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Reactor configuration influences microbial community structure during high-rate, low-temperature anaerobic treatment of dairy wastewater

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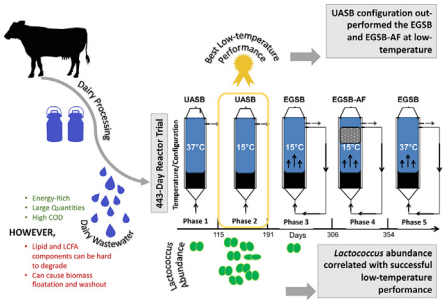
GRAPHICAL ABSTRACT



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ABSTRACT

Low temperature anaerobic digestion remains in its infancy, despite increasing interest for the treatment of complex wastewaters. In this study, the feasibility of low-temperature anaerobic treatment of dairy wastewater was assessed during a 443-day laboratory-scale bioreactor trial. The bioreactors were operated in triplicate at organic loading rates of 7.5–9 $kgCODm^{-3}d^{-1}$ throughout five operational phases. The structure of the microbial community was analysed using quantitative real-time PCR and amplicon sequencing of 16S rRNA genes from DNA and rRNA. The results indicated that low-temperature treatment of dairy wastewater is feasible at 15 °C, but that reactor configuration remains extremely important. The upflow anaerobic sludge bed (UASB) configuration out-performed the expanded granular sludge bed (EGSB)-based configurations. Decreased temperatures resulted in significant reductions in microbiome diversity. *Methanosaeta* was identified as a dominant genus throughout the trial, while *Lactococcus* was identified as an important bacterial genus at low-temperatures. However, the relative abundance of *Lactococcus* was significantly influenced by reactor configuration.

1. Introduction

For over four decades, high-rate anaerobic digestion (AD) has been successfully applied for the treatment of industrial and municipal wastewater (van Lier et al., 2015). AD processes not only remove complex organic pollutants from wastewater, but in doing so, also generate a methane-based biogas as a renewable energy source. Success of these high-rate AD systems is underpinned by a highly settleable and

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active granular biomass, which can be retained in the system for up to months (Hulshoff Pol et al., 2004). Each of these anaerobic granules contain the entire microbial community necessary to completely mineralise complex suspended organic material to methane. These granular-based reactor configurations, namely the upflow anaerobic sludge bed (UASB) and expanded granular sludge bed (EGSB), have been successful at a range of temperatures (Kim et al., 2002; McKeown et al., 2009) – the EGSB often outperforming the UASB due to increased granule-wastewater contact (Kato et al., 1998). Moreover, the hybrid expanded granular sludge bed anaerobic filter (EGSB-AF) incorporates a filter in the top of the reactor, trapping biomass which may otherwise be lost (Keating et al., 2018). With many attractive advantages, AD has become a highly attractive treatment option for a wide variety of wastewater types (Lettinga, 1995).

Among these, dairy wastewater provides significant challenges and opportunities for AD. Not only is the dairy industry one of the largest sources of industrial wastewater in Europe – generating 2–10 times the volume of actual milk that is produced (Buntner et al., 2013) – but the wastewater is generally energy-rich (thus having a high-methane potential), although not always containing easily biodegradable chemical constituents. Composition is highly variable, dependent upon the type of dairy product produced, but is generally characterisied by high chemical oxygen demand (COD), consisting of carbohydrates, dairy proteins and dairy lipids, as well as nutrient components such as ammonia and phosphates (Sarkar et al., 2006). The carbohydrate component is primarily comprised of lactose, which is readily and almost completely degradable (Pavlostathis and Giraldo-Gomez, 1991). Dairy proteins account for up to 40% of the COD and usually consist of 20% whey proteins and 80% casein proteins (Vidal et al., 2000). Notably, previous studies have reported protein hydrolysis as the main bottleneck during low-temperature AD of dairy wastewater (Bialek et al., 2014, 2012). Finally, lipids present the final metabolic challenge for AD of dairy wastewaters (Vidal et al., 2000). In some cases, a fatty scum layer can accumulate at the top of reactor, preventing biogas release (Passeggi et al., 2012). In most cases, lipids are hydrolysed to long-chain fatty acids (LCFAs), but further degradation of LCFA is difficult, and accumulation can result in process inhibition (Angelidaki and Ahring, 1992). Moreover, LCFA can adsorb to the surface of the granular biomass, causing severe flotation episodes and leading to biomass washout (Hwu et al., 1997).

In temperate climates, many dairy wastewaters are discharged at ambient temperatures (< 18 °C); however, the majority of treatment systems are operated under mesophilic (25–45 °C) or thermophilic (45–65 °C) conditions (Lettinga et al., 2001). A significant energy input is therefore required for heating, and maintaining AD systems at these temperatures is often costly and unfeasible (Smith et al., 2015). In such cases, low-temperature AD (< 25 °C) is a more attractive alternative – allowing treatment to proceed at ambient discharge temperatures. Laboratory-scale studies on low-temperature AD have investigated its potential use for several substrates including: domestic (Keating et al., 2016), acidified (McKeown et al., 2009), brewery (Connaughton et al., 2006) and dairy (Bialek et al., 2013a; Buntner et al., 2013). These studies have demonstrated the feasibility of low-temperature AD – in some cases as low as 4–6 °C (McKeown et al., 2009; Smith et al., 2015) – and yet, knowledge gaps still remain concerning the appropriate reactor design and operating parameters. Moreover, and importantly, there is a lack of understanding regarding the structure and function of the microbial community when treating dairy wastewater at low temperatures.

The AD microbiome comprises several distinct microbial trophic groups from the two evolutionarily distinct domains of bacteria and archaea. They function in concert, and sometimes in competition, to convert complex organic compounds ultimately into methane and carbon dioxide. Each trophic group is reliant upon another for a carbon and/or electron source, and the loss of any one trophic group can cause a bottleneck in the AD process – ultimately resulting in reactor failure. Understanding the resilience, constraints, and limitations of the microbial community can help to fundamentally improve the AD process and encourage its widespread application (Lee et al., 2009). Further application of advanced molecular techniques, such as high-throughput sequencing, when targeted and applied appropriately, can provide deeper insights into the changes in structure and function of the microbiome as it responds to changing environmental or operational conditions.

The objectives of this study were threefold: (i) to assess the feasibility of low-temperature anaerobic treatment of dairy wastewater; (ii) to compare the efficiency of various reactor configurations and operating temperatures on the process; and (iii) to find links between reactor performance and the dynamics of the microbial community. To this end, a laboratory-scale reactor trial was designed to compare UASB, EGSB, and EGSB-AF configurations at both mesophilic (37 °C) and low (15 °C) temperatures.

2. Materials and methods

2.1. Source of biomass

Anaerobic sludge granules were sourced from a full-scale (1500 m^3), mesophilic internal circulation reactor at Carbery Milk Products (Ballineen, Co. Cork, Ireland) treating wastewater generated from ethanol production. The granules were stored in a 20 L sealed plastic container at 4 °C prior to inoculation.

2.2. Reactor design and operation

Triplicate, laboratory-scale (3.5-L working volume) reactors were operated under identical conditions for a 443-day trial. The reactors were inoculated with 20 g volatile solids (VS) L^{-1} of granular biomass. The reactors were supplied a synthetic dairy wastewater, consisting of skimmed milk powder at a concentration of 2.5 g COD L^{-1}, and supplemented with both macronutrients and micronutrients (Shelton and Tiedje, 1984). The pH was buffered using 1.2 g L^{-1} $NaHCO_3$. The synthetic wastewater consisted of 35% protein, 56% carbohydrates, 1% fat, and 8% minerals/ash.

The 443-day reactor trial consisted of a start-up phase lasting for the first 74 days, followed by five experimental phases (Table 1), during which, the reactor configuration and temperature were strategically changed. During the start-up phase the hydraulic retention time (HRT) was decreased stepwise from 48 h to 8 h. Following the start-up phase, the reactors were operated at an organic loading rate (OLR) of 7.5–9 kg COD m^{-3} d^{-1} and HRT of 6.6–8 h. Phase 1 (P1) was characterised by mesophilic (37 °C) UASB operation, while in Phase 2 (P2) the temperature was decreased to 15 °C to test the feasibility of AD at low temperatures. Phase 3 (P3) maintained low temperatures, however the reactor configuration was changed to an EGSB by fitting a recycle line, controlled using peristaltic pumps (Watson and Marlow 300 series), at an upflow velocity of 3.5 m h^{-1}. Following this, Phase 4 (P4) tested low-temperature EGSB-AF operation through the addition of an inert pumice stone filter described by Keating et al. (2016). In Phase 5 (P5), the filter was removed and the temperature of the EGSB reactors was increased to 37 °C until the end of the trial to investigate the capacity of the microbial community for recovery when the temperature is increased back to 37 °C.

2.3. Analytical techniques to monitor performance

For the duration of the trial, reactor influent and effluent were collected and analysed every 1–2 days for total COD (COD_{tot}), soluble (COD_{sol}), volatile fatty acids (VFA), and pH according to standard methods (APHA, 2005). Biogas samples were collected every 7–10 days, and the methane content of the biogas was determined using a VARIAN CP-3800 gas chromatograph (Varian, Inc., Walnut Creek, CA). VFA

Table 1
Reactor operating parameters.

Phase	Start-up	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
Days	0–74	75–114	115–190	191–305	306–354	355–443
COD [a]	2.5 ± 0.3	2.6 ± 0.3	2.6 ± 0.2	2.4 ± 0.4	2.4 ± 0.2	2.5 ± 0.3
HRT [b]	48–8	8	8	8	6.6	8
Temp [c]	37	37	15	15	15	37
Up-flow [d]	–	–	–	3.5	3.5	3.5
OLR [e]	1.25–7.5	7.5	7.5	7.5	9	7.5
Type [f]	UASB	UASB	UASB	EGSB	EGSB-AF	EGSB
SMA [g]	–	D110	D190	D276	–	D443
LCFA [h]	–	D110	D190	D276	–	D443

[a] Influent chemical oxygen demand, total (g l^{-1}).
[b] Hydraulic retention time (h).
[c] Temperature (°C).
[d] Up-flow velocity (m h^{-1}).
[e] Organic loading rate (kg COD m^{-3} d^{-1}).
[f] Reactor type (upflow anaerobic sludge bed, expanded granular sludge bed, expanded granular sludge bed with anaerobic filter).
[g] Specific methanogenic activity assays performed.
[h] Long chain fatty acid biomass attachment quantified.

components were separated and quantified using a Varian Saturn 2000 GC/MS system (Varian Inc., Walnut Creek, CA). LCFA content was determined using a method adapted from Neves et al. (2009). Finally, proteins were quantified using the method previously described by Lowry et al. (1951). Statistical differences were determined using a Kruskal-Wallace test followed by Dunn's multiple comparison test.

2.4. Specific methanogenic activity (SMA) assays

Specific methanogenic activity (SMA) assays were performed using biomass samples from the seed inoculum and from the reactors at four strategic timepoints during the trial: day 110 (end of P1), day 190 (end of P2), day 276 (end of P3), and day 443 (end of trial). These were performed at both 15 °C and 37 °C as described by Colleran et al. (1992). Statistical differences were determined using two-way analysis of variance (ANOVA).

2.5. DNA/RNA co-extraction & complimentary DNA synthesis

DNA and RNA were co-extracted from duplicate samples of granular biomass collected from each reactor at the same four timepoints used for the SMA assay. For each sample, nucleic acids from 2 to 4 g of wet biomass were extracted on ice following the procedure described by Griffiths et al. (2000). Briefly, this method is based on bead-beating in 1% (w/v) cetyl trimethylammonium bromide (CTAB; Sigma-Aldrich) buffer, immediately followed by a phenol-chloroform based extraction. Washed and purified nucleic acids were resuspended in nuclease-free water. The integrity of these samples was assessed by agarose gel electrophoresis and a nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). Concentrations were determined using a Qubit flurorometer (Invitrogen, Carlsbad, CA, USA). An aliquot of DNA was set aside and stored at −80 °C.

cDNA was synthesised from the RNA in three steps: (i) DNA was removed through a DNAse treatment using the Turbo DNA-free kit (Ambion – Invitrogen, Carlsbad, CA, USA), following the recommendations from the manufacturer, and (ii) PCR, with universal bacterial and archaeal primers 515F and 806R (Caporaso et al., 2011), confirmed the samples to be DNA-free; and (iii) cDNA was reverse transcribed from the RNA using the SuperScript III Reverse Transcriptase Kit (Thermo Fisher, Waltham, MA, USA), following the manufacturers recommendations. Successful cDNA generation was confirmed by PCR amplification using the same primer pair previously used. cDNA was then stored at −80 °C.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed on both DNA and cDNA samples using a Light-Cycler 480 instrument (Roche, Penzberg, Germany). The assay separately targeted and quantified both bacterial and archaeal domains. For bacterial quantification, the primer set 1369F (GGTGAATACGTTCY-CGG) and 1492R (GGWTACCTTGTTACGACTT) and the Taqman probe TM1389F (CTTGTACACACCGCCCGTC) were used (Suzuki et al., 2000). For archaeal quantification the primer set 787F (ATTAGATACCCSBG-TAGTCC) and1059R (GCCATGCACCWCCTCT) and the Taqman probe TM915F (CTTGTACACACCGCCCGTC) were used (Yu et al., 2005). Quantitative standard curves were made using *Escherichia coli* as a representative bacterial isolate and *Methanosarcina barkeri* as the representative archaeal isolate. The standards were generated by polymerase chain reaction (PCR) amplification using the above primers and subsequently purified using the QIAQuick PCR Clean Up kit (Qiagen, Hilden, Germany). To generate standard curves, a 10-fold serial dilution series of 10^{-9}–10^{-1} copies μL^{-1} of each PCR product was analysed in duplicate by real-time PCR. qPCR reactions were prepared using the LightCycler 480 Probes Master mix (Roche, Penzberg, Germany). Each reaction contained 10 µL 2x master mix, 0.8 µL of each primer (400 µM final concentration), 0.4 µL TaqMan probe (200 µM final concentration), 7 µL qPCR-grade water and 1 µL of template DNA or cDNA. Amplification was performed on the Light-Cycler 480 using a two-step thermal cycling protocol which consisted of denaturing at 95 °C for 10 min, followed by 40 cycles of 10 seconds at 95 °C and 30 seconds at 60 °C. Standard curves were generated by plotting the CT-values (cycle threshold) against the logarithm of the dilution factors and calculating the regression line through these points. The 16S rRNA gene copy concentrations were then estimated against the standard curves and their concentrations converted to copies g^{-1} biomass.

2.7. High-throughput sequencing

Nucleic acids ($n = 2$) from each time point and each reactor were quantified using the Qubit HS dsDNA Assay (Thermo Fisher Scientific, Waltham, MA, USA), then normalised to 20 µL mL^{-1}. Normalised samples were combined and run in triplicate on a 2% agarose gel. The ~300 bp bands were excised and purified using the Wizard SV gel and PCR clean-up kit (Promega, Madison, Wisconsin, USA) in order to remove primer dimers and other PCR artefacts. The purified PCR products were normalised to 7.1 ng μL^{-1}. Amplification of the V4 region of the 16S rRNA gene was performed on the Illumina MiSeq platform by the Centre for Genomic Research in the University of Liverpool (Liverpool, UK) using the universal bacterial and archaeal primer set: 515F and

806R (Caporaso et al., 2011), with indexed barcodes on the forward primer.

2.8. Bioinformatics and statistical analysis

Following Illumina sequencing, raw fastq files were trimmed and reads shorter than 10 base pairs were removed, as were non-paired-end reads. The fastq files were then processed according to the Mothur protocol (Kozich et al., 2013). Chimeric, eukaryotic, and mitochondrial sequences were identified and removed. Operational taxanomic units (OTU) were clustered according to the average neighbour algorithm with a cut-off of 0.03 and were generated against the SILVA reference library (Quast et al., 2013). All taxonomic classifications were assigned using the naïve Bayesian algorithm developed for the RDP classifier (Kozich et al., 2013). Singletons were removed using QIIME (Caporaso et al., 2010). Sequence abundance for each sample was calculated and used to create an OTU table. Further statistical processing was performed in R. Species richness was calculated using the Chao1 richness estimator and Shannon entropy was used to assess diversity with statistical differences tested using ANOVA. Non-metric multidimensional scaling (nMDS) was performed using the Bray-Curtis distance metric. This was performed and plotted in R (version 3.3.1) using the Picante and Vegan packages (Kembel et al., 2010; Oksanen et al., 2018). Finally, similarity percentages (SIMPER) analysis was performed to determine the level of similarity and dissimilarity between timepoints, using the top 200 most abundant OTUs. These data were log normalised and the Kruskal–Wallis one-way ANOVA test used to identify the discriminant OTUs.

3. Results and discussion

3.1. Effect of temperature and configuration on reactor performance

By the end of the start-up phase all three reactors were performing efficiently with average COD_{tot} removal efficiencies of 88% (± 2%) and COD_{sol} removal efficiencies of 92%. COD removal remained high throughout P1 (Table 2), which was distinguished by stable operation and the highest COD removal efficiencies of the five phases. The carbohydrate and protein concentrations in the effluent remained low with average values of 38 and 241 mg L^{-1}, compared to influent concentrations of 1380 and 865 mg L^{-1} respectively (Fig. 1; Table 2). This compares favourably to similar studies involving low-strength dairy wastewater treatment in UASB reactors under mesophilic conditions (Banu et al., 2008; Passeggi et al., 2012; Vidal et al., 2000).

The decrease in temperature to 15 °C in P2 demonstrated that anaerobic treatment of dairy wastewater is feasible at low temperature. For the duration of P2 all reactors displayed high rates of COD_{sol} removal (~90%), protein degradation, and efficient conversion of VFA intermediates to methane. Total COD removal was, however highly variable (39–84%) and biomass loss due to pistoning and flotation of granules frequently occurred. The washout of biomass accounted for a large part of the high total COD concentrations measured in the effluent. However, COD_{sol} removal remained high – only slightly lower than achieved by Buntner et al. (2013) during treatment of dairy wastewater at ambient temperatures (17–25 °C), and better than some mesophilic UASB treatment systems (Nadais et al., 2005). The COD_{sol} removal was also higher than that reported by Bialek et al. (2012) and Bialek et al. (2013b) when treating similar wastewater in an EGSB configuration at low temperatures. This phase, therefore indicated a promising development for low-temperature AD of dairy wastewater.

Conversion from a UASB to an EGSB in P3 did not improve reactor performance, and in fact, COD removal decreased. P3 was also characterised by the highest concentrations of protein and carbohydrates in the effluent (Table 2). P3 had the highest degree of variation in COD_{tot} removal (Fig. 1), ranging between 40 and 76%, with an average of 64.2 ± 7.9%. Low removal of COD_{sol} was also recorded, averaging at 83% across the three reactors – significantly lower than in P2 ($p < 0.0001$). Moreover, pistoning (periodic floatation) of the sludge bed occurred frequently in all three reactors, ultimately resulting in biomass washout of quantities of greater than 0.3 g VS L^{-1} d^{-1}. Increases in COD removal commonly associated with EGSB designs were not observed during this study, and surprisingly, reactor performance decreased significantly with respect to COD_{sol} removal and protein degradation. This was unexpected, and counter-intuitive, as a number of previous studies demonstrated that using EGSB reactors had a positive impact on low-strength wastewaters when treated at low temperatures (Kato et al., 1998). Granular shearing due to high upflow velocities had previously been identified as a problem at low temperature, resulting in the loss of active biomass and impacting system performance (McKeown et al., 2009; Rebac et al., 1995).

During this study it is suspected that the increased shear velocity associated with the recirculation system selectively removed, inhibited, or washed out, key microbial taxa, specifically *Lactococcus* (Fig. 4) – ultimately, having a detrimental effect on performance. Correlated to the decrease in *Lactococcus* abundance, mean protein concentration in the effluent peaked at 511 mg L^{-1} during P3. Tommaso et al. (2003) reported that hydrolysis of proteins and lipids decreases rapidly as temperature approaches 15 °C. Furthermore, hydrolysis of protein was suspected to be the rate-limiting step in previous studies involving low-temperature treatment of dairy wastewater (Bialek et al., 2014, 2012). Overall, the hydraulic changes had a detrimental effect on the AD process. It is likely that key proteolytic microbial groups or enzymes

Table 2
Average reactor performance and standard deviation during each phase of operation.

Phase	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
COD_{tot} [a]	421 ± 132	853 ± 257	881 ± 195	565 ± 138	495 ± 146
$\%RE_{tot}$ [b]	82.9 ± 5.4	65.3 ± 10.4	64.2 ± 79	77 ± 5.6	79.9 ± 6
COD_{sol} [c]	122 ± 31	174 ± 65	289 ± 138	228 ± 70	199 ± 82
$\%RE_{sol}$ [d]	92.9 ± 1.8	89.9 ± 3.8	83.2 ± 8	87.1 ± 4.1	88.4 ± 4.8
Carb [e]	37.7 ± 15.7	51.3 ± 22.9	35.6 ± 17.3	22.5 ± 11.1	27.9 ± 16.8
Prot [f]	241 ± 35	344 ± 81	511 ± 193	447 ± 156	394 ± 201
$\%CH_4$ [g]	68.9 ± 5.2	55.5 ± 6.3	52.6 ± 7.3	44.5 ± 14.4	59.7 ± 4.6
pH [h]	7.6 ± 0.2	7.5 ± 0.2	7.4 ± 0.3	7.2 ± 0.2	7.3 ± 0.3

[a] Effluent COD, total (mg l^{-1}).
[b] Total COD removal efficiency (%).
[c] Effluent COD, soluble (mg l^{-1}).
[d] Soluble COD removal efficiency (%).
[e] Effluent carbohydrate concentration (mg l^{-1}).
[f] Effluent protein concentration (mg l^{-1}).
[g] Methane in biogas (%).
[h] Effluent pH.

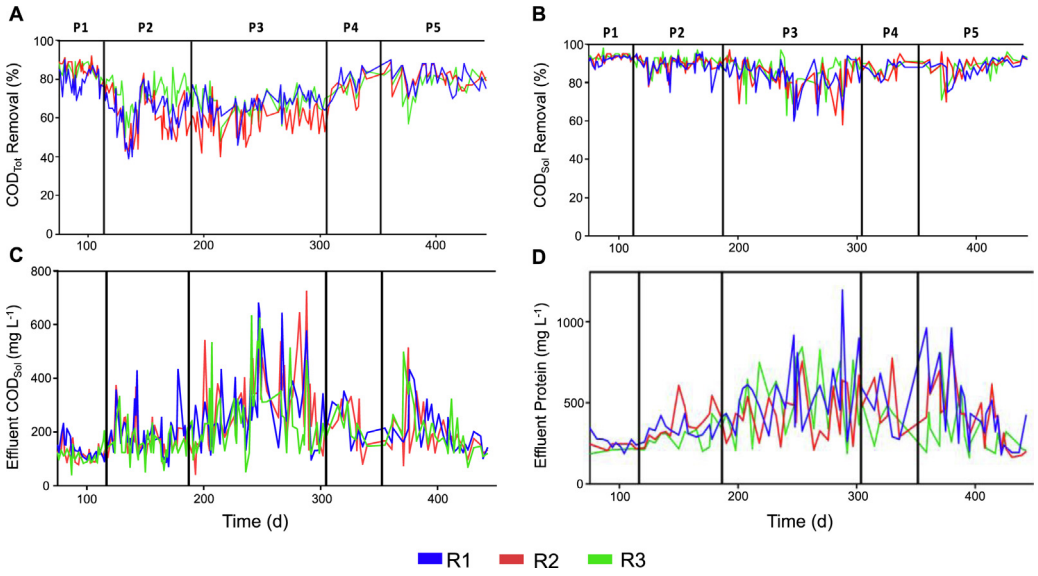

Fig. 1. Reactor process performance data for R1, R2 and R3, over the five phases, showing (a) the total COD removal; (b) the soluble COD removal; (c) the soluble COD concentration in the effluent; and (d) the protein concentration in the effluent.

were washed out due to increased upflow velocity, thereby creating a build-up of protein within the system.

Addition of the pumice stone filter in P4 resulted in a notable increase in average COD removal efficiencies (Table 2), also observed during municipal wastewater treatment (Keating et al., 2016). Washout of biomass decreased, however biomass continued to float just underneath the filters, until they became blocked and were removed. Filter removal, coupled with the temperature increase to 37 °C, eventually resulted in stabilised performance and increased COD removal efficiencies of 80 ± 6% for COD_{tot} and 88 ± 4.8% for COD_{sol}.

Interestingly, LCFA were not detected in the influent or effluent at any point during the trial. LCFA were, however, detected from the biomass. The average total LCFA concentration increased from 63 ± 18.3 mg gTS^{-1} at the end of P1 to 109.6 ± 32.6 mg gTS^{-1} at the end of the trial, and could partially explain the floatation of the granules. Furthermore, a white layer was observed on the surface of the granules, potentially caused by adsorption of LCFA.

The conditions employed during this trial resulted in biomass loss and may have implications for the sustainability of long-term treatment. Pistoning, granule floatation, and subsequent biomass washout are common challenges associated with many types of reactor disturbances such as a sudden reduction in temperature (Bialek et al., 2013a), accumulation of fats (Passeggi et al., 2012) and build-up of VFA (McKeown et al., 2009). In this case, the sudden decrease in temperature likely caused the sludge to "thicken" and get "sticky," resulting in biogas trapped within the sludge bed and subsequent pistoning events. During larger pilot, or full-scale operation such a sudden drop in temperature would not be advised, rather, gradual temperature decreases would likely circumvent many of these operational issues. Overall, the trial yielded three important findings with respect to process performance: (i) that low-temperature treatment of dairy wastewater is indeed feasible at 15 °C, but that (ii) reactor configuration had a significant effect on performance, with the UASB, surprisingly, outperforming the EGSB, and finally that (iii) protein hydrolysis was likely a rate-limiting step under low-temperature conditions.

3.2. Effect of temperature on methanogenic activity

Activity against all substrates, and at each phase, was significantly lower ($p < 0.0001$) at 15 °C than at 37 °C (Fig. 2). However, throughout the trial the activity against all substrates continued to increase at both temperatures. At both temperatures the biomass from the end of P5 displayed the highest rates of methane generation. Indeed, the increase in activity between the inoculum and P5, against each of the substrates, was highly significant ($p < 0.0001$) at both temperatures – suggesting adaptability of the microbial community to low-temperatures.

3.3. Microbiome diversity and reactor performance

Differences in gene copy numbers from the qPCR were observed between DNA and cDNA, with cDNA yielding higher 16S rRNA gene copy numbers for both archaea and bacteria over each of the phases (Fig. 3A–B). Bacterial gene copy numbers from the DNA did not significantly change, but the archaeal gene copy numbers did fluctuate significantly ($p = 0.0038$) with archaeal numbers significantly higher during P1 than during P2 ($p < 0.05$).

Amplicon sequencing of the 16S rRNA gene on the Illumina MiSeq platform yielded a total of 6,627,947 paired-end reads. Clean-up and removal of singletons left a total of 24,318 OTUs across the 48 samples analysed.

By the end of P1, a rich and well-balanced microbial community had become established (Fig. 3C-D). The sudden decrease in temperature to 15 °C resulted in a significant, and permanent (for the duration of the trial), decrease in diversity, particularly the evenness of the active community – although roughly the same overall number of OTUs were still detected. This indicated that the change in temperature did not completely remove any of the microbial groups, just that at lower temperatures a sub-group of the active community became more dominant, shifting the evenness. The performance, however remained stable throughout P2, regardless of the shift in diversity. A study by Carballa et al. (2011) concluded that in anaerobic digesters, the microbial community with a higher evenness and diversity produced more methane. The decrease in evenness of the active community during P2 meant that the process was then reliant on a few, now dominant guilds – and could be more vulnerable to perturbations if, for example, the population of one of these groups was reduced or lost. Such a loss in functional redundancy has been shown to result in less stable system performance (Briones and Raskin, 2003), a finding which is mirrored in this study considering the decrease system performance following operational changes in P3.

Beta diversity analysis using Bray-Curtis distance metrics and non-metric multidimensional scaling (NMDS) ordination, revealed significant differences in both the total and active community structure for

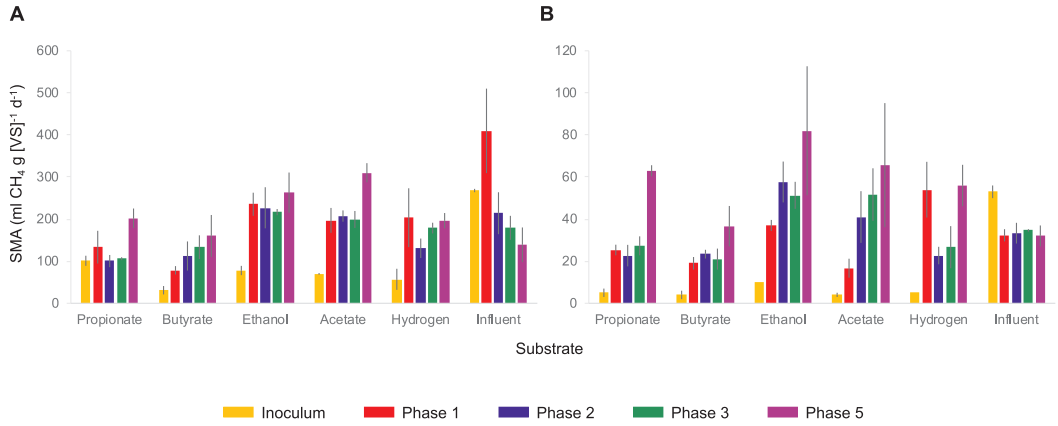

Fig. 2. Average SMA (n = 3) of reactor biomass from the seed inoculum (day 0), and samples taken from P1, P2, P3, and P5, performed at (a) 37 °C and (b) 15 °C against methanogenic substrates: propionate, butyrate, acetate, hydrogen (H_2/CO_2), and the reactor influent.

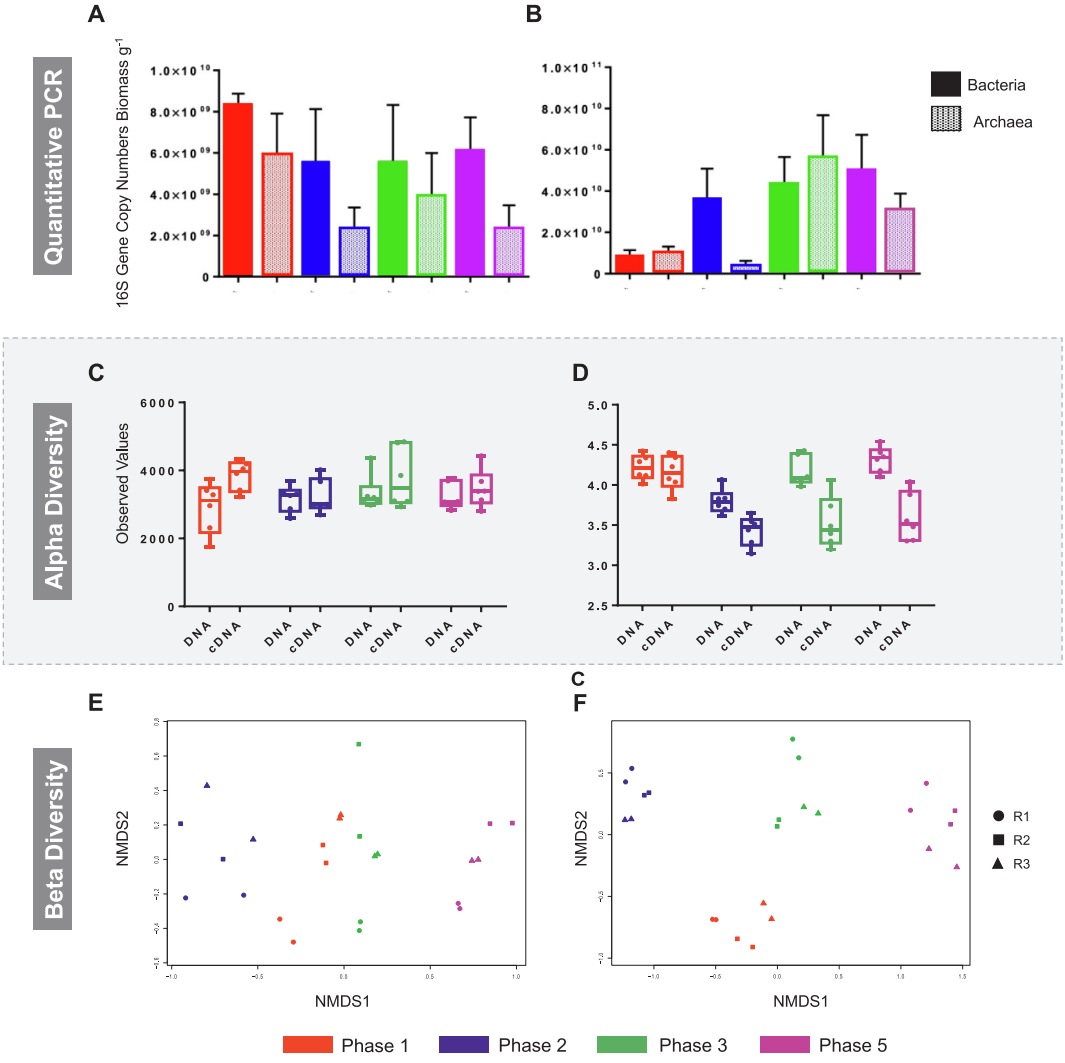

Fig. 3. Average microbial diversity of biomass samples (mean of biological and technical replicates, n = 6) according to variances in the 16S rRNA gene, from the end of P1, P2, P3 and P5 of the reactor trial. *qPCR data*: bar graph of average bacterial and archaeal 16S rRNA gene copy numbers per g of biomass in absolute numbers for (a) DNA and (b) cDNA. *Alpha diversity*: box plots of (c) rarefied species richness and (d) Shannon Entropy of both DNA and cDNA. *Beta diversity*: Non-Metric Multidimensional Scaling (NMDS) using Bray-Curtis dissimilarity of (e) DNA and (f) cDNA.

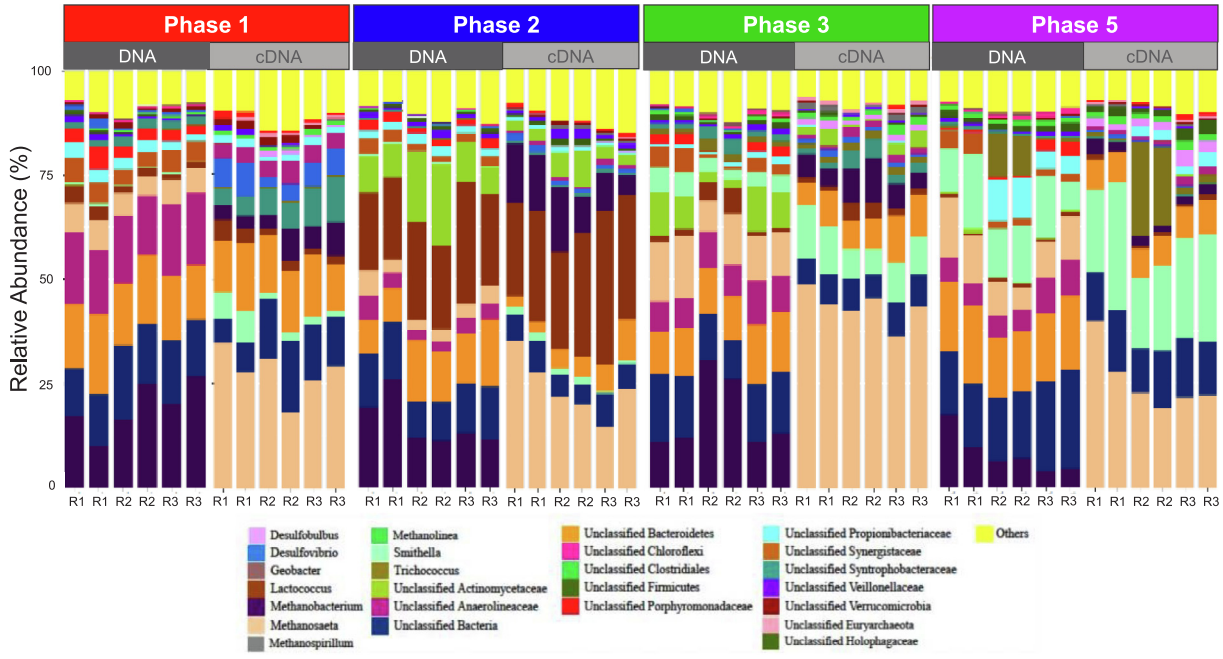

Fig. 4. Stacked bar chart of the total (DNA) and active (cDNA) microbial community structure based on the relative abundance of the top-25 most abundant genera across samples taken at the end of P1, P2, P3, and P5, according to variances in the 16S rRNA gene, where 'others' refers to anything that is not in the top-25.

each phase (Fig. 3E-F). ANOSIM analysis indicated that the phases were significantly different from one another according to DNA ($p = 0.001$) and cDNA ($p = 0.001$).

Shifts in relative abundance of the top 25 genera were evident between the total (DNA) and active (cDNA) communities (Fig. 4). *Methanobacterium*, for example, was relatively high in abundance in the total community, but relative abundance decreased in the active proportion of the community. The opposite was true for *Methanosaeta*, which was highly active, but only represented a relatively small proportion of the total community structure. Such differences between the total and active communities were expected. AD microbiomes are famously functionally redundant, containing many different taxa which can perform the same functions (Campanaro et al., 2019), but not all of the present groups are able to compete under the given conditions. DNA-based analysis will detect dead, senescent, dormant or otherwise inactive cells, while cDNA analysis targets only the community members who are actively producing RNA (De Vrieze et al., 2018).

Differences in community structure were also observed between the phases. Seven discriminants from the DNA analysis and 9 discriminants from the cDNA (Fig. 5) were identified. Notably, P2 saw significant increases in the relative abundance of *Lactococcus* in both the total and active community. Additionally, *Smithella* increased during P3, and continued to increase in relative abundance through P5. Finally, unclassified *Actinomycetaceae* increased in relative abundance during P2 and P3, for both the total and active communities.

As expected, the microbiome was made up of a metabolically diverse group of OTUs spanning the key trophic groups involved in the AD process, which fluctuated in relative abundance depending on reactor operation. Key hydrolysers included members of the phylum *Bacteroidetes*, a cDNA discriminant, which was present throughout, but became relatively less active as the trial progressed – perhaps increasingly pressured by reactor operational conditions. *Bacteroidetes* are common in AD systems and have been linked to hydrolytic and fermentative reactions for a range of organic substrates (Bialek et al., 2014, 2012). Acidogens, responsible for VFA production, were represented by *Actinomycetaceae* and *Trichococcus* – both of which were identified as discriminant taxa. *Actinomycetaceae* were more relatively abundant at low temperatures and may therefore be important for low-temperature acidogenesis. *Trichococcus* are psychrotolerant lactic acid bacteria, which have been previously identified in dairy wastewater treatment systems at 10 °C (Bialek et al., 2013b). The initial reduction in temperature during P2 decreased the relative abundance of active *Trichococcus* initially, but the group recovered during P3 (still at low temperature), suggesting that these bacteria may require a longer adjustment period for low-temperature activity.

Throughout the trial, no VFA were detected in the effluent, suggesting a healthy and active community of acetogens, which utilise VFA to produce acetate, hydrogen and carbon dioxide. Indeed, the SMA assays indicated a high activity of methane production against VFA substrates. A notable and active member of this trophic group, which were active during this trial were *Smithella,* syntrophic propionate degraders (Lueders et al., 2004). They decreased in relative abundance after the initial temperature reduction, but recovered in P3 indicating a degree of psychrotolerance.

Finally, the acetate, hydrogen and carbon dioxide, which are produced by the acetogens are utilised by the methanogenic archaea to produce methane. *Methanosaeta* were the predominant active methanogen throughout the trial. They have long been recognised as an integral part of the AD process (Hulshoff Pol et al., 2004). They are known acetoclastic methanogens and are associated with low acetate concentrations (Schmidt and Ahring, 1996). In the current study acetate concentrations remained at extremely low levels, likely due to the high conversion rates from acetate to methane by *Methanosaeta* at both 37 °C and 15 °C. Overall, an active community was established at low temperatures and managed efficient biochemical conversions at each trophic level.

3.4. The role of Lactococcus

This study potentially identified *Lactococcus* as a key genus during the low-temperature anaerobic treatment of dairy wastewater. *Lactococcus* are lactic acid bacteria that rapidly ferment sugars and proteins to lactic acid (Guillot et al., 2003). *Lactococcus* became the dominant genus in P2, at low temperatures, when the relative

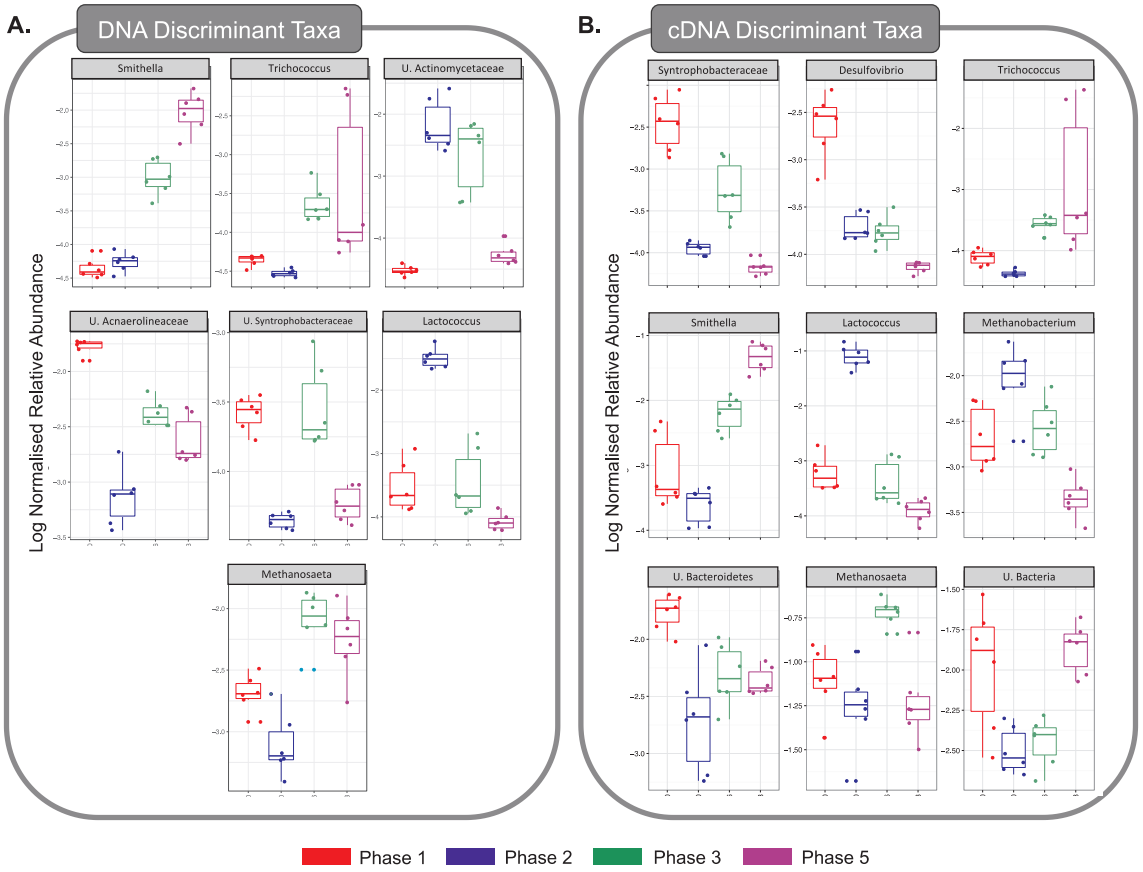

Fig. 5. Box plots of the discriminant taxa across samples (n = 6) from P1, P2, P3 and P5 from (a) DNA, and (b) cDNA, identified using Kruskal-Wallis one-way Analysis of Variance (ANOVA).

abundance of the active community increased from 2.5% to 27.9%, and the system performed efficiently. However, addition of the recirculation system in P3 resulted in a significant decrease in the relative abundance of active *Lactococcus*, down to 2.2% and further to 0.8% by the end of P5. Considering the bacterial cDNA qPCR gene copy numbers, this pattern is similarly reflected in approximate numbers of active *Lactococcus*.

It is notable that the P3 decrease in *Lactococcus* corresponded to a significant reduction in performance of the reactor evidenced by reduced COD removal, methane production and protein degradation – indicating an important role that *Lactococcus* play during low-temperature treatment of dairy wastewater. In addition to fermentation, *Lactococcus* have been shown to hydrolyse casein to free amino acids and peptides – the source of essential amino acids responsible for cell growth in this genus (Smid et al., 1991). The synthetic dairy wastewater in the current study was comprised of 35% protein, of which casein constituted 80% (Vidal et al., 2000). The relatively low level of effluent protein detected during P2 correlates with the high relative abundance of *Lactococcus*. Moreover, the significant reduction in relative abundance of *Lactococcus* during P3 correlated with an increase in effluent protein detected at this time. This may reflect the importance of *Lactococcus* for efficient protein degradation, and stable reactor performance at 15 °C.

Finally, the change in reactor configuration from UASB to EGSB during P3 significantly reduced *Lactococcus* numbers. It is therefore hypothesised that *Lactococcus* attach loosely to the exterior of the anaerobic granule and so, are susceptible to shear flow forces and washout. Additionally, the recirculation and mixing conditions may displace and expel extracellular proteins required to initialise casein hydrolysis. *Lactococcus* numbers did not recover and remained low for the remainder of the trial, which may be due to the continued use of the recirculation system. Interestingly, the addition of the anaerobic filters improved protein degradation and COD removal. This may have provided a location for an attached anaerobic biofilm – away from the disruptive shear forces caused by mixing – which allowed *Lactococcus* to re-establish and repopulate.

4. Conclusions

This study demonstrated that high rate anaerobic treatment of dairy wastewater was feasible at 15 °C. The microbial community changed at low-temperature: the alpha diversity of the active community was reduced, however the reactors continued to perform well. The *Lactococcus* genus was dominant signifying its importance during low-temperature operation. However, they were highly susceptible to the applied reactor configuration and the change from UASB to EGSB caused a significant reduction in Lactococcal numbers. This was accompanied by a significant decrease in reactor performance, with the UASB out-performing the EGSB.

5. Data availability

The sequencing data from this study are available through the NCBI database under the project accession number PRJNA601960 or at the following link: http://www.ncbi.nlm.nih.gov/bioproject/601960.

CRediT authorship contribution statement

Paul G. McAteer: Conceptualization, Data curation, Investigation, Methodology, Writing - original draft. **Anna Christine Trego:** Data curation, Writing - original draft, Writing - review & editing. **Camilla Thorn:** Formal analysis. **Therese Mahony:** Resources, Validation,

Writing - review & editing. **Florence Abram:** Supervision, Writing - review & editing. **Vincent O'Flaherty:** Supervision, Funding acquisition, Project administration, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.123221.

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