour. Technol*. 100, 5103–5108. https://doi.org/10.1016/j.biortech.2009.03.081

McCarty, P. L., Bae, J., Kim, J. (2011). Domestic wastewater treatment as a net energy producer-can this be achieved? *Environ. Sci. Technol*. 45, 7100–7106. https://doi.org/10.1021/es2014264

Meslé, M., Dromart, G., Haeseler, F., Oger, P. M. (2015). Classes of organic molecules targeted by a methanogenic microbial consortium grown on sedimentary rocks of various maturities. *Front. Microbiol*. 6, 1–15. https://doi.org/10.3389/fmicb.2015.00589

Meslé, M., Dromart, G., Oger, P. (2013). Microbial methanogenesis in subsurface oil and coal. *Res. Microbiol*. 164, 959–972. https://doi.org/10.1016/j.resmic.2013.07.004

Miranda-Tello, E., Fardeau, M. L., Thomas, P., Ramirez, F., Casalot, L., Cayol, J. L., Garcia, J. L., Ollivier, B. (2004). *Petrotoga mexicana* sp. nov., a novel thermophilic, anaerobic and xylanolytic bacterium isolated from an oil-producing well in the Gulf of Mexico. *Int. J. Syst. Evol. Microbiol*. 54, 169–174. https://doi.org/10.1099/ijs.0.02702-0

Morita, M., Malvankar, N. S., Franks, A. E., Summers, Z. M., Giloteaux, L., Rotaru, A. E., Rotaru, C., Lovley, D. R. (2011). Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates. *MBio* 2, 5–7. https://doi.org/10.1128/mBio.00159-11

Nettmann, E., Bergmann, I., Pramschüfer, S., Mundt, K., Plogsties, V., Herrmann, C., Klocke, M.(2010). Polyphasic analyses of methanogenic archaeal communities in agricultural biogas plants. *Appl. Environ. Microbiol*. 76, 2540–2548. https://doi.org/10.1128/AEM.01423-09

Noike, T., Endo, G., Chang, J. E., Yaguchi, J. I., Matsumoto, J. I., (1985). Characteristics of carbohydrate degradation and the rate-limiting https://doi.org/10.1002/bit.260271013

Penner, T. J., Foght, J. M., Budwill, K. (2010). Microbial diversity of western Canadian subsurface coal beds and methanogenic coal enrichment cultures. *Int. J. Coal Geol*. 82, 81–93. https://doi.org/10.1016/j.coal.2010.02.002

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A. (2009). Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *ISME J*. 3, 700–714. https://doi.org/10.1038/ismej.2009.2

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol. Mol. Biol. Rev.* 61, 262–280. http://dx.doi.org/10.1111/j.1574-6941.2001.tb00879.x

Schlüter, A., Bekel, T., Diaz, N. N., Dondrup, M., Eichenlaub, R., Gartemann, K. H., Krahn, I., Krause, L., Krömeke, H., Kruse, O., Mussgnug, J. H., Neuweger, H., Niehaus, K., Pühler, A., Runte, K. J., Szczepanowski, R., Tauch, A., Tilker, A., Viehöver, P., Goesmann, A. (2008). The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. *J. Biotechnol*. 136, 77–90. https://doi.org/10.1016/j.jbiotec.2008.05.008

Schnürer, A., Zellner, G., Svensson, B. H. (1999). Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. *FEMS Microbiol. Ecol*. 29, 249–261. https://doi.org/10.1016/S0168-6496(99)00016-1

Smith, A. L., Stadler, L. B., Love, N. G., Skerlos, S. J., Raskin, L. (2012). Perspectives on anaerobic membrane bioreactor treatment of domestic wastewater: a critical review. *Bioresour. Technol.* 122, 149–159. https://doi.org/10.1016/j.biortech.2012.04.055

Strąpoć, D., Mastalerz, M., Dawson, K., Macalady, J., Callaghan, A. V., Wawrik, B., Turich, C., Ashby, M. (2011). Biogeochemistry of microbial coal-bed methane. *Annu. Rev. Earth Planet. Sci.* 39, 617–656. https://doi.org/10.1146/annurev-earth-040610-133343

Strapoć, D., Picardal, F. W., Turich, C., Schaperdoth, I., Macalady, J. L., Lipp, J. S., Lin, Y. S., Ertefai, T. F., Schubotz, F., Hinrichs, K. U., Mastalerz, M., Schimmelmann, A. (2008). Methane-producing microbial

community in a coal bed of the Illinois basin. *Appl. Environ. Microbiol.* 74, 2424–32. https://doi.org/10.1128/AEM.02341-07

Struchtemeyer, C. G., Elshahed, M. S. (2012). Bacterial communities associated with hydraulic fracturing fluids in thermogenic natural gas wells in North Central Texas, USA. *FEMS Microbiol. Ecol.* 81, 13–25. https://doi.org/10.1111/j.1574-6941.2011.01196.x

Vanwonterghem, I., Jensen, P. D., Ho, D. P., Batstone, D. J., Tyson, G. W. (2014). Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. *Curr. Opin. Biotechnol*. 27, 55–64. https://doi.org/10.1016/j.copbio.2013.11.004

Warwick, P. D., Breland Jr., F. C., Hackley, P. C. (2008). Biogenic origin of coalbed gas in the northern Gulf of Mexico Coastal Plain, USA. *Int. J. Coal Geol*. 76, 119–137. https://doi.org/10.1016/j.coal.2008.05.009

Wawrik, B., Mendivelso, M., Parisi, V. a, Suflita, J. M., Davidova, I. a, Marks, C. R., Van Nostrand, J. D., Liang, Y., Zhou, J., Huizinga, B. J., Strąpoć, D., Callaghan, A. V. (2012). Field and laboratory studies on the bioconversion of coal to methane in the San Juan Basin. *FEMS Microbiol. Ecol*. 81, 26–42. https://doi.org/10.1111/j.1574-6941.2011.01272.x

Weiland, P., 2010. Biogas production: current state and perspectives. *Appl. Microbiol. Biotechnol*. 85, 849–860. https://doi.org/10.1007/s00253-009-2246-7

Weiss, A., Jérôme, V., Freitag, R., Mayer, H. K. (2008). Diversity of the resident microbiota in a thermophilic municipal biogas plant. *Appl. Microbiol. Biotechnol*. 81, 163–173. https://doi.org/10.1007/s00253-008-1717-6

Wery, N., Lesongeur, F., Pignet, P., Derennes, V., Cambon-Bonavita, M. A., Godfroy, A., Barbier, G. (2001). Marinitoga camini gen. nov., sp. nov., a rod-shaped bacterium belonging to the order *Thermotogales*, isolated from a deep-sea hydrothermal vent. *Int. J. Syst. Evol. Microbiol*. 51, 495–504. https://doi.org/10.1099/00207713-51-2-495

Widdel, F., Zengler, K., Richnow, H. H., Rosselló-Mora, R., Michaelis, W. (1999). Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* 401, 266–269. https://doi.org/10.1038/45777

Yamane, K., Hattori, Y., Ohtagaki, H., Fujiwara, K. (2011). Microbial diversity with dominance of 16S rRNA gene sequences with high GC contents at 74 and 98°C subsurface crude oil deposits in Japan. *FEMS Microbiol. Ecol*. 76, 220–235. https://doi.org/10.1111/j.1574-6941.2011.01044.x