www.czasopisma.pan.pl PAN POLSKA AKADEMIA NAUK www.journals.pan.pl

Archives of Environmental Protection
Vol. 43 no. 3 pp. 53–60

PL ISSN 2083-4772
DOI 10.1515/aep-2017-0033

DE G DE GRUYTER OPEN

Microbiota of anaerobic digesters in a full-scale wastewater treatment plant

Piotr Świątczak*, Agnieszka Cydzik-Kwiatkowska, Paulina Rusanowska

University of Warmia and Mazury in Olsztyn

*Corresponding author's e-mail: piotr.swiatczak@uwm.edu.pl

Keywords: methane fermentation, next-generation sequencing (NGS), IHT, *Meniscus glaucopsis*.

Abstract: Anaerobic digestion is an important technology for the bio-based economy. The stability of the process is crucial for its successful implementation and depends on the structure and functional stability of the microbial community. In this study, the total microbial community was analyzed during mesophilic fermentation of sewage sludge in full-scale digesters.

The digesters operated at 34–35°C, and a mixture of primary and excess sludge at a ratio of 2:1 was added to the digesters at 550 m^3/d, for a sludge load of 0.054 $m^3/(m^3 \cdot d)$. The amount and composition of biogas were determined. The microbial structure of the biomass from the digesters was investigated with use of next-generation sequencing.

The percentage of methanogens in the biomass reached 21%, resulting in high quality biogas (over 61% methane content). The abundance of syntrophic bacteria was 4.47%, and stable methane production occurred at a Methanomicrobia to Synergistia ratio of 4.6:1.0. The two most numerous genera of methanogens (about 11% total) were *Methanosaeta* and *Methanolinea*, indicating that, at the low substrate loading in the digester, the acetoclastic and hydrogenotrophic paths of methane production were equally important. The high abundance of the order *Bacteroidetes*, including the class *Cytophagia* (11.6% of all sequences), indicated the high potential of the biomass for efficient degradation of lignocellulitic substances, and for degradation of protein and amino acids to acetate and ammonia.

This study sheds light on the ecology of microbial groups that are involved in mesophilic fermentation in mature, stably-performing microbiota in full-scale reactors fed with sewage sludge under low substrate loading.

Introduction

Activated sludge technology is the most common and widely used method of biological treatment of urban and industrial wastewater (Klimiuk and Łebkowska 2003). Typical sewage sludge generated in this process comprises primary sludge separated from wastewater during pre-settling and biological excess sludge from the activated sludge system. The most popular method of sludge stabilization is methane fermentation, which is a multi-track process that transforms organic compounds into biogas and digested sludge. Methane fermentation has become an alternative energy source because methane from biogas can be used to produce heat during combustion and energy cogeneration (Pokój et al. 2014, Amani et al. 2010).

Sludge fermentation starts with a hydrolysis stage, in which hydrolytic enzymes break down complex organic compounds. In the acidic stage, enzymes produced by microorganisms further break down organics into organic acids, alcohols, aldehydes, molecular hydrogen and carbon dioxide. In both these stages, the predominant microorganisms are obligatory anaerobes and facultative bacteria. The third stage of methane fermentation is acetogenesis. Acetogenic bacteria produce hydrogen but require an environment with low hydrogen concentration. Therefore, they are in symbiosis with methanogenic Archaea, which consume hydrogen and decrease the partial pressure of this gas (syntrophy or interspecies hydrogen transfer – IHT, de Bok et al. 2004). The final stage of fermentation is methanogenesis, which is conducted by strictly anaerobic methanogenic microorganisms that convert acetic acid, other organic acids, hydrogen and carbon dioxide to methane. Methanogens belong to six established orders (Methanobacteriales, Methanococcales, Methanomicrobiales, Methanocellales, Methanopyrus and Methanosarcinales) and one proposed order (Methanomassiliicoccus) (Borrel et al. 2013), and at least 31 genera (Liu and Whitman 2008).

For stable fermentation, it is important that the microbial community is diverse and has a balanced composition (Fernandez et al. 1999). To reveal the microbial structure of complex communities in wastewater treatment systems, next-generation sequencing (NGS) methods can be used as a cost-effective approach (Caporaso et al. 2012). NGS gives a cross-section of the entire microbiota, including microorganisms sparsely represented in the sample. An

NGS-based approach to identify the microbial structure and metabolic pathways of methane fermentation is particularly important because experimental study of the physiology of methanogenic Archaea and the ecosystems in which they exist is difficult and some properties of the organism can only be inferred based on the properties of the family with which they are grouped in the phylogenetic tree. Research on microorganisms in the aerobic stage preceding fermentation has shown that biomass is usually dominated by Proteobacteria, followed by Bacteroidetes and Actinobacteria, while Rhodocyclales, Xanthomonadaceae, Sphingomonadales and Rhizobiales produce extracellular polymeric substances that support biomass formation (Cydzik-Kwiatkowska 2015, Zhang et al. 2015). Studies on full-scale methane fermentation reactors treating sewage sludge point to high diversity in the kingdom Archaea (Sundberg et al. 2013). Guo et al. (2015) observed that in digested sludge from a full-scale wastewater treatment plant treating wastewater with a 10–20% share of industrial inflow, *Methanosaeta* and *Methanosarcina* were the dominant genera, and acetoclastic methanogenesis was the dominant methanogenesis pathway.

The complexity of the relationships between different trophic groups in methane fermentation makes the process prone to disturbances. Methanogenesis is regarded as the rate-limiting step in anaerobic digestion, therefore optimization of reactor performance is usually focused on increasing the efficiency of the methanogenic phase. However, the overall efficiency of methanogenesis depends on optimal activity and growth conditions for microorganisms involved in the earlier phases of fermentation. The balance between methanogens and microorganisms involved in acido- and acetogenesis is crucial because if the activity of the latter is too high, the concentrations of acids in the digester will also be too high, and anaerobic digestion will fail. It has been shown that acido- and acetogenic microorganisms are more sensitive to changes in substrate loading than methanogens (Rincón et al. 2008). Despite the importance of these microorganisms in the first stages of fermentation, there is limited information about their species structure and the conditions that favor their growth in fermentation reactors in comparison to what is known about methanogens. Thus, the aim of this study was to investigate the microbiota of full-scale mesophilic fermentation reactors fed with sewage sludge, using the most reliable molecular technique for identification of microorganisms in environmental microbiology (NGS), as well as to draw conclusions about the ecology of microbial groups that are involved in the process in a mature, stably-performing biocenosis under low substrate loading.

Material and Methods

Characteristics of the facility

The samples were taken from mesophilic digesters of the "Łyna" WWTP in Olsztyn (Poland). The plant operates under low organics load in activated sludge technology to treat wastewater in an amount corresponding to approximately 190 000 PE. The average wastewater flow is 60 000 m^3/day. Loads that are introduced into the activated sludge bioreactors are 11 397 kg BOD_5/d, 7 396 kg TSS/d, 3 478 kg N/d and 521 kg P/d. Activated sludge retention time is 11 days. At the WWTP, all types of sludge generated during wastewater treatment are processed in the sewage sludge treatment section. Sludge from primary settling tanks is directed to a digester and then to two gravity thickeners that together constitute a system for generation of volatile fatty acids (VFAs). The separated supernatant containing VFAs is returned to biological treatment line. Pre-thickened primary sludge is mixed with mechanically dewatered excess sludge in 2:1 ratio and the mixture is fed to the closed chambers of mesophilic fermentation. In the chambers, biogas, which is used as a fuel for heating of WWTP buildings is produced. Two anaerobic digesters are operated at a sludge load of 0.054 $m^3/(m^3 \cdot d)$, sludge retention time of 18.5 days, and the temperature of 34–35°C. The total solids constituted 2.0% of digested sludge while volatile solids constituted 68.1% of total solids.

The pH and redox potential in the digester remained at 7.3 and -210 mV, respectively. Biogas composition was measured using GA 2000 PLUS analyzer (Geotechnical Instruments); the results presented in the manuscript are averages from two independent measurements. All other measurements were performed at the end of study according to Polish Standards (PN-81/G-04516, PN-EN 872:2007, PN-EN ISO 1716, PN-93/M-53950/01).

Molecular analysis

The microbiota of the two digesters was analyzed for 6 months in 2-month intervals using PCR-DGGE targeting bacterial 16S rDNA genes. Since the DGGE patterns did not change in time (data not shown), two samples for NGS were taken 3 months apart from both of the digesters. The collected samples were stored at -20°C. After thawing the samples were mixed and DNA isolation was done using the FastDNA® SPIN Kit for Soil (MP Biomedicals). Purity and concentration of the isolated DNA was measured on a Lite NanoDrop spectrometer (Thermo Scientific). A 939F/1492R primer set was used to amplify the V6 to V8 region of the bacterial 16S rDNA gene. The amplicons were sequenced in duplicate using the MiSeq Illumina platform in Research and Testing Laboratory (USA). Because Phred quality scores for both sets of sequences differed slightly, for further analysis the sample of better quality was taken. Over 110 thousand of full sequences were obtained (over 38 million of nucleotide sequences).

To detect chimeras and remove them from the raw reads, UCHIME (Edgar et al. 2011) was executed in *de novo* mode on the clustered, denoised data. After denoising (a minimum average quality score of 30 for the base) and chimera-checking, the reads were condensed into FASTA format. Then, sequences with low quality tags, less than half the expected amplicons length or less than 250 bp in length, were removed. For determination of the taxonomic information for each sequence, the sequences were clustered into operational taxonomic units (OTUs) with 100% sequence identity using USEARCH global alignment (Edgar 2010). To query FASTA formatted file with seed sequences for each cluster against a database of NCBI derived sequences, a .NET algorithm that utilizes BLASTN+ was used. Sequences with identity scores greater than 97% were resolved at the species level; those between 95% and 97%, at the genus level; those from 90% to 95%, at the family level; between 85% and 90%, at the order level; 80 to 85% at the class level; and 77% to 80% at the phyla level. Alignment of the obtained sequences was performed by Infernal (Nawrocki and Eddy 2013); clustering, by Complete

Linkeage Clustering using modules of the RDPipeline. These sequences were assigned to phylotype clusters at five cutoff levels: 1%, 3%, 5%, 7% and 10%. RDP modules were also used for rarefaction analysis, the Evenness index, and richness indices such as the Shannon-Wiener index of diversity (H') and the Chao1 Estimator (all sequences were taken for the analysis).

The sequences have been deposited in the Sequence Read Archive (SRA) NCBI within BioProject PRJNA309482 as an experiment "Full-scale mesophilic methane fermentation of sludge" (Accession: SRX1592670).

Results

The study was carried out in full-scale mesophilic digesters fed with sewage sludge operated at a low loading of 0.054 $m^3/(m^3 \cdot d)$. The production of biogas in the full-scale digesters was 7 000 m^3/d and characteristics of the biogas is given in Table 1. The biogas had a density of 1.2 kg/m^3, a calorific value of 22.04 MJ/m^3 and heat of combustion of 24.47 MJ/m^3. The methane content was at a level of 61%.

The results of NGS at the kingdom, phylum, class, order, family, genus and species level are presented in Table 2 and Table 3. In summary, 118276 sequences were analyzed and the rarefaction plot (Fig. 1) shows that the sampling was done correctly and allowed for an in-depth analysis of the microbial community at the species (3%) and genus levels (5–7%). The Shannon-Wiener index, Chao 1 Estimator and Evenness index were 814,77.0, 10.0 ± 0.0 and 0.975, respectively. About 22% of the identified sequences belonged to Archaea, 54% to Bacteria and the rest of the sequences was not recognized pointing out to great diversity and richness of unexpected and hitherto uncultured bacteria in the biomass from full-scale installation.

Table 1. Composition of biogas

Compound	Unit	Value
Methane	% vol	61.260
Carbon dioxide	% vol	37.280
Hydrogen	% vol	0.005
Hydrogen sulfide	% vol	0.060
Nitrogen	% vol	1.400
Oxygen	% vol	0.070

Predominant phyla belonging to Bacteria were Bacteroidetes (16.99%), Proteobacteria (9.61%), Firmicutes (7.74%) and Chloroflexi (7.23%). Cytophagia was the most numerous class of Bacteria and had over 11.86% of all sequences (Table 2). The second and third most numerous classes of Bacteria were Clostridia (7.03%) and Anaerolineae (6.73%). In the digested sludge, 98 families were identified, but the abundance of most of them did not exceed 1% of all identified sequences (Table 2).

In the investigated biomass, Euryarchaeota was the predominant Archaea phylum, accounting for 21.95% of total sequences. The predominant orders within Euryarchaeota were acetotrophic and methylotrophic Methanosarcinales (11.21%) and Methanomicrobiales (10.42%). Acetotrophic archaea constituted 11.21% of all sequences and the only identified genus was *Methanosaeta*. The second most numerous genus of methanogenic Archaea was hydrogenotrophic *Methanolinea* (9.92%).

In our study, efficient and stable methane fermentation was observed at the ratio of class Methanomicrobia to Synergistia of about 4.6:1.0. Both *Synergistes* sp. and *Syntrophus* sp. occurred in the biomass. Acetogenic *Synergistes* sp. accounted for 2.10% of all microorganisms while the strictly anaerobic genus *Syntrophus* constituted 2.37% of all microorganisms. *Meniscus glaucopsis* (4.30%), *Gracilibacter thermorolerans* (2.78%) and *Leptolinea tardivitalis* (2.32%) (Table 3) were the most abundant bacterial species in digested sludge.

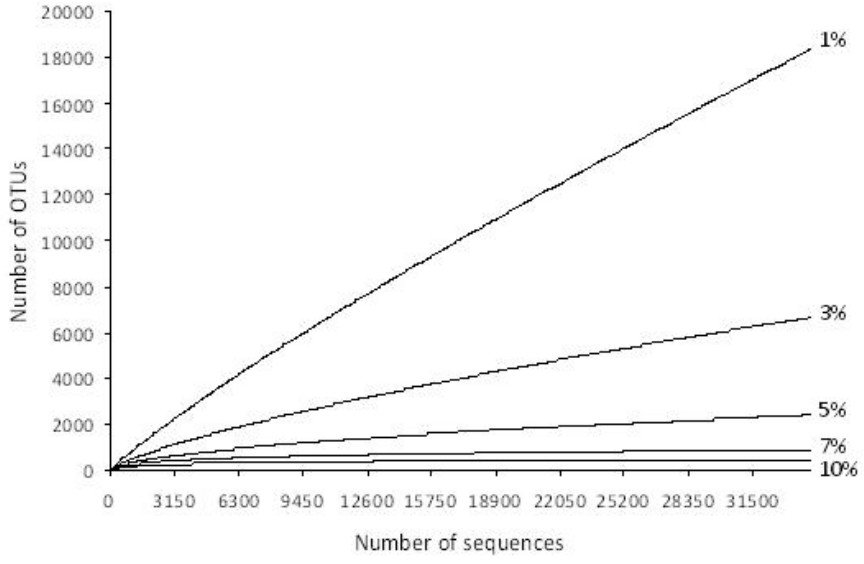

Fig. 1. Rarefaction curves of samples from full-scale anaerobic digesters at cutoff levels of 1%, 3%, 5%, 7% and 10%. The rarefaction curve was computed using RDP pyrosequencing rarefaction tool. The samples were arranged descendingly on the basis of the OTUs numbers

Table 2. Percentage of microorganisms on different taxonomic levels

Kingdom/phylum	%	Class	%	Order	%	Family	%
Archaea							
Euryarchaeota	21.95	*Methanomicrobia*	21.63	*Methanosarcinales*	11.21	*Methanosaetaceae*	11.21
				Methanomicrobiales	10.42	*Methanomicrobiales*	9.92
Bacteria							
Bacteroidetes	16.99	*Cytophagia*	11.86	*Cytophagales*	11.86	*Cytophagaceae*	11.86
		Bacteroidia	5.02	*Bacteroidales*	5.02	*Bacteroidaceae*	4.95
Proteobacteria	9.61	*Deltaproteobacteria*	4.09	*Syntrophobacterales*	2.66	*Syntrophaceae*	2.37
				Deltaproteobacteria	1.07	*Syntrophorhabdaceae*	1.07
		Betaproteobacteria	2.92	*Burkholderiales*	2.41	*Comamonadaceae*	1.02
						Burkholderiales	0.59
		Gammaproteobacteria	1.84	*Xanthomonadales*	1.27	*Xanthomonadaceae*	1.04
Firmicutes	7.74	*Clostridia*	7.03	*Clostridiales*	7.03	*Gracilibacteraceae*	2.78
						Clostridiaceae	1.43
						Eubacteriaceae	1.11
						Clostridiales	1.03
Chloroflexi	7.23	*Anaerolineae*	6.73	*Anaerolineales*	6.73	*Anaerolineaceae*	6.73
Synergistetes	4.61	*Synergistia*	4.61	*Synergistales*	4.61	*Synergistaceae*	4.61
Actinobacteria	3.37	*Actinobacteria*	3.37	*Actinomycetales*	2.62	*Intrasporangiaceae*	1.27
				Actinobacteria	0.66	*Actinobacteria*	0.65
Spirochaetes	2.48	*Spirochaetia*	2.48	*Spirochaetales*	2.48	*Spirochaetaceae*	2.00
Verrucomicrobia	0.65	*Verrucomicrobiae*	0.65	*Verrucomicrobiales*	0.65	*Verrucomicrobiaceae*	0.65

Table 3. The most abundant genera and species of microorganisms

Genus	Species	%
Methanosaeta		11.21
Methanolinea		9.92
Cytophaga		7.54
Bacteroides		4.95
Meniscus	*Meniscus glaucopis*	4.3
Gracilibacter	*Gracilibacter thermotolerans*	2.78
Longilinea		2.69
Thermovirga		2.41
Syntrophus		2.37
Leptolinea	*Leptolinea tardivitalis*	2.32
Synergistes		2.1
Spirochaeta		1.98
Clostridium		1.34

Genus	Species	%
Bellilinea	*Bellilinea caldifistulae*	1.13
Eubacterium		1.09
Syntrophorhabdus	*Syntrophorhabdus aromaticivorans*	1.07
Sedimentibacter		0.80
Acidovorax		0.87
Tetrasphaera		0.88
Microthrix	*Microthrix parvicella*	0.56
Verrucomicrobium		0.65
Xylophilus	*Xylophilus ampelinus*	0.58
Acidobacterium		0.57
Pseudoxanthomonas	*Pseudoxanthomonas mexicana*	0.51
*Summary**		62.08

Discussion

Currently, one of the main goals of microbial ecology is to associate the molecular information from environmental samples with the physiological properties of the relevant organisms, to learn about the role these organisms play in ecosystems. In the present study, microbial communities were analyzed during digestion of sewage sludge to draw conclusions about species structure and interrelations between microorganisms in stable biocenoses in full-scale methane fermentation reactors operated at mesophilic conditions under low substrate loading. The use of NGS in our study allows for in-depth analysis of both the Archeal and the Bacterial communities; in the literature, information on these communities in full-scale digesters is scarce.

The production of biogas in the full-scale digesters was 7000 m^3/d and the concentrations of CH_4 and CO_2 in the biogas were similar to typical values observed during fermentation of sludge from municipal wastewater treatment plants. The amount of hydrogen sulfide, which reduces the quality of biogas, was slightly lower than typical values during anaerobic digestion (0.2–3.0%). Usually, the calorific value of biogas varies from 15.9 to 28.89 MJ/m^3 with an average value of about 25.8 MJ/m^3 (Salunkhe 2012). The calorific value of the biogas in our study is slightly lower than the average, which may have resulted from low loading of the WWTP and production of VFAs from primary sludge, which diminishes the amount of organics introduced to methane fermentation digesters.

To assess the complexity of microbial populations in the full-scale digesters, the Shannon-Wiener index, Chao 1 Estimator and Evenness index were calculated. Both the Chao indicator, based upon the number of rare OTUs found in a sample, and the Shannon-Wiener index indicated a highly diverse community with even distribution of particular bacterial groups. Diversity and even distribution of species in anaerobic digesters causes the reactor to be less prone to environmental disturbances and break-down of fermentation.

The high Archaea to Bacteria ratio (1:2.5) in biomass may have decided on the effective production of biogas with a high methane content. The quantitative ratio of Archaea to Bacteria in anaerobic digesters is usually lower and can be influenced by pH changes. This ratio equaled 1:16 in anaerobic digesters treating sewage sludge (Guo et al. 2015) and changed from 1:16 at pH 4, to 1:41 at pH 10, and 1:9 under uncontrolled pH conditions (Yuan et al. 2015). The highest Archaea to Bacteria ratios are obtained in anaerobic systems with thermal-alkaline pretreatment of sludge. During digestion of pretreated biomass, the Archaea to Bacteria ratio increased with increasing volatile solids to total solids ratio (VS/TS) from 1.6:1 at VS/TS 0.35 to 6.5:1 at VS/TS 0.56 (Wang et al. 2016).

Our results indicate that the Bacteroidetes, Proteobacteria, Firmicutes and Chloroflexi conducting specific processes that are integral parts of initial phases of fermentation constituted over 40% of the biomass. Relatively even distribution of microorganisms within identified phyla in the biomass may have resulted from a very long time for biomass adaptation (many years of operation) and environmental conditions in the reactors that are not very restrictive and enable the growth of various groups of microorganisms. This even distribution is in contrast to other research. Guo et al. (2015) indicated that in mesophilic full-scale digester treating sewage sludge Proteobacteria predominated (41%) over Firmicutes (12.5%), Bacteroidetes (9.6%) and Actinobacteria (5.2%). Yuan et al. (2015) noted that during fermentation of seed sludge under uncontrolled pH conditions, Proteobacteria predominated consisting of 47.3% Bacteria. Chloroflexi was a predominant phylum in mesophilic digesters (42.58% and 63.47%) treating difficult-to-degrade hydrocarbons with both a high (24%) and low (6%) content of solids (Lu et al. 2016), while Firmicutes(53%) predominated in co-digesting of various wastes from restaurants, slaughterhouses, manure and households (Sundberg et al. 2013, Sun et al. 2015). Firmicutes and Bacteroidetes communities were very stable during the digestion of the substrates in biogas reactors fed with casein, starch, and cream and only starving periods, changes of pH values, or long-time continuous feeding caused species shifts in bacterial community (Kampmann et al. 2012). A predominance of Bacteroidetes (even over 58% of all bacteria) was noted in reactors treating protein-rich substrates such as casein or bovine serum albumin (Tang et al. 2005, Kampmann et al. 2012). In the investigated sewage-sludge fed digesters they probably played an important role in degradation of protein and amino acids to acetate and ammonia.

It is interesting to note that over 22.48% of the identified sequences belonged to phyla that include homoacetogenic microbes such as Spirochaetaes, Firmicutes, Chloroflexi, Deltaproteobacteria and Spirochaetia. The total abundance of homoacetogenic microbes suggests the high potential of the biomass for acetate production. Microorganisms belonging to Chloroflexi are also known as efficient decomposers of hard-to-biodegrade hydrocarbons and other organic compounds (Lu et al. 2016).

It was pointed out in this study that the low substrate loading of digester favored the growth of Cytophagia and Clostridia. Cytophagia are able to endure long periods of nutrient limitation (Kampmann et al. 2012), which may explain their high abundance in digesters operated under low organic loading. Cytophagia are also able to hydrolyze cellulose and use the products from this process as their carbon source for energy production, so their high abundance in the biomass provides efficient degradation of lignocellulitic substances during anaerobic digestion. The second most numerous class of Bacteria was Clostridia. Within the class Clostridia there are cellulose-hydrolyzing bacteria and bacteria able to catabolize proteins (Lu et al. 2016). In Schlüter et al. (2008), Clostridia abundance reached over 50% of Bacteria in mesophilic anaerobic digesters fed with cellulose- or protein--rich substrates such as maize silage, green rye, and chicken manure. Fermentative microorganisms such as Clostridia or Bacteroidia (in our study 5.02%) perform acidogenesis and produce short-chain fatty acids, CO_2 and H_2, so their presence in anaerobic digesters is associated with a high-rate hydrolysis and VFA fermentation. Anaerolineae, the third most numerous class of Bacteria, conduct chemoorganotrophic decomposition of hydrocarbons and aminoacids as a source of substrates for growth.

The species structure of the biomass suggests that a wide range of substrates may have been used for methane generation in the investigated full-scale digesters. Methanomicrobiales are usually less numerous than Methanosarcinales during the methane fermentation (Tabatabaei et al. 2010), however, in this study, under low loading of digesters, the percentage Methanomicrobiales in the biomass (10.42%) was comparable to the abundance of Methanosarcinales. There was balance between the abundance of acetotrophic, and of hydrogenotrophic and methylotrophic Archaea, pointing to the equal role of these groups in methane production at a low organic loading. The sensitivity of *Methanosaeta* to organic loading is unclear. Rincón et al. (2008) have observed that this genus was present in the digesters at wide range of organic loading from 0.7 to 9.1 kg VS/(m^3·day), while Montero et al. (2008) noted an increase in the share of Methanosaeta in the community from 1 to 30% with an increase in organic loading from 4.4 to 7.2 kg VS/(m^3·day). *Methanosaeta* sp. usually predominate during fermentation of organic-rich substrates – in an up--flow anaerobic sludge bed reactor (UASB), *Methanosaeta* sp. abundance was higher in the bottom part of the reactor, where

the granules contained more polysaccharides and protein than granules in the upper part (Amin and Vriens 2014). In the investigated digesters, the predominance of *Methanosaeta* sp. with a high affinity for acetate, a central metabolite resulting from the anaerobic fermentation of substances (Narihiro et al. 2009), indicates their success within the niche of methane production from acetate. The high abundance of the genus *Methanosaeta* may have also been favored by the mesophilic conditions in the digesters that covered the optimum temperature for the growth of these microorganisms (Whitman et al. 2006). *Methanoculleus*, *Methanobrevibacter* and *Methanobacterium* were the predominant microorganisms in anaerobic digesters operated at high loadings and under thermophilic conditions, during co-digestion of manure and waste whey at an organic loading rate of 60.4 g COD/(m^3·day) *Methanoculleus* abundance reached over 40% (Li et al. 2015, Lv et al. 2013). *Methanosarcina* sp., which are very versatile microorganisms that often occur in anaerobic digesters (Wu et al. 2013, Cydzik-Kwiatkowska et al. 2012), were not identified in the investigated samples. *Methanosarcina* sp. produce methane using all three known methanogenesis pathways (Liu and Whitmann 2008) therefore they are more competitive than *Methanosaeta* sp. The lack of *Methanosarcina* sp. was probably caused by the fact that they prefer higher temperatures (55–60°C, Narihiro et al. 2009) than those observed in the digesters. In anaerobic digesters methanogens are dominant hydrogenotrophs since they have a lower threshold for H_2 than acetogens, and the energy that yield from the conversion of CO_2 to H_2 to methane is greater than for conversion to acetate. Under such conditions, acetogens have a highly diverse metabolic menu including polymers, lignins, sugars, alcohols, aldehydes, aromatic compounds and inorganic gases CO, H_2 and CO_2 (Ragsdale and Pierce 2008).

This syntrophic cooperation is essential for the proper functioning of both H_2-producing syntrophs and methanogenic Archaea, as it affects their metabolism. Methanogenic microorganisms consume hydrogen and decrease the partial pressure of this gas, which stimulates acetogenic bacteria activity. However, Lykidis et al. (2011) point out that syntrophic interactions are more complex than pairwise syntroph-methanogen relationships and probably include other members of community that maintain and regulate interspecies hydrogen transfer, which improves community stability.

Our technological results indicate that the ratio of class Methanomicrobia to Synergistia of about 4.6:1.0 allows for efficient methane fermentation. In our study, both *Synergistes* sp. and *Syntrophus* sp. occurred in the biomass, which favors efficient generation of biogas because they closely cooperate with methanogens conducting interspecies hydrogen transfer. Acetogenic *Synergistes* sp., is able to degrade amino acids, especially during IHT, or to a lesser extent in the presence of an external electron acceptor (Diaz et al. 2010), while *Syntrophus* sp. can convert benzoates and some fatty acids in co-culture with methanogenic Archaea, which results in the production of acetate and methane, and they have the genetic potential to oxidize butyrate to CO_2/H_2 and acetate (Lykidis et al. 2011).

Meniscus glaucopsis is rarely reported in the literature and our report is the first one showing the preference of this species for mesophilic temperatures and a low substrate loading in the digester. The growth of the genus *Meniscus* is favored at 15 to 35°C, with an optimum around 30°C and pH 7. Its growth requires vitamin B_{12}, thiamin, and CO_2. Good growth has been observed in the presence of ammonia as a nitrogen source (Irgens 2015). *Gracilibacter thermotolerans* is involved in acidogenesis stage (Shah et al. 2014). The acetate, lactate, ethanol, CO_2 and H_2 produced by this species (Lee et al. 2006) can be further converted to acetate by obligate H_2-producing acetogenic bacteria or homoacetogenic bacteria that reduce CO_2 with the acetyl--CoA synthase as the key enzyme. In the continuous process of acidogenesis, *Gracilibacter thermotolerans* grew because of its thermotolerance, whereas other microorganisms were washed out of the reactor (Kim et al. 2010). *Leptolinea tardivitalis* is filamentous microorganism that is able for aerobic respiration (Ward et al. 2015). The optimal temperature for the growth of *Leptolinea tardivitalis* is around 37°C so thermal conditions in the reactor favored its presence. This species can metabolize a number of carbohydrates (Yamada et al. 2006), so in the anaerobic digesters they could have been involved in degradation of extracellular polymers, which are major constituents of activated sludge. This species has been observed in sulphidogenic bioreactors fed with a mixture of municipal wastewater and acid mine drainage. It these reactors, the redox potential was from -71 up to -545 mV (Deng et al. 2016), indicating that *Leptolinea tardivitalis* can tolerate such redox potentials. In the investigated digester another filamentous microorganism namely aerobic *Microthrix parvicella* was present (0.56%). This species occurs in aerated activated sludge chambers of the investigated WWTP because the low organic loading causes that the system is prone to bulking and foaming. *Microthrix parvicella* is introduced to anaerobic digesters with sewage sludge and may disturb their proper functioning due to foam formation.

Foster and Foot (1997) studied the abundance of the filamentous bacteria *Microthrix pavicella* by measuring the length of the filaments in relation to the time that activated sludge was aerated. They found that the threads were longer in anaerobic conditions, when the redox potential ranged between 0 and -50 mV. Thus, the presence of *M. pavicella* in our samples may be due to the decrease in oxygen and the concurrent decrease in the redox potential.

In the present study, a relatively large number of sequences have not been assigned to a taxon (approximately 24%). This indicates that the diversity of microbial communities in full--scale installations is high, and that many microorganisms in environmental samples are still to be discovered and described in the literature.

Conclusions

This study investigated the abundances and species structures of methanogens, hydrolyzing bacteria and acetate-producing bacteria in full-scale mesophilic digesters treating sewage sludge under low substrate loading. Acetotrophic and hydrogenotrophic methanogens played equally important roles in methane generation. The high abundance of methanogens belonging to Methanomicrobiales and Methanosaetaceae was reflected in the high quality of the biogas produced (methane content over 61% vol.). *Synergistes* sp. and *Syntrophus* sp. were involved in interspecies hydrogen transfer; at the observed Methanomicrobia to Synergistia ratio of 4.6:1.0, methane production was stable.

Acknowledgement

This work was supported the statutory project 18.610.006-300, University of Warmia and Mazury in Olsztyn, Poland and the project 2013/09/B/NZ9/01811 of National Science Centre, Poland.

References

Amani, T., Nosrati, M. & Sreekrishnan, T.R. (2010). Anaerobic digestion from the viewpoint of microbiological, chemical, and operational aspects – a review, *Environmental Reviews,* 18, pp. 255–278.

Amin, G.A. & Vriens, L. (2014), Optimization of up-flow anaerobic sludge blanket reactor for treatment of composite fermentation and distillation wastewater, *African Journal of Biotechnology*, 13, pp. 1136–1142.

de Bok, F.A.M., Plugge, C.M. & Stams, A.J.M. (2004). Interspecies electron transfer in methanogenic propionate degrading consortia, *Water Research*, 38, pp. 1368–1375.

Borrel, G., O'Toole, P.W., Harris, H.M.B., Peyret, P., Brugère, J.F. & Gribaldo, S. (2013). Phylogenomic data support a seventh order of methylotrophic methanogens and provide insights into the evolution of methanogenesis, *Genome Biology and Evolution,* 5, pp. 1769–1780.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G. & Knight, R. (2012). Ultra--high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms, *The ISME Journal,* 6, pp. 1621–1624.

Cydzik-Kwiatkowska, A. (2015). Bacterial structure of aerobic granules is determined by aeration mode and nitrogen load in the reactor cycle, *Bioresource Technolology,* 181, pp. 312–320.

Cydzik-Kwiatkowska, A., Zieliński, M. & Jaranowska, P. (2012). Microwave radiation and reactor design influence microbial communities during methane fermentation, *Journal of Industrial Microbiology and Biotechnology,* 39(9), pp. 1397–1405.

Deng, D., Weidhaas, J.L. & Lin, L.S. (2016). Kinetics and microbial ecology of batch sulfidogenic bioreactors for co-treatment of municipal wastewater and acid mine drainage, *Journal of Hazardous Materials*, *305*, pp. 200–208.

Díaz, C., Baena, S., Patel, B.K.C. & Fardeau, M.L. (2010). Peptidolytic microbial community of methanogenic reactors from two modified UASBS of brewery industries, *Brazilian Journal of Microbiology*, 41, pp. 707–717.

Edgar, R.C. (2010). Search and clustering orders of magnitude faster than BLAST, *Bioinformatics*, 12, pp. 1–3.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. & Knight, R. (2011). UCHIIME improves sensitivity and speed of chimera detection, *Bioinformatics*, 27, 6, pp. 2194–2200.

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

Forster, C.F. & Foot, R.J. (1997). The operation of a selector for the control of foam-foaming bacteria in activated sludge, *Environmental Technolology*, 18, pp. 237–241.

Guo, J., Peng, Y., Ni, B.-J., Han, X. & Yuan, L.F.N. (2015). Dissecting microbial community structure and methane-producing pathways of a full-scale anaerobic reactor digesting activated sludge from wastewater treatment by metagenomic sequencing, *Microbial Cell Factories,* 14, pp. 33.

Irgens, R.L. (2015). Meniscus, *Bergey's Manual of Systematics of Archaea and Bacteria*, Wiley Online Library, pp. 1–3.

Kampmann, K., Ratering, S., Kramer, I., Schmidt, M. & Zerr, W.S. (2012). Schnell unexpected stability of Bacteroidetes and Firmicutes communities in laboratory biogas reactors fed with different defined substrates, *Applied and Environmental Microbiology*, 78, 7, pp. 2106–2119.

Kim, W., Hwang, K., Shin, S.G., Lee, S. & Hwang, S. (2010). Effect of high temperature on bacterial community dynamics in anaerobic acidogenesis using mesophilic sludge inoculums, *Bioresource Technol*, 101, pp. 17–22.

Klimiuk, E. & Łebkowska, M. (2003). *Biotechnology in environmental protection*, Wyd. Naukowe PWN, Warszawa 2003. (in Polish)

Lee, Y.J., Romanek, C.S., Mills, G.L., Davis, R.C., Whitman, W.B. & Wiegel, J. (2006). Gracilibacter thermotolerans gen. nov., sp. nov., an anaerobic, thermotolerant bacterium from a constructed wetland receiving acid sulfate water, *International Journal of Systematic and Evolutionary Microbiology,* 56, 9, pp. 2089–2093.

Li, Y.-F., Abraham, Ch., Nelson, M.C., Chen, P.-H., Graf, J. & Yu, Z. (2015). Effect of organic loading on the microbiota in a temperature-phased anaerobic digestion (TPAD) system co-digesting dairy manure and waste whey, *Applied Microbiology and Biotechnology*, 99, pp. 8777–8792.

Liu, Y. & Whitman, W.B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea, *Annals of the New York Academy of Sciences,* 1125, pp. 171–189.

Lu, Q., Yi, J. & Yang, D. (2016). Comparative analysis of performance and microbial characteristics between high solid and low solid anaerobic digestion of sewage sludge under mesophilic conditions, *Journal of Microbiology and Biotechnology*, 26, 1, pp. 110–119.

Lv, W., Zhang, W. & Yu, Z. (2013). Evaluation of system performance and microbial communities of a temperature-phased anaerobic digestion system treating dairy manure: Thermophilic digester operated at acidic pH, *Bioresource Technology*, 142, pp. 625–632.

Lykidis, A., Chen, C.-L., Tringe, S.G., McHardy, A.C., Copeland, A., Kyrpides, N.C., Hugenholtz, P., Macarie, H., Olmos, A., Monroy, O. & Liu, W.-T. (2011). Multiple syntrophic interactions in a terephthalate-degrading methanogenic consortium, *The ISME Journal*, 5, pp. 122–130.

Montero, B., Garcia-Morales, J.L., Sales, D. & Solera, R. (2008). Evolution of microorganisms in thermophilic-dry anaerobic digestion, *Bioresource Technology*, 99, pp. 3233–3243.

Narihiro, T., Terada, T., Kikuchi, K., Iguchi, A., Ikeda, M., Yamauchi, T., Shiraishi, K., Kamagata, Y., Nakamura, K. & Sekiguchi, Y. (2009). Comparative analysis of bacterial and archaeal communities in Methanogenic sludge granules from upflow anaerobic sludge blanket reactors treating various food--processing, high-strength organic wastewaters, *Microbes and Environments*, 24, pp. 88–96.

Nawrocki, E.P. & Eddy, S.R. (2013). Infernal 1. 1, 100-fold faster RNA homology searches, *Bioinformatics*, 29, pp. 2933–2935.

Pokój, T., Gusiatin, Z.M., Bułkowska, K. & Dubis, B. (2014). Production of biogas using maize silage supplemented with residual glycerine from biodiesel manufacturing, *Archives of Environmental Protection*, 40, 4, pp. 17–29.

Ragsdale, S.W. & Pierce, E. (2008). Acetogenesis and the Wood--Ljungdahl pathway of CO(2) fixation, *Biochimica et Biophysica Acta*, 1784, 12, pp. 1873–1898.

Rincón, B., Borja, R., González, J.M., Portillo, M.C. & Sáiz-Jiménez, C. (2008). Influence of organic loading rate and hydraulic retention time on the performance, stability and microbial communities of one-stage anaerobic digestion of two-phase olive mill solid residue, *Biochemical Engineering Journal*, 40, pp. 253–261.

Salunkhe, D.B. (2012). Biogas technology, *International Journal of Engineering Science and Technology*, 4, 12, pp. 4934–1940.

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 analyzed by the 454-pyrosequencing technology, *Journal of Biotechnology*, 136, 1–2, pp. 77–90.

Shah, F.A., Mahmood, Q., Shah, M.M., Pervez, A. & Asad, S.A. (2014). Microbial ecology of anaerobic digesters: the key players of anaerobiosis, *The Scientific World Journal*, pp. 1–21.

Sun, L., Pope, B.P., Eijsink, V.G.H. & Schnürer, A. (2015). Characterization of microbial community structure during continuous anaerobic digestion of straw and cow manure, *Microbial Biotechnology*, 8, 5, pp. 815–827.

Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sørensen, S.J. & Karlsson, A. (2013). 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters, *FEMS Microbioogyl Ecology*, 85, pp. 612–626.

Tabatabaei, M., Rahim, R.A., Abdullah, N., Wright, A.-D.G., Shirai, Y., Sakai, K., Sulaiman, A. & Hassan, M.A. (2010). Importance of the methanogenic archaea populations in anaerobic wastewater treatments, *Process Biochemistry*, 45, pp. 1214–1225.

Tang, Y.Q., Shigematsu, T., Morimura, S. & Kida, K. (2005). Microbial community analysis of mesophilic anaerobic protein degradation process using bovine serum albumin (BSA)-fed continuous cultivation, *Journal of Bioscience and Bioengineering*, 99, pp. 150–164.

Wang, X., Duan, X., Chen, J., Fang, K., Feng, L., Yan, Y. & Zhou, Q. (2016). Enhancing anaerobic digestion of waste activated sludge by pretreatment: effect of volatile to total solids, *Environmental Technology*, DOI:10.1080/09593330.2015.1120783.

Ward, L.M., Hemp, J., Pace, L.A. & Fischera, W.W. (2015). Draft genome sequence of Leptolinea tardivitalis YMTK-2, a mesophilic anaerobe from the Chloroflexi class Anaerolineae, *Genome Announcements*, 3, 6.

Whitman, W.B., Bowen, T.L. & Boone, D.R. (2006). The methanogenic bacteria, *Prokaryotes*, 3, pp. 165–207.

Wu, J.-H., Chuang, H.-P., Hsu, M.-H. & Chen, W.-Y. (2013). Use of a hierarchical oligonucleotide primer extension approach for multiplexed relative abundance analysis of methanogens in anaerobic digestion systems, *Applied and Environmental Microbiology*, 79, 24, pp. 7598–7609.

Yamada, T., Sekiguchi, Y., Hanada, S., Imachi, H., Ohashi, A., Harada, H. & Kamagata, Y. (2006). Anaerolinea thermolimosa sp. nov., Levilinea saccharolytica gen. nov., sp. nov. and Leptolinea tardivitalis gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes Anaerolineae classis nov. and Caldilineae classis nov. in the bacterial phylum Chloroflexi, *International Journal of Systematic and Evolutionary Microbiology*, 56, 6, pp. 1331–1340.

Yuan, Y., Wanga, S., Liu, Y., Li, B., Wang, B. & Peng, Y. (2015). Long-term effect of pH on short-chain fatty acids accumulation and microbial community in sludge fermentation systems, *Bioresource Technology*, 197, pp. 56–63.

Zhang, X., Qu, Y., Ma, Q., Zhang, Z., Li, D., Wang, J., Shen, W., Shen, E. & Zhou, J. (2015). Illumina MiSeq sequencing reveals diverse microbial communities of activated sludge systems stimulated by different aromatics for indigo biosynthesis from indole, *PLoS ONE*, 10, 4, e0125732. doi:10.1371/journal.pone.0125732.

PN-81/G-04516 – Polish standard of determination of combustion heat and calculation of calorific value.

PN-EN 872:2007 – Polish standard of determination of total solids – Method by filtration through glass fiber filters

PN-EN ISO 1716 – Polish standard of determination of heat of combustion (calorific value)

PN-93/M- 53950/01 – Polish standard of determination of density of gases

Struktura mikrobiologiczna osadu z komór fermentacji mezofilowej w skali technicznej

Streszczenie: Fermentacja metanowa jest ważnym elementem biogospodarki. Efektywna eksploatacja reaktorów zależy od stabilności procesu determinowanej składem gatunkowym mikroorganizmów. W pracy badano strukturę mikrobiologiczną biomasy podczas mezofilowej fermentacji osadów ściekowych w skali technicznej. Do komór fermentacyjnych eksploatowanych w 34–35°C wprowadzano mieszaninę osadu wstępnego oraz nadmiernego w stosunku 2:1, w ilości 550 m^3/d (0,054 m^3/(m3·d)). Badane były ilość i skład wytwarzanego biogazu. Biomasę z fermentorów poddawano badaniom metagenomowym z wykorzystaniem wysokosprawnego sekwencjonowania. Wysoka jakość biogazu (ponad 61% zawartości metanu) była determinowana odsetkiem metanogenów w biomasie wynoszącym 21%. Udział bakterii syntroficznych w biomasie wyniósł 4,47%, a stabilną produkcję metanu zaobserwowano przy stosunku Methanomicrobia do Synergistia wynoszącym 4,6:1,0. Wśród metanogenów najliczniejsze były rodzaje Methanosaeta i Methanolinea, co wskazuje, że przy niskim obciążeniu komór fermentacyjnych acetoklastyczny i hydrogenotroficzny szlak produkcji metanu są równie ważne. Wysoka liczebność Bacteroidetes, w tym klasy Cytophagia (11,6% wszystkich sekwencji), wskazuje na wysoką zdolność biomasy do efektywnego rozkładu substancji lignocelulozowych oraz rozkładu białek i aminokwasów do octanu i amoniaku. Badania dostarczają danych na temat ekologii mikroorganizmów we wpracowanych, stabilnie funkcjonujących reaktorach fermentacji mezofilowej w skali technicznej zasilanych osadami ściekowymi w warunkach niskiego obciążenia substratowego.