Water Research xxx (2015) 1–9

Contents lists available at ScienceDirect

Water Research

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

Comparing the inhibitory thresholds of dairy manure co-digesters after prolonged acclimation periods: Part 2 – correlations between microbiomes and environment

Leticia Regueiro [a, b, 1], Catherine M. Spirito [a, 1], Joseph G. Usack [a], Denina Hospodsky [a], Jeffrey J. Werner [c], Largus T. Angenent [a, *]

[a] Department of Biological and Environmental Engineering, Cornell University, Ithaca, NY 14853, USA
[b] Department of Chemical Engineering, School of Engineering, University of Santiago de Compostela, Rúa Lope Gómez de Marzoa s/n, E-15782 Santiago de Compostela, Spain
[c] Chemistry Department, SUNY-Cortland, Cortland, NY 13045, USA

ARTICLE INFO

Article history:
Received 31 January 2015
Received in revised form
9 May 2015
Accepted 23 May 2015
Available online xxx

Keywords:
Bacteroidetes
Clostridia
Co-digestion
Ammonia
Volatile fatty acids

ABSTRACT

Here, we studied the microbiome succession and time-scale variability of four mesophilic anaerobic reactors in a co-digestion study with the objective to find links between changing environmental conditions and the microbiome composition. The changing environmental conditions were ensured by gradual increases in loading rates and mixing ratios of three co-substrates with a constant manure-feeding scheme during an operating period longer than 900 days. Each co-substrate (*i.e.*, alkaline hydrolysate, food waste, and glycerol) was co-digested separately. High throughput 16S rRNA gene sequencing was used to examine the microbiome succession. The alkaline hydrolysate reactor microbiome shifted and adapted to high concentrations of free ammonia, total volatile fatty acids, and potassium to maintain its function. The addition of food waste and glycerol as co-substrates also led to microbiome changes, but to a lesser extent, especially in the case of the glycerol reactor microbiome. The divergence of the food waste reactor microbiome was primarily linked to increasing free ammonia levels in the reactor; though, these levels remained below previously reported inhibitory levels for acclimated biomass. The glycerol reactor microbiome succession included an increase in Syntrophomonadaceae family members, which have previously been linked to long-chain fatty acid degradation. The glycerol reactor exhibited rapid failure and limited adaptation at the end of the study.

1. Introduction

The application of anaerobic co-digestion technology for the treatment of organic wastes has increased in recent years. Benefits of co-digestion over mono-substrate anaerobic digestion include a better nutrient balance, a shortened payback period due to a more efficient use of the reactor volume, and a reduction of the negative effects of toxic compounds (Mata-Álvarez et al., 2000). Several co-digestion studies have noted distinct differences in the methane yields and other environmental parameters based on the type of substrate fed (Cirne et al., 2007; Weiland, 2010).

Studies have also found that the microbiota composition in anaerobic reactors is greatly influenced by the composition of the input substrates (Regueiro et al., 2014; Zhang et al., 2014). Sundberg et al. (2013) found differences in the microbial communities between full-scale mono-substrate reactors treating sewage sludge *vs.* co-digestion of wastes from a combination of sources. The latter study only used a single sampling point for each reactor. The emergence of next-generation sequencing technologies has enabled researchers to carry out large-scale sequencing surveys in a cost-effective way (Caporaso et al., 2012). As a result, researchers

Abbreviations: ANOVA, Analysis of Variance; COD, chemical oxygen demand; HRT, hydraulic retention time; LCFA, long chain fatty acids; OLR, organic loading rate; OTU, operational taxonomic unit; QIIME, Quantitative Insights into Microbial Ecology; PCoA, principal coordinate analysis; db-RDA, constrained distance-based redundancy analysis; TKN, Total Kjeldahl Nitrogen; VFA, volatile fatty acids; VIF, Variance Inflation Factor; AD, anaerobic digester; CT, co-digestion treatment; AH, alkaline hydrolysate; FW, food waste; GY, crude glycerol; MN, manure.

* Corresponding author. Biological and Environmental Engineering, 226 Riley Robb Hall, Ithaca, NY 14853-5701, USA.
E-mail address: la249@cornell.edu (L.T. Angenent).

[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.watres.2015.05.046
0043-1354/

have begun to utilize these technologies to perform time-series studies of multiple anaerobic reactors (Vanwonterghem et al., 2014; Werner et al., 2011a, 2011b) to monitor microbiota changes during the operating period.

Fernández et al. (1999) suggested that microbial communities change even during stable operating periods when no apparent changes in the operating conditions are made. In addition, changes in operating and environmental parameters besides the organic composition have been shown to substantially influence the microbial communities present in a reactor (Regueiro et al., 2014; Town et al., 2014). By using statistical methods, Werner et al. (2011a, b) found that differences in the bacterial community composition of full-scale anaerobic reactors were correlated with operating conditions (*i.e.*, reactor temperature and feed-to-biomass rates) and function (*i.e.*, substrate removal efficiency and methanogenic activity). Importantly, finding this correlation was only possible because of a time-series study with multiple reactors, resulting in a sufficient amount of environmental metadata in parallel to an in-depth microbiome characterization by DNA sequencing.

Here, our main objective was to examine how the reactor microbiomes changed in response to co-substrate addition, especially when the addition of the co-substrate led to potentially inhibitory environmental conditions in the reactor. The controlled conditions that are necessary to achieve this objective were ensured by gradual increases in loading rates and mixing ratios of three co-substrates in separate reactors and a control reactor with only the base substrate (dairy manure). We tested three co-substrates: alkaline hydrolysate, food waste, and crude glycerol with a constant manure-feeding scheme during an operating period exceeding 900 days at mesophilic conditions. 180 biomass samples from the time series were sequenced *via* Illumina MiSeq paired-end sequencing of the 16S rRNA gene to investigate changes in the reactor microbiome composition. Constrained ordination was used to identify which of the operating and environmental parameters significantly correlated with microbial community succession. Specific indicator populations were linked to individual reactors *via* machine learning.

2. Materials and methods

2.1. Lab-scale anaerobic reactor operation

Four mesophilic continuously-stirred anaerobic reactors with a working volume of 4.5 L and a constant hydraulic retention time (HRT) of 25 days were operated for more than 900 days. One reactor was fed only dairy manure (MN) and was used as a control reactor (manure only), while the remaining three reactors received alkaline hydrolysate (AH), food waste (FW), or crude glycerol (GY) as co-substrates. All manure and co-substrates batches were homogenized and stored at −23 °C until use. The reactor biomass was conditioned to operate at an initial target organic loading rate (OLR) of 2.0 g VS L·Day^{-1} and a hydraulic retention time (HRT) of 25 days. Reactors were inoculated with anaerobic digester (AD) effluent from a local dairy farm and fed the same manure semi-continuously (*i.e.*, once every two days). Following start-up (Days 1–295), steady state conditions (SS) were established over three HRT periods (Days 296–370, SS). On Day 371, the co-digestion experiment began with seven co-digestion treatment (CT) periods based on gradually increasing the mixing ratios of co-substrate (CT-I – CT-VII). Alkaline hydrolysate, food waste, and crude glycerol were incrementally added to the manure-fed base operating conditions such that the OLR was increased by 10% (VS basis) for each increment, while maintaining a 25-day HRT. Three batches of manure (Batch #1: Days 145–300, Batch #2: Days 300–697, and Batch #3: Days 697–932), two batches of alkaline hydrolysate (Batch #1: Days 371–603 and Batch #2: Days 603–865), one batch of food waste, and one batch of crude glycerol were used in this study. Reactors treating hydrolysate and glycerol were terminated on Days 865 and 895 of the operating period, respectively. The average values for the environmental parameters monitored in the reactors are reported with the associated 95% confidence interval error.

2.2. Sampling of anaerobic reactors

We selected 180 samples from the four reactors during the entire operating period for DNA extraction. The samples were selected at least once per HRT per reactor, with additional samples selected during times of system disturbance. Detailed information of the 180 selected samples is displayed in Fig. S1 along with the OLR applied in each reactor. After collection, samples were centrifuged, and approximately 3 g of the biomass was stored at −80 °C until further processing.

2.3. DNA extraction, PCR, and sequencing

Genomic DNA was extracted from the 180 reactor biomass samples using the PowerSoil®-htp 96 Well Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the protocol of the manufacturer. The extracted DNA was PCR amplified using 515-forward primers and 806-reverse Golay barcoded primers targeting the V4 region of the 16S rRNA gene according to the protocols of Gilbert et al. (2010). Duplicate 50 μL PCR reactions were performed using: 0.5 μL of 10 μM 515f forward primer, 0.5 μL of 10 μM 806r barcoded reverse primer, 20 μL of 5PRIME Hot Master Mix (5 Prime Hot Master Mix, 5 Prime, Fisher Scientific, USA), 28 μL of molecular grade H_2O, and 1 μL of DNA template. The PCR cycle conditions consisted of 94 °C for 3 min for initial denaturation; 25 cycles of denaturation at 94 °C for 45 s followed by annealing at 50 °C for 60 s, and elongation at 72 °C for 90 s; and finally elongation for 10 min at 72 °C. The duplicate PCR products were then pooled and confirmed by gel electrophoresis. The PCR products were cleaned using the MagBind E-Z pure cleaning kit (Omega Bio-tek Inc., Norcross, GA, USA). The DNA concentration of the amplicons was quantified using Invitrogen Quant-It Pico Green DNA quantification kit (Life technologies, USA), and samples were subsequently pooled at equimolar ratios (approximately 100 ng of each sample). Libraries were sent for paired-end sequencing (2 × 250 bp) on the MiSeq platform (Illumina, San Diego, CA, USA) at the Cornell University Biotechnology Resource Center (Ithaca, NY, USA). Sequences were submitted to the EBI database under the following accession number ERP010564. Sequences and study metadata are also publically available in QIITA, which is an open-source microbiome storage and analysis resource, under the study number 10137.

2.4. Amplicon sequence analysis

Computational analysis of the reactor sequencing reads was performed using the Quantitative Insights into Microbial Ecology (QIIME v1.7) platform (Caporaso et al., 2010). Paired-end reads were joined using the fastq-join program created by Aronesty (2011) with the default options. Quality filtering and demultiplexing was performed (*via* the split_libraries_fastq.py script in QIIME; default values were used with the exception that the minimum acceptable Phred quality score was set to 25). In total, for the 180 samples extracted we obtained 11,213,454 sequences after filtering out low quality sequences (*i.e.*, approximately 14% of the total number of sequences). The number of assigned reads per sample ranged between 12,154 and 135,747 with an average of 52,144 reads

Please cite this article in press as: Regueiro, L., et al., Comparing the inhibitory thresholds of dairy manure co-digesters after prolonged acclimation periods: Part 2 – correlations between microbiomes and environment, Water Research (2015), http://dx.doi.org/10.1016/j.watres.2015.05.046