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Long-term microbial community dynamics in a pilot-scale gas sparged anaerobic membrane bioreactor treating municipal wastewater under seasonal variations

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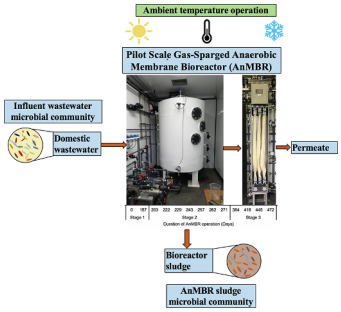
GRAPHICAL ABSTRACT



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ABSTRACT

This study evaluates the microbial community development in the suspended sludge within a pilot-scale gas sparged Anaerobic membrane bioreactor (AnMBR) under ambient conditions, as well as understand the influence of microbial signatures in the influent municipal wastewater on the bioreactor using amplicon sequence analysis. The predominant bacterial phyla comprised of *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, and *Chloroflexi* demonstrated resiliency with ambient temperature operation over a period of 472 days. Acetoclastic *Methanosaeta* were predominant during most of the AnMBR operation. Beta diversity analysis indicated that the microbial communities present in the influent wastewater did not affect the AnMBR core microbiome. Syntrophic microbial interactions were evidenced by the presence of the members from *Synergistales*, *Anaerolineales*, *Clostridiales,* and *Syntrophobacterales*. The proliferation of sulfate reducing bacteria (SRB) along with sulfate reduction underscored the competition of SRB in the AnMBR. Operational and environmental variables did not greatly alter the core bacterial population based on canonical correspondence analysis.

1. Introduction

Anaerobic membrane bioreactors (AnMBRs) are an engineered environmental biotechnology platform that promise to offer a sustainable solution to treat wastewater with its improved energy efficiency and simultaneous recovery of indirect potable water and nutrients (Harb et al., 2015; Seib et al., 2016b; Lim et al., 2019). AnMBRs combine anaerobic biological treatment and membrane filtration to effectively degrade the organic matter, while producing high quality permeate. The degradation of organic matter is performed by a diverse group of

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anaerobic microorganisms (mainly *Bacteria* and *Archaea*) within the bioreactor to form methane rich biogas (methanogenesis), thereby contributing to the energy neutrality potential of AnMBR operation.

Different microbial groups anaerobically decompose the organic matter at different sequential stages, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis wherein bacteria are responsible for achieving the first three processes while *Archaea* alone carry out methanogenesis (Zinder, 1984). Bacterial phyla such as *Chloroflexi*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* are known to be ubiquitous and predominant in full scale anaerobic digesters (Ariesyady et al., 2007). The presence of methanogenic *Archaea* in anaerobic digesters are of great importance as methane generation could offset a significant portion of the energy demand of a wastewater treatment plant (McCarty et al., 2011). Methane generation in mesophilic anaerobic digesters is mainly contributed by acetolactic methanogenesis including members of *Methanosaetaceae* family, specifically *Methanosaeta* (Nelson et al., 2011). However, the predominant methanogenesis pathway(s) under psychrophilic conditions or under ambient operation subject to seasonal temperature swings still remains unclear, although hydrogenotrophic methanogenesis was seen to be predominant under psychrophilic temperatures (Bialek et al., 2011).

The microbial metabolism involved in the anaerobic degradation of complex organic substrates are driven by syntrophic interactions between fermentative bacteria and methanogens (McInerney et al., 2007). Such microbial interactions are essential to maintain steady state anaerobic digestion operations, as primary and secondary syntrophic bacteria degrade organic compounds such as alcohols, volatile fatty acids, and sugars to produce hydrogen. Furthermore, the methanogenic *Archaea* consume the hydrogen to produce methane, thereby making the overall syntrophic interactions thermodynamically favorable under steady state operation. Identifying the microbial community members that facilitate completion of syntrophic processes is essential to understand the underlying mechanisms of interspecies hydrogen/formate transfer or direct interspecies electron transfer (DIET) from an AnMBR perspective operated under ambient conditions.

Although previous AnMBR studies have reported the microbial composition of the biofilm and suspended sludge within the system (Smith et al., 2013; Xie et al., 2014; Seib et al., 2016a; Cheng et al., 2019; Inaba et al., 2020), not much is known about the long term response of the AnMBR core microbial population to the microbial signatures of real influent wastewater. Seib et al. (2016a) observed a shift in AnMBR microbial communities when fed with primary effluent municipal wastewater. Similarity in bacterial community structures of raw sewage and sludge samples were also observed by Liu et al. (2007) in conventional wastewater treatment plants (WWTPs) and WWTPs with Biological Nutrient Removal (BNR) systems but suggested that the dominant bacterial phyla in raw sewage did not play a major role in the treatment process.

Apart from syntrophic bacteria and methanogenic *Archaea*, sulfate reducing bacteria (SRB) also compete for the complex substrates within anaerobic reactors. SRB reduce sulfate in the influent municipal wastewater to form corrosive hydrogen sulfide by competing with methanogens for substrate, impeding methane generation and energy recovery (Giménez et al., 2011). SRB show greater substrate affinity for both hydrogen and acetate than hydrogenotrophic methanogens and acetoclastic methanogens, respectively (Lens et al., 1998). Thus, understanding the population dynamics and interactions of competing microbial communities within the AnMBR is essential for long term stable process operation.

The main challenges impeding AnMBR operation include its ability to operate at low temperatures and treat low strength wastewaters. While many bench-scale AnMBR related studies have successfully treated low strength domestic wastewaters (Lew et al., 2009; Ho and Sung, 2010; Smith et al., 2013, 2015; Seib et al., 2016b), only a few studies have been performed at ambient and psychrophilic temperatures (Smith et al., 2013, 2015; Gouveia et al., 2015; Seib et al., 2016a). The current study focuses on microbial community dynamics in one of the first pilot scale AnMBR treatment systems treating 1000 gallons per day of municipal wastewater under ambient temperature fluctuations due to seasonal changes over a long duration of operation and having demonstrated successful operation (Lim et al., 2019). Furthermore, evaluating the response of the microbial community within the reactor to operational variables including temperature, pH, organic loading rate (OLR), bioreactor volatile solids, and influent characteristics is important for successful microbial management for steady state AnMBR operation. This study evaluates microbial community development and its temporal evolution in response to varying operational or environmental parameters including ambient temperature fluctuations, the impact of microbial groups in influent wastewater, as well as competing sinks, such as sulfate reduction, in a pilot scale gas sparged AnMBR treating municipal wastewater. Additionally, bacterial genera performing key functional roles were identified based on relative bacterial abundances determined from the 16S rRNA gene-based Illumina Miseq high throughput sequencing analysis.

2. Materials and methods

2.1. AnMBR operation and chemical analysis:

A pilot scale gas sparged AnMBR system treating municipal wastewater under ambient conditions was operated at Fort Riley, Kansas, USA. The system design and operating conditions are described in detail in a previous publication by Lim et al. (2019). A summary of key operational parameters is shown in Table 1.

2.2. Sampling and DNA extractions

Following inoculation of the reactor using seed sludge collected from the Topeka WWTP mesophilic anaerobic digester, representative bioreactor samples were collected from the middle sampling port of the

Table 1
Key operational and process parameters corresponding to days when biomass samples were collected from the primary bioreactor of the pilot scale gas-sparged AnMBR.

Process and operational parameters	DNA sampling during different stages of AnMBR operation (days)												
	Stage 1		Stage 2							Stage 3			
	0	157	203	222	229	243	257	262	271	384	416	445	472
pH	–	7.1	6.95	6.96	6.7	–	6.88	7.49	6.89	6.93	6.84	6.77	6.92
Temperature (°C)	25.2	13.9	16.4	18.9	17.1	15.6	18	17.7	20	26.3	26.5	25.6	20.5
Bioreactor VS (mg/L)	–	5820	7069	8667	5531	4337	9931	–	8153	2810	2847	1190	1853
HRT (h)	8.6	13.2	14.3	14.6	12.6	13.5	10.3	10.6	10	7.14	8.10	11.73	11.7
SRT (days)	–	68.4	68	68.4	68.4	68.6	65.8	66.3	65.5	43.1	50.2	69.5	51.5
OLR ($kg/m^3/d$)	0.88	1.48	0.9	1	1.88	1.5	0.73	1.15	1.3	1.56	1.36	1.09	1.27
Net flux (LMH)	13	6	5.9	6	7	7	8	8	8	8.12	8.8	8.8	6.06

reactor at various times during the operational period (Days 0, 157, 203, 222, 229, 243, 257, 262, 271, 384, 416, 445, and 472). Additionally, influent feed samples were collected for DNA extractions during the later phase of operation (Days 384, 416, 432, 445, 458, and 472) to understand the impact of the influent feed on the reactor microbial community. The sampling points were grouped into three stages of reactor operation: Stage 1 (Day 0 to 157), Stage 2 (Days 203 to 272), and Stage 3 (Days 384 to 472).

2.3. DNA extraction

50 mL bioreactor samples were collected for each sampling point and centrifuged in an Eppendorf centrifuge 5920 R (Hauppauge, New York, USA) at 21,000 RCF (relative centrifugal force) to concentrate the biomass. The supernatant was discarded, and the settled biomass pellets were either immediately used for DNA extraction (samples collected after Day 384) or stored in a freezer at −20 °C (samples collected before Day 384). The biomass pellets (after thawing frozen pellets) were subsequently weighed out for DNA extraction. The extractions were carried out using E.Z.N.A® Water DNA kit (Omega bio-tek, Norcross, Georgia, USA) as per the manufacturer's protocol. Further, an optimization process to compare the extraction efficiency and quality of DNA using different weights of the freeze thawed pellets versus immediately extracted samples was also performed. The DNA extraction results (data not included) showed some limitations for the stored samples as the freshly extracted samples resulted in higher DNA concentrations for similar weights of biomass pellets, although the storage method did not have an impact on the DNA quality (260/280 absorbance ratio).

2.4. High throughput sequencing and data processing

To determine the structure of the Bacterial and Archaeal community in the AnMBR, DNA was sequenced at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on an Illumina MiSeq (Illumina, USA) platform. 16S rRNA universal prokaryotic primers 519F and 806R, with barcode on the forward primer, were used to amplify the V3 and V4 hypervariable region of this highly conserved gene (Takai and Horikoshi, 2000). The reads were paired end sequenced with DNA fragments consisting of 2 × 300 bp reads using an Illumina MiSeq with the MiSeq Reagent Kit v3. MR DNA provided sequencing data in pr.FASTA and pr.QUAL files containing joined reads which were further combined into FASTQ files using the "Combine FASTA and QUAL" tool on Galaxy. The FASTQ files were uploaded to the Beocat Research Cluster at Kansas State University for subsequent analysis. Reads were sorted by sample ID into separate FASTQ files using grep. The sorted sequence files were imported into QIIME2 (Bolyen et al., 2019) and denoised with qiime deblur denoise-16S to obtain representative sequences. After denoising, the sequences were clustered into OTUs in QIIME2 based on 99% similarity threshold (Edgar, 2018). Phylogenetic analysis of representative sequences was performed using qiime phylogeny fast tree and qiime phylogeny midpoint-root.

Representative sequences were classified in QIIME 2 with a Naive Bayes classifier trained with the Greengenes 13_8 99_otu_taxonomy and 515F/806R reference sequences from Greengenes 13_8 99% OTUs. The qiime taxa barplot function was used to visualize the resulting taxonomy and results were exported from QIIME2View as a .csv file for further processing in Microsoft Excel.

2.5. Statistical analysis

The taxonomic table containing the sequence reads at different taxonomical levels in each sample was used to calculate the relative bacterial abundances and relative archaeal abundances separately in Microsoft Excel (Version 16.30). Both bacterial and archaeal relative abundances at different taxonomic levels were determined by normalizing against the total number of bacterial sequences and archaeal sequences, per sample, respectively. Taxonomically unassigned reads were excluded from the relative abundance calculations and only taxa representing ≥1% relative abundance in at least one of the sampling points were reported in this study. The impact of influent wastewater microbial composition on AnMBR microbial community was investigated with beta diversity analysis performed using QIIME2 based on weighted unifrac metric, visualized as Principal Coordinate Analysis (PCOA) plots and neighbor joining dendrograms. The resulting emperor plot output of PCOA was viewed on QIIME2View (https://view.qiime2.org) and the dendrogram was visualized using iTOL (Letunic and Bork, 2019). Canonical correspondence analysis (CCA) and Pearson's correlation analysis was carried out to correlate the phylum level *Bacteria* and genus level *Archaea* with environmental and operational variables [pH, temperature, organic loading rate (OLR), Bioreactor volatile solids (VS)]. Sequence reads of *Bacteria* and *Archaea* > 1% relative abundance in different samples were input to XLSTAT software to construct CCA biplots and determine the Pearson's correlation coefficients. Pearson's correlation was also performed with potential syntrophic genera in the microbial community.

2.6. Sample characterization

Biogas generation from the bioreactor exhaust, hollow-fiber gas transfer membrane (vacuum pump discharge), and combined gas exhaust was quantified using a variable gas flow meter (Alicat Scientific). An online gas flow sensor was used to measure the methane content of the biogas (Nova Analytical Systems Inc). Total chemical oxygen demand (TCOD) was analyzed with HACH® kits using a HACH DR3900 (Loveland, CO, USA) spectrophotometer.

BOD_5, Total solids (TS), Total suspended solids (TSS), fixed and volatile solids were analyzed following methods 5210B, 2540B, 2540D, and 2540E, respectively of the Standard Methods for the Examination of Water and Wastewater, 22nd Edition. Sulfide and sulfate concentrations were measured using standard HACH methods (HACH methods 8051 and 8131, respectively) and measured on a HACH (Loveland, CO, USA) DR3900 spectrophotometer. Sulfide measurements were done immediately after sampling in order to minimize the oxidation or escape of sulfides in the sample. Additional sulfate measurements were made using an ion chromatograph (ICS1000, Thermo Fisher, Waltham, Massachusetts, USA). Organic loading rate (OLR) was calculated based on the influent wastewater COD values, reactor volume, and the influent flowrate measured by an online flow meter. All chemical analyses were done using Milli-Q water.

3. Results and discussion

3.1. Overall microbial community diversity in pilot-scale AnMBR

3.1.1. Distribution of predominant bacterial communities

The distribution of bacterial communities in the AnMBR sludge are shown in Fig. 1. The AnMBR was dominated by a core group of bacterial phyla comprised of *Bacteroidetes*, *Proteobacteria*, *Firmicutes,* and *Chloroflexi* throughout the operational period (Fig. 1A). This is consistent with previous studies on microbial communities in mesophilic anaerobic systems and psycrophilic AnMBRs (Regueiro et al., 2012; Smith et al., 2013). *Proteobacteria and Bacteroidetes* together consistently constituted > 50% of average relative bacterial abundance in each of the three stages of reactor operation. *Bacteroidetes* are known to metabolize proteins and carbohydrates to produce volatile fatty acids (propionate and acetate) (Seo et al., 2019). *Firmicutes* are volatile fatty acid degrading syntrophic bacteria that produce hydrogen (Riviere et al., 2009) increasing up to 24.2% on day 257. The relative bacterial abundance of *Chloroflexi* decreased with the reactor operation and temperature decrease. The functional role of *Chloroflexi* are still unclear, but studies have identified their probable role in the anaerobic degradation of glucose and other complex organic substances

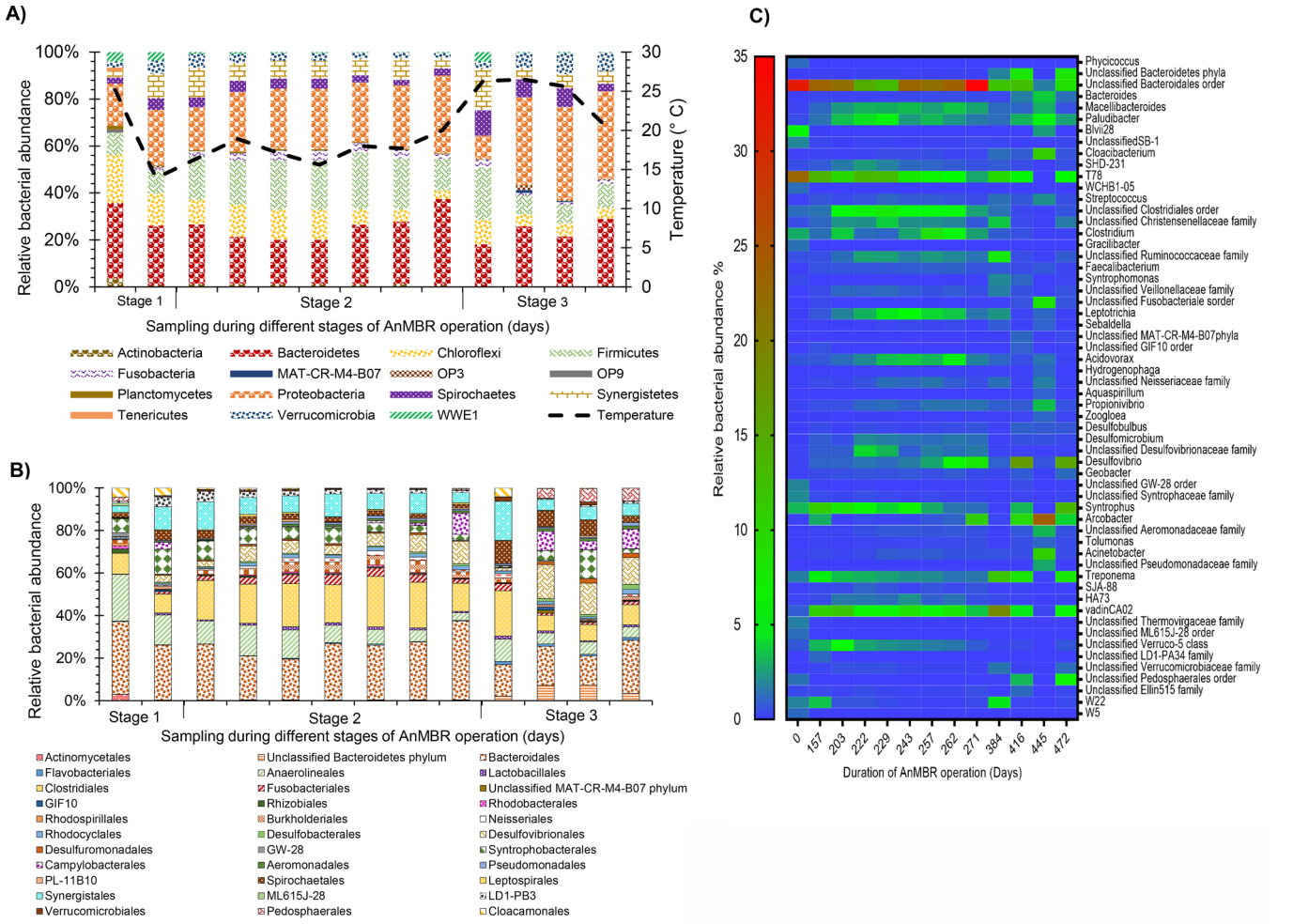

Fig. 1. A) Distribution of bacterial phyla with ≥1% relative bacterial abundance in bioreactor sludge samples; B) Distribution of order level bacteria with ≥1% relative bacterial abundance in bioreactor sludge samples; C) heat map showing distribution of genus level bacteria with ≥1% relative bacterial abundances.

(Ariesyady et al., 2007). Phyla such as *Synergistetes, Spirochaetes*, and *Verrucomicrobia* together constituted only a low fraction of the bacterial phyla in the first two stages but increased up to 22.5% of the total bacterial abundance during stage 3 of operation. *Actinobacteria* and *Tenericutes* were present as a minor fraction in the seed sludge but eventually diminished during the later stages of reactor operation.

The order level bacterial community showed ample diversity and was well represented by members of the predominant phyla (Fig. 1B). *Bacteroidales*, a member of the *Bacteroidetes* phylum were very stable (13.63–37.15%) and were always present in the community and their relative abundance did not diminish with time of operation in the reactor. *Clostridiales* (affiliated to *Firmicutes* phylum) increased with reactor operation increasing from 9.7% (Stage 1) → 19.1% (Stage 2) and were a significant part of the bioreactor, although its abundance decreased and levelled off during the final stage (11.6%) of reactor operation.

Bacterial genera at ≥1% of average relative bacterial abundance in at least one of the three stages were identified as the key members of the AnMBR system. The key bacterial microbiome of the AnMBR system was represented by the phyla *Bacteroidetes, Proteobacateria, Chloroflexi, Firmicutes, Fusobacteria, Spirochaetes, and Synergistetes* and their associated general. Overall, the key members of the microbial community and their likely role in the AnMBR system have been identified.

3.1.2. Distribution of predominant archaeal communities

High-throughput sequencing revealed that the relative abundance of *Euryarchaeota* consisting of the methanogenic *Archaea* in the AnMBR system was consistently below 2.5% of the overall microbial community profile (Fig. 2A). This is similar to previously reported archaeal abundance values in AnMBR studies (Smith et al., 2015; Zamorano-López et al., 2019). The inoculum was initially predominant with unclassified *WSA2* genera belonging to the *Methanobacteriales* order and were completely out competed over the course of AnMBR operation. Although *WSA2* is classified as a family of *Methanobacteriales* order, it is considered a class-level monophyletic lineage within Euryarchaeota distinct from the *Methanobacteriales*, and likely perform methylated thiol reduction to drive methanogenesis (Riviere et al., 2009; Wilkins et al., 2015; Nobu et al., 2016). The genus *Methanosaeta* (also known as *Methanothrix*) belonging to the *Methanosaetacea*e family was a minor fraction (4.73% relative archaeal abundance) in the inoculum on Day 0 of the AnMBR operation (Fig. 2B). However, it continued to increase and was at a significant level for most of the reactor operation reaching up to 82.23% and 76.93% during Stages 2 and 3, respectively, indicating that temperature did not have a big impact on its metabolic abilities. *Methanosaeta* are acetoclastic methanogens capable of forming methane from acetate cleavage and is the dominant acetoclastic methanogen under low acetate concentrations (van Haandel et al., 2014). The increase in *Methanosaeta* abundance indicated a shift from methyl group reducing methanogenesis by unclassified *WSA2* genera to acetoclastic methanogenesis.

The bioreactor witnessed a slow and progressive increase in *Methanospirillum,* reaching appreciable relative archaeal abundance

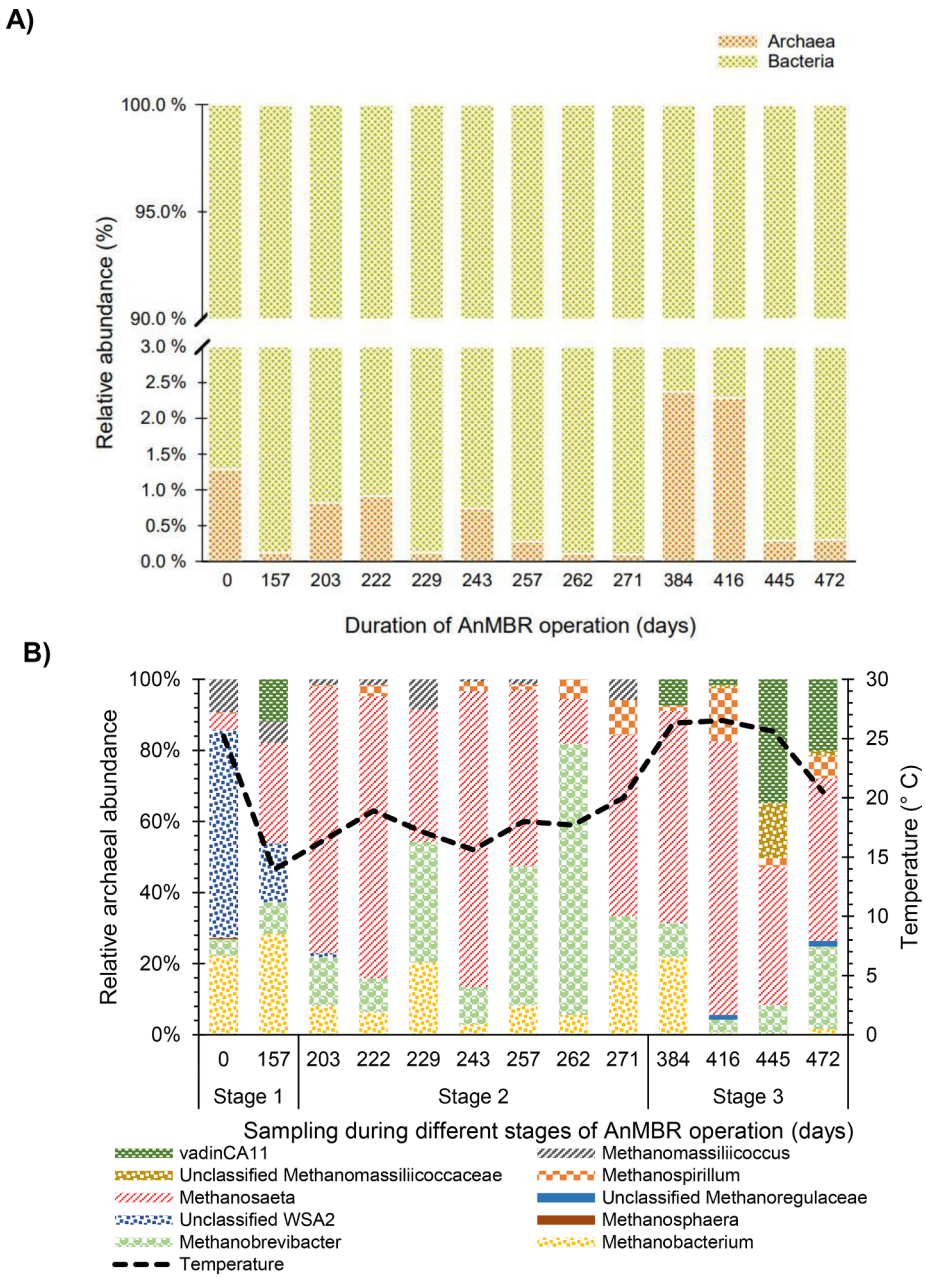

Fig. 2. Distribution of A) total relative abundance of *Archaea* and *Bacteria* in bioreactor samples; B) *Archaea* with ≥1% relative archaeal abundance at genus level in the bioreactor sludge samples.

levels between day 262 (5.45%) and day 416 (15%) of reactor operation. *Methanospirillum* are psychrotolerant hydrogenotrophic methanogens that produce methane from H_2 and CO_2 or from formate (Oren, 2014). *VadinCA11*, an archaeal genus whose metabolic functions are unclear and has been previously found in other anaerobic systems (Buhlmann et al., 2019), also surged in abundance around days 445 (34.84%) and 472 (20.16%) as shown in Fig. 2B. Hydrogenotrophic methanogens such as *Methanobrevibacter* and *Methanobacterium* were consistently present in the reactor, with *Methanobrevibacter* abundance exceeding *Methanosaeta* around day 262 of reactor operation. The genus *Methanobacterium*, generates methane from CO_2 and H_2 or formaldehyde (H_2CO). Based on the microbial community profile, there was a competition between acetoclastic methanogenesis and hydrogenotrophic methanogenesis with acetoclastic methanogenesis dominating for the most part. In the present study, the surge in abundance of hydrogenotrophic methanogens (*Methanobrevibacter* and *Methanobacterium*) around days 257 (39.2%) and 262 (76.3%) could likely be attributed to the preceding low temperatures in the bioreactor which would have increased the availability of dissolved hydrogen in the reactor. Overall, the taxonomic profile of archaeal community demonstrated synergistic occurrence of different methanogenesis pathways including acetoclastic, hydrogenotrophic and possibly methylotrophic indicating methanogenic adaptation to ambient temperature AnMBR operation.

3.2. Role of syntrophic interactions leading to methanogenesis

Syntrophic oxidation–reduction reactions involving syntrophic bacteria and methanogens play an important role in methane generation in anaerobic systems. High-throughput sequencing data of the AnMBR samples revealed presence of several syntrophic bacterial orders including *Synergistales*, *Anaerolineales*, *Clostridiales,* and

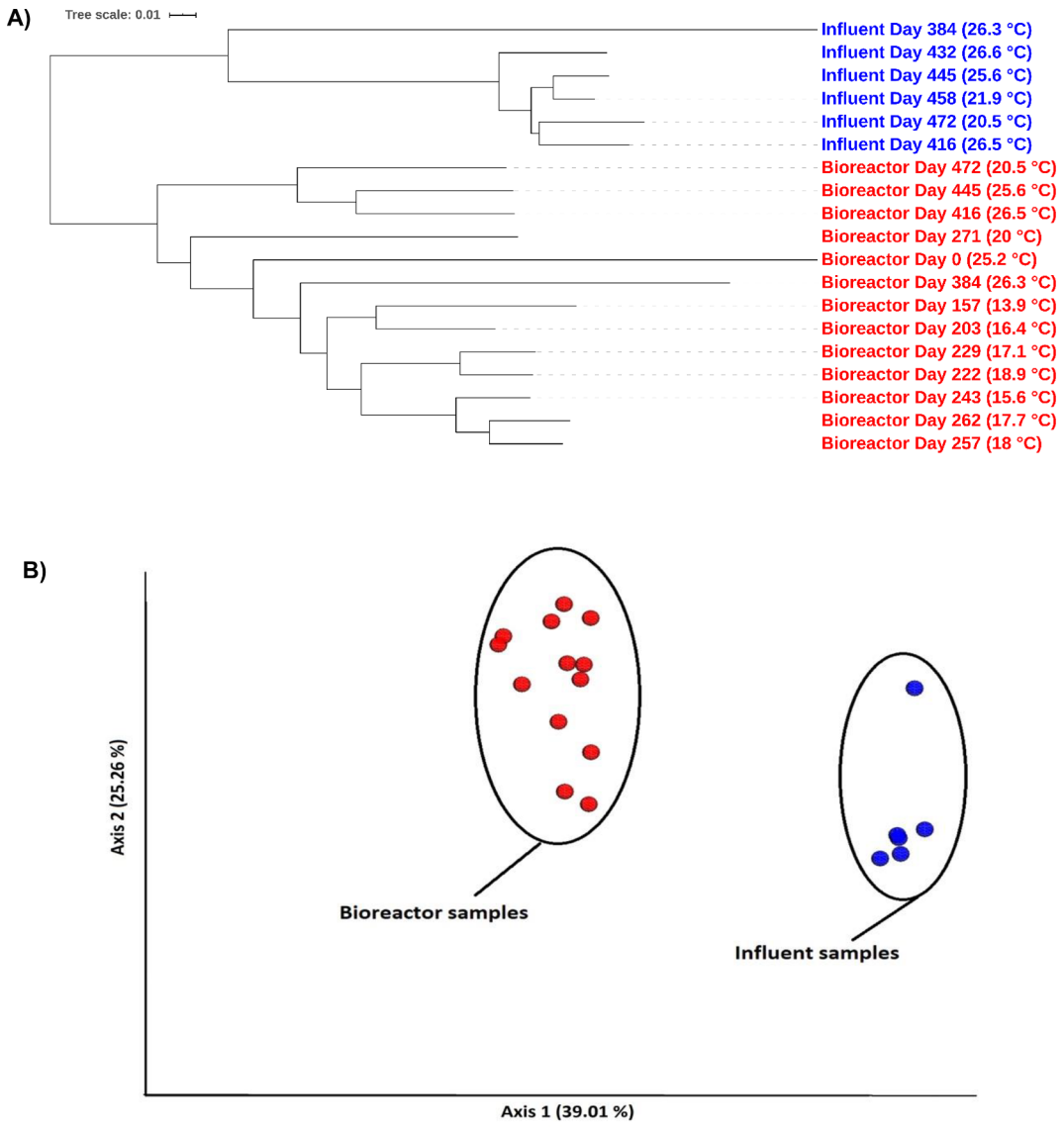

Fig. 3. A) Weighted Unifrac dissimilarity cluster analysis dendrogram of microbial populations in the influent and bioreactor sludge using neighbor joining method.; B) Principal coordinates analysis (PCoA) plot showing clustering of bioreactor samples and influent samples as determined by Weighted Unifrac analysis.

Syntrophobacterales (Fig. 1B). *Synergistales* are syntrophic fermentative bacteria in the *Synergistetes* phylum that have the ability to degrade amino acids into volatile fatty acids and contribute to acidogenesis and acetogenesis via syntrophic relationships with methanogens (Ferguson et al., 2016). The genus *VadinCA02* within *Synergistales* was present as a major constituent in AnMBR and their relative abundance reached up to 21% on Day 384. *Bacteria* assigned to *vadinCA02* and the genus *HA73* (also belonging to *Synergistales*) are likely to degrade peptone and amino acids into acetate (Yamashita et al., 2016; Yin et al., 2017).

Syntrophobacterales represented by the genus *Syntrophus* was consistently present in the reactor and was not a trivial community (relative bacterial abundance 3.6% at Day 0 and 14.4% at Day 452). *Syntrophus* can degrade butyrate, benzoate, and other fatty acids through synergistic interaction with hydrogenotrophic methanogens (Jackson et al., 1999). The bacterial order *Anaerolineales* were a bigger portion of the community during the initial start-up phase of reactor operation but gradually declined in abundance with time (decreased from 26% at Day 0 → 6.4% Day 472) as shown in Fig. 1B. The *Clostridiales* order (belonging to *Clostridia* class) was represented by the syntrophy promoting genus *Clostridium* and the known syntrophic genus, *Syntrophomonas* (Narihiro et al., 2015). *Clostridium* was steadily present for most of the reactor operation, but its relative bacterial abundance declined during the later stages of reactor operation (reduced to 0.18% on Day 472). *Syntrophomonas* (*Syntrophomonadaceae* family) grow in obligate syntrophy with hydrogenotrophic methanogens and SRBs to oxidize butyrate into acetate and H_2 (Sousa et al., 2009). In this study, Syntrophomonas was present in low abundance (0.44% on Day 0 → 2.37% (Day 384) → 0.3% (Day 472)) but their presence was positively correlated with hydrogenotrophic *Methanobacterium* (Pearson's correlation coefficient r = 0.731, p = 0.005) and *Methanobrevibacter* (Pearson's correlation coefficient r = 0.729, p = 0.005 indicating that the genus may have contributed to hydrogen production in the reactor. Interestingly, *Syntrophomonas* also showed strong positive correlation with acetoclastic *Methanosaeta* which could be possibly be due to interspecies acetate transfer which has been postulated as the basis for Direct interspecies electron transfer (DIET).

The genus *T78* (member of *Anaerolinieae* family) metabolize alcohols and carbohydrates through syntrophic interactions (Praveckova et al., 2016). Although present in high relative abundance during the initial reactor operation phase (23.5% at Day 0), *T78* steadily declined to reach 5.8% at Day 472 but were still one of the dominant genera in the biomass suspension. The genus *Treponema* was present throughout

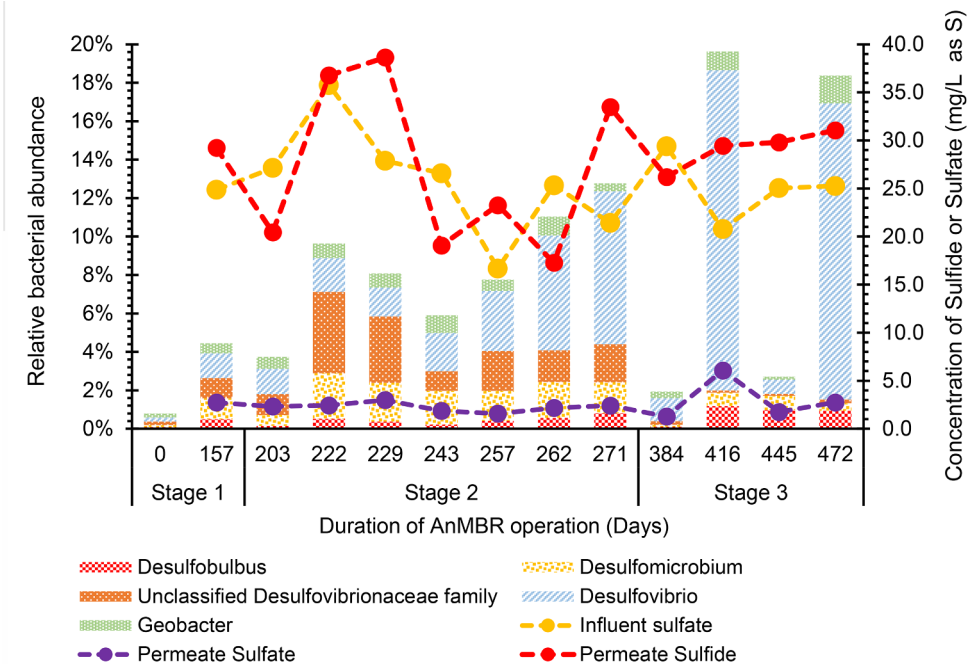

Fig. 4. Key sulfate reducing bacteria (SRB) genera at ≥1% relative bacterial abundance in the bioreactor sample over the long term AnMBR operation under ambient operational conditions. Gradual proliferation of selected key SRB ((*Desulfovibrio, Desulfobulbus* and *Geobacter*) while other SRB (*Desulfomicrobium*, unclassified *Desulfovibrionales*) gradually declined in relative abundance.

the AnMBR operation and their relative bacterial abundance spiked during the final stage of AnMBR operation (11.94% on day 384 and 7.91% on Day 472) (Fig. 1C). *Treponema* consists of likely homoacetogenic strains that produce acetate from H_2 and CO_2 which could work in synergy with acetoclastic methanogens to produce methane (Zhang et al., 2009). Interestingly, the surge in *Treponema* during the later AnMBR operation stages corresponded to a parallel surge in acetoclastic *Methanosaeta* (Fig. 2B), likely confirming a coordinated synergy between the homoacetogen and acetoclastic methanogen. The synergy was further confirmed based on the statistically significant positive correlation between the two genera (Pearson's correlation coefficient, r = 0.677 and p = 0.011. Additionally, genus *Geobacter*, whose relative abundance reached up to 1.4%, may have also been involved in syntrophic interactions with or without DIET (Rotaru et al., 2014).

3.3. Impact of influent municipal wastewater on AnMBR microbial community dynamics

The dendrogram comparing the samples from the influent and suspended sludge, shown in Fig. 3A, revealed that the influent feedwater samples grouped distinctly separate from the bioreactor samples, indicating that it did not impact the microbial dynamics in the reactor. Based on the PCOA plot (Fig. 3B), the sample clusters had a maximum variation in 39.01% (Axis 1) and 25.26% (Axis 2) with separate clustering of the influent group and the bioreactor group samples. The variation of clustering patterns indicates significant differences in community structure in samples from the two sources with the bioreactor samples clustering separately, away from the influent wastewater. Similar difference in clustering pattern between the influent and bulk sludge samples was observed in a study on granular activated carbon fluidized AnMBR and it was concluded that the influent wastewater had little impact on the sludge microbial community (Evans et al., 2018). Although samples from the bioreactor and influent wastewater grouped distinctly, the spatial variation in clustering pattern of microbial communities in samples within each group could possibly be due to the effect of temperature.

The microbial differences in the observed sample clustering were further investigated with taxonomic analysis of the influent, which revealed that *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* together representing > 98% relative bacterial abundance in all the influent samples. The predominant phyla level distribution of *Bacteria* reported in this study is consistent with previously reported studies on influent wastewater microbial composition (McLellan et al., 2010; Lee et al., 2015), although their relative abundances were not similar. Comparison of key bacterial orders (≥1% bacterial relative abundance) revealed sixteen overlapping bacterial orders in samples from the two sources. Of these orders, *Campylobacterales* (range 6.4–25.5%), *Flavobacteriales* (range 3.2–15.4%) and *Bacteroidales* (range 3.1–18.7%) were the main candidates in the influent and may be considered as generalists that can possibly survive in both sewerage drains and activated sludges (Lee et al., 2015). Within the bioreactor, *Campylobacterales* may likely have originated from the influent as its abundance remained small throughout the AnMBR operation. However, they progressively increased after 257 days of reactor operation which could likely be a transient surge within the AnMBR possibly due to their dominant abundance in the influent. Furthermore, the genus *Arcobacter* (belonging to *Campylobacterales* order), a potential enteric human pathogen (Vandenberg et al., 2004), was present in the reactor at low abundances (< 1.5%) but spiked towards the final stages (10.5% at Day 445) of reactor operation. *Arcobacter* is reported to grow under both aerobic and anaerobic conditions, and often found in raw sewage (Fisher et al., 2014).

The archaeal abundance in the influent samples was negligible; *Methanobacteriales* and *Methanomassilliicoccales* were the only identified orders and therefore are unlikely to have influenced the bioreactor archaeal community. Overall, the core microbial population within the AnMBR reactor and the inherent shifts in the community dynamics is unlikely to be attributed to the influent wastewater microbial signature. The influence of influent wastewater could be limited to a mere transient surge in minor microbial genera for a few days but no influence on the core AnMBR bacterial or archaeal community composition.

3.4. Proliferation of sulfate reducing bacteria (SRB)

Sulfate reducing bacteria proliferated with the duration of the AnMBR operation, although their relative abundance in the seed sludge was below 1% on Day 0. *Desulfovibrio* genus (*Desulfovibrionaceae* family) was the dominant SRB with relative abundance increasing from 0.2% (Day 0) → 3.1% (Day 257) → 15.4% (Day 472). *Desulfovibrio* utilizes

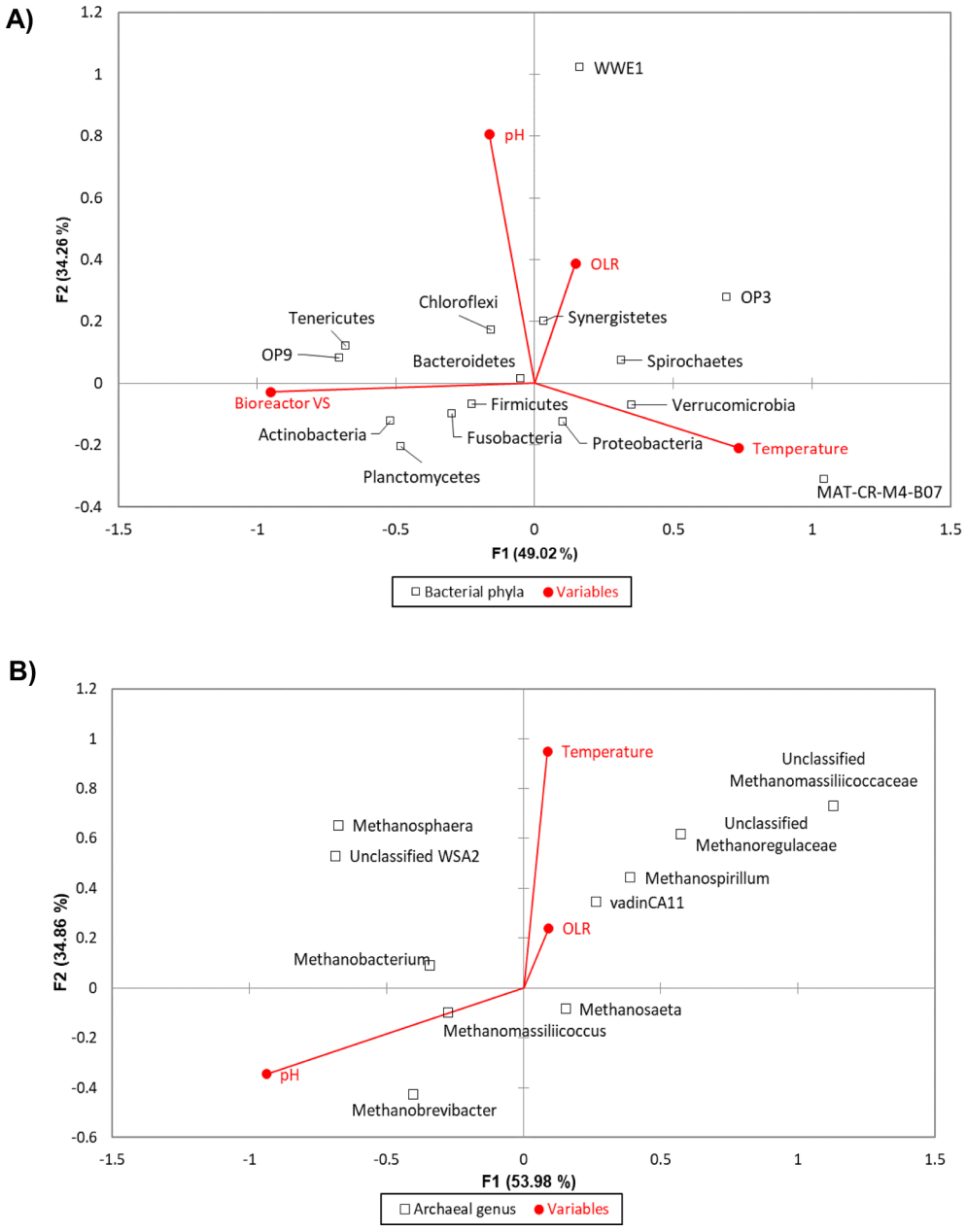

Fig. 5. Canonical correspondence analysis (CCA) plot to investigate the relationship between relative abundance of AnMBR microbial communities and key operational parameters (pH, temperature, OLR, and bioreactor VS) for A) *Bacteria* at phylum level, and B) *Archaea* at genus level.

sulfate as a terminal electron acceptor while oxidizing H_2, formate, ethanol, and lactate (Devereux et al., 1990). The genus *Desulfomicrobium*, which utilizes H_2 as an electron donor and acetate as carbon source was consistently present in the reactor, but their abundance decreased after Day 384 from 0.1% (Day 0) → 1.6% (Day 257) → 0.4% (Day 472). The proliferation of SRB in the bioreactor can be correlated with the increase in bioreactor sulfide, represented in Fig. 4, which shows a reduction in sulfate concentrations and concomitant biological production of sulfide in the permeate. In addition, the sulfide concentration in the influent feed wastewater was always below detection indicating the role of SRB as the most likely factor for the sulfide generation, further evidenced from the high throughput sequencing data. Overall, the increasing abundance of SRB with an increasing production of sulfide (as hydrogen sulfide) causes concern because of the potential of microbially induced sulfide corrosion that could degrade the inner metallic parts of the AnMBR system. Additionally, certain SRB genera including *Desulfovibrio* are capable of shifting from being sulfidogenic to syntrophic VFA fermenters under sulfate limiting conditions (Plugge et al., 2011). Therefore, the metabolic flexibility of SRB could add to the microbial community redundancy in the AnMBR system.

3.5. Influence of environmental and operational variables on microbial population

In the 2-dimensional canonical correspondence analysis biplots (Fig. 5), the environmental and operational variables are represented as vector lines. The length of the lines indicates the significance of the environmental and operational variables on respective bacterial phyla and archaeal genera within the bioreactor. The cosine angle between the vector variables lines indicates their correlation with the environmental variables. The phyla level bacterial CCA biplot (Fig. 5A) had 49.02% variation along the first axis (F1) and 34.26% variation along the second axis (F2) and together represented 83.28% variation of

relative bacterial abundances of the phyla. Bioreactor VS was shown to have the most significant correlation on the relative abundances of phyla. Bacterial phyla with minor relative abundance such as *Actinobacteria and Planctomycetes* positively correlated with bioreactor VS while *Spirochaetes* correlated negatively. Synergistetes correlated positively with OLR. At the genus level of *Archaea* (Fig. 5B), *Methanobrevibacter* and M*ethanomassiliicoccus* showed a positive correlation with pH, while *VadinCA11* and *Methanospirillium* showed positive correlation with OLR. Temperature was negatively correlated with *Methanobrevibacter* which possibly explains their surge in abundance in response to low temperature conditions. Additionally, *Methanosaeta* was also found to be negatively correlated to temperature. However, due to it being positioned closer to the origin, it may not have been strongly impacted by the different operational parameters including temperature and was therefore seen to be present throughout the duration of AnMBR operation.

To confirm the trends in correlation between environmental variables and the taxa as well as to analyze correlations between different taxa, Pearson's correlation analysis was also performed. The Pearson's correlation analysis revealed that the Bioreactor volatile solids strongly correlated with *Actinobacteria* ($r = 0.826$, $p = 0.003$). Although the Pearson's correlation results did not exactly match with all the CCA correlation trends, it established crucial correlations between environmental variables and sample taxa. Interestingly, the core bacterial phyla *Bacteroidetes, Firmicutes, Chloroflexi, and Proteobacteria* clustered close to the origin in the CCA plot, indicating that they are not really affected by environmental variables. This could be indicative of the resilience of the core microbial community to the environmental variables including temperature along with the likely presence of psychrotolerant mesophilic bacteria originating from the seed sludge.

4. Conclusion

The core of the AnMBR microbial community represented by *Bacteroidetes*, *Proteobacteria*, *Firmicutes, Chloroflexi* phyla and acetoclastic methanogenic genus, *Methanosaeta* is unique and not influenced by the influent wastewater microbial community. This core microbiome also exhibited resilience to operational variables – temperature, Bioreactor VS, OLR, and possibly pH. Proliferation of SRB with long term operation might necessitate sound microbial community management or long-term reactor modifications to enable high microbial efficiency. Overall, the findings of this study will be useful in elucidating the resilience and redundancy of the sludge microbiome to maximize treatment performance efficiency of AnMBRs.

CRediT authorship contribution statement

Arvind Damodara Kannan: Writing - original draft, Software, Formal analysis, Data curation, Visualization. **Patrick Evans:** Supervision, Funding acquisition. **Prathap Parameswaran:** Conceptualization, Methodology, Resources, Writing - review & editing, Project administration.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.123425.

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