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Characterizing the growing microorganisms at species level in 46 anaerobic digesters at Danish wastewater treatment plants: A six-year survey on microbial community structure and key drivers

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ABSTRACT

Anaerobic digestion (AD) is a key technology at many wastewater treatment plants (WWTPs) for converting primary and surplus activated sludge to methane-rich biogas. However, the limited number of surveys and the lack of comprehensive datasets have hindered a deeper understanding of the characteristics and associations between key variables and the microbial community composition. Here, we present a six-year survey of 46 anaerobic digesters, located at 22 WWTPs in Denmark, which is the first and largest known study of the microbial ecology of AD at WWTPs at a regional scale. For three types of AD (mesophilic, mesophilic with thermal hydrolysis pretreatment, and thermophilic), we present the typical value range of 12 key parameters including operational variables and performance parameters. High-resolution bacterial and archaeal community analyses were carried out at species level using amplicon sequencing of >1,000 samples and the new ecosystem-specific MiDAS 3 reference database. We detected 42 phyla, 1,600 genera, and 3,584 species in the bacterial community, where 70% of the genera and 93% of the species represented environmental taxa that were only classified based on MiDAS 3 *de novo* placeholder taxonomy. More than 40% of the bacterial species were found not to grow in the mesophilic and thermophilic digesters and were only present due to immigration with the feed sludge. Ammonium concentration was the main driver shaping the bacterial community while temperature and pH were main drivers for the archaea in the three types of ADs. Sub-setting for the growing microbes improved significantly the correlation analyses and revealed the main drivers for the presence of specific species. Within mesophilic digesters, feed sludge composition and other key parameters (organic loading rate, biogas yield, and ammonium concentration) correlated with specific growing species. This survey provides a comprehensive insight into community structure at species level, providing a foundation for future studies of the ecological significance/characteristics and function of the many novel or poorly described taxa.

1. Introduction

Anaerobic digestion (AD) is successfully employed worldwide to convert organic feedstocks into biogas by anaerobic mixed microbial communities. As a key technology at wastewater treatment plants (WWTPs), AD is used to reduce and stabilize the primary and waste-activated sludge by generating methane for bioenergy production. Moreover, AD can be used as a platform for the recovery of value-added compounds (e.g., phosphorus, nitrogen, volatile fatty acids) (Nielsen, 2017; Puyol et al., 2017). Thus, it is an important step in the development of a circular economy at the WWTPs. The conversion of organic feedstock is carried out by the AD microbial community, a complex network of hydrolyzing and ferment-

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ing bacteria, specialized acidogenic and acetogenic syntrophs, and methanogenic archaea (Briones and Raskin, 2003). This community is shaped by stochastic (birth-death immigration) and deterministic (microbial competition, operation and environment) factors (Ofiţeru et al., 2010; Vanwonterghem et al., 2014a). A good understanding of the microbial ecology in digesters is essential for informed control and manipulation of the process for optimal performance.

The complex microbial network in ADs is ideal for identifying diversity trends in constrained microbial community structures. Research has shown that the operational parameters, including temperature, substrate type, organic loading rate (OLR), and sludge retention time (SRT) are vital factors for determining the microbial community structure (Carballa et al., 2011; Campanaro et al., 2018; De Vrieze et al., 2015; Ho et al., 2014; Kim et al., 2002; Kirkegaard et al., 2017; Lucas et al., 2015; Vanwonterghem et al., 2015). Other parameters, such as ammonia concentration and salinity, are also thought to be significant drivers shaping the community (De Vrieze et al., 2015; Mei et al., 2017; Schnürer and Nordberg, 2008; Sundberg et al., 2013). Additionally, the microorganisms immigrating with the feed sludge should not be overlooked. Most of them do not grow or contribute to the ecological functions in the system, but they still account for a significant fraction of sequencing reads identified by 16S rRNA gene amplicon sequencing (Kirkegaard et al., 2017; Mei et al., 2016).

Most of the findings described above are based on investigations across various AD substrate types, such as manure, food waste, and wastewater sludge, where large differences in growth conditions are observed. It is unclear whether the same drivers are also important among digesters at WWTPs, as the operational conditions are regarded to be very similar. However, AD performance can be highly variable between different WWTPs, and how performance links to different microbial communities and growth conditions is poorly described for full-scale systems.

The quantitative relationships between specific microorganisms and key parameters in AD can be evaluated by multiple linear regression (MLR). Most studies have focused on linear associations between methanogenic populations (i.e., characterized by the *mcrA* gene) and specific methanogenic activities (Bocher et al., 2015; Morris et al., 2014; Tale et al., 2011; Venkiteshwaran et al., 2015). However, the traditional MLR fails when the number of predictors is comparable to, or larger than, the number of observations, and when there is high collinearity in predictors. Projection-based methods for analysis of multivariate data, such as Partial Least Squares (PLS) regression, stand as promising techniques to combine elements from principal component analysis (PCA) and MLR, lessening the shortcomings of traditional methods (Kucheryavskiy, 2020). PLS regression has been widely applied in various high-dimensional datasets, such as in chemometrics and spectrometrics (Carrascal et al., 2007). More recently, PLS has also been successfully applied in microbial ecology to link high-dimensional microbial data to environmental factors (Li et al., 2016; Frindte et al., 2019; Rudi et al., 2007).

To provide insightful links between the AD community and its performance, it is crucial to obtain high phylogenetic resolution and good taxonomic classification at all ranks. High phylogenetic resolution can be obtained by using amplicon sequence variants (ASVs) (Callahan et al., 2017; García-García et al., 2019) instead of operational taxonomic units (OTUs), typically clustered at 97% similarity thresholds, and by using an ecosystem-specific, high-quality 16S rRNA gene reference database for taxonomic classification. We recently developed MiDAS 3, a comprehensive ecosystem-specific reference database for activated sludge and anaerobic digesters that contains a seven-rank taxonomy (domain to species level) for all reference sequences based on an improved and automated classification system (AutoTax) (Dueholm et al., 2020; Nierychlo et al., 2020). The MiDAS 3 reference database is based on a comprehensive set of full-length 16S rRNA gene ASVs (FL-ASVs) obtained from Danish WWTPs and digesters, but can be applied to similar systems worldwide (Dueholm et al., 2020). MiDAS 3 improves the classification of prokaryotic microorganisms found in AD compared to other public reference databases, such as SILVA (Quast et al., 2013), Greengenes (DeSantis et al., 2006), and RDP (Cole et al., 2014), which lack reference sequences for many taxa and high taxonomic resolution, often resulting in poor classification (Dueholm et al., 2020). In particular, the classification at species-level is a game changer as it provides stable taxa identifiers independent of the dataset, thus allowing cross-study comparisons. In addition, it offers the possibility of finding the link between identity and function of novel or poorly classified species-level taxa.

The aims of our study are threefold. Firstly, we describe the typical operational parameters and performance values of three different types of AD at WWTPs (i.e., mesophilic AD, mesophilic AD with pre-treatment (thermal hydrolysis) of waste activated sludge, and thermophilic AD). Secondly, we carry out reproducible species-level classification of the entire microbial communities in the AD systems with focus on the growing microbes, and make this publicly available on the MiDAS website (https://www.midasfieldguide.org/guide). And thirdly, by focusing on species-level community structure, we analyse the correlations between key AD parameters and community structure in mesophilic digesters, which are the most common digesters at WWTPs in Denmark. To our knowledge, our work presents the largest known study of the microbial ecology of AD at WWTPs at a regional scale.

2. Methods

2.1. Anaerobic digesters and sample collection

The survey was conducted during the period 2011 – 2016 in 46 anaerobic digesters at 22 WWTPs across Denmark, which were operated under mesophilic (MAD), mesophilic with thermal hydrolysis pretreatment of feedstock (THP-MAD), or thermophilic (TAD) conditions (see Table S1 for information of digesters). During the six years of survey, all plants reported minor fluctuations in substrate amounts and composition, but no major changes of operating conditions were introduced, except for Aaby and Aalborg East, which switched from mesophilic to thermophilic operation (Table S1). A total of more than 50,000 observations, including operational, physicochemical, and performance parameters were obtained from the records of individual plants, except for volatile fatty acids (VFAs), which were determined in our laboratories. Temperature, total solids (TS), volatile solids (VS), pH, total ammonia nitrogen (TAN), and alkalinity were determined at the plants according to standard methods (APHA et al., 2005). Each key variable had at least 1,087 observations, except for VFAs (Table 1).

Digester sludge samples were obtained 2-4 times a year (every three or six months) during the investigation period (2011-2016). VFA measurements were performed with the samples from 2016. All samples were transported to the laboratory within 24 h and processed immediately upon arrival. After homogenization, the biomass samples were stored as 2 mL aliquots at -80°C before DNA extraction. Samples for VFA analysis were filtered with 0.22 µm filters (Frisenette, Knebel, Denmark) and stored at -20°C until analysis, which is described elsewhere (Hao et al., 2020).

2.2. DNA extraction, 16S rRNA gene amplicon sequencing, and bioinformatics processing

The microbial communities of a total of 1,010 AD sludge samples (418 for archaea and 592 for bacteria) were analyzed using 16S

Table 1
Operational variables and performance parameters: Intervals and median values for ADs at WWTPs in Denmark.

Category	Variable	Unit	MAD*		THP-MAD*		TAD*	
			Interval[1]	Median	Interval	Median	Interval	Median
Operational variables	Temperature	°C	35.6 ~ 39.9	38.0	38.2 ~ 39.0	38.6	51.1 ~ 55.40	53.6
	OLR[2]	kg VS/m^{3}d	0.84 ~ 1.13	0.96	1.66 ~ 2.30	2.04	1.63 ~ 2.49	2.15
	SRT*	Day	24.8 ~ 35.6	29.4	27.3 ~ 34.9	30.1	15.8 ~ 20.7	17.3
Performance parameters	TS*	g/L	21.1 ~ 38.4	31.0	41.1 ~ 65.9	44.8	31.2 ~ 39.0	35.8
	VS*	g/L	12.1 ~ 19.9	16.2	25.9 ~ 34.0	27.7	21.0 ~ 25.5	23.6
	VS	TS%	56.0 ~ 61.6	58.6	54.0 ~ 63.5	60.2	57.0 ~ 60.9	58.0
	pH	-	7.06 ~ 7.38	7.19	7.64 ~ 7.86	7.75	7.50 ~ 7.80	7.70
	TAN	mg N/L	603 ~ 972	745	2691 ~ 3100	2888	1070 ~ 1430	1215
	Alkalinity	mM	50.0 ~ 73.0	60.8	148 ~ 186	168.6	67.9 ~ 87.7	78.4
	Total VFA*	mM	0.28 ~ 1.11	0.50	0.45 ~ 2.34	0.73	0.92 ~ 2.18	1.30
	Acetate	mM	0.10 ~ 0.40	0.21	0.25 ~ 0.44	0.30	0.53 ~ 1.18	0.79
	Biogas yield[2]	Nm^3/kg VS	0.39 ~ 0.53	0.46	0.38 ~ 0.56	0.49	0.25 ~ 0.36	0.29
	Methane content	%	61.0 ~ 63.5	61.7	63.0 ~ 65.0	65.0	53.7 ~ 64.3	57.6
	Methane production[3]	Nm^3/m^{3}d	-	0.27	-	0.65	-	0.36

[1] Interval shows the range of first quantile and third quantile of each variable.
[2] OLR and biogas yield are normalized using an average volatile solids value of influent feed (74.5%).
[3] Methane production is calculated on median value of biogas yield, OLR, and methane content.
* MAD = mesophilic, THP-MAD = mesophilic with thermal hydrolysis pretreatment, TAD = thermophilic, OLR = organic loading rate, SRT = solids retention time, TS = total solids, VS = volatile solids, TAN = total ammonia nitrogen, Total VFA = total volatile fatty acid.

rRNA gene amplicon sequencing. 50 µl of AD sample was used for DNA extraction with the FastDNA® Spin Kit for soil (MP Biomedicals, Solon, OH, USA), following the optimized protocol for anaerobic digesters (Kirkegaard et al., 2016). The library preparation for 16S rRNA amplicon sequencing was performed as previously described (Kirkegaard et al., 2017), targeting the V1-3 variable regions for bacteria and V3-5 variable regions for archaea. The bacterial primers used were 27F (AGAGTTTGATCCTGGCTCAG) (Lane, 1991) and 534R (ATTACCGCGGCTGCTGG) (Muyzer et al., 1993), which amplify a DNA fragment of ~500 bp of the 16S rRNA gene (V1–3). The archaeal primers used were 340F (CCCTAHGGGGYGCASCA) (Pinto and Raskin, 2012) and 915R (GWGCYCCCCCGYCAATTC) (Pinto and Raskin, 2012), which amplify a DNA fragment of ~ 560 bp of the 16S rRNA gene (V3–5). The choice of separate primers is due to the better coverage of the known diversity within the ecosystem compared to the primers covering both domains. Furthermore, the bacterial V1-V3 amplicon carries much more phylogenetic information than short-read amplicons for other regions of the 16S rRNA gene, so it allows for species-level resolution of more taxa (Dueholm et al., 2020). The amplicon libraries were paired-end sequenced (2 × 300 bp) on the Illumina MiSeq as previously described (Albertsen et al., 2015).

The archaeal and bacterial read data were analyzed separately using USEARCH (v.11.0.667) (Edgar, 2010). For the V1-3 amplicons, raw fastq files were filtered for phiX sequences using -filter_phix, trimmed to 250 bp using -fastx_truncate -trunclen 250, and quality filtered using -fastq_filter with -fastq_maxee 1.0. The sequences were dereplicated using -fastx_uniques with -sizeout -relabel Uniq. ASVs were generated using UNOISE3 (Edgar, 2016), and ASV-tables were created by mapping the raw reads to the ASVs using -otutab with the -zotus and -strand both options. Taxonomy was assigned using the MiDAS 3 reference database (Dueholm et al., 2020; Nierychlo et al., 2020) using sintax with the -strand both and -sintax_cutoff 0.8 (Edgar, 2018). The V3-5 amplicon data were analyzed in the same way except that only the reverse read was used and the primer binding site was removed during trimming using –fastx_truncate –stripleft 18 –trunclen 250.

2.3. Data processing and statistical analysis

Downstream statistical analyses and visualization were mostly performed in the R environment (v3.6.2) (R Core Team, 2019) using *ampvis2* (v2.5.8) (Albertsen et al., 2015) and *ggplot2* (v3.2.1) (Wickham, 2016), unless indicated otherwise. Non-parametric *dunn.test* was used to identify significant differences between AD types. The correlations between all the variables were explored by Spearman correlation, where correlations greater than ±0.5 and false discovery rate (FDR) corrected $P > 0.05$ were visualized in Gephi (v0.9.2) (Bastian et al., 2009), using Force Altas2 and manual tweaking to generate the network. For sequence data, samples were randomly subsampled to 10,000 sequences per sample, yielding a final dataset of 402 archaeal and 564 bacterial samples. For the growing bacteria datasets, after removing non-growing ASVs from the ASV table, samples were also randomly subsampled to 10,000 sequences per sample for downstream analysis and comparison. Boxplot and heatmaps were made with the *amp_boxplot* and *amp_heatmap* functions in *ampvis2*. Alpha-diversity was calculated with *amp_alphadiv* function in *ampvis2*. The Wilcoxon rank-sum test was applied to compare the alpha-diversity between types of AD. The linear regression between alpha-diversity (using Shannon's index) and each operational and performance variable was used to pick the key variables most correlated. Weighted UniFrac distance, calculated by *beta_diversity.py* script in QIIME (v1.9.0) (Caporaso et al., 2010), was applied for all beta-diversity comparisons. For ordination visualizations, the non-metric multidimensional scaling (NMDS) was performed with *amp_ordinate* in ampvis2 to show the dissimilarities of microbial profiles. Based on weighted UniFrac distance matrix, ANOSIM was applied to assess similarities for categorical variables using *compare_categories.py* in QIIME with 999 permutations. A PERMANOVA analysis using adonis in QIIME was used to describe the strength and significance for continuous variables. The significant difference of species between two groups of feed sludge was explored by Wilcoxon rank-sum test.

PLS regression was performed using R package *mdatools* v0.10.1 (Kucheryavskiy, 2020) to validate quantitative relationship between operational and performance parameters, and the microbial community, as well as to identify specific microbes which correlate to each variable the most. All bacterial species with median relative abundance >= 0.01% and archaeal ASVs with median relative abundance >= 0.05% were used to perform the PLS analysis. The model was trained using all samples and validated by segmented cross-validation (CV) with systematic splits (venetian blinds). Determination coefficient (R^2) and root mean square error (RMSE) were used to assess performance of the model. The contribution of individual predictors was evaluated using regression coefficients

and corresponding inferential analysis carried out by Jack-Knifing approach (Martens and Martens, 2000).

3. Results and discussion

3.1. Characterization of key parameters of AD

Key operational and performance parameters of the 46 anaerobic digesters during the six-year survey are summarized in Table 1. The digesters are classified into three types, based on the operational temperature and pretreatment of the feed sludge. MAD is the most common configuration (78% of all digesters) followed by TAD (15%) and THP-MAD (7%). The most common digester type is single-stage continuously stirred tank reactor (CSTR). The anaerobic digesters surveyed were running stably without major process complications for six years, therefore common ranges of operation and performance conditions are described for each digester type. As presented in Table 1 and described in Appendix B, values of several performance parameters are very different from other AD systems treating manure, crops, food waste, and industrial waste (Calusinska et al., 2018; De Vrieze et al., 2015; Fotidis et al., 2014; Lucas et al., 2015; Luo et al., 2016; Martí-Herrero et al., 2019; Mata-Alvarez et al., 2014; Sundberg et al., 2013), with generally lower or much lower concentrations of total ammonia nitrogen (TAN) and VFAs.

The median temperature values of the three types of digesters were 38.0°C, 38.6°C, and 53.6°C, for MAD, THP-MAD, and TAD, respectively. Other operational variables (OLR and SRT) and the performance parameters (pH, TAN, alkalinity, TS, VS, biogas yield, and methane content) were found to be significantly different across all three types of AD (Fig. S1). For more details on the description of each parameter, please see Appendix B. In general, the same overall correlations between operational and performance parameters across digesters treating different types of substrates could also be observed specifically for digesters among WWTPs. Strong positive correlations (Spearman, $r > 0.7$, false discovery rate (FDR) $P < 0.05$) were observed between TAN and TS or VS, OLR and alkalinity, and methane production and SRT (Fig. S2). Strong negative correlations between the OLR and methane production and biogas yield were also revealed (Spearman, $r > 0.65$, FDR $P < 0.05$), and between methane production and TAN (Fig. S2), indicating that these operational variables are linked to the performance of the digesters. It is interesting that VFAs only related weakly to SRT and the ratio of VS to TS, which have previously been considered as important variables (Chen et al., 2008). This may be due to the low concentration of VFAs in the digesters surveyed and the low organic loading at the WWTPs.

The digesters surveyed were all running at low OLR and long SRT, which is usually considered “suboptimal” operational conditions, and may lead up to a 30% profitability loss (Fotidis et al., 2014; Tsapekos et al., 2017). Increasing the OLR seems promising, but a number of operational problems needs to be considered, such as foaming and acidosis, due to the imbalance between operational and microbial processes. Thus, a better understanding of microbial communities and their function may help to control or manipulate the processes to decrease the potential risks of operational failures.

3.2. Bacterial and archaeal communities at species level

We obtained 33,047 bacterial and 878 archaeal unique ASVs, which were classified using sintax and the MiDAS 3 database. A total of 42 phyla, 1,600 genera, and 3,584 species were detected in the bacterial communities, where 1,117 (70%) genera and 3,336 (93%) species were novel or previously unclassified and could only be assigned genus and species name based on the MiDAS 3 *de novo* placeholder taxonomy. The *de novo* species in the MiDAS 3 database are defined using a 98.7% identity threshold (Dueholm et al., 2020; Yarza et al., 2014) which gives better resolution compared to the often used identity level of 97% applied for OTUs in many studies. For archaea it was not possible to analyze the methanogenic archaea at the species level because the phylogeny of most of these species cannot be resolved using the 16S rRNA gene, even with full-length sequences (Dueholm et al., 2020). As a result, only 26 species were classified, and most of the archaeal populations are shown at ASV level.

The application of the MiDAS 3 reference database and the AutoTax approach provided reliable and reproducible genus and species identification, often based on the placeholder taxonomy. Species-level classifications cannot be provided by other references databases such as SILVA, Greengenes, or RDP, and often genus-level classifications are prevented by the high degree of novel or unclassified AD taxa (Dueholm et al., 2020). An example of using SILVA compared to MiDAS 3 is shown in Fig. S3, demonstrating the great improvement even at genus-level by using MiDAS 3. Despite the fact that the reference database is only built on full-length 16S rRNA gene sequences from Danish ADs (and activated sludge plants), it covers the global diversity well and performs better than other reference databases (Dueholm et al, 2020). We are currently working on expanding the MiDAS 3 database with full-length 16S rRNA gene references from WWTP and AD from across the world (https://www.midasfieldguide.org/global). These updates are expected to be introduced in spring 2021 to further improve the MiDAS reference database. Classification at the species-level by the MiDAS taxonomy provides stable taxa identifiers independent of the dataset, which enables cross-study comparisons, and offers the possibility to link identity with function for novel or unclassified species-level taxa.

3.3. Growing and non-growing bacterial and archaeal communities

AD at WWTPs are complex systems, as they receive substantial amounts of microorganisms via feed streams in the form of primary sludge (PS) or surplus activated sludge (AS). Many of these microorganisms are not growing in the digester, presumably inactive or dying off (Kirkegaard et al., 2017; Mei et al., 2016; Petriglieri et al., 2018). The growing and non-growing microorganisms can be differentiated directly based on 16S rRNA gene amplicon data and the ratio between the relative read abundances in digester and in the feed as previously described (Kirkegaard et al., 2017). We compared this ratio-based method to the calculation of growth rates based on a mass-balance of influent and digester biomass (modified from Saunders et al., 2016), and the two methods showed very similar results (Appendix D).

The bimodal distribution of ratios clearly split at a threshold around 20 (Fig. 1A), showing two distinct groups of ASVs. The group with a ratio >20 represents ASVs enriched in digesters compared to the feed sludge, here designated as “growing microorganisms”. The group with a ratio <20 represents ASVs with unchanged or lower relative read abundance in digesters, compared to the feed sludge, here designated as “non-growing microorganisms”. The THP-MAD acted as a perfect control for the method. Since these digesters were fed with sterilized sludge, essentially all microorganisms observed must be growing, and accordingly the relative read abundance ratio distribution lacked the bimodal distribution and contained only one group with a ratio > 20.

The total ASVs (>0.01% median relative abundance) were divided into four groups across each type of AD: growing/non-growing ASVs with high abundance (>0.1%) and growing/non-growing ASVs with low abundance (<0.1%). It was observed that the growing highly abundant ASVs only accounted for 7.6%, 23.2%,

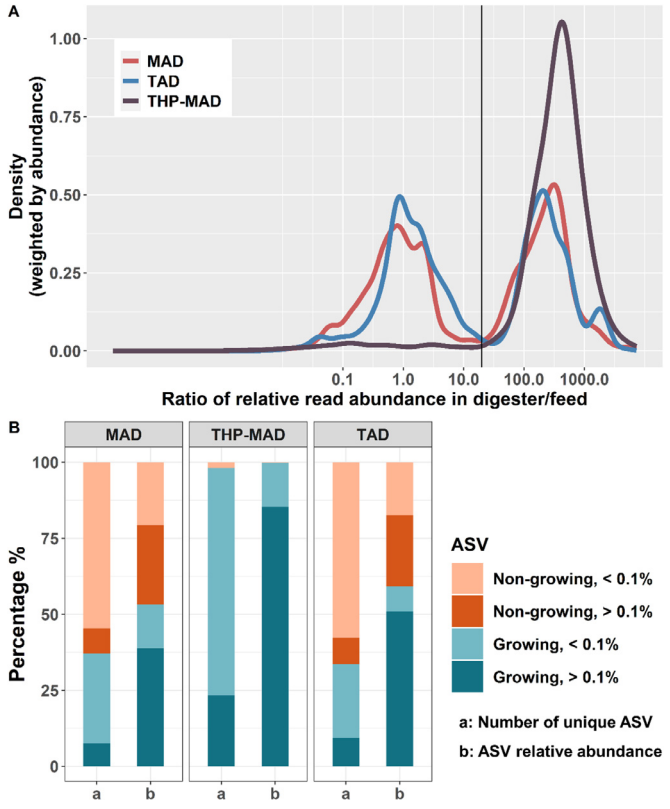

Fig. 1. (A) Distribution of ratio of relative read abundance in digester/feed for each ASV. The black vertical line indicates the ratio threshold at 20. The group with ratio >20 represents the ASVs highly enriched in the digester compared to the feed sludge, and named as the growing group. The read abundance of the ASVs in the group with ratio <20 is unchanged or lower compared to the feed sludge, here named as non-growing. **(B) Composition of growing and non-growing ASVs in Danish ADs at WWTPs.** The total ASVs (median relative abundance > 0.01%) divided into four groups based on growth ratio (growing/non-growing) and relative abundance (high/low abundant, 0.1% indicates the cutoff value). a and b show the composition of number of unique ASV and ASV relative abundance for these four groups in MAD, THP-MAD, and TAD, respectively. MAD = Mesophilic AD, THP = Mesophilic with thermal hydrolysis pretreatment, TAD = Thermophilic AD.

and 9.4% of the total number of unique ASVs in MAD, THP-MAD, and TAD, respectively (Fig. 1B). However, they accounted for 38.8%, 85.3%, and 50.9% of the community according to relative read abundance in MAD, THP-MAD, and TAD, respectively. This suggests that the performance and functionality of AD at WWTPs are driven by only a small number of the microbial phylotypes detected by amplicon sequencing. Immigration has also been shown to strongly affect ADs with other feed streams (Mei et al., 2016). We therefore recommend that this phenomenon is always considered when linking microbial community structure with functions in ADs systems.

The five most abundant bacterial phyla were Firmicutes, Proteobacteria, Chloroflexi, Actinobacteria, and Bacteroidetes, which accounted for 75.7% (median value) of all amplicon sequences across all samples. These phyla are typical for digesters at WWTPs (Calusinska et al., 2018; De Vrieze et al., 2015; Lee et al., 2018, 2012; Nelson et al., 2011; Rivière et al., 2009; Sundberg et al., 2013). The three types of AD in our study showed variations in the dominant bacterial taxa (Fig. S4). Among the 25 most abundant species, 11 species in MAD and 9 species in TAD belonged to the group of non-growing microorganisms (Fig. 2A and 2B). These included species in genera belonging to the polyphosphate-accumulating organisms (PAO) *Tetrasphaera*, the putative PAO *Dechloromonas* (Stokholm-Bjerregaard et al., 2017), the filamentous genus *Ca.* Microthrix (McIlroy et al., 2013), and the genera *Romboutsia,* and *Trichococcus*. These all belong to the top-most abundant reported genera in activated sludge in Danish WWTPs (Nierychlo et al., 2020), thereby showing carry-over to the digesters with the feed sludge. Since the top 100 species in MAD and TAD, respectively, were very similar across the digesters surveyed, these lists may likely be used as a representative reference of abundant growing and non-growing organisms in digesters at WWTPs across the world (Fig. S5 and S6). These results demonstrate that many species, 44% and 54% of the top 100, were non-growing in MAD and TAD, respectively. The top 25 species in THP-MAD all belonged to growing microorganisms in good agreement with the presence of the THP pretreatment, which caused a decay of essentially all organisms coming with the feed sludge (Fig. S7A).

The growing microorganisms are considered to be responsible for the most important ecological functions within AD. Among the dominant growing bacterial species there were many known fermenters, such as species belonging to the genera *Thermovirga, Ca.* Fermentibacter, and *Leptolinea* in MAD, and *Coprothermobacter* and *Acetomicrobium* in TAD. There were also several syntrophic genera in MAD, where the most abundant species were members of *Smithella* and *Ca.* Cloacimonas which were also identified in other studies (Pelletier et al., 2008; Lam et al., 2020). However, a large fraction of the most abundant growing species were novel or previously unclassified taxa without any known function. They were only identified due to the MiDAS 3 species-level taxonomy, which contains stable *de novo* placeholder names for many environmental taxa that have not yet obtained official classification. Due to their high relative abundance, some placeholder genera in a number of families and orders are of special interest: midas_g_12 (family Prolixibacteraceae), midas_g_19 (family Bacteroidetes vadinHA17), midas_g_156 (family Anaerolineaceae), and midas_g_789 (family Anaerolineaceae) in MAD; midas_g_88 (family Syntrophomonadaceae), midas_g_112 (order MBA03), and midas_g_16 (family Lentimicrobiaceae) in TAD, and midas_g_13 (order D8A-2) in THP-MAD. Some of these genera encompass very abundant species, such as midas_g_156 and midas_g_789 (up to 8% median abundance, family Anaerolineaceae) which incorporate species midas_s_156, midas_s_876, midas_s_956, midas_s_1462, midas_s_467, and midas_s_1625. These abundant undescribed taxa should be investigated in future studies, as their physiology and ecological role in AD are completely unknown while likely important.

Moreover, compared with the MiDAS 2 taxonomy (which is an ecosystem-curated version of the SILVA release 1.23 taxonomy) (McIlroy et al., 2017a), MiDAS 3 provides a much higher resolution to classify sequences and introduces species-level names for the first time for most microorganisms in AD ecosystem. For example, genus T78 (family Anaerolineaceae) in MiDAS 2 encompassed sequences that are split into midas_g_156 and midas_g_467 in MiDAS 3 (the abundant genera mentioned above). These genera are both diverse, each having three abundant species present in MAD (Fig. S8). *Ca.* Cloacimonas and *Pelotomaculum* and the newly discovered syntrophic genus midas_g_995 (Hao et al., 2020) also had high species diversity, with several abundant species showing random distributions (Fig. S8).

Euryarchaeota was the dominant archaea in the digesters (99.9% relative abundance of all archaea, median value). The acetoclastic genus *Methanothrix* (previously named *Methanosaeta*) dominated in MAD (71.8%) and THP-MAD (93.8%), whereas the genera *Methanothermobacter* (70.7%) and *Methanosarcina* (24.8%) dominated in TAD (Fig. S9B). *Methanosarcina* was in very low abundance in MAD (0.1%) and THP-MAD (0.01%), in contrast to other mesophilic full-scale studies of manure-based AD where it was dominant (De Vrieze et al., 2015; Leclerc et al., 2004; Nelson et al., 2011). The abundance of *Methanosarcina* in MAD and TAD may depend on the specific species composition as shown in a recent study by Lam et al., (2020), probably explaining the differ-

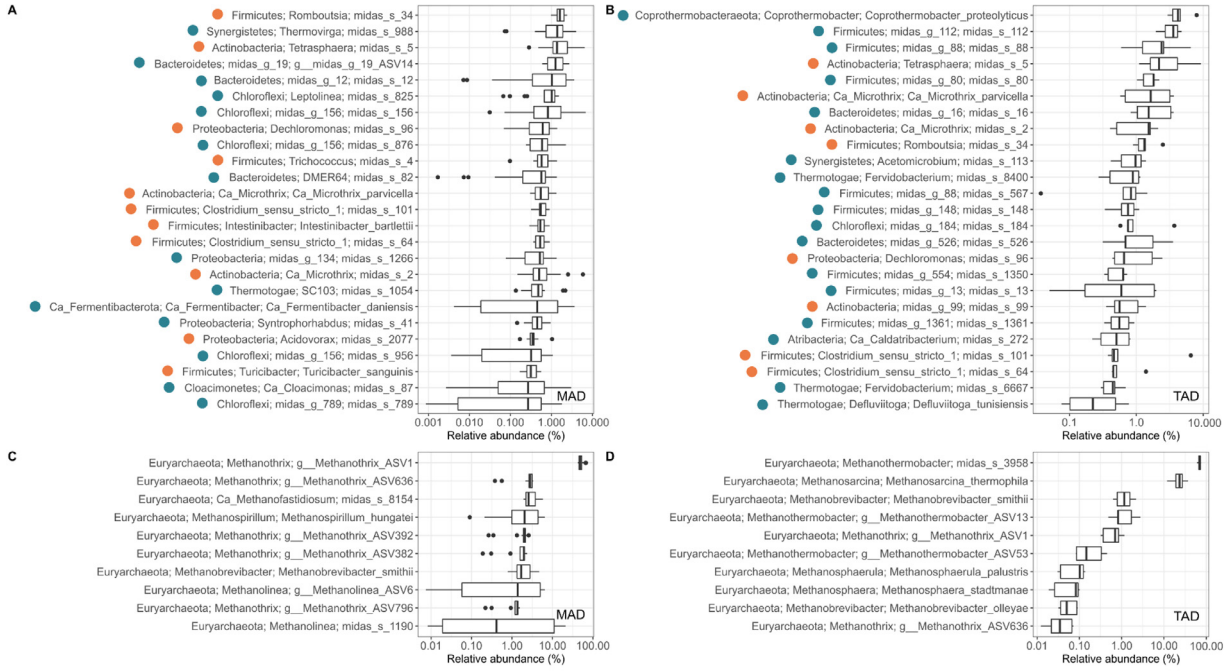

Fig. 2. Boxplots of the most abundant species/ASVs in Danish ADs at WWTPs. (A) The 25 most abundant bacterial species/ASVs in MAD, (B) The 25 most abundant bacteria species/ASVs in TAD, (C) The 10 most abundant archaeal species/ASVs in MAD, (D) The 10 most abundant archaeal species/ASVs in TAD. The dots at the left in A and B indicate whether the species/ASVs are growing (ratio >20, blue), non-growing or dying off (ratio <20, orange). MAD = Mesophilic AD, TAD = Thermophilic AD. The relative abundance is calculated based on the median abundance in each plant. Ratio refers to the digester to influent relative read abundance ratio (please see **Appendix C**). The taxa is shown at respective phylogenetic level (phylum, genus, and species). Sequences not possessing species-level classification are shown as individual ASVs.

ent observations in various studies when only classifying at genus level. The low concentrations of VFAs (<1 mM) in the mesophilic AD may explain why *Methanothrix* dominated (Hori et al., 2006). The hydrogenotrophic methanogenic *Methanoculleus* was the predominant genus (4.2%) in THP-MAD, which is in line with other lab-scale and pilot-scale THP digesters studies (Choi et al., 2018; Wandera et al., 2019).

Acetoclastic and hydrogenotrophic methanogenic species/ASVs were quite abundant in all the types of AD (Fig. 2C, 2D, and Fig. S7B). *Methanothrix* ASV1 was dominant in both MAD and THP-MAD, followed by species in the genus *Methanolinea* (midas_s_1190 and ASV6) in MAD and *Methanoculleus* midas_s_880 in THP-MAD. *Methanothermobacter tenebrarum* and *Methanosarcina thermophila* were the second most abundant species in TAD (Fig. 2). *M. tenebrarum* was also abundant in TAD in the study by Lam et al., (2020), while *M. thermophila* was not detected and *M. flavescens* was dominant instead. This discrepancy may be due to the lack of a proper reference database, the OTU-clustering of their full-length 16S rRNA reads, and the best hit taxonomy assignment that was used in their study despite many OTUs shared percent identities below the species-level threshold (98.7%) (Yarza et al., 2014).

3.4. Microbial diversity in different AD types

The use of common measures for richness and diversity in the digesters surveyed has only limited value, as abundant non-growing immigrating bacteria that arelikely without any functional role in the systems, will influence the diversity measures and produce misleading results. This is illustrated by comparing the diversity measures calculated for all bacteria and for the growing bacteria only (Fig. 3A). When the non-growing fraction was removed, the median values of observed ASVs decreased from 1935 to 928 in MAD, and from 1486 to 534 in TAD. Shannon index median values were reduced from 6.22 to 5.11 in MAD and 5.59 to 4.33 in TAD (Fig. 3A). THP-MAD only had a minor change in observed ASVs (from 832 to 741) and the median Shannon index (from 4.63 to 4.47), reflecting, as expected, that these communities were not strongly influenced by immigration. The adjusted diversity measures showed the same trend for archaea (Fig. S10) with the THP-MAD diversity between MAD and TAD. The thermophilic reactors were found to harbor a lower number of microbes compared to mesophilic reactors in accordance to other full-scale surveys (Lee et al., 2018; Sundberg et al., 2013), where the exact values are strongly dependent on the inclusion of the immigrating microbes. Higher alpha-diversity measures for bacterial communities compared with archaea is in agreement with other full-scale WWTPs studies (Calusinska et al., 2018; Lee et al., 2018; Sundberg et al., 2013). The diversity in thermophilic AD has been shown to be lower than in mesophilic digesters (Karakashev et al., 2005; Levén et al., 2007; Li et al., 2015), which is also supported by our data.

The total bacterial (including growing and non-growing fraction) and archaeal communities seemed relatively stable in each digester across all 22 WWTPs during the six-year survey as indicated by tight clustering visualized by non-metric multidimensional scaling (NMDS) (Fig. 4). This is also reported in other time-series studies of full-scale digesters mainly treating manure, agricultural waste, and municipal solid waste (Calusinska et al., 2018; De Vrieze et al., 2015), suggesting that stable communities are common in full-scale digesters during steady-state operation. However, as a major part of the microorganisms are immigrants, they may strongly affect the beta-diversity measures. Therefore, it is important to compare the diversity of both the total and the growing fraction of the population. The overall dissimilarity between plants was not statistically affected by the non-growing bacteria for MAD (ANOSIM; Total bacteria: $R = 0.65$, $P = 0.001$; Growing bacteria: $R = 0.63$, $P = 0.001$) and THP-MAD (ANOSIM; Total bacteria: $R = 0.45$, $P = 0.001$; Growing bacteria: $R = 0.49$, $P = 0.001$). However, the bacterial community in TAD was more similar across plants for the growing bacteria alone compared to the total bac-

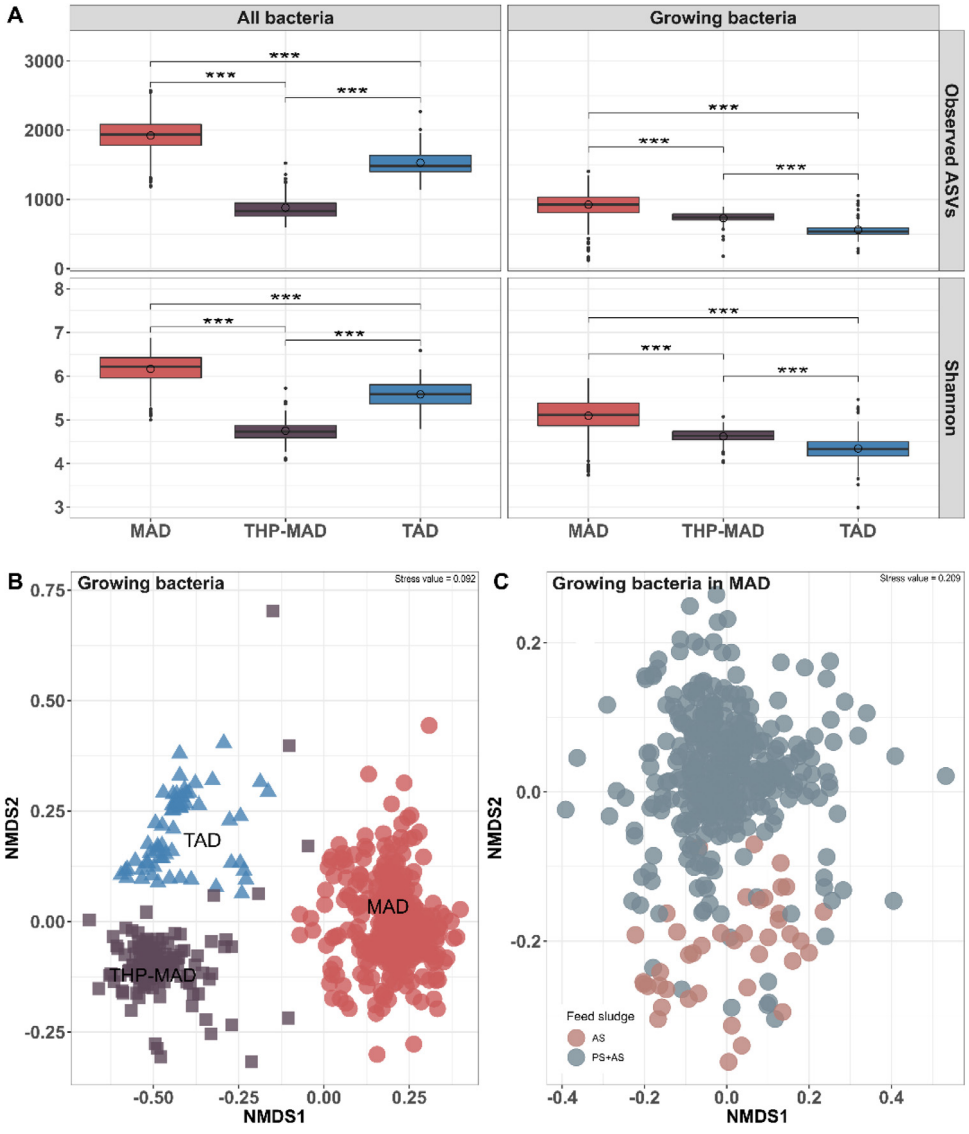

Fig. 3. Alpha and beta-diversity of bacterial and archaeal communities of three types of AD. (A) Boxplots of observed ASVs and Shannon index of totaltotal and growing bacterial community, significant differences are indicated (Wilcoxon rank-sum test; ***, p < 0.001). (B) Non-metric multidimensional scaling (NMDS) plots of growing bacterial community structure based on weighted UniFrac matrix, (C) NMDS plots of growing bacterial community structure of MAD based on weighted UniFrac matrix. MAD = Mesophilic AD, THP-MAD = Mesophilic with thermal hydrolysis pretreatment process, TAD = Mesophilic AD, AS = Activated sludge, PS = Primary sludge.

terial population (ANOSIM; Total bacteria: R = 0.64, P = 0.001; Growing bacteria: R = 0.40, P = 0.001). This shows that the inclusion of non-growing bacteria in community analyses may lead to misleading results and erroneous conclusions.

Several factors can influence the microbial communities in AD (Carballa et al., 2011; De Vrieze et al., 2015; Ho et al., 2014; Kim et al., 2002; Kirkegaard et al., 2017; Lucas et al., 2015; Vanwonterghem et al., 2015), however, no studies have clearly identified their effect on the bacterial and archaeal community separately. In our study, analysis of beta-diversity of bacterial and archaeal community revealed three distinct clusters corresponding to MAD, THP-MAD, and TAD (Fig. 3B and Fig. S11A). Clear separation dictated by AD type was evident for all bacteria (ANOSIM: R = 0.95, P = 0.001), the growing bacteria (ANOSIM: R = 0.97, P = 0.001), and the archaeal community (ANOSIM: R = 0.83, P = 0.001), reflecting the huge effects of operational conditions on the resulting variation in the community structure. Permutational multivariate analyses of variance showed that TAN contributed to shaping the structure of the total bacterial community (adonis: R^2 = 32%, P = 0.001, Table S2, Fig. S11B) with the range from 603 to 3100 mgN/L. This has also been observed in full-scale digesters treating different kinds of substrates but with a larger concentration range (128-6427 mgN/L) (De Vrieze et al., 2015). In contrast to the bacterial community, the overall structure of the archaeal community was separated mainly by temperature (adonis: R^2 = 66%, P = 0.001, Table S2), with a separate cluster for THP-MAD alongside MAD (Fig. S11C). pH was the second strongest factor influencing the archaeal community (adonis: R^2 = 27%, P = 0.001, Table S2), which may explain the separated cluster of THP-MAD (pH range: 7.64-7.86) from MAD (pH range: 7.06-7.38) (Fig. S11D).

3.5. Main drivers of MAD microbial communities

Since most digesters surveyed were MAD, we further applied the correlation analysis between key parameters and microbial diversity and structure to determine the main drivers, with special focus on the growing bacterial community, as non-growing microorganisms may mask the influence of key drivers on the active community in correlation analyses. In general, a bigger difference was observed for the linear regression of key parameters against

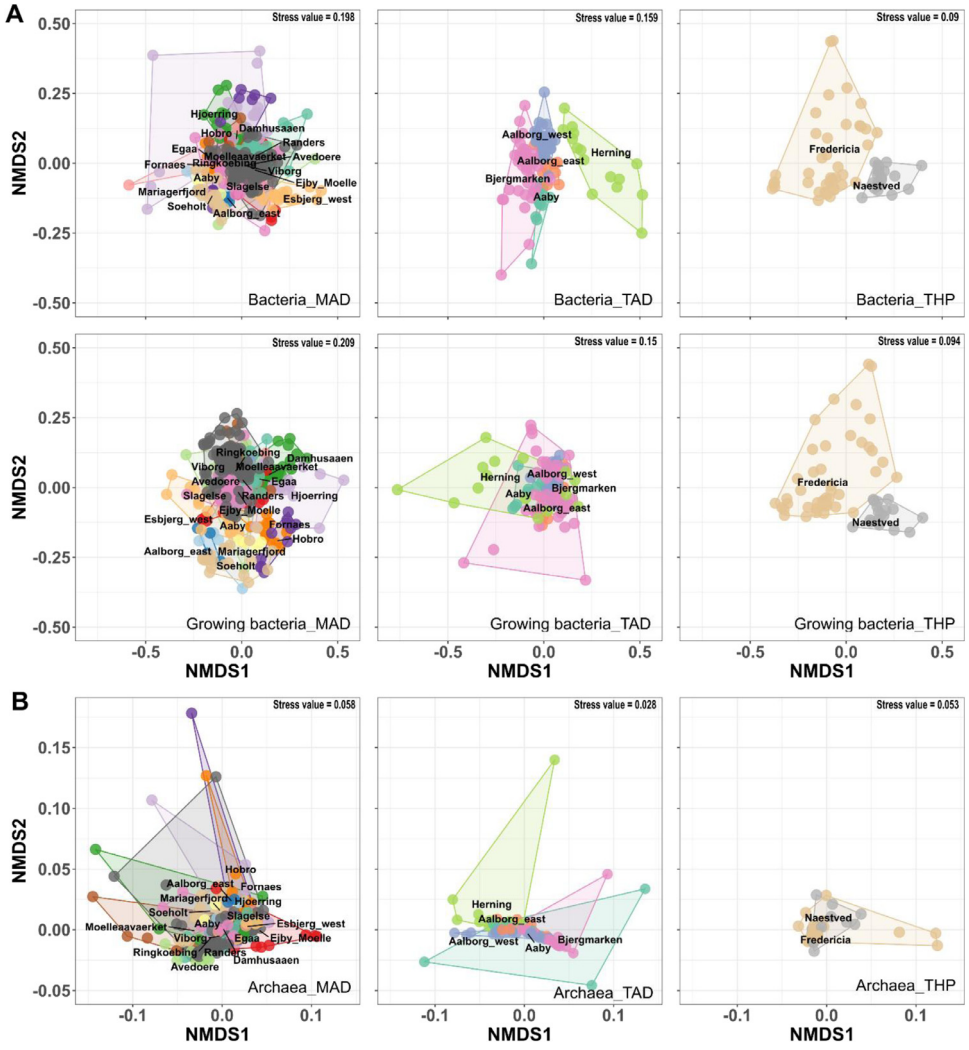

Fig. 4. Non-metric multidimensional scaling (NMDS) plots of bacterial and archaeal community structure based on weighted UniFrac matrix colored by WWTPs. (A) The total ntreand growing bacterial communities colored by WWTPs in MAD, TAD, and THP. (B) The archaeal communities colored by WWTPs in MAD, TAD, and THP. MAD = Mesophilic AD, TAD = Mesophilic AD, THP = Mesophilic with thermal hydrolysis pretreatment process.

the alpha-diversity between the total and growing bacterial community, compared with the permutational multivariate analysis of beta-diversity (Table S3 and Table S4).

It is well-known that temperature is a very important factor for shaping the microbial diversity and community structure in full-scale digesters (Calusinska et al., 2018; De Vrieze et al., 2015; Sundberg et al., 2013), but this importance is unclear for mesophilic AD at WWTPs. In our study, the temperature range in MAD was small (35.6-39.9) and was only considered to be most important to the total bacterial alpha-diversity in MAD (25%, linear regression, FDR $P < 0.001$, Table S3), but not the alpha-diversity of growing bacteria (16%, FDR $P < 0.001$). This indicates that temperature may not be the most important factor in MAD. Instead, the correlation coefficient of OLR improved significantly by subsetting the growing bacterial alpha-diversity (31%, $P < 0.001$) compared to the total bacterial alpha-diversity (9%, $P > 0.05$). OLR also shaped the microbial community structure (beta-diversity) of growing bacteria (adonis: $R^2 = 21\%$, $P = 0.001$, Table S4). Although OLR is widely accepted as a deterministic factor for any type of AD microbial community (Gou et al., 2014; Goux et al., 2015; Razaviarani and Buchanan, 2014; Xu et al., 2018), our study further strengthened this observation when OLR was only correlated with growing bacteria. Moreover, the biogas yield exhibited strong correlation with the growing bacterial community both on alpha-diversity (46%, $P < 0.001$) and beta-diversity (adonis: $R^2 = 31\%$, $P = 0.001$), as well as archaeal beta-diversity (adonis: $R^2 = 23\%$, $P = 0.008$), supporting the observation that AD performance depends on the activity of the microbial community composition (Vanwonterghem et al., 2014b). Similarly, TAN was observed to be more correlated to growing bacterial alpha-diversity compared with the total bacterial population (Table S3). Regarding the archaeal community, no strong correlation was found between parameters and alpha-diversity in MAD. However, apart from biogas yield, acetate concentration (adonis: $R^2 = 18\%$, $P = 0.04$) was also found to have significant correlation with the archaeal community structure.

Samples from MAD digesters treating only surplus AS and without PS (Fornæs, Mariagerfjord, and Søholt) formed a small separate cluster, compared to MAD treating a mixture of both types of substrates for all bacteria (ANOSIM: $R = 0.44$, $P = 0.001$, Fig. S12A) and for the growing bacteria (ANOSIM: $R = 0.57$, $P = 0.001$, Fig. 3C). The higher dissimilarity of growing bacteria in MAD supports the observation that substrate characteristics (e.g., biodegradability, composition, concentration) from PS shape the growing bacterial community structure (De Vrieze et al., 2015; Sundberg et al., 2013). This is different from the growing bacteria in the three types of

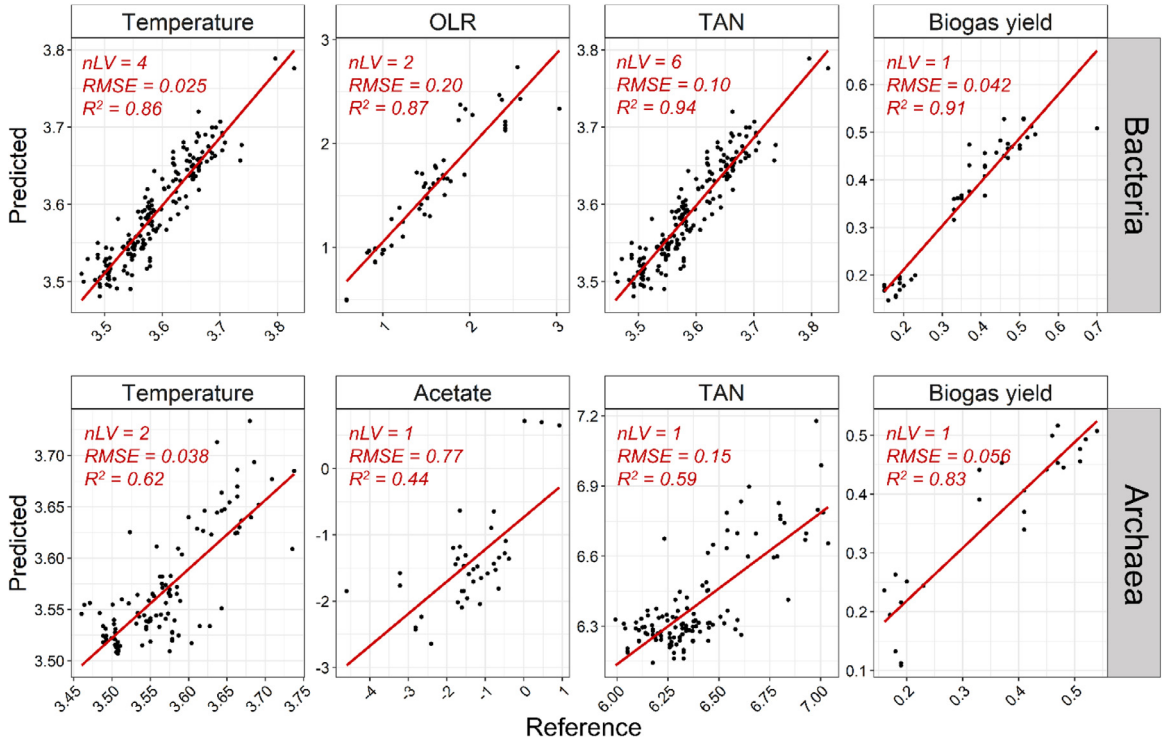

Fig. 5. Prediction plots of main drivers based on bacterial and archaeal communities in MAD by partial least squares regression. MAD = mesophilic AD, OLR = organic loading rate, TAN = total ammonium nitrogen. nLV = number of selected components, RMSE = root mean squared error.

AD, which were mainly driven by operational parameters (Fig. 3B). For all bacterial communities, the relative abundance of 18 of the 25 most abundant species showed a significant difference between the two clusters depending on the feed sludge (Wilcoxon rank-sum test, $P < 0.05$, Fig. S12B). Species non-growing in digesters but abundant in AS (i.e., species belonging to genus *Tetrasphaera, Ca.* Microthrix, or *Dechloromonas*) were found in higher relative abundance in MAD treating only AS compared to MAD treating AS and PS. These results underline that, besides substrate characteristics, the immigrating bacterial load has a strong impact on the total community structure in AD. Additionally, we also observed the influence of feed sludge on the archaeal community (Fig. S13A), shown by the significant difference between the digesters with different feed sludge (ANOSIM: $R = 0.23$, $P = 0.001$). It is interesting to see that species belonging to genus *Methanolinea* were rare in the digesters only fed with AS and not PS and AS (Fig. S13B), but the exact reason is not known.

3.6. Relationship between MAD microbial community and its driving factors predicted by PLS regression model

We applied PLS regression to predict key operational variables and performance parameters and their relationship to the microbial community in MAD. Separate PLS models were built on the bacterial community at species level and archaeal ASVs for each factor (for bacteria: temperature, OLR, TAN, and biogas yield; for archaea: temperature, TAN, acetate, and biogas yield). Very good prediction accuracy was observed for the bacterial community, where the CV R^2 of all four PLS models exceeded 0.85 (Fig. 5). However, none of the models based on archaeal ASVs had an R^2 over 0.80, except for biogas yield (Fig. 5). PLS regression models were also carried out on pH and SRT, since they are important AD parameters (Fig. S14 and S15). The growing bacterial species and archaeal ASVs, which most significantly contributed to each PLS regression model (the contribution was estimated using inferential statistics for corresponding regression coefficients, $P < 0.05$) for both positive and negative contributions, are shown in Fig. 6.

Most growing bacterial species exhibiting significant correlations were represented by novel or previously unclassified taxa (Fig. 6A). Species belonging to the same genus were correlated to different operational or performance parameters, which was the case for two species in the family Anaerolineaceae (midas_s_467 and midas_s_1462 belonging to genus midas_g_467). The positive correlation of midas_s_467 with TAN and biogas yield, and the negative correlation of midas_s_1462 with temperature and OLR, could explain the abundance variability and trend across MAD (Fig. S8). Similar observations were found for three species belonging to genus midas_g_12 (family Prolixibacteraceae, phylum Bacteroidetes). The ecological function of these novel or unclassified species is unknown so the PLS correlation results provide hypotheses that could aid the design of experiments to reveal the role of these undescribed taxa in AD (Peces et al., 2018). Among the known species, *Ca.* Brevefilum fermentans showed a positive correlation with TAN, which may indicate a preference or tolerance to slightly higher TAN conditions. This hypothesis is supported by their genome blueprint indicating that *Ca.* B. fermentans can ferment proteinaceous substrates to VFAs with ammonium as a by-product of protein degradation (McIlroy et al., 2017b). This species is filamentous and was recently discovered to be an indicator species for foam-formation in MAD (Jiang et al., 2021). Other studies have also seen either positive or negative correlations to TAN (e.g., Campanaro et al., 2018), but the application of other reference databases or MAGs without the 16S rRNA gene makes it difficult to compare. Additionally, species belonging to the known syntrophic genera *Ca.* Cloacimonas, *Smithella, Syntrophomonas*, and *Syntrophorhabdus* (Ariesyady et al., 2007a, 2007b; Hao et al., 2016; Narihiro et al., 2015) were mostly negatively correlated with TAN and temperature, thereby confirming the high response of this group to environmental conditions as previously observed by Hao et al., (2016).

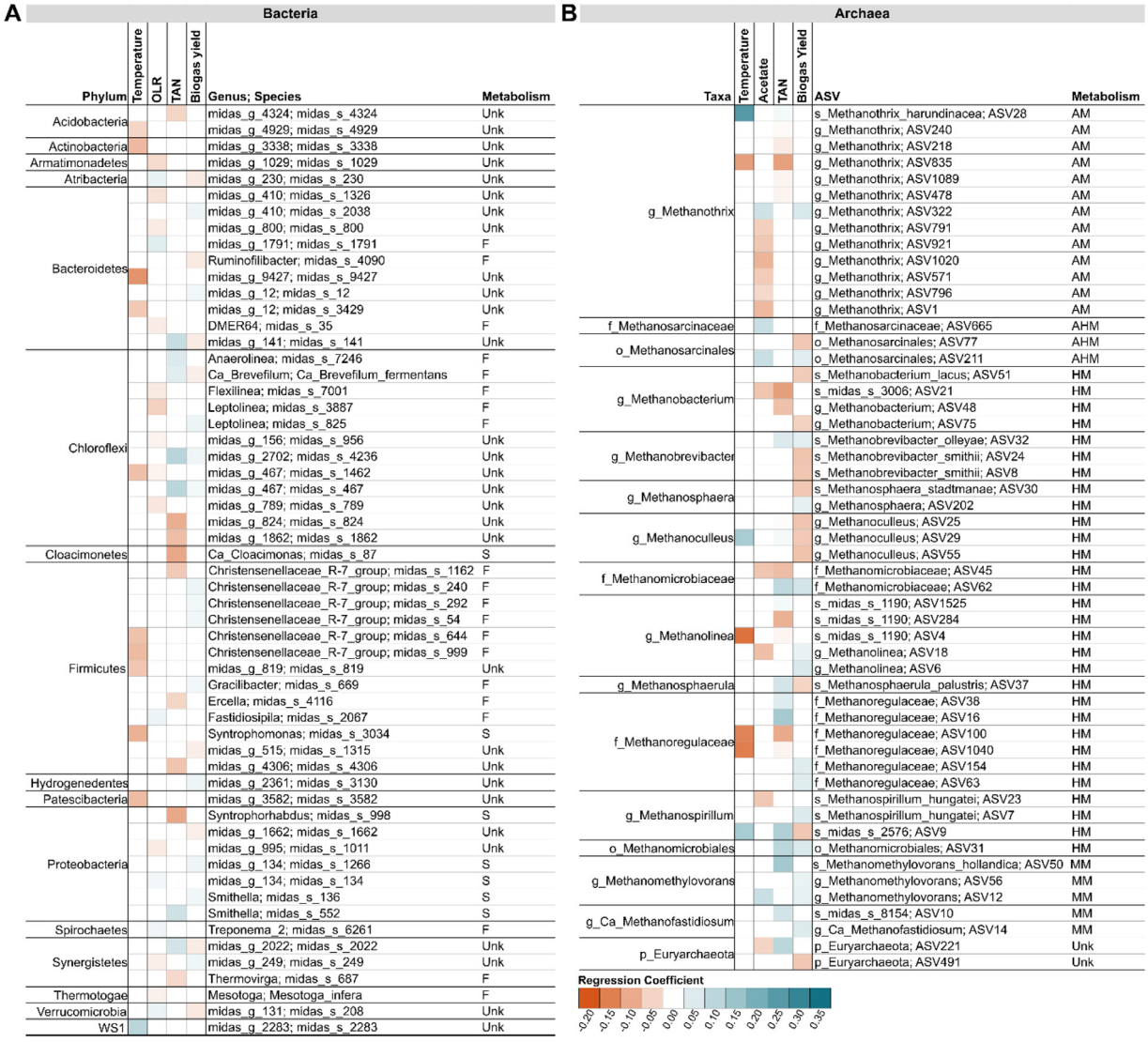

Fig. 6. Partial least squares estimation for main driver for important growing bacterial species (A) and archaeal ASVs (B) in MAD. $P < 0.05$, positive correlation in blue, negative correlation in orange. F = Fermenters, S = Syntrophic bacteria, AM = Acetoclastic methanogens, AHM = Acetoclastic or Hydrogenotrophic methanogens, HM = Hydrogenotrophic methanogens, MM = Methylotrophic methanogens, Unk = Unknown.

Non-growing bacterial species (primarily immigrating with the feed sludge) showed negative correlations to some key parameters (Fig. S16), especially for biogas yield and SRT, suggesting that they were not directly involved in the conversion of feed stocks to biogas and were probably degraded or washed out of the digesters. This is exemplified by *Tetrasphaera* midas_s_5, the most abundant non-growing species in MAD, which was negatively correlated with SRT together with *Tetrasphaera elongata* and midas_s_299. *Tetrasphaera* is very abundant in Danish WWTP (Stokholm-Bjerregaard et al., 2017) and is introduced into the digester with the AS. The negative correlation with the SRT indicates that *Tetrasphaera* was dying off in the digesters despite the potential for surviving or growing under anaerobic conditions as fermenters, and polyphosphate accumulators (Kristiansen et al., 2013).

Correlation results for archaeal ASVs are shown in Fig. 6B. Generally, ASVs belonging to the same genus showed the same trends. For example, ASVs classified to family Methanosarcinaceae and order Methanosarcinales known as acetoclastic and hydogenotrophic methanogens, positively correlated with acetate. This is consistent with other findings (Jetten et al., 1992) where *Methanosarcina* was most abundant in digesters with higher acetate concentration. In contrast, most *Methanothrix* ASVs correlated negatively with acetate, supporting the dominance of *Methanothrix* at low acetate concentrations (Gehring et al., 2016). Many *Methanothrix* ASVs also showed negative correlation with TAN, which is in agreement with studies that show that *Methanothrix* is predominantly found in ADs with low TAN, being notably sensitive to free ammonia inhibition (Capson-Tojo et al., 2020). It is interesting that even small TAN variations as seen here for Danish digesters (603-972 mg N/L, MAD – Table 1) could affect individual *Methanothrix* ASVs in different ways.

For hydrogenotrophic methanogens, different ASVs from the same genus or species showed diverse correlations with AD parameters. The second most abundant archaeal species in MAD belonging to genus *Methanolinea* (midas_s_1190) included three ASVs, which correlated with TAN differently, suggesting that species microdiversity can influence process performance. The negative correlation of many hydrogenotrophic methanogens with biogas yield, such as *Methanoculleus,* could be due to their ability to

survive/compete in ADs operated at sub-optimal conditions (e.g., increased TAN or high VFAs) as recently reviewed by Capson-Tojo et al., (2020).

Overall, the PLS regression models enabled the elucidation of the relationships between important AD parameters and the main drivers shaping AD communities at very high resolution in the digesters surveyed. The results for known taxa agree with present knowledge (mentioned above), thus verifying the robustness of the PLS application in microbial community studies. Importantly, a combination of the PLS regression with species-level microbial data provides the first insight into potential functional importance of several novel or previously unclassified microorganisms, where little or no description of their ecology and physiology is available. Based on our observations, hydrolytic-fermentative bacteria, and acetogenic syntrophs along with archaeal methanogens, all have significant and quantitative relationships with important parameters in MAD. This shows great promise for improved models to optimize functional performance of AD.

Conclusion

A six-year survey of 46 anaerobic digesters located at 22 Danish WWTPs provided a comprehensive overview of typical operational and performance parameters, enabled the high-resolution identification of the AD microbial community at species level, and elucidated relationships between specific taxa and key parameters in AD. The anaerobic digesters surveyed were running stably but operated at low intensity, a common feature across digesters at WWTP. Non-growing species migrating from the feed sludge were abundant in mesophilic and thermophilic AD, but did not seem to contribute to the functionality of AD. Many growing species belonged to previously unclassified taxa that could only be identified using the MiDAS 3 taxonomy, and their physiological and ecological roles in AD remain to be described. The microbial community of the three types of AD surveyed (mesophilic, thermophilic, and thermal hydrolysis pre-treatment-mesophilic) showed high stability within plants, forming separate clusters for all bacteria, growing bacteria, and archaea depending on the operational parameters. The variations of growing bacteria within mesophilic digesters were related to organic loading rate, ammonium concentration, feed sludge characteristics, and biogas yield. Multiple correlations between growing bacteria and archaea at species level and key parameters were found. This study provides a foundation for linking species-level identity of novel or poorly described taxa to studies of their function and dynamics in full-scale ADs across studies. This is a necessary and important step towards improving surveillance, optimization, performance, and management of ADs at WWTPs.

Authors' contributions

PHN and CJ conceived and designed the work. CJ, MP, and PHN wrote the manuscript. CJ, MP, SK, and EY performed bioinformatic analysis, statistical analysis, and data visualization. KSA and MSD provided bioinformatics support. MHA, MN, RHK, and LH performed data collection, sampling, and lab procedures. JH and AAH contributed to sample and plant record collection. CJ, MP, PHN, SK, MN, and MSD contributed to data interpretation. All co-authors read and approved the final manuscript.

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Availability of data and materials

The raw amplicon sequences generated and analyzed during the current study were deposited in NCBI under the bioproject accession number PRJNA637463.

Appendix information

Appendix A: Supplementary tables and figures

Appendix B: Characterization of key parameters of anaerobic digestions

Appendix C: Digester to influent relative read abundance ratios for each ASV

Appendix D: Anaerobic digester biomass mass-balance on identifying growing and non-growing microorganisms

Declaration of Competing Interest

The authors declare that they have no competing interests

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.116871.

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