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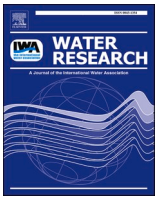

Sludge retention time in anaerobic digestion affects Archaea by a cascade through microeukaryotes

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ABSTRACT

Anaerobic digestion is a crucial process for treating organic waste, such as wastewater sludge, agricultural residues and food waste. While the influence of physicochemical parameters on the prokaryotic community composition in anaerobic digesters has been extensively characterized, the role of biotic interactions in shaping the prokaryotic communities remains poorly understood. This study addresses this knowledge gap by analyzing the complete active microbiome of nine full-scale anaerobic digesters. Our findings reveal that eukaryotes, consisting primarily of protists and fungi, account for approximately 40 % of RNA sequence reads alongside dominant Archaea, indicating their substantial role in the digestion process. Our results suggest that the chosen sludge retention time during anaerobic digestion indirectly affects the archaeal community composition and thus treatment efficacy by cascading through eukaryotes, highlighting their integral role in the system. This study highlights the critical role of eukaryotes in regulating prokaryotic communities and their indirect contribution to the optimization of anaerobic digestion efficiency.

1. Introduction

Anaerobic digestion is a biological process in which organic matter is decomposed in an oxygen-free environment by a microbial consortium, producing biogas and nutrient-rich digestate (Holm-Nielsen et al., 2009; Pain and Hepherd, 1985). Each year, substantial quantities of waste are produced from municipal wastewater treatment, agriculture, forestry, food production, and other industries, posing significant environmental and health risks. Anaerobic digestion offers a sustainable approach to waste management, simultaneously enhancing energy production by converting biomass waste into bioenergy products (Yu and Schanbacher, 2010). Large bioreactors, or anaerobic digesters (ADs), are used for anaerobic digestion, which is generally cost-effective and environmentally friendly. Nonetheless, ADs present challenges, such as system instability and limited bioenergy efficiency (Xu et al., 2018). Currently, a deeper understanding of microbial community composition and function within ADs is recognized as crucial for optimization (Yang et al., 2022).

Most studies to date have focused on prokaryotic community dynamics and interspecies interactions in ADs, examining factors such as substrate composition (Xie et al., 2007), digester operating conditions (Castillo M. et al., 2006), eco-thermodynamics (Nobu et al., 2020), metabolic inhibition (Glenn, 1976), and quorum sensing (Anburajan et al., 2023). Accordingly, four main phases of anaerobic digestion are characterized – hydrolysis, acidogenesis, acetogenesis, and methanogenesis – all of which are recognized to be facilitated by distinct prokaryotic species (Gujer and Zehnder, 1983). During hydrolysis, complex organic waste is broken down into monomers, such as amino acids, long-chain fatty acids, and sugars, by extracellular enzymes from hydrolytic Bacteria (Gujer and Zehnder, 1983; Menzel et al., 2020). This step may be inhibited by the accumulation of intermediates, including amino acids and sugars, due to suppressed enzyme production and activity (Glenn, 1976). The soluble monomers are then used as substrates by acidogenic Bacteria, which convert them into volatile fatty acids (VFAs), acetate, hydrogen (H_2), and carbon dioxide (CO_2) (Gujer and Zehnder, 1983). Next, acetogenic Bacteria further degrade these products, also producing acetate, H_2, and CO_2. Methanogenic Archaea complete the final step by converting acetate to CO_2 and methane through acetotrophic methanogenesis or by reducing CO_2 with H_2 to methane and water via hydrogenotrophic methanogenesis. These stages are interdependent, with methanogens relying on acidogens and acetogens for substrates, and acetogens depending on methanogens to maintain low H_2 partial pressure through interspecies H_2 transfer (Boone et al., 1989; Harper and Pohland, 1986; Liu et al., 2020; Winter and

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Knoll, 1989).

Next to prokaryotic communities, anaerobic environments are also inhabited by eukaryotes. Several studies have characterized eukaryotic communities in anoxic environments, revealing they are predominantly composed of protists and fungi (Fenchel and Finaly, 1990; Hackstein et al., 1999; Hirakata et al., 2019). Anaerobic protists and fungi can directly degrade organic matter, accelerating hydrolysis (Mishra et al., 2020; Priya et al., 2007). For example, in the rumen, the yeast *Candida* assimilates fatty acids (Ando et al., 2006), while ciliates break down complex carbohydrates to produce VFAs, lactate, CO_2, and H_2 (Li et al., 2022; Williams, 1986). In anaerobic reactors, ciliates have been observed to consume particulates directly, thus facilitating the solubilization of particles, which is generally considered a rate-limiting step during treatment (Priya et al., 2007). Despite this knowledge, eukaryotic functions in ADs are poorly understood (Bareither et al., 2013; Hirakata et al., 2016; Li et al., 2021; Matsubayashi et al., 2017; Mishra et al., 2020; Priya et al., 2007; Staley et al., 2018; Yang et al., 2022).

A variety of interactions, including competition, syntrophy, and predation, exist among bacterial, archaeal, and eukaryotic communities in waste decomposition (Yang et al., 2022). Protistan predation is known to exert top-down control on prokaryotic abundance, community structure, function, and diversity across ecosystems (Hirakata et al., 2016; Jürgens et al., 1999). Although little is known about how predation affects anaerobic microbial communities, one study found that anaerobic ciliates significantly modified prokaryotic community structure and function, particularly methanogenesis, in upflow anaerobic sludge blanket reactors (Hirakata et al., 2016). There is further evidence that certain protists selectively prey on Archaea (Ballen-Segura et al., 2017). However, these studies investigated the effects of individual species. Given the complexity of species interactions, which often involve more than one-to-one relationships, examining cross-kingdom interactions at the community level may reveal a higher level of ecological complexity. Here, we hypothesize that eukaryotes as a community play a critical role in shaping the prokaryotic community composition and thus process efficacy in ADs.

The aim of this study is to identify and disentangle the biotic and abiotic factors influencing microbial community composition in full-scale ADs. This includes mapping the most abundant active prokaryotic and eukaryotic species and investigating interactions between them. To achieve this, we re-analyzed a publicly available metatranscriptomic dataset from previously studied ADs (Mei et al., 2016; Nobu et al., 2020), gaining comprehensive insights into the microbial community composition, including Archaea, Bacteria, and eukaryotes.

2. Material and methods

2.1. Sample collection and sequencing

We analyzed data from previously investigated ADs (Mei et al., 2016; Nobu et al., 2020). While Mei et al. (2016) used 16S rRNA gene sequencing to characterize the microbial communities, Nobu et al. (2020) used the same sludge samples for RNA extraction, which were here analyzed for the whole active microbiome. In brief, sludge samples were selected in triplicate from ADs at nine full-scale municipal wastewater treatment plants (WWTPs) across two countries (called ADurb, USST, USRA, USMO, USDV (USA) and JPTR, JPNA, JPMR and JPHW (Japan)): Eight of the WWTPs were operated with the conventional primary-secondary (activated sludge) treatment scheme, while one plant (USRA from the USA) was only configured with primary treatment before AD treatment (Table 1). Most ADs operated at mesophilic temperatures (approximately 35 °C), except for JPTR, which ran at a slightly elevated temperature (approximately 40 °C), JPHW and USRA, which were operated at slightly lower temperature (< 30 °C), and JPMR, which was operated at thermophilic conditions (> 50 °C). Additionally, three ADs (JPHW, JPNA and USDV) were operated in series (with the same retention time) with the first digester treating primary/secondary clarifier sludge, and the second treating sludge produced by the first. ADurb, JPNA, and USST also treated non-sewage-derived waste, including food waste, green waste and sludge from other sources. Different sludge samples were collected at different time points (e.g. 1 month apart), as documented in Mei et al. (2016). To assess active gene expression, RNA was extracted and analyzed through metatranscriptomic sequencing, using the Illumina HiSeq-2000 1 TB platform. For further details on the sampling process and sequencing, see Mei et al. (2017) and Nobu et al. (2020).

2.2. Data processing

The raw metatranscriptome data from Nobu et al. (2020) are available on the Joint Genome Institute Integrated Microbial Genome and Metagenome (IMG/M) database (project IDs can be found in Nobu et al., 2020). The sequence data files were downloaded in compressed SRA format using the SRA toolkit v.3.0.1 (SRA Toolkit Development Team, 2022) with prefetch, then converted to the standard FASTQ format using fasterq-dump. To address potential biases introduced during the sampling, RNA extraction, and sequencing processes, the quality of the FASTQ files was controlled using FASTQC v0.11.9 (Andrews, 2010). To minimize potential biases, the paired-end FASTQ files were trimmed using Trim Galore v.0.6.4_dev (Krueger, 2015). Specifically, bases with a quality score below 30 were removed, and the last ten bases were trimmed from the 3′ end. Mothur v.1.45.3 (Schloss et al., 2009) was employed to assemble the overlapping paired-end reads into a single contiguous sequence (contig) with a maximum expected error threshold and maximum ambiguity of 2 bp. SortMeRNA v.4.3.4 (Kopylova et al., 2012) was used to filter the contigs for rRNA, using several reference databases (e.g., SILVA 16S, 23S, 18S, and 28S). The sorted rRNA contigs were then assembled into longer contigs using Trinity v.2.14.0 (Grabherr et al., 2011). The contigs were subsequently utilized for taxonomic assignment by screening with BLASTN+ 2.10.0 (Camacho et al., 2009) against the ribosomal gene sequence databases PR^2 v4.13.0 (Guillou et al., 2012) for eukaryotes and SILVA 138 Ref NR. 99 (Pruesse et al., 2007) for prokaryotes. The following filtering criteria were employed to ensure precise taxonomic assignment: (i) Only the best hit of the query sequence to a reference sequence of the respective database was retained and (ii) an expectation value for saving hits was set to a threshold of $1e^{-30}$ for both databases. After assembling the reads into

Table 1
Metadata for anaerobic digester samples. The table provides key operational and environmental parameters for the AD samples analyzed in this study.

AD name	Sample ID	State	Country	Operation temperature	Feed sludge type	Sludge retention time	pH
ADurb	SRR5466728, SRR5467090, SRR5467091	Illinois	USA	35	Primary+ Secondary	26	7.1
USST	SRR6960509, SRR6960551, SRR6960797	Illinois	USA	35	Primary+ Secondary	23	7.1
USRA	SRR6960540, SRR6960549, SRR7976299	Illinois	USA	30	Primary	20	7.5
USMO	SRR6960507, SRR7976295, SRR7976300	Illinois	USA	35	Primary+ Secondary	72	7.3
USDV	SRR6960550, SRR7976301, SRR7976310	Illinois	USA	35	Primary+ Secondary	NA	7.3
JPTR	SRR6960803, SRR7976356, SRR7976357	Kansai	Japan	40	Primary+ Secondary	27.8	7.3
JPNA	SRR6960802, SRR7976323, SRR7976324	Chubu	Japan	35	Primary+ Secondary	39.6	NA
JPMR	SRR6960800, SRR6960801, SRR7976327	Kanto	Japan	50	Primary+ Secondary	20	7.6
JPHW	SRR6960799, SRR7976325, SRR7976326	Kyushu	Japan	30	Primary+ Secondary	23.1	7.5

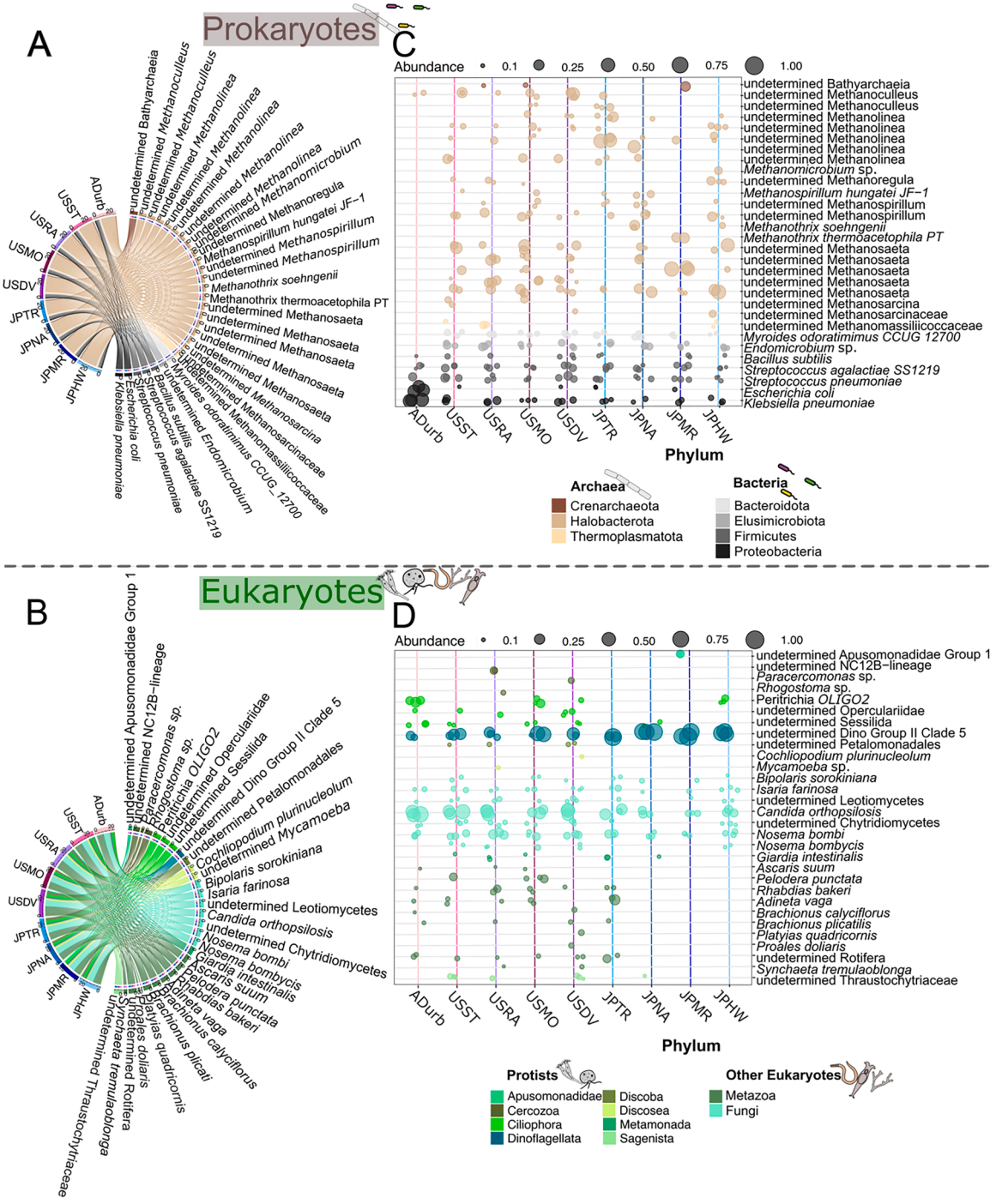

Fig. 1. Microbial community composition in anaerobic digesters. (*A* + *B*) Chord charts showing the 30 most abundant **(A)** prokaryotic and **(B)** eukaryotic species with a relative abundance >1 %. Each line represents the presence of the annotated species in the corresponding AD, and the scale refers to the sum of connections between sampling location and found number of species. **(C + D)** Bubble plots showing the relative abundance of the 30 most abundant **(C)** prokaryotic and **(D)** eukaryotic species.

contigs using Trinity, the original reads were mapped back to the assembled transcripts using Bowtie2v.2.4.1 (Langmead and Salzberg, 2012). Backmapping allowed us to validate the accuracy and completeness of the assembled contigs. Salmon v.1.9.0 (Patro et al., 2017) was employed to quantify the Bowtie2 read counts of each Trinity transcript. The "Number of Reads" provided by Salmon was used as read counts. Read counts of distinct transcripts were then binned if being assigned to the same species for all further analyses. Streptophyta, chloroplasts, and macroscopic Metazoa were removed as they are presumed to be remnants of dying material within the sludge derived from macroscopic organisms, rather than actual active organisms. The microbial community was distinguished in prokaryotes (Bacteria and Archaea) and eukaryotes (protists, fungi, and microscopic Metazoa). To minimize the impact of background noise, species with read counts below four were excluded. To consider only data of high quality, contigs shorter than 400 bp were removed, as they were considered to be too fragmented or the result of artifacts. The data were processed in R v.4.3.2 (2023–10–31), making use of the following packages: lattice v. 0.22–6 (Deepayan, 2008), tidyr v. 1.3.1 (Wickham et al., 2024), dplyr v. 1.1.4 (Wickham et al., 2023), tidyverse v. 2.0.0 (Wickham et al., 2019),

circlize v. 0.4.16 (Gu et al., 2014), rlist v. 0.4.6.2 (Ren, 2021), funrar v. 1.5.0 (Grenié et al., 2017) and vegan v. 2.6–6.1 (Oksanen et al., 2024). Visualization was performed using ggplot2 v. 3.5.1 (Wickham, 2016), ggthemes v. 5.1.0 (Arnold, 2024), and kableExtra v. 1.4.0 (Zhu, 2024). To determine whether the sequencing depth was adequate to capture the diversity in each sample, rarefaction curves were generated (function rarefy, package vegan). The rarefaction curves (Hurlbert, 1971) showed an adequate saturation in sequencing depth, indicating a high level of comparability between samples and thus no rarefying was required (Supplementary Figure S 1).

2.3. Community analyses

To gain an overview of the microbial community composition at varying levels of richness and evenness, alpha diversity was assessed using Hill Numbers (Hill, 1973) for $q = 0$ (species richness), $q = 1$ (exponential of Shannon entropy), and $q = 2$ (Inverse Simpson index) (function renyi, package vegan, Supplementary Figure S 2). These alpha diversity indices were used to assess differences within each sample. Subsequently, chord charts were constructed to provide an overview of the most abundant species and their distribution (Fig. 1*A* + *B*). Bubble plots were generated to analyze the relative abundance of the most prevalent taxa with an abundance greater than 1 % (Fig. 1C + D). Principal Coordinate Analysis (PCoA) (Gower, 1966) based on Bray-Curtis dissimilarities was employed to quantify the community structure of archaeal, bacterial, and eukaryotic communities. For each community, the first axis (PCoA1) of the eigenvectors, which explained 56 % for eukaryotes, 29 % for Bacteria, and 19 % of the variation for Archaea served as a measure of community structure (function pcoa, package ape, Supplementary Table S 3, Table S 4, Table S 5). PERMANOVA (Anderson, 2001) was used to disentangle the influence of abiotic factors and biotic factors, represented by the PCoA eigenvectors of the microbial communities, on the composition of archaeal, bacterial and eukaryotic communities (function adonis, package vegan, Fig. 2). To further investigate the correlations between the factors influencing the community composition, multivariate dispersion based on normalized and Bray-Curtis transformed data was calculated and an NMDS plot for each community composition (archaeal, bacterial and eukaryotic) was generated (metaMDS function, package vegan, Fig. 3A–C). No potential outliers were identified. A two-dimensional NMDS was employed for ease of interpretation, but for the archaeal community, a three-dimensional NMDS with a lower stress value (0.198) was also obtained to enhance ordination accuracy (Supplementary Figure S 5). To determine the influence of eukaryotic and prokaryotic species on the AD microbiome, the eukaryotic species were fitted onto the archaeal and bacterial NMDS plots, and the prokaryotic species were fitted onto the eukaryotic NMDS plot. The ten species exhibiting the most robust correlations, as indicated by the highest R^2-values, were displayed (function envfit, package vegan, Fig. 3A–C). To test which abiotic factors shape the microbial communities the measured abiotic factors (operation temperature, sludge retention time, and pH) were fitted onto the ordination (Fig. 3A–C). To further identify potential correlations between metabolic capacities we accessed particularly prominent physiological capabilities of respective prokaryotic communities, as determined by Nobu et al. (2020), and also plotted them onto the ordination. In brief, Nobu et al. (2020) constructed a matrix containing the species-level metagenome-assembled genomes (MAG) clusters with values representing their corresponding presence, absence, or diversity (number of protein families or number of pathways present in the target MAG cluster). Subsequently, all metagenomes were annotated and specifically analyzed for a range of processes, including sugar degradation, amino acid degradation, electron transduction mechanisms, respiration (in the presence of oxygen and nitrogen species), H_2 metabolism, formate metabolism, and polymer hydrolysis (glycosyl hydrolase, extracellular peptide, and extracellular lipase families). Curation included an analysis of the functional domains, signal peptides, transmembrane domains, carbohydrate-active enzymes, peptidases, lipases, and hydrogenases (see Nobu et al., 2020 for more details).

Given the observed biological effects, variance partitioning analysis (Anderson and Cribble, 1998; Cushman and McGarigal, 2002; Økland, 2003) was conducted to disentangle interactions (function varpart, package vegan). To test differences in specific species abundance between ADs from different regions, we used Welch Two Sample *t*-tests (function t.test, package stats) and assessed differences in community composition between ADs from the USA and Japan, via PERMANOVAs on both the eukaryotic and prokaryotic communities (function adonis, package vegan). Network analysis was conducted following alpha- and beta-diversity analysis to further elucidate and enumerate the relationships among microbial communities (Fig. 4). The network analysis was performed using the Sparse and Compositionally Robust Inference of Microbial Ecological Networks (SPIEC-EASI) pipeline (Kurtz et al., 2015) with non-normalized count data as the input. The networks were generated using the sparse Meinshausen-Bühlmann neighborhood selection (mb) method (function spiec.easi, package SpiecEasi). In consideration of the density of the networks, the default scaling factor determining the minimum sparsity (lambda.min.ratio) was set to 0.001

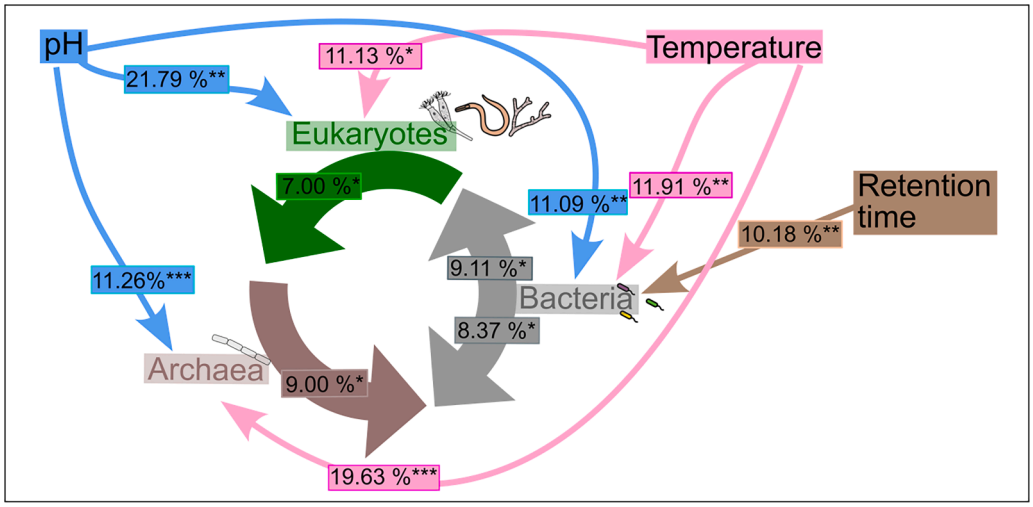

Fig. 2. Overview of explained variation in prokaryotic (Bacteria and Archaea) and eukaryotic community composition. Arrows indicate significant influences on the eukaryotic and prokaryotic communities according to PERMANOVA, respectively. Percentages represent the R^2-values of explained variation. Asterisks represent significant codes: * for $p \leq 0.05$, ** for $p \leq 0.01$, and *** for $p \leq 0.001$.

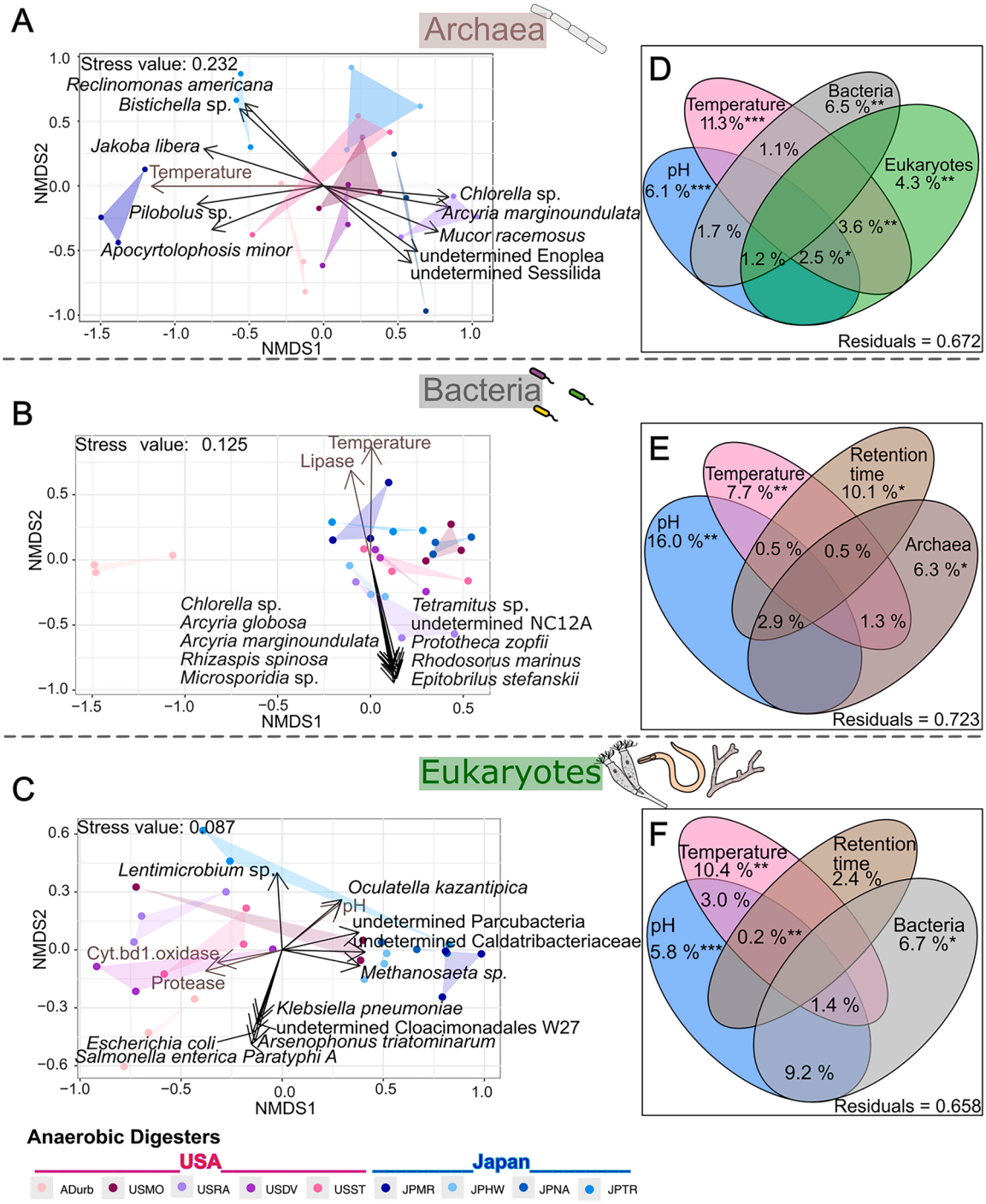

Fig. 3. Influences on prokaryotic (Archaea and Bacteria) and eukaryotic community composition in anaerobic digesters. (A-C) The non-metric multidimensional scaling (NMDS) plots showing the multidimensional data in two dimensions. Each point represents one independent sample. The proximity of two points (samples) is indicative of a high degree of similarity in terms of their microbial communities. The color of each point and polygon indicates the sampled AD. The arrows showing the direction of the fitted factors which were displayed by a maximum estimated p-value of 0.05. Weak predictors have shorter arrows than strong predictors. **(A)** The archaeal community is shown, with the significant abiotic shaping force, temperature, and the eukaryotic shaping species, highlighted. **(B)** The bacterial community is displayed, with the significant abiotic shaping factors, temperature and lipase as well as the shaping eukaryotic species. **(C)** NMDS plot of the eukaryotic community composition shows the significantly shaped abiotic factor pH and the prokaryotic (archaeal and bacterial) shaping species. **(D-F)** Venn diagrams showing the partitioned variance explaining the composition of the **(D)** archaeal, **(E)** bacterial and **(F)** eukaryotic community. Significant effects according to ANOVA are marked by stars: * for $p \leq 0.05$, ** for $p \leq 0.01$, and *** for $p \leq 0.001$. Note that here **(D)** also a significant effect of the eukaryotic community composition on the archaeal community was found.

and the parameter nlambda was set to 50, with the objective of achieving a stability threshold closer to 0.05. The resulting network was visualized using Cytoscape v. 3.8.0 (Shannon et al., 2003). To facilitate visualization, the complexity of the network was reduced by aggregating nodes at the taxonomic order level, with edges representing the number of species with detected associations (Supplementary Figure 4). Further, taxonomic orders were grouped into functional groups - defined as fungi, microscopic Metazoa, protists, Bacteria and Archaea - with their

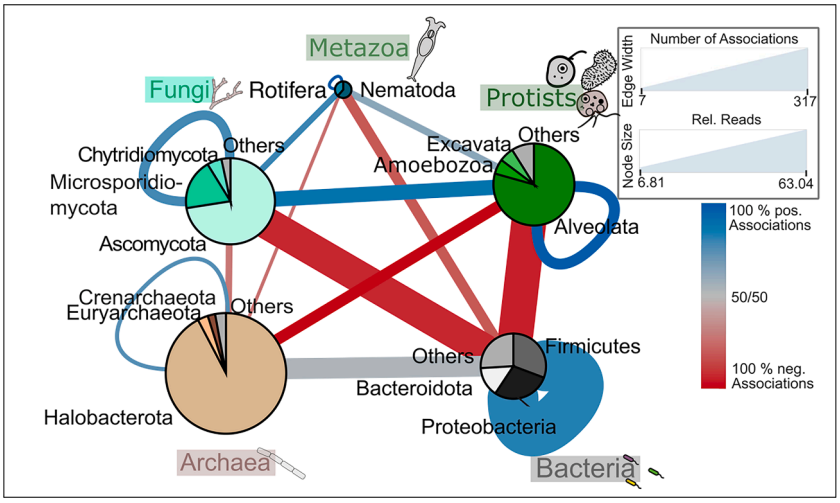

Fig. 4. Specific microbial associations in the anaerobic digester microbiome, shown by the summarized core co-occurrence network. The associations are summarized for different taxonomic groups, i.e. fungi, microscopic Metazoa, protists, Bacteria and Archaea. Node size is proportional to the relative number of reads for prokaryotes and eukaryotes. Edge thickness indicates the number of associations between taxonomic groups. The color codes for the ratio of negative (red) to positive (blue) associations.

respective nodes displayed as pie charts, reflecting the relative abundance of each order (Fig. 4). The size of the nodes was adjusted in accordance with the number of reads, while the thickness of the edges corresponded to the number of individual connections between the orders. The color of the edge indicated the sign of the association.

3. Results

3.1. Eukaryotic communities in ADs are more diverse than prokaryotic communities

Following quality filtering and Trinity contig assembly, a total of 110,615,484 SSU rRNA contiguous sequence reads were identified. These sequence reads were assigned to 6185 distinct species. Prokaryotes represented the majority of sequence reads and species richness with approximately 62.8 % and 66.6 %, respectively. Among prokaryotes, only a small number of archaeal species (approx. 6.75 % of all prokaryotic species) contributed to about 61.45 % of all prokaryotic reads (Fig. 1, Supplementary Table S 2). Eukaryotes (37.2 % of all reads) were dominated by protists with approximately 60.2 % of all eukaryotic species contributing about 45.7 % of all eukaryotic reads. Fungi accounted for 31.1 % of all eukaryotic species representing approx. 43.5 % of all eukaryotic sequence reads and microscopic Metazoa represented approx. 8.7 % of the eukaryotic species richness and contributed approximately 10.7 % of all eukaryotic sequence reads.

Given that the vast majority of sequence reads were represented by only a few species-level taxa (hereafter called species) across all ADs (Fig. 1*A + B*), we specifically investigated the relative abundance of the 30 most abundant prokaryotic and eukaryotic species (Fig. 1C + D). Of the 30 most abundant prokaryotic species, 21 species were Archaea belonging to the phylum Halobacterota, three species represented Firmicutes (Bacteria), and two species Proteobacteria (Bacteria) (Fig. 1A + C). A single species was identified within each of the following taxonomic groups: Crenarchaeota (Archaea), Thermoplasmatota (Archaea), Bacteroidota (Bacteria), and Elusimicrobiota (Bacteria) (Fig. 1A + C). Twenty-six of the top 30 species were identified in all nine ADs (Fig. 1A). Overall, species from the archaeal phylum Halobacterota exhibited the highest relative abundance in all ADs, except for ADurb, where species of the Proteobacteria dominated (Fig. 1C).

In contrast to prokaryotes, which were heavily dominated by species of only one phylum, the thirty most abundant eukaryotes were more evenly composed of 13 protistan, nine metazoan, and seven fungal species. Within protists, three of the most prevalent species belonged to the phyla Ciliophora and Cercozoa, while two were assigned to the phylum Discosea. Apusomonadidae, Dinoflagellata, Discoba, Metamonada and Sagenista were each represented by a single species. An undescribed species of the Dino-Group II Clade 5 (Dinoflagellata) exhibited the highest relative abundance in many ADs, with up to 80 % of eukaryotic sequence reads in ADs from Japan (Supplementary Figure S 4). Additionally, the yeast *Candida orthopsilosis* was also found in high abundance in many ADs, with a higher relative abundance of approximately 32 % in the USA compared to 10.2 % in Japan (Welch's *t*-test: $p < 0.001$, Supplementary Figure S 4, therein referred to as "Fungi").

Differences in community composition between ADs from the USA and Japan were evident in both eukaryotic and prokaryotic communities. However, regional variation was more pronounced in eukaryotes (PERMANOVA: $R^2 = 0.421$, $p < 0.001$) compared to prokaryotes (PERMANOVA: $R^2 = 0.098$, $p < 0.002$) (Fig. 1C+D).

3.2. Biotic and abiotic factors contribute similarly to community composition variation

Anaerobic digestion is monitored and steered by changes in abiotic factors, such as sludge retention time (SRT), operation temperature, and pH, which were given special attention during our analyses (Table 1). However, the main interest here is the investigation of biotic influences as a shaping factor and a response to environmental changes. The majority of environmental factors exhibited a similar degree of explanatory power comparable to that of biotic factors (Fig. 2). Measured abiotic factors in the tightly controlled ADs collectively explained approximately 30 % of the variation in respective community composition, while individual biotic factors accounted for around 10 % of the variation of eukaryotes, Archaea, and Bacteria, respectively (Fig. 2).

To further explore and disentangle abiotic and biotic influences, we investigated how biotic influences correlate and interact with abiotic influences while shaping the community compositions of Archaea, Bacteria, and eukaryotes (Fig. 3). The technical replicates grouped closely, confirming that the respective microbial communities in each AD were stable and consistent across repeated sampling and processing. We show that eukaryotic species shaped the archaeal community in dependence on environmental temperature (Fig. 3A). To further disentangle the effects of temperature and biotic effects on the archaeal community composition, variance partitioning was employed, which revealed a significant overlap of 3.6 % of the explained variation by

temperature and eukaryotes, next to independent effects of pH and the bacterial community (Fig. 3D).

For the bacterial communities, NMDS plotting revealed a strong influence of temperature and lipase activity, i.e. the degradation of lipids, both following the same axis (Fig. 3B). The bacterial communities in the AD ADurb were distinct, which was not explained by measured environmental factors (Fig. 3B, see also Fig. 1C). Temperature negatively correlated with all ten shown eukaryotic species.

Variance partitioning revealed that the archaeal community structure exerted an independent effect on the bacterial community composition, explaining 6.3 % of the variation (Fig. 3E, Supplementary Table S 3).

For the eukaryotic community composition, pH negatively correlated with cytochrome bd oxidase (a terminal oxidase for aerobic respiration) and protease (degradation of proteins) (Fig. 3C). The eukaryotic communities from the US American ADs correlated with enzyme activity, whereas eukaryotic communities from Japanese ADs correlated with pH. The bacterial community composition influenced the eukaryotic community by 6.7 % (Fig. 3F).

3.3. From community analyses to specific microbial interactions

To lift our analyses from the community level to the species level, network analyses were performed (Fig. 4). In total, 1277 edges were identified, representing associations between functional groups. The predominant associations were observed among Bacteria, with 317 edges (~24.8 %), followed by those between Bacteria and protists (214 edges, ~16.8 %), Bacteria and fungi (194, ~15.2 %), and Bacteria and Archaea (122, ~9.6 %) (Fig. 4). Associations involving microscopic Metazoa were fewer in comparison to the other functional groups.

Functional groups showed distinct differences in the sign of associations. The analyses revealed predominantly negative associations between protists and Bacteria (67.3 %), suggesting strong predator-prey interactions between both groups. Similarly, predominantly negative associations were observed between fungi and Bacteria (66.5 %), indicating strong competition between these groups. In contrast, predominantly positive associations were identified among the bacterial community (75.1 %) and between protists and fungi (78.8 %).

4. Discussion

This study reveals that AD microbial communities are prokaryote-dominated, supporting their recognized role as primary agents in anaerobic digestion (Supplementary Table S2; Godon et al., 1997). The most prevalent prokaryotes include members of the archaeal phylum Halobacterota, notably *Methanothrix* (formerly Methanosaeta) and other Halobacterota/Euryarchaeota species such as *Methanoculleus* and *Methanospirillum*, which contribute to terminal end-product degradation (Fig. 1; Nobu et al., 2020). Among Bacteria, the classes Bacilli, Gammaproteobacteria, and Bacteroidales (within Firmicutes, Proteobacteria, and Bacteroidota, respectively) rank among the top 30 most abundant taxa, playing key roles in fat and carbohydrate degradation (Li et al., 2013; Fig. 1). Consistent with prior research, we identified a core prokaryotic community, with 26 of the top 30 species detected across all nine ADs (Chouari et al., 2005; Fig. 1).

Historically, eukaryotic communities in ADs have been overlooked, primarily due to difficulties in accurately detecting microeukaryotes. Biases in universal eukaryotic primers can exclude entire phyla, such as Amoebozoa (Fiore-Donno et al., 2016; Hong et al., 2009; Jeon et al., 2008). Moreover, DNA-based methods may not reliably reflect active community members, potentially distorting interpretations of community structure (Zakrzewski et al., 2012). Using ribosomal RNA from metatranscriptomes, which better represents active community members due to rRNA's correlation with microbial activity (Wagner, 1994), we found that active eukaryotes, primarily protists, constitute 40 % of the sequence reads in ADs (Supplementary Table S 2).

Via whole microbiome analyses, this study reveals a complex interkingdom loop among Bacteria, Archaea, and eukaryotes within ADs (Fig. 2). Unlike temperature and pH, which can fluctuate due to biochemical processes by the microorganisms' activity within the reactor, SRT can be more precisely controlled through active management of sludge input and removal. We show that changes in SRT significantly shift bacterial communities (Lee et al., 2011) with subsequent effects on both archaeal and eukaryotic composition. Our findings indicate that subsequently, eukaryotes shape the archaeal community through primarily negative correlations. Finally, Archaea influence bacterial community structure through a mix of positive and negative associations. This interkingdom loop, modulated by SRT, reveals that shifts in the archaeal community cascade through eukaryotes, highlighting their integral role in the system. The indirect effects of SRT through this interkingdom loop represent a novel finding, complementing the known impacts of temperature and pH.

The question arises by which mechanisms do eukaryotes shape the prokaryotic core microbiome of ADs. These mechanisms can be categorized into several fundamental ecological interactions: predation, competition, and symbiosis/syntrophy. While AD operational controls and prokaryotic self-regulation are influential, the interplay between eukaryotes and prokaryotes involves complex relationships that collectively impact microbial community structure and function. It is important to acknowledge that predator-prey dynamics can result in positive and/or negative associations requiring a careful interpretation of associations.

Predation emerge as a primary interaction type, where eukaryotes, as voracious microbial predators, exert top-down control in these microbial food webs, supported by numerous negative associations of eukaryotes with bacterial and archaeal species (Fig. 4). Despite this, predation appears relatively unselective, as indicated by its limited impact on overall bacterial community composition (Fig. 2). Sessile peritrich ciliates, especially species related to *Campanella umbellaria, Opercularia* sp., and the yet undescribed species assigned to Sessilida_X, rank among the 30 most abundant eukaryotes. Known for their high filtration rates in activated sludge, sessile peritrich ciliates significantly contribute to prokaryotic population regulation and water clarification (Dubber and Gray, 2011; Macek, 1989; Martín-Cereceda et al., 2001). Although predation on Archaea by these ciliates is undocumented, it is plausible given their low selectivity in particle filtration.

A key question is why eukaryotes specifically alter archaeal composition without similarly affecting Bacteria. Rapid bacterial turnover may allow these communities to swiftly recover from predation. Certain taxa, such as acetogenic Bacteria, display high metabolic flexibility, allowing efficient growth across diverse conditions and thus resilience to protistan predation (Lever, 2012). In contrast, slower-growing Archaea, including dominant methanogens that rely on energetically less favourable pathways (Schink, 1997), may be more vulnerable to predation in energy-limited environments. This growth disparity may explain why bacterial composition remains stable while archaeal communities shift under predation.

In addition to predation, competition between prokaryotes and eukaryotes shapes community dynamics. As Bacteria initiate AD by converting organic matter into monomers, which are further degraded into VFAs, hydrogen, and acetate (Mata-Alvarez, 2002), some eukaryotes – particularly fungi such as *Candida* and the fungus-like non-photosynthetic green alga *Prototheca* – compete with acidogenic and acetogenic Bacteria for monomers and VFAs (Anderson, 1945; Bernalier et al., 1992). Furthermore, most predatory protists consume preferentially smaller food particles like prokaryotes (Estermann et al., 2023; Geisen et al., 2016), allowing fungi an advantage in competing for shared resources, further reflected in the negative associations observed between fungi and Bacteria (66.5 %).

Symbiotic and syntrophic interactions also likely occur between eukaryotes and prokaryotes in ADs, particularly involving archaeal methanogens. This is evidenced by taxa such as *Paracercomonas* sp. and

Giardia intestinalis among the 30 most abundant eukaryotes. *Paracercomonas* produces acetate and hydrogen (Hirakata et al., 2020), essential metabolites for methanogens. Similarly, *Giardia intestinalis*, which lacks hydrogenosomes, produces hydrogen nonetheless, potentially supporting methanogenic Archaea (Benchimol et al., 2022; Lloyd et al., 2002). These potential interactions, though poorly understood, highlight the complex relationships between eukaryotes and Archaea. The relative importance of each interaction type likely fluctuates depending on specific environmental conditions and community compositions within individual AD systems. Understanding these diverse ecological interactions is crucial for optimizing AD performance and stability for which this study is an important stepping stone.

An intriguing finding in our analysis that must be addressed is the high abundance of an undescribed species from the Dino-Group II Clade 5 (Syndiniales, Alveolata) in multiple ADs (Supplementary Figure S 4, therein referred to as "Dinoflagellata"). Although widely distributed and likely ecologically significant, little is known about its biology due to limited culturable isolates (Stoeck et al., 2006). Syndiniales, encompassing five major MALV groups (MALV I–V or called Dino-Groups I–V in the PR^2 database), are frequently found in anoxic habitats and are likely heterotrophic, though their ability to prey on prokaryotes remains uncertain (Guillou et al., 2008; Stoeck et al., 2006).

5. Conclusion

In conclusion, the results of our study support that eukaryotes play a crucial role as regulators of the archaeal community composition and thus treatment efficacy and efficiency. The herein-reported interkingdom loop, modulated by SRT, reveals that shifts in the archaeal community cascade through eukaryotes, highlighting their integral role in the system.

Data availability

All underlying data is publicly available under the accession number Gp0197952 at the JGI database.

CRediT authorship contribution statement

Maria Badra: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Jule Freudenthal:** Writing – review & editing, Supervision, Software, Conceptualization. **Kenneth Dumack:** Writing – review & editing, Visualization, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123371.

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