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# Performance and Microbial Community Dynamics in Anaerobic Digestion of Waste Activated Sludge: Impact of Immigration

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Abstract: Waste activated sludge (WAS) is a byproduct of municipal wastewater treatment. WAS contains a large proportion of inactive microbes, so when it is used as a substrate for anaerobic digestion (AD), their presence can interfere with monitoring of active microbial populations. To investigate how influent cells affect the active and inactive microbial communities during digestion of WAS, we operated model mesophilic bioreactors with conventional conditions. Under six different hydraulic retention times (HRTs; 25, 23, 20, 17, 14, and 11.5 d), the chemical oxygen demand (COD) removal and CH<sub>4</sub> production of the AD were within a typical range for mesophilic sludge digesters. In the main bacteria were proteobacteria, bacteroidetes, and firmicutes in both the WAS and the bioreactors, while in main archaeal methanogen group was Methanosarcinales in the WAS and methanomicrobiales in the bioreactors. Of the 106 genera identified, the estimated net growth rates were negative in 72 and positive in 34. The genera with negative growth included many aerobic taxa. The genera with positive growth rates included methanogens and syntrophs. In some taxa, the net growth rate could be positive or negative, depending on HRT, so their abundance was also affected by HRT. This study gives insights into the microbial dynamics of a conventional sludge anaerobic digester by distinguishing potentially active (growing) and inactive (non-growing, dormant) microbes and by correlating population dynamics with process parameters.

**Keywords:** sewage sludge; hydraulic retention time; high-throughput sequencing; real-time PCR; net growth rate

# 1. Introduction

Modern municipal wastewater treatment plants (MWWTPs) generate a large quantity of waste sludge. Efficient management of the waste sludge, comprising the primary and the secondary sludges, is one of the major issues in wastewater treatment practices. Typically, a MWWTP consumes a large



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amount of energy for aeration, pumping, and sludge management; however, a MWWTP can be a net energy producer by extracting energy from the wastewater and sludge [1]. Several sludge-to-energy technologies are available today such as combustion, pyrolysis, gasification, and anaerobic digestion (AD) [2]. Among these, AD is a proven technology to reduce the footprint of and produce bioenergy, in the form of methane (CH<sub>4</sub>)-rich biogas, from sewage sludge [3]. AD of organic substrates, including sewage sludge, involves complex biochemical reactions such as hydrolysis, acidogenesis, acetogenesis, and methanogenesis. These reactions are mediated by a complex microbial community that cooperates and sometimes competes to utilize the organic substrates. Understanding the composition and growth of the microbial populations is one of the key prerequisites for fundamental improvement of AD because the bioconversion of the feedstock depends on the harmonious activity of the anaerobic microorganisms [4].

Between the two major streams of the sewage sludge, secondary sludge consists of the undigested biomass that remains after biological treatment units such as the activated sludge process. The secondary sludge, or the waste activated sludge (WAS), is mainly composed of cellular biomass. Thus, when used a major component of the influent to the anaerobic digesters at MWWTPs, the WAS can be regarded both as the organic substrate and as a source of immigrant, which are inactive in the digesters [5,6]. Organic materials in the WAS contain large proportions of cell walls and extracellular polymeric substances, and therefore often resist biochemical attack, so sludge digesters require a long retention time [7]. This high content of cellular biomass in a sludge digester obscures the distinction between cells that are included in the WAS, but that function only as substrate, and cells that thrive in the digester and that produce enzymes during AD. Culture-independent molecular techniques such as high-throughput sequencing have revealed the microbial communities in different anaerobic digesters [8–10]. Substrate type [11,12] and digester operation conditions [13,14] are regarded as important factors that determine the microbial population patterns in AD. However, the identity of the microbes that constitute the mixed biomass in WAS digesters is still unclear, particularly at different retention times. Furthermore, single-time applications of these techniques cannot distinguish inactive microbes from those that participate in the AD process.

AD of sludge sometimes involves pretreatment of the substrate [15]. However, the pretreatment requires extensive use of chemicals, heat, electricity, or some combination of these, so its application is limited. An alternative is co-digestion of mixed organic wastes; this procedure often promotes biogas production by synergistic effects [16], but it relies on the availability of a secondary substrate. Therefore, the basic process (mono-digestion of WAS without pretreatment) as a model system should be studied to learn of the microbial components of sludge digestion. Applying different retention times (or dilution rates) can be beneficial in such investigations because the dilution rate governs the washout rate of microbes in bioreactors.

The goal of this study was to investigate microbial community dynamics, and how they correlate with chemical oxygen demand (COD) removal and  $CH_4$  generation during AD of WAS. Anaerobic continuous stirred-tank reactors (CSTRs) were operated at a wide range of retention times. The microbial community dynamics were monitored using high-throughput sequencing and real-time polymerase chain reaction (PCR). Net growth rates of the identified taxa were estimated to infer whether or not they grow actively during the AD process. This study provides new information on which microbes are potentially active (growing) or inactive (non-growing, dormant), as well as which populations are preferred at higher or lower dilution rates, in the dynamic environment of anaerobic sludge digestion

#### 2. Material and Methods

#### 2.1. Collection of Sludge

WAS and anaerobic sludge (AS) samples were collected from a local sewage treatment plant that serves a population of ~400,000. The WAS (Table 1) was collected in a single batch (approximately

300 L), sieved through a 2 mm mesh, then kept at 4 °C until use. The AS was sieved through a 850- $\mu$ m mesh to remove impurities before use. The WAS had total solids (TS) of 23.0  $\pm$  0.6 g/L, and volatile solids (VS) of 10.2  $\pm$  0.2 g/L; the AS had TS of 34.0  $\pm$  0.1 g/L, and VS of 17.4  $\pm$  0.1 g/L.

| Parameter                       | Unit | Value  | Standard Deviation |
|---------------------------------|------|--------|--------------------|
| pH                              | _    | 6.9    | 0.3                |
| Chemical oxygen demand (COD)    | mg/L | 15,189 | 227                |
| Soluble COD (SCOD)              | mg/L | 107    | 118                |
| Total solids (TS)               | mg/L | 23,032 | 568                |
| Volatile solids (VS)            | mg/L | 10,173 | 202                |
| Total suspended solids (TSS)    | mg/L | 13,575 | 260                |
| Volatile suspended solids (VSS) | mg/L | 8930   | 42                 |
| Crude carbohydrate              | mg/L | 2056   | 304                |
| Crude protein                   | mg/L | 5478   | 31                 |
| Crude lipid                     | mg/L | 429    | 35                 |
| Volatile fatty acids (VFAs)     | mg/L | 86.6   | 3.5                |
| Ethanol                         | mg/L | ND     | ND                 |
| Total ammonia nitrogen (TAN)    | mg/L | 24.2   | 2.0                |
| Sodium (Na <sup>+</sup> )       | mg/L | 2619   | 2                  |
| Chloride (Cl <sup>-</sup> )     | mg/L | 5595   | 3                  |

Table 1. Characteristics of the WAS used in this study.

ND, not detected.

# 2.2. Bioreactor Operation

Two CSTRs were operated for 330 d at six hydraulic retention times (HRTs). Reactor R1 was sequentially operated at HRT = 25, 23, and 11.5 d; reactor R2 was sequentially operated at HRT = 20, 17, and 14 d. Steady state was assumed when no significant variation was observed for residual chemical oxygen demand (COD) and CH<sub>4</sub> production rate after at least three volume changes. Before continuous operation, a batch operation was conducted using a mixture of AS (as seed inoculum; 1% v/v) and WAS (as substrate; 99%) for 25 d to acclimatize the biomass to the substrate. Temperature was maintained at 35 °C and the pH was not corrected. Each bioreactor had a working volume of 6 L. The surface of the bioreactors was covered with aluminum foil to block light and prevent the growth of phototrophs.

# 2.3. Sampling and Analyses

Mixed liquor sampling was conducted at least twice a week for COD and VFA analyses. The substrate (WAS) was additionally tested to determine the total and volatile solids (TS and VS), total and volatile suspended solids (TSS and VSS), crude carbohydrate, crude protein, crude lipid, total ammonia nitrogen (TAN), Na<sup>+</sup> and Cl<sup>-</sup> contents (Table 1). COD and solids were measured following standard methods [17]. Volatile fatty acids (VFAs; C<sub>2</sub>–C<sub>6</sub>) and ethanol concentrations were quantified using a gas chromatograph (6890 plus, Agilent, United States) with a flame ionization detector and a capillary column (Innowax, Agilent) [6]. The crude carbohydrate content was determined using the phenol–sulfuric acid method [18]. The crude lipid concentration was analyzed using gravimetry after extraction with a solvent (chloroform: methanol, 1:2 v/v) [19]. The crude protein concentration was estimated by assuming 6.25 g protein for 1 g organic nitrogen, where the organic nitrogen was analyzed by subtracting the TAN from the Total Kjeldahl nitrogen (TKN) [17]. For VFA and TAN measurements, samples were used after filtering through a 0.45 µm syringe filter. All physicochemical analyses were conducted in duplicate.

Biogas production was monitored using a gas-tight bag attached to each bioreactor. Biogas volume was measured at least three times a week, and its composition was measured at least once a week. Biogas composition was determined using a second gas chromatograph (6890 plus, Agilent) equipped with a thermal conductivity detector and a capillary column (GS-Carbon Plot, Agilent) [6].

## 2.4. DNA Extraction and High-Throughput Sequencing

Total DNA was extracted from the WAS (at the beginning (0 d) and the end (330 d) of the experiment, from the AS, and from bioreactor samples at the six HRT conditions (three times during steady state at each HRT). A sub-sample of 0.2 mL was centrifuged to remove potential PCR inhibitors in the supernatant. Total DNA was extracted from the subsample in duplicate using a Magtration System 6GC (Precision System Science, Japan).

Nine samples (two WAS, one AS, and six temporal bioreactor samples) were processed for high-throughput sequencing after pooling relevant DNA samples at equal volumes. After pooling, PCR was performed to amplify the V4 region of the 16S rRNA gene using modified F515 and F806 primers [9]. The reaction mixture composition [20] and the cycling conditions [21] were described previously.

The high-throughput sequencing was performed using an Ion PGM instrument (Life Technologies) as described previously [21]. Short reads (<200 bp) and low-quality reads were excluded and operational taxonomic units (OTUs) were defined as being under 3% sequence dissimilarity level by UPARSE software (v8.1.1861) [22]. The OTUs were taxonomically assigned to the database at 50% confidence threshold by using the Ribosomal Database Project pipeline [23].

# 2.5. Real-Time PCR

The abundance of the methanogen-related taxa was determined using real-time PCR for the domain Archaea and the orders Methanobacteriales, Methanococcales, Methanomicrobiales, and Methanosarcinales [24]. Real-time PCR with LightCycler 480 system (Roche, Germany) was conducted for individual DNA samples. The composition of the reaction mixture ( $20 \mu$ L) and the cycling conditions were described previously [25].

# 2.6. Statistical Analyses and Estimation of Net Growth Rates of the Sequencing Data

The analyses and visualization in this section were performed with R software using vegan, scales, optparse, gclus, and ggplot2 libraries. Bray-Curtis distance measure of the sequencing data at the genus-level was used to visualize the beta diversity by ordination (non-metric multidimensional scaling, NMDS). To investigate the correlations between process and sequencing data, Spearman coefficients were evaluated. The net growth rates were estimated using mass balance [5]. Population growth and decay was assumed to be explained by first-order equation during steady-state operation:

$$\frac{dN_{X,rxr}}{dt} = kN_{X,rxr} + n_{X,WAS} - n_{X,eff} \tag{1}$$

where  $N_{X,rxr}$  is the number of microorganism X in the bioreactor (estimated as VSS × relative abundance of X),  $k [d^{-1}]$  is the rate constant of net growth or decay,  $n_{X,WAS} [n \cdot d^{-1}]$  is the number of microorganism X in the WAS that enter the bioreactors, and  $n_{X,eff} [n \cdot d^{-1}]$  is the number of microorganism X that leave the bioreactors in the effluent.

# 3. Results

#### 3.1. Bioreactor Operation

The WAS had a neutral pH ( $6.9 \pm 0.3$ ), with average VS ( $10.2 \pm 0.2 \text{ g/L}$ ) was 44.2% to the TS ( $23.0 \pm 0.6 \text{ g/L}$ ), so the difference, which represents fixed solids (FS) was significant (Table 1). This high FS is attributable to the high salinity (8.2 g NaCl/L) of the WAS, which is likely caused by seawater inputs into the sewage collection stream [26]. Most (87.8%) of the VS was in the suspended form; this result suggests that the organic materials in the secondary sludge require breakdown of barriers such as the cell wall [7]. The most abundant macromolecules in the VS were crude proteins; their abundance (53.8%) was similar level to the protein content (55%) of a typical prokaryotic cell [27]. Crude carbohydrate (20.2%) and crude lipid (4.2%) were also present.

Both digesters operated stably with constant reduction of COD and production of CH<sub>4</sub> during the 330 d period (Figure 1). In both reactors, the residual COD and CH<sub>4</sub> production rate increased as HRT decreased. The COD removal efficiency was estimated as 33.5%–14.9% and the CH<sub>4</sub> production rate as 42.4–62.6 mL/L/d at steady state (Figure 2). The total CH<sub>4</sub> yield generally decreased as the HRT decreased (Figure 2b). The pH of the bioreactors was maintained between 7.0–7.5 without external chemical addition (data not shown). The VFA concentrations in R1 and R2 were maintained at 70  $\pm$  29 mg as COD/L during the operation (data not shown); this value is only 0.5% of the influent COD (Table 1). Overall, the lab-scale WAS digesters were stable during operation, and the COD removal and CH<sub>4</sub> production showed a continuous pattern according to the HRT applied (Figure 2), with no significant difference between the two bioreactors.



**Figure 1.** The chemical oxygen demand (COD) (**a**) and CH4 production rate (**b**) profiles of the WAS bioreactors.



Figure 2. The COD removal efficiency (a) and CH4 production rate and yield (b) at the steady states.

# 3.2. Microbial Community Results

Microbial populations in the WAS, AS, and bioreactors were analyzed using high-throughput sequencing (Figure 3). Two WAS samples were collected, one at the beginning (0 d, WAS1) and one at the end (330 d, WAS2) of the experiment. Six samples were collected from the bioreactors at the steady state of each HRT condition (total six conditions). High-throughput sequencing for the nine samples collectively yielded 132,773 high-quality reads, which were assigned to 1284 OTUs. The Ribosomal Database Project database pipeline was used to assign OTUs to the most likely taxa [23].



**Figure 3.** Relative abundance of the identified taxa in the samples at the phylum level. Taxa < 1% at all samples are collectively shown as 'minor groups'.

Overall, 17 phyla were identified in the nine samples. Proteobacteria, bacteroidetes, and firmicutes were the most prominent phyla (summed relative ratio =  $69.1 \pm 8.6\%$ ). Eight phyla with relative abundance  $\geq 1\%$  (Euryarchaeota, Woesearchaeota, Actinobacteria, Chloroflexi, Planctomycetes, Synergistetes, Tenericutes, and Thermotogae) were considered numerically significant. The six remaining phyla are referred to collectively "minor groups" (Figure 3).

Proteobacteria was the most abundant (35.3  $\pm$  6.9%) phylum in all nine samples. The average abundance of Proteobacteria was higher in the WAS samples (n = 2; 46.9  $\pm$  3.6%) than in the bioreactor samples (n = 6; 32.0  $\pm$  2.3%) (p = 0.07, Student's t-test). Bacteroidetes (overall 22.5  $\pm$  4.3%) was the second-most-abundant member; these were also more abundant in the WAS (28.3  $\pm$  4.0%) than in the bioreactors (21.7  $\pm$  1.7%). Firmicutes (11.3  $\pm$  5.8%) was more abundant in the bioreactors (13.7  $\pm$  5.2%) than in the WAS (6.0  $\pm$  5.2%), however no significant statistical difference was confirmed (p > 0.2). Among the other phyla in Figure 3, Euryarchaeota, Chloroflexi, Synergistetes, and Thermotogae were significantly (p < 0.05) more abundant in the bioreactors than in the WAS.

NMDS ordination and a dendrogram (Figure A1) clearly separated the six bioreactor samples from the WAS and AS samples. The Bray-Curtis distances (dissimilarity) were <0.15 between bioreactor samples, but >0.30 between the bioreactors and the WAS, and between the bioreactors and the AS (Figure A1b). Interestingly, the distance between the AS sample (the inoculum) and the bioreactor samples was as high as between the AS and the WAS samples, although both the AS and the bioreactor samples were obtained from anaerobic environments.

Due to the low relative abundance of the methanogenic taxa (Euryarchaeota) (Figure 3), real-time PCR was additionally performed for the domain Archaea and the methanogen groups Methanobacteriales, Methanomicrobiales, Methanosarcinales, and Methanococcales to quantify the methanogen communities. The Methanobacteriales, Methanomicrobiales, and Methanosarcinales primer-probe sets successfully amplified their target taxa (Figure 4). The results showed that the bioreactors (4.3–11.1 × 10<sup>7</sup> copies/mL) had one order of magnitude higher levels of Archaea than the WAS ( $5.3 \times 10^6$  copies/mL). Among the methanogen orders, Methanomicrobiales ( $6.3 \pm 2.0 \times 10^7$  copies/mL) was dominant (25 times higher than the sum of others) in the bioreactor samples, whereas Methanosarcinales ( $4.9 \pm 2.8 \times 10^6$  copies/mL) was the most abundant (three times higher than the sum of the others) in the WAS.



**Figure 4.** Real-time polymerase chain reaction (PCR) quantification of the archaeal 16S rRNA genes at the domain and the order levels. Methanococcales was not detected.

#### 3.3. Estimation of Net Growth Rates

The net growth rates (Equation (1)) of the identified microbial taxa were analyzed to estimate how immigrant microbes affected the microbial community in the anaerobic bioreactors. The number

of microorganisms was estimated by multiplying the average VS value of the corresponding sample (i.e., WAS or bioreactor at the steady states) by the proportion of the taxa at the genus level. The two WAS samples were averaged in this calculation. The mean estimated net growth rates for the six HRT conditions were plotted (Figure 5), along with their mean relative abundance in the bioreactors.



**Figure 5.** Mean relative abundance of identified genera in the bioreactors and the estimated net growth rate. Taxa > 1% in the waste activated sludge (WAS; influent) are labelled in black, whereas taxa that were not detected in the WAS are labelled in blue.

Among the 106 genera identified in this study, 72 genera had negative net growth rates, and 34 had positive net growth rates (Figure 5). All genera with relative abundance >0.5% in the WAS had negative net growth rates (Figure 5, red). Genera that were not detected in the WAS must have actively grown in the bioreactors; these genera include *Methanocalculus, Methanogenium, Garciella, Defluviitoga, Chlorobium, Coprothermobacter, Methanothrix, Candidatus* Hydrogenedens, and *Phycisphaera.* Overall, the abundance of a taxon in the influent correlated with its estimated net growth rate in the anaerobic bioreactors.

# 3.4. Relationship between Process Parameters and Abundance of Microbial Taxa

Spearman correlation coefficients  $\rho$  were calculated between process parameters and the proportion of microbial taxa at the genus level. Several general showed significant correlations with HRT, COD removal efficiency, CH<sub>4</sub> production rate, and CH<sub>4</sub> yield (Table 2). These genera could be assigned to two groups.

| Group  | Genus            | HRT      | COD Removal | MPR <sup>a</sup> | MY <sup>b</sup> |
|--|------------------|----------|-------------|------------------|-----------------|
| Methanogenium<br>Acetobacteroides<br>Mesotoga<br>Thermovirga<br>Variovorax<br>Aquabacterium<br>Soehngenia<br>Arcobacter<br>Fusibacter<br>Spirochaeta | Methanogenium    | 0.83 *   | 0.83 *      | -0.83 *          | 0.66            |
|  | Acetobacteroides | 0.89 **  | 0.89 **     | -0.89 **         | 0.94 **         |
|  | Mesotoga         | 0.83 *   | 0.83 *      | -0.83 *          | 0.94 **         |
|  | Thermovirga      | 0.94 **  | 0.94 **     | -0.94 **         | 1.00 ***        |
|  | Variovorax       | 0.83 *   | 0.83 *      | -0.83 *          | 0.77            |
|  | Aquabacterium    | 1.00 *** | 1.00 ***    | -1.00 ***        | 0.94 **         |
|  | Soehngenia       | 0.94 **  | 0.94 **     | -0.94 **         | 1.00 **         |
|  | Arcobacter       | 0.94 **  | 0.94 **     | -0.94 **         | 1.00 **         |
|  | 0.94 **          | 0.94 **  | -0.94 **    | 0.83*            |                 |
|  | 0.83 *           | 0.83 *   | -0.83 *     | 0.94 **          |                 |

**Table 2.** Spearman correlation coefficients  $\rho$  and the level of the *p* values (\*\*\* <0.01; \*\* <0.05; and \* <0.1) between selected process parameters and relative abundance at the genus level.

| Group | Genus  | HRT   | COD Removal   | MPR <sup>a</sup>                                   | MY <sup>b</sup>                                  |
|-------|--|---|---|--|--|
| Ш     | Gelidibacter<br>Chondromyces<br>Phaeodactylibacter<br>Clostridium<br>Mycobacterium | -0.89 **<br>-0.94 **<br>-0.83 *<br>-0.89 **<br>-0.89 ** | -0.89 **<br>-0.94 **<br>-0.83 *<br>-0.89 **<br>-0.89 ** | 0.89 **<br>0.94 **<br>0.83 *<br>0.89 **<br>0.89 ** | -0.71<br>-0.83 *<br>-0.89 **<br>-0.77<br>-0.83 * |
|       | Methanocalculus  | -0.83 *   | -0.83 *   | 0.83 *   | -0.77  |

Table 2. Cont.

<sup>a</sup> MPR, CH<sub>4</sub> production rate. <sup>b</sup> MY, CH<sub>4</sub> yield.

Group I includes 10 genera for which populations were positively correlated with HRT and COD removal efficiency. These genera were *Methanogenium*, *Acetobacteroides*, *Mesotoga*, *Thermovirga*, *Variovorax*, *Aquabacterium*, *Soehngenia*, *Arcobacter*, *Fusibacter*, and *Spirochaeta*. The populations of group I taxa increased as HRT increased, and were associated with high COD removal efficiency and high CH<sub>4</sub> yield.

Group II includes six genera for which populations were negatively correlated with HRT, COD removal efficiency, and CH<sub>4</sub> yield. These genera were *Gelidibacter*, *Chondromyces*, *Phaeodactylibacter*, *Clostridium*, *Mycobacterium*, and *Methanocalculus*, Populations of group II taxa decreased as HRT increased, and were associated with low COD removal efficiency and low CH<sub>4</sub> yield.

## 4. Discussion

The WAS is essentially composed of inactive aerobic cells that are generated by the activated sludge process in MWWTPs [7]. Cellular materials and extracellular polymeric substances resist hydrolytic enzymes, so the efficiency of AD of WAS is relatively low if it is not pretreated [28,29]. The anaerobic bioreactors in this study showed a typical range of COD removal, CH<sub>4</sub> production, and microbial community compositions for AD of WAS. The COD removal efficiencies of R1 and R2 (Figure 2) were comparable to those of sludge AD under mesophilic conditions [13,30,31], but lower than those of sludge AD under thermophilic conditions [3,32]. The higher organic degradation and biogas production by thermophilic digesters compared to mesophilic has been documented in the literature [33,34]. The CH<sub>4</sub> yield generally decreased as the HRT decreased from 25 to 11.5 d (Figure 2b); this result suggests that the higher CH<sub>4</sub> production rate at shorter HRTs was due to increased substrate input rate to the bioreactors. This observation has been reported in previous literature [35–38]; use of a pretreatment [15,29] or a co-substrate [16,39] can increase CH<sub>4</sub> productivity. However, a conventional process (WAS digestion without pretreatment) was used in this study to achieve the objective of investigating microbial community dynamics for AD of WAS. The results of this study could be extrapolated to other sludge AD processes that use additional treatment, secondary substrate, or both [11].

The sequencing analysis clearly showed the compositions of the microbial populations at different taxonomic levels in this study. At the phylum level, Proteobacteria, Bacteroidetes, and Firmicutes were the major members (Figure 3). The dominant abundance of Proteobacteria in the bioreactors is similar to previous studies on sewage sludge and sludge AD [11,13,40], Firmicutes or Bacteroidetes have been reported to be the most abundant phyla in studies on AD of food waste [21,41], distillery wastewater [9], and mixed farm wastes [42].

The dominance of Proteobacteria in activated sludge processes has been reported [43]. This result implies that the abundance of Proteobacteria in the sludge digesters might be linked to its high concentration in the influent. This possibility is supported by the higher abundance of Proteobacteria in the WAS (46.9  $\pm$  3.6%) than in the bioreactors (32.0  $\pm$  2.3%) (Figure 3). While the top three phyla (proteobacteria, bacteroidetes, and firmicutes) were not statistically (*p* < 0.05) more abundant in the WAS than in the bioreactors, or vice versa, the levels of Euryarchaeota, Chloroflexi, Synergistetes, and Thermotogae were significantly (*p* < 0.05) higher in the bioreactors than in the WAS (Figure 3).

Euryarchaeota includes all of the methanogens, which are strict anaerobes that perform methanogenesis, which is the final step of the AD food chain [44]. Chloroflexi has been reported as the major bacterial population in AD of sludge in a large-scale international survey [45]. Synergistetes consists of anaerobic, amino-acid-degrading bacteria that have been reported in many anaerobic digesters [45–47]. Thermotogae includes a mesophilic genus, *Mesotoga*, which has been suggested to be a significant member in AD of sludge [11,48]. Overall, the sequencing analysis identified the core anaerobic communities, as well as potential groups derived from the influent WAS, in sludge AD.

Failure to disrupt cell walls and barriers can leave the influent microbes intact, yet dormant, during the AD of WAS without pretreatment. This phenomenon has resulted in the detection of many inactive WAS-borne microbes in the digesters (Figure 5). The 72 out of 106 genera that were estimated to have negative net growth rates contributed 42.1% of the total reads in the WAS, whereas the other 34 with positive net growth rates contributed 2.8%. Excluding the remaining 55.1% that could not be identified to genus, the proportion of the former and the latter genera were 93.8% and 6.2%, respectively. The relative abundance of the genera with negative and positive net growth rates were 32.2% (65.4% of the sum of the two) and 17.1% (34.6%), respectively, in the bioreactors. These results indicate the prevalence of potentially inactive microbes in the sludge digesters as well as in the influent sludge [49]. These microorganisms were likely being diluted during continuous operation of the process, possibly due to exposure to environments unfavorable for their growth [11]. Many of these organisms are considered aerobic [50–54]; this trait is consistent with their net negative population growth under anaerobic conditions. Interestingly, all the genera identified in the WAS (n = 97) were also detected in the anaerobic bioreactors in this study; this observation indicates that complete washout of a taxon is not easily achieved under the HRT conditions (11.5–25 d) applied in this study.

Among the 34 genera classified as net positive growers in this study (Figure 5), many were documented as active anaerobic taxa in previous studies. For example, all five methanogen genera (*Methanocalculus, Methanogenium, Methanothrix, Methanobacterium,* and *Methanobrevibacter*) identified in this study had positive estimated net growth rates. Syntrophic bacteria such as *Smithella* and *Syntrophus* are also likely to have grown actively in the WAS bioreactors [55,56]. Other examples include *Coprothermobacter* and *Mesotoga*, which are presumably proteolytic bacteria in AD [48,57]. While a significant proportion of the influent microbes was inactive, the WAS digesters had dozens of active genera that can perform anaerobiosis. Among these, nine genera (Figure 5, blue) were non-immigrating active bacteria. To summarize, the influent sludge can affect the microbial populations in sludge digesters by both inoculating it with active taxa and feeding it with inactive taxa [11].

The net growth rates of the taxa in each of groups I and II (Table 2) included both positive and negative values. For example, both *Methanogenium* (group I) and *Methanocalculus* (group II) were identified as net growers in the bioreactors (Figure 5). The identified correlations of group I taxa indicate that the abundances of these organisms likely increased as HRT increased; these trends are orthogonal to their overall growth or decay during AD. For example, an actively-growing taxon could be more competitive, compared to its substrate competitors, either being more competitive at short HRT with high residual substrate concentration (R strategy), or by being more competitive at longer HRT with depleting substrate conditions (K strategy) [58,59]. Considering their correlation to HRT, group I microorganisms are most likely to be K-strategists, whereas group II are most likely to be r-strategists; however, the given sequencing data do not allow a firm conclusion. The co-migrating pattern of HRT, COD removal efficiency, CH<sub>4</sub> production rate, and CH<sub>4</sub> yield, indicates that the lab-scale anaerobic sludge digestion process has shown a conventional pattern of COD removal and CH<sub>4</sub> production, in which substrate-removal efficiency increases as HRT increases.

Methanogen populations were further quantified using real-time PCR (Figure 4). The hydrogenotrophic pathway mediated by Methanomicrobiales was the significant methanogenic pathway during AD of WAS in this study. The dominance of hydrogenotrophic methanogens, especially members within Methanomicrobiales, has been recently documented in commercial-scale sludge digesters [25,60]. However, this trend is controversial because other reports [3,45] have claimed

that Methanosarcinales is the major methanogenic group. In this study, the genera *Methanocalculus* and *Methanogenium* were identified within Methanomicrobiales, the genus *Methanothrix* within Methanosarcinales, and the genera *Methanobacterium* and *Methanobrevibacter* within Methanobacteriales. The real-time PCR and the sequencing data suggest that methanogenesis in these bioreactors was likely mediated mainly by *Methanocalculus* and *Methanogenium*, potentially by the hydrogenotrophic pathway in combination with the activities of their syntrophic partners.

Overall, this study identified bacterial and archaeal groups that were numerically important in the influent and the bioreactors of sludge AD. Based on their numerical patterns (higher or lower in the influent and higher or lower at longer HRT) and their characteristics documented in the literature, some of the microbes could be suggested as incoming inactive microbes and others as core anaerobic microbes in AD. The potential role of the incoming microbes in sludge digestion has been rarely documented [13,45,49], although different scenarios may be developed using microbial ecology theories [61,62]. The results of this study could be used to assess which microbes are potentially active (growing) or inactive (non-growing and dormant), as well as which populations are preferred at longer or shorter HRTs, in the dynamic environment of sludge AD. On the other hand, to improve the COD removal and CH<sub>4</sub> production of sludge AD, a pretreatment unit could be designed to disintegrate the former microbes, and the anaerobic digester can be kept at conditions favorable for the latter. Conventional, high-strength ultrasonication could be applied to the influent sludge for pretreatment, and low-strength ultrasonication could be used to the improve the activities of anaerobic organisms in the AD process [63]. Future study is required to confirm these reported microbial dynamics in WAS AD, and to investigate the effects of different strategies to increase the COD removal and CH<sub>4</sub> production of the process.

## 5. Conclusions

The model WAS digesters were operated under conventional conditions; their COD removal and  $CH_4$  production were within a typical range for mesophilic sludge digesters. Predominant bacteria were Proteobacteria, Bacteroidetes, and Firmicutes in both the WAS and the bioreactors; the major methanogen groups were Methanosarcinales in the WAS and Methanomicrobiales in the bioreactors. Out of the 106 genera identified, the estimated net growth rate was negative for 72 genera and positive for 34 genera. The 72 that had negative estimated growth rates include many aerobic taxa. The 34 that had positive estimated growth rates included methanogens and syntrophs. Abundances of some taxa (with both positive and negative net growth rates) were affected by HRT.

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# Appendix A



**Figure A1.** NMDS ordination plot (**a**) and dendrogram (**b**) for the identified taxa in the samples at the genus level. Bray-Curt.

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