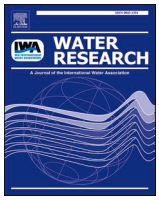
Water Research 253 (2024) 121354

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Dead in the water – Role of relic DNA and primer choice for targeted sequencing surveys of anaerobic sewage sludge intended for biological monitoring

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ARTICLE INFO

Keywords:
Renewable biogas
Anaerobic digestion
Circular economy
Resource recovery
Foaming, Methanogenesis
DNA surveillance

ABSTRACT

DNA-based monitoring of microbial communities that are responsible for the performance of anaerobic digestion of sewage wastes has the potential to improve resource recoveries for wastewater treatment facilities. By treating sludge with propidium monoazide (PMA) prior to amplicon sequencing, this study explored how the presence of DNA from dead microbial biomass carried over with feed sludge may mislead process-relevant biomarkers, and whether primer choice impacts such assessments. Four common primers were selected for amplicon preparation, also to determine if universal primers have sufficient taxonomic or functional coverage for monitoring ecological performance; or whether two domain-specific primers for Bacteria and Archaea are necessary. Anaerobic sludges of three municipal continuously stirred-tank reactors in Victoria, Australia, were sampled at one time-point. A total of 240 amplicon libraries were sequenced on a Miseq using two universal and two domain-specific primer pairs. Untargeted metabolomics was chosen to complement biological interpretation of amplicon gene-based functional predictions. Diversity, taxonomy, phylogeny and functional potentials were systematically assessed using PICRUSt2, which can predict community wide pathway abundance. The two chosen universal primers provided similar diversity profiles of abundant Bacteria and Archaea, compared to the domain-specific primers. About 16 % of all detected prokaryotic genera covering 30 % of total abundances and 6 % of PICRUSt2-estimated pathway abundances were affected by PMA. This showed that dead biomass in the anaerobic digesters impacted DNA-based assessments, with implications for predicting active processes, such as methanogenesis, denitrification or the identification of organisms associated with biological foams. Hence, instead of running two sequencing runs with two different domain-specific primers, we propose conducting PMA-seq with universal primer pairs for routine performance monitoring. However, dead sludge biomass may have some predictive value. In principal component analysis the compositional variation of 239 sludge metabolites resembled that of 'dead-plus-alive' biomass, suggesting that dead organisms contributed to the potentially process-relevant sludge metabolome.

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https://doi.org/10.1016/j.watres.2024.121354
Received 5 October 2023; Received in revised form 20 February 2024; Accepted 21 February 2024
Available online 22 February 2024

1. Introduction

Anaerobic digestion of wastewater sludge remains an essential biological treatment process for water utilities, enabling the reduction of solid mass and pathogen load, as well as the recovery of renewable biogas or nutrients (Batstone and Virdis, 2014). Studying the biology of anaerobic digestion to understand how relevant microorganisms can be better managed is becoming increasingly important for water utilities that seek to improve their carbon footprint and increase resource recoveries from our sewage wastes (Vanwonterghem et al., 2014). High-throughput sequencing of sludge DNA is a promising approach to profile and catalogue relevant microorganisms and their metabolism, which is fundamental for enabling innovations in process stability and bioenergy recoveries of anaerobic digestion (Carballa et al., 2015; Jiang et al., 2021b; Krohn et al., 2022). DNA-based assessments of microbial communities in anaerobic digesters are often conducted through targeted short-read sequencing, sometimes referred to as amplicon sequencing (Hugerth and Andersson, 2017). Amplicon sequencing of environmental DNA remains a popular, readily available, established and low-cost method to profile microbial populations. However, it remains unknown how the presence of inactive and dead organisms impact on performance of the digestion process.

Overall, it is known that a significant number of microorganisms enter the treatment process with the feed sludge (Jiang et al., 2021b; Kirkegaard et al., 2017). As the engineered ecological niches change throughout the wastewater treatment process, microbial cells die. This dead biomass in anaerobic reactors from the feed sludge (becoming a carbon source itself) may interfere with the quantification of active organisms and their metabolic potentials (Nagler et al., 2021). An important knowledge gap this study aimed to address is how the presence of dead biomass impacts DNA-based biomarker development.

One way to distinguish between dead and alive organisms is through the treatment of sludge with propidium monoazide (PMA) prior to PCR amplification and subsequent sequencing of amplicons, dubbed PMA-seq (Fittipaldi et al., 2012; Nocker et al., 2007)'. Propidium monoazide works by penetrating compromised (damaged) cell membranes of dead organisms and by randomly and covalently cross-linking to their DNA (also called relic DNA), preventing its amplification in PCR (Marotz et al., 2021). As a consequence of this mode of action, amplicon length impacts PMA-seq because longer relic fragments have a higher probability to be suppressed from PCR amplification (Fittipaldi et al., 2012). Yet, it remains unclear if primer choice (and hence amplicon length) has any bearing on compositional analysis for PMA-seq approaches on anaerobic sludge. Hence, one aim of this study was to assess the effect of primer choice on PMA-seq.

Another important aspect, especially for DNA-based monitoring of anaerobic digestion is primer selection to appropriately cover both Bacteria and Archaea. Internationally, there is no harmonised use of universal or domain-specific primers for research in the water sector. The use of universal prokaryote primers, covering both Bacteria and Archaea, is desirable to reduce costs associated with frequent surveillance efforts. Universal prokaryote primers can further provide greater coverage compared to bacteria or archaea-specific primer sets, and in some cases with lower bias towards specific taxa (Takahashi et al., 2014).

A bacteria-specific primer pair (targeting the V1-V3 region at ~500 bps) was commonly used for profiling microbial communities in activated sludge (Albertsen et al., 2015). Albertsen et al. (2015) showed that phylum-level abundance distributions with the V1/V3 primer pair agreed more with metagenomes derived through PCR-free library construction, compared to universal primer pairs targeting the V4 and V3/V4 regions. Hence, this V1/V3 primer pair was subsequently used more commonly for sequencing surveys, even on anaerobic sludge (Jiang et al., 2021b). However, targeting bacteria with the bacteria-specific V1/V3 primer pair in anaerobic sludge requires an additional sequencing run with archaea-specific primers to cover methanogens, which are a key group of microorganisms that facilitate the production of renewable methane gas during the anaerobic digestion of wastewater sludge. Hence, some researchers additionally used a primer pair that targets the V3-V5 region (~ 560 bp amplicon), opting to do two sequencing runs for one survey (Jiang et al., 2021b).

Alternatively, the universal V4 primer pair (targeting a ~ 292 bp amplicon) is commonly used because they produce an appropriate amplicon size for next-generation sequencing and provide a wide coverage of both Archaea and Bacteria (Apprill et al., 2015). V4 primers are used for large-scale environmental surveys, while also integrated into the protocol for the Earth Microbiome Project (https://earthmicrobiome.org) (Caporaso et al., 2010). Another commonly used universal primer pair amplifies a longer fragment of the 16S rRNA gene in the V3-V4 region, also covering Bacteria and Archaea. The V3/V4 primer pair (targeting a ~ 457 bp amplicon) performed well in pig faecal samples (Takahashi et al., 2014) and was amended recently to better cover anammox organisms (Mazzoli et al., 2020). Both, the V4 and the V3/V4 primer sets were recently included in a global sequencing effort of anaerobic digesters (Dueholm et al., 2023). Therefore, such universal primer pairs are potential candidates for routine sludge surveillance efforts for anaerobic digestion. Hence, this study explored whether universal primers are sufficient for researchers and operators that monitor ecological performance of anaerobic digestion through sequencing of anaerobic sludge.

To address these aims PMA-seq was performed on sludge from three full-scale anaerobic digesters in Victoria, Australia. DNA-based diversity indices, phylogeny and metabolic potentials were systematically assessed and compared to 223 sludge primary metabolites, derived from untargeted metabolomics, to understand how relic DNA impacts on such analyses. Four commonly used primer pairs were chosen to compare functional predictions of domain specific versus universal primers, including two universal primers (V4 and V3-V4 regions), one bacteria-specific (V1-V3 region) and one Archaea specific primer pair (V3-V5 region).

We hypothesised that the presence of relic DNA does have important implications on diversity coverage and that universal primers cover sufficient phylogenetic and metabolic diversity to inform process, based on amplicon sequence variants (ASVs) and their functional predictions using PICRUSt2.

2. Material and methods

2.1. Sludge sampling and properties

Anaerobic digestate was sampled from three municipal continuously stirred tank reactors operated by two urban wastewater treatment plants (WWTPs) in Melbourne, Australia (Denoted X and Y). These three different digesters (denoted MesoX, ThermoY and MesoY) were chosen to control for different sludge habitats when assessing the effects of primers and propidium monoazide (PMA) on diversity analyses. MesoX (37.5 °C) was fed with a mix of thickened primary sludge and thickened waste activated sludge (TWAS). ThermoY and MesoY (55.5 and 37 °C respectively), were operated sequentially, with the thermophilic digester being fed a mix of primary and TWAS, and the mesophilic digester fed the thermophilic outflow. Hence, ThermoY biomass functioned as feed for MesoY, which provided an opportunity to assess survival of thermophiles in MesoY and validate the method used to do so (detailed below). Digester operating parameters are presented in Table S1. A total of 15 samples of anaerobic sewage sludge (each in five 1 L containers per digester - denoted technical replicates) were drawn on 9th May 2022 from flushed pipes, transported on ice and stored at 4 °C. DNA extraction and library preparation was performed within 24 h. Additional aliquots (40 mL) were freeze-dried for metabolite extractions and 1.5 mL aliquots stored at −20 °C. Common sludge properties were measured (Table 1).

Table 1
Sludge properties of sludge received in laboratory after sampling three continuously stirred anaerobic digesters from two urban wastewater treatment plants (WWTPs) in Victoria, Australia.

Digester	WWTP	Sludge type	pH	TS g L^{-1}	VS g L^{-1}	COD g L^{-1}	TN g L^{-1}
$Meso_X$	X	Mesophilic	7.02 ± 0.02	23.2 ± 0.22	16.9 ± 0.16	25.0 ± 0.90	1.6 ± 0.08
$Thermo_Y$	Y	Thermophilic	7.31 ± 0.01	19.9 ± 0.13	15.8 ± 0.14	23.5 ± 0.46	1.5 ± 0.02
$Meso_Y$	Y	Mesophilic	7.25 ± 0.13	17.3 ± 0.05	13.4 ± 0.09	19.2 ± 0.04	1.6 ± 0.06

Values ± SE, ThermoY sludge (55°C) feeds into MesoY (37°C); TS; Total solids, VS; Volatile solids, COD; Chemical oxygen demand, TN; Total nitrogen.

2.2. DNA extraction, propidium monoazide treatment and sequencing

Four primers were selected (two universal, one Bacteria-specific and one Archaea-specific: V4_U, V3V4_U, V1V3_B and V3V5_A respectively) to study the relevance of primer choice on alpha, beta, taxonomic and functional diversity indices of Bacteria and Archaea. Primer details and definitions are available in Table S2, as well as a summary of number of samples and treatments in Table S3. To explore how relic DNA affected these metrics, one set of sludge samples was treated with propidium monoazide (PMA) prior to DNA extraction and library preparation. Two PMA products were used, PMA and PMAxx, both from Biotium (Fremont, USA) to test for differences between PMA formulations. As no differences in diversity indices between these two sets of PMA-treated sludge were observed, their data was grouped together and denoted as "PMA-treated", increasing replication per sludge type ($n = 10$ per sludge type) (Table S3).

Sludge DNA from a total of 60 samples was extracted prior to sequencing with four primer pairs. DNA of 45 sludge samples (0.5 mL), treated with (PMA) and without viability dye (No-PMA control) (100 μM PMA, Biotium, CA, USA) (1.25 μL of 20 mM PMA stock), were extracted (DNeasy Powersoil Pro, Qiagen, Hilden, Germany) on the day of sampling. Another set of the 15 untreated samples of the same sludge was extracted two weeks later and denoted as "Untreated Control" (storage −20 °C). For all sets, sludge was centrifuged (Eppendorf 5424 R, Hamburg, Germany) at 5000 rpm for 5 min. For PMA-treated and No-PMA controls, supernatant was replaced with phosphate buffered saline (PBS, pH ~7.4) and the volume adjusted to approximately 0.5 mL. Viability dye was added to the treated set only, followed by a 10 min incubation prior to the light treatment (PMA-Lite LED Photolysis device, Biotium, USA) for PMA-treated and No-PMA controls. This followed centrifugation (5000 rpm, 5 min), removal of supernatant and addition of beads for DNA extraction in the same 2 mL tube, following the kit's standard protocol. DNA of the Untreated Controls was extracted from the centrifuged pellet without PBS buffer or light treatment.

Extracts (PMA and No-PMA control) were stored at 4 °C prior to library preparation within 5 days. DNA purity was measured with a spectrophotometer (Nanodrop One™, Thermo Scientific, USA). Concentration of double stranded DNA was quantified and normalised to 10 ng μL^{-1} using a Qubit 4.0 fluorometer (Invitrogen, USA). To compare 16S rRNA copy numbers of PMA and non-PMA treated sludge, non-normalised sludge DNA was quantified using real-time PCR with relevant primer details and PCR conditions available in Supplementary Materials (Fig. S1).

Details about amplicon sequencing, data processing, quality control, as well as generation of amplicon sequence variants, phylogenetic trees and PICRUSt2-estimted pathways are available in Supplementary Materials. Briefly, a total of 240 libraries (3 sludge types x 5 replicates x 4 treatments x 4 primers), plus a negative control were pooled. The pool was sequenced twice at 6 pM on an Illumina MiSeq (2 × 300 bp), including ~ 15–20 % phiX, after library preparations using Nextera XT indices, resulting in a total of 21,000,000 reads. Quality plots and summaries of all trimming and processing parameters, as well as feature and read frequencies are shown in Table S4 and Fig. S2.

2.3. Metabolomics

Untargeted metabolomics was chosen to complement biological interpretation of amplicon gene-based functional predictions. The method can semi-quantitatively quantify organic acids, intermediates of the tricarboxylic acid cycle, fatty acids, sterols, amino acids, sugars or sugar phosphates and alcohols. All fifteen freeze-dried sludge samples were broken up in a mortar and 30 mg each transferred to 2 mL cryomill tubes containing 1000 μL methanol, spiked with internal standards (0.2 mg mL^{-1} $^{13}C_6$ Sorbitol, $^{13}C_5$–^{15}N-Valine in water). Samples were homogenised for 60 s at 6.5 M/s at 21 °C (FastPrep-24 Classic, MP Biomedicals) then incubated at 70 °C for 5 min, centrifuged at 14,000 rpm for 5 mins and 100 μL aliquots were dried in vacuo for subsequent TMS (trimethylsilyl) polar metabolite derivatisation. Details on derivatisation, GC–MS conditions and data processing are available in Supplementary Materials.

2.4. Statistics

All analyses were performed in R version 4.2.2 (2022–10–31) with data managed in packages phyloseq (v1.44.0) or tidyverse (v2.0.0) and visualised with ggtree (v3.8.0) and ggpubr (v0.6.0) (McMurdie and Holmes, 2013; Yu et al., 2017). A detailed description of statistical approach and parameters used is provided in Supplementary Materials. Briefly, alpha diversity indices were calculated from rarefied abundances on a subset of the data for each primer pair and digester separately, each at selected resampling depths (Figs. S3 and S4). PERMANOVA was conducted on Bray-Curtis dissimilarities using the *adonis2* function in vegan (Oksanen et al., 2022). Abundances were converted to ratios, for all other abundance-related analyses (Aitchison et al., 2000). For example, principal component analysis (PCA) and redundance analysis (RDA), was performed on centred log-ratios transformed abundances. Mantel correlation analysis was conducted with the help of vegan function *mantel* after conversion of all abundances to Aitchison distances (centred log-ratios), and the metabolite abundances (log ratios of internal standard) to Euclidean distances. This was followed by regularised canonical correlation analysis using the cross-validation approach of the mixOmics package (http://mixomics.org/methods/rcca/) to assess differences in metabolite/pathway correlation between primers. To identify differences in population abundances and metabolic potentials between digesters and primer pairs, differential abundance analysis was performed using packages Phylofactor (Washburne et al., 2017) and ANCOMBC (Analysis of Compositions of Microbiomes with Bias Correction) (Lin and Peddada, 2020). Unless stated otherwise, data of untreated and noPMA controls were combined, as well as data of PMA and PMAxx-treated sludge. Diversity indices and community composition was similar among these treatment groups (Figs. S5 and S6).

3. Results

3.1. Alpha and beta diversity

3.1.1. Differences between digesters

Sludge from two anaerobic mesophilic digesters ($Meso_X$ and $Meso_Y$) and one thermophilic digester ($Thermo_Y$) were studied. The two

universal primers used in this study showed that the thermophilic anaerobic digester contained the lowest median ASV richness (putative species richness) of Bacteria and Archaea, compared to $Meso_X$ and $Meso_Y$ (Fig. 1 and Table 2). This resulted in the effective diversity, or the diversity of abundant organisms based on Shannon's diversity index, to be lowest in $Thermo_Y$. Furthermore, Pielou's Evenness was lowest, indicating that abundance distributions were skewed towards a few dominant organisms in $Thermo_Y$ compared to $Meso_X$ and $Meso_Y$ (Fig. 1).

Sequencing of the untreated and noPMA controls (capturing dead-plus-alive organisms) showed that the prokaryote composition was different between the three digesters. In PCA 70.3 % of compositional variance was explained by digesters (Fig. 2, top left). However, removal of relic DNA from the sequencing pool of the same sludge revealed that the composition of the active mesophilic community was in fact highly similar (Fig. 2). The variance (70.5 %) of the active microbial community was largely explained by the two different temperature regimes (mesophilic and thermophilic) and not by differences inherent to the digester-specific communities.

Consequently, $Meso_X$ and $Meso_Y$, which were part of two different WWTPs in different regions of Victoria, shared more active organisms

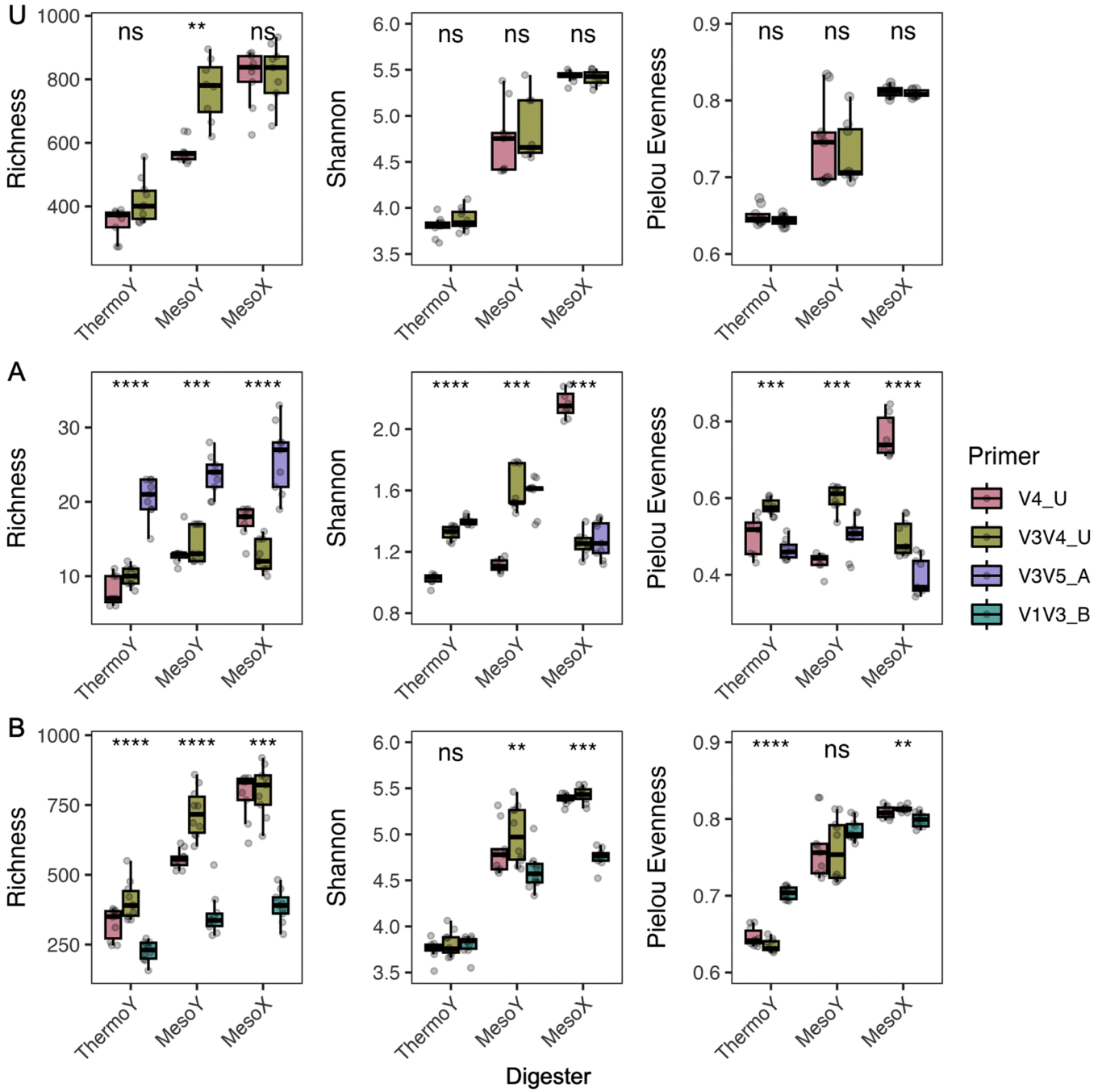

Fig. 1. Selected alpha diversity indices of three full-scale digesters (ThermoY, MesoY, MesoX). Calculated from normalised (rarefied) abundances of amplicon sequence variants (ASVs) of bacteria and archaea in untreated sludge (Untreated control and noPMA controls, $n = 10$ per primer) using two universal primers (V4_U and V3V4_U) and two domain-specific primers (V1V3_B, V3V5_A). Samples were normalised within each digester separately. Indices are shown for all ASVs (including Archaea and Bacteria) **(U)** and after subsetting to Archaea **(A)** and Bacteria **(B)**. A large percentage (42–50 %) of ASVs derived from the V3V5_A primer were unclassified and are not represented in this figure. Significance of Kruskal-Wallis tests is indicated above each group (ns = not significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Table 2
Summary of normalised (rarefied) amplicon sequence variants (ASVs) of three full scale anaerobic digesters (MesoX, ThermoY, MesoY). ThermoY effluent was directly fed to digester MesoY as part of the same treatment plant.

	$Meso_X$ (n = 8–10)			$Thermo_Y$ (n = 10)			$Meso_Y$ (n = 9–10)		
V4 (Universal)	Archaea	Bacteria	Unassigned	Archaea	Bacteria	Unassigned	Archaea	Bacteria	Unassigned
ASV (total count)	31	1497	40	15	686	45	37	1393	36
ASV (mean count)	18 (±2)	832 (±65)	7 (±5)	8 (±2)	350 (±39)	5 (±4)	13 (±2)	556 (±26)	5 (±4)
Total abundance	5261	164,629	138	16,572	171,625	721	21,033	148,729	266
Median abundance	673	18,218	5	1624	17,176	46	1825	16,992	18
V3V4 (Universal)	Archaea	Bacteria	Unassigned	Archaea	Bacteria	Unassigned	Archaea	Bacteria	Unassigned
ASV (total count)	25	1728	17	16	907	9	32	1970	34
ASV (mean count)	13 (±2)	822 (±71)	1 (±1)	10 (±1)	390 (±49)	4 (±3)	14 (±2)	716 (±62)	2 (±2)
Total abundance	12,962	231,602	76	35,458	270,313	29	45,071	260,586	ß
Median abundance	1664	28,914	6	3731	27,186	1	1817	25,505	8
V1V3 (Bacteria)	Archaea	Bacteria	Unassigned	Archaea	Bacteria	Unassigned	Archaea	Bacteria	Unassigned
ASV (total count)	0	963	11	4	592	11	3	1155	13
ASV (mean count)	0	390 (±46)	4 (±2)	0	230 (±26)	2 (±2)	0	336 (±52)	3 (±1)
Total abundance	0	268,599	116	21	297,743	516	44	298,407	85
Median abundance	0	29,856	20	0	29,780	84	0	29,846	24
V3V5 (Archaea)	Archaea	Bacteria	Unassigned	Archaea	Bacteria	Unassigned	Archaea	Bacteria	Unassigned
ASV (total count)	42	170	216	37	125	120	52	130	144
ASV (mean count)	26 (±4)	NA	26 (±6)	21 (±2)	NA	30 (±20)	24 (±2)	NA	87 (±39)
Total abundance	54,055	4573	1371	131,122	3114	721	101,487	2788	724
Median abundance	13,480	1176	319	14,583	351	72	14,609	337	54

Note: ±95 % confidence interval of the mean; Non-PMA and untreated control samples only; Number of eukaryotic ASVs were negligible and are not shown.

than was apparent from conventional sequencing of untreated sludge. After treating sludge with PMA and subsequently removing relic DNA prior to sequencing the same sludge, digester $Meso_X$ and $Meso_Y$ shared 43 % bacterial ASVs (± 0.03 SE, 95 % CI: 31–55 %) taking all primers into account. This 15 % increase of shared species after PMA treatment of mesophilic sludge, likely represents the removal of feed-specific relic DNA from mesophilic digesters $Meso_X$ and $Meso_Y$. This observation was consistent across all primers for Bacterial and Archaeal ASVs (Figs. S7 and S8).

Furthermore, PMA treatment indicated that most sequences that were shared between the thermophilic $Thermo_Y$ (which feeds into the mesophilic $Meso_Y$ as part of the same treatment plant) and $Meso_Y$ were dead cells, as compositional similarities drastically dropped once DNA from dead cells was removed from the sequencing pool (Fig. 2). On average, only 1.5 % (±0.5 SE) of bacterial ASVs were shared between $Thermo_Y$ and $Meso_Y$ after relic DNA was removed from the sludge through PMA-treatment (Figs. S7 and S8), while without PMA treatment this value was 10 %. In other words, the sequenced thermophilic organisms from $Thermo_Y$ did not survive once fed into the mesophilic $Meso_Y$, and thus this diversity is overrepresented in digester $Meso_Y$. Again, this observation was consistent across all primers (Fig. 3).

While removal of relic DNA was critical to compare the active microbial composition, the dead sludge biomass may nonetheless have some predictive value. Principal component analysis showed that metabolite composition explained as much variation between digesters as the `dead-plus-alive` microbial diversity (Fig. 2, bottom). Seeing that each digester had a different metabolite composition (sample points well separated on PCA) it resembled more the ASV composition that included dead organisms. The Mantel correlation analysis of ASV abundance tables and the metabolite abundance tables showed that the composition of sludge that included relic DNA was more correlated to the metabolic composition, compared to sludge that was treated with PMA. This observation was independent of the primers used (Table 3).

3.1.2. *Effect of primers*

Based on the relatively low sequencing depths in this study (~87,500 sequences per sample across four primers), primers had minimal impact on the core alpha diversity indices of different sludge environments. There was no difference in diversity indices between the two universal primers V4 and V3V4 (Fig. 1), except for ASV richness, which was lower with the V4 primer in digester $Meso_Y$ only. Both Shannon's and Pielou's evenness indices remained equal across all three digesters (Fig. 1) based on V4 and V3/V4 primer sets. Furthermore, the two universal primers used in this study produced a very similar numerical community composition as evident from principal components analysis which showed clearly overlapping variance of the community composition based on V4 and V3/V4 primer sets (Fig. 2). Results of PERMANOVA at genus-level on untreated/nonPMA controls showed that universal primers had no effect on community composition across all three digesters (PERMANOVA, Bray-Curtis ~ Primer type, df = 1, $F = 1.93$, $R^2 = 0.03$, $p = 0.15$, $n = 28$ per each primer - V4 and V3/V4).

Removal of relic DNA from the sequencing pool of the same sludge resulted in small compositional differences between two universal primers V4 and V3/V4. After PMA treatment of the sludge, 7.5 % of the variance of the active community was explained by primers V4 and V3/V4 (Fig. 2, right). Notably, the primer effect was stronger for the mesophilic diversity and less pronounced for the thermophilic digester. This primer effect on the composition was marginal across all digesters, based on PERMANOVA on Bray-Curtis dissimilarities after normalising abundances (PERMANOVA, Bray-Curtis ~ Primer type, df = 1, $F = 2.17$, $R^2 = 0.04$, $p = 0.13$, $n = 28$ per each primer - V4 and V3/V4).

However, domain-specific primers resulted in different diversity indices. The archaea-specific primer set (V3/V5) resulted in an increase of 80 % (± 29 SE) identified archaeal ASVs (Fig. 1, Table 2) compared to universal primer pairs V4 and V3/V4 across all three digesters. Diversity indices were compared on a subset of ASVs to select Archaea only (Kingdom = Archaea), which showed that the archaea-specific primer pair (V3/V5) produced the greatest richness of archaeal ASVs (Fig. 1). The true archaeal richness was expected to be even greater as the majority of potential archaeal ASVs (42–50 %) remained unclassified with this primer pair (Table 2). Based on classified reads only, the V4 primer pair resulted in the greatest 'effective' archaeal diversity as indicated by the significantly higher Shannon index and Pielou's evenness (Fig. 1). In other words, the V4 primer appeared to capture a greater diversity of the common archaeal ASVs (Fig. 1).

The bacteria-specific primer set (V1/V3) resulted in a 25 % (±2 SE) reduction of bacterial ASVs across all three digesters compared to amplicons from universal primers (Fig. 1, Table 2). For example, 151 and 170 genera were covered for Firmicutes with universal primers V4 and V3/V4 respectively, while 101 with the V1/V3 primer pair.

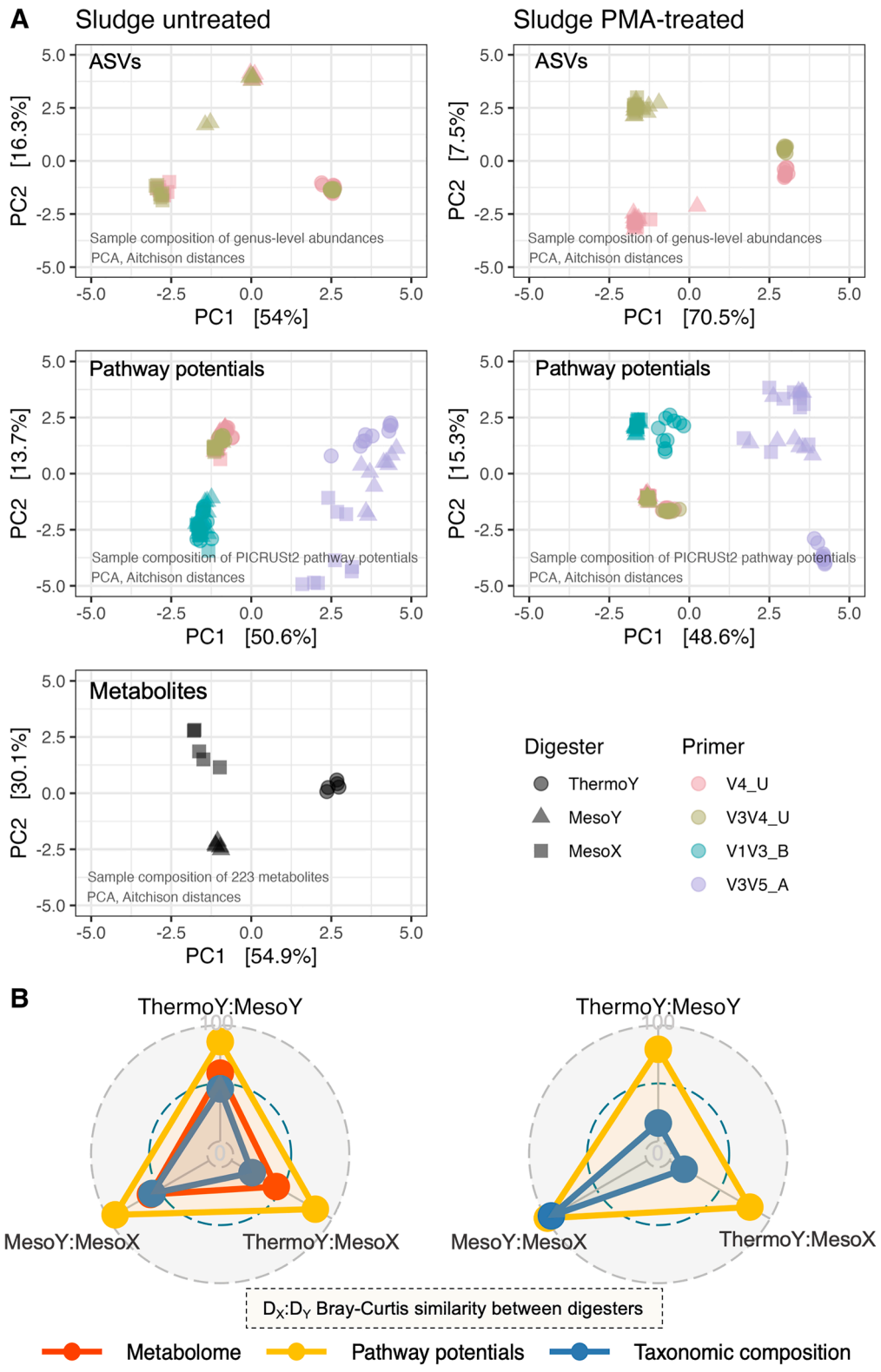

Fig. 2. A: Principal component analyses (PCA) of sludge community, Picrust2-estimated pathways and the metabolome, where each symbol represents the composition of one sample. Compositions of untreated/noPMA controls and sludge treated with propidium monoazide (PMA), using PMA and PMAxx (Biotum) are compared. Abundances of amplicon sequence variants (ASVs) were agglomerated to genus-level and transformed to centred-log ratios (Aitchison distances). **B:** Compositional similarities (before and after PMA treatement from left to right) of the metabolome, metabolic pathway potentials (PICRUSt2) and genus-level taxonomic composition between digesters based on Bray-Curtis similarities.Values further outward show greater compositional similarities between digesters.

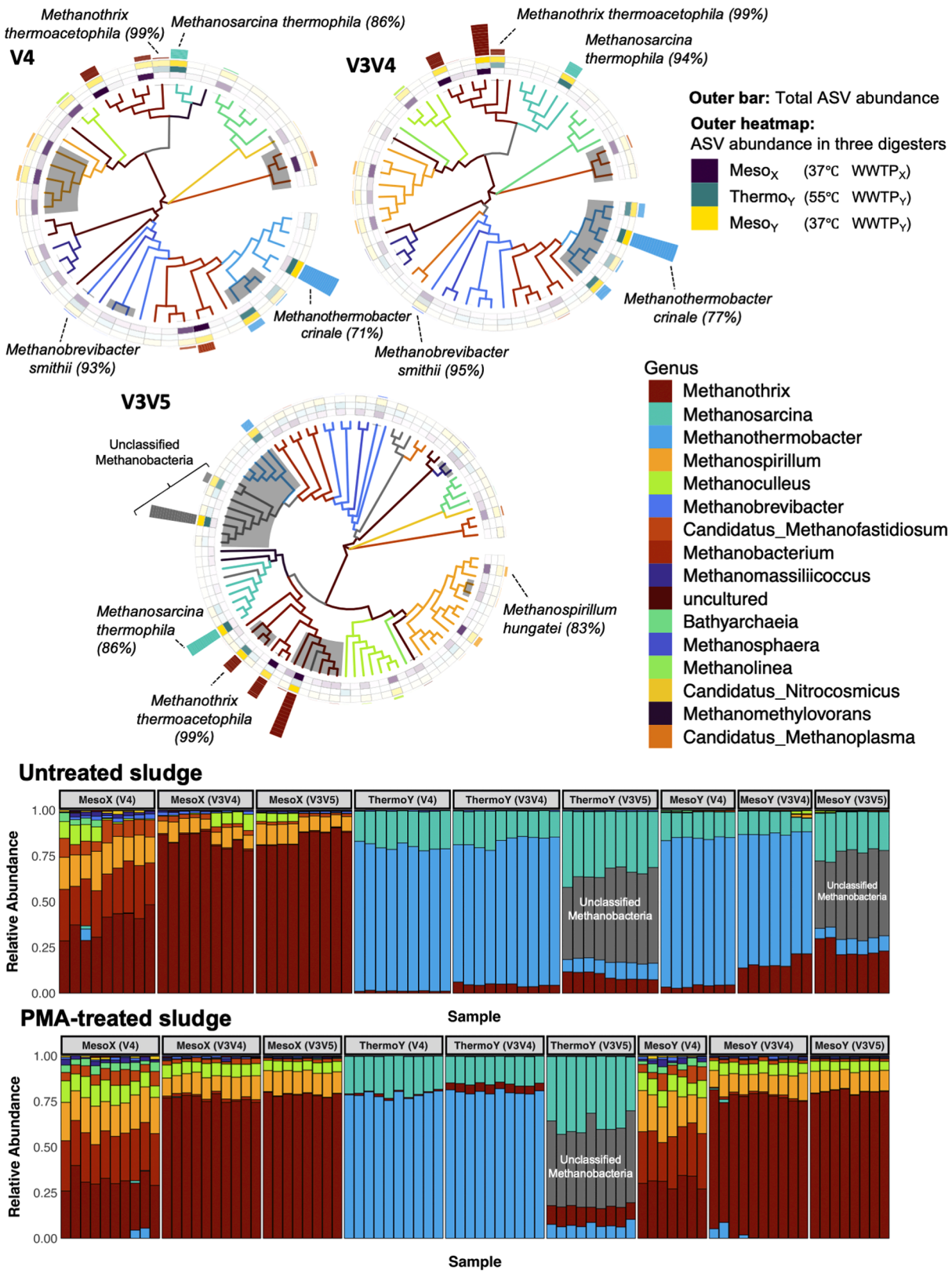

Fig. 3. Phylogenetic profiles of Archaea. Top: Phylogenetic trees of amplicon sequences aligned to a Silva reference tree, showing a subset of archaeal reads of anaerobic sludge samples (untreated and noPMA controls) from three full-scale municipal digesters. Each tree-tip represents an amplicon sequence variant (ASV). Each ASVs is coloured by Genus. Some ASVs were classified to species level with classification confidence shown in brackets (%). Grey-shaded areas represent clades or single ASVs with significantly different abundances between digesters according to Phylofactor analysis. Digester abundances are indicated by the outer heatmap. Relative abundances are shown as the outermost bar. Bottom: Relative abundance barplots of the same subset of archaeal reads. A large percentage (42–50 %) of ASVs derived from the V3V5 primer had no domain-classification and were removed from this analysis.

Table 3
Mantel correlations of ASV abundances and pathway potential matrices (Aitchison distances) with metabolite abundances (Euclidean distances).

Primer pair	Mantel correlations (Spearman R^2)	Sludge Untreated / No PMA	PMA treated sludge
V4	ASVs vs Metabolome	0.12**	0.00
V3V4	ASVs vs Metabolome	0.07**	0.00
V1V3	ASVs vs Metabolome	0.13**	0.02
V3V5	ASVs vs Metabolome	0.25**	0.01
V4	Pathways vs Metabolome	0.43***	0.30***
V3V4	Pathways vs Metabolome	0.39**	0.28***
V1V3	Pathways vs Metabolome	0.43***	0.21**
V3V5	Pathways vs Metabolome	0.34**	0.18**

Note: ** $p = < 0.01$; *** $p = < 0.01$; $n = 15$; Amplicon Sequence Variants (ASVs) filtered to > 100 reads; pathway abundances filtered to > 20,000 reads.

Similarly, for Proteobacteria, 147 and 167 genera were covered with V4 and V3/V4, while only 109 with the V1/V3 primer. Thus, a lower bacterial diversity (Richness, Shannon, and Pielou Evenness) was captured with the bacteria-specific primer pair (V1/V3) (Fig. 1). Overall, the V4 and V3/V4 amplicons also covered a greater genus richness of the phylum Chloroflexi (32–33 ASVs with V4 and V3/V4 vs 23 ASVs with V1/V3). However, one (unknown) Chloroflexi genus in the family Anaerolineacea was covered at greater depth with the V1/V3 amplicons compared to the universal primers, making up the main difference in of this phylum when comparing abundance profiles (Figs. S9 and S10).

The observed lower diversity with the V1/V3 amplicons did not reflect the true (lower) coverage of this primer and was likely an artefact from quality filtering and the removal of poor-quality reverse reads of V1/V3-based amplicons during pairing of forward and reverse reads with DADA2. Once diversity of only forward reads (without pairing with lower quality reverse reads) were assessed, the V1/V3 primer produced the greatest mean richness compared to the universal V4 and V3/V4 primer pairs (Fig. S4).

3.2. Taxonomy and metabolic potentials

Taxonomic profiles and metabolic potentials relevant to anaerobic digestion were assessed. As mentioned, the DNA of abundant thermophilic organisms were detected in mesophilic $Meso_Y$. $Thermo_Y$ effluent (55 ℃) was fed directly into mesophilic $Meso_Y$ for residual digestion at 37 ℃ as part of the same WWTP. Hence, the detected thermophilic organisms in $Meso_Y$ were likely dead cells as evident from their disappearance after treating the sludge with PMA (Figs. 3 and 4). This was further evident from ANCOMBC analysis, which showed that 16 % of genera (representing 30 % of total abundances) were affected by PMA treatment of sludge (ANCOMBC2, $p < 0.05$ holm-corrected, $n = 60$, $N = 120$). The differences in abundance profiles before and after PMA treatment are consistent with observations from PCA (Fig. 2) and Venn diagrams (Figs. S7 and S8), showing that sludge of the two mesophilic digesters $Meso_X$ and $Meso_Y$ share a very similar active phylogenetic diversity, which was only detectable after DNA from inactive organisms was removed. These observations were made independent of primer pairs used.

3.2.1. Archaea

In terms of methane production, as expected the acetoclastic *Methanothrix* phylotypes were most abundant in $Meso_X$ and $Meso_Y$, while hydrogenotrophic *Methanothermobacter* ASVs contributed most to methanogenesis in the $Thermo_Y$, as well as the metabolically versatile *Methanosarcina* spp. (Figs. 3 and 4). Overall, the consortia of thermophilic $Thermo_Y$ carried the greatest relative potential for methanogenesis from acetate and from H_2/CO_2 (Fig. 5), indicating an increased potential for biomethane production at 55 ℃, albeit at the cost of lower phylogenetic diversity. It was observed that *Methanotrix* ASVs were more abundant with V3/V4 and V3/V5 amplicons compared to V4 amplicons (Figs. 3, 4 and S11).

In terms of abundances, the additional taxonomic coverage provided by archaea-specific V3/V5 primers was marginal. Over 99 % of all classified archaeal reads were covered across the three primers, the two universal primers (V4, V3/V4) and the archaea-specific primer pairs (V3/V5). Thirteen methanogenic archaeal genera were identified with V4 and V3/V4 primers in the anaerobic sludge across all three full-scale digesters, with eleven of them identical between the two primers (Fig. 3). Nonetheless, with a total of fifteen genera a greater richness of Archaea was captured by sequencing amplicons with the archaea-specific primer pair (V3/V5), which included the same eleven genera covered with the universal primer pairs. *Methanolinea* and *Candidatus* Methanoplasma (not detected with V4), *Candidatus* Nitrosocosmicus (not detected with V3V4), as well as *Methanomethylovorans* (not detected with V3/V4) were all detected with the V3/V5 primer pairs. The phylogenetic profiles of PMA-treated sludge are supplied with Supplementary Materials (Figure S11). It is important to note that a large percentage (42–50 %) of ASVs derived from the V3/V5 primer had no domain-classification and were removed from the taxonomic analysis.

3.2.2. Bacteria

The phylogenetic profile of Bacteria showed that Proteobacteria (184 genera), Firmicutes (178 genera) and Bacteroidota (80), were the most diverse bacterial phyla across all three anaerobic digesters (Figs. S9 and S10). Members of the fermenting Firmicutes and Bacteroidota were most abundant. For example, the Firmicute *Ruminococcus*, a diverse genus of fermenters and acetate producers, were most abundant in mesophilic digesters $Meso_X$ and $Meso_Y$ but were not present at 55 °C in the $Thermo_Y$ (Fig. 4). The commonly found fermenter *Canditatus* Cloacimonas and the lesser known methanotrophic Verrucomicrobiota in the genus *Pedosphaeraceae* were also among the most abundant Bacteria in $Meso_X$ and $Meso_Y$ (Fig. 4). Generally, abundances of mesophilic Proteobacteria were low and evenly spread across a diversity of species, compared to other major phyla (Figs. S9 and S10). By contrast, $Thermo_Y$ was dominated by few Bacteria that were highly abundant. Here, most abundant were fermenting or acetate oxidising genera *Defluviitoga, Fervidobacterium* and *Coprothermobacter, Candidatus* Caldatribacterium and *Diclyoglomus, Acetomicrobium* and *Lentimicrobium* (Fig. 4).

Furthermore, clustering abundances of abundant genera of sludge that was treated with PMA highlighted differences in coverage between the two universal primer pairs (V4, V3/V4), with some more or less abundant with each primer pair (Fig. 4). This highlighted for example, that *Mycobacterium* was the most abundant and active Actinobacteria (Fig. 4). The genus *Mycobacterium* contains foam-associated species that are part of the 'Mycolata' subgroup known for their extremely hydrophobic, mycolic acid containing, cell-membrane. Other genera that are believed to be associated to foam potentials during anaerobic digestion were detected in all digesters, including *Nocardioides, Gordonia, Candidatus* Microthrix, *Skermania* and *Rhodococcus*. However, these were present only at marginal abundances and likely non-viable, as their abundances reduced significantly after PMA treatment (Figs. S12, S13 and S14).

PMA treatment of sludge prior to sequencing improved the resolution of various rare and active bacteria. One example was the methylotrophic Proteobacteria *Hyphomicrobium*, present in $Meso_X$ and $Meso_Y$, which were abundant, hence functionally relevant for denitrification (12,313 ASVs, 0.23% relative abundances with universal primers). Similarly, the nitrite-oxidising *Nitrospira* sp. were present (7.157 ASVs, 0.13% relative abundances with universal primers). PMA treatment indicated that ASVs of this important nitrifier were present and active. By contrast, 10,097 ASVs (0.19% relative abundances with universal primers) of nitrogen-fixing *Azospira* spp. were removed with PMA, hence unlikely to have contributed to nitrogen metabolism (Fig. S12). Furthermore, the phosphorus accumulating organisms *Candidatus* Accumulibacter (8230 total reads in $Meso_X$ and $Meso_Y$) and *Microlunatus* (2078 reads in $Thermo_Y$) were present. The abundances of *Candidatus*

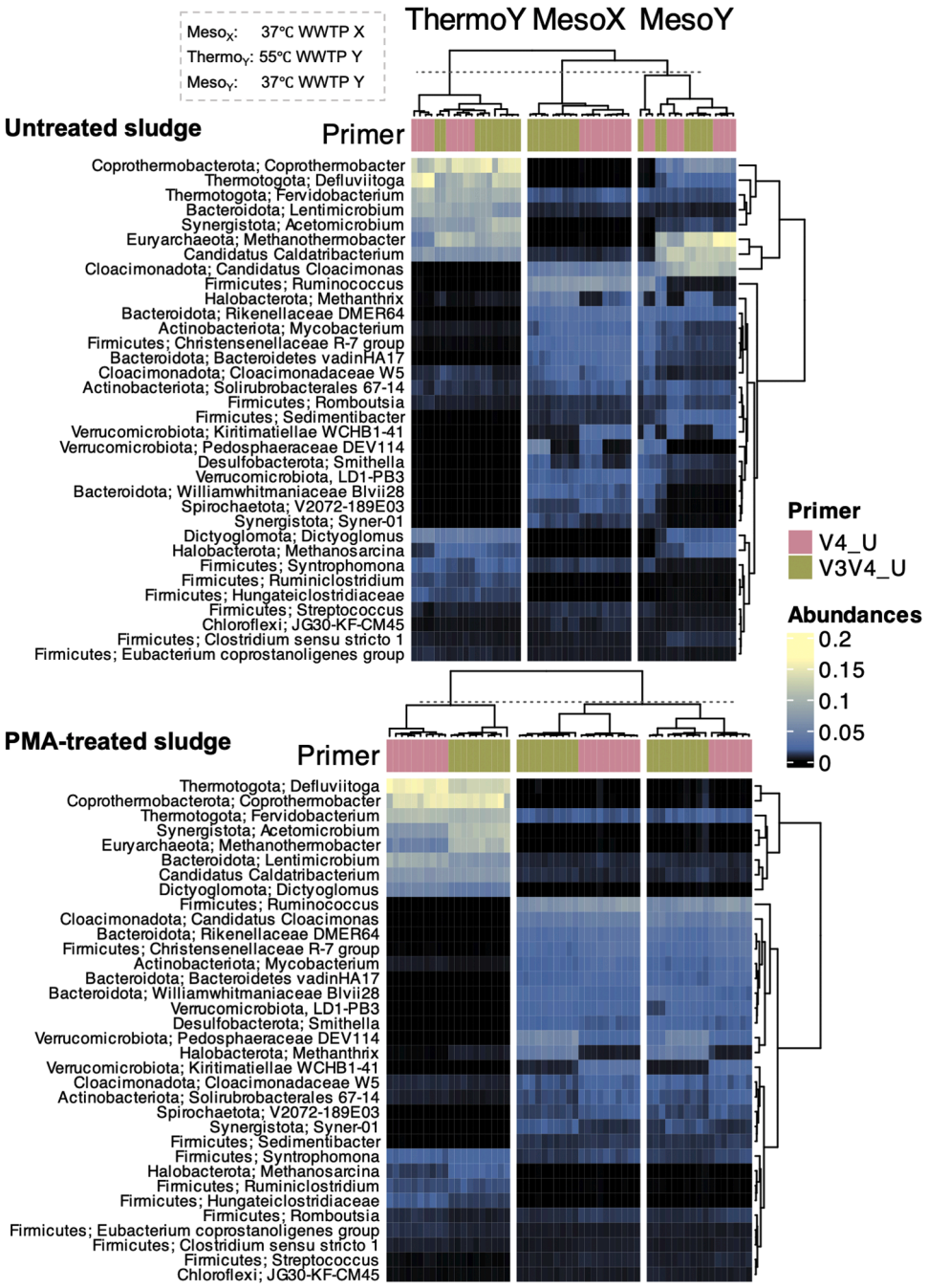

Fig. 4. Heatmap of relative abundances in three full-scale anaerobic digesters (Meso$_X$, Thermo$_Y$, Meso$_Y$). Only the most abundant Bacteria and Archaea are shown on genus level (> 0.5% relative abundance), representing approximately 75 % of total abundances, before and after treatment with propidium monoazide (PMA). Effluent of Thermo$_Y$ was fed into Meso$_Y$ as part of the same treatment plant. Not including unknown genera which made up ~9 % of all taxonomically assigned amplicon sequence variants. Hierarchical clustering performed on rows and columns (Euclidean distances, Ward's minimum variance).

Accumulibacter were not reduced, while the reads from *Microlunatus* were reduced after treating sludge with PMA and is further discussed below (ANCOMBC2, $p < 0.05$ holm-corrected, $n = 60$, $N = 120$).

3.2.3. Metabolic potentials

Overall, the pathway potentials predicted from ASVs with PICRUSt2 indicated that the consortia of all three digesters had the potential to ferment sludge into a range of intermediate metabolites and subsequently into methane through acetoclastic and hydrogenotrophic methanogenesis (Figs. 5 and 6). Pyruvate, l-lycine and acetyl-CoA fermentation potentials were greater in Thermo$_Y$, as was methanogenesis from acetate and H_2/CO_2 (Figs. 5 and 6). This coincided with increased prevalence of the amino acid glycine in Thermo$_Y$, compared to alanine and valine in Meso$_X$ and Meso$_Y$ (Figs. 6 and 7).

Treatment of sludge with PMA affected the prediction of 6 % of total abundances of metabolic pathway potentials (ANCOMBC2, $p < 0.05$ holm-corrected, $n = 60$, $N = 120$). Accordingly, abundances of 105 out of 403 detected metabolic pathways, were significantly reduced in sludge treated with PMA (Fig. S15).

Once sludge was treated with PMA, it was further observed that the active prokaryote populations of the two mesophilic sludges contained a very similar composition of metabolic pathways, as none of the 420 metabolic pathways were differentially abundant between the mesophilic digesters Meso$_X$ and Meso$_Y$ (ANCOMBC2, $p > 0.05$ holm-

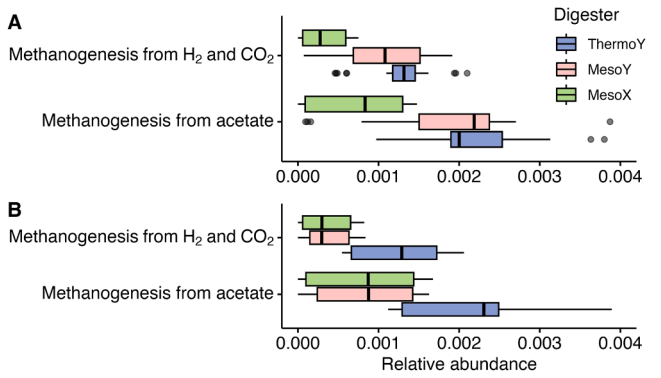

Fig. 5. Boxplots of relative abundances of methanogenesis pathway potentials predicted with PICRUSt2 based on the MetaCyc database before (**A**) and after (**B**) PMA treatment. The line inside the 50 % distribution bar represents the median of the combined abundances from sequence reads using universal primers V4 and V3V4 and an archaea-specific primer (V3V5) ($n = 30$).

corrected, $n = 10$, $N = 120$) (Fig. 2). Untreated sludge, sequenced including non-viable cells, contained 33 metabolic pathways with different abundances between $Meso_X$ and $Meso_Y$ (ANCOMBC2, $p < 0.05$ holm-corrected, $n = 10$, $N = 120$). PMA treatment prior to sequencing subsequently removed these differences. Among the 33 pathways were methanogenesis pathway potentials (Fig. 5) and a range of nutrient cycling and degradation pathways, such as nitrate reduction I (MetaCyc ID: DENITRIFICATION-PWY) and the degradation of potentially hazardous compounds such as catechol (MetaCyc ID PWY-5417) or toluene (MetaCyc ID: PWY-5178).

One hundred and four metabolic potentials were significantly different between the thermophilic and mesophilic digesters (ANCOMBC2, $p > 0.05$ holm-corrected, $n = 10$, $N = 120$, PMA treated). For example, the potential for pyruvate fermentation to acetone (MetaCyc ID PWY-6588), flavin biosynthesis (MetaCyc PWY-6167) as well as propylene glycol degradation (MetaCyc ID PWY-7013), were markedly increased in $Thermo_Y$ compared to $Meso_X$ and $Meso_Y$. In contrast, nitrifier denitrification (MetaCyc PWY-7084) and the degradation of specific sugar derivatives (MetaCyc ID PWY-6507, 4-deoxy-L-threo-hex-4-enopyranuronate degradation), alcohols (e.g., MetaCyc ID GLYCOL-GLYOXDEG-PWY, glycol metabolism and degradation) and aromatic compounds (e.g., MetaCyc ID PWY-6210, 2-aminophenol degradation) were more prevalent in the mesophilic digesters.

Furthermore, the metabolome (239 classified and unclassified metabolites) was reviewed. It was found that pathway potentials were overall more predictive of primary metabolites compared to ASV abundances based on Mantel matrices correlation analysis (Table 3). However, with five sludge replicates and three sludge types in this study it was not possible to perform pair-wise correlation analysis with sufficient statistical power (as a guide R package `pwr', $n = 5$, $R = 0.5$, $p =$

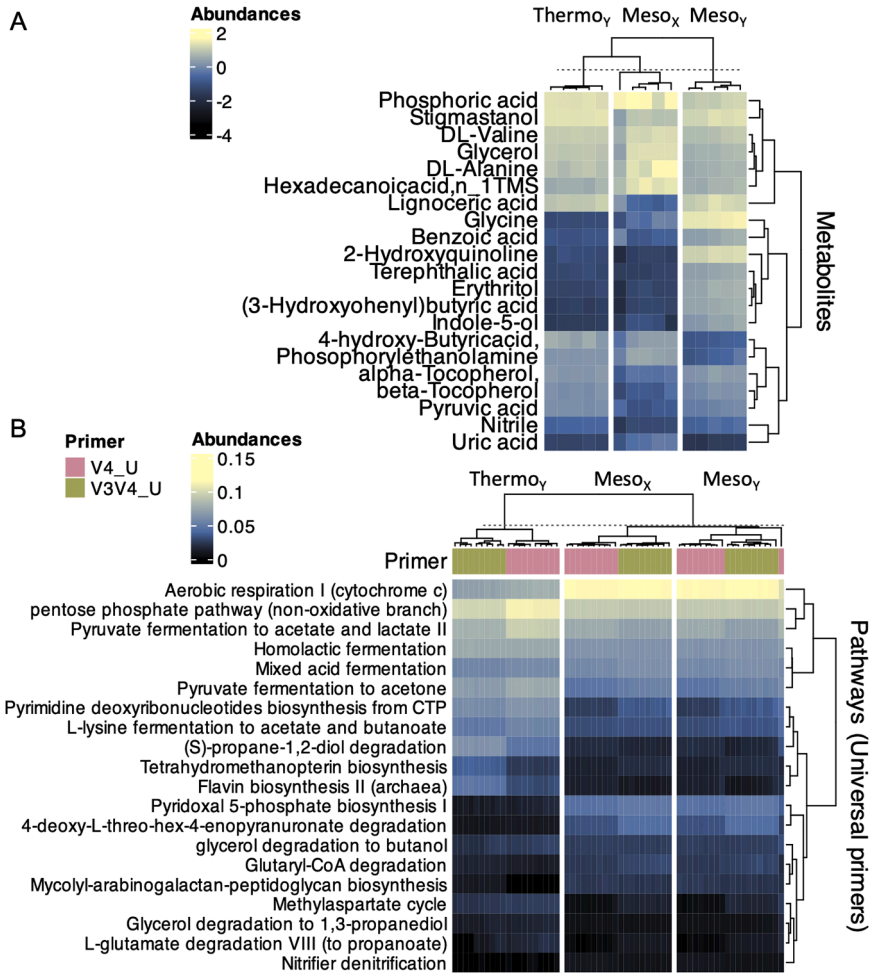

Fig. 6. A: Heatmaps of metabolite abundances of three full-scale digesters ($Thermo_Y$, $Meso_Y$ and $Meso_X$), shown as log-ratios of an internal standard. Metabolites were significantly different between digesters according to ANCOMBC. **B:** Heatmap of selected MetaCyc pathway potentials (Hellinger transform), predicted from amplicon sequences with PICRUSt2, observed in sludge post removal of relic DNA with propidium monoazide (PMA) ($n = 10$ per primer and digester). Hierarchical clustering performed on rows and columns (method `complete linkage`).

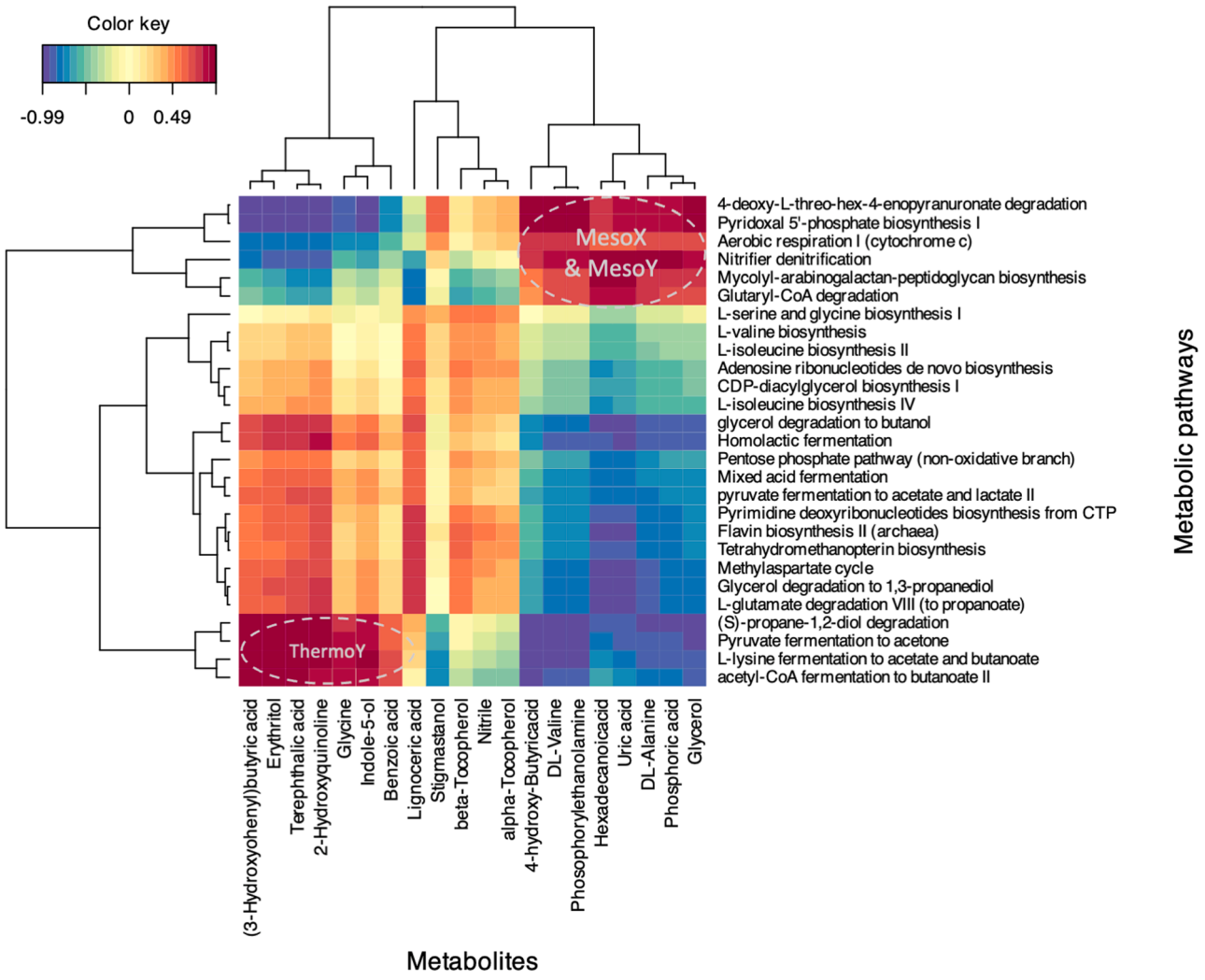

Fig. 7. Correlations of two datasets using regularised canonical correlation analysis (rcc) with help of the mixOmics package in R (rcc, method = "shrinkage", $n = 15$ per dataframe). Correlations coefficients shown are between manually selected variables of the first two components from rcc with the most abundant pathway potentials of untreated sludge and the most abundant metabolites. Data from all three different digesters was included with pathway abundances merged ($n = 5$ per digester). Pathway potentials were estimated from amplicon sequence variants (universal V4 and V3V4 primers) with PICRUSt2.

0.05, power = 0.14). Nonetheless, regularised canonical correlation analysis created clusters of pathway potentials and genus abundances that coincided with groups of metabolite abundances (Figs. 7 and S16). For example, nitrifier denitrification (MetaCyc ID PWY-7084), a pathway that is known to produce nitrous oxide in activated sludge, was observed in $Meso_X$ and $Meso_Y$ and coincided with the prevalence of uric acid and the amino acids alanine and valine in those digesters (Figs. 6 and 7). Although, most correlation clusters were co-incidental, such as the presence of a group of xenobiotics (terephthalic acid, hydroxyquinoline, benzoic acids and erythritol) that were associated with propylene glycol degradation (MetaCyc ID PWY-7013) and three fermentation pathways.

4. Discussion

This study examined the viable procaryote biomass of sludge from three full-scale anaerobic digesters in Victoria, Australia. For simplicity we refer to the viable organism as 'active', noting that not all viable cells are metabolically active. Independent of primers used in this study, it was found that the presence of dead prokaryotes in anaerobic sludge can mislead DNA-based assessments that aim at predicting active digester performance. However, the results also indicated that dead cells may still have predictive value, given that their cell content influence the metabolite composition of sludge. The evidence for these two observations, as well as their implications for the development of DNA-based monitoring strategies are discussed.

4.1. The presence of relic DNA impacts DNA-based surveillance

Overall, sixteen percent of all detected genera, covering thirty percent of total reads across all three investigated digesters, were affected by PMA treatment, representing presumptive dead organisms carried over with feed sludge that influenced reactor bio-composition. In comparison, based on AD-to-feed abundance ratios (to estimate growing and non-growing organisms) in Danish ADs, Kirkegaard et al. (2017) found that fifteen percent of abundant reads were non-growing. Furthermore, these results agreed with findings from De Vrieze et al. (2018), who compared DNA (all genes) and RNA (active genes) in anaerobic digesters. Similarly, the presence of dead biomass in this study represented 'DNA noise' that affected comparisons of process-relevant taxonomy and metabolic potentials between digesters.

For example, the two mesophilic anaerobic digesters ($Meso_X$ and $Meso_Y$, ~37 °C) appeared to have a different microbial composition (Fig. 2). That was somewhat expected given they were located at different sites in Victoria, receiving different feed qualities and operated at different retention times (~16 vs 11, $Meso_X$ vs. $Meso_Y$&$Thermo_Y$).

However, removal of relic DNA from the sequencing pool through PMA treatment, resulted in the (presumably) active microbial diversity and their functional potentials to be almost identical in composition and taxonomy (Figs. 2–4). Between $Meso_X$ and $Meso_Y$, there was no observable difference in the presence of the most dominant Bacteria and Archaea, representing three quarters of total measured abundances. Hence, it was only the presence of DNA from dead Archaea and Bacteria, likely immigrating with the feed, that explained the differences between these two digesters. Furthermore, the effect of this dead biomass would likely be more noticeable under shorter retention times, partly explaining why it was more noticeable between $Thermo_Y$ and $Meso_Y$.

However, it is important to note that the digesters studied here represented a special case of dead biomass content, seeing that the main taxonomic differences between $Meso_X$ and $Meso_Y$ were driven by the thermophilic feed of $Meso_Y$. This was because $Meso_Y$ was fed with thermophilic biomass from $Thermo_Y$, as part of the treatment process. Hence, dead thermophilic Archaea and Bacteria in $Meso_Y$, such as the genera *Methanothermobacter, Caldatribacterium, Coprothermobacter, Defluviitoga* and *Acetomicrobium*, largely explained the differences between $Meso_X$ and $Meso_Y$.

Nonetheless, any other feed sources, such as primary and activated sludge are likely to have background DNA. It is well established that immigrating Bacteria with feed sludge have an impact on community structure (Jiang et al., 2021b; Kirkegaard et al., 2017; Nagler et al., 2021). Kirkegaard et al. (2017) et al. also highlighted that a failure to consider immigration will interfere with correlation analysis. However, in contrast to the AD-to-feed ratios used in their study, sequencing PMA-treated sludge reveals viable cells directly, meaning even if cells are non-growing, they are assumed to be alive. Hence, based on the improved fidelity and resolution of rare but active organisms, it can be argued that the removal of relic DNA is a critical requirement to make comparisons of metagenomic functional assessments reliable. A closer examination of the specific type of potentially dead organisms and their metabolic potential revealed that some important functions may be misrepresented, unless relic DNA is removed prior to sequencing.

For example, the biomass potential to generate biogas is of interest to operators. Without PMA treatment, the total potential for methanogenesis was overestimated for digester $Meso_Y$ (Figs. 3 and 5); in particular the hydrogenotrophic methanogens in genus *Methanothermobacter* (Fig. 3) with their syntrophic partners, the acetate-oxidiser *Coprothermobacter*, which both are relics from the thermophilic reactor (Fig. 4). While the dominant presence of these two organisms under thermophilic conditions was expected (Gagliano et al., 2015; Ho et al., 2016), a significant quantity of non-viable carry-over DNA was also detected in $Meso_Y$ under mesophilic conditions (Fig. 3). A DNA-based comparison between $Meso_X$ and $Meso_Y$ of methanogenisis potentials without considering this carry-over DNA, would lead to the conclusion that $Meso_Y$ biomass had a greater potential to produce methane compared to $Meso_X$ (Fig. 5), and possibly the relative role of acetate oxidation overpredicted vs acetoclastic methanogenesis. However the active methanogen relative abundance were in fact very similar between $Meso_X$ and $Meso_Y$, which aligns with the digester loading rates (Fig. 5 and Table S1).

Another priority for operators is to understand the role of microbial ecology in disruptive foams during anaerobic digestion. The mechanisms of biological foaming are largely unknown, although commonly associated with filamentous Actinobacteria immigrating from activated sludge feed (Jiang et al., 2021a; Petrovski et al., 2011). While many of the known foam-associated organisms were present in all three digesters (*Nocardioides, Gordonia, Candidatus* Microthrix, *Skermania* and *Rhodococcus*), albeit at marginal abundances, they were likely non-viable cells as they were significantly reduced after PMA-treatment. Phylogenetic abundance analysis further showed that most other Actinobacteria, as well as a large group of Burkholderiales were part of the necromass (Figs. S12 and S13). Their presence may still indicate foam risk, as it was found that dead filamentous and hydrophobic cells can lead to foaming, due to their role in gas bubble entrapment (Petrovski et al., 2011). However, it is unknown if actively growing anaerobic organisms play a more or less important role in foaming during anaerobic digestion.

One exception was actinobacterial members in the genus *Mycobacterium*, which were highly abundant and active in mesophilic digesters (Fig. 4) and able to synthesise unique 'mycolyl-arabinogalactan-peptidoglycan' membrane complexes (Fig. 6). Although none of the digesters were foaming at the time of sampling, the presence of mycobacteria indicated a potential for foaming, due to their waxy, lipid-rich cell-membranes that may stabilise foams (Petrovski et al., 2011). PMA-treatment indicated that mycobacteria were active in the anaerobic digesters, despite most of the known species being obligate aerobes. Curiously, aerobic respiration pathways (with oxygen as final electron acceptor) were prevalent in mesophilic digesters, indicating that active organisms were able to utilise oxygen (likely limited by availability of dissolved oxygen or alternative electron acceptors) and that obligatory respiration was as dominant as fermentation (Fig. 6, MetaCyc aerobic respiration PWY-3781).

Operators may further seek to monitor nitrogen cycling. Two aerobic N-cycling genera, *Nitrospira and Hyphomicrobium,* were abundant and active in mesophilic anaerobic digesters, occupying a yet-to-be explored niche. Their presence coincided with 'Nitrifier denitrification' potentials, as well as increased concentrations of uric acid (Fig. 7, MetaCyc Id: PWY-7084). *Nitrospira,* a diverse genus of nitrite oxidisers, have shown to reduce nitrate under anaerobic conditions (Koch et al., 2015; Latocheski et al., 2022). *Hyphomicrobium* spp. with their strict respiratory metabolism, were likely reducing nitrite and nitrate instead of oxygen (Timmermans and Van Haute, 1983). *Hyphomicrobium* sp. also have a unique filamentous physiology (hyphae) making them potential causative foam agents (Urakami et al., 1995). PMA-treatment has provided more confidence to these assertions, enabling the creation of new hypotheses.

Overall, our results showed that PMA removed relic DNA from the analysis in such a way that was expected given the feed regimes of digesters studied. Hence, this validated PMA-seq as a useful approach to improve DNA-based monitoring strategies. Primer choice made no apparent difference for our assessments, despite the different amplicon lengths (292 bp V4 primers to 560 bp for V3V5 primers), which have shown to impact viability PCR studies (Holm et al., 2021). However, it is noted that efficiency of PMA-seq is not fully validated on municipal sludge yet, and while appropriate within sample types, has shown to be highly variable across different types of environmental samples and inaccurate in quantifying viable taxa (Wang et al. 2021). Additionally, not all dead cells have damaged membranes, highlighting the need for further PMA-validation on a case-by-case basis (Cangelosi and Meschke, 2014).

4.2. *Does the necromass have any predictive value?*

Despite PMA improving comparability of active organisms, DNA of the total, 'dead-plus-alive' biomass may still have predictive value. Digester performance is impacted by non-viable cells through the release of metabolite contents, and in this study, it was observed that the untreated DNA-based diversity was more representative of the metabolome compared to PMA-treated sludge (Fig. 2 and Table 3). In other words, the cell contents of dead organisms (sequenced as relic DNA) likely contributed to the metabolic composition. Hence, the presence of DNA of these dead Bacteria and Archaea may still indirectly inform how metabolites of dead cells influence the performance of anaerobic digestion.

A robust correlation analysis between the metabolome and microbial abundances was not within the scope of this study design. However, to illustrate the argument (while needing extensive validation), the presence of dead cells of polyphosphate-accumulating-organisms (PAOs) in anaerobic sludge would support elevated phosphate levels. Non-viable *Microlunatus* ASVs were detected in ThermoY and sequencing DNA of

their dead cells may still provide information about the potential presence of polyphosphates in ThermoY. Although, it was observed that the higher abundances of an active PAO (*Candidatus* Accumulibacter) in mesophilic digesters coincided with higher phosphate concentrations (Fig. S16), with some phosphate caused by release of intracellular phosphate from the PAOs, which supports their survival under anaerobic conditions (Acevedo et al., 2012; Flores-Alsina et al., 2016).

A second example are *Azospira* ASVs, which were detected $Meso_X$ and $Meso_Y$ but were likely dead as evidenced with PMA. *Azospira* sp. are N-fixing rhizobia and were carried in with feed sludge. They form specialised storage structures consisting of polyhydroxybutyrate when conditions become unfavorable for their growth (Mikes et al., 2021; Reinhold-Hurek and Hurek, 2006). Hence, the presence of their relic DNA may partly explain the presence of the metabolite 4-hydroxybutyric acid in digesters $Meso_X$ and $Meso_Y$ as polyhydroxybutyrate is released from ruptured cell walls (Fig. 6).

4.3. Universal primers cover process-relevant organisms

Four commonly used primer pairs were chosen for this study to test if universal primers cover Archaea and Bacteria sufficiently for routine DNA-based sequencing surveys of anaerobic digesters. Using two universal primers, a bacteria-specific and an archaea-specific primer pair (V4, V3/V4, V1/V3, V3/V5 respectively, Table S2) we found that the universal primer pairs provided similar phylogenetic abundance profiles, compared to bacteria or archaea-specific primers Figs. 3 and S9). While a greater domain-specific diversity was covered with Bacteria and Archaeal primers, the most abundant organisms were well represented with universal primers. For example, all known methanogens were covered at similar relative abundance, as were their syntrophic partners, such as *Methanothermobacter* in the thermophilic digester $Thermo_Y$.

An exception where domain-specificity was important were Bacteria in the Anaerolineaceae family (phylum Chloroflexi), which were highly abundant and mostly captured with bacteria-specific V1/V3 primer pairs (Fig. S9), but not with universal primers. Through metagenomic sequencing it is known that Anaerolineaceae can be highly abundant primary fermenters, hence their lack of coverage with universal primers is an important consideration when choosing primers (McIlroy et al., 2017).

For Archaea, *Methanothermobacter* reads were classified only at order-level (*Methanobacteriales*) with reads based on the V3/V5 primer pair, while read classification improved with the universal primers to genus-level (Fig. 3). This was likely due to the poor read quality of these relatively long V3/V5 amplicons (560 bps), which were sequenced on one flowcell together with the shorter amplicons used in this study (e.g., 292–456 bps). Longer amplicons clustered less effectively on the Miseq flowcell compared to the shorter amplicons, with error rates increasing with each cluster cycle (Schirmer et al., 2015). Furthermore, at 560 bps the forward and reverse reads cannot be paired for the V3/V5 amplicons, with only forward reads classified, potentially resulting in overall lower confidence in taxonomic classifications. Hence, ironically longer-amplicons, which could provide greater phylogenetic resolution, resulted in poorer taxonomic classification, highlighting the technical limitations of Miseq short-read sequencing technologies.

In terms of the two universal primers V4 and V3/V4 there was no apparent advantage in the use of one over the other. Although it was found that *Methanothrix* reads where lower with the V4 primer pair, while abundant members of yet-to-be described Kiritimatiellae order WCHB1–41, where better covered with V3V4 primers (Fig. 4). We further highlight that removing the background of relic DNA made this primer comparison easier (Figs. 4 and 6). Yet, both taxa were clearly detectable with both primer pairs and therefore remain viable option in either case.

Compared to the V4 primer, the abundance distribution of archaea with V3/V4 primers was more like that of the archaea-specific primer pair (V3/V5) (Figs. 1 and 3). The main difference was that archaeal reads from the V4 primers were more evenly distributed, compared to V3/V4 and V3/V5 samples. This may be an artefact of the relatively shorter amplicon length of the V4 primer pair (292 vs 457 and 560 bps, V4, V3/V4 and V3/V5 respectively) and the higher read quality available for pairing of forward and reverse reads. For Miseq sequencing platforms with a read length of 2×300 bp, the length of amplicons affects the bioinformatic processing of the sequencing reads, with shorter amplicons at an advantage for ensuring read confidence after overlapping of forward and reverse reads. This has implications for the estimation of diversity indices and highlights that cross-study comparisons of diversity indices are contingent on the use of identical primers.

Overall, while primer-specific taxonomic biases were observed, most of the abundant amplicon sequences of the two universal primer pairs provided similar phylogenic profiles compared to domain-specific primer pairs and provided comparable process-related or biological conclusions. The main fermenters and methanogens, as well as their functional potentials were clearly differentiated between mesophilic and thermophilic conditions, independent of primers (Figs. 3–6). Hence, a universal primer with lower coverage of Archaea and Bacteria, compared to more specific domain-level primers, may still provide diversity profiles and valid biomarkers that explain biological processes relevant for specific research aims and hypotheses or are critical for reactor performance.

4.4. Conclusions

Removing relic DNA from the sequencing pool improved fidelity for the detection of active, functionally relevant Bacteria and Archaea. Hence, PMA-seq may improve biological surveillance strategies that aim to predict process performance of anaerobic digestion from microbial abundances. PMA-seq may further benefit longitudinal studies where feed quality is variable over time.

However, the results further indicated that the DNA of dead Bacteria and Archaea may still indirectly inform how metabolites present in dead cells influence the performance of anaerobic digestion, supporting some predictive value of relic DNA.

The choice of primers used in this study tested their broad utility, and demonstrated that, while the use of specific primers is critical for specific research objectives, they may be less critical for the generic monitoring of digester performance. The results suggested that it may be more important to improve comparability of samples by removing DNA from functionally irrelevant organisms. Although, we acknowledge that, depending on the aims, PMA-treatment may not always be a realistic option as it doubles the DNA extraction and sequencing costs, unless validation through a non-PMA control is not required. However, for researchers intending to use two domain-specific primers for their project, resources may be better spent by doing PMA-seq with one universal primer.

Data availability

The data files supporting this study are openly available at the Sequencing Read Archive (SRA) of The National Center for Biotechnology Information (NCBI) under accession number PRJNA1077478.

CRediT authorship contribution statement

Christian Krohn: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Kraiwut Jansriphibul:** Conceptualization, Methodology, Data curation. **Daniel A. Dias:** Formal analysis, Methodology, Writing – review & editing. **Catherine A. Rees:** Resources, Supervision, Writing – review & editing. **Ben van den Akker:** Resources, Supervision, Writing – review & editing. **Jennifer C. Boer:** Supervision, Validation, Writing – review & editing. **Magdalena Plebanski:** Supervision, Validation, Writing – review & editing. **Aravind Surapaneni:**

Resources, Supervision, Funding acquisition. **Denis O'Carroll:** Resources, Supervision, Writing – review & editing. **Stuetz Richard:** Project administration, Supervision. **Damien J. Batstone:** Supervision, Validation, Writing – review & editing. **Andrew S. Ball:** Funding acquisition, Supervision, 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.

Funding

This research was funded by an Australian Research Council Industrial Transformation Training Centre Grant (IC190100033).

Acknowledgments

We much appreciate the guidance of all project partners and steering committees across the ARC Training Centre for the Transformation of Australia's Biosolids Resource. Thanks also to Sneha Gupta for her assistance with processing of the GC–MS data.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121354.

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