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Integrated biological system for remediation and valorization of tannery wastewater: Focus on microbial communities responsible for methanogenesis and sulfidogenesis

P.J. Welz^{a,*}, N. De Jonge^c, M. Lilly^a, W. Kaira^a, A.B. Mpofu^{a,b}

^a Applied Microbial and Health Biotechnology Institute, Cape Peninsula University of Technology, Symphony way, Bellville, Cape Town 7535, South Africa

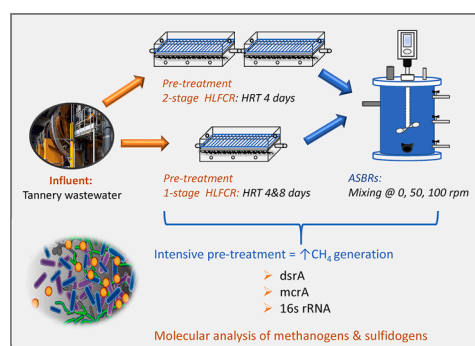
^b Department of Chemical Engineering, Cape Peninsula University of Technology, Symphony way, Bellville, Cape Town 7535, South Africa

^c Department of Chemistry and Bioscience, Aalborg University, Fredrik Bajers vej 7H, Aalborg DK-9220, Denmark

HIGHLIGHTS

- Ammonia was the primary driver of sulfate reducing bacterial community composition.
- Sulfide, silica, pH were the primary drivers of methanogenic archaeal composition.
- Only incomplete organic oxidizing sulfate reducers dominated during pre-treatment.
- Incomplete and complete oxidizing sulfate reducers dominated anaerobic digestion.
- Concurrent sulfidogenesis was not detrimental to methanogenesis.

GRAPHICAL ABSTRACT



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ABSTRACT

Microbial communities in hybrid linear flow channel reactors and anaerobic sequencing batch reactors operated in series for remediation and beneficiation of tannery wastewater were assessed. Despite concurrent sulfidogenesis, more intensive pre-treatment in hybrid linear flow channel reactors reduced methanogenic inhibition usually associated with anaerobic digestion of tannery effluent and promoted efficiency (max 321 mLCH₄/gCOD_{consumed}, 59% biogas CH₄). Nitrification and biological sulfate reduction were key metabolic pathways involved in overall and sulfate reducing bacterial community selection, respectively, during pre-treatment.

Abbreviations: AD, anaerobic digestion; Alk, alkalinity; AM, acetoclastic methanogens; ANOSIM, analysis of similarity; ASBR, anaerobic sequencing batch reactor; ASV, amplicon sequencing variant; BC, Bray-Curtis; BMP, biochemical methane potential; bp, base pair; BSR, biological sulfate reduction; CASP, conventional activated sludge process; CO, complete oxidizers; COD, chemical oxygen demand; DNA, deoxyribonucleic acid; dsrB, dissimilatory sulfite reductase; ED, Euclidian distance; FSB, floating sulfur biofilm; HLFCR, Hybrid linear flow channel reactor; HM, hydrogenotrophic methanogens; HRT, hydraulic retention time; IO, incomplete oxidizers; MA, methanogenic archaea; mcrA, methyl co-enzyme M reductase; ML, mixed liquor; nMDS, non-metric multidimensional scaling; OLR, organic loading rate; PCA, principal component analysis; PCR, polymerase chain reaction; qPCR, quantitative PCR; RA, relative abundance; rpm, revolutions per minute; SO, sulfide oxidation; SRB, sulfate reducing bacteria; SS, suspended solids; SRT, solids retention time; TWW, tannery wastewater; TOC, total organic carbon; VOA, volatile organic acids; VS, volatile solids; WAS, waste activated sludge; zOTU, zero rated operational taxonomic unit.

* Corresponding author.

E-mail addresses: welzpj@cput.ac.za (P.J. Welz), ndj@bio.aau.dk (N. De Jonge), lillym@cput.ac.za (M. Lilly), ashton.mpufu@isleutilities.com (A.B. Mpofu).

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Taxonomic selection could be explained by the proteinaceous and saline character of tannery effluent, with dominant genera being protein and/or amino acid degrading, halotolerant and/or ammonia tolerant. Complete oxidizers dominated the sulfidogenic populations during pre-treatment, while acetoclastic genera dominated the methanogenic populations during anaerobic digestion. With more intensive pre-treatment, the system shows promise for remediation and recovery of biogas and sulfur from tannery wastewater in support of a bio-circular economy.

1. Introduction

Tanneries provide employment and contribute to the economies of many developing countries. However, the tanning process generates large volumes of toxic effluents, especially from the beamhouse processes of soaking, unhairing/liming and delimiting/bating. The tannery wastewater (TWW) from these preparatory beamhouse operations differs from the TWW generated from the downstream tanning operations (Swartz et al., 2017). Volumetrically, for every metric ton of skins/hides that is processed, 20–25 m³ of beamhouse effluent is generated (Buljan and Král, 2019). In the general (mixed) tannery wastewater (TWW), around three quarters of the organics emanate from beamhouse processing (Buljan and Král, 2019), with chemical oxygen demand (COD) measurements ranging from 3 to 27 g/L (Mpofu et al., 2023). Sulfates (SO₄²⁻) (270–2400 mg/L), sulfides (HS⁻/S²⁻) (250–525 mg/L), ammonia/ammonium (NH₃/NH₄⁺) (96–865 mg/L) and chlorides (Cl⁻) (900–9025 mg/L) dominate the inorganic pollutant profile (Mpofu et al., 2023). Conventional physicochemical and biological process combinations are used to remediate TWW at most larger tanneries, but the conventional activated sludge process (CASP) that is traditionally used requires high energy inputs for aeration, generates excess sludge, and the effluent quality is often non-compliant with discharge standards (Swartz et al., 2017). More advanced and expensive TWW remediation technologies such as activated carbon adsorption, ion-exchange, reverse-osmosis, electro-dialysis, and membrane filtration can be quite effective, but there is no opportunity for valorization and excess potentially toxic sludge is generated that requires disposal to landfill sites (Buljan and Král, 2019; Saxena et al., 2019). Anaerobic digestion (AD) is an old technology that has been revived as a renewable source of energy that concurrently remediates waste, protects the environment, and preserves resources. The process generates significantly less sludge than aerobic biological systems such as the CASP (Sodhi et al., 2021). Historically, there was a perception that AD of TWW was not feasible because it contains chemicals that either directly or indirectly inhibit the growth and function of the sensitive methanogenic archaeal (MA) populations (Horn et al., 2022a). Methanogenic inhibition has been attributed to high respective concentrations of HS⁻/S²⁻ (>260 mg/L, Song et al., 2001) and SO₄²⁻ (≥1960 mg/L, Kibangou et al., 2022) in TWW. Historically, the perception was that sulfate reducing bacteria (SRB) outcompete MA for organic substrates as they tolerate wider pH ranges, have higher energy yields, and higher affinities for hydrogen (H₂) and acetate (CH₃COO⁻) (Wu et al., 2023). Pre-treatment of TWW has been advanced as a strategy to overcome inhibition of AD. However, classical two-stage AD strategies that promote organic substrate hydrolysis in the first stage are unlikely to promote effective methanogenesis of TWW containing inhibitory concentrations of HS⁻/S²⁻ and/or SO₄²⁻. For example, Saxena et al. (2019) applied hydrodynamic cavitation upstream of AD of TWW co-digested with food waste. Although the authors reported increased COD reduction rates of 43 % with 8.4–10 gCOD/L influent, the specific biogas yield was poor (69 mL/gVS) with low methane (CH₄) composition (max. 27 %) and the final effluent was still organic-rich. Despite these challenges, biochemical methane potential (BMP) testing and/or laboratory sequencing batch reactor experiments have demonstrated that biogas recovery from TWW may be feasible. Provided that the inoculum is well acclimated to TWW, applied in a high ratio (Horn et al., 2022a; Saxena et al., 2019), and the HS⁻/S²⁻ concentration is not excessive (Song et al., 2001), mono digestion and/or co-digestion of

TWW with other substrates have been successful in laboratory studies including tannery solid wastes (Berhe and Leta, 2018; Mpofu et al., 2023;) and food waste (Saxena et al., 2019). However, in 'real world' scenarios, the quality of TWW is inherently variable and it is difficult to consistently maintain the low levels of HS⁻/S²⁻ required for efficient AD unless the TWW is pre-treated to reduce the HS⁻/S²⁻ concentration (Mpofu et al., 2023; Swartz et al., 2017). Physicochemical pre-treatments are an option. For example, Song et al. (2001) successfully used coagulants to reduce (among other parameters), the COD, suspended solids (SS) and HS⁻/S²⁻ by 32 %, 64 % and 80 %, respectively before AD and achieved final effluent COD values of < 0.8 g/L, and a CH₄ yield of 210 mL/gCOD_{removed}. The problem with coagulant addition is the unavoidable generation of copious amounts of spent sludge that needs to be disposed to landfill. The alternative is to apply biological sulfate reduction (BSR) and partial sulfide oxidation (SO) under anoxic and aerobic conditions, respectively, to remove and recover sulfur from TWW. For example, Sabumon (2008) successfully integrated these processes in a hybrid upflow anoxic reactor that was sparged with air from the bottom. In other studies, novel hybrid linear flow channel reactors (HLFCRs) have been assessed experimentally for pre-treating TWW for AD and recovering elemental sulfur (S⁰) (Horn et al., 2022a; Mpofu et al., 2023). These reactors are semi-passive systems that spatially separate anoxic and aerobic zones while maintaining functional interactions between microbial species. A floating sulfur biofilm (FSB) containing harvestable S⁰ forms at the air-liquid interface. This study describes the microbial community composition and function in a novel integrated biological system consisting of HLFCRs and anaerobic sequencing batch reactors (ASBRs) in series with the potential to recover sulfur, biogas, biofertilizer and process/irrigation water from TWW. The results of this study provide key information required for scaling-up the process for industrial implementation of this biological system which supports a circular economy. The detailed performance and kinetics of the system have been described elsewhere (Mpofu et al., 2023).

2. Materials and methods

2.1. Set-up and operation of integrated biological system

The HLFCRs and ASBRs were set up and operated as previously described in detail (Horn et al., 2022a; Kibangou et al., 2022; Mpofu et al., 2023) and as shown in Table 1 and depicted in Fig. 1. Briefly, two HLFCRs (HLFCR1, HLFCR2) were inoculated with appropriately acclimated microbial consortia and then fed with raw beamyard TWW and operated in 1-stage (Experiment 1) followed by 2-stage (Experiment 2) fed-batch modes at hydraulic retention times (HRT) of 4 days. The inoculum was obtained from HLFCRs used in previous experiments that was sourced from saline estuary sediments and a tannery wastewater treatment plant that contained indigenous BSR communities (Horn et al., 2022a). The ASBRs were operated until biogas generation ceased (maximum 35 days). Two ASBRs that had previously been operated with TWW and contained acclimated microbial consortia were fed with the pooled effluent from HLFCR1 and HLFCR2 and operated in batch mode. To ascertain the effects of mixing and mixing speed on AD efficiency after 1-stage HLFCR pre-treatment, ASBR runs were conducted with no mixing and continual mixing at 50 and 100 revolutions per minute (rpm) (Experiment 1, Fig. 1). Based on these results, ASBR runs treating TWW after treatment in 2-stage HLFCRs were conducted with

Table 1
Characteristics of selected parameters measured in tannery influent and effluent.

Parameter	Influent	Effluent			
	TWW	Experiment 1, 3* 1-stage HLFGR	ASBR	Experiment 2 2-stage HLFGR	ASBR
Duration (days)	NA	0–46, 78–86*	27–32	50–70	27–30
HRT (days)	NA	4	27, 30, 32	4	27,30
rpm	NA	NA	0, 50, 100	NA	50
COD (g/L)	22.8 ± 3.7	8.72 ± 1.48	2.10–4.57	6.37 ± 1.68	2.51–3.01
Alk (g/L)	3.78 ± 0.42	3.89 ± 0.58	1.69–2.55	1.41 ± 0.38	2.01–3.09
VOA (g/L)	1.36 ± 0.03	0.72 ± 0.10	2.30–2.47	2.97 ± 1.17	0.85–2.28
SO ₄ ²⁻ (g/L)	1.95 ± 0.31	0.91 ± 0.24	0.18–3.12	0.96 ± 0.04	0.21–0.23
HS ⁻ (mg/L)	1118 ± 0.02	461 ± 60	77–308	81 ± 29	68–172
NH ₄ ⁺ (mg/L)	43.6 ± 39.0	51.9 ± 16.5	176–474	232 ± 53.8	232–248
pH	12.4	13.1	7.0 ± 0.5	7.44	7.0 ± 0.5
NO ₃ ⁻ (mg/L)	11.5 ± 1.0	10.5 ± 3.1	3.0–4.7	4.0 ± 1.6	3.3–5.7
NO ₂ ⁻ (mg/L)	4.5 ± 1.0	1.26 ± 0.23	0.60–0.82	0.50 ± 0.29	0.34–0.65
Cl ⁻ (g/L)	6.72 ± 0.05	8.26 ± 2.90	3.88–9.23	8.26 ± 2.91	7.85–8.20
Na (g/L)	2.14±	2.01 ± 0.83	1.51–2.77	4.83	2.39–5.40
P (mg/L)	2.67	2.34 ± 1.21	1.71–4.50	0.72 ± 0.21	0.80–1.84
Ca (mg/L)	660	485 ± 206	20–296	425 ± 229	125–299

TWW = tannery wastewater; ASBR = anaerobic sequencing batch reactor; HLFGR = hybrid linear flow channel reactor; HRT = hydraulic retention time; rpm = revolutions per minute; COD = chemical oxygen demand; Alk = alkalinity; VOA = volatile organic acids; SO₄²⁻ = sulfate; HS⁻ = hydrogen sulfide; NH₄⁺ = ammonium; NO₃⁻ = nitrate; NO₂⁻ = nitrite; Cl⁻ = chloride ions; Na = sodium; P = phosphorus; Ca = calcium.

continuous mixing at 50 rpm (Experiment 2, Fig. 1). At the end of Experiment 1, a study was conducted to determine the effect of increased HRT (from 4 to 8 days) on HLFGR pre-treatment and ASBR efficiency at 50 rpm (Experiment 3, Fig. 1) followed by a study to determine whether the volume of waste sludge generated from the HLFGRs could be reduced by downstream AD (Experiment 4, Fig. 1). The selected average influent and effluent parameters for Experiments 1–3 are provided in Table 1. The ASBR runs were operated at an inoculum to substrate ratio (ISR) of 2.5 ((volatile solids (VS)/VS)), at 37 ± 2 °C, and pH 7 ± 0.5 based on previous experimental outcomes (Mpofu et al., 2022).

2.2. Sampling

Samples were taken at the start and end of each run to determine the process efficiency. The physicochemical parameters were determined as previously described (Mpofu et al., 2023).

2.2.1. Hybrid linear flow channel reactors

Nineteen HLFGR ML samples were taken for Experiment 1. Samples were taken at day 0 and then every 3–4 days until day 46 (HLFGR1) and day 57 (HLFGR2). Four HLFGR ML samples were taken for Experiment 2 at days 58 and 62 (HLFGR1) and day 66 and 79 (HLFGR2). Two HLFGR ML samples were taken for Experiment 3 at days 78 (HLFGR1) and 86 (HLFGR2). Three samples of FSB were harvested after Experiment 1 (HLFGR1 day 78, HLFGR2 day 86) and Experiment 2 (HLFGR1 day 141).

2.2.2. Anaerobic sequencing batch reactors

Eight ASBR ML samples for Experiment 1 and 2 ASBR ML samples for Experiment 2 were taken from dedicated sampling ports on day 0 (start) and when biogas generation started to decrease at day 14 for all runs except the unmixed (0 rpm) run which was taken on day 30. An inoculum sample was taken at the start of Experiment 1. For Experiment 3, samples of HLFGR waste sludge were taken before and after 30 days AD.

2.3. Microbial analyses

2.3.1. Extraction of deoxyribonucleic acid

All samples were immediately frozen at -20 °C until required for deoxyribonucleic acid (DNA) extraction. Total genomic DNA was extracted from 0.5 g of dried FSB samples and centrifuged pellets of ML,

ASBR inoculum, HLFGR sludge and HLFGR digestate using the Qiagen (Hilden, Germany) DNeasy Powerlyzer PowerSoil DNA isolation kit according to the manufacturers' instructions. Each extraction was performed in duplicate, and the DNA concentrations were measured using a Jenway Genova (Bibby Scientific, Staffordshire, United Kingdom) NanoDrop spectrophotometer. Equimolar amounts of each duplicate were combined for molecular studies.

2.3.2. Amplicon sequencing

Amplicon sequencing was performed using an Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA) at Molecular Research laboratories (MR DNA) (Shallowater, TX, USA) according to their established in-house protocols as previously described (Horn et al., 2022b, Kibangou et al., 2022). Briefly, metagenomic DNA was used to amplify: (i) the V4 region of the small subunit of the 16S rRNA gene using the primer pairs 515F-Y and revised 806-R, (ii) a ~350 base-pair (bp) fragment of the β-subunit of the dissimilatory sulfite reductase (dsrB) gene using the primer pairs dsr2061F and dsr4R, and a 464 to 491 bp fragment of the methyl co-enzyme M reductase (mcrA) gene using a forward and reverse mcrA primer pair. The forward primers for each amplification were barcoded. The raw sequence data was analyzed via MR DNA as well as custom pipelines as previously described (Horn et al., 2022b, Kibangou et al., 2022). Briefly, the raw data was demultiplexed and subsequently formatted for use with the AmpProc pipeline version 5.1 (<https://github.com/eyashiro/AmpProc>). The pipeline was used in paired-end processing mode, and all reads were quality checked, merged and clustered into amplicon sequencing variants (ASVs) and taxonomically classified as previously described (Kibangou et al., 2022).

2.3.3. Quantitative polymerase chain reaction

Copy numbers of the mcrA gene fragment were determined in 20 μL reactions using a CFX96 thermal cycler (Bio-Rad, Hercules, USA) according to the described in detail by Kibangou et al. (2022) while copy numbers of the dsrB gene were determined using the same equipment according to the method described in detail by Horn et al., (2022b). The same primer pairs as per the amplicon sequencing (Section 2.3.2) were used for both quantitative polymerase chain reaction (qPCR) reactions.

In order to interpret the melt curve results, standard graphs were plotted as described by Kibangou et al. (2022) using plasmids containing amplicons from mcrA and dsrB polymerase chain reaction (PCR) products that were purified using the NucleoSpin kit (Machery-Nagel GmbH

& Co., Düren, Germany), ligated into pGEM®-T and pGEM®-T Easy vectors (Promega, Madison, USA), transformed into *Escherichia coli* JM109 high-efficiency competent cells (Promega), and isolated using the High Pure plasmid isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) following the respective manufacturers' instructions. For amplicon verification, Sanger sequencing of the putatively positive clones was conducted at Inqaba Biotech (Pretoria, South Africa).

2.3.4. Statistical analyses of data

Diversity indices were determined from relevant 16S rRNA, *dsrB* and *mcrA* amplicon zero rated operational taxonomic units (zOTUs) data using Primer 7® software. The relative abundances (RA) of zOTUs and the physicochemical data were analyzed using Primer 7® software (Primer-e, Auckland, New Zealand). Sequencing data was square root transformed and used to construct Bray-Curtis (BC) similarity plots. Based on BC similarity: (i) one way analysis of similarity (ANOSIM), and (ii) non-metric multidimensional scaling (nMDS) was performed. Similarity matrices based on Euclidian distance (ED) of fourth root transformed and normalized physicochemical data was analyzed using principal component analyses (PCA) and one-way ANOSIM. 'BEST' analyses of Spearman rank correlations between the transformed and normalized physicochemical data and the BC similarity of the biotic data were conducted to establish the most significant abiotic drivers of microbial community selection. The 'best' correlated parameters were used to construct LINKTREE plots using Primer 7® software. Heatmaps were generated using statistical software R version 4.2.2, wrapped by RStudio version 2023.06.2 (<https://rstudio.com/>) and the R package *ampvis2*. Significance levels for all statistical data are defined as: $<0.05^* \geq 0.01 > **0.005 \geq ***$ throughout the manuscript unless

otherwise stated.

3. Results and discussion

3.1. Statistical analysis of microbial community composition

In conjunction with ANOSIM (Table 2), the nMDS results (Fig. 2) indicated that: (i) both reactor environments (HLFCR/ASBR) were able to support relatively stable microbial populations, (ii) the environmental conditions present during either HLFCR pre-treatment or AD in ASBRs supported significantly different microbial populations, and (ii) the environment in the ASBRs was more favorable for growth of microbial species from the well-acclimated inoculum than those from the HLFCR effluent during AD. These findings applied to all the microbial communities that were tested, namely the overall bacterial, MA and SRB.

Amplicon sequencing analysis yielded a total of 19 471 796, 2 602 195, and 10 912 635 high-quality reads for the 16S rRNA, *mcrA* and *dsrB* gene amplicons across 41 samples, respectively. Horizontal asymptotes were achieved in the rarefaction curves of all the samples, showing that the sequencing depth was sufficient to capture the diversity. Ratios between observed and estimated richness (Chao 1) of 0.84, 0.66 and 0.71 were obtained for 16S rRNA, *mcrA* and *dsrB* gene amplicons, respectively, indicating high quality sequencing data. In terms of univariate indices (see Supplementary material), the diversity of the overall bacterial and MA communities in the ASBRs were highly similar and reflected the diversity in the inoculum. However, the diversity of the SRB populations was lower in the ASBR runs fed with influent from the more intensive pre-treatment (2-stage HLFCRs) than

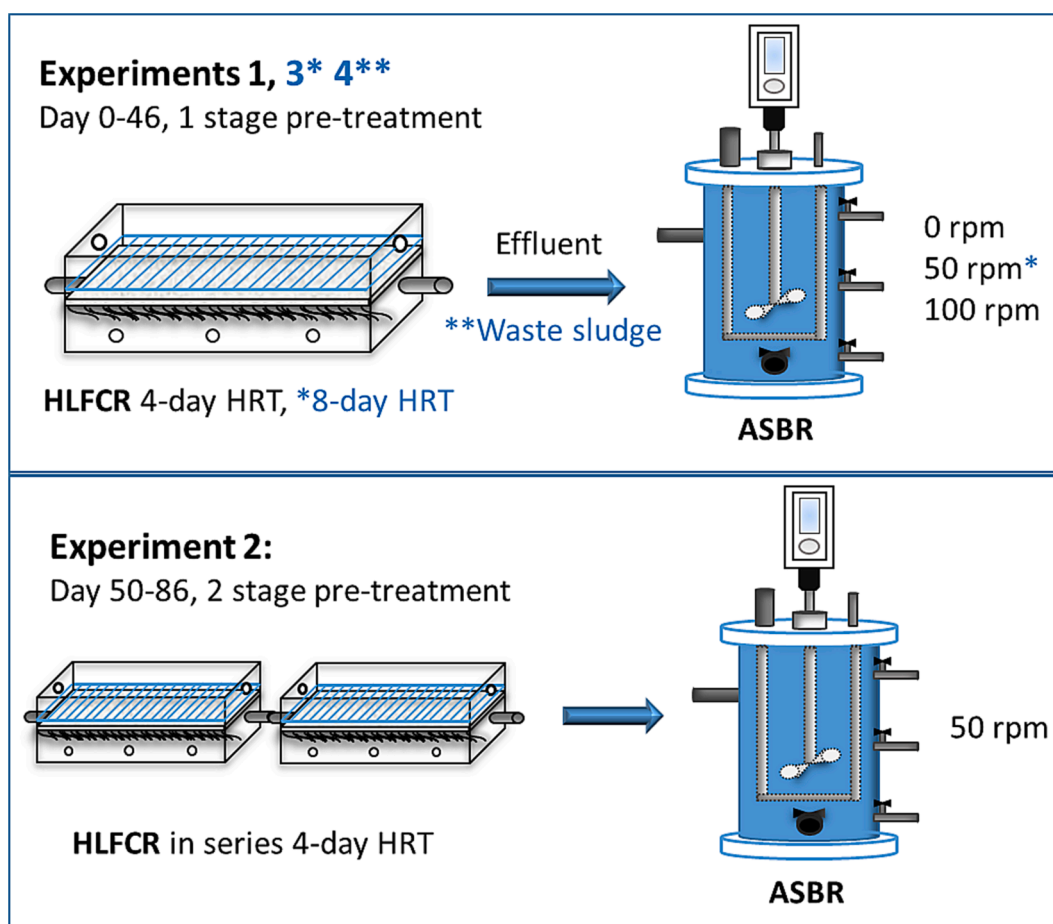


Fig. 1. Experimental set-up showing the operational variables of the hybrid systems.

Table 2

One-way Analysis of similarity between mixed liquor samples from anaerobic sequencing batch reactors and hybrid linear channel reactors (4-day hydraulic retention time).

	16S rRNA Global R = 0.463***		mcrA Global R = 0.397***		dsrB Global R = 0.194***	
	HLFCR1	HLFCR2	HLFCR1	HLFCR2	HLFCR1	HLFCR2
ASBR	0.635***	0.747***	0.376***	0.740***	0.682***	0.602***
HLFCR1		0.031		0.053	0.010	

Level of significance: *** \leq 0.005.

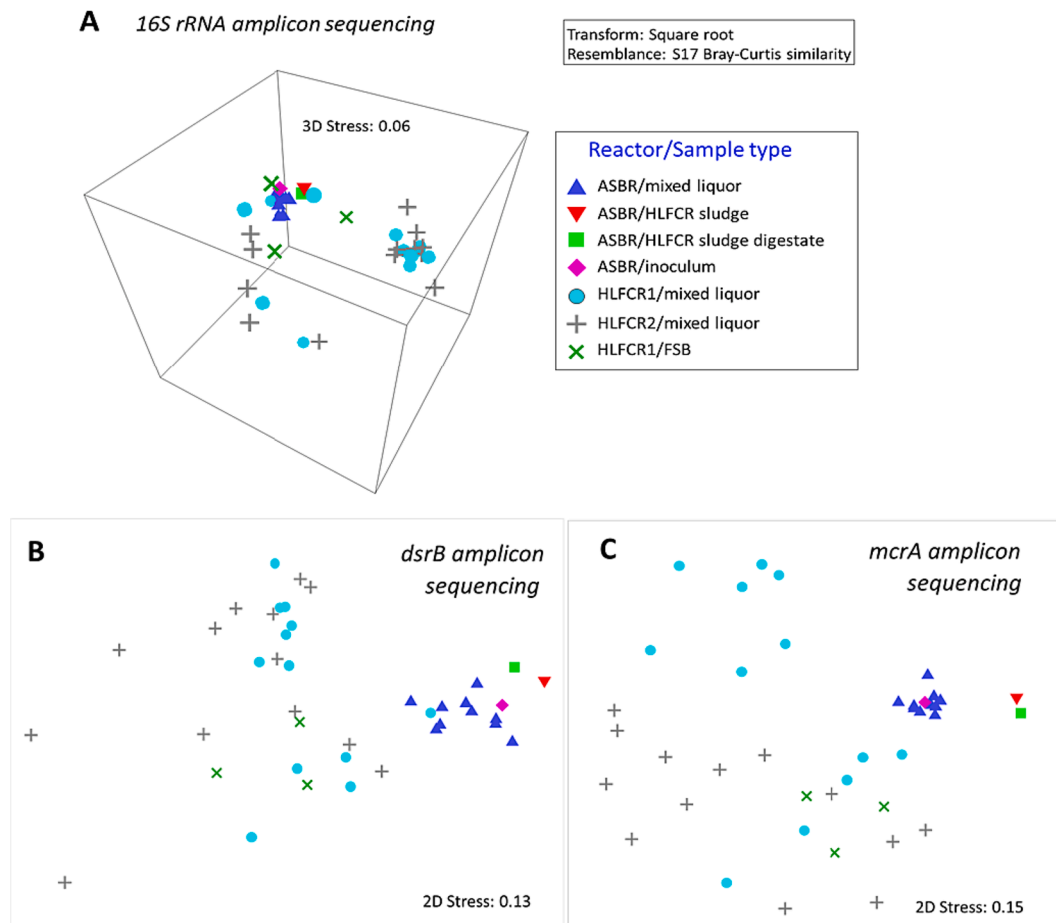


Fig. 2. Non-metric multidimensional scaling plots depicting the bray-curtis similarity of square root transformed amplicon sequencing data using primer sets for: (A) the 16S rRNA (B), the dsrB (C), the mcrA gene sequences.

those fed with influent from the 1-stage HLFCRs. Conversely, Increasing the HRT from 4 to 8 days appeared to stimulate higher diversity in the SRB populations in the HLFCRs.

3.2. Functional analysis of methanogens and sulfidogens in the hybrid system

3.2.1. Analysis of results from anaerobic sequencing batch reactors

In a previous study it was found that the CH₄ yield from raw TWW increased significantly after pre-treatment in HLFCRs (Mpofu et al., 2023). This study investigated the effect of mixing and HLFCR intensity on AD efficiency in conjunction with bacterial community composition using the protocol described in Section 2.1 for Experiments 1–4. In the ASBR runs treating the effluent from less intensive, (1-stage, 4-day HRT) HLFCR pre-treatment (Experiment 1), the highest average CH₄ yield was found with continual mixing at 50 rpm (225 mLCH₄/gCOD_{consumed}, 62 % biogas CH₄). Under the same conditions, AD of the TWW pre-treated more intensely in 2-stage HLFCRs (Experiment 2) showed a notably higher yield (321 mLCH₄/gCOD_{consumed}, 59 % biogas CH₄). Similar

results (314 mLCH₄/gCOD_{consumed}, 52 % biogas CH₄) were achieved for AD of TWW that had been pre-treated in 1-stage HLFCRs with increased HRT (from 4 to 8 days, Experiment 3). Overall, these results showed that AD efficiency was promoted by increasing the intensity of HLFCR pre-treatment.

In terms of AD of the HLFCR sludge (Experiment 4, Fig. 1), the results were not promising as only low CH₄ yields (88 mL/gVS) were obtained after a lag phase of 10 days. The poor performance was assumed to be due to the presence of high concentrations of metals that had partitioned into the sludge, and lower amounts of readily biodegradable organics available after pre-treatment (Mpofu et al., 2023). The close proximity of data points representing the HLFCR sludge before and after AD indicated that the microbial populations remained highly similar throughout the AD process (Fig. 2).

In terms of methanogenic etiology, the inoculum to the ASBRs was continuously fed with the same batch of TWW between successive runs (Section 2.1). The copy numbers of the mcrA gene in the ASBRs varied at the start of each run, reflecting that the MA population within the side-stream inoculum was in an unavoidable state of flux (Fig. 3A). In all

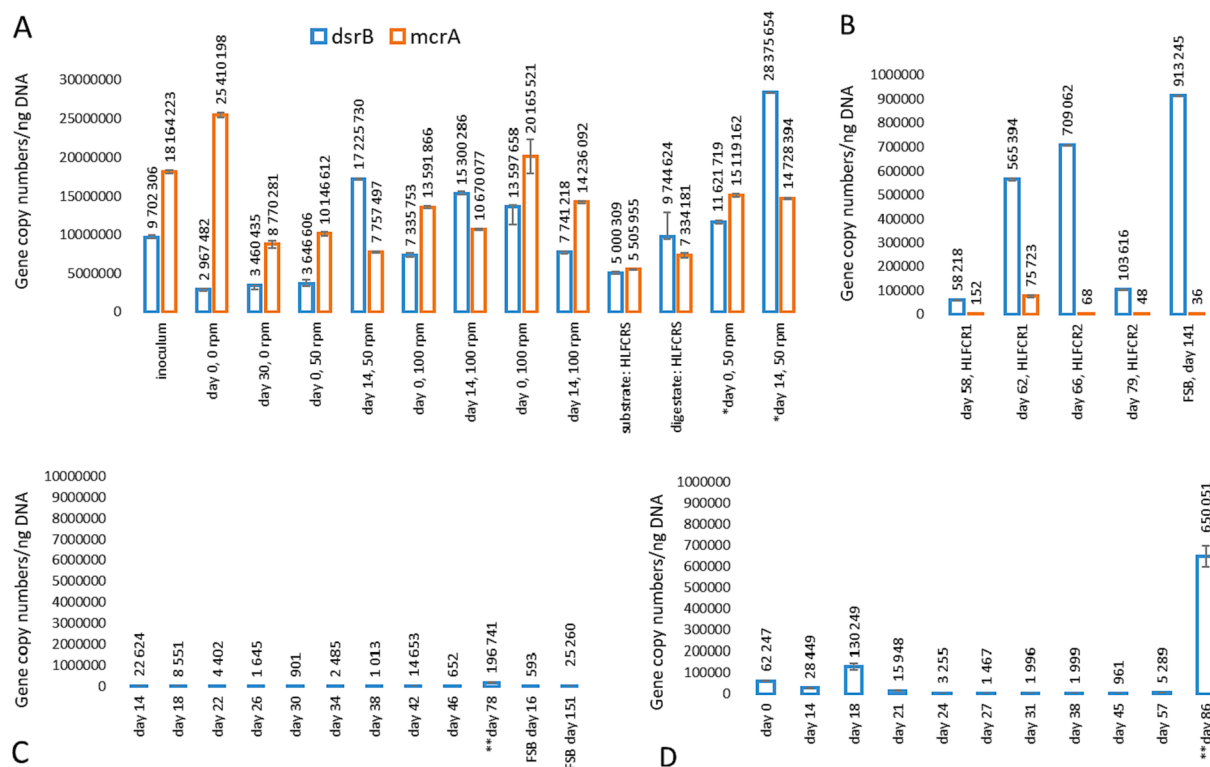


Fig. 3. Copy numbers of *dsrB* and *mcrA* amplicons in: (A) anaerobic sequencing batch reactors, and (B–D) hybrid linear flow channel reactors: 2-stage HLFCHR (B), 1-stage HLFCHR1 (C), 1-stage HLFCHR2 (D). All samples are mixed liquor unless otherwise stated. *Influent from 2-stage HLFCHR, **8-days HRT. HLFCHR = hybrid linear flow channel reactor sludge. Error bars represent standard deviation from the mean.

ASBR reactors, there were temporal decreases in copy numbers of the *mcrA* gene amplicon during AD (Fig. 3A). However, the decrease was negligible in the ASBR runs treating effluent after more intensive (2-stage) pre-treatment, supporting the results obtained in terms of CH_4 yield. While significant positive correlations between *mcrA* gene amplicon copy numbers and methanogenesis have been found in AD reactors digesting anaerobic biomass (Morris et al., 2014), coffee wastewater (Cetecioglu et al., 2019) and TWW (Kibangou et al., 2022), differences in CH_4 generation in ASBRs treating TWW may also be related to the MA community composition independent of *mcrA* gene copy numbers. For example, it has been postulated that methanogenic efficiency is increased by high RA of the highly functional and adaptable species *Methanosarcina mazei* (Kibangou et al., 2022). Selection of highly functional methanogenic taxa during AD may explain the *mcrA* qPCR results obtained in this study.

In contrast to the *mcrA* gene copy numbers, there was a temporal increase in *dsrB* gene copy numbers (Fig. 3A) in the majority of samples taken from the ASBR reactors, indicating that concurrent BSR and methanogenesis took place in the ASBRs (refer to Section 3.2.2 for detailed discussion).

3.2.2. Analysis of results from hybrid linear flow channel reactors and downstream effects on anaerobic digestion

Average 53 % and 59 % reductions in SO_4^{2-} and HS^- from raw TWW were obtained after pre-treatment in 1-stage HLFCHR at 4-day HRT (Experiment 1), with average effluent concentrations of 910 ± 240 mg/L and 461 ± 60 mg/L, respectively (Table 1). More intensive pre-treatment, either 2-stage HLFCHR operation (Experiment 2) or increased HRT (Experiment 3) did not have significant effects on the SO_4^{2-} concentrations, but significantly decreased the HS^- concentrations, showing overall 90–94 % and 89–96 % reductions, respectively. The lower residual HS^- in the effluent after more intensive pre-treatment (81 ± 29 mg/L in the 2-stage HLFCHR, Table 1) almost certainly contributed

to the higher downstream methanogenic rates achieved in the ASBRs.

The *dsrB* amplicon copy numbers in HLFCHR after more intensive pre-treatment (Experiments 2 and 3) were orders of magnitude higher than those from Experiment 1, namely the 1-stage HLFCHR operated at 4-day HRT (Fig. 3B–D). The notably higher residual HS^- and lower *dsrB* abundance findings are supported by the results obtained by Kibangou et al. (2022) who found a significant negative correlation between HS^- and *dsrB* abundance in BMP reactors treating TWW with a range of influent SO_4^{2-} concentrations. Copies of the *mcrA* gene amplicon were found in the samples taken from the 1-stage HLFCHR, but results were not analyzed further because copy numbers were low (0.93–94 copies/ngDNA). Higher copy numbers were measured in a sample taken from the 2-stage HLFCHR (75723 copies/ngDNA, Fig. 3B). It was postulated that although the redox potential in the bulk liquid of HLFCHR is consistently low enough to support methanogenesis (<380 mV, Horn et al., 2022a), the strict anaerobic conditions needed for robust growth of MA would have been prevented by O_2 and/or inhibitory concentrations of HS^- in the influent. The lower HS^- concentrations and O_2 ingress during 2-stage operation would theoretically be more conducive to growth of MA.

The qPCR results are interesting in terms of substrate competition by SRB and MA, because high sulfidogenic activity and low methanogenic activity may be expected with an increase in *dsrB* and decrease in *mcrA* during AD. However, good CH_4 yields were obtained after intensive HLFCHR pre-treatment (Section 3.2.1). These results suggest that MA and SRB competition during AD of TWW may not have significant detrimental effects on process efficiency, particularly if organic substrates are not limited. Indeed, provided the COD/ SO_4 ratio is not too low, controlled sulfidogenesis has previously been shown to increase AD process stability by promoting hydrolysis-acidification and subsequent aceticlastic methanogenesis (Lu et al., 2016). In this study, the residual COD concentrations in the effluent from the 1-stage and 2-stage HLFCHR were 8720 ± 1480 mg/L and 6370 ± 1680 mg/L, respectively (influent

22800 ± 3700 mg/L), while the volatile organic acid (VOA) concentrations were 720 ± 100 mg/L and 2970 ± 1170 (Table 1). The average concentration of VOAs in the ASBRs treating effluent from the 1-stage HLFGR increased more than 3-fold, indicating VOA accumulation. Conversely, there was an average 3.5-fold decrease in the VOA concentration in the ASBRs treating effluent from the more intensive 2-stage HLFGR pre-treatment. Accumulation of VOAs can inhibit hydrogenotrophic methanogens (HM) and acetoclastic methanogens (AM) (Wang et al., 2023), and, together with other factors like HS⁻ inhibition, already alluded to, may have contributed to the comparatively low methanogenic efficiency in the ASBRs treating effluent from the less intensive (1-stage, 4-day HRT) HLFGR pre-treatment.

3.2.3. Key drivers of bacterial community selection in hybrid linear channel reactors

A wide range of physicochemical parameters were measured in ML samples from the 4-day HRT HLFGRs: COD, total organic carbon (TOC), Alk, VOA, SO₄²⁻, HS⁻, NH₄⁺, pH, NO₃⁻, NO₂⁻, Cl⁻, Na, P, phosphate (PO₄²⁻), calcium (Ca), magnesium (Mg), silica (Si), potassium (K), aluminium (Al), iron (Fe), zinc (Zn), strontium (Sr), barium (Ba) and the COD:SO₄²⁻ and C:N ratios (data not shown). There were highly significant differences between the physicochemical profiles in the 1-stage and 2-stage HLFGRs (global ANOSIM R = 0.926**). The physicochemical data was analyzed in conjunction with overall bacterial and SRB microbial data to assess which parameters were the major drivers of community compositions (Fig. 4A). The highest Spearman rank correlations (BEST analyses) were noted for NH₄⁺ (r = 0.613 and 0.499, respectively for the overall bacterial and SRB populations), while the 'best' correlated combinations of parameters were: NH₄⁺, PO₄²⁻, NO₃⁻, Si, pH (r = 0.786) and HS⁻, Si, pH (r = 0.709) for the overall bacterial and SRB populations, respectively. Binary divisive cluster (LINKTREE) plots using these parameters were compiled for each population (Fig. 4B–C). The 1-stage HLFGRs were characterized by significantly higher NH₄⁺ and Si concentrations and lower NO₃⁻, than the 2-stage HLFGRs, which were the primary drivers of differences in the overall bacterial populations (Fig. 4B). The results suggest that nitrification plays a crucial functional role in HLFGRs. The SRB results were more complex but showed that HS⁻ concentration played a pivotal role in SRB community selection (Fig. 4C). Lower overall rates of BSR naturally expected with less intensive pre-treatment not only had a knock-on negative effect on AD of the pre-treated TWW, but also led to selection of different SRB more suited to the high HS⁻ and lower Si environment. The metabolic role of Si on the SRB in HLFGRs merits further investigation.

3.3. Analysis of dominant overall bacterial, methanogenic archaeal, and sulfate reducing taxa

3.3.1. Overall bacterial community composition

In the ASBRs, *Firmicutes*, *Synergistota*, *Bacteroidota*, and to a lesser extent, *Thermotogota* co-dominated the phyla, while *Firmicutes* dominated in the HLFGRs, followed by *Proteobacteria*. Many *Firmicutes* and *Bacteroidota* are hydrolytic and these phyla are commonly found in high RA in AD reactors (Arelli et al., 2023; Yamamoto-Ikemoto et al., 2023), while *Thermotogota* are capable of inter-species H-transfer with methanogens and are often found in moderately high RA during AD of different substrates under a variety of conditions (Arelli et al., 2023; Yamamoto-Ikemoto et al., 2023). *Synergistota* may (Deng et al., 2023) or may not (Arelli et al., 2023; Yamamoto-Ikemoto et al., 2023) form part of the dominant overall bacterial phylum profile in AD, and it appears that this may be related to the type of organic substrate/s available for conversion to VOAs by this synergistic group of organisms (Deng et al., 2023). Somewhat uncharacteristically for AD, *Proteobacteria* and *Actinobacteria* were found in low RA, and *Chloroflexi* did not even rank among the top 10 dominant phyla in the ASBRs (Arelli et al., 2023; Yamamoto-Ikemoto et al., 2023). This may have been driven by the complex nature and potential toxicity of TWW (Mpofu et al., 2023; Kibangu et al., 2022).

There was also a relatively high abundance of *Desulfobacterota* (0.5 % to 23 %) in the ASBRs, the 1-stage HLFGRs (only after long term operation) and the 2-stage HLFGRs. This group of organisms typically prefer anoxic or anaerobic growth conditions and many use S species as terminal electron acceptors and/or donors during heterotrophic fermentation or chemolithotrophic disproportionation (Murphy et al., 2021). In this study, selection of these SRB during more intensive or long-term pre-treatment was associated with lower HS⁻ concentrations when analyzed in conjunction with the LINKTREE results (Section 3.2.2).

The genera *Aminobacterium* (phylum *Synergistota*), *Aminirod* (phylum *Synergistota*) and family *Rikenellaceae* (phylum *Bacteroidata*) co-dominated in the ASBRs (Fig. 5) but were only present in low RA (0–2.5 %, data not shown) in the 1-stage HLFGRs. These taxa have also been found in high RA during AD of municipal sludge (Li et al., 2022). Other genera present in high RA in the ASBRs were *Proteiniphilum* (phylum *Bacteroidata*) and *Mesotoga* (phylum *Thermotogae*), followed by *Sulfospirillum* (phylum *Proteobacteria*), *Thermovirga* (phylum *Firmicutes*) and *Anaerobaculum* (phylum *Firmicutes*). In the 1-stage HLFGRs, different patterns of dominant genera emerged (Fig. 5). In the majority of ML samples from HLFGR1 and HLFGR 2 at 4-day HRT, *Halomonas* (phylum *Proteobacteria*), and/or, *Alkalibacillus* (phylum *Firmicutes*) and a series of unidentified bacilli co-dominated. In other ML samples from the 1-stage HLFGRs (day 4 and day 8 HRT) and the FSB samples, different combinations of *Dethiosulfobaculum* (phylum *Proteobacteria*), *Marinobacterium* (phylum *Proteobacteria*), *Denitrovibrio* (phylum *Deferribacteres*), *Oceanotoga* (phylum *Proteobacteria*), *Sulfospirillum* (phylum *Proteobacteria*) and *Clostridium* (phylum *Firmicutes*) co-dominated, while different combinations of *Proteiniphilum*, *Halomonas*, *Marinobacterium*, *Oceanotoga*, *Clostridium*, *Aminirod*, *Alcaligenes* (phylum *Proteobacteria*) and *Macellibacteroides* (phylum *Bacteroidata*) co-dominated transiently in the 2-stage HLFGRs. Although the overall bacterial genera RA profiles in the 1-stage and 2-stage HLFGRs differed, increasing the intensity of pre-treatment by increasing the HRT did not affect overall bacterial dominance. However, these genera are not typically associated with nitrification (Section 3.2.2).

Taxa that were present in high RA in the HLFGRs and continued to thrive in ASBRs (after day 0) included *Aminirod*, *Proteiniphilum*, *Dethiosulfobaculum*, *Desulfobaculum* and *Halomonas*, taxa which are either involved in protein degradation, S metabolism or nitrification/denitrification. As the name infers, *Halomonas* species are extremely salt tolerant and can proliferate in hypersaline environments contaminated with toxic metals and/or high concentrations of S²⁻ (200 mg/L according to Liu et al., 2016). *Halomonas salifodinae* and other *Halomonas* species have been found in high RA in other wastewater treatment systems such granular reactors treating synthetic saline wastewaters (Liu et al., 2016), and an activated sludge system treating pharmaceutical effluent (Hu et al., 2022). Members of the genus are metabolically versatile. They are capable of heterotrophic organic metabolism as well as simultaneous (heterotrophic) nitrification and (aerobic) denitrification (Hu et al., 2022; Liu et al., 2016).

Apart from general organic hydrolytic and/or acidogenic metabolic capabilities, other members of the dominant taxa (family/genera) found in the ASBR and HLFGR samples have been reported to harbor one or more specific metabolic capabilities that may explain their competitive selection in the saline TWW milieu which typically contains relatively high amounts of fats, proteins, NH₃/NH₄⁺, and S-species. These include: (i) taxa that have the ability to degrade and/or utilize proteins and/or amino acids as substrates such as *Alkalibacillus* (Abdel-Hamed et al., 2016) (ii) taxa that are notably halophilic/halotolerant such as *Alkalibacillus* (Abdel-Hamed, 2016), *Aminobacterium* (Wu et al., 2023) and *Marinobacterium* (Huang et al., 2020) (iii) taxa that are NH₃/NH₄⁺ tolerant such as *Rikenellaceae* (Li et al., 2022) and *Proteiniphilum* (Feng et al., 2023), (iv) taxa capable of simultaneous nitrification–denitrification: *Halomonas* (Hu et al., 2022), *Marinobacterium* (Huang et al., 2020) or dissimilatory denitrification: *Denitrovibrio* (Myhr and Torsvik, 2000), and (v) taxa dependent on or able to oxidize or

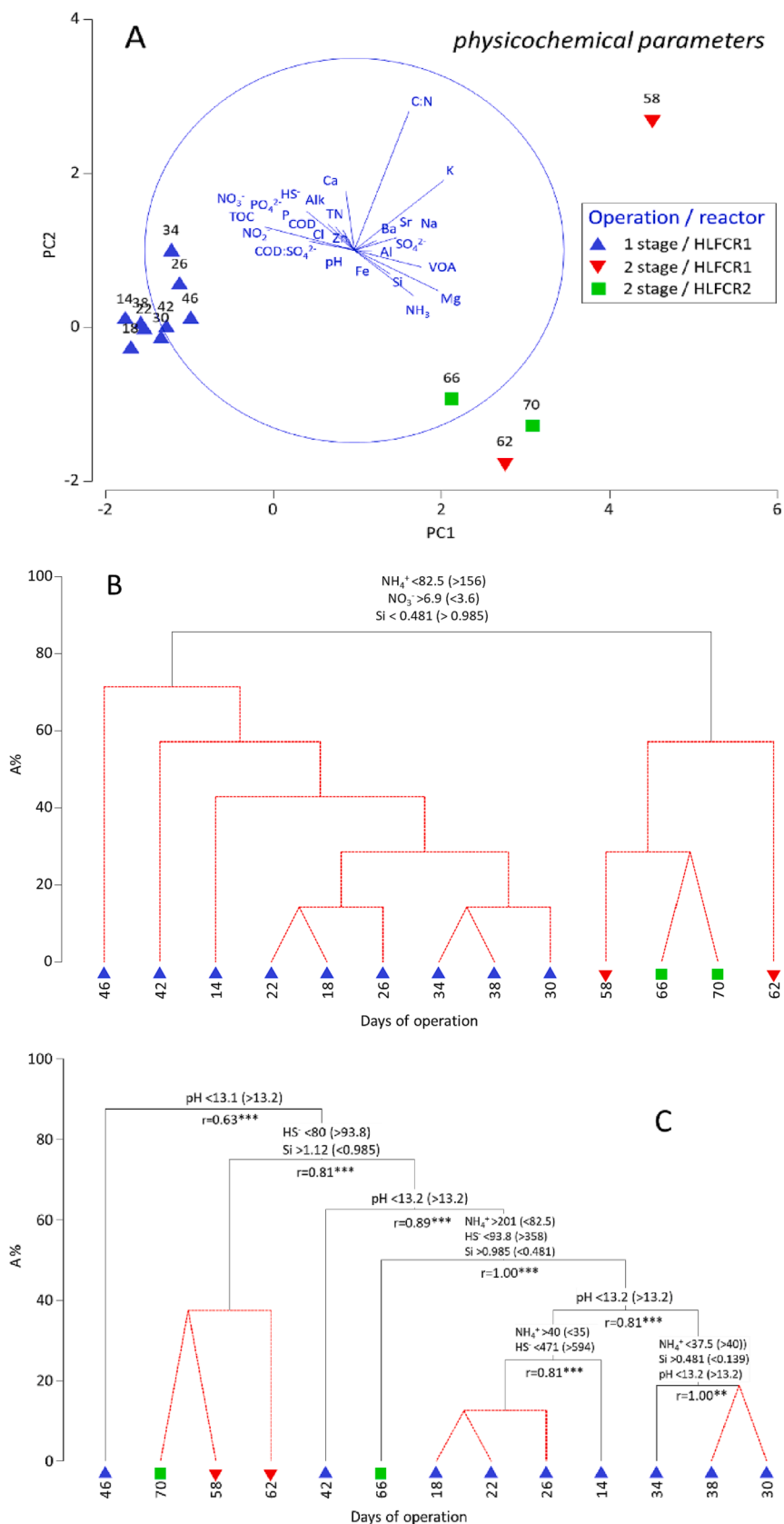


Fig. 4. Principal component analysis plot of physicochemical data (A) and LINKTREE plots of the physicochemical parameters driving significant differences in the overall bacterial community composition (B), and the sulfate reducing bacterial community composition (C).

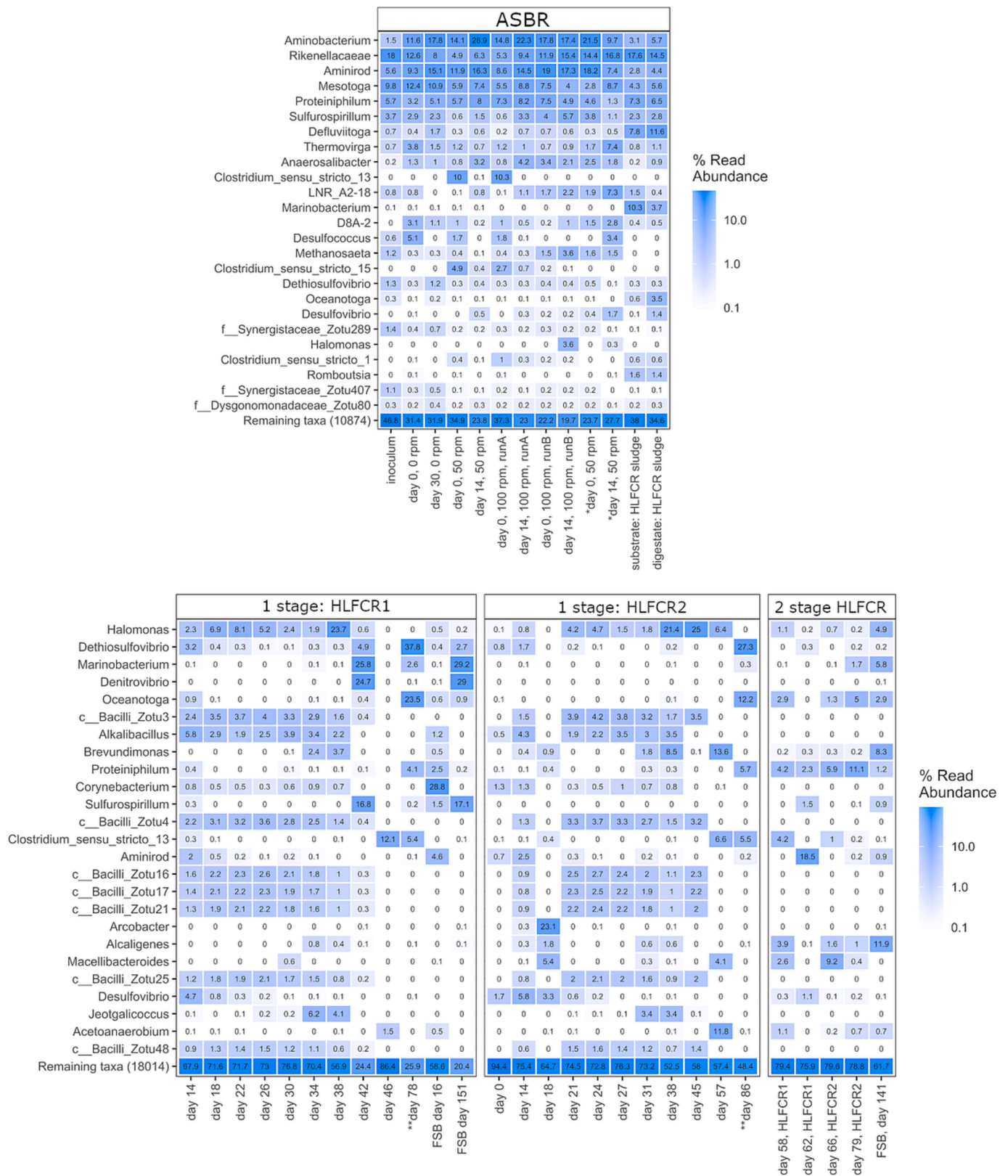


Fig. 5. Heatmaps showing the most dominant genera in the anaerobic sequencing batch reactors and the hybrid linear channel reactors determined using 16S rRNA gene amplicon sequencing. **8 days HRT. All samples are mixed liquor unless otherwise stated.

reduce S-species for energy such as *Dethiosulfovibrio* (Horn et al., 2022b), *Sulfopirillum* (Guerrero-Barajas et al., 2014) and *Desulfovibrio* (Guerrero-Barajas et al., 2014).

3.3.2. Methanogenic archaeal community composition

The qPCR results indicated that the absolute abundance of the MA in the ML samples from the 1-stage 4-day HRT HLFGRs were low (Section 3.2.2), and results from these samples have been excluded from the

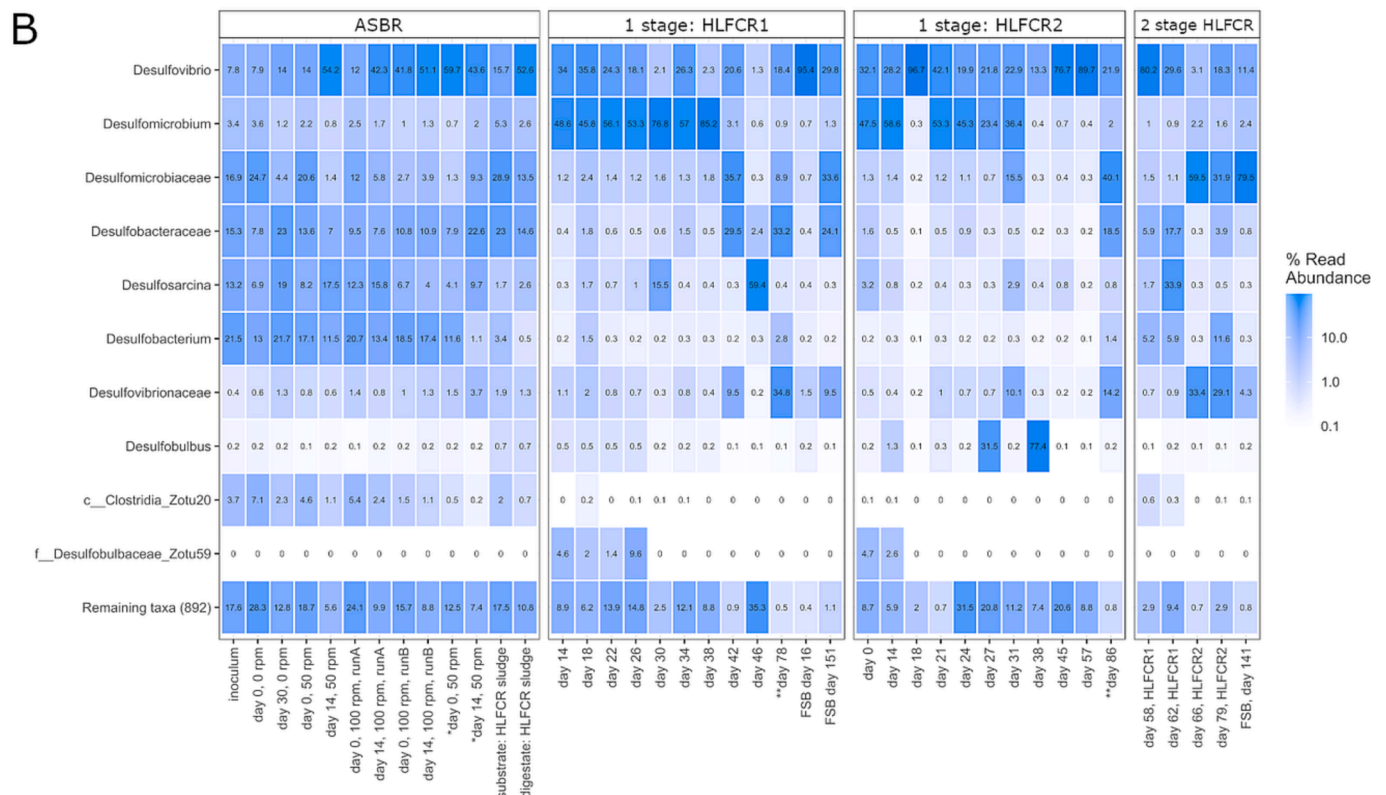
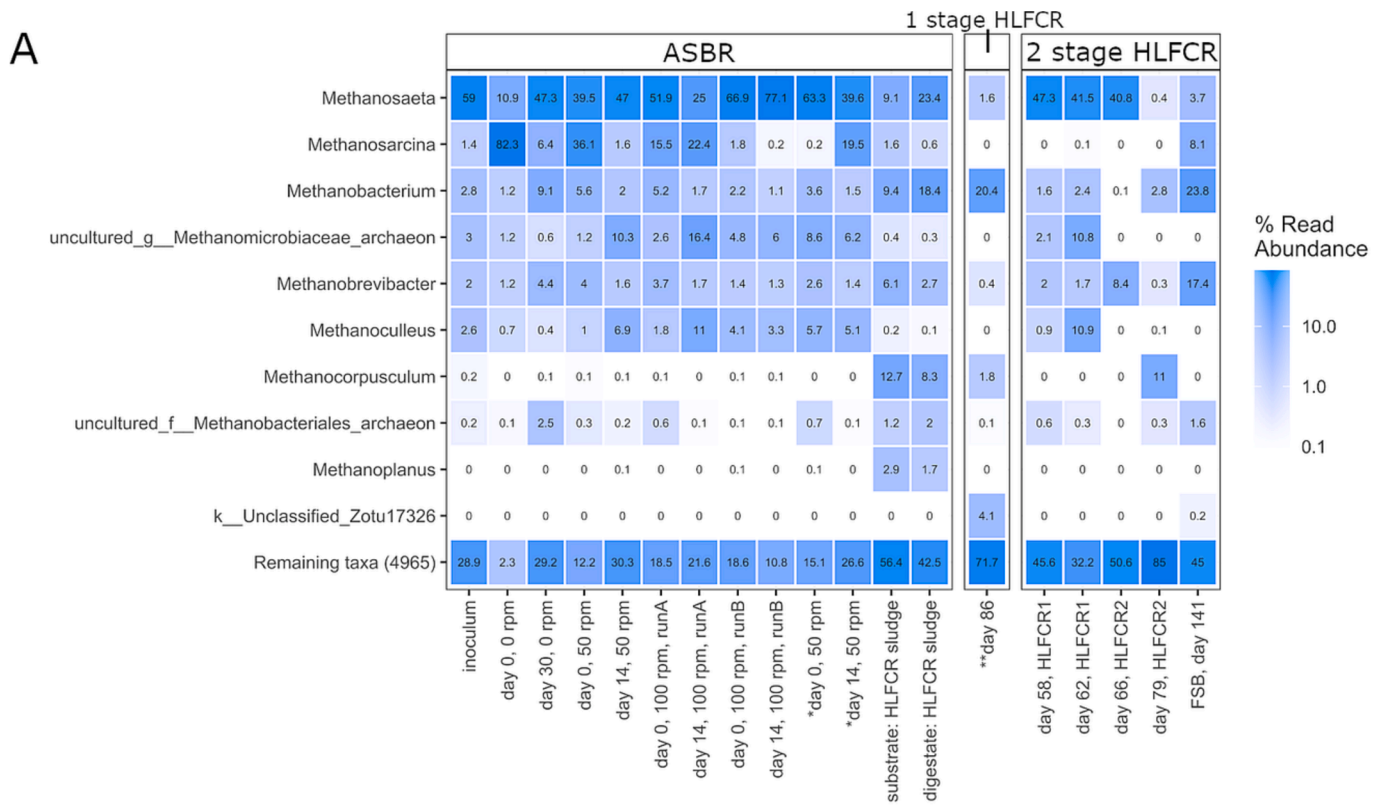


Fig. 6. Heatmaps showing the ten most dominant genera determined using mcrA gene amplicon sequencing (A) and the most dominant genera determined using dsrB gene amplicon sequencing (B) *2-stage HLFCR, **8 days HRT. All samples are mixed liquor unless otherwise stated.

discussion on the MA community composition.

In the initial inoculum and the majority (10/11) of the ML samples taken from the ASBRs, *Methanosaeta* was the most dominant genus (25–77 % RA), usually followed by *Methanosarcina* (Fig. 6). Mixing did not appear to influence preferential selection of *Methanosaeta* or *Methanosarcina*. In a previous study, strong selection of *Methanosarcina mazei* in ASBRs treating TWW was thought to be linked to continuous mixing (Kibangu et al., 2022). However, although *Methanosaeta* and *Methanosarcina* were the dominant genera in both studies, the etiology of the TWW was different, which could explain this finding. *Methanosaeta* was also the most dominant genus in 3 (of 4) ML samples from 2-stage HLFGRs and the digestate from AD of HLFGR sludge, indicating strong selection of this genus in the TWW used in this study under a variety of different operational conditions.

Methanosarcina and *Methanosaeta* (also designated as *Methanothrix*) are the only two AM genera. *Methanosarcina* are also capable of methylophilic methanogenesis and HM (Chen and He, 2015). Members of both genera are typically associated with high CH₄ generation during AD (Kibangu et al., 2022; Yan et al., 2020). *Methanosarcina* have higher maximum specific growth rates (μ_{max}) and half-saturation coefficients (K_s) than *Methanosaeta* species when grown in acetate-containing media (Chen and He, 2015; Conklin et al., 2006). Most studies have found that *Methanosaeta* are present in higher abundance during AD of a variety of substrates, but more efficient and stable performance has been associated with the presence of *Methanosarcina* (Conklin et al., 2006), and they often prevail in environments that are toxic to other AM (Yan et al., 2020) which could explain their selection in reactors treating TWW. It has been clearly demonstrated that enrichment of *Methanosarcina* over *Methanosaeta* can be achieved by increasing organic loading rates (OLR) and reducing solids retention times (SRT) (Conklin et al., 2006; Mathai et al., 2020). In previous studies, *Methanosarcina* have also been preferentially selected in AD reactors with high fats and volatile fatty acid contents (Capson-Tojo et al., 2018) typically encountered in TWW. In the past, it was thought that *Methanosaeta* spp. can only utilize acetate as a substrate, but members of the genus have since been shown to be more metabolically versatile (Rotaru et al., 2014; Feng et al., 2023). In addition, direct interspecies electron transfer from *Geobacter metallireducens* (Rotaru et al., 2014) and *Synthrophomonas* (Zhao et al., 2018) to *Methanosaeta* have been demonstrated, a mechanism that promotes the reduction of CO₂ to CH₄ (Zhao et al., 2018). Both *Methanosaeta* and *Methanosarcina* can acclimate to high concentrations of NH₃/NH₄⁺ (Capson-Tojo et al., 2018; Feng et al., 2023; Nordgård et al., 2017), which would also be key to their dominance during AD of TWW. In addition, co-dominance of *Proteiniphilum* (found in high RA in this study) and *Methanosarcina* has been demonstrated in AD reactors operated under NH₃/NH₄⁺ stress (Feng et al., 2023). However, another study found that *Methanosarcina* dominated at lower NH₄⁺ concentrations, while *Methanosaeta* dominated at higher NH₄⁺ concentrations (1.9 g/L NH₄⁺ and 3.7 NH₄⁺ g/L, respectively in upflow anaerobic sludge blanket reactors treating pig manure supernatant) (Nordgård et al., 2017). Clearly, there are many factors that affect selection and proliferation of *Methanosarcina* and/or *Methanosaeta* during AD as another study showed that AM by *Methanosaeta thermophila* is inhibited by NH₃/NH₄⁺ (≥ 1.7 g/L) when compared with CH₄ generation by syntrophic acetate oxidation (SAO) and HM (Kato et al., 2014). This SAO-HM shift from AM by *Methanosaeta* to HM by members of the fast-growing and NH₃/NH₄⁺ tolerant *Methanoculleus* genus has also been seen during accumulation of VOA brought on by high OLR (Mathai et al., 2020; Yang et al., 2018). It is possible that there may be competition between HM by *Methanoculleus* and AM by *Methanosarcina* in reactors operated with high OLR (Conklin et al., 2006; Mathai et al., 2020).

In light of these previous findings, the dominance of *Methanosarcina* and *Methanosaeta* in the ASBRs suggests that the primary CH₄ generation mechanism during AD of TWW was via AM. The VOA and NH₄⁺ concentrations in the ASBR ML samples ranged from 0.85 to 2.47 g/L and 0.18–0.47 g/L, respectively (Table 1), so NH₃/NH₄⁺ inhibition on AM is

unlikely, but transiently high OLRs and VOA concentrations (Table 1) may have been key drivers in the shifts in RA between *Methanosarcina* and *Methanosaeta*. In some of the HLFGR samples, the HM (*Methanobacterium*, *Methanoculleus*, *Methanomicrobiaceae*, *Methanobrevibacter*) dominated or co-dominated in different ratios (Fig. 6). These were also present as secondary dominant MA in the ASBRs, suggesting that HM was the major CH₄ generation mechanism in the HLFGRs and also played an important function in the ASBRs (Bharathi et al., 2020; Jiao et al., 2022; Yang et al., 2018).

3.3.3. Sulfate reducing bacterial community composition

Different genera of SRB oxidize organics completely to CO₂, or incompletely to acetate (or both) using a variety of sulfurous and/or non-sulfurous electron acceptors (Zhang et al., 2022). There are a number of studies that support the notion that incomplete oxidizers (IO) dominate over complete oxidizers (CO) during AD because they provide acetate for AM while CO compete with AM (Hao et al., 2014; Zhang et al., 2022). However, Du et al. (2023) showed that IO/CO selection may be substrate dependent: while IO (*Desulfuromonas*) dominated in ethanol fed reactors, CO (*Desulfococcus*) dominated in acetate-fed reactors. In this study, both IO and CO SRB families and genera dominated or co-dominated in the ASBRs: *Desulfovibrio* (IO), *Desulfomicrobiaceae* (IO), *Desulfobacteraceae* (different genera IO or CO), *Desulfosarcina* (CO), *Desulfobacterium* (CO) and two less dominant genera (*Desulfomicrobium* (IO), and *Desulfovibrio* (only member of *Desulfovibrionaceae* (IO) (Fig. 6). It was hypothesized that the high prevalence of both IO and CO SRB was possible because there was sufficient biodegradable organic substrate available for both CO and IO SRB, with the latter still being able to provide sufficient acetate for the AM in the ASBRs (Table 1). Previously, in ASBRs treating TWW, three of the same dominant genera (*Desulfovibrio*, *Desulfomicrobium*, and *Desulfobacterium*) and one additional genus (*Desulfobulbus*) were identified (Kibangu et al., 2022), suggesting that both TWW and HLFGR pre-treatment influences the SRB community composition.

Some of the dominant genera have also been found to selectively proliferate during AD of other forms of SO₄²⁻ rich waste: *Desulfovibrio* during AD of high SO₄²⁻ vegetable waste (Zhang et al., 2023), organic agro-industrial effluent (Olivera et al., 2022) and WAS (Lippens and Vrieze, 2019), *Desulfobacterium* during AD of high SO₄²⁻ pig manure (Du et al., 2021), *Desulfomicrobium* during AD of high SO₄²⁻ WAS (Lippens and Vrieze, 2019), slaughterhouse wastewater (Yan et al., 2018), and *Desulfobulbus* during AD of high SO₄²⁻ WAS (Lippens and Vrieze, 2019).

There were typically two co-dominant genera in the HLFGRs, namely, *Desulfovibrio* and *Desulfomicrobium*. A literature review has established that these IO are amongst the four most common genera found in bioreactors treating SO₄²⁻ rich effluents (Hao et al., 2014). There was more sample-to-sample variation in the SRB RA genus profiles from the HLFGRs than from the ASBRs, although *Desulfovibrio* (IO) was dominant or co-dominant in most of the samples and *Desulfomicrobium* (IO) was co-dominant in the 1-stage HLFGRs after the first month of operation, after which other genera filled the co-dominant niche. The SRB profiles in the samples from the 2-stage HLFGRs exhibited similarities with those from the 1-stage HLFGRs after 38 days' operation. The lower diversity in dominant SRB genera in the HLFGRs may render them less resilient to environmental changes than the ASBRs.

Overall, as with the MA, the most abundant SRB genera in the ASBRs and HLFGRs harbored other characteristics that would theoretically allow them to adapt to the SO₄²⁻ rich TWW milieu. For example, saline tolerance by *Desulfobacterium*, (Marietou et al., 2021); *Desulfosarcina* (Kleindienst et al., 2014; Watanabe et al., 2020), and *Desulfovibrio* (Kumar et al., 2020) and utilization of protein or amino acids as substrates by *Desulfobacterium* (Marietou et al., 2021).

4. Conclusion

Anaerobic digestion of tannery effluent is inhibited by high

concentrations of HS⁻, among other parameters. Hybrid linear flow channel reactors are novel systems that have only recently been evaluated at laboratory scale for pre-treatment of tannery effluent in order to render it more amenable to AD. This study showed that more intensive treatment in HLFGRs selected microbial communities that effectively reduced the HS⁻ concentration, promoting efficient AD despite the co-occurrence of sulfidogenesis. The results of this study are key to understanding the fundamental function of HLFGRs in order to successfully scale-up the process for future industrial implementation.

Data availability: Data Availability Statement: Raw sequence data has been submitted to the European Nucleotide Archive (ENA) database under Project number PRJEB67997. All other raw data is available on the CPUT Institutional eSango repository <https://esango.cput.ac.za>.

CRedit authorship contribution statement

P.J. Welz: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. **N. De Jonge:** Writing – original draft, Software, Methodology, Investigation, Data curation. **M. Lilly:** Methodology, Investigation, Formal analysis. **W. Kaira:** Validation, Investigation, Formal analysis. **A.B. Mpofu:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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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.2024.130411>.

References

- Abdel-Hamed, A.R., Abo-Elmatty, D.M., Wiegel, J., Mesbah, N.M., 2016. Biochemical characterization of a halophilic, alkalithermophilic protease from *Alkalibacillus* sp. NM-Da2. *Extremophiles* 20, 885–894. <https://doi.org/10.1007/s00792-016-0879>.
- Arelli, V., Mamindlapelli, N.K., Anupaju, G.R., 2023. Influence of solids concentration on microbial diversity and methane yield in the anaerobic digestion of rice husk. *Bioresour. Technol. Rep.* <https://doi.org/10.1016/j.biteb.2023.101455>.
- Berhe, S., Leta, S., 2018. Anaerobic co-digestion of tannery wastewater and tannery solid waste using two-stage anaerobic sequencing batch reactor: focus on performances of methanogenic step. *J. Mater. Cycles Waste Manage.* 20, 1468–1482. <https://doi.org/10.1007/s10163-018-0706-9>.
- Bharathi, M., Kumar, N.S., Chellapandi, P., 2020. Functional prediction and assignment of *Methanobrevibacter ruminantium* M1 operome using combined bioinformatic approach. *Front. Genet.* 11, 593990. <https://doi.org/10.3389/fgene.2020.593990>.
- Buljan J, Král I (2019) The framework for sustainable leather manufacture (No. 2). Vienna.
- Capson-Tojo G, Trably E, Rouez M, Crest M, Bernet N, Steyer J.-P., Delgen'es J.-P., Escudie R (2018) *Methanosarcina* plays a main role during methanogenesis of high solids food waste and cardboard. *Waste Management* 76, 423–430. <https://doi.org/10.1016/j.wasman.2018.04.004>.
- Cetecioglu, Z., Dol, J., Taylor, J., Purdy, K.J., Eyiced, Ö., 2019. COD/sulfate ratio does not affect the methane yield and microbial diversity in anaerobic digesters. *Water Res.* 155, 444–454. <https://doi.org/10.1016/j.watres.2019.02.038>.
- Chen, S., He, Q., 2015. Persistence of methanosaeta populations in anaerobic digestion during process instability. *Environ. Microbiol.* 42, 1129–1137. <https://doi.org/10.1007/s10295-015-1632-7>.
- Conklin, A., Stensel, H.D., Ferguson, J., 2006. Growth kinetics and competition between methanosarcina and methanosaeta in mesophilic anaerobic digestion. *Water Environ. Res.* 78, 486–496. <https://doi.org/10.2175/106143006X95393>.
- Deng, Y., Liu, M., Fang, T., Ma, H., Beadham, I., Ruan, W., Wang, S., Zhang, X., Zhang, C., 2023. Enhancement of anaerobic digestion of rice straw by amino acid-derived ionic liquid. *Bioresour. Technol.* 380, 129076. <https://doi.org/10.1016/j.biortech.2023.129076>.
- Du, H., Sun, T., Liu, Y., An, S., Xie, H., Wang, D., Igarashi, Y., Imanaka, T., Luo, F., Ma, M., 2021. Bacteria and archaea involved in anaerobic mercury methylation and methane oxidation in anaerobic sulfate-rich reactors. *Chemosphere* 274, 129773. <https://doi.org/10.1016/j.chemosphere.2021.129773>.
- Du, J., Zhou, X., Yin, Q., Zuo, J., Wu, G., 2023. Revealing impacts of operational modes on anaerobic digestion systems coupling with sulfate reduction. *Bioresour. Technol.* 305, 129431. <https://doi.org/10.1016/j.biortech.2023.129431>.
- Feng, G., Zeng, Y., Wang, H.-Z., Chen, Y.-T., Tang, Y.-Q., 2023. *Proteiniphilum* and *Methanotheroxiphilum* become dominant acetate utilizers in a methanogenic reactor operated under strong ammonia stress. *Front. Microbiol.* 13, 1098814. <https://doi.org/10.3389/fmicb.2022.1098814>.
- Guerrero-Barajas, C., Ordaz, A., Garibay-Orijel, C., Garcia-Solares, S.M., Bastida-Gonzalez, F., Zarate-Segura, P.B., 2014. Enhanced sulfate reduction and trichloroethylene (TCE) biodegradation in a UASB reactor operated with a sludge developed from hydrothermal vents sediments: process and microbial ecology. *Int. Biodeter. Biodegr.* 94, 182–191. <https://doi.org/10.1016/j.ibiod.2014.07.015>.
- Hao, T., Xiang, P., Mackey, H.R., Chi, K., Lu, H., Chui, H., van Loosdrecht, M.C.M., Chen, G.-H., 2014. A review of biological sulfate conversions in wastewater treatment. *Water Res.* 65, 1–21. <https://doi.org/10.1016/j.watres.2014.06.043>.
- Horn, E.J., Oyekola, O.O., Welz, P.J., van Hille, R.P., 2022a. Biological desulfurization of tannery effluent using hybrid linear flow channel reactors. *Water* 14, 32. <https://doi.org/10.3390/w14010032>.
- Horn, E.J., van Hille, R.P., Oyekola, O.O., Welz, P.J., 2022b. Functional microbial communities in hybrid linear flow channel reactors for desulfurization of tannery effluent. *Microorganisms* 10, 2305. <https://doi.org/10.3390/microorganisms10112305>.
- Huang, F., Pan, L., He, Z., Zhang, M., Zhang, M., 2020. Culturable heterotrophic nitrification-aerobic denitrification bacterial consortia with cooperative interactions for removing ammonia and nitrite nitrogen in mariculture effluents. *Aquaculture* 523, 735211. <https://doi.org/10.1016/j.aquaculture.2020.735211>.
- Jiao, Y., Yuan, Y., He, C., Liu, L., Pan, X., Li, P., 2022. Enrichment culture combined with microbial electrochemical enhanced low-temperature anaerobic digestion of cow dung. *Bioresour. Technol.* 360, 127636. <https://doi.org/10.1016/j.biortech.2022.127636>.
- Kibangou, V.A., Lilly, M., Mpofu, A.B., de Jonge, N., Oyekola, O.O., Welz, P.J., 2022. Sulfate-reducing and methanogenic microbial community responses during anaerobic digestion of tannery effluent. *Bioresour. Technol.* 347, 126308. <https://doi.org/10.1016/j.biortech.2021.126308>.
- Kleindienst, S., Herbst, F.-A., Stagars, M., von Netzer, F., von Bergen, M., Seifert, J., Peplies, J., Amann, R., Musat, F., Luedes, T., Knittel, K., 2014. Diverse sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* clade are the key alkane degraders at marine seeps. *ISME J.* 8, 2029–2044. <https://doi.org/10.1038/ismej.2014.51>.
- Kumar, S.S., Kuman, V., Gude, V.G., Malyan, S.K., Pugazhendhi, A., 2020. Alkalinity and salinity favor bioelectricity generation potential of clostridium tetrathioabacter and desulfovibrio in microbial fuel cells (MFC) treating sulfate-laden wastewater. *Bioresour. Technol.* 306, 123110. <https://doi.org/10.1016/j.biortech.2020.123110>.
- Li, M.-T., Rao, L., Wang, L., Gou, M., Sun, Z.-Y., Xia, Z.-Y., Song, W.-F., Tang, Y.-Q., 2022. Bioaugmentation with syntrophic volatile fatty acids-oxidizing consortia to alleviate the ammonia inhibition in continuously stirred anaerobic digestion of municipal sludge. *Chemosphere* 288, 132389. <https://doi.org/10.1016/j.chemosphere.2021.132389>.
- Lippens, C., De Vrieze, J., 2019. Exploiting the unwanted: Sulfate reduction enables phosphate recovery from energy-rich sludge during anaerobic digestion. *Water Res.* 163, 114859. <https://doi.org/10.1016/j.watres.2019.114859>.
- Liu, C., Zhao, D., Ma, W., Guo, Y., Wang, A., Wang, Q., Lee, D.-J., 2016. Denitrifying sulfide removal process on high-salinity wastewaters in the presence of *Halomonas* sp. *Appl. Microbiol. Biotechnol.* 100, 1421–1426. <https://doi.org/10.1007/s00253-015-7039-6>.
- Lu, X., Zhen, G., Ni, J., Hojo, T., Kubota, K., Li, Y.-Y., 2016. Effect of influent COD/SO4²⁻ ratios on biodegradation behavior of an upflow anaerobic sludge blanket (UASB) reactor. *Bioresour. Technol.* 214, 175–183. <https://doi.org/10.1016/j.biortech.2016.04.100>.
- Marietou, A., Kjeldsen, K.U., Glombitza, C., Jørgensen, B.B., 2021. Response to substrate limitation by a marine sulfate-reducing bacterium. *ISME J.* 16, 200–210. <https://doi.org/10.1038/s41396-021-01061-2>.
- Mathai, P.P., Nicholes, M.S., Venkiteswaran, K., Brown, C.M., Morris, R.L., Zitomer, D.H., 2020. Make JS (2020) Dynamic shifts withing volatile fatty acid-degrading microbial communities indicate process imbalance in anaerobic digesters. *Environmental Biotechnology* 104, 4563–4575. <https://doi.org/10.1007/s00253-020-10552-9>.
- Morris R, Schauer-Gimenez A, Bhattad U, Kearney C, Struble CA, Zitomer D, Maki JS (2014) Methyl coenzyme M reductase (*mcrA*) gene abundance correlates with activity measurements of methanogenic H₂/CO₂-enriched anaerobic biomass. *Microbial Biotechnology* 7, 77–84. <https://dx.doi.org/10.1111%2F1751-7915.12094>.

- Mpofu, A.B., Kaira, W.M., Holtman, G.A., Oyekola, O.O., van Hille, R.P., Welz, P.J., 2023. Resource recovery from tannery wastewater using an integrated biological system: towards a circular bioeconomy and net positive tannery operations. *J. Clean. Prod.* 387, 135872 <https://doi.org/10.1016/j.jclepro.2023.135872>.
- Murphy, C.L., Biggerstaff, J., Eichorn, A., Ewing, E., Shahan, R., Soriano, D., Stewart, S., VanMol, K., Walder, R., Walters, P., Elshahed, M.S., Youssef, N.H., 2021. Genomic characterization of three novel *Desulfobacterota* classes expand the metabolic and phylogenetic diversity of the phylum. *Environ. Microbiol.* 23, 4326–4343. <https://doi.org/10.1111/1462-2920.15614>.
- Myhr, S., Torsvik, T., 2000. *Denitrivibrio acetiphilus*, a novel genus and species of dissimilatory nitrate-reducing bacterium isolated from an oil reservoir model column. *Int. J. Syst. Evol. Microbiol.* 50, 1611–1619. <https://doi.org/10.1099/ijs.0.032508-0>.
- Nordgård, A.S.R., Bergland, W.H., Vadstein, O., Mironov, V., Bakke, R., Østgaard, K., Bakke, I., 2017. Anaerobic digestion of pig manure supernatant at high ammonia concentrations characterized by high abundances of *Methanosaeta* and non-euryarchaeotal archaea. *Sci. Rep.* 7, 15077. <https://doi.org/10.1038/s41598-017-14527-1>.
- Olivera, C., Tondo, M.L., Giradi, V., Fattobene, L., Herrero, S., Pérez, L.M., Salvatierra, L. M., 2022. Early-stage response in anaerobic bioreactors due to high sulfate loads: Hydrogen sulfide yield and other organic volatile sulfur compounds as a sign of microbial community modifications. *Bioresour. Technol.* 350, 126947 <https://doi.org/10.1016/j.biortech.2022.126947>.
- Rotaru, A.-E., Shrestha, P.M., Liu, F., Shrestha, M., Shrestha, D., Embree, M., Zengler, K., Wardman, C., Nevin, K.P., Lovely, D.R., 2014. A New Model for Electron Flow during Anaerobic Digestion: Direct Interspecies Electron Transfer to *Methanosaeta* for the Reduction of Carbon Dioxide. <https://doi.org/10.1039/c3ee42189a>.
- Sabumon, P.C., 2008. Development of the Sulphidogenesis Cum Ammonia Removal Process for treatment of tannery effluent. *Water Sci. Technol.* 58, 391–397. <https://doi.org/10.2166/wst.2008.369>.
- Saxena, S., Saharan, V.N., George, S., 2019. Modeling and simulation studies on batch anaerobic digestion of hydrodynamically cavitated tannery waste effluent for higher biogas yield. *Ultrasonics – Sonochemistry* 58, 104692. <https://doi.org/10.1016/j.ultsonch.2019.104692>.
- Sodhi, V., Babsak, A., Jha, M.K., 2021. Investigation of activated sludge characteristics and their influence on simultaneous sludge minimization and nitrogen removal from and advanced biological treatment for tannery wastewater. *Environ. Technol. Innov.* 24, 102013 <https://doi.org/10.1016/j.eti.2021.102013>.
- Song, Z., Williams, C.J., Adyvean, R.G.J., 2001. Coagulation and anaerobic digestion of tannery wastewater. *Transactions of the Institute of Chemical Engineers* 79, 23–28.
- Swartz CD, Jackson-Moss C, Roswell R, Mpofu AB, Welz PJ (2017) Water and Wastewater Management in the Tanning and Leather Finishing Industry. Water Research Commission, Pretoria. ISBN 978-1-4312-0881-4 <https://doi.org/10.13140/RG.2.2.35505.02402>.
- Wang, S., Li, D., Zhang, K., Ma, Y., Liu, F., Li, Z., Gao, X., Gao, W., Du, L., 2023. Effects of initial volatile fatty acid concentrations on process characteristics, microbial communities, and metabolic pathways on solid-state anaerobic digestion. *Bioresour. Technol.* 369, 128461 <https://doi.org/10.1016/j.biortech.2022.128461>.
- Watanabe, M., Higashioka, Y., Kojima, H., Fukui, M., 2020. Proposal of *Desulfosarcina ovata* subsp. *sediminis* subsp. *nov.*, a novel toluene-degrading sulfate-reducing bacterium isolated from tidal flat sediment of Tokyo bay. *Syst. Appl. Microbiol.* 43, 126109 <https://doi.org/10.1016/j.syapm.2020.126109>.
- Wu, Y., Yue, X., Zhou, A., Song, X., Su, B., Cao, F., Ding, J., 2023. Simultaneous recovery of short chain fatty acids and phosphorus during lipid-rich anaerobic fermentation with sodium hydroxide conditioning. *Chemosphere* 312, 137227. <https://doi.org/10.1016/j.chemosphere.2022.137227>.
- Yamamoto-Ikemoto, R., Matsuura, N., Honda, R., Yamamura, H.H., Some, K., Prak, S., Koike, K., Togari, T., 2023. Ammonia tolerance and microbial community in thermophilic co-digestion of sewage sludge initiated with lignocellulosic biomass. *Bioresour. Technol.* 376, 128834 <https://doi.org/10.1016/j.biortech.2023.12>.
- Yan, M., Fotidis, I.A., Jeglot, A., Treu, L., Tian, H.A., Palomo, Zhu, X., Angelidaki, I., 2020. Long-term preserved and rapidly revived methanogenic cultures: Microbial dynamics and preservation mechanisms. *J. Clean. Prod.* 263, 121577 <https://doi.org/10.1016/j.jclepro.2020.121577>.
- Yan, L., Ye, J., Zhang, P., Xu, D., Wu, Y., Liu, J., Zhang, H., Fang, W., Wang, B., Zheng, G., 2018. Hydrogen sulfide formation control and microbial competition in batch anaerobic digestion of slaughterhouse wastewater sludge: Effect of initial sludge pH. *Bioresour. Technol.* 259, 67–74. <https://doi.org/10.1016/j.biortech.2018.03.011>.
- Yang, Y., Yang, F., Huang, W., Huang, W., Li, F., Lei, Z., Zhang, Z., 2018. Enhanced anaerobic digestion of ammonia-rich swine manure by zero-valent iron: With special focus on the enhancement effect on hydrogenotrophic methanogenesis activity. *Bioresour. Technol.* 270, 172–179. <https://doi.org/10.1016/j.biortech.2018.09.008>.
- Zhang, S., Chen, Y., Zhang, Z., Ping, Q., Li, Y., 2023. Co-digestion of sulfur-rich vegetable waste with waste activated sludge enhanced phosphorus release and hydrogenotrophic methanogenesis. *Water Res.* 242, 120250 <https://doi.org/10.1016/j.watres.2023.120250>.
- Zhang, Z., Zhang, C., Yang, Y., Zhang, Z., Tang, Y., Su, P., Lin, Z., 2022. A review of sulfate-reducing bacteria: Metabolism, influencing factors and application in wastewater treatment. *J. Clean. Prod.* 376, 134109 <https://doi.org/10.1016/j.jclepro.2022.134109>.
- Zhao, Z., Y, I., Yu, Q., Zhang, Y., 2018. Ferroferric oxide triggered possible direct interspecies electron transfer between *Syntrophomonas* and *Methanosaeta* to enhance waste activated sludge anaerobic digestion. *Bioresour. Technol.* 250, 79–85. <https://doi.org/10.1016/j.biortech.2017.11.003>.