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Exploring Anaerobic Digestion from Mesophilic to Thermophilic Temperatures—Operational and Microbial Aspects

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Abstract: Digesters at water resource recovery facilities (WRRFs) operating at different temperatures within the mesophilic and thermophilic temperature range is a flexibilization concept to contribute to heat management. Four 25 L digesters were fed with sewage sludge from a municipal WRRF and were operated at 37, 43, 47 and 53 °C, respectively, to describe changes in the overall process performance and the microbiota. Specific methane yield and COD degradation rates were the highest at 47 °C, only being up to 7% higher compared with at 37 °C. The increase in pH and concentrations of NH_4-N and PO_4-P above 43 °C were statistically significant. The effect on the microbial community was strong, indicating both a constant specialization towards thermophilic organisms as well as a change from acetoclastic to hydrogenotrophic/methylotrophic methanogenesis. The influence of temperature on process-engineering and physicochemical aspects was rather small compared with the changes in the microbiota.

Keywords: biogas; digester; methanogenesis; microbiome; process stability; sludge water; temperature; 16S rRNA

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1. Introduction

Temperature takes on a decisive role in the operation of digesters at water resource recovery facilities (WRRFs), as it is one of the essential milieu conditions for a stable anaerobic digestion (AD) process influencing both physicochemical parameters and microbiota. Digesters are commonly operated at 35 to 39 °C or 50 to 55 °C in the mesophilic or thermophilic operation modes, respectively [1,2]. These two operation modes have been proposed as the only two alternatives in the context of the identification of optimal temperature levels, process stability, energetic assessments, as well as microbial structure and adaptability [1,2]. However, recent studies show an efficient and stable AD process even at other temperature levels between the mesophilic and thermophilic temperature range for different substrates, e.g., swine manure and liquid cattle manure with wheat straw [3–5], but not for sewage sludge. Digester operation at a temperature between the ranges of classical mesophilic and thermophilic processes might have operational (e.g., enhancement of gas yield and degradation capacity, operating conditions such as hydraulic retention time or organic loading rate), energetic (e.g., seasonal variation in the digester temperature to balance heat deficits and surplus heat) and, thus, ecological and economic advantages.

Contradictory results of mesophilic and thermophilic AD, like specific methane yields and content as well as process stability (partially with different process conditions such as hydraulic retention time (HRT), organic loading rates (OLR) and constancy of the temperature) possibly indicate that neither of the temperature regimes might be ideal. Only

a few publications report constancy of specific methane yields, methane yields and process stability of the AD process of sewage sludge in between mesophilic and thermophilic temperature regimes [6–10].

Temperature influences numerous parameters of chemical, physical and biochemical reactions (in particular gas solubility, dissociation, equilibrium constants, partial pressure of water vapor at saturation, density of wet gas, viscosity and diffusion) [11]. Additionally, a stable AD process requires an active microbial community, whereby the milieu conditions such as temperature, pH, substrate concentration and composition, as well as toxic and inhibitory components, influence the structure of the community and the entire degradation process [12–14]. Temperature's influence on the biological processes is shaped by both the activity and growth of different groups of microorganisms and the enzyme activities, enzyme reactions and substrate diffusion rates which might differently respond to chosen temperature levels [15].

Species richness and diversity of the microbiota reflect the process stability and flexibility of AD, with higher species diversity in mesophilic than in thermophilic conditions [16,17]. However, the process stability can also be described by the parameter gas production, methane content, concentrations of volatile fatty acids (VFAs) and the ratio of VFAs to alkalinity.

The microbial structure changes with temperature owing to different specific growth optima concerning temperature and/or associated growth conditions. Fundamentally, it is assumed that the growth rate, and thus the degradation rates, of the microorganisms at thermophilic temperatures are two to three times higher than at mesophilic conditions [18].

Structural differences of the biocenosis were attributed to process temperature by several authors for various substrates [3,17,19,20], but not for sewage sludge.

In mesophilic digesters, representatives of bacterial phyla Firmicutes, Proteobacteria, Bacteriodetes and Chloroflexi [21–23], and in thermophilic digesters, Firmicutes, Proteobacteria, Chloroflexi and Actinobacteria, often dominate among the bacteria [23,24].

Microorganisms producing methane are all among the archaea and are obligate anaerobes. Depending on temperature, methane is either produced by acetoclastic methanogens (especially *Methanosarcina* and *Methanothrix/Methanosaeta*), even though most known methanogens convert CO_2/H_2 (hydrogenotrophic methanogenesis), or methyl compounds (methylotrophic methanogenesis) [16]. However, both acetoclastic and hydrogenotrophic/methylotrophic methanogens are essential for a stable AD process [25]. In digesters, methane-forming archaea of the order Methanosarcinales are typically found, with *Methansaeta* mainly appearing in mesophilic and *Methanosarcina* in thermophilic systems [26,27]. In contrast to mesophilic temperatures, hydrogenotrophic methanogenesis dominates at thermophilic temperature levels [5,7,20,28], where archaea of the orders Methanobacteriales (family: Methanobacteriaceae, genus: *Methanothermobacter*) and Methanomicrobiales (family: Methanomictobiaceae, genus: *Methanoculleus*) often prevail [27,29]. Nevertheless, depending on the environmental conditions (e.g., high pH and/or ammonium content), acetoclastic methanogens may even dominate at thermophilic temperatures [30,31]. Usually, small numbers of thermophilic methanogens are already present in mesophilic sludge and become dominant when switching to higher temperatures [18,32,33]. It can be assumed that this is also the case vice versa and that mesophilic methanogens can survive thermophilic conditions but do not dominate. However, there are different views on this, and [32], for example, stated that no mesophilic bacteria are present in thermophilic sludge.

The investigations in this article are an essential part of the research in the operating concept 'digester as heat storage', in which the digester temperature fluctuates within mesophilic and thermophilic temperatures during the year, as described in [34]. Thus, this article aims to describe the AD process for digesters fed with sewage sludge from a municipal WRRF in Germany at temperature levels between 37 and 53 °C in the context of the process performance, process stability and microbial community structure.

2. Materials and Methods

2.1. Substrate

The substrate fed to the lab digesters was a 1:2 mixture (*w*/*w*) of primary and secondary sludge (PS: SS) from a WRRF in Germany, consisting of mechanical and biological wastewater treatment serving 50,000 population equivalents (PEs). Mixtures of substrate were frozen and thawed before feeding. Digesters were fed six days a week once a day. Each batch of the substrate mixture was fed for at least one HRT. The characteristics of the fed mixtures of sewage sludge are shown in Table 1.

Table 1. Characteristics of two feeding periods and the fed substrate (TS: total solids, TVS: total volatile solids, COD_T: total chemical oxygen demand, TKN: total Kjeldahl-nitrogen, HRT: hydraulic retention time, PS: SS: mixture of primary and secondary sludge).

Aspect	Parameter	Unit	Period I (PS: SS #1)	Period II (PS: SS #2)
Digester operation	HRT	d	20	20
	Temperature	°C	37, 43, 47, 53	37, 43, 47, 53
Substrate	Feeding period	d	27	75
	TS	%	5.4 ± 0.1	4.5 ± 0.1
	TVS	%	81.0 ± 2.0	84.0 ± 2.0
	COD_T	mg/L	60,800 ± 2430	52,800 ± 2110
	COD_T/TS	g COD/kg TS	1126 ± 55	1173 ± 59
	TKN	mg/L	2730 ± 136	2290 ± 115
	TKN/TS	g TKN/kg TS	51 ± 3	51 ± 3

2.2. Design and Operation of Lab Digesters

Four identical digesters with a working volume of 25 L were operated in parallel, one each at 37, 43, 47 and 53 °C, to cover the range of digester temperatures required for seasonal variation to balance phases of heat deficits in winter and surplus heat in summer [35]. The HRT was 20 days in each digester. The inoculum was sampled from the same WRRF as the mixtures of PS: SS. The lab digesters were continuously stirred at 20 rpm. A schematic diagram of the lab digesters is shown in Figure 1. Online measurement of gas quantity and pH was integrated.

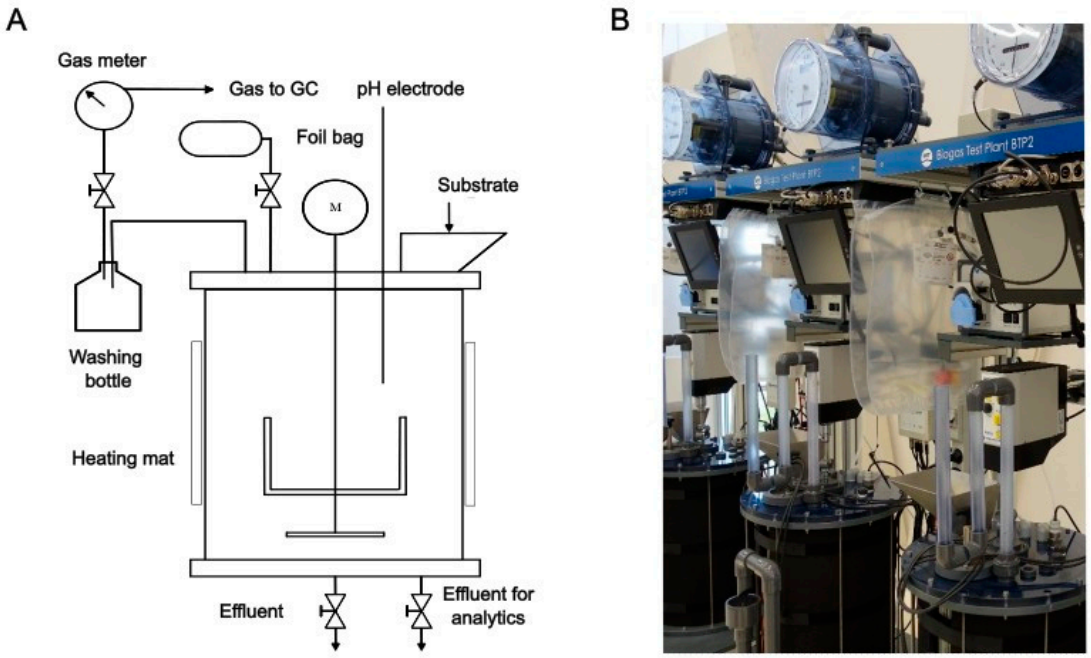

Figure 1. Schematic diagram (**A**) and photo of the lab digesters (**B**).

More information about start-up and digester operation can be found in a previous article by [10]. However, digesters were operated at each temperature level for more than two years before the data evaluation and sampling presented here. Thus, steady-state conditions and acclimatization to each temperature level are assumed.

2.3. Analysis

2.3.1. Operation of the Lab Digesters

Substrates were characterized by total solids (TS), total volatile solids (TVS), total chemical oxygen demand (COD_T) and total Kjeldahl-nitrogen (TKN)). Gas quantity and pH were continuously measured, while TS and TVS in the effluent were determined daily. COD was analyzed using cell tests (Spectroquant, Merck, Darmstadt, Germany) complying with the recommendations of Schaum et al. (2016). Soluble chemical oxygen demand (COD_S), ammonium (NH_4-N) and phosphate (PO_4-P) were determined after 0.45 µm filtration. Concentrations of NH_4-N and PO_4-P were measured with a continuous flow analyzer (CFA, Bran + Luebbe Auto Analyzer III, Norderstedt, Germany).

The concentrations of organic acids (single acids from C2 to C6) were determined as volatile fatty acids (VFAs) after 0.45 µm filtration by using gas chromatography (GC; Agilent Technologies 6890N; capillary column Agilent J&W HP-FFAP, Santa Clara, CA, USA). Concentrations of total and COD_T, COD_S, NH_4-N, PO_4-P and organic acids were measured once a week.

Additionally, concentrations of organic acids were determined as volatile fatty acids (FOS) using titration (TitraLab AT1000 Series; Hach Lange GmbH, Düsseldorf, Germany) using sulfuric acid (0.1 N) after centrifugation of the samples for 15 min at 12,500 rpm. The concentrations of total alkalinity (TAC) were measured with the same sample preparation and titrator as used for the determination of FOS. Determination of concentrations of FOS and TAC took place at the end of periods I and II, while VFA concentrations were measured every week.

The gas quantity was measured with an online gas meter (Ritter TG 0.5, Bochum, Germany) as accumulated values every 15 min. Gas quantities were normalized to standard temperature (273 K) and pressure (1013 hPa) and were corrected with the Magnus formula as recommended in [36]. The gas composition was determined once a week using a micro GC (Agilent Technologies 490 Micro GC, Santa Clara, CA, USA).

2.3.2. DNA Extraction

Samples for the microbiological analysis were taken at three different time points close to the end of feeding period II: day 65, day 70 and day 72. DNA was extracted out of frozen samples using a NucleoSpin Soil DNA extraction kit (Macherey-Nagel, Düren, Germany) and following the kit's instruction manual, with these modifications: After thawing, samples were centrifuged for 10 min at 10,000× *g* and the supernatant was partially removed. The pellets were then dissolved in the remaining supernatant to increase the cell density, and 400 mg of these mixtures was transferred into Bead Tubes. After adding SL1 buffer, samples were homogenized at room temperature using a FastPrep-24 machine (MP Biomedicals, Irvine, CA, USA) and following the suggested settings (5 m/s, 30 s). In course of contaminant precipitation, samples were incubated for 15 min at 4 °C and centrifuged for 3 min at 11,000× *g*. Finally, DNA was eluted in 40 µL PCR-grade water (Rotipuran Low Organic Water, Roth, Karlsruhe, Germany) and stored at −20 °C in low-binding tubes (Eppendorf, Hamburg, Germany). The success of the DNA extraction was proven with gel electrophoresis (10 min, 100 V) on a 1% (*w/v*) agarose gel and DNA concentrations were determined using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Quantus system (Promega, Fitchburg, WI, USA).

2.3.3. Amplicon Sequencing

Next-generation sequencing was performed by the Microsynth (Balgach, Switzerland) on an Illumina MiSeq machine (San Diego, CA, USA). A universal primer pair was used to

target the archaeal and bacterial V4 region of the 16S rRNA with a 2 × 250 bp approach, 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806r (5′-GGACTACHVGGGTWTCTAAT-3′), as suggested by [37]. Raw data were then analyzed with CoMA [38].

First, paired-end reads were assembled and linker- as well as primer sequences were truncated. During quality filtering, reads with a mean Phred quality <30, a length deviation >5% compared with the median read length and any unambiguous bases (N) were excluded from further analysis. Amplicon sequence variants (ASVs) were assigned with DADA2 and the taxonomic assignment was conducted with the BLAST algorithm using SILVA SSU (v. 138) as the primary and Greengenes (v. 13_5) as the backup database. Finally, rare ASVs with <5 hits within all samples were removed.

2.4. Calculations

2.4.1. COD Balances

Balances of COD for the lab digesters were used to determine specific methane yields, methane contents and COD degradation rates for each feeding period following the procedure described by [35]. It considers a load of COD of the substrate (COD_{input}), the load of COD in the digested sludge ($COD_{effluent}$), the load of degraded COD calculated by subtraction of COD_{input} and $COD_{effluent}$ ($COD_{degraded}$) and, for plausibility check, a load of COD resulting in biogas (COD_{biogas}). Taking into consideration that up to 10% of COD was used for the formation of new biomass, for calculation of COD_{biogas} it was assumed that 1 kg degraded COD equals 320 L_N CH_4, as the COD balance was based on added COD and produced methane [36]. For the COD balances, the 1st to 27th day of period I and the 1st to 75th day of period II were evaluated.

2.4.2. Statistical Analyses

Statistical analyses were performed for the methane content and physicochemical parameters to determine significant differences between the means of each parameter at 37, 43, 47 and 53 °C. Other parameters (e.g., specific methane yields, COD degradation rates and OLR) were based on COD balances, so no statistical analysis could be performed. All statistical analyses were carried out at the 95% confidence level (a = 0.05); thus, significance was determined at $p \leq 0.05$.

Significance tests were selected for each parameter after prior testing of normal distribution (modified Kolmogorov–Smirnov test and the Shapiro–Wilk test) and variance homogeneity (Levene test). In the case that there is no normal distribution, the Kruskal–Wallis test was selected which does not require further testing for variance homogeneity. In the case of normal distribution, single-factor analysis of variance (ANOVA) was performed if variance homogeneity was applicable, and Welch ANOVA if variance homogeneity was not applicable. IBM SPSS Statistics 25 statistical software was used to perform the significance tests.

2.4.3. Microbiome Analyses

Shannon–Wiener diversity (H′) and Pielou's evenness (J′) as indicators for alpha diversity were calculated based on ASVs with Equations (1) and (2), respectively:

$$H' = -\sum_{i=1}^{R} p_i \times \log_2 p_i \quad (1)$$

$$J' = \frac{H'}{H'_{max}} \quad (2)$$

where p_i stands for the frequency of reads for each ASV and R for the ASV count. H_{max} represents the maximum H′ and can be calculated by $\log_2(R)$. The significance of the alpha diversity results was tested with a Kruskal–Wallis test, followed by a Conover post hoc test with Benjamini–Hochberg correction. For beta diversity, a principal coordinates analysis (PCoA) based on weighted UNIFRAC distance was computed, and results were statistically

tested using ANOSIM and PERMANOVA with 1000 permutations each. Graphics were created with in-house Python scripts. For the taxonomic plots, families/genera with an abundance <1% were ignored. For all analyses, significance was determined at $p \leq 0.05$.

3. Results and Discussion

3.1. Performance of the Lab Digesters

3.1.1. COD Balances of Lab Digesters

Figure 2 shows the results of the COD balance for each lab digester operated at 37, 43, 47 and 53 °C. The load of COD_{input} was almost similar for each digester within each period. For both periods, the load of degraded COD was almost identical, while the load of COD in the biogas differed within each period. The comparison of $COD_{degraded}$ and COD_{biogas} shows deviations below 15% for both periods (cf. Figure 2).

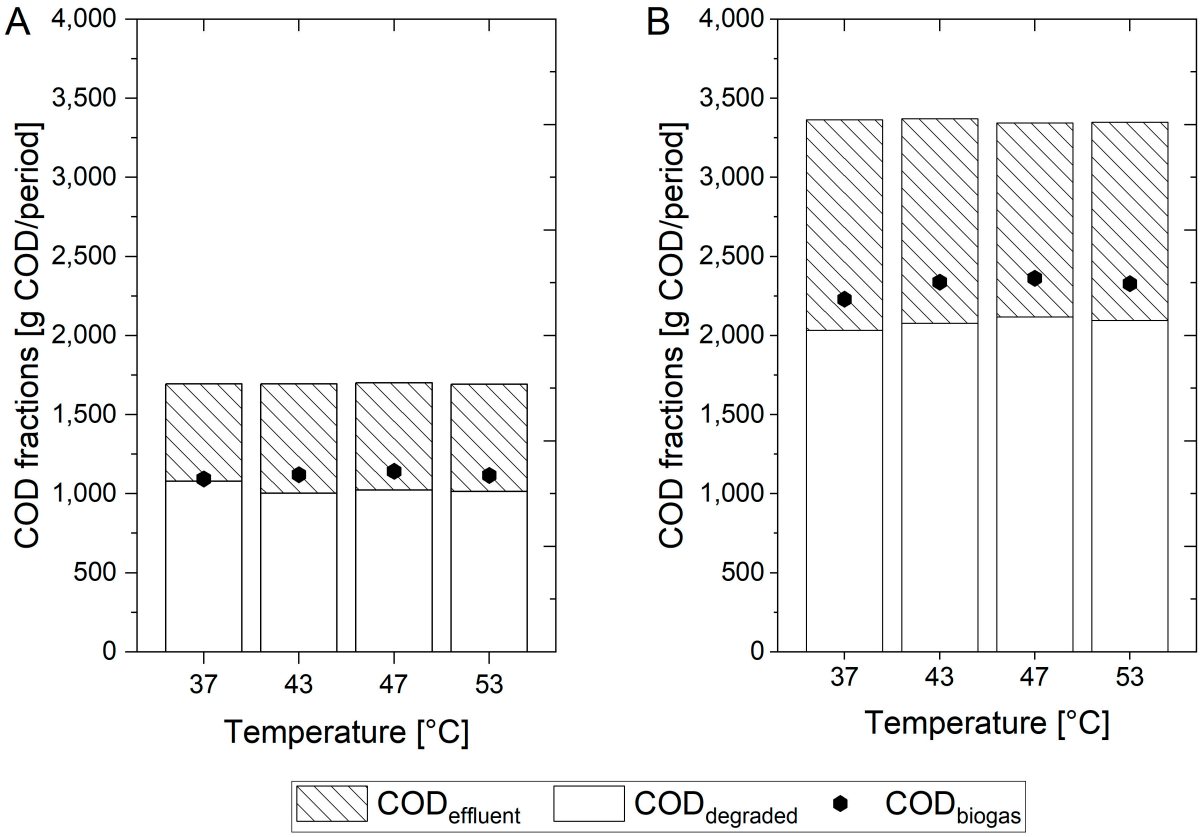

Figure 2. Chemical oxygen demand (COD) balance of lab digesters during period I, with a feeding period of 27 days (**A**), and period II, with 75 days (**B**). The sum of $COD_{effluent}$ and $COD_{degraded}$ equals $COD_{influent}$.

From the COD balances, specific methane yields, COD degradation rates and OLR were calculated to describe the performance of the lab digesters. In Table 2, characteristic parameters from the COD balances for the lab digesters operated at 37, 43, 47 and 53 °C are summarized for periods I and II.

The experiments were conducted at an HRT of 20 days and with an OLR around 1.6 g $COD_{degraded}$/(L·d) in both periods. Specific methane yields varied between 207 and 215 L_N CH_4/kg COD_{added} resp. 325 and 358 L_N CH_4/kg $COD_{degraded}$ in period I and between 212 and 226 L_N CH_4/kg COD_{added} resp. 351 and 360 L_N CH_4/kg $COD_{degraded}$ in period II. In total, the specific methane yields remained almost at a constant level. The methane content varied between 61.0 and 61.5% in period I and between 61.6 and 62.6% in period II. The methane content showed statistically significant differences between 37 and 53 °C, 43 and 47 °C as well as 47 and 53 °C in period I ($p \leq 0.05$). In period II, significance analyses

indicated statistically significant differences for 47 °C in comparison with 37, 43 and 53 °C, as well as between 37 and 53 °C.

Table 2. Summary of relevant data of the COD balances of periods I and II in lab digesters.

Period	Parameter	Unit	Temperature in °C			
			37	43	47	53
I	specific methane yield	L_N CH_4/kg COD_{added}	207	212	215	211
		L_N CH_4/kg $COD_{degraded}$	325	358	357	352
	methane content [(a)]	%	61.2	61.4	61.0	61.5
	COD degradation rate	%	64.1	62.6	63.6	63.0
	OLR	g COD_{deg}/(L·d)	1.7	1.5	1.6	1.6
	HRT	d	20	20	20	20
II	specific methane yield	L_N CH_4/kg COD_{added}	212	222	226	223
		L_N CH_4/kg $COD_{degraded}$	351	360	357	356
	methane content [(a)]	%	62.6	62.1	61.6	62.1
	COD degradation rate	%	63.4	65.5	67.0	66.1
	OLR	g $COD_{degraded}$/(L·d)	1.5	1.5	1.5	1.5
	HRT	d	20	20	20	20

[(a)] period I: 61.2 ± 0.6%, 61.4 ± 0.6%, 61.0 ± 0.5% and 61.5 ± 0.4% (for 37, 43, 47 and 53 °C); period II: 62.6 ± 1.6%, 62.1 ± 0.9%, 61.6 ± 0.7% and 62.1 ± 0.8% (for 37, 43, 47 and 53 °C).

The COD degradation rates varied between 62.6 and 64.1% in period I and between 63.4 and 67.0% in period II, with the highest values at 47 °C. Values of specific methane yields, methane contents and COD degradation rates correspond with values reported in the literature [1,2,39] and complement the previously published data in [10].

However, specific methane yields, methane contents and the COD degradation rates of the process data initially indicate both a stable degradation process and no prominent temperature dependence on the overall performance of the lab digesters up to 1.6 g $COD_{degraded}$/(L·d) and an HRT of 20 days at 37, 43, 47 and 53 °C.

3.1.2. Physicochemical Parameters

Temperature influences the AD process in chemical, physical and biochemical reactions. The process quality is described in detail with the physicochemical parameters COD_T, TS, TVS and pH measured in the digested sludge as well as organic acids, COD_S, NH_4-N and PO_4-P measured in the sludge water (cf. Figure 3).

Means of COD_T remained between 909 and 933 mg/g TS in period I and between 853 and 965 mg/g TS in period II. The COD_T in period I showed no statistically significant difference between the temperature levels ($p > 0.05$). In period II, COD_T at 53 °C was significantly higher compared with 37 °C.

The means of TS declined with increasing temperature from 3.2% at 37 °C to 2.9% at 53 °C in period I and from 2.7% at 37 °C to 2.6% at 53 °C in period II. Means of TVS varied between 63.4% at 37 °C and 62.1% at 47 °C in period I and between 64.9% at 37 °C and 64.0% at 47 °C in period II. The TS in period I showed statistically significant differences between each temperature level, except comparing 47 and 53 °C ($p > 0.05$). In period II, there were no statistically significant differences ($p > 0.05$). Additionally, the TVS in periods I and II showed statistically significant differences ($p \leq 0.05$ between 37 and 43 °C, 47 and 53 °C in period I; $p \leq 0.05$ between 37 and 47 °C, 37 and 53 °C in period II). The pH increased with the digester temperature from means of 7.3 at 37 °C to 7.6 at 53 °C in period I and from 7.2 at 37 °C to 7.5 at 53 °C. The increase in the pH can be attributed to a shift in the equilibrium between ammonium and ammonia as well as between carbonate and hydrogen-carbonate with increasing temperature [2,11]. The increase in the pH with the digester temperature was statistically significant above 43 °C ($p > 0.05$ between 37 and 43 °C).

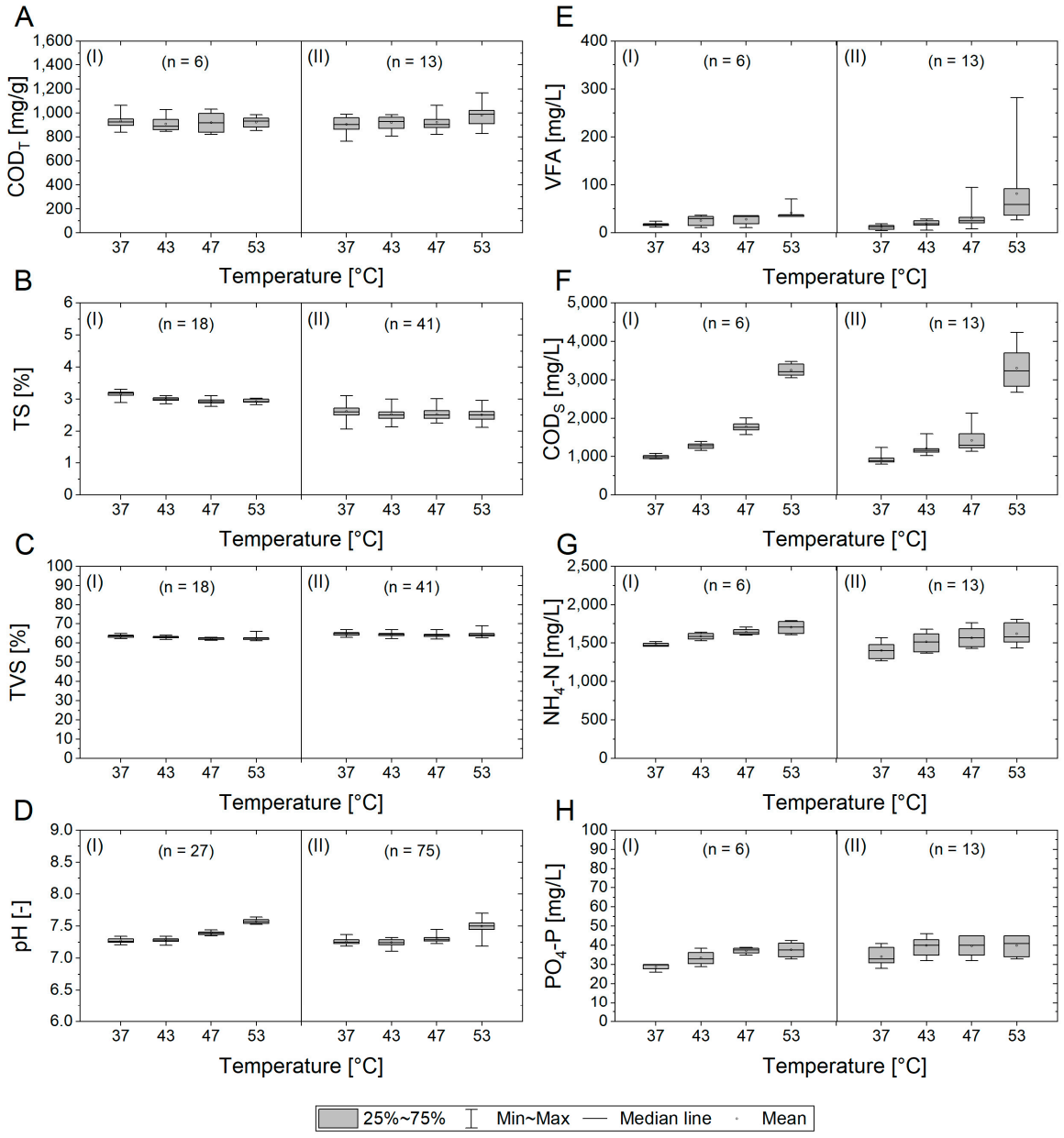

Figure 3. Physicochemical parameters measured in the digested sludge (**A**–**D**) and the sludge water (after 0.45 µm filtration) (**E**–**H**) for periods I and II (n = amount of data for each temperature level) (COD_S: soluble chemical oxygen demand, COD_T: total chemical oxygen demand).

The parameters VFA, COD_S, NH_4-N and PO_4-P were used to classify the dissolved substances in the sludge water. With increasing digester temperatures, these parameters increased to varying degrees. The means of VFA concentration increased from means of 16 mg/L at 37 °C to 42 mg/L at 53 °C in period I and from 12 mg/L at 37 °C to 115 mg/L at 53 °C in period II. At each temperature, VFA concentrations were below 200 mg/L, indicating a stable AD process [40]. An increase in VFAs with the digester temperature agrees with earlier publications on anaerobic sewage sludge digestion [41,42], and may occur due to increased hydrolysis and slowed methanogenesis [43]. which Statistically significant differences only occurred between 53 °C and the other temperature levels in both periods ($p \leq 0.05$). The means of COD_S increased by a factor up to 3.6 from 995 mg/L at 37 °C to 3250 mg/L at 53 °C in period I and from 926 mg/L at 37 °C to 3310 mg/L at 53 °C in period II. As already shown for the VFA concentration, an increase in COD_S is also partially attributed to an increase in soluble proteins and organic acids due to increased

hydrolysis or slowed methanogenesis [43], as well as to the destruction of sludge flocs at higher temperatures [44]. The increase in COD_S with the temperature was statistically significant for period I ($p \leq 0.05$). In period II, COD_S were significantly different at all temperature levels, except between 37 and 43 °C as well as 43 and 47 °C.

Additionally, means of NH_4-N increased in both periods from 1478 mg/L at 37 °C to 1703 mg/L at 53 °C resp. from 1402 mg/L at 37 °C to 1620 mg/L at 53 °C, which is an increase of up to 15%. The increase in the concentration of NH_4-N can be caused by the increased degradation of proteins. Irrespective of temperature and period, NH_4-N remained below 1700 mg/L, above which an initial inhibition by free ammonia is expected [45]. Significance analyses showed statistically significant differences of NH_4-N between 37 and 47 °C as well as 37 and 53 °C in both periods ($p \leq 0.05$). The mean concentrations of PO_4-P varied at a low level between 29 mg/L at 37 °C and 38 mg/L at 53 °C in period I and between 34 mg/L at 37 °C and 40 mg/L at 53 °C in period II. There only were statistically significant differences of PO_4-P between 37 and 47 °C as well as 37 and 53 °C in both periods ($p \leq 0.05$). The increase in PO_4-P at higher temperatures can be traced back to the increased redissolution of phosphate under anaerobic conditions and has been reported before when comparing mesophilic with thermophilic temperatures at an HRT between 20 and 30 days [46,47]. The increase in pH and the concentration of soluble components can raise the potential of struvite precipitation at higher temperatures. However, [48] showed slightly lower phosphorous, magnesium and calcium precipitation at thermophilic temperatures in comparison with mesophilic temperatures for digesters operated at an HRT of 20 days.

The additional parameters can be used to identify the temperature influence on both the physicochemical and microbiological aspects of the AD process. In total, the influence of the temperature is rather small, still indicating a stable degradation process.

3.1.3. Organic Acids and Alkalinity as Indicators for Process Stability

The quantification and characterization of organic acids (FOS/TAC via titration as well as VFAs via GC) enables a closer look into the temperature dependence of the intersection of acetogenesis and methanogenesis. The mean VFA concentration increased with the digester temperature, as did FOS, TAC and the FOS/TAC ratio (cf. Table 3).

Table 3. Concentrations of FOS, TAC and VFAs as well as the FOS/TAC ratio for each temperature level in lab digesters (FOS: volatile fatty acids measured with titration, TAC: total alkalinity, VFAs: volatile fatty acids measured with gas chromatography after 0.45 µm filtration).

Period	Parameter	Unit	Temperature [°C]			
			37	43	47	53
I	VFAs [a]	mg/L	16 ± 3	26 ± 9	29 ± 7	42 ± 16
	FOS [b]	mg/L	450	614	651	792
	TAC [b]	mg/L	4340	5040	5360	5170
	FOS/TAC [b]	-	0.10	0.12	0.12	0.15

[a] measured with GC. [b] measured with titration

Higher concentrations of organic acids with increasing digester temperatures have been published before by [41,42], indicating enhanced hydrolysis or slowed methanogenesis [43]. However, concentrations of acetic acid equivalent below 300 or 500 mg/L indicate a well-established AD process [1,40]. This complies with the GC measurements, but concentrations of VFAs slightly exceed these values at temperatures of 43, 47 and 53 °C in period I. However, the concentrations of organic acids measured via titration are influenced by cross-sensitivities with other buffer systems, showing generally higher levels in comparison with those measured with GC (sum of single acids C2 and C6).

Furthermore, concentrations of TAC were slightly above values between 2500 and 5000 mg/L, usually indicating well-established digesters [40]. Higher alkalinity at thermophilic temperature levels has been reported before and can be traced back to the degra-

dation of nitrogenous organic compounds, sulfate reduction, release of orthophosphate and an increase in VFAs [49,50].

The FOS/TAC ratios were below 0.2 at each temperature level, indicating that the AD process was not overloaded [51]. A slight increase with increasing digester temperatures was observed before [7,44]. This agrees well with the OLR in the lab digesters (around 1.6 and 1.5 g $COD_{degraded}$/(L·d) in periods I and II, respectively) being in the lower range of the recommended OLR for large-scale digesters at WRRFs up to 100,000 PE, according to [1].

In total, considering the concentrations of VFAs, TAC and FOS/TAC, a stable degradation process at each temperature level is proven, especially in combination with the results of specific methane yields, methane contents and COD degradation rates.

3.2. Microbial Community Analysis

3.2.1. Microbial Diversity

Looking at the microbial communities, Shannon–Wiener diversity ($p = 0.02$), Pielou's evenness ($p = 0.02$) and ASV richness ($p = 0.02$) clearly decreased with temperature (cf. Figure 4A–C).

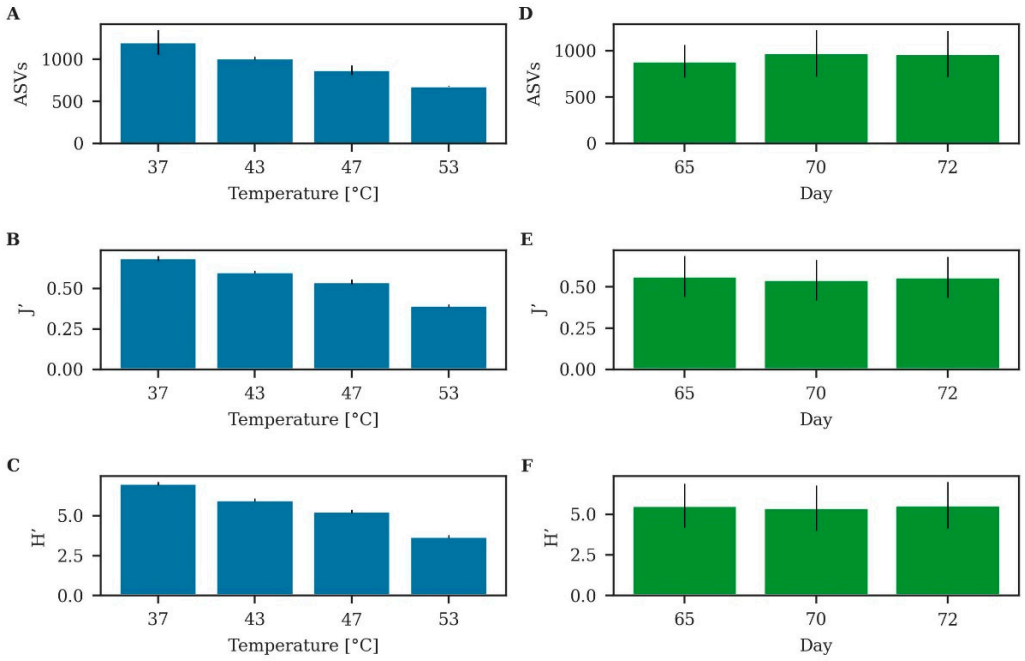

Figure 4. ASV richness (**A**,**D**), Pielou's evenness J′ (**B**,**E**) and Shannon–Wiener diversity H′ (**C**,**F**) in lab-scale biogas reactors operated at four different temperatures and sampled at three different time points. Calculations are based on 16S rRNA amplicon sequencing data. Bars indicate means ± standard deviation. (ASV: amplicon sequence variant).

This is in line with several other studies, where thermophilic communities were always less diverse compared with mesophilic ones [52,53]. The range between mesophilic and thermophilic temperatures represents a transition phase and is characterized by thermo-flexible mesophilic and thermophilic organisms, and by taxa specifically thriving at this temperature [20]. In the presented study, alpha diversity measures were higher at 43 °C and 47 °C than at 53 °C, but lower than at 37 °C, indicating a specialized but still versatile microbial community at these temperatures. Some degree of specialization is important to allow for an efficient substrate conversion into biogas under the given environmental conditions. On the other hand, diversity makes the microbiota resilient to disturbances such as temperature fluctuations due to the diverse growth optima of species with redundant functions [54,55]. Even though Pielou's evenness steadily decreased with temperature,

the drop from 47 °C (J′ = 0.54) to 53 °C (J′ = 0.39) was remarkable and indicated that only a small number of highly abundant taxa dominated at thermophilic conditions, while the abundance of minor taxa decreased significantly. This is particularly interesting since microbial diversity and evenness can be seen as an indicator for process stability, although specific methane yield, methane content, VFA and FOS/TAC analysis show process stability at each temperature level.

In contrast to temperature, time did not show any influence on alpha diversity, and neither Shannon–Wiener diversity ($p = 0.79$) nor Pielou's evenness ($p = 0.74$) or ASV richness ($p = 0.93$) were affected (cf. Figure 4D–F). This was expected, since the three sampling points all laid within a single week and no fundamental changes in the microbial community were expected in such a short period. In addition, the experiment already ran over three HRT at this point, a duration after which a stable microbiota can be assumed [12].

The conclusions drawn from alpha diversity analyses were confirmed with ordination analysis. Samples clearly clustered together in a PCoA based on weighted UNIFRAC distance depending on temperature (ANOSIM: $p = 0.001$, PERMANOVA: $p = 0.001$) and irrespective of the sampling time (ANOSIM: $p = 0.96$, PERMANOVA: $p = 0.095$) (cf. Figure 5). Interestingly, samples digested at 37 °C were the most distant from all other temperatures with respect to the *x*-axis, explaining 69.1% of the total variation. For the separation of the remaining three temperatures, on the other hand, mainly the *y*-axis (25.0%) was decisive. In total, 94.1% of the variation could be explained. Within each temperature, sample points were located in close proximity, again indicating that changes in the microbial communities over the study period were negligible and only the different temperatures evoked changes.

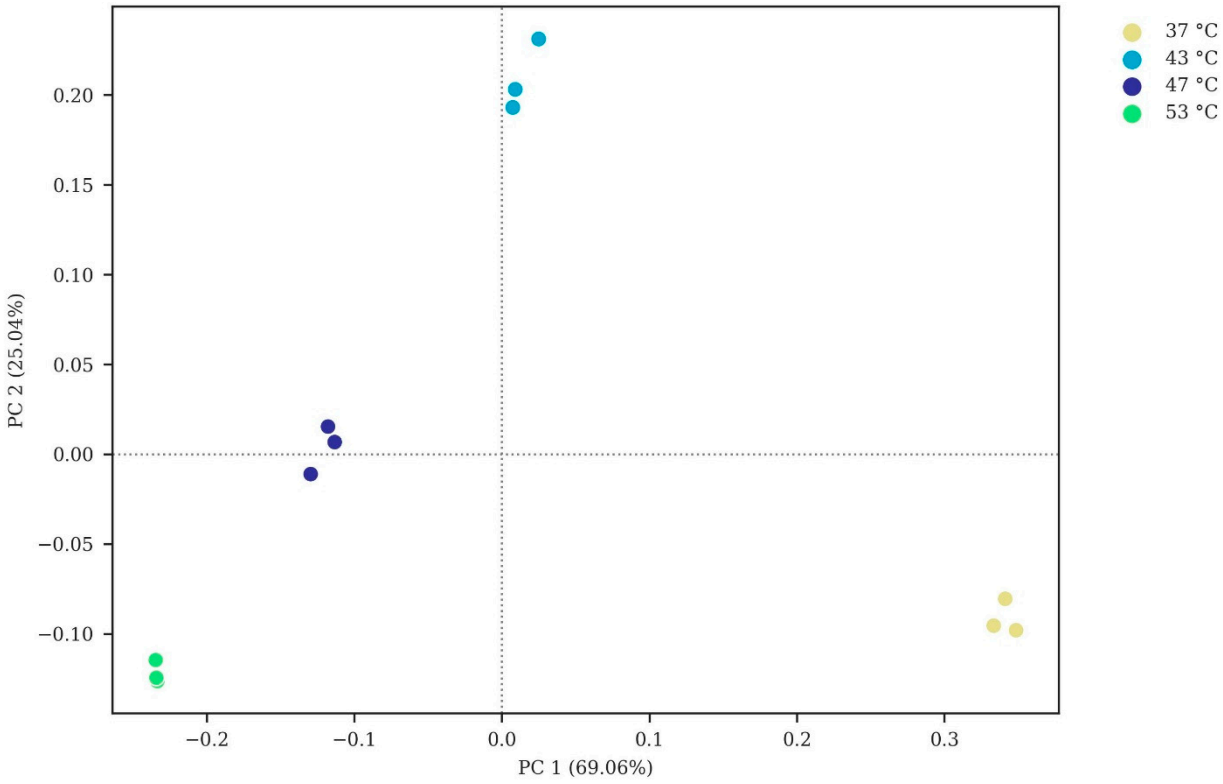

Figure 5. Principal coordinates analysis showing the difference/similarity in microbial communities in lab-scale biogas reactors operated at 37, 43, 47 and 53 °C. Samples were collected after 65, 70 and 72 days of operation. (PC: principal coordinate). Both axes explained 94.1% of the total variation.

3.2.2. Microbial Community Composition

The above observation was further strengthened when comparing the presence/absence of taxa in different samples. Ninety-four percent of all detected families (Figure S1) and ninety-three percent of all detected genera (Figure S2) were found irrespective of the

sampling time. This is a clear hint for systems being equilibrated and supports former studies reporting that a stabilization of the microbial community can be expected after two to three HRTs [12,20]. When comparing process temperatures, in turn, only 67% of all families (Figure S3) and 62% of all genera (Figure S4) were found in all treatments, clearly demonstrating the temperature-driven adaptations of the microbiota that were already assumed after alpha- and beta diversity analyses. However, one should not imagine a clear demarcation between the examined temperatures; rather, fluent transitions were observed. By far, the most common families were shared between 37 and 43 °C (85%), followed by 43 and 47 °C (77%) and 37 and 47 °C (77%). The smallest overlap was observed for the 53 °C samples, sharing only 71–72% of all detected families with each of the other three temperatures. This indicates that microbial communities at 43 and 47 °C were much more closely related to those at 37 °C, and, hence, a classical mesophilic microbiota, rather than to those at 53 °C, representing thermophilic conditions. Only 2% of all families were found exclusively at 43 and 47 °C, and neither under mesophilic conditions nor under thermophilic conditions. These results thus confirm the frequently held assumption that microbial communities in this temperature range are merely a mixture of mesophilic and thermophilic organisms, with only a few exceptions [56].

When checking the collected reads against the taxonomic databases, it turned out that 6% and 4% of all families at 37 and 53 °C, respectively, could not be assigned. At the other two temperatures, allocation rates were even lower and 9% of all families could not be assigned. This indicates that the temperature range in between is understudied and not as well-covered as mesophilic and thermophilic conditions in conventional databases such as SILVA and Greengenes, and that several characteristic taxa are not yet included since the databases so far are biased towards classical meso- and thermophilic process conditions.

3.2.3. Bacterial Taxonomy

Indicator organisms are taxa that are either found exclusively or at least at a much higher rate in the investigated sample or sample group. This was the case for Bacteroidetes vadinHA17 (12%), Prolixibacteraceae (6%), Peptostreptococcaceae (5%) and Sedimentibacteraceae (5%) at 37 °C (cf. Figure 6). All these families are well-known inhabitants of mesophilic digesters [57–59], covering three of the four main steps of AD. While Prolixibacteraceae is mainly involved in the initial hydrolysis [60], Bacteroidetes vadinHA17 has been described to decompose organic material further down to acetic acid, propionate and hydrogen/carbon dioxide [61]. Peptostreptococcaceae belongs to the group of syntrophic acetate-oxidizing bacteria (SAOB) [62] and is tightly connected to hydrogenotrophic methanogens. The most abundant family at 37 °C, however, was Anaerolineaceae (16%), which is known to be involved in the hydrolysis of polysaccharides [63]. Despite its frequent appearance, it was not characteristic for 37 °C since it also appeared at the other investigated temperatures. While it was still considerably abundant at 43 °C (11%), numbers significantly decreased and ended up at an abundance <0.1% at 53 °C. This is consistent with a study by Hupfauf, Plattner, Wagner, Kaufmann, Insam and Podmirseg [20], in which Anaerolineaceae was found in a temperature range of 10 °C and 55 °C for bioreactors fed with a mixture of cattle slurry and ground maize straw. However, in contrast to the present study, bacterial counts were not affected by temperature and similar abundances were reported over the whole investigated temperature range.

In addition to Anaerolineaceae, Synergistaceae (17%) and Caldatribacteriaceae (8%) were most abundant at 43 °C, both being well-known members of SAOB [64,65]. Their frequent occurrence at this temperature in the present study was associated with a simultaneous shift among methanogens, where acetoclastic methanogenesis was replaced by hydrogenotrophic methanogenesis. Since both families have been frequently found in mesophilic AD systems [63,66] and no strong temperature-specificity is expected, it can be assumed that their occurrence at 43 °C (and also at 47 and 53 °C) was mainly driven by their syntrophic association with methanogens rather than by the process temperature. This will be discussed in more detail later in the discussion of Archaea. Generally, Synergistaceae

were similarly abundant at temperatures beyond 37 °C and no correlation with temperature was observed. Different was the situation for Caldatribacteriaceae, where the abundances significantly increased with temperature (47 °C: 20%, 53 °C: 24%).

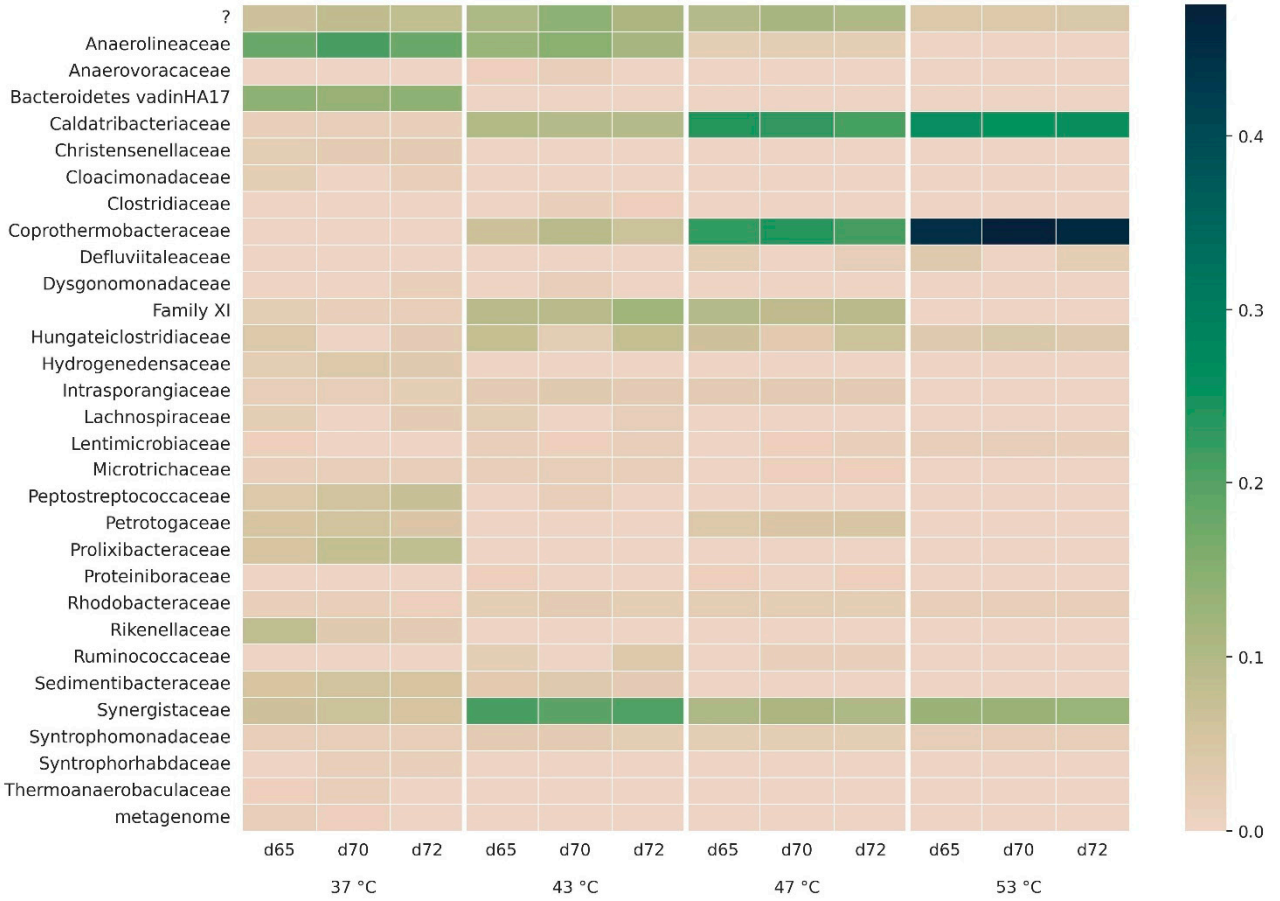

Figure 6. Bacterial families detected in biogas reactors operated at four different temperatures after 65, 70 and 72 days. The color code indicates the relative abundance within each sample. Families with an abundance <1% were excluded.

For lab digesters operated at 43 and 47 °C, Family XI (Clostridiales) was revealed as the indicator family. While 9% and 8% of all families at 43 and 47 °C belonged to this group, respectively, only very small abundances were detected at 37 (1%) and 53 °C (0.1%). Members of these families, like *Anaerococcus*, *Ezakiella*, *Gallicola*, *Murdochiella* and *Peptoniphilus*, are proteolytic and can produce lactic acid from peptones and amino acids [67]. As with Caldatribacteriaceae, counts of Coprothermobacteraceae increased with temperature. However, Coprothermobacteraceae was only found at 47 (20%) and 53 °C (43%), but not at lower temperatures. This is in line with previous studies, detecting the family in thermophilic- but not at mesophilic conditions [68,69] and corresponds well with [70], who reported growth until 75 °C with an optimum at 63 °C. While originally being assigned to Firmicutes, Coprothermobacteraceae now belongs to the new phylum Coprothermobacterota, which was introduced in 2018 [71]. *Coprothermobacter* (formerly named *Thermobacteroides*), as the most important representative, is an important SAOB [72], fermenting sugars and proteins to produce acetate, H_2 and CO_2 [73].

Overall, higher temperatures evoked a higher degree of specialization of the bacterial community, with thermophiles outcompeting mesophilic organisms. This could be shown with an alpha diversity analysis as well as when comparing the taxonomy. While the three most abundant families accounted for 34% of the total abundance at 37 °C, already 79% were

observed at 53 °C. This growing specialization correlated directly with the temperature, without indicating a sudden point at which the microbiota changed fundamentally.

3.2.4. Archaeal Taxonomy

In terms of Archaea, 37 °C was mainly characterized by *Methanosaeta* (68%), cand. *Methanofastidiosum* (11%) and *Methanobacterium* (9%) (cf. Figure 7). While the latter two utilize methylated compounds [74] and H_2/CO_2 [75] for methane production, respectively, *Methanosaeta* (also known as *Methanothrix*) uses the acetoclastic pathway, where acetate is cleaved into methane and carbon dioxide. Hence, there was a co-existence of methylotrophic-, hydrogenotrophic- and acetoclastic methanogenesis at this temperature regime. In contrast to Bacteria, increasing the temperature to 43 °C evoked a fundamental change in the archaeal community, where *Methanosaeta* was completely replaced by *Methanosarcina* (97%). These, likewise, use the acetoclastic pathway, but are also capable of utilizing H_2/CO_2, methanol and methylated amines [76]. Since no other parameters have changed, it is assumed that the temperature or cross-effects with temperature caused this sudden change. It is not clear which methanogenic pathway was used by *Methanosarcina* in the present study. However, increasing numbers of SAOB as described before indicate that most methane was produced via hydrogenotrophic methanogenesis. A direct connection between *Methanosarcina* and Caldatribacteriaceae has already been reported [64].

Figure 7. Genera of Archaea detected in biogas reactors operated at four different temperatures after 65, 70 and 72 days. The color code indicates the relative abundance within each sample. Families with an abundance <1% were excluded. (Candidatus Diapher.: Candidatus Diapherotrites archaeon ADurb.Bin253).

At 47 (92%) and 53 °C (69%), *Methanosarcina* dominated, as at 43 °C, albeit less strongly. While significant numbers of *Methanobacterium* (7%) were found at 47 °C, *Methanothermobacter* (30%) was the second most important genus at 53 °C. Both genera belong to the Methanobacteriaceae and are, hence, closely related despite their different temperature

optima. However, this is not true in terms of their preferred temperature range. *Methanobacterium* was described as a typical mesophilic methanogen, thriving at 37–45 °C [75], while *Methanothermobacter* prefers temperatures >55 °C [77]. This corresponds well with the results of the present study, where *Methanobacterium* was found at temperatures up to 47 °C, and was then replaced by the thermophilic *Methanothermobacter*.

3.2.5. Process Stability

The data confirm that with increasing temperature, diversity, evenness and richness declines. On the level of Bacteria, a constant specialization towards thermophilic organisms was observed. Many of these organisms belonged to the SAOB and their occurrence was directly connected to the presence of hydrogenotrophic methanogens, acting as syntrophic partners. Among Archaea, temperature also evoked changes; however, these changes were not linear, as seen for Bacteria, but happened at a distinct point somewhere between 37 and 43 °C. A higher degree of specialization, as seen particularly for 53 °C, generally reduced the stability of the reactor system. Microbes that are not thriving in these challenging conditions are either deadened or dormant. A re-activation is often not possible, or is at least time-consuming. On the other hand, diverse microbial consortia typically cover a broad range of growth conditions, and changes in process conditions (e.g., temperature) can be tolerated much easier/faster. In the case of methanogens, the shift from (partially) acetoclastic to hydrogenotrophic methanogenesis is another important factor influencing process stability. The complex syntrophic interactions between different partners makes adaptability more difficult, whereas individual acetoclasts are able to adapt to new conditions much faster.

4. Relevance of Application

The operation at different temperature levels between 37 and 53 °C enables the use of digesters both for sludge stabilization and, from an energetic perspective, as a seasonal heat storage. The operation mode involves an increase in the digester temperature with excessive heat in summer to compensate for heat deficits occurring in winter by decreasing the digester temperature [34]. Thus, this is suitable for WRRFs in temperate climate zones with distinct seasons and (heated) digesters with insulation according to the state of the art.

Both the process-engineering and the microbiological aspects confirm that digester operation with raw sludge from municipal WRRFs is possible without the loss of gas production and maintaining the process stability between 37 and 53 °C at an HRT of 20 days. However, a reduction below 35 °C, at the most below 30 °C, can lead to a generally undesirable reduction in gas production. For large-scale applications, the influence on the dewatering behavior and load of the process water when recirculated into the water line of the WRRF must be considered.

The adjustment of the digester temperature beyond the original temperature requires prior verification of both the static construction of the digester, especially in concrete structures, and the specific boundary conditions of the digester (such as HRT, OLR, co-digestion, etc.).

5. Conclusions

The flexibilization of the digester temperature is a concept to contribute to the heat management at WRRFs, allowing for operation at different temperatures and, thus, potentially using digesters as heat storage units. With this study, we were able to demonstrate a stable anaerobic process at four different temperatures between 37 and 53 °C when treating sewage sludge from a municipal WRRF. Despite distinct temperature-induced changes in the microbial community, particularly a shift among archaea between 37 and 43 °C, the data suggest that the microbiota easily adapts to new temperature regimes within the range of 37 to 53 °C. This high adaptability guarantees a stable degradation process, a well-digested sludge, as well as high biogas yields at each temperature, and points out the potential of flexible operating strategies in terms of digester temperature. However, in order to fully capture the microbiota of the hitherto little-studied gap between mesophilic

and thermophilic temperatures, further research is required, as the databases commonly used today have proven insufficient for this purpose. For the operation of the digester as a heat storage unit, further research is necessary in the adaptability of the microbiota to changes in temperature over time.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9090798/s1, Figure S1: Venn plot for comparing the microbiota of days 65, 70 and 72 at family level; Figure S2: Venn plot for comparing the microbiota of days 65, 70 and 72 at genus level; Figure S3: Venn plot for comparing the microbiota at different temperatures at family level; Figure S4: Venn plot for comparing the microbiota at different temperatures at genus level.

Author Contributions: Conceptualization, B.S. and C.S.; Formal analysis, B.S. and S.H.; Funding acquisition, C.S.; Investigation, B.S.; Methodology, B.S.; Project administration, S.H. and C.S.; Supervision, C.S.; Validation, S.H.; Visualization, B.S. and S.H.; Writing—original draft, B.S. and S.H.; Writing—review and editing, H.I. and C.S. The operation, operational analysis and evaluation of process engineering data of the lab digesters were carried out at the Bundeswehr University Munich. Microbiological analyses and data evaluation took place at the Universität Innsbruck. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AD	anaerobic digestion;	
ASV	amplicon sequence variant;	
COD_S	soluble chemical oxygen demand	mg/L;
COD_T	total chemical oxygen demand	mg/L;
FOS	volatile fatty acids (titration)	mg/L;
GC	gas chromatography;	
HRT	hydraulic retention time;	
OLR	organic loading rate	g $COD_{degraded}$/(L·d);
PCoA	principal coordinate analysis;	
PE	population equivalent;	
PS:SS	mixture of primary and secondary sludge;	
SAOB	syntrophic acetate-oxidizing bacteria;	
TAC	total alkalinity	mg/L;
TKN	total Kjeldahl-nitrogen	mg/L;
TS	total solids	%;
TVS	total volatile solids	%;
VFAs	volatile fatty acids (GC)	mg/L (acetic acid equivalents);
WRRF	water resource recovery facility.	

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