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# Comparative Analysis of Bacterial and Archaeal Community Structure in Microwave Pretreated Thermophilic and Mesophilic Anaerobic Digesters Utilizing Mixed Sludge under Organic Overloading

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**Abstract:** The effects of microwave (MW) pretreatment were investigated by six anaerobic digesters operated under thermophilic and mesophilic conditions at high organic loading rates (4.9–5.7 g volatile solids/L/d). The experiments and analyses were mainly designed to reveal the impact of MW pretreatment and digester temperatures on the process stability and microbial community structure by correlating the composition of microbial populations with volatile fatty acid (VFA) concentrations. A slight shift from biogas production (with a reasonable methane content) to VFA accumulation was observed in the thermophilic digesters, especially in the MW-irradiated reactors. Microbial population structure was assessed using a high-throughput sequencing of 16S rRNA gene on the MiSeq platform. Microbial community structure was slightly affected by different MW pretreatment conditions, while substantially affected by the digester temperature. The phylum *Bacteroidetes* proliferated in the MW-irradiated mesophilic digesters by resisting high-temperature MW (at 160 °C). Hydrogenotrophic methanogenesis (mostly the genus of *Methanothermobacter*) was found to be a key route of methane production in the thermophilic digesters, whereas aceticlastic methanogenesis (mostly the genus of *Methanosaeta*) was the main pathway in the mesophilic digesters.

**Keywords:** anaerobic digestion; municipal sludge; thermal pretreatment; microwave; microbial community structure; Illumina high-throughput sequencing, volatile fatty acids; bioenergy

## 1. Introduction

There is a significant effort to develop sustainable approaches and technologies for the minimization of excess sludge production all over the world. As a widely used sludge stabilization method in the wastewater treatment plants (WWTPs), anaerobic digestion (AD) is applied as an economical and environmentally friendly method by converting wastewater treatment sludge into methane-enriched bioenergy and reducing the amount of sludge to be disposed or land applied as stable biosolids [1,2]. The potential usage of recovered methane (as electricity and/or waste heat) and the beneficial usage of highly nutritious biosolids for agricultural land application have made anaerobic sludge digestion more favorable, specifically for medium/large-scale facilities [3]. AD includes multistage biochemical processes in the absence of free oxygen by several groups of microorganisms through four subsequent degradation steps; namely, hydrolysis and acidogenesis by hydrolytic fermentative bacteria, acetogenesis by proton-reducing acetogenic bacteria, and lastly, methanogenesis by hydrogenotrophic, aceticlastic and methylotrophic methanogens [4]. The complex microbial floc structure and the limited

degradability of waste activated sludge (WAS) is creating relatively unfavorable substrate for hydrolysis (as a rate-limiting step) during the conventional AD [5,6]. Microwave (MW) irradiation is an effective thermal pretreatment method that has been proven to disrupt the floc structures and microbial cell membranes. Hereby, it improves sludge disintegration and the AD performance, while also destroying pathogens and enhancing sludge dewaterability [7]. In the literature, many studies were conducted to investigate the impacts of different MW pretreatment conditions (e.g., temperature, sludge retention time (SRT), or heating ramp rate) on sludge disintegration and the performance of anaerobic digesters in batch and continuous flow mode [8–12]. In the study by Mehdizadeh et al. [9], MW temperature was a statically significant factor on sludge cake solubilization and methane production at SRTs of 20, 10, and 5 days. At MW pretreatment temperatures of 80, 120, and 160 °C, as MW temperature increased, solubilization of chemical oxygen demand (COD) and biopolymers increased. At the SRT of 5 days, semi-continuous flow mesophilic and thermophilic control digesters utilizing non-irradiated sludge stopped generating biogas due to elevated volatile fatty acid (VFA) concentrations, while digesters utilizing MW-irradiated sludge continued generating biogas. The effects of MW temperature (80, 120, and 160 °C) and heating ramp rate (3, 6, and 11 °C/min) on sludge solubilization and performance of thermophilic batch AD were also evaluated in the study of Hosseini Koupaie and Eskicioglu [8]. Among the tested pretreatment conditions, the maximum solubilization ratio was obtained when MW pretreatment was applied to the dewatered mixed sludge cake at the heating ramp rate of 3 °C/min and MW temperature of 160 °C. The maximum specific biodegradation rate was obtained under MW temperature of 120 °C at the slowest heating ramp rate. In their study, thermophilic AD revealed higher sensitivity to the inhibitory effects of thermal pretreatment at the elevated temperatures in comparison to mesophilic AD. The literature also suggests that the benefits of sludge pretreatment technologies are more discernable and economically feasible when AD is operated under high organic loading rates (OLRs) corresponding to short SRTs (< 8 days), since the control (non-irradiated) AD is challenged to complete acid and methane formation [3,13]. Stability of the AD process is linked to a balanced interaction between microbial consortia during degradation of organic matter. This is essential for efficient biogas generation from an AD operated especially under high OLRs [14].

High-throughput sequencing is commonly applied for a deeper understanding of the diversity of microbial community structures and population dynamics in complex biological systems. It is a cost-effective technology that allows the collection of thousands of sequences from a large number of samples to be run simultaneously [14,15]. Among several sequencing technologies, Illumina is currently used as a promising platform to describe the phylogenetic classification and functional potential of complex microbial populations (such as soil cores, ocean, human gut microbes, cow rumen, and sludge samples) [15,16]. However, to our knowledge, a limited number of studies in the literature reported about the applications of Illumina sequencing technology in anaerobic sludge digestion [14,16,17]. Furthermore, there is very limited knowledge on bacterial and archaeal microbial community structures of anaerobic culture in AD utilizing MW-irradiated sludge [18,19], even though an extensive number of studies have examined the impact of MW pretreatment on sludge disintegration and many conventional operational parameters (e.g., solids destruction, biogas production, and digestate dewaterability) [3,10,13]. In this context, the objective of this study was to explore the impact of MW pretreatment on the relationship between process stability and microbial community structure in the mixed microbial culture samples from thermophilic and mesophilic anaerobic sludge digesters operated at high OLRs. The microbial community analysis by high-throughput sequencing of the 16S rRNA gene (on the Illumina MiSeq platform) was performed to explore to what extent MW pretreatment and digester operating temperature were effective in changing the microbial population structure in AD systems.

## 2. Materials and Methods

### 2.1. Sludge Sample Collection

The Westside Regional Wastewater Treatment Plant located in West Kelowna (British Columbia, Canada), which serves an approximately 44,500 people, was selected for the bi-weekly collection of fermented primary sludge and thickened WAS samples. At this plant, the wastewater undergoes preliminary and primary treatment processes, followed by secondary (biological nutrient removal) and tertiary (filtration and UV disinfection) treatments.

### 2.2. Experimental Approach

A Milestone 2.45 GHz MW Lab Station (ETHOS-EZ: maximum pressure of 35 bar, maximum temperature of 300 °C, and maximum power of 1200 W) equipped with ATC-400-CE thermocouple was used for MW irradiation. A total of 540 g of dewatered WAS samples, which had a total solids (TS) concentration of 10.5% (by weight) via the addition of a cationic polymer and centrifugation, was exposed to MW irradiation at desired temperatures of 80 °C and 160 °C. After reaching the desired temperatures, the samples were held at these temperatures for 30 min. After MW-irradiated sludge samples were brought to ambient room temperature in the pressure-sealed vessels, they were blended with raw (non-irradiated) fermented primary sludge in a 67:33% (by volume) ratio after dilution with centrate collected during centrifugation. Table 1 summarizes the key characteristics of non-irradiated and MW-irradiated sludge feed samples prepared for control and pretreated anaerobic digesters, respectively.

**Table 1.** Mixed sludge feed characteristics for bench-scale anaerobic digesters.

| Parameters | Mixed Sludge Digester Feed ([1]FPS:TWAS = 33:67% by Volume) | | |
|---|---|---|---|
| | Non-irradiated | [2]MW-irradiated | |
| | Control (Raw Sludge) | MW 1—80 °C | MW 2—160 °C |
| [3]TS (% by wt.) | [4]3.94 ± 0.24 (16) | 3.40 ± 0.35 (16) | 3.37 ± 0.25 (16) |
| [5]VS (% by wt.) | 3.38 ± 0.20 (16) | 2.90 ± 0.33 (16) | 2.87 ± 0.24 (16) |
| [6]Ammonia (mg N/L/%VS by wt.) | 207 ± 51 (3) | 259 ± 54 (3) | 219 ± 18 (3) |
| pH (-) | 5.63 ± 0.13 (5) | 5.64 ± 0.05 (5) | 5.52 ± 0.20 (5) |
| [6]Alkalinity (mg as $CaCO_3$/L/%VS by wt.) | 502 ± 116 (3) | 590 ± 38 (3) | 531 ± 106 (3) |
| | Volatile Fatty Acids (VFAs) | | |
| [6,7]Acetic acid (mg/L/%VS by wt.) | 316 ± 79 (3) | 415 ± 104 (3) | 451 ± 104 (3) |
| [6,7]Propionic acid (mg/L/%VS by wt.) | 241 ± 80 (3) | 337 ± 103 (3) | 322 ± 93 (3) |
| [6,7]Butyric acid (mg/L/%VS by wt.) | 174 ± 64 (3) | 208 ± 86 (3) | 203 ± 66 (3) |
| [6,7]Total VFAs (mg/L/%VS by wt.) | 731 ± 223 (3) | 960 ± 293 (3) | 976 ± 264 (3) |

[1]FPS: fermented primary sludge; TWAS: thickened waste activated sludge; [2]MW: microwave; [3]TS: total solids; [4]Arithmetic mean ± standard deviation (number of data points); [5]VS: volatile solids; [6]Since centrifugation, pretreatment, and resuspension of thickened waste activated sludge created slight differences in VS concentrations of MW-irradiated mixed sludge compared to non-irradiated sludge, ammonia, alkalinity, and VFA concentrations were reported after normalization based on the VS content in the samples; [7]Samples were analyzed each time in duplicate.

A total number of 6 bench-scale anaerobic digesters were set up with the inocula from existing bench-scale digesters utilizing the mixed sludge from the same WWTP for more than one year. The digesters used were two-liter side-armed Erlenmeyer flasks with an effective volume of 1 L. A two-hole rubber stopper was placed into the mouth of each flask: one to collect the digestate and the other to collect the biogas in Tedlar® bags for testing. The biogas volume obtained was measured daily by a U-Tube-type manometer. Each digester was fed semi-continuously (once a day, 7 days/week) through the side-arm of the flask. The thermophilic and mesophilic digesters were kept in constant

temperature-controlled shakers at 55 ± 1 °C and 35 ± 1 °C, respectively. The summary of MW pretreatment and anaerobic digester conditions is given in Table 2.

**Table 2.** The experimental design used for MW pretreatment and anaerobic digesters.

| [1]MW Pretreatment Conditions | | | Anaerobic Digester Conditions | | |
|---|---|---|---|---|---|
| [2]Final Temp. (°C) | Holding Time (min) | Mixed Sludge Feed | Digester | Temperature (°C) | [3]SRT (days) |
| - | - | Control(non-irradiated) | [4]T1—Control<br>[5]M1—Control | 55 ± 1<br>35 ± 1 | 6 |
| 80 | 30 | MW 1—80 °C | T2—80 °C<br>M2—80 °C | 55 ± 1<br>35 ± 1 | 6 |
| 160 | 30 | MW 2—160 °C | T3—160 °C<br>M3—160 °C | 55 ± 1<br>35 ± 1 | 6 |

[1]MW: microwave; [2]Samples were pretreated at a constant heating ramp rate of 2.25 °C/min; [3]SRT: sludge retention time; [4]T: thermophilic; [5]M: mesophilic.

### 2.3. Analytical Procedures

Characterization of digester performance was performed by measuring multiple parameters at steady-state conditions from the waste sludge streams (i.e., effluent and influent of each digester) (Table 3).

**Table 3.** Characterization of conventional parameters and frequency of analysis[a].

| Sample | Parameter | Frequency |
|---|---|---|
| **Digester influent** | | |
| Mixed sludge feed | [1]TS, [2]VS, total & soluble [3]COD, pH, alkalinity, ammonia, and total [4]VFAs | Upon preparation of feed with fresh substrates (bi-weekly) |
| | Biopolymers (protein, humic acids, and sugar) | [5]Minimum three sets of data |
| **Digester Effluent** | | |
| Digestate | pH | Daily |
| | TS, VS and total & soluble COD | Every three days |
| | Alkalinity, ammonia, and total VFAs | Once a week |
| | Biopolymers (protein, humic acids, and sugar) | [5]Minimum three sets of data |
| Digester biogas | Biogas volume | Daily |
| | Biogas composition | Once a week |

[1]TS: total solids; [2]VS: volatile solids; [3]COD: chemical oxygen demand; [4]VFAs: total volatile fatty acids; [5]at steady state.

Measurements of TS, volatile solids (VS), pH, alkalinity, and ammonia were conducted using Standard Methods [20] procedures 2540 B, 2540 E, 4500-$H^+$B, 2320B, and 4500-$NH_3$D, respectively. COD concentrations in terms of total and soluble were carried out using the closed reflux colorimetric method as defined in the Standard Methods [20] procedure 5250 D. A modified Lowry protein assay was used to measure protein and humic acid concentrations in the soluble and total phases of sludge samples [21]. Quantification of sugar concentrations was implemented using a procedure proposed by DuBois et al. [22]. The supernatants of the sludge samples were filtered through membrane filters with 0.45-µm pore sizes before soluble COD and biopolymer analyses. Total VFAs (acetic, propionic, and butyric acids) were measured by an Agilent 7890A gas chromatograph with a flame ionization detector and Agilent 19091F-112 capillary column (0.32 mm internal diameter; nitroterephthalic acid-modified polyethylene glycol stationary phase) [23]. The initial temperature conditions were

70, 220, and 300 °C for oven, inlet, and detector, respectively, while the final temperature for each aforementioned component was 200, 220, and 300 °C, respectively. Helium was used as a carrier gas with a flow rate of 40 mL/min gas. The supernatants were filtered through a 0.22-µm pore size membrane filters before VFA measurements. Biogas composition was analyzed by an Agilent 7820A gas chromatograph equipped with a thermal conductivity detector [24].

### *2.4. Microbial Community Analysis*

Samples were collected from each digester effluent stream and stored at −20° C until further analyses. Genomic DNAs from anaerobic culture samples were extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) as per the manufacturer's instructions. V4 16S rRNA gene amplification, polymerase chain reaction (PCR) conditions, primer selection, and sequence library preparation were done using the Earth Microbiome Project protocols and standards (EMP 16S Illumina Amplicon Protocol) [15,25] using Thermo Fisher Platinum® *Taq* DNA Polymerase (Thermo Fisher Scientific—catalog number: 10966026). Sequencing libraries were run on the Illumina MiSeq platform using services provided by the University of British Columbia Sequencing and Bioinformatics Consortium. A custom pipeline leveraging USEARCH [26] and Quantitative Insights into Microbial Ecology (QIIME) [27] modules was applied in order to determine microbial community structure based on V4 16S rRNA gene sequences. UCHIME was used to identify potential chimeric sequences using the RDP gold database (https://drive5.com/uchime/gold.fa) [28,29]. QIIME's "pick_otus.py" was used to identify operational taxonomic units (OTUs) de novo at 97% sequence similarity from the chimera-filtered fragments using SUMACLUST. A representative sequence set was selected from the de novo OTUs by "pick_rep_set.py" prior to taxonomic assignment using the RDP classifier against QIIME's default database. Chimeras and singletons were removed to avoid Type 1 errors ultimately resulting in the OTU table used for downstream analyses and data visualization. The sequence reads are available at the Sequence Read Archive under accession number PRJNA564408 (runs between SRR10083155 and SRR10083160).

### *2.5. Statistical Analysis*

The experimental data were analyzed with an analysis of variance (ANOVA) considering a 95% confidence interval ($\alpha = 0.05$), using Minitab™ 17 statistical software.

## 3. Results and Discussion

### *3.1. Sludge Solubilization by Microwave Pretreatment*

The degree of sludge solubilization was examined by analyses of COD and biopolymers (i.e., sugar, protein, and humic acids). The soluble phase concentrations, solubilization ratios, and the fold increase after MW pretreatment in solubilization (over the control) are listed in Table 4. Results showed that increasing the final MW temperature (applied for 30 min) improved the mixed sludge solubilization. According to a One-way ANOVA test, the final MW temperature had a statistically significant effect on improvement (over control) in COD, protein, humic acids, and sugar solubilization ($p$-value < 0.05). After the pretreatment condition of MW2—160 °C was applied, the soluble phase concentrations of COD, protein, humic acids, and sugar in the mixed sludge increased to 4028 ± 688, 199 ± 1.9, 276 ± 9.6, and 38.4 ± 1.3 mg/L normalized by % VS by weight, whereas these ratios were found as 2107 ± 444, 30 ± 0.41, 61 ± 1.3, and 4.6 ± 0.2 mg/L/%VS by weight before the pretreatment (control). This means that the disintegration of the complex sludge structure was achieved by MW pretreatment and extracellular (inside the polymeric network) and intracellular (inside the microorganism) biopolymers that were released into the soluble phase, resulting in faster hydrolysis and, therefore, higher AD improvement compared to conventional digestion. A detailed explanation of the sludge solubilization analysis was given in the previous publication of the authors [10].

**Table 4.** Effect of MW pretreatment on mixed sludge solubilization.

| Parameters | Mixed Sludge Digester Feed | | |
|---|---|---|---|
| | Non-irradiated | [1]MW-irradiated | |
| | Control(Raw Sludge) | MW1—80 °C | MW2—160 °C |
| [2]COD: | | | |
| $COD_{soluble}$ (mg/L/%[3]VS by wt.) | [4]2107 ± 444 (9) | 2917 ± 349 (9) | 4028 ± 688 (9) |
| [5]The solubilization ratio (%) | 13 ± 0.4 (9) | 19 ± 1.3 (9) | 27 ± 0.8 (9) |
| [6]The fold increase in solubilization (-) | - | 1.5 | 2.1 |
| Protein: | | | |
| $Protein_{soluble}$ (mg/L/%VS by wt.) | 30 ± 0.41 (2) | 94 ± 2.1 (2) | 199 ± 1.9 (2) |
| The solubilization ratio (%) | 4 ± 0.1 (2) | 20 ± 1.1 (2) | 39 ± 0.1 (2) |
| The fold increase in solubilization (-) | - | 4.8 | 9.4 |
| Humic acids: | | | |
| $Humic\ acids_{soluble}$ (mg/L/%VS by wt.) | 61 ± 1.3 (2) | 131 ± 8.5 (2) | 276 ± 9.6 (2) |
| The solubilization ratio (%) | 8 ± 0.2 (2) | 23 ± 1.2 (2) | 51 ± 1.7 (2) |
| The fold increase in solubilization (-) | - | 2.8 | 6.2 |
| Sugar: | | | |
| $Sugar_{soluble}$ (mg/L/%VS by wt.) | 4.6 ± 0.2 (2) | 8.4 ± 0.6 (2) | 38.4 ± 1.3 (2) |
| The solubilization ratio (%) | 0.9 ± 0.1 (2) | 2.1 ± 0.1 (2) | 9.2 ± 0.8 (2) |
| The fold increase in solubilization (-) | - | 2.2 | 10.4 |

[1]MW: microwave; [2]COD: chemical oxygen demand; [3]VS: volatile solids; [4]Arithmetic mean ± standard deviation (number of data points); [5]The solubilization ratio (%) = soluble phase concentration/total phase concentration × 100%; [6]Over the non-irradiated mixed sludge after MW pretreatment.

### *3.2. Performance of Bench-Scale Anaerobic Digesters by Microwave Pretreatment*

The bench-scale digesters (three mesophilic and three thermophilic) were operated at longer SRTs of 20 and 12 days (low to medium OLRs of 1.45–2.50 g VS/L/d) for a duration of seven months prior to an operation at SRT of 6 days (high OLRs: 4.9–5.7 g VS/L/d) to allow for gradual transition to high OLRs and sufficient acclimation time to by-products of MW irradiation. Detailed information about the acclimation and steady-state AD performance parameters at low/medium OLRs is not provided here but can be found in Kor-Bicakci [30]. The steady-state conditions (less than ±10% variation in solids concentrations, biogas yield, biogas composition, and pH for all digesters) was reached after 20 days of reducing SRT from 12 to 6 days and was maintained for an additional period of 38 days at the high OLRs. The steady-state performance parameters and digestate characteristics of anaerobic digesters operated under SRT of 6 days are summarized in Table 5.

All digesters attained relatively stable performances in terms of organics removal efficiency (between 52% and 54% at thermophilic temperature and 45% and 53% at mesophilic temperature) at a low-SRT. Daily pH values were also stable during the AD operations at an SRT of 6 days. The pH values were in line with literature values [5] and ranged between 7.7 and 8.0 in the thermophilic digesters and were typically greater than the pH of the mesophilic digester (between 7.3 and 7.4). The alkalinity concentrations of the thermophilic and mesophilic digesters were in the range of 3900–4800 and 3000–3500 mg/L as $CaCO_3$, respectively. As expected, the ammonia and soluble COD levels in the effluents of the thermophilic digesters were also higher than mesophilic levels (Table 5).

**Table 5.** Operating conditions and steady-state performances of bench-scale anaerobic digesters fed with non-irradiated (control) and MW-irradiated mixed sludge.

| Parameters | Thermophilic | | | Mesophilic | | |
|---|---|---|---|---|---|---|
| | [1]T1 Control | T2 80 °C | T3 160 °C | [2]M1 Control | M2 80 °C | M3 160 °C |
| | **Retention Time and Loading Conditions** | | | | | |
| [3]SRT (days) | 6 | 6 | 6 | 6 | 6 | 6 |
| [4]OLR (g [5]VS/L/d) | 5.72 ± 0.32 (5)[b] | 5.12 ± 0.15 (5) | 4.91 ± 0.28 (5) | 5.72 ± 0.32 (5) | 5.12 ± 0.15 (5) | 4.91 ± 0.28 (5) |
| OLR (g [6]COD/L/d) | 8.71 ± 1.28 (3) | 7.81 ± 0.48 (3) | 7.56 ± 0.33 (3) | 8.71 ± 1.28 (3) | 7.81 ± 0.48 (3) | 7.56 ± 0.33 (3) |
| | **Removal Efficiencies** | | | | | |
| [3]TS (% by wt.) | [4]45 ± 2.5 (9) | 44 ± 2 (9) | 46 ± 4 (9) | 38 ± 3.7 (9) | 40 ± 2.1 (9) | 45 ± 2.4 (9) |
| [5]VS (% by wt.) | 52 ± 3.0 (9) | 52 ± 2.4 (9) | 54 ± 3.8 (9) | 45 ± 3.6 (9) | 47 ± 1.8 (9) | 53 ± 2.4 (9) |
| | **Methane Production** | | | | | |
| [6]Daily specific methane yield (mL $CH_4$/g $VS_{fed}$) | 319 ± 17 (32) | 317 ± 16 (32) | 318 ± 17 (32) | 346 ± 18 (32) | 369 ± 16 (32) | 384 ± 25 (32) |
| $CH_4$ content in the biogas (%) | 65.3 ± 0.8 (4) | 65.4 ± 1.1 (4) | 64.9 ± 0.7 (4) | 65.8 ± 0.3 (4) | 66.9 ± 0.4 (4) | 65.1 ± 1.0 (4) |
| | **Digestate (Effluent) Characteristics** | | | | | |
| pH (-) | 8.0 ± 0.04 (32) | 7.8 ± 0.03 (32) | 7.7 ± 0.07 (32) | 7.4 ± 0.02 (32) | 7.3 ± 0.03 (32) | 7.4 ± 0.04 (32) |
| VS (% by wt.) | 1.64 ± 0.1 (9) | 1.46 ± 0.1 (9) | 1.37 ± 0.1 (9) | 1.87 ± 0.1 (9) | 1.63 ± 0.1 (9) | 1.45 ± 0.1 (9) |
| | **Digestate Supernatant Characteristics** | | | | | |
| Alkalinity (mg/L/% VS by wt. as $CaCO_3$) | 2888 ± 109 (3) | 2440 ± 192 (3) | 2589 ± 392 (3) | 1990 ± 164 (3) | 1850 ± 84 (3) | 2220 ± 275 (3) |
| Ammonia (mg N/L/% VS by wt.) | 781 ± 32 (3) | 709 ± 107 (3) | 798 ± 158 (3) | 538 ± 2.3 (3) | 504 ± 44 (3) | 636 ± 85 (3) |
| [7]$COD_{soluble}$ (mg/L/% VS by wt.) | 2474 ± 263 (5) | 2906 ± 175 (5) | 4535 ± 687 (5) | 515 ± 74 (5) | 488 ± 50 (5) | 1452 ± 179 (5) |
| [8]Total [9]VFAs (mg/L/%VS by wt.) | 607 ± 219 (5) | 1031 ± 253 (5) | 1341 ± 297 (5) | 27 ± 4 (5) | 25 ± 3 (5) | 104 ± 13 (5) |

[1]T: thermophilic; [2]M: mesophilic; [3]SRT: sludge retention time; [4]OLR: organic loading rate; [5]VS: volatile solids; [6]COD: chemical oxygen demand. [3]TS: total solids; [4]Arithmetic mean ± standard deviation (number of data points); [5]VS: volatile solids; [6]at standard temperature and pressure (0°C and 1 atm); [7]COD: chemical oxygen demand; [8]Samples were analyzed each time in duplicate; [9]VFAs: volatile fatty acids (summation of acetic acid, butyric acid, and propionic acid).

3.2.1. The Relationship between Process Stability and Volatile Fatty Acids Accumulation

The evolution of anaerobic digesters' specific biogas production under thermophilic and mesophilic conditions through the operating period (while at a steadystate) is shown in Figure 1. It is clearly seen that the mesophilic control digester (532 ± 13 mL/g $VS_{fed}$) had substantially higher biogas production compared to the thermophilic control digester (489 ± 15 mL/g $VS_{fed}$) operated at an SRT of 6 days ($p$-value < 0.05). Regarding the effect of MW pretreatment on AD under mesophilic temperatures, MW-irradiated digesters accomplished higher biogas generation compared to the respective control ($p$-value < 0.05). The digester "M3—160 °C" which was fed with the most intensive MW-irradiated sludge (160 °C for 30 min), reached the maximum biogas production (590 ± 23 mL/g $VS_{fed}$), as seen in Figure 1. The improvement in biogas production in this digester was 11% ± 4% over the control. The comparatively high biogas yield (551 ± 21 mL/g $VS_{fed}$) was also obtained from the digester "M2—80 °C", with a biogas enhancement of 4% ± 5% over the control.

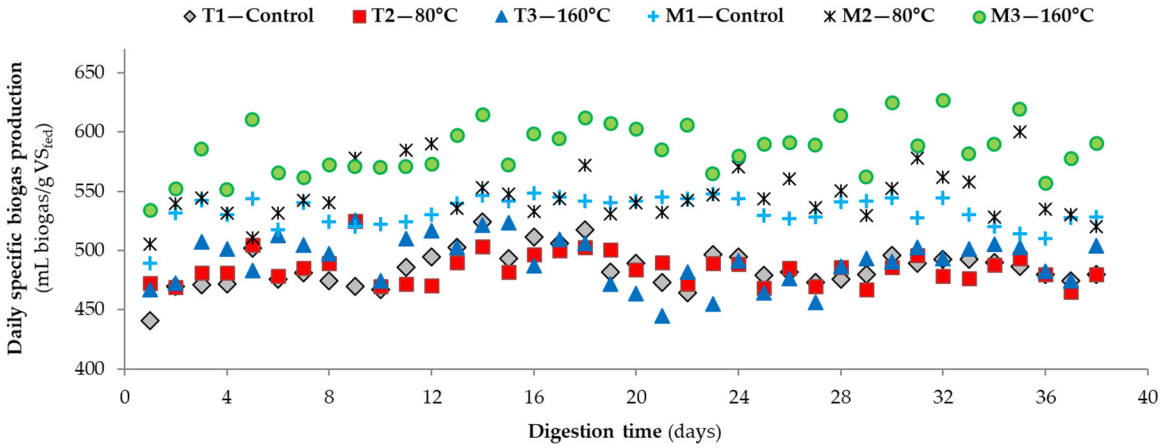


**Figure 1.** Evolution of specific biogas yields of thermophilic and mesophilic digesters during the operating period after reaching a steady state (0°C and 1 atm).

Under thermophilic temperatures, the digester "T1—Control" was able to reach up to a biogas production of 489 ± 15 mL/g $VS_{fed}$ only. This is possibly due to the accumulation of VFAs during thermophilic AD. The VFA accumulation is usually related to microbial stress and the inhibition of methanogens. These may have arisen from substrate overloading, the existence of toxic compounds, or temperature variations [31]. In this situation, there may not be enough time for methane conversion of all organic compounds by methanogens within 6 days at high OLRs; however, there may be adequate time for the acidification of complex organics to intermediates (including VFAs) by fermentative bacteria. Even though the kinetics of methane production are typically slower than kinetics of VFAs production, an adequate methanogenic population generally establishes in an anaerobic environment at steady-state conditions. This results in a stable and healthy AD operation with low VFA concentrations (inside the acceptable range: between 0–250 mg/L) and the optimum pH range of 6.5–7.6 [31]. In this context, increasing levels of VFAs (up to 607 ± 219 mg/L) may have caused a slight inhibition in the digester "T1—Control", even under relatively stable operations (VS removal efficiency of 52% ± 3.0% and reasonable biogas production with methane contents of 65.3% ± 0.8%).

In contrast to mesophilic MW-irradiated digesters, thermophilic MW-irradiated digesters did not achieve any improvements in biogas generation compared to the respective control; thus, similar specific biogas productions were obtained from digesters of "T2—80 °C" (485 ± 13 mL/g $VS_{fed}$) and "T3—160 °C" (491 ± 20 mL/g $VS_{fed}$). Similar to the digester "T1—Control", the VFA accumulations took place in the MW-irradiated thermophilic digesters, and the VFA concentrations increased further, up to 1341 ± 297 mg/L. It can be postulated that MW pretreatment at high exposure times (i.e., 30 min) may trigger the accumulation of VFAs under thermophilic temperatures as a result of increased disintegration

and the solubilization of organic compounds (consistent with the results summarized in Table 4). As seen in Figure 2, thermophilic digesters had a markedly high level of VFA concentrations (between 607 and 1341 mg/L), whereas the VFA concentrations of MW-irradiated mesophilic digesters were found between 25 and 104 mg/L ($p$-value < 0.05). In the digesters of "M2—80 °C" and "M3—160 °C", fermentative and acetogenic anaerobic microorganisms efficiently utilized the solubilized organic materials obtained after MW pretreatment.

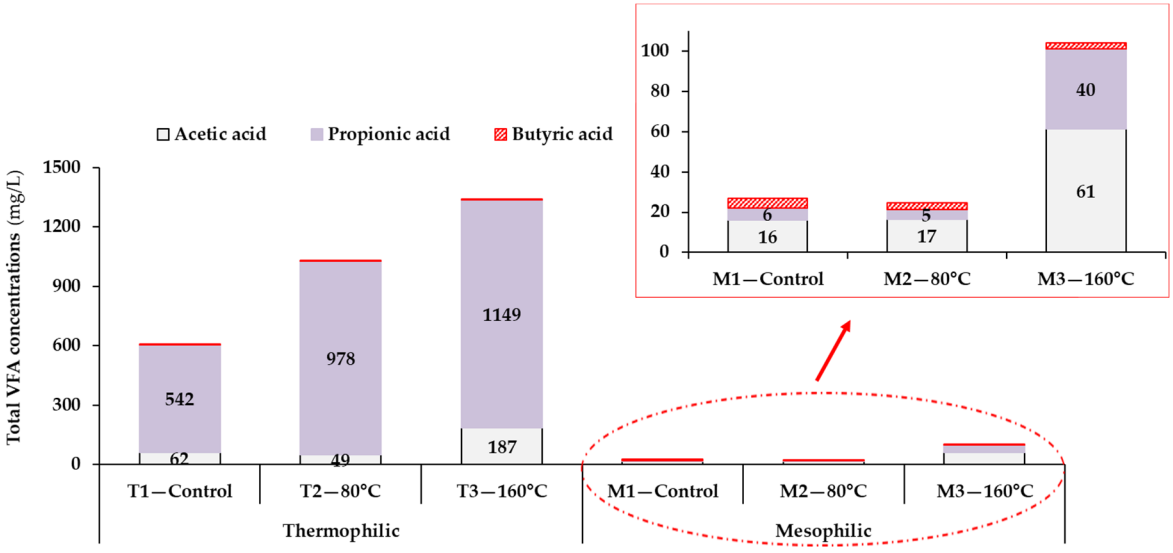


**Figure 2.** Concentrations of acetic, butyric, and propionic acids in the thermophilic and mesophilic digesters operated under high organic loading rates.

Among the short chain fatty acids (e.g., acetate, propionate, butyrate, and valerate), acetate and propionate have been generally regarded as the major intermediates in AD [32]. Hobson and Shaw [33] reported that acetate or butyrate did not have toxicity on hydrogen-utilizing *Methanobacterium formicicum* at concentrations up to 10,000 mg/L, while propionate was inhibitory above certain concentrations (>1000 mg/L) and would inhibit digestion. The propionate accumulation is known as a drawback of thermophilic AD operation [34]. Kim et al. [35] and Speece et al. [36] attribute this situation to the following three possible reasons: (1) single-stage continuously stirred tank reactor configuration where all stages of digestion occur in a dispersed common environment, which is not ideal for all members of the consortia, (2) the possible deficiency in micro/macro inorganic nutrients to sustain effective enzymatic processing, and (3) the lack of microbial consortia (acetate and $H_2$/formate-consuming methanogens and obligate $H_2$-producing acetogens) in close proximity, which is thermodynamically favorable only at $H_2$ concentrations between $10^{-4}$–$10^{-6}$ atm, resulting in an $H_2$ concentration increase and, subsequently, the propionate accumulation [37]. High propionate concentrations (e.g., from 1000 to 9600 mg/L) are frequently reported in anaerobically treated effluents at thermophilic temperatures [36]. Moreover, Aitken et al. [38] stated that the VFA levels were relatively high in the effluents of AD under thermophilic temperatures (51–55 °C) at the short SRTs (4 to 6 days) and that the most abundant VFA concentration in the effluent was propionate.

In agreement with the results in the literature, the propionic acid accumulation in thermophilic digester effluents (supernatants), especially combined with MW pretreatment, increased significantly compared to acetic acid and butyric acid at the SRT of 6 days. Figure 2 also represents the contribution of individual fatty acids in the total VFAs monitored during thermophilic and mesophilic AD. The propionic acid levels were significantly higher in the thermophilic ADs in comparison to the mesophilic ADs ($p$-value < 0.05). The propionic acid concentration in the digester effluent of "T3—160 °C" increased remarkably to 1149 ± 277 mg/L even at a neutral reactor pH of 7.7 ± 0.07, although the acetic and butyric acids concentrations were merely up to 187 ± 31 and 5.6 ± 1.3 mg/L, respectively (Figure 2).

On the contrary, the digester of "M3—160 °C" had 40 ± 8 mg/L propionic acid concentrations with very low butyric acid (<5 mg/L) in addition to 61 ± 7 mg/L acetic acid concentrations.

"A ratio of propionic to acetic acids" in digester effluent can be also considered as one of the key indicator parameters for evaluating the process imbalance caused by organic overloading in an AD system [39,40]. Hill et al. [40] reported that acetic acid concentrations higher than 800 mg/L or a propionic to acetic acids ratio greater than 1.4 indicate impending digester failure. As can be seen in Figure 3, the ratios of propionic to acetic acids were in the range of 6.19–19.84 for thermophilic digesters. These extremely high ratios (> 1.4) can be explained by a mild inhibition that may have occurred during the operation of control and MW-irradiated digesters under thermophilic conditions. This ratio was found to be higher for the digester of "T2—80 °C" because of the lower acetic acid concentrations (49 ± 8 mg/L) in the digester of "T2—80 °C" than that of the digesters of "T1—Control" (62 ± 15 mg/L) and "T3—160 °C" (187 ± 31 mg/L). Conversely, under mesophilic temperatures, the ratios of propionic to acetic acids in the control and MW-irradiated digesters (between 0.34 and 0.65) were considerably lower than the threshold ratio (Figure 3).

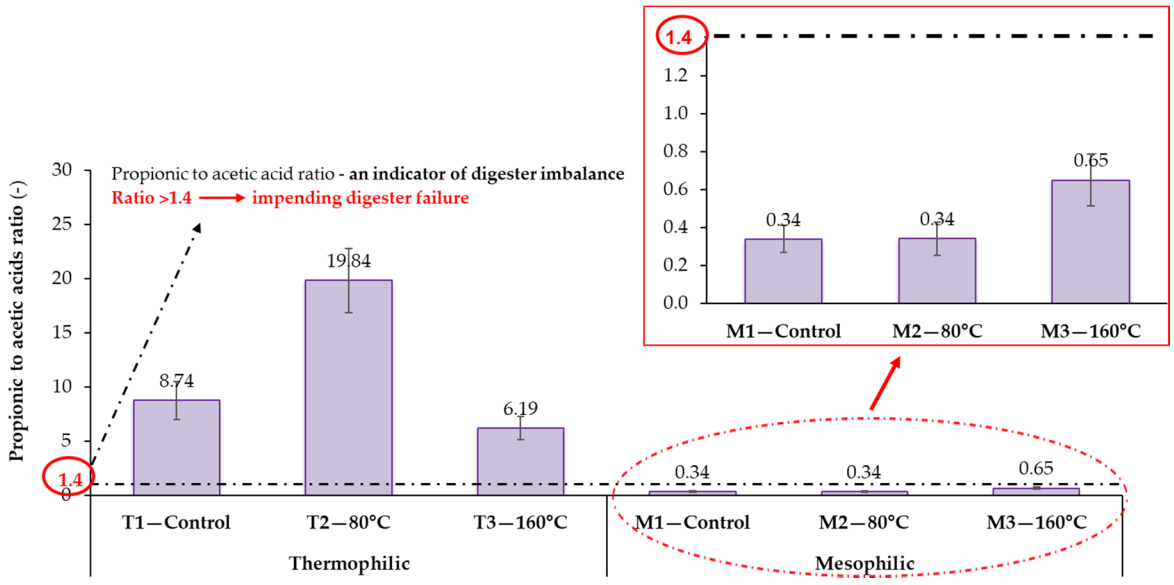


**Figure 3.** Ratios of propionic to acetic acids in the thermophilic and mesophilic digesters.

In spite of a mild inhibition that occurred in thermophilic digesters, methane producers tolerated the accumulation of the VFAs in the system and were still able to achieve reasonable biogas yields with a typical methane content (65%). This can be explained by the buffering capacity of the digesters. Specifically, the pH of the digesters were higher than 7.6, and the alkalinity concentrations were around 4200 ± 500 mg/L as $CaCO_3$.

### 3.2.2. The Relationship between Process Stability and Microbial Community Structure

A total of 327,999 high-quality sequences were clustered at the 97% identity threshold (after singleton removal). The abundance of each OTU was normalized to the total number of reads recovered per sample and expressed on a percentage (%) basis due to the unequal numbers of sequences recovered from each sample. Overall, 4736 bacterial OTUs containing 99.34% of the quality-controlled reads and 26 archaeal OTUs containing 0.66% of the quality controlled reads were identified. An additional 2 OTUs containing 0.002% of the quality-controlled reads had no identifiable database annotation.

The average relative abundance (% of the total sequences) of the most abundant bacterial and archaeal phyla in the thermophilic and mesophilic digesters fed with non-irradiated and MW-irradiated sludge is shown in Figure 4. Under mesophilic temperatures, the microbial culture sample from digester "M1—Control" was mainly dominated by the Candidate Division *WWE1* (Wastewater of

Evry 1, belonging to the class of *Cloacamonae*) with an abundance of 46.4%. Chouari et al. [41] discovered this novel bacterial candidate division *WWE1* within a mesophilic anaerobic biomass from a municipal AD (in the fermentation and acidogenesis stages) at the Evry WWTP (France). According to Pelletier et al. [42], *WWE1* easily adapted to anaerobic conditions, because it had several proteins typical of anaerobic bacteria and was detected in 32 (out of 43) anaerobic digesters. Moreover, the *WWE1* bacteria was proposed to accomplish syntrophic propionate oxidation in addition to amino acid fermentation [17,42]. Members of phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* accounted for 14.8%, 12.2%, and 10.0%, respectively, in this digester. Our findings regarding the dominant bacterial communities were in accordance with the literature [16,17,43,44]. Mei et al. [17] stated that the predominant bacterial phyla were *Bacteroidetes*, candidate division *WWE1*, *Firmicutes*, *Spirochaetes*, and *Tenericutes* in anaerobically digested sludge samples taken from different digesters (37 °C) in a full-scale water reclamation plant at Chicago, Illinois. A study by Riviere et al. [43] observed *Chloroflexi*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* as the main bacterial phyla from microbial communities of seven full-scale mesophilic ADs (in France, Germany, and Chile).

In the mesophilic AD operation in tandem with MW pretreatment, the amounts of *Firmicutes* increased gradually according to MW pretreatment intensity applied to sludge. The relative abundance of *Firmicutes* increased from 14.8% ("M1—Control") to 21.6% and 28.4% in the digesters of "M2—80 °C" and "M3—160 °C", respectively. This increase is consistent with previous research conducted by Westerholm et al. [18], which was related to a comparative analyses of bacterial and archaeal community dynamics in three mesophilic digesters during start-up and operation using non-irradiated and MW-irradiated WAS. Their results proposed that increased substrate availability induced by MW pretreatment stimulated growth of members within *Firmicutes*. Furthermore, many *Firmicutes* can produce endospores resistant to extreme conditions including temperature etc. On the other hand, the bacterial members of *Proteobacteria* phylum decreased noticeably in number from 12.2% ("M1—Control") to 3.2% in the digester of "M2—80 °C". They also continued to decrease down to 1.5% in the digester of "M3—160 °C". Significant changes in some bacterial phyla was observed in the microbial culture sample taken from the digester fed with high temperature MW-irradiated sludge (160 °C). For example, *Bacteroidetes* (mostly belonging to the class of *Bacteroidia*) increased to an abundance of 37.9%. This means that it has resistance to stress conditions during digestion such as high OLRs and VFAs accumulation as previously reported [45,46]. In contrast, Candidate Division *WWE1* decreased to 14.1% (Figure 4). Additionally, the increases of *Synergistetes* (4.0%) and *Verrucomicrobia* (4.3%) were observed in the "M3—160 °C" system.

Under thermophilic temperature, the microbial community in the digester of "T1—Control" was primarily dominated by the phylum *Firmicutes* (mostly belonging to the class of *Clostridia*), as they accounted for 42.3% of the total population, as shown in Figure 4. *Clostridia* is a fermentative microorganism known to play a main role in the production of short-chain fatty acids, carbon dioxide, and hydrogen via acidogenesis; thus, its presence in anaerobic biomass can be correlated to a high-rate of hydrolysis and VFAs fermentation [14]. The phylum *Thermotogae* (belonging to the class of *Thermotogae*) was another predominant phylum in this digester with an abundance of 15.2%. It has been known as the predominant group within anaerobic culture at thermophilic temperatures [47,48]. As one of the thermophilic anaerobes, it has the ability to catalyze a wide range of polysaccharides to acetate, $CO_2$, and $H_2$ by excreting hydrolytic enzymes [48]. When MW pretreatment was applied prior to the thermophilic AD operation, moderate changes were observed in bacterial community profiles of the MW-irradiated digestate samples. For instance, the members of *Synergistetes* phylum (belonging to the class of *Synergistia*) increased gradually from 3.9% (control) to 6.7% and 11.1% in the samples from the digesters of "T2—80 °C" and "T3—160 °C", respectively (Figure 4). In the study by Godon et al. [49], the genus *Synergistes* was explored in 93 anaerobic environments (e.g., guts, soils, digesters, etc.). They reported that this phylum seemed to be anaerobic amino acid degraders and are related to anaerobic digesters/soils or thermophilic conditions. Moreover, a decrease of the phylum *Proteobacteria* from 6.3% to 3.8% and 1.5%, respectively, was observed in reserve correlation with pretreatment

intensity. Furthermore, the level of *Thermotogae* slightly increased to 16.4% and 19.3% in the digesters of "T2—80 °C" and "T3—160 °C", respectively, while the levels of *Firmicutes* remained at similar abundances (42%) (Figure 4).

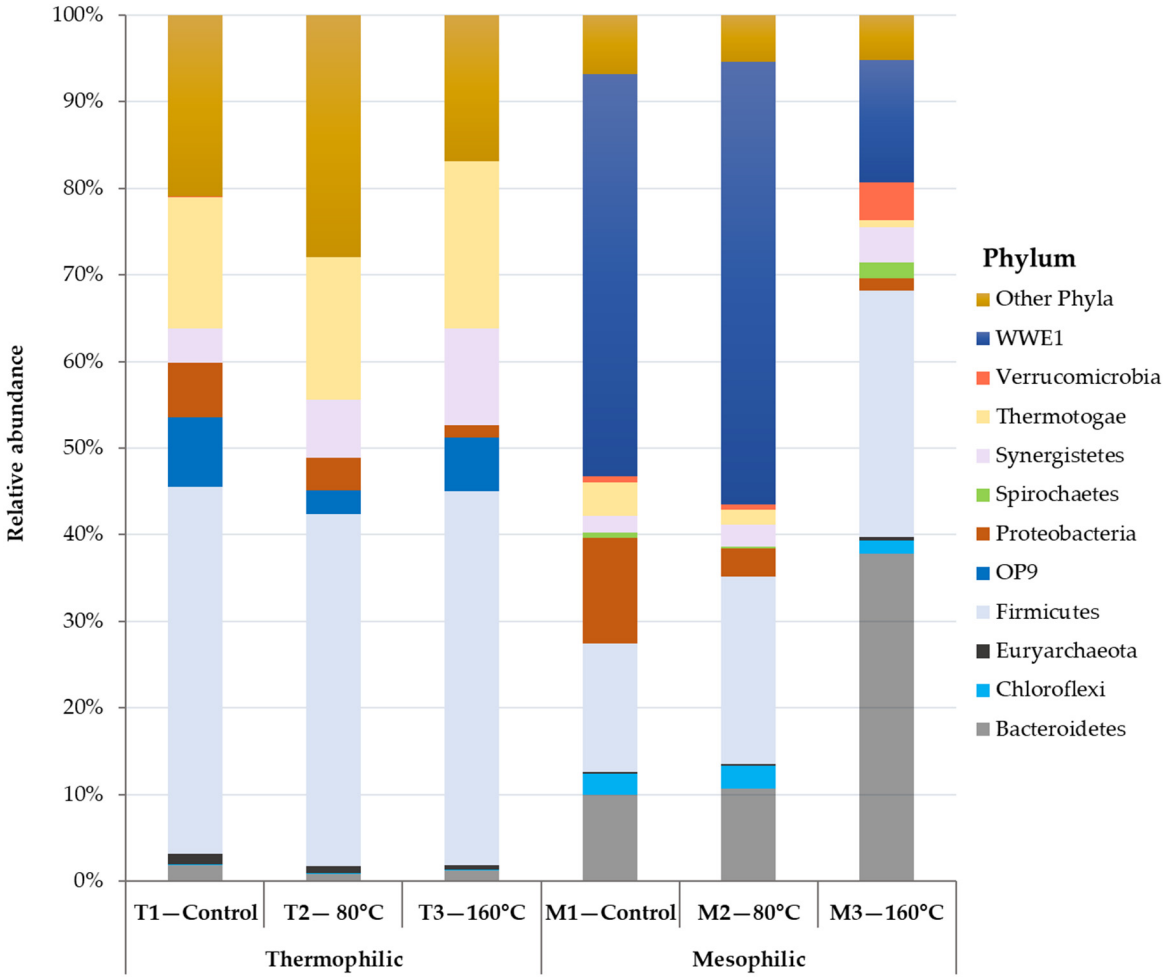


**Figure 4.** Taxonomic classification of the most abundant bacterial and archaeal phyla found in the mesophilic and thermophilic digesters (Phyla that were more abundant than 1% in either one of the mesophilic or thermophilic digesters effluents' samples were plotted. Phyla that accounted for <1% of the total population were grouped into "Other Phyla".).

An analysis of archaeal community structure is the key to provide more insight about a stable and balanced AD operation. The average relative abundance (% of total bacterial and archaeal sequences in each sample) of methanogens (affiliated to the phylum of *Euryarchaeota*) was between 0.18–0.37% in mesophilic and 0.51–1.24% in thermophilic mixed microbial culture samples. The samples from mesophilic digesters mostly belonged to the members of *Methanosaetaceae*, *WSA2*, and *Methanospirillaceae* families, whereas the samples from thermophilic digesters only belonged to *Methanobacteriaceae*, and *Methanosarcinaceae*. The average relative abundance (% of total archaeal sequences in each sample) of methanogenic *Archaea* at the genus level is shown in Figure 5.

The most dominant genus of thermophilic methanogens in the sludge sample from digester of "T1—Control" was *Methanothermobacter* with an abundance of 81.3% (Figure 5a). This suggests that at SRT of 6 days methane production was possibly achieved through a hydrogenotrophic methanogenesis pathway that mostly used $H_2$ as an electron acceptor [4]. Demirel and Scherer [4] reported that thermophilic conditions and some other environmental factors (e.g., short SRTs in a biomass reactor) could favour the growth of rod like or coccoid hydrogenotrophic methanogens.

In our study, the only hydrogenotrophic methanogen species identified was *Methanothermobacter thermautotrophicus*, which is generally found as a member of methanogenic *Archaea* in many anaerobic systems under thermophilic conditions [50,51]. In the study by Hori et al. [52], the *Methanothermobacter* sp. became the predominant hydrogenotrophic methanogen during the propionate accumulation in a thermophilic anaerobic digester, while the other parameters (i.e., acetate concentrations or pH) changed slightly. The findings of Hori et al. [52] are consistent with our results given in Figures 2 and 5a. The members of *Methanothermobacter* continued to dominate the microbial community when the AD operation was coupled with MW pretreatment. The relative occurrence (74.3% and 77%, respectively) of *Methanothermobacter* was slightly lower in MW-irradiated digesters at 80 °C and 160 °C compared to control. Our findings were in agreement with the previously conducted study by Gagliano et al. [50]. In their study, the microbial community dynamics were investigated in two semi-continuous flow ADs fed with un-pretreated and thermally pretreated sludge. Additionally, in our study, an acetate utilizer methanogen, belonging to the genus *Methanosarcina*, constituted 12.0% and 14.7% (of total archaeal population) in the digesters of "T2—80 °C" and "T3—160 °C" respectively; however, this genus accounted for only 3.3% in the digester of "T1—Control" (Figure 5a). *Methanosarcina* spp. are very versatile microorganisms that often develop in anaerobic digesters, especially under elevated temperatures (i.e., 55–60 °C) [14].

*Methanosarcina* spp. can produce methane using all three methanogenesis pathways (i.e., aceticlastic, hydrogenotrophic, and methylotrophic) at high levels of acetate [14]; thus, they are known to be more competitive than *Methanosaeta* species. Demirel and Scherer [4] reported that high concentrations of toxic ionic agents (e.g., ammonia, hydrogen sulphide, and VFAs) allow for the growth of *Methanosarcina* sp. during AD operation. This advantage can explain why the member of genus *Methanosarcina* increase in the MW-irradiated digesters with regards to the VFAs accumulation (Figures 2 and 5a). However, the relative abundance of *Methanosarcina* was very limited in the thermophilic digesters when compared to *Methanothermobacter*. This was possibly due to methane generation through syntrophic acetate oxidation pathway at high acetate (i.e., VFAs) concentrations. This process consists of two reactions: (1) the conversion of acetate to $H_2$ and $CO_2$ by syntrophic acetate oxidizing bacteria via syntrophic acetate oxidation, and (2) the subsequent conversion of these products to methane by hydrogenotrophic methanogens [53]. Thus, the syntrophs of the acetate oxidizing bacteria and hydrogenotrophic methanogens (such as the genus *Methanothermobacter* in this study) could overcome the aceticlastic methanogens and the hydrogenotrophic methanogens become the dominant methane formers [53,54]. Ho et al. [55] also reported that the majority of methane was produced by non-aceticlastic pathways in high-rate thermophilic AD of WAS, indicating syntrophic acetate oxidation as a key pathway at elevated temperatures. Another study by Świątczak et al. [14] revealed that the acetogenic *Synergistes* sp. promoted efficient biogas production because they closely cooperated with methanogens (i.e., *Methanomicrobia*) by conducting interspecies hydrogen transfer. Additionally, as seen in Figure 5a, a minor increase was also observed in the abundance of genus *Methanomassiliicoccus* in reverse correlation to MW pretreatment intensity, but the relative abundance of this methylotrophic methanogen was lower than 5% (between 0.7–3.1%). The recently described genus *Methanomassiliicoccus*, belonging to the family of *Methanomassiliicoccaceae* (the class *Thermoplasmata*), was isolated from human feces, and discovered to be a methanol-reducing, mesophilic, slightly alkaliphilic methanogen [56].

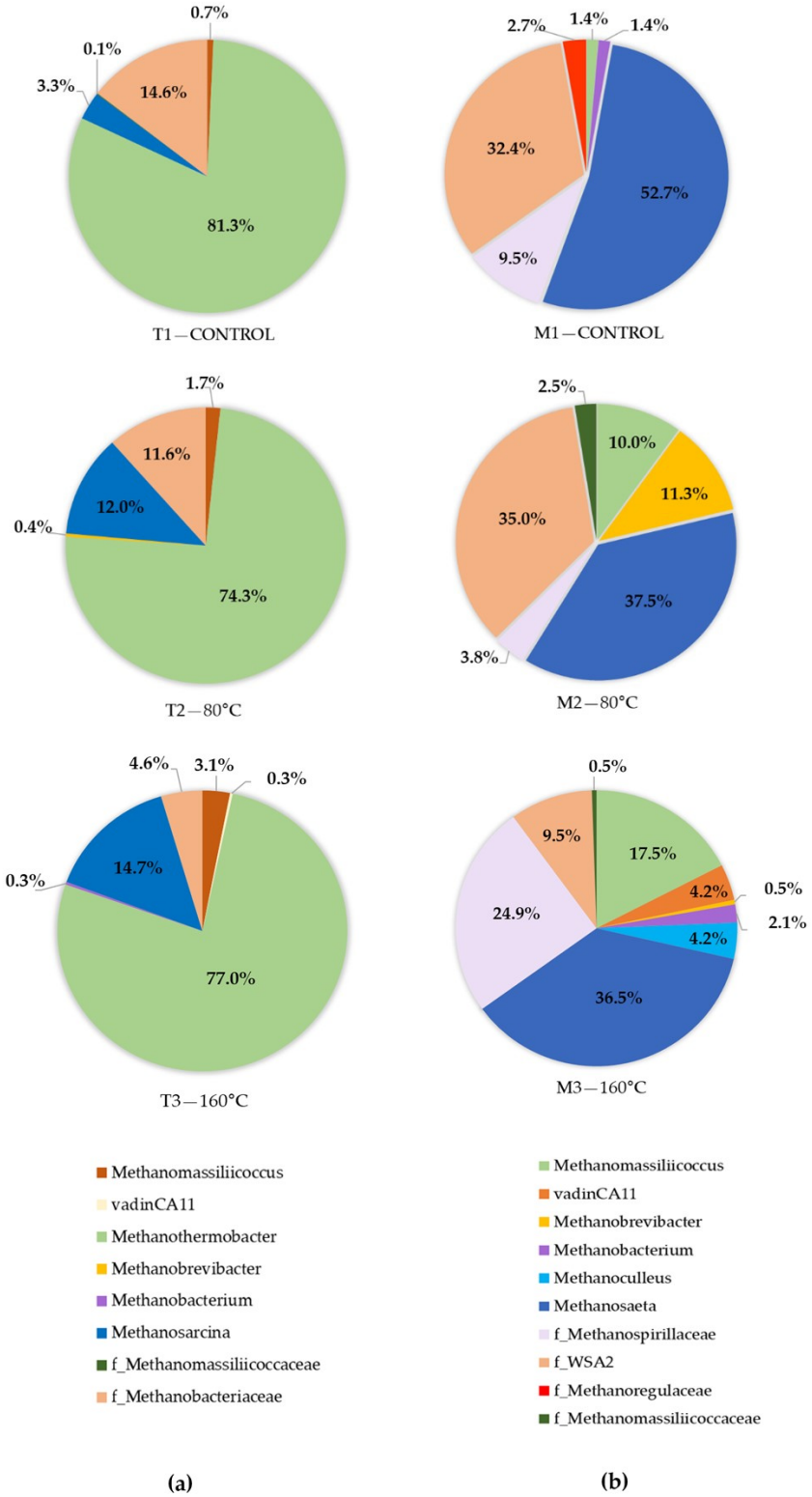


**Figure 5.** Taxonomic distribution of methanogens found in the mesophilic and thermophilic digesters at the genus level (Undefined genera in database were shown as family (f_) level as the lowest taxonomic level designated.).

Under mesophilic conditions, the high abundance of the genus *Methanosaeta* (52.7%) was observed in the digestate sample taken from the digester of "M1—Control". However, the percentage of *Methanosaeta* decreased to 37% in the digesters coupled with MW pretreatment under the same

operating conditions (Figure 5b). As a strict aceticlastic methanogen, *Methanosaeta* (formerly called *Methanothrix*) can only utilize acetate as a substrate for methanogenesis and its existence is strictly associated with acetate concentration in the environment [50,57]. The highly abundant *Methanosaeta* sp. can grow only at low acetate concentrations in a variety of anaerobic reactors [4]. The moderate decrease in the abundance of *Methanosaeta* found in the MW-irradiated digestate samples was most probably due to fluctuations of higher VFA concentrations observed during digestion (Figures 2 and 5b). However, in consistence with the results of MW-irradiated digesters' biogas/methane production (Figure 1 and Table 5), the genus *Methanosaeta* was still dominant in these digesters. This indicated effective acetate degradation in stable AD operation with overall low levels of VFAs (i.e., acetate). In the study by McMahon et al. [58], microbial population dynamics were investigated in mesophilic anaerobic laboratory-scale co-digesters treating municipal solid waste and sludge (including primary sludge and WAS). According to their findings, *Methanosarcina* spp. were the most abundant aceticlastic methanogens under the unstable operation of co-digesters with high levels of acetate, while *Methanosaeta concilii* was dominant in stable systems with low levels of acetate. On the other hand, the relative abundances of *WSA2* (belonging to the order of *Methanobacteriales*) were between 32% and 35% in the digesters of "M1—Control" and "M2—80 °C", whereas this novel methanogen decreased to 9.5% in the digester of "M3—160 °C" (Figure 5b). Similar to thermophilic digesters, the percentage of genus *Methanomassiliicoccus* gradually increased from 1.4% (control) to 10.0% and 17.5% in the mesophilic digesters fed with MW-irradiated sludge at 80 °C and 160 °C, respectively. Other hydrogenotrophic methanogens encountered in the mesophilic microbial culture samples belonged to the genera of *Methanobrevibacter* (up to 11.3%) and *Methanobacterium* (up to 2.1%) (Figure 5b).

## 4. Conclusions

Anaerobic digesters utilizing non-irradiated and MW-irradiated sludge under high organic loading rates (4.9–5.7 g VS/L/d) indicated that the archaeal community structure was closely related to the levels of VFAs produced, triggering a syntrophic acetate oxidation as a main route for methane production under thermophilic temperatures. The thermophilic digesters had higher levels of VFAs than the mesophilic digesters. The propionic acid concentrations in the MW-irradiated thermophilic digesters increased up to 1150 mg/L; however, these digesters still maintained a steady-state operation by producing reasonable biogas generation (485–491 mL biogas/g $VS_{fed}$) with a typical methane content (65%). The genus *Methanosaeta* was the most abundant aceticlastic methanogen in the mesophilic digesters with low levels of VFAs, while the hydrogenotrophic *Methanothermobacter* dominated in the thermophilic digesters with high VFAs concentrations. AD operation tandem with MW pretreatment did not induce a shift in the dominant bacterial phylum of *Firmicutes* in the microbial population under thermophilic conditions. However, the dominant phylum shifted to *Bacteroidetes* (38%) in mesophilic digesters utilizing MW-irradiated sludge at high temperatures. This study underlined the importance of molecular analysis as a fundamental tool to gain insight and deeper understanding of anaerobic digester performances, especially under high OLRs.

**Author Contributions:** Conceptualization and methodology, G.K.-B., E.U.-C., and C.E.; formal analysis, investigation, and writing—original draft preparation, G.K.-B.; writing—review and editing, E.U.-C. and C.E.; supervision, E.U.-C. and C.E.; and funding acquisition, G.K.-B., E.U.-C., and C.E. All authors have read and agreed to the published version of the manuscript.

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