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Increased loading stress leads to convergence of microbial communities and high methane yields in adapted anaerobic co-digesters

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ABSTRACT

Enhancing biogas production, while avoiding inhibition of methanogenesis during co-digestion of grease interceptor waste (GIW), can help water resource recovery facilities reduce their carbon footprint. Here we used pre-adapted and non-adapted digesters to link microbial community structure to digester function. Before disturbance, the pre-adapted and non-adapted digesters showed similar methane production and microbial community diversity but dissimilar community composition. When exposed to an identical disturbance, the pre-adapted digester achieved better performance, while the non-adapted digester was inhibited. When re-exposed to disturbance after recovery, communities and performance of both digesters converged, regardless of the temporal variations. Co-digestion of up to 75% GIW added on a volatile solids (VS) basis was achieved, increasing methane yield by 336% from 0.180 to 0.785 L-methane/g-VS-added, the highest methane yield reported to date for lipid-rich waste. Progressive perturbation substantially enriched fatty acid-degrading *Syntrophomonas* from less than 1% to 24.6% of total 16S rRNA gene sequences, acetoclastic *Methanosaeta* from 2.3% to 11.9%, and hydrogenotrophic *Methanospirillum* from less than 1% to 6.6% in the pre-adapted digester. Specific hydrolytic and fermentative populations also increased. These ecological insights demonstrated how progressive perturbation can be strategically used to influence methanogenic microbiomes and improve co-digestion of GIW.

1. Introduction

The anaerobic co-digestion of energy-rich grease interceptor waste (GIW) integrates waste management and resource recovery in a potentially cost-effective way. GIW consists of the entire contents of a grease interceptor (GI), including fat, oil and grease (FOG), food solids and associated wastewater (Wang et al., 2013). A key question in GIW co-digestion is how to substantially increase biogas production, while avoiding overloading with GIW and inhibition of methanogenesis. Major metabolic processes in anaerobic digestion, including hydrolysis, acidogenesis (fermentation), acetogenesis, and methanogenesis, are carried out by different consortia of microbes with distinct ecological roles. When treating high-strength lipid waste, one major challenge is to ensure efficient degradation of intermediates such as long chain fatty acids (LCFAs), acetate, propionate, and butyrate. Excessive substrate input can lead to accumulation of these intermediates and decreases in pH. High levels of LCFA can also lead to inhibition of substrate and product transport, damage to cell membrane, increased lag phase of methane production, loss of methanogenic activity, and sludge flotation and washout (Palatsi et al., 2010; Rinzema et al., 2013).

The co-digestion of sewage sludge with lipid wastes from different sources such as FOG from a receiving facility, a meat processing plant and restaurants has been widely studied. A wide range of organic loading rates (OLRs) of FOG on a volatile solids (VS) basis has been evaluated, ranging from 0.05 (4% FOG (w/w) added) to 7.47 g-VS-FOG/L/day (90% FOG (w/w) added) with methane yields from 0.2 to 0.6 L-methane/g-VS-added (Davidsson et al., 2008; Kabouris et al., 2009; Luostarinen et al., 2009; Martín-González et al., 2011; Noutsopoulos et al., 2013; Wan et al., 2011; Wang et al., 2013). Process failure has been documented at 1.45, 2.5, 4.0 and 7.47 g-VS-FOG/L/day, but some experiments demonstrated operation between 1.3 and 3.0 g-VS-FOG/L/day. No general

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conclusion can be drawn based on empirical limitations of FOG addition, especially at OLRs of FOG higher than 1.3 g-VS-FOG/L/day where the performance profiles differed markedly even at the same FOG loading rate. The limit of FOG co-digestion (threshold of FOG loading rate that can be achieved without process upset) appears to vary depending on the sources of inoculum, base substrate and co-substrate, temperature, solids retention time (SRT) and mixing intensity.

Results from a study of co-digestion between sewage sludge and carbon-rich beverage waste showed that there was an OLR threshold where the function and resilience of the anaerobic microbiomes could be maintained (Nguyen et al., 2018). However, whether the threshold can be further "pushed" to improve performance remains unclear. The microbial community dynamics in digester sludge are largely driven by deterministic factors, such as substrate selection (De Francisci et al., 2015; Wagner et al., 2013), immigration from feedstock (Kirkegaard et al., 2017; Mei et al., 2017), control of inoculum, and operational conditions (OLR and SRT) (Ju et al., 2017; Peces et al., 2018). To link microbial community to improved digester performance, a widely studied approach is feeding strategy, for example, through changes in FOG loading rates (e.g. incremental step increases or a pulse/sudden shock of higher OLR) (Goux et al., 2015; Luostarinen et al., 2009; Wang et al., 2013; Ziels et al., 2016) or feeding frequency (e.g. continuous, daily, or intermittently) (De Vrieze et al., 2013; Nadais et al., 2006; Ziels et al., 2017). Through the use of different feeding strategies, microbial adaptation (Chen et al., 2008; Palatsi et al., 2010), particularly shifts in abundance of syntrophic fatty acid-degrading populations, has been shown to correlate to reduced inhibition and improved community performance in FOG-disturbed environments (Amha et al., 2017; Ziels et al., 2017, 2016). However, results from such environmental surveys are limited to community-function correlations (De Los Reyes et al., 2015; Reed and Martiny, 2007), rather than direct demonstrations of how composition drives function. Only two studies showed improved performance of co-digestion of grease waste (Silvestre et al., 2011) and LCFAs (Kougias et al., 2016) due to the presence of pre-adapted microbial populations in the inoculum. However, both were short-term disturbance analyses (approximately 25 days) and did not examine community dynamics in response to post-failure recovery, or re-exposure at similar or higher intensity.

In this study, we used a pre-adapted digester with a disturbance-to-failure experience and a non-adapted digester with no prior contact to the disturbance and subsequently exposed them to a common overloading disturbance. This approach, similar to a "common garden" experiment (De Los Reyes et al., 2015; Reed and Martiny, 2007), allowed us to more directly test how compositional differences due to past disturbance experience lead to functional differences, compared to environmental treatment studies. Following process failure, post-failure recovery, and re-exposure to a second perturbation, we examined how different perturbation events direct long-term community dynamics in pre-adapted and non-pre-adapted anaerobic co-digesters.

2. Materials and methods

2.1. Digester setup, seeding, environment and performance monitoring

To link the microbial community dynamics to digester performance and environmental conditions, two 8 L lab-scale anaerobic digesters with a working volume of 6 L were set up in parallel. The schematic overview of reactor set-up was described in detail previously (Wang et al., 2013). Mixing was provided using a peristaltic pump that generated a circular mixing pattern. Feeding and decanting were conducted every other day in a draw-and-fill semi-continuous mode. Every other day after biogas analysis, 600 ml of effluent was removed for characterization and an equal amount of feedstock was fed into the digesters to achieve an SRT of 20 days.

Digesters were inoculated with anaerobic digester biosolids from the South Durham Water Reclamation Facility (SDWRF) in North Carolina (NC). The SDWRF digester was operated under mesophilic condition with a working volume of 732,000 gallons and was fed every other day using primary sludge and thickened sludge from a gravity belt thickener. Thickened waste activated sludge (TWAS) from the North Cary Water Reclamation Facility in NC was used as base substrate, and GIW from a restaurant in Cary, NC was used as co-substrate. The GIW is defined as the entire contents of a GI and therefore comprises three primary components: FOG, food particles, and wastewater. These components were collected separately from the GI and stored at 4 °C immediately after collection. Digesters were supplied with a mixture of TWAS and GIW every other day in a temperature-controlled room to maintain mesophilic conditions (37 °C). Detailed description of feedstock characteristics was provided previously (Wang et al., 2013).

Biogas production was recorded daily using a wet tip gas meter (Wet Tip Gas Meter.com, Nashville, TN) and normalized to standard temperature and pressure (273 K and 101,325 Pa) conditions. The methane content in biogas was analyzed daily using a gas chromatograph (GC, SRI 8610C, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a CTR 1 column (Alltech, Deerfield, IL) maintained at 75 °C. Helium was used as the carrier gas at a flow rate of 88 mL/min, and the valve, injector and TCD temperatures were 90, 100 and 100 °C, respectively. The injection volume was 5 ml for each chromatography. The detection limits for methane, carbon dioxide, nitrogen and oxygen were 4.81%, 1.26%, 1.00% and 1.02%, respectively. Effluent sludge was analyzed for total solids (TS), VS, alkalinity, and pH according to Standard Methods (American Public Health Association (APHA), 2012). Chemical oxygen demand (COD) was analyzed using HACH High Range Plus COD vials (HACH, Loveland, CO) according to manufacturer's instructions. Concentration of total volatile fatty acids (TVFAs) up to six carbon atoms was measured following Method 5560 C in Standard Methods. A blank and a reference standard samples were included in each test batch to improve quality control.

2.2. Digester operation during the training phase

To create a pre-adapted digester community to compare to a non-adapted community, the pre-adapted digester was subjected to discrete loading steps from 0% GIW (w/w VS-added) to 46% and 66% then 84%, as described previously (Wang et al., 2013). Process inhibition was observed at 84% GIW (w/w VS-added), after which the pre-adapted digester was allowed to recover at 100% TWAS (0% GIW). Stable biogas production and methane content and returns of effluent pH, alkalinity, and concentrations of VS, COD and TVFAs back to the original ranges were achieved in the pre-adapted digester at the end of the training phase. During the training phase, the non-adapted digester was operated at 100% TWAS (0% GIW). In this study, sludge samples collected during the training phase (Fig. S1) were analyzed to investigate how pre-adaptation influenced the microbial populations and subsequent digester performance during the disturbance phases.

The progressive perturbation approach used for the pre-adapted digester was replicated in a subsequent Experiment II. The same reactor setup was used with a different source of GIW. Progressive perturbations from 0% GIW (w/w VS-added) to 30% and 70% then 90% (Fig. S2) were used to drive shifts in the digester communities.

2.3. DNA extraction, 16S rRNA gene amplicon sequencing, and bioinformatics

A total of 68 composite samples were collected from which genomic DNA components were extracted using an aluminum sulfate DNA extraction method (Staley et al., 2011). Forward and reverse primer pair sequences, 341F and 806R, respectively, were used to amplify a DNA fragment of ~460 bp length flanking the V3 and V4 regions of the 16S rRNA gene of bacteria and archaea (Yu et al., 2005). To improve primer coverage for the Ribosomal Database Project (RDP) database, the base 5 from the 5′ end of the forward primer was modified from C to Y and the bases 8 and 9 from the 5' end of the reverse primer were modified from YV to NN, as described previously (Sundberg et al., 2013). Library preparation, quantification, normalization, and pooling were performed according to the Illumina 16S metagenomics protocol (Illumina, 2013). Library quantity and quality were assessed using the High Sensitivity Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA). Pooled libraries were run on an Illumina MiSeq platform for 300 bp paired-end read sequencing at the Genomic Sciences Laboratory, North Carolina State University, NC. Sequences were deposited to the National Centre for Biotechnology Information Sequence Read Archive (accession number SRP077521).

Amplicon sequence pairs were merged, trimmed to remove primer sequences, and quality filtered using the QIIME pipeline (Caporaso et al., 2010a) and Trimmomatic 0.33 (Bolger et al., 2014). OTU clustering was performed at the ≥97% sequence similarity level using open-reference OTU picking workflows (Edgar, 2010; Rideout et al., 2014). RDP Classifier 2.2 (Wang et al., 2007) was used to assign taxonomy to each cluster representative based on the Greengenes taxonomy and reference database (McDonald et al., 2012; Werner et al., 2012). Sequences were aligned based on the Greengenes core reference alignment (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010b). Chimeric sequences were identified using ChimeraSlayer (Haas et al., 2011) and removed from the alignment to build a phylogenetic tree using FastTree 2.1.3 (Price et al., 2010). Sequences with less than 0.005% of the total number of sequences were further removed from the datasets to reduce possible spurious OTUs, as described previously (Navas-Molina et al., 2013). Quality filtered and chimera-free sequences were rarefied to correct for differences in sampling results (Knight et al., 2018). The compositional differences between microbiomes were assessed by principal coordinate analysis (PCoA) using R phyloseq (McMurdie and Holmes, 2013) applying the Bray-Curtis distance on rarefied datasets (Navas-Molina et al., 2013). To assess alpha diversity, we calculated measures of species richness (observed OTU counts) and combined richness and evenness (Shannon's index) using R phyloseq (McMurdie and Holmes, 2013) on rarefied datasets. Sludge samples from the replicate experiment were processed, sequenced and analyzed with samples from the first experiment to reduce biases from library preparation, sequencing platforms and bioinformatics when comparing individual microbiome studies, as identified previously (Allali et al., 2017).

3. Results

3.1. Digester performance during the disturbance and the 2nd disturbance phases

To investigate the influence of pre-adaptation on subsequent digester performance when exposed to a sudden overloading shock, we spiked the non-adapted and pre-adapted digesters with 66% GIW (w/w VS-added) at an OLR of 2.16 g-VS/L/day (Disturbance phase, Fig. 1). The non-adapted digester responded poorly

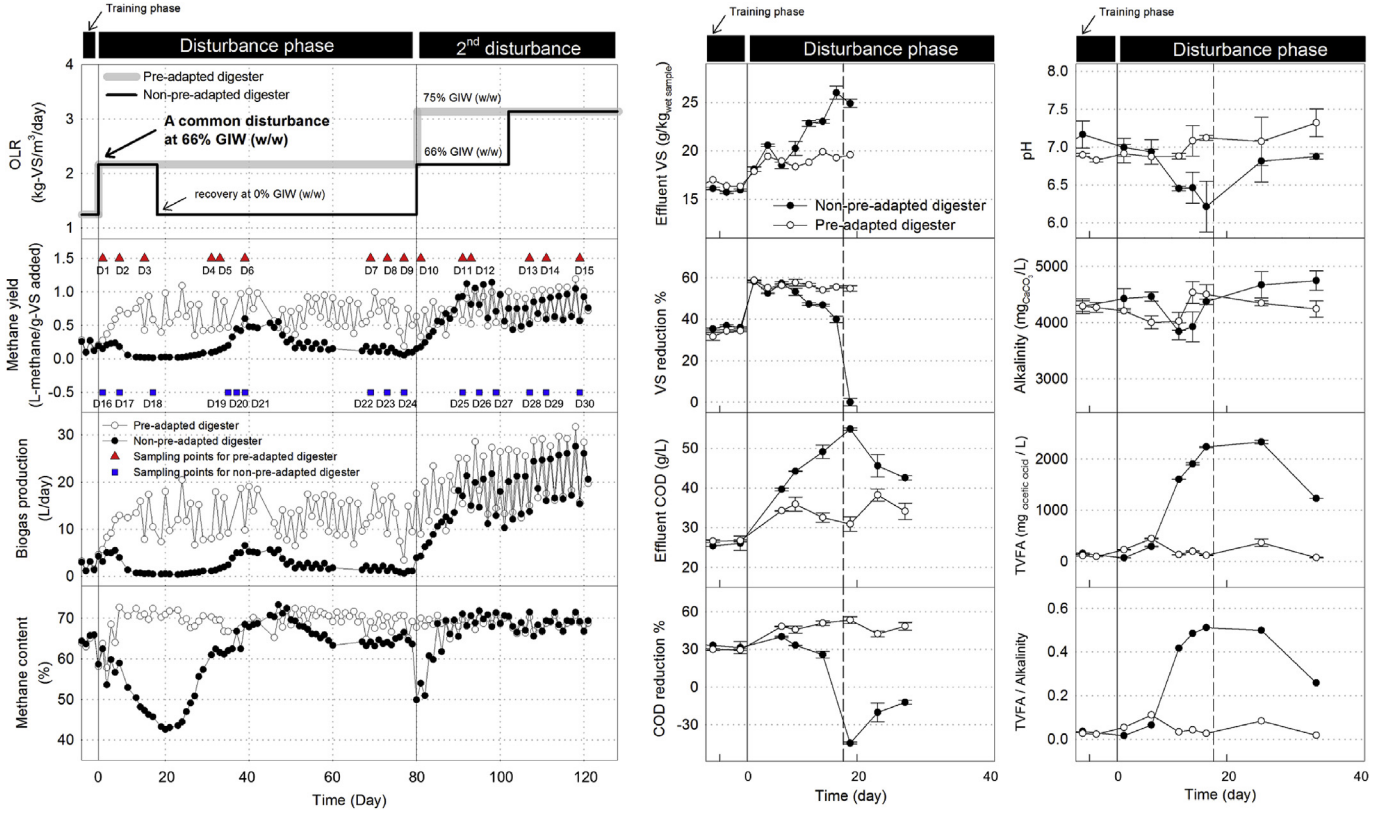

Fig. 1. Organic loading rate (OLR), methane yield, biogas production, methane content, effluent quality data and the collection time of each sample from the pre-adapted and non-pre-adapted digesters during the disturbance and the 2nd disturbance phases.

compared to the pre-adapted digester when faced with GIW perturbation of identical intensity. A severe digester upset was observed shortly after 66% GIW (w/w VS-added) was introduced into the non-adapted digester. Immediate decreases in methane yield, methane content, biogas production, reduction rates of COD and VS, pH, and alkalinity, as well as increases in effluent VS, COD, and concentrations of TVFAs, and TVFA/alkalinity ratio indicated buildup of undigested substrates and inhibition of methanogenesis in the non-adapted digester (Fig. 1). On day 18 the 66% GIW (w/w) feed was discontinued and replaced by 100% TWAS to allow recovery. As the non-adapted digester recovered, the methane yield gradually peaked at 0.599 L-methane/g-VS-added on day 39 (Fig. 1). This increase in methane yield was due to the consumption of accumulated substrates (undigested FOG in GIW). It required approximately three SRTs (60 days) for the non-adapted digester to regain stable biogas production and methane content. On the other hand, the pre-adapted digester stabilized within 10 days when re-stressed at 66% GIW (w/w as VS), producing biogas at 12.6 L/day, methane yield at 0.665 L-methane/g-VS-added, and methane content at 69.6% (Fig. 1).

During the 2nd disturbance phase, we tested the recovery capacity of post-failure anaerobic digesters. Specifically, we examined (1) if the pre-adapted digester can tolerate the 2nd disturbance at higher GIW addition (75% GIW (w/w)), and (2) if the non-adapted digester overloaded at 66% GIW (w/w) during the disturbance phase can survive the same GIW feed this time after recovery. On day 79, the pre-adapted digester was challenged with 75% GIW (w/w), increasing methane yield from 0.665 to 0.785 L-methane/g-VS-added and biogas production from 12.6 to 21.6 L/day (Fig. 1). At the same time, the non-(pre-)adapted digester was spiked with 66% GIW (w/w) which increased methane yield from 0.197 to 0.740 L-methane/g-VS-added and biogas production from 2.3 to 16.5 L/day. The non-pre-adapted digester was further pushed on Day 102 to 75% GIW (w/w), producing 0.783 L-methane/g-VS-added and biogas at 21.4 L/day. Both the pre-adapted and non-pre-adapted digesters achieved similar levels of digester performance and could be further pushed to a higher GIW loading rate after recovery. However, the regular fluctuation of methane production associated with the semi-continuous feeding mode (Wang et al., 2013) was more substantial when re-stressed, suggesting less stable operation in both digesters.

Overall, the implementation of progressive perturbation in the pre-adapted digester, up to 75% GIW (w/w), increased biogas production from 2.2 to 21.6 L/day, methane content from 60.2% to 68.6% and methane yield from 0.180 to 0.785 L-methane/g-VS-added.

3.2. *The training phase: the influence of progressive perturbation on microbial community dynamics and digester performance*

A total of 68 samples were sequenced and processed, generating 5,957,812 quality filtered and chimera-free sequences with an average of 99,296 sequences per sample and a total of 370 operational taxonomic units (OTUs) (97% sequence similarity) identified. The taxonomic distribution showed that the majority of the OTUs remained low in relative abundance (sub-dominant) throughout the experiment (Figs. 2 and 3). Only 15 OTUs (4% of the populations) individually constituted more than 6% of total sequences in at least one sample, but collectively accounted for 40–70% of total sequences in different samples. The 6% cutoff was chosen to improve

Fig. 2. The relative abundances of all archaeal (top) and 15 most prevalent bacterial (bottom) OTUs during the training phase when the pre-adapted digester was subjected to progressive perturbation and the non-pre-adapted digester remained as the control. Samples in the pre-adapted digester were collected (Fig. S1) during GIW additions (w/w VS-added) from 0% (T1 to T3) to 46% (T4 to T9) and 66% (T10 to T18), then at 84% during overloading (T19 to T21), followed by 0% during recovery (T22 to T24). Samples in the non-pre-adapted digester were collected during 0% GIW additions (w/w VS-added) (T25 to T30). Bacterial OTUs with more than 6% relative abundance in at least one sample are shown. Sub-dominant OTUs that individually accounted for less than 6% of total sequences in any sample are grouped into one bar. Bars without numbers represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.

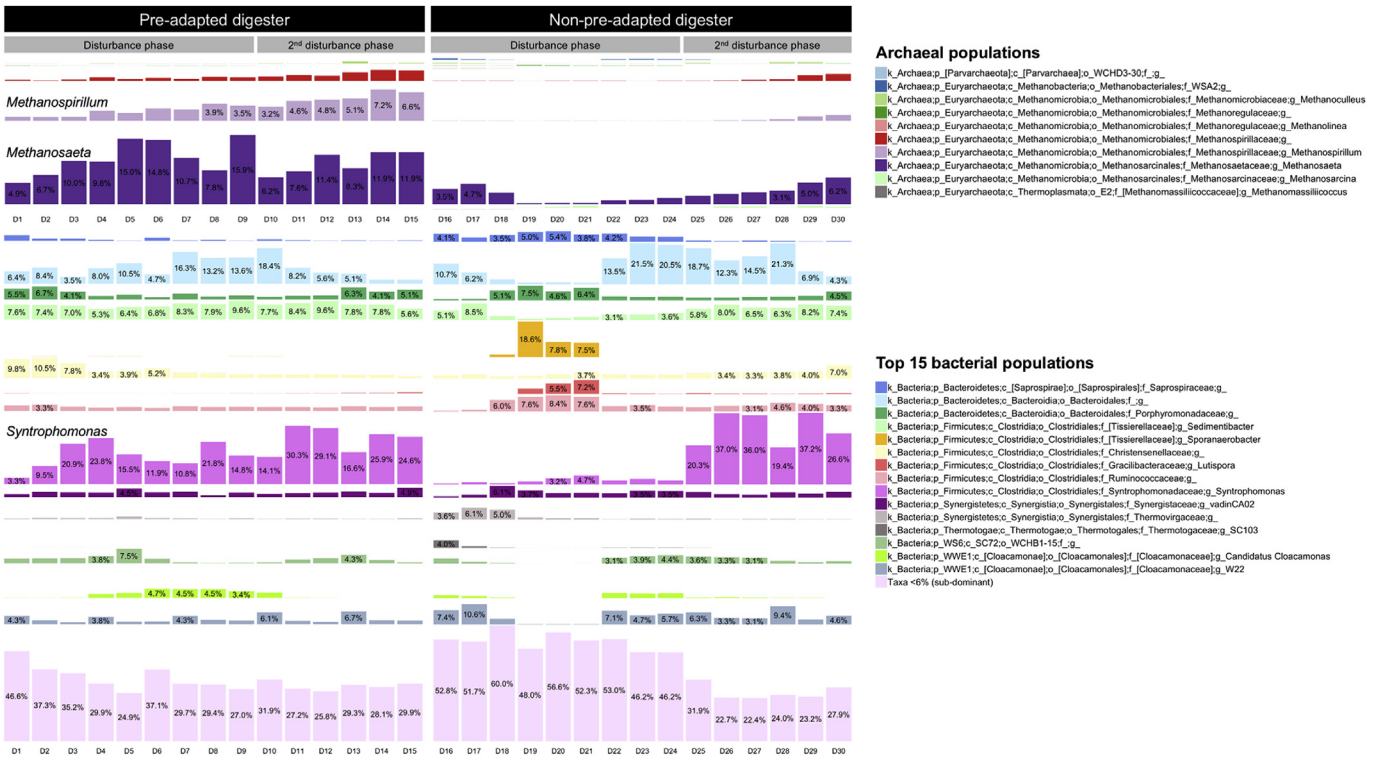

Fig. 3. The relative abundances of all archaeal (top) and 15 most prevalent bacterial (bottom) OTUs during the disturbance and the 2nd disturbance phases. Bacterial OTUs with more than 6% relative abundance in at least one sample are shown. Sub-dominant OTUs that individually accounted for less than 6% of total sequences in any sample are grouped into one bar. Bars without numbers represent less than 3% relative abundance. p_: phylum, c_: class, o_: order, f_: family, g_: genus.

visualization of taxonomic profiles and identification of sub-dominant populations that were low in abundance.

Dynamic changes of microbial community were observed throughout the experiment (Figs. 2–4). During startup (100% TWAS, 0% GIW, not shown on Fig. 1), communities from both digesters were similar (Fig. 4). The most abundant bacterial populations during startup were *Saprospiraceae* and Bacteroidales of the phylum Bacteroidetes, *Thermovirgaceae* of the phylum Synergistetes, SC103 of the phylum Thermotogae, *Candidatus* Cloacamonas and W22 of the phylum WWE1 (Fig. 2). The most abundant archaeal population during startup was acetoclastic *Methanosaeta*. During startup these populations detected appeared to be phylogenetically and functionally diverse and involved in hydrolysis and utilization of carbohydrates, proteins, and VFAs (SI).

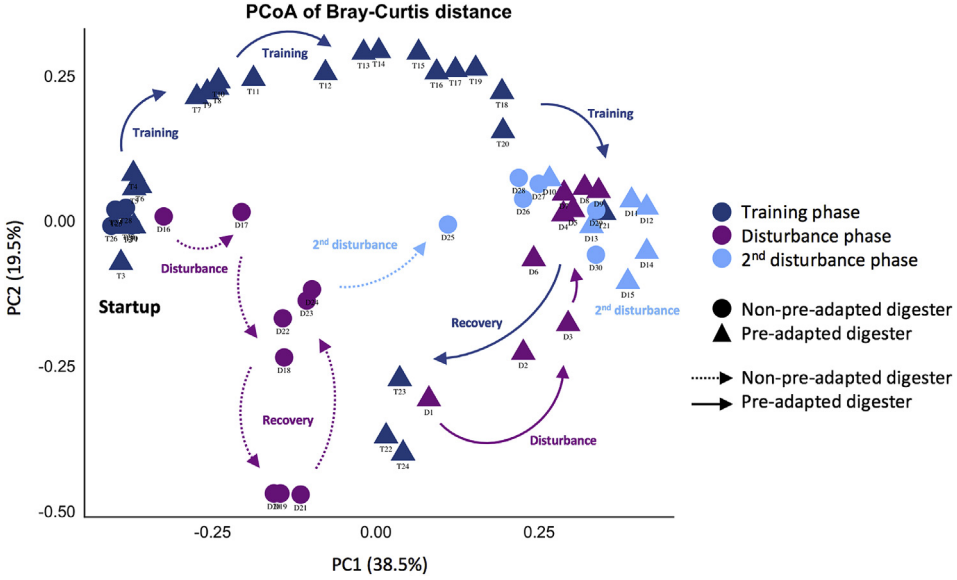

Fig. 4. Microbial community dynamics applying principal coordinate analysis (PCoA) based on Bray-Curtis distance. Datasets were rarefied at 13,000 sequences per sample and reflected both bacterial and archaeal populations during the training, disturbance and the 2nd disturbance phases.

Communities during startup also showed higher community richness (observed species) and evenness (Shannon's index), compared to other communities (Fig. 5). In agreement with this observation, over 50% of the sequences obtained during startup belonged to sub-dominant populations with less than 6% relative abundance (Fig. 2), suggesting the long-tailed existence of low-abundance populations in both digesters (Galand et al., 2009; Lynch and Neufeld, 2015).

During the training phase (before Day 0 on Fig. 1), the relative abundances of the pre-dominant bacterial populations during startup decreased drastically with increasing GIW addition. Since 16S rRNA gene amplicon sequencing measures relative abundances of taxa, the observed succession does not reflect absolute taxon abundance and could be due to the pre-dominant populations becoming relatively less abundant. Progressive perturbation of GIW led to dynamic shifts of methanogenic microbiomes away from the startup community (Fig. 4), decreasing sub-dominant populations (from 57.4% to 27.4%) (Fig. 2) and whole-community diversity (Fig. 5). Studies have suggested that higher community diversity or evenness resulted in more robust function (Werner et al., 2014, 2011; Wittebolle et al., 2009) because it ensures more suites of populations with a wide range of metabolic possibilities. However, in the face of major disturbances that led to dynamic successions of microbial populations, we observed decreasing community richness and evenness with increasing digester performance (Fig. 5). Decreases in diversity associated with enhanced population function were also observed in other studies (Jia et al., 2016; Nguyen et al., 2018).

Progressive perturbation selectively enriched a group of populations over time, including Bacteroidales within Bacteroidetes, *Sedimentibacter*, *Christensenellaceae*, *Ruminococcaceae* and *Syntrophomonas* of the phylum Firmicutes, vadinCA02 of phylum Synergistetes and WCHB1-15 of phylum WS6, while other populations decreased (Fig. 2). The syntrophic relationship between methanogens and Firmicutes family *Syntrophomonadaceae* capable of using fatty acids with carbon chain lengths ranging from C4 to C18 has been well established (McInerney et al., 2009; Sieber et al., 2012). Importantly, *Syntrophomonas* became substantially prevalent and increased in abundance from less than 1% to 21.7% during the training phase (Fig. 2). Acetoclastic methanogenesis became more dominated by *Methanosaeta* (from 2.3% to 18.9% relative abundance), while more versatile *Methanosarcina* remained sub-dominant with less than 0.1% relative abundance. Hydrogenotrophic Methanomicrobiales members *Methanospirillum* increased from <1% to 8.8%, while other hydrogenotrophs decreased in relative abundance. These observations are consistent with a previous study where *Syntrophomonas*, *Methanosaeta* and *Methanospirillum* dominated lab-scale mesophilic digesters treating waste cooking oil (Ziels et al., 2016).

At the end of the training phase (before Day 0 on Fig. 1), the pre-adapted digester recovered from the 84% GIW addition (Wang et al., 2013) and the relative abundances of the most pre-dominant communities, including *Syntrophomonas*, *Methanosaeta* and *Methanospirillum*, decreased drastically. Approximately 45–50% of the community was re-populated by the sub-dominant members (Fig. 2). Interestingly, the populations in the pre-adapted digester were dissimilar from the startup communities (samples T22 to 24, Fig. 4), although the community richness and evenness increased back to the original level observed during startup (Fig. 5). A smaller subset of bacterial populations continued to thrive and became dominant, even after the process failure and the change of feeds during recovery, including *Porphyromonadaceae*, *Sedimentibacter*, *Christensenellaceae* and *Ruminococcaceae*, as well as vadinCA02 of Synergistetes (Fig. 2). At the end of the training phase, *Syntrophomonas* also became slightly more abundant compared to the startup communities. As a result, the community remained dissimilar and did not return to the previous structure during startup (Fig. 4).

3.3. The disturbance phases: the influence of adaptation history on microbial community dynamics and digester performance

During the disturbance phase, both the pre-adapted and non-pre-adapted digesters were subjected to 66% GIW (w/w) addition. The non-pre-adapted digester encountered major process failure (Fig. 1), after which the majority of the community remained populated by sub-dominant populations with less than 6% relative abundance (Fig. 3). However, the microbial structure of the non-pre-adapted digester became dissimilar from the original structure (Fig. 4). During post-failure recovery with 100% TWAS feeds, the microbial community further shifted as the accumulated substrates were consumed. A group of populations emerged during recovery, including *Porphyromonadaceae* (up to 7.5%), *Sporanaerobacter* (up to 18.6%), *Lutispora* (up to 7.2%) and *Ruminococcaceae* (up to 8.4%) (Fig. 3). By the end of the recovery, several pre-dominant organisms during startup, including Bacteroidales and the populations within the phylum WWE1, regained their prevalence. The community richness and evenness also increased back to the original level during startup (Fig. 5). However, the microbial

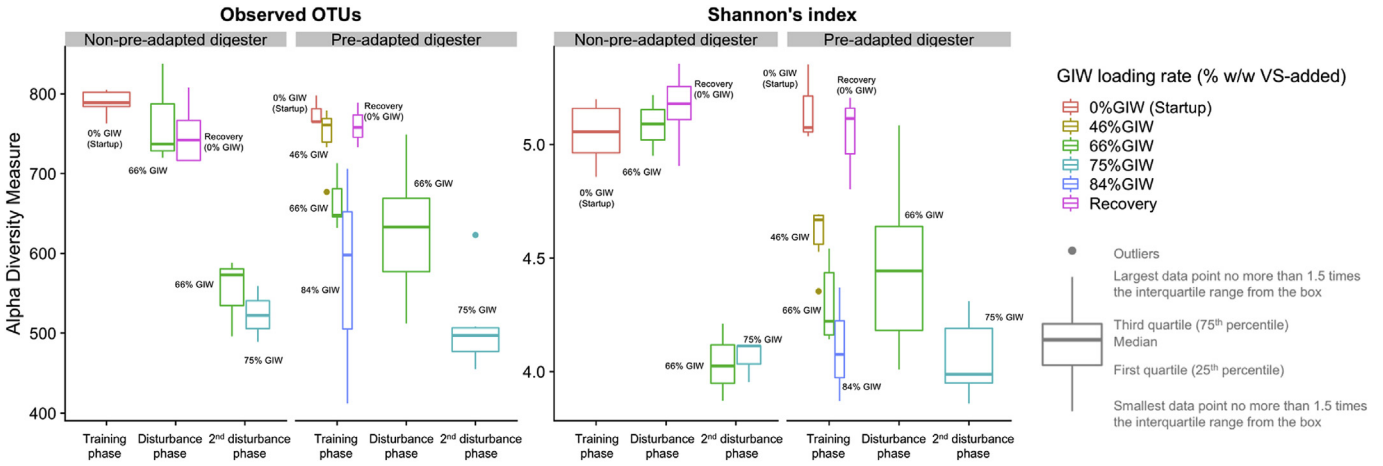

Fig. 5. Microbial community diversity based on observed numbers of OTUs and Shannon's index. Datasets were rarefied at 13,000 sequences per sample and reflected both bacterial and archaeal populations.

community in the non-pre-adapted digester did not return to the original structure during startup (samples D22 to 24, Fig. 4). *Syntrophomonas*, *Porphyromonadaceae*, *Sedimentibacter*, *Christensenellaceae*, *Ruminococcaceae* and vadinCA02 (Synergistetes) became noticeably more abundant after recovery, compared to the startup communities in the non-pre-adapted digester. These populations were the same group of organisms detected in the recovered pre-adapted digester and their increased abundance remained even after 60 days of continuous 100% TWAS feeding by the end of the recovery.

On the other hand, during the disturbance phase the pre-adapted digester showed increased methane production (Fig. 1). Increased GIW addition and prior adaptation history also led to substantial increases of pre-dominant *Syntrophomonas* (from less than 1% to 14.8%), *Methanosaeta* (from 3.5% to 15.9%) and *Methanospirillum* (from less than 1% to 2.9%) (Fig. 3), compared to the non-pre-adapted digester. Continuous prevalence of *Porphyromonadaceae*, *Sedimentibacter*, *Christensenellaceae*, *Ruminococcaceae* and vadinCA02 was also observed. The pre-adapted digester was able to rebound (Fig. 4), generating similar amounts of methane as observed during the training phase despite more fluctuations (Fig. 1). As anticipated, the disturbance also led to decreases of community richness and evenness (Fig. 5) and total sub-dominant populations (from 50.2% to 27.0%) (Figs. 2 and 3).

During the 2nd disturbance, the non-pre-adapted digester showed enhanced digester performance (Fig. 1). As the community diversity decreased (Fig. 5), relative abundances of *Syntrophomonas*, *Porphyromonadaceae*, *Sedimentibacter*, *Christensenellaceae*, *Ruminococcaceae* and vadinCA02 (Synergistetes) continued to increase with increasing methane production (Fig. 3). Both the pre-adapted and non-pre-adapted digesters were able to produce similar amounts of methane at 66% GIW (w/w) and be further pushed to 75% GIW (w/w) (Fig. 1). The microbial community structure in both digesters converged (Fig. 4), and *Syntrophomonas*, *Methanosaeta* and *Methanospirillum* were dominant, along with other populations such as *Porphyromonadaceae*, *Sedimentibacter*, *Christensenellaceae*, *Ruminococcaceae*, and vadinCA02 that showed higher relative abundance compared to other populations (Fig. 3). As anticipated, sub-dominant populations decreased to as low as 22.4% total relative abundance when re-stressed at 75% GIW (w/w) when the lowest community richness and evenness were observed, compared to communities during other phases (Fig. 5).

3.4. Progressive perturbations led to similar microbial community shifts and increased methane production in experiment II

The implementation of progressive perturbation during Experiment II, up to 70% GIW (w/w), increased biogas production from 2.0 to 18.6 L/day, methane content from 57.9% to 66.8% and methane yield from 0.141 to 0.704 L-methane/g-VS-added (Fig. S2). These results were in good agreement with the performance at 75% GIW (w/w) in Experiment I. A total of 8 sludge samples were sequenced and analyzed, generating 562,878 quality filtered and chimera-free sequences with an average of 70,359 sequences per sample. A consistent trend of selective enrichments in the archaeal and bacterial populations was observed. With increasing GIW loading rates, *Syntrophomonas*, *Methanosaeta* and *Methanospirillum* thrived, while other populations subsided (Fig. S3). After overloading (sample R5), a smaller subset of bacterial populations continued to increase and became dominant, including *Porphyromonadaceae*, *Sedimentibacter*, *Christensenellaceae* and *Ruminococcaceae*, as well as vadinCA02 of Synergistetes (Fig. S3). An additional fermenter *Prevotella* was also identified (Purushe et al., 2010; Strobel, 1992). Similarly, increasing GIW disturbance levels correlated with the community dynamics (Fig. S4). After overloading, microbial populations of the replicate digester (Experiment II) converged with other communities in recovery (Experiment I). In Experiment II, decreases in diversity were also associated with increasing perturbation and digester performance (Fig. S5). Results from Experiment II supported and were in agreement with the findings in Experiment I.

4. Discussion

4.1. The link between community composition and digester function

During startup, digester sludge was dominated by phyla Bacteroidetes, Synergistetes, Thermotogae and WWE1, whereas Firmicutes remained sub-dominant. Before large proportions of GIW were introduced, carbohydrates and proteins from the base substrate TWAS could be utilized by *Saprospiraceae* and other Bacteroidales populations (Rosenberg et al., 2014), amino acids by Synergistetes *Thermovirgaceae* (Göker et al., 2012; Vartoukian et al., 2007), fatty acids by *Syntrophomonas* and perhaps unconventional syntrophic acetate degraders Thermotogae (also fermenters of carbohydrates) (Lykidis et al., 2011; Nobu et al., 2015; Sieber et al., 2012). More versatile WWE1 populations could perform cellulose hydrolysis of substrates (Limam et al., 2014), syntrophic metabolism of amino acid and oxidative degradation of propionate (Pelletier et al., 2008; Sieber et al., 2012).

After pre-adaptation through progressive perturbation, a specialized microbial community displaced the original community in the digester sludge that showed improved performance. In addition to known *Syntrophomonas* as fatty acids degraders, several key populations were selectively enriched in the high-yield digesters including *Porphyromonadaceae*, *Sedimentibacter*, *Christensenellaceae*, *Ruminococcaceae* and vadinCA02 (Synergistetes). Their prevalence was observed consistently when (1) the pre-adapted digester recovered during the training phase, (2) the non-pre-adapted digester recovered during the disturbance phase, (3) the pre-adapted digester showed enhanced performance during the disturbance phase and (4) the non-pre-adapted digester showed enhanced performance during the 2nd disturbance phase. These selection-driven shifts could be linked to enhanced digester performance. During co-digestion of GIW, monosaccharides could be utilized by saccharolytic *Christensenellaceae* and versatile *Porphyromonadaceae*. Family *Christensenellaceae* of the phylum Firmicutes has been identified to be strictly anaerobic and saccharolytic with acetic acid and a small amount of butyric acid as the end products of fermentation (Morotomi et al., 2011). *Porphyromonadaceae* members of the phylum Bacteroidetes can utilize a wide range of substrates such as glucose, fructose, glycogen, soluble starch and gelatin. Some species are asaccharolytic and utilization of a few substrates such as starch and gelatin are not detected in some strains. Fermentation products include butyric, propionic, acetic, succinic acids, hydrogen and carbon dioxide (Rosenberg et al., 2014). In lab-scale digesters of chemically enhanced primary treatment sludge, Bacteroidales members were identified as stronger competitors for carbohydrates than Cloacamonales (WWE1) and Saprospirales populations (Ju et al., 2017). In our digester sludge, Bacteroidales members *Porphyromonadaceae* outcompeted Saprospirales populations *Saprospiraceae* after GIW disturbance.

The predominance of Bacteroidetes, Synergistetes and WWE1 during startup was replaced by members of Firmicutes after GIW disturbance, because of the substantial growth of *Christensenellaceae*, *Ruminococcaceae*, *Sedimentibacter* and *Syntrophomonas*. Since GIW is rich in FOG and food solids, the addition of polysaccharides (e.g., starch and cellulose from fruits and vegetables) likely selected for *Ruminococcaceae* and some Bacteroidales members,

proteinaceous substrates (e.g. from meats) and associated amino acids for *Sedimentibacter* and vadinCA02 (Synergistetes), as well as fatty acid-rich materials (e.g. FOG from cooking oil and meats) for *Syntrophomonas*. *Ruminococcaceae* has been studied in diverse gut communities and identified as polysaccharide degraders (Abell et al., 2008; Ding et al., 2001; Flint et al., 2008; Ze et al., 2012). In digester sludge disturbed with alpha-cellulose, members of the order Bacteroidales and genus *Ruminococcus* had a significant correlation to higher concentrations of volatile fatty acids (VFAs) and were likely the main cellulose degraders during the initial hydrolysis (Vanwonterghem et al., 2014). *Sedimentibacter* is capable of utilizing amino acid and pyruvate, producing acetate and butyrate as the main fermentation products, and propionate, lactate and traces of isobutyrate and isovalerate as the minor products (Breitenstein et al., 2002). Within Synergistetes, vadinCA02 became dominant after GIW disturbance, while predominant *Thermovirgaceae* subsided. Synergistetes can be found in a wide variety of habitats such as anaerobic digestion sludge and wastewater, particularly when high levels of amino acids are present (Göker et al., 2012; Rosenberg et al., 2014; Vartoukian et al., 2007). Synergistetes members are mostly asaccharolytic and can utilize amino acids, peptides, and proteins. Selective members can grow in syntrophic interactions with hydrogenotrophic methanogens. Additionally, acetate utilization by *Synergistes* group 4 affiliated with Synergistetes has been identified in anaerobic digester sludge, and may compete with acetoclastic *Methanosaeta* at high acetate concentrations (Ito et al., 2011). Subsequently, as more VFAs and other intermediates were produced, syntrophic oxidizers such as *Syntrophomonas*, acetoclastic *Methanosaeta* and hydrogenotrophic *Methanospirillum* increased in abundance to further convert these substrates to acetate, and/or hydrogen and carbon dioxide and finally methane and carbon dioxide.

Overall, the implementation of progressive perturbation and pre-adaptation directed community dynamics and led to a specific selection of microorganisms. The increased methane yield of the pre-adapted community (from 0.180 to 0.785 L-methane/g-VS-added) is the highest value reported to date, compared to other studies at closely identical OLRs of FOG-based materials (Amha et al., 2017; Davidsson et al., 2008; Kabouris et al., 2009; Luostarinen et al., 2009; Noutsopoulos et al., 2013; Silvestre et al., 2011; Wan et al., 2011; Ziels et al., 2016).

4.2. Microbial populations from the feedstock

From an ecological perspective, the successions from *Saprospiraceae*, *Thermovirgaceae*, Thermotogae and WWE1 during startup to *Porphyromonadaceae*, *Sedimentibacter*, *Christensenellaceae*, *Syntrophomonas*, *Ruminococcaceae* and vadinCA02 after GIW perturbation could be due to niche preferences of these populations when switched to a high-fat, food solids-rich environment. However, although progressive perturbation of GIW led to proliferation of these populations, it is unclear whether this was due to selective growth in the startup assemblage, or populations originated from the feedstock ('immigration'), or both. Additional studies are necessary to evaluate the impact of feedstock-originated populations on post-disturbance digester microbiomes. For example, pre-defined criteria have been used to identify a target community (e.g., residue or "non-growing" populations) and study their influence on digester microbiomes (Mei et al., 2017). Alternatively, the change in abundance of all influent populations could be calculated to profile their distribution and tendency to die off, survive, or grow after being introduced (Kirkegaard et al., 2017). Pre-exposing inocula to increasing disturbance of (non-sterile) sewage sludge and grease waste has been shown to reduce initial lag phase and increase acetoclastic methanogenic and synthrophic acetogenic activities, compared to the original community (Silvestre et al., 2011). A previous metagenomic study using synthetic LCFAs (Na-Oleate) and cattle manure (sterilized using autoclave) also showed that the presence of specialized consortia in the LCFA-acclimated inoculum led to improved process efficiency, compared to the non-acclimated inoculum (Kougias et al., 2016). Since waste materials are most likely not pre-sterilized before co-digestion, regardless of the variations in ascertaining the effect of feedstock-originated populations, the common finding from the current and previous studies demonstrates the feasibility of enriching a group of specialized microbial populations through adaptation to achieve better co-digestion of lipid-rich substrates.

5. Conclusions

In this study, we evaluated the long-term microbial community dynamics in lab-scale methanogenic digesters challenged by a series of disturbance-to-failure experiments. Pre-adaptation through progressive perturbation resulted in a specialized microbial community in digester sludge that showed improved performance and tolerance to high GIW loading rates. Fermenters *Porphyromonadaceae*, *Christensenellaceae*, *Ruminococcaceae*, *Sedimentibacter*, and vadinCA02 (Synergistetes), along with fatty acid-degrading *Syntrophomonas*, acetoclastic *Methanosaeta* and hydrogenotrophic *Methanospirillum* became dominant after adaptation and post-disturbance recovery, suggesting their niche preferences under a high-fat, food waste-rich environment. At high GIW loadings, well-adapted communities converged and became less diverse and less even, but more specialized with increased methane yield. This convergence occurred regardless of stress history - reactors that were pre-adapted or that have recovered after previous failure had communities that converged once well-adapted to high GIW addition. Implementation of progressive perturbation, up to 75% GIW (w/w) addition, achieved the highest methane yield reported to date for co-digestion of GIW, increasing methane yield by 336% from 0.180 to 0.785 L-methane/g-VS-added.

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.watres.2019.115155.

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