Enhanced Anaerobic Digestion Performance via Combined Solids- and Leachate-based Hydrolysis Reactor Inoculation

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Abbreviations: AD¹, DWWTP², OFMSW³, OTU⁴, MDS⁵, TAN⁶, T-RFLP⁷, T-RFs⁸, OLR⁹

¹ Anaerobic digestion

² Drake Municipal Wastewater Treatment Plant

³ Organic fraction municipal solid waste

⁴ Operational taxonomic unit

⁵ Non-metric multi-dimensional scaling

⁶ Total ammonia nitrogen

⁷ Terminal restriction fragment length polymorphism

⁸ Terminal restriction fragments

⁹ Organic loading rate

ABSTRACT

Suboptimal conditions in anaerobic digesters (e.g., presence of common inhibitors ammonia and salinity) limit waste hydrolysis and lead to unstable performance and process failures. Application of inhibitor-tolerant inocula improves hydrolysis, but approaches are needed to establish and maintain these desired waste-hydrolyzing bacteria in high-solids reactors. Herein, performance was compared for leach bed reactors (LBRs) seeded with unacclimated or acclimated inoculum (0-60% by mass) at start-up and over long-term operation. High quantities of inoculum (~60%) increase waste hydrolysis and are beneficial at start-up or when inhibitors are increasing. After start-up (~112 days) with high inoculum quantities, leachate recirculation leads to accumulation of inhibitor-tolerant hydrolyzing bacteria in leachate. During long-term operation, low inoculum quantities (~10%) effectively increase waste hydrolysis relative to without solids-derived inoculum. Molecular analyses indicated that combining digested solids with leachate-based inoculum doubles quantities of Bacteria contacting waste over a batch and supplies additional desirable phylotypes *Bacteriodes* and *Clostridia*.

KEYWORDS

Anaerobic digestion, ammonia, multi-stage, salinity, leachate, inoculation, acclimation

1. Introduction

Anaerobic digestion (AD) is an environmentally attractive technology for conversion of various wastes to energy. However, despite numerous benefits, AD applications to the organic fraction of municipal solid waste (OFMSW) remain limited in North America due to economic barriers with existing technologies. To achieve economic viability, improved reactor performance is needed to increase bioenergy generation. One challenge to optimizing bioenergy generation is the presence of microbial inhibitors that can lead to suboptimal performance or even process failures. Food waste is highly digestible, and 83-91 VS% destruction typically can be achieved if conditions are optimal (Nagao et al., 2012; Yi et al., 2014; Zhang et al., 2007). However, elevated levels of inhibitors, including ammonia and salinity, are often found in AD systems that recycle leachate (Chen et al., 2008; Kayhanian, 1994; Shahriari et al., 2012). Leachate recycle leads to build up of these inhibitors, which are present in feedstocks including food waste and manure. Inhibition of AD performance due to elevated ammonia and salinity has been demonstrated to occur at concentrations >1.7 g total ammonia nitrogen (TAN)/L and 3.5 g Na^+ /L, respectively (Chen et al., 2008). Leachate recycle may be employed to retain methanogens in single-stage AD configurations and to reduce water usage in both single- and multi-stage systems. Thus, strategies are needed to maintain high energy generation even when inhibitors are present.

Multi-stage AD technologies are advantageous because these systems use separate reactors for each stage, allowing for individual optimization of hydrolysis and methanogenesis processes (Voelklein et al., 2016). For OFMSW, hydrolysis is preferably conducted in leach bed reactors (LBRs) operated in dry batch mode because LBRs can accept high solids waste ($>15\%$ solids) (Mata-Alvarez et al., 2000). Methanogenesis is conducted in high-rate reactors including fixed film (FF) or upflow anaerobic sludge blanket (UASB) reactors (Najafpour et al., 2006).

Numerous studies have focused on improving methanogenesis under inhibitory conditions (Chen et al., 2008), but studies focused on enhancing hydrolysis in the presence of inhibitors are lacking. Hydrolysis is the first and often rate-limiting step in high-solids anaerobic digestion (Xu et al., 2011). Thus, advancements in hydrolysis processes have the potential to improve AD performance and economic viability.

Previous research has demonstrated that hydrolysis rates can be increased in the presence of elevated ammonia and salinity via inoculation (Wilson et al., 2013). Further, inoculum acclimation has been shown to enhance hydrolysis rates in wet reactors treating food waste and manure by selecting for optimal microbial communities capable of performing under inhibitory conditions (Wilson et al., 2013). However, inoculation with hydrolyzers is not used in practice in high-solids systems. Since the quantity of microorganisms in LBRs can limit reactor performance, establishing and maintaining desirable microbial communities in reactors is key to optimal operation. To promote establishment of desirable microorganisms in LBRs, acclimated inoculum may be used as initial seed. After start-up, LBRs may benefit from re-inoculation with each new batch because substantial time (several days to weeks) is required to develop inhibitor-tolerant hydrolyzing bacterial communities (Wilson et al., 2016). Thus, development of successful inoculation approaches for high-solids systems is needed.

To maintain desired microorganisms in AD systems, inocula derived from previously digested waste are promising because surface-attached bacteria are known to mediate hydrolysis of the recalcitrant feedstock cellulose (Wang et al., 2010). Digested waste inoculum has been shown to increase hydrolysis rates in LBRs under low ammonia conditions. Xu et al. (2012) recommended 20% inoculum (by mass) for LBRs, although that study used artificial food waste and LBRs were run in isolation (i.e., not connected to methanogenesis reactors). Another study

suggested leachate recycle (as opposed to single pass) may provide inoculum for improving process performance in LBRs treating manure (El-Mashad et al., 2006). By contrast, Degueurce et al. (2016) suggested that the beneficial role of leachate recirculation in LBR-based AD is abiotic and mainly due to leachate nutrient content, pH, and buffering capacity. However, none of these studies investigated the impact of inhibitors, and studies only examined one batch period. Thus, further research was needed to investigate optimal inoculation approaches and amounts for post-consumer waste in multi-stage systems during start-up and long-term operation with elevated ammonia and salinity.

Optimal inoculation approaches, including use of digested waste inoculum or leachate recycle, will most readily be developed with a fundamental understanding of the composition and distribution of microbial communities present in digested waste, leachate and fresh waste. Different microbial communities are selected for as a function of different reactor conditions including inhibitor concentrations and feedstock (Wilson et al., 2013). Thus, by extension, microbial communities are expected to differ substantially between digested waste, leachate and fresh waste, but the distribution and composition of microbial communities in multi-stage AD systems over long-term operation has yet to be investigated. This knowledge will support optimization of operational methods, potentially leading to improved economic viability via increased methane production, or reduced reactor volumes and batch times.

Thus, the objectives of this study were to (1) evaluate initial and long-term inoculation methods to establish enriched inhibitor-tolerant hydrolyzing communities and enhance performance of LBRs, and (2) to advance understanding of hydrolyzing microbial communities in multi-stage AD systems. The approach was to operate laboratory-scale, multi-stage AD systems fed post-consumer OFMSW under elevated ammonia and salinity conditions. The

impact of operational approaches on hydrolysis performance was investigated by comparing performance for LBRs seeded with unacclimated inoculum and different ratios of acclimated inoculum at start-up. Additionally, the effect of seeding methods was examined during long-term operation by identifying the optimal ratio of fresh waste to previously digested waste in multi-stage systems incorporating leachate recycle. Molecular tools (e.g., terminal restriction fragment length polymorphism [T-RFLP] and 16S rRNA gene amplicon sequencing) were utilized to track microbial community development in the inoculum, feedstock, and leachate.

2. Materials and Methods

2.1. Feedstock and inoculum collection

The OFMSW feedstock utilized in this study was a combination of food and yard waste. Food waste was collected from the Colorado State University Ram's Horn dining facility as previously described; in this facility, food waste is separated, pulped and de-watered (Wilson et al., 2013). Pulped waste was used to produce a homogenized waste and increase reproducibility. Characterization of this food waste source also has been previously reported (Wilson et al., 2013). Food waste was stored in bins for less than 2 days prior to experimental use. Yard waste (leaves and grass clippings) and wood chips (used as a bulking material) were collected from a local landscaping company (Hageman Earth Cycle, Fort Collins, CO). In high-solids AD systems, a bulking agent must be added to promote optimal hydraulics (Xu et al., 2011). After collection, OFMSW was stored at 4°C until subsequent use the following day. Immediately prior to experimental use, food waste (82% by wet mass), grass clippings (7.25%), leaves (3.5%), and wood chips (7.25%) were mixed thoroughly by hand to create the OFMSW feedstock loaded into LBRs. This food waste to yard waste ratio is consistent with ratios that may be used in the field (Beanie, 2013). Original microbial inoculum was collected from the Drake Municipal

Wastewater Treatment Plant (DWWTP) mesophilic anaerobic digester (Fort Collins, CO) and used immediately after collection. Waste and inoculum were analyzed for total and volatile solids (TS and VS, respectively) content prior to digestion (Table S1).

2.2. Reactor configurations

2.2.1. Start-up test configuration

Two different configurations were used in this study to investigate the effects of inoculum percentage at start-up and over time. For experiments focused on reactor start-up, 4 LBRs were connected to individual leachate storage tanks (LSTs) (Fig. 1a). In this set-up, the LSTs were not connected to methanogenic reactors and instead recycled leachate directly to the top of the LBRs. Methanogenic reactors were not used to simplify the experimental setup and because hydrolysis was the study focus. Utilizing separate LSTs allowed for isolation of the effects of inoculum percentages without the influence of microbial community development from the other LBRs or methanogenic reactors. LBRs were clear polycarbonate pipe with a diameter and height of 20 cm and 91 cm, respectively. The LBRs were sealed with Cherne adjustable pipe caps that were fitted with 5 evenly-spaced ports for leachate distribution (Fig. 1b). The volume of waste in each LBR varied depending on the percentage of inoculum used and was ~10 - 25L. 19 L plastic carboys (~15 L leachate) were utilized as LSTs. Leachate from each LBR was gravity-fed into an individual LST, and then pumped (via Cole Parmer Masterflex L/S Digital Drive peristaltic pumps) back to the top of the LBR at a rate of 20 ml/min. Flow rates were digitally controlled, and the accuracy of the flow rate was manually confirmed three times during the experimental period. 1.27 cm and 0.32 cm vinyl tubing (inside diameter) were used for leachate delivery to the LSTs and LBRs, respectively. LBRs were refilled every 16 days. A relatively short (16-day) batch digestion period was selected to investigate the impact of

operational changes during the early to middle portion of a digestion period; batch digestion times of ~3 weeks are common at full scale.

2.2.2. Long-term operation configuration

For long-term operation, a multi-stage system was utilized that contained a triplicate set of LBRs (previously described in *Section 2.2.1*), 1 LST (54.6 cm x 38.1 cm x 66 cm), and 1 fixed film reactor (cylindrical drum with a radius and height of 23 cm and 79 cm, respectively) (Fig. 1c). The LST and FF had working volumes of ~38 and 114 L, respectively. Leachate from the 3 LBRs flowed into 1 LST and then was pumped to the FF at a rate of 9 ml/min. Effluent from the FF was pumped at the same rate back to the LST. Leachate also was pumped from the LST to the top of the LBRs as described in *Section 2.2.1*. Vinyl tubing (1.27 cm inside diameter) was used for leachate delivery to and from the FF. The FF was filled with plastic packing material (Bioportz moving media, Entex Technologies). Leachate samples for liquid analysis were collected from the LST and FF reactor effluent (post-treatment). Samples were collected from the LST, rather than LBR effluent directly, as a means of monitoring pH and volatile fatty acid (VFA) concentrations in the composited leachate delivered to the LBRs and FF to determine if pH and VFA concentrations were inhibitory (e.g., >6.5 pH in the LST) (Ahn et al., 2010). pH and VFA levels were not found to be inhibitory at any point during operation.

2.3. Microbial acclimation for LBRs and FF

To develop a large volume of acclimated inoculum to seed LBRs for tests under elevated ammonia and salinity concentrations, 2 LBRs were loaded with 3600 g (wet weight) of OFMSW and inoculated with DWWTP AD sludge (20% of total material by mass) by layering (*Section 2.1*) (Fig. 1d, Table S1). During digestion, leachate generated was collected in one LST (initially filled with nitrogen-purged deionized [DI] water) and recirculated back to the top of each LBR

using the configuration in Fig. 1a. Leachate was analyzed at least twice a week for dissolved chemical oxygen demand (DCOD), VFAs, ammonia, pH, and electrical conductivity (EC). After the first batch (16 days), the LBRs were emptied and refilled by layering fresh waste with previously digested waste (60% wet weight). Fresh waste and inoculum were alternately layered (4 and 5 layers of fresh waste and inoculum, respectively) to provide contact between waste and inoculum; layers were separated by mesh (5 mm) so that they could be distinguished post-digestion. Fresh waste layers were 900 g (wet weight) each, and the mass of inoculum layers varied depending on the inoculum percentage utilized. Columns were purged with nitrogen gas after each refilling to avoid prolonged exposure of the inoculum to oxygen. LBRs were operated over a 16-week period, and ammonia and salinity concentrations in leachate gradually were elevated artificially to 3.5 g TAN/L and a conductivity of 45 mS/cm (surrogate measure of salinity, ~6 g Na⁺/L), respectively, by adding ammonium chloride and sodium chloride. Acclimation was considered complete when final solids destruction did not vary by more than 10% for consecutive batches.

To acclimate a microbial inoculum for the FF reactor (Fig. 1c), AD sludge was incubated at 35 °C for 2 weeks and fed glucose while the ammonia and salinity concentrations were increased gradually to 1.5 g TAN/L and 3 g Na⁺/L using ammonia chloride and sodium chloride. Next, the inoculum was mixed with a nutrient solution (50% v/v basis) (Owen et al., 1979) to ensure that nutrients were not limiting in the system, and the reactor was fed glucose or leachate generated from the LBRs at an organic loading rate (OLR) of 1 g COD/L·day while ammonia and conductivity concentrations were slowly increased further to 3.5 g TAN/L and 45 mS/cm. A low OLR was used during acclimation to prevent the build-up of VFAs in the FF in the case that conversion of VFAs to methane was slow due to the increasing ammonia and salinity

concentrations. The effluent was recycled continuously at a rate of 50 ml/min, and the pH was adjusted to 7 using NaOH as needed. Throughout the 16-week period, the methanogenic reactor was operated as a “wet” (low-solids) digester (i.e., recycled liquid flowed through a sludge of suspended biomass) rather than as a FF reactor to avoid sub-optimal microorganisms (non-inhibitor-tolerant microorganisms) attaching to the plastic packing. After 16 weeks, packing material was added to the reactor, and the FF reactor was operated for 3 weeks prior to subsequent experiments.

2.4. Reactor operation

2.4.1. Determining optimal seeding methods at start-up

To determine optimal seeding methods at start-up or when inhibitors become elevated, LBRs operating under elevated ammonia and salinity (3.5 g TAN/L and 45 mS/cm, respectively) were seeded by layering initially with unacclimated (60% by wet mass) or different percentages of acclimated inoculum (60%, 10%, and 0% by mass) and operated for a total of 3 batches with the set-up described in *Section 2.2.1* (Fig. 1a, d). **In full-scale systems that incorporate recycle, it can take many batches (~months of operation) for inhibitors to build up in leachate. Thus, ammonia and salinity were artificially elevated using ammonium chloride and sodium chloride as described in Sections 2.3. to accelerate our study.** The unacclimated inoculum utilized was previously digested waste from an identical multi-stage system operating under low ammonia and salinity. The source of acclimated inoculum was previously digested waste from the acclimated LBRs (*Section 2.3*). For all 3 batches, the mass of fresh waste (3600 g) loaded into each LBR remained constant. Since a FF reactor was not utilized in this set-up, leachate was manually diluted 50% by volume daily (Xu et al., 2011) with nitrogen-purged DI water to simulate the FF function (i.e., to reduce the concentration of soluble organics typically consumed

in the FF). The DI water was spiked with appropriate concentrations of ammonium chloride and sodium chloride to maintain elevated ammonia and sodium in the system.

2.4.2. Determining optimal seeding methods over time

To determine the optimal ratio of previously digested waste to fresh waste over time, LBRs operating under elevated ammonia and salinity were seeded initially with a high percentage (60% by mass) of acclimated inoculum with the set-up described in *Section 2.2.2* (Fig. 1c). After each batch (16 days), the LBRs were emptied and refilled with fresh and previously digested waste as described in *Section 2.3*. LBRs were reseeded with decreasing percentages of inoculum (40%, 20%, 10%, and 0%) to determine the minimum amount of inoculum needed to maintain optimal microorganisms in the system. For each inoculum percentage, LBRs were operated for 2 (at 60%, 20%, and 0% inoculum) or 3 (at 40% and 10% inoculum) batches. Post-digestion, separate inoculum and digested waste layers were mixed thoroughly by hand for 5 minutes, and 5 random samples were analyzed for TS and VS. Thus, for each inoculum percentage, hydrolysis performance results were based on a minimum of 30 samples (3 columns x 2 batches x 5 samples).

2.5. Chemical analyses

TS and VS of the inoculum and waste were measured pre- and post-digestion according to Standard Method 2540 (APHA, 1995). TS and VS removal efficiencies were calculated according to Xu et al. (2011). Liquid samples extracted from the LST and FF effluent were analyzed for pH and EC using probes (VWR #89231-604 and #11388-382, respectively). DCOD concentrations were determined using Hach's COD High Range Vials and digestion colorimetric method (Hach, Loveland, CO). VFAs were determined using Hach's Volatile Acids TNTplus Reagent Set and esterification method. Samples were filtered through a 0.2 µm syringe filter

prior to DCOD and VFA analyses. Hach's Nitrogen-Ammonia High Range Reagent Set was utilized weekly to verify that ammonia concentrations remained constant over time. Biogas volume was measured utilizing a wet tip gas meter (Speece Co., Nashville, TN), and biogas composition was determined via gas chromatography using a thermal conductivity detector as described previously (Wilson et al., 2013).

2.6. *Microbial community analyses*

Motivated by the long-term study results, experiments were conducted to compare quantities and compositions of the microbial communities among the leachate, inoculum, and fresh waste. Samples used for quantification of Bacteria in leachate and previously digested inoculum were collected on day 192 of the long-term experiment. To prepare samples for DNA extraction, 50-ml leachate samples were centrifuged at 5,000 g for 10 minutes at 4°C. The supernatant was discarded, and the remaining pellet was used for DNA extraction. For solid samples, 25 g of fresh waste or inoculum were blended with 125 ml of sterile DI water for 1 minute to ensure representative samples. The blended mixture was then centrifuged at 5000 g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was used for DNA extraction. For all samples, DNA was extracted using MoBio's PowerSoil[®] DNA isolation kit according to the manufacturer's instructions.

Bacterial 16 rRNA genes were quantified by qPCR generally according to previously described methods. Details are provided in supplemental information. For all inoculum percentages, bacterial 16S rRNA gene quantities were estimated based on a single sample (192-day sample) to eliminate the impact of changes over time. The number of 16S rRNA genes per mass of solid inoculum was determined, and the total quantity of Bacteria added to the LBRs was projected for each inoculum percentage. All numbers are expressed as a function of the

amount of fresh waste added, which was constant over time. For leachate, the quantity of 16S rRNA genes was determined per volume of leachate, and multiplied by the amount of leachate distributed to the LBRs over the experimental period.

Community composition was determined via T-RFLP analyses of DNA isolated from leachate, fresh waste, and previously digested inoculum and waste. Previously digested waste was collected from three LBRs operated with fresh waste and inoculum (40% by mass) layered (LI), fresh waste and inoculum (40% by mass) thoroughly mixed by hand (MI), and fresh waste only with no inoculum (NI) utilizing the set-up described in *Section 2.2.2*. To determine microbes present early in the batch period, digested samples were extracted from each layer of the LBRs after 5 days of operation; leachate and previously digested inoculum samples were collected as described above. To track changes to bacterial, fermenting, and hydrolyzing communities, T-RFLP analyses were conducted targeting the 16S rRNA gene, *hydA* gene (encodes the alpha subunit of iron hydrogenase), and *cel48* gene (encodes glycoside hydrolases of family 48). T-RFLP analysis targeting the 16S rRNA gene was conducted as described by Wilson et al. (2013). T-RFLP analyses targeting the *hydA* gene and *cel48* gene were conducted as previously described by Lefèvre et al. (2013).

DNA from leachate (collected on day 192), fresh waste, and inoculum (collected on day 192) were submitted to Research and Testing Laboratories LLC (Lubbock, Texas) for bacterial 16S rRNA gene amplicon sequencing using Illumina MiSeq. Primers 28f and 388R were used, targeting the V1-V2 hypervariable regions of 16S rRNA genes (Sundquist et al., 2007; Turnbaugh et al., 2009). Bioinformatic analysis is described in the supplemental information.

2.7. Statistical analyses

Analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) tests were conducted using R Statistical Software (R Core Team, 2012) to determine if the slope of the fitted regression line for VS reduction as a function of inoculum percentage was statistically different from 0 as well as to determine statistical differences in average VS reduction for a range of inoculum percentages. Results yielding a p-value less than 0.05 were considered to be significant. T-RFLP data were analyzed and similarity matrices were calculated according to Wilson et al. (2013), except terminal restriction fragments (T-RFs) representing less than 1% of the total area were excluded (Rees et al., 2004). Pairwise distances were calculated on aligned sequences, followed by clustering and estimation of Shannon diversity.

3. Results and Discussion

3.1. Impact of inoculum percentage on waste hydrolysis at start-up

We hypothesized that waste reduction would be limited by the number of organisms provided as inoculum in the LBRs under elevated ammonia and salinity conditions. As expected, results demonstrated that higher percentages of inoculum are beneficial and improve VS reduction. Initially, with acclimated inoculum at 60% and a digestion period of 16 days, reactors demonstrated an average VS reduction of 69.5% (± 3.5) (Fig. 2a). Results from our study demonstrated that hydrolysis performance was better than or comparable to previously reported performances for dry digesters operating under lower ammonia and salinity, despite the elevated levels present. For example, Xu et al. (2014) achieved 45.4 - 69.4% VS reduction in LBRs treating food waste inoculated with WWTP AD sludge with a 16-day residence time. Similarly, Yan et al. (2014) demonstrated 58.7 – 68.1% VS reduction in LBRs fed simulated food waste and inoculated with AD sludge and cow manure. Lu et al. (2008) demonstrated 63% VS reduction in LBRs connected to a UASB with leachate recirculation treating vegetable and

flower waste over a 10 day period. Thus, results indicated that high solids conversion can be achieved even in the presence of inhibitors, if required organisms are provided in sufficient quantities via inoculation.

For a lower acclimated inoculum percentage (10%) or for no inoculum (0%), average VS reduction was reduced ~1.5- and 2-fold, respectively (Fig. 2a). An ANOVA test on triplicate batches at each inoculum percentage indicated that significant differences exist among means for VS reduction (p -value = 0.0001). The significant reduction in solids removal with reduced inoculum percentages demonstrates that the quantity of hydrolyzing bacteria present in LBRs limits performance. VS reduction improved by the end of batch 3 for all inoculum percentages (with VS reduction increasing to 73% for the 60% acclimated inoculum). Performance increases for successive batches likely indicate that the concentration of inhibitor-tolerant hydrolyzing bacteria increased in the systems (e.g., in the digestate). However, VS reduction with 0% inoculum was still 1.9-fold lower than VS reduction with 60% inoculum for the third batch. In the only other study to investigate the impact of inoculum percentage at start-up in LBRs, Xu et al. (2012) also observed a decrease in waste hydrolysis with reduced inoculum percentages (80%, 20%, 5%, and 0%), albeit a less dramatic trend than observed in our study. The less dramatic decrease was likely because Xu et al. (2012) investigated low ammonia and salinity conditions. Specifically, their study demonstrated 1.13- and 1.14-fold lower VS removals for inoculum percentages of 20% and 5%, respectively, compared to an inoculum percentage of 80%. Xu et al. (2012) recommended a 20% inoculum; however, the study did not test multiple batches over time or 10% inoculum. Ultimately, the substantial reduction in VS removal observed in our study at lower inoculum percentages suggests that higher volumes of acclimated

inoculum are needed at start-up, or when salinity and ammonia are observed to increase in leachate, for optimal performance.

To determine if acclimated inoculum enhanced performance compared to unacclimated inoculum, both inocula were tested in parallel at 60%. Interestingly, acclimated inoculum initially demonstrated 68% VS reduction, compared to 56% VS reduction for unacclimated inoculum (Fig. 2a). However, both reactors performed similarly by the end of batch 3. Thus, results suggest that inhibitor-tolerant organisms accumulated in as little as 3 batches when a high quantity of inoculum was used (60%). Several studies have demonstrated that acclimated inoculum performs better than unacclimated inoculum in wet systems (Calli et al., 2005; Chen et al., 2008; Omil et al., 1995; Sung & Liu, 2003). For example, we previously demonstrated that an AD sludge-derived inoculum acclimated to elevated ammonia (5 g TAN/L) fed manure and food waste performed significantly better than unacclimated sludge directly exposed to elevated ammonia concentrations in wet batch reactors (Wilson et al., 2013). Thus, results herein extend this finding to LBRs, although performance improvements were marginal in the high-solids system. Additionally, data provide guidance on the quantity of inoculum needed to maximize solids reduction at start-up (~40-60%) or when inhibitors build up.

3.2. Impact of inoculum percentage on system performance during long-term operation

To determine how inoculum percentages and connection to a FF affect maintenance of desired organisms and performance over long-term operation, experiments were conducted with multi-stage reactors that incorporated leachate recycle for over 190 days. Reactors were only run with acclimated inoculum because results of the start-up experiments indicated that microbial populations within the hydrolysis reactors seeded with unacclimated inoculum acclimated to inhibitors over only three batches. Hydrolysis performance and methane generation were

monitored across a range of inoculum percentages (60% - 0%). Average VS reduction for LBRs with low ammonia and salinity at 60% inoculum was slightly better (1.17-fold) than for LBRs with high ammonia (~3.5 g TAN/L) and salinity (45 mS/cm) (Fig. 2b). This slight inhibition is consistent with the observations of others (Chen et al., 2008); however, in our study inhibition likely was minimized via the usage of acclimated inoculum. In one of the first studies to determine the effects of elevated ammonia, Kayhanian (1994) demonstrated that high solids digesters performed best at TAN concentrations in the range of 0.6-1 g/L. More recently, Duan et al. (2012) reported significant inhibition at 3 - 4 g TAN/L in high solids AD of sewage sludge with the highest VS reduction reaching only 40% (compared to ~60% for uninhibited reactors) and VFA concentrations exceeding 10 g/L (Song et al., 2004). In contrast, our study demonstrated VS reduction levels up to an average of 62 (± 7)% at 60% inoculum under elevated ammonia and salinity, indicating a benefit for use of inoculums acclimated to elevated ammonia and salinity.

Interestingly, in contrast to start-up, over time VS reduction remained relatively constant for decreasing inoculum percentages (Fig. 2b). At 60% initial inoculum, VS reduction averaged 62 (± 7)% over the first two batches. When inoculum percentages were then decreased to 40% and 20%, VS reduction appeared to decrease slightly. Unexpectedly, VS reduction increased to an average of 67 (± 7)% when the inoculum percentage was lowered further to 10%, which was the highest VS reduction demonstrated for any of the percentages. Moreover, this performance at 10% inoculum is comparable to performance demonstrated in reactors operating under low ammonia and salinity. However, at 0% inoculum, VS reduction once again decreased and demonstrated the lowest reductions of any inoculum percentages. Despite a decreasing trend for VS reduction as a function of inoculum percentage (with the exception of 10% inoculum), the

slope of the linear regression for all percentages with acclimated inoculum was not significantly different from zero, suggesting that inoculum percentage did not substantially affect VS reduction over time. However, an ANOVA test revealed significant differences in mean VS reductions among the various inoculum percentages (p -value = 0.0074), and Tukey's HSD test identified significant differences between the mean VS reduction at 10% and 0% inoculum (p -value = 0.003). No other significant differences between mean VS reductions were observed. Thus, regardless of inoculum percentage, over time reactors demonstrated VS reduction comparable to systems running under optimal conditions despite the elevated ammonia and salinity levels (Lu et al., 2008; Xu et al., 2014; Yan et al., 2014).

Consistent with solids reduction data, VFA concentrations demonstrated steady performance for decreasing inoculum percentages over time (Fig. 3a). At the start of a new batch, leachate VFA concentrations spiked in the LST due to the influx of fresh waste but gradually reduced as readily hydrolysable substrates were converted to soluble products and methane. Steady, low VFA concentrations in FF effluent demonstrated the FF was effective despite elevated inhibitor levels. pH values ranged from 6.6 – 8.3 and 7.8 - 8.5 in the LST and FF effluent, respectively, over the course of the experiment (Fig. S1).

Overall, our reactors demonstrated comparable or higher methane generation to previous literature values for reactors operating under low ammonia and salinity conditions (Fig. 3b). The highest average methane yield, observed with the greatest inoculum percentage, was 0.260 [$\pm$ 0.01] L CH₄/ g VS_{fresh waste}) (Fig. 3b). By contrast, lower methane production (0.182 L CH₄/ g VS) has been demonstrated in LBRs coupled with a UASB fed simulated food waste for a 17-day digestion period at start-up (Xu et al., 2011). Additionally, Han et al. (2002) observed 0.27 L CH₄/ g VS for sequential batch, two-phase anaerobic composting systems fed food waste (with

impurities removed) and inoculated with rumen microorganisms with a 10 day solids retention time. Dearman et al. (2007) demonstrated similar or slightly lower methane yields (0.214, 0.229 L CH₄/ g VS) for dry food waste digesters using 10% (w/w) inoculum despite an extremely long digestion period (73 days). Further, herein 48-63% of the theoretical methane yield was achieved based on measured biochemical methane potential (0.41 L CH₄/g VS) previously reported for the source of food waste use (Wilson et al., 2013), which is similar to food waste methane potentials reported by others (Cho et al., 1995; Lin et al., 2011). Higher percentages of the theoretical yields (>55%) were observed in reactors operating under 60% and 10% inoculum, whereas reactors operating under 40, 20, and 0% demonstrated ≤52% of theoretical yields. Yields could be increased by increasing digestion time (e.g., to 3 weeks). Additionally, the methanogenic (FF) reactor was likely not fully optimized because hydrolysis was the focus on this study. However, achieved yields were similar to those reported by others (47 – 85%) (Carucci et al., 2005; Davidsson et al., 2007; Voelklein et al., 2016) despite suboptimal reactor conditions. Solids reduction is strongly impacted by feedstock composition, and thus lower rates reported by others may also be due, in part, to use of more recalcitrant feedstocks. However, superior microbial populations likely improved methane production herein.

Interestingly, despite remaining within the range of reported methane yields, average yields in our study decreased with decreasing inoculum at 40% and 20% (0.212 [±0.01] and 0.195 [±0.01] L CH₄/ g VS_{fresh waste}, respectively), but then increased at 10% inoculum and decreased at 0% inoculum, consistent with solids reduction data (Fig. 3b). At 10% inoculum, methane yield was 0.230 (±0.02) L CH₄/ g VS_{fresh waste}, and at 0% inoculum yield was 0.207 [±0.01] L CH₄/ g VS_{fresh waste}). Thus, collectively our results suggest that after an initial start-up period, low quantities of solid inoculum (~10%) are sufficient under elevated ammonia and

salinity, and importantly, that even after start-up, solid-phase inoculum addition improves performance relative to no inoculum. The increase in performance observed at 10% was unexpected considering the declining trend for higher percentages and the idea that hydrolyzers are solids-associated (Wang et al., 2010; Zhang et al., 2007). These results led us to hypothesize that desired microorganisms built up in the leachate over time, and thus leachate recirculation could also provide a source of hydrolyzing bacteria. Similarly, others have speculated that leachate recirculation enhances performance based on results from a high-solids, single-stage AD system fed cattle waste operated at 40°C and 50°C (El-Mashad et al., 2006). To test this hypothesis and develop a mechanistic understanding of microbial community dynamics in multi-stage AD systems, bacterial communities were characterized over long term operation.

3.4. Quantification of leachate-derived and solids inoculum bacteria

We sought to determine if leachate recirculation could provide a source of hydrolyzing bacteria and first quantified Bacteria in the leachate and solid waste inoculum to determine how the quantities of Bacteria present compared. qPCR data revealed 60% inoculum resulted in the addition of 3.1×10^{11} bacterial 16S rRNA genes/ g fresh waste to the LBR (without including bacteria contributed by leachate), while 10% inoculum provided 2.3×10^{10} bacterial genes/ g fresh waste (Fig. 4). Interestingly, data indicate that the quantity of Bacteria in 10% inoculum (2.3×10^{10} 16S rRNA genes/ g fresh waste) and in the leachate (0% inoculum) after 192 days of operation (2.1×10^{10} 16S rRNA genes/ g fresh waste) are very similar. These data are 16S rRNA gene-based and thus do not indicate the type or activity of Bacteria, but these results support the hypothesis that leachate is a good source of inoculum for hydrolysis after sufficient time has allowed microorganisms to build up in the leachate. Notable improvements in performance were not seen until day 112, so a high percentage of inoculum may be beneficial initially for at least 2-

4 batches. The inoculum initial percentage used likely will affect the number of batches required to establish sufficient hydrolyzing populations because high initial volumes of inoculum will add greater quantities of microorganisms to the system. The inoculum percentage could also be decreased slowly after 2 batches as was done here to continue building up desired organisms, and simultaneously avoid system overload by slowly introducing higher quantities of OFMSW. Further work is necessary to clarify the most advantageous method of decreasing inoculum over time. However, within ~3-4 months, results suggest that low volumes of inoculum may be utilized consistently. Importantly, molecular data also indicate that addition of 10% inoculum doubles the quantity bacteria that contact fresh waste over the course of a batch; this finding is consistent with the superior performance observed at 10% inoculum compared to leachate alone.

3.4.2. *Microbial community composition dynamics*

Because the type, as well as the quantity, of Bacteria control performance, we sought to determine if microbial community compositions between the solid inoculum and leachate after 192 days of operation were similar. Thus, we examined the total bacterial (via 16S rRNA gene-targeted T-RFLP), fermenting (via *hydA* gene-targeted T-RFLP), and hydrolyzing (via *cel48* gene-targeted T-RFLP) microbial communities among fresh waste, leachate, and inoculum. Additionally, digested samples were analyzed after a 5 day digestion period to determine microbes present at the early stages of a batch.

Ribosomal and functional gene-targeted non-metric multi-dimensional scaling (MDS) analysis demonstrated that over time the leachate microbial communities became similar to digestate-based communities (Fig. 5), while the microbial communities in the feedstock remained distinct. Initially, through day 80, leachate bacterial communities were distinct from other communities. However, by day 112, the communities had undergone a large shift, and by

day 192, the leachate communities clearly clustered with inoculum and digested waste communities (Fig. 5a, b). Similarly, by day 192, hydrolyzing communities in leachate converged with digested waste communities, even though hydrolyzers have been considered solids-associated (Fig. 5d). Interestingly, *cel48* microbial communities did not cluster as tightly as the bacterial or fermenting communities (Fig. 5c), which indicates that cellulose hydrolyzers are less different among fresh waste, inoculum, and leachate. Thus, molecular data suggest that recirculated leachate can indeed be a useful inoculum for hydrolysis after a lag period if the leachate microbial communities are properly developed. Interestingly, in one of the few studies to investigate microbial community dynamics in AD leachate, Degueurce et al. (2016) concluded there was little transfer of microorganisms between leachate and solid waste in LBRs digesting manure, and, instead, microorganisms inherently present in the manure strongly impacted reactor performance. The contrasting results observed in our study may be explained by the difference in feedstocks; manure often is used as an inoculum due to its high quantity of microorganisms, which likely dominated reactor communities, whereas OFMSW microorganisms were readily outcompeted by optimized inoculum microbes. The OFMSW feedstocks likely did not contain high levels of bacteria tolerant of elevated salinity and ammonia. Additionally, lower redox in the AD system likely contributed to selection of different microbes despite storage of the food waste in large bins that may have become anaerobic.

Results also indicated that the inoculum delivery approach impacted development of desired microbial communities within fresh waste during the digestion process. For LI, original inoculum and 5-day digested waste formed a tight cluster when compared to fresh waste and early leachate, indicating relatively successful transfer of inoculum microbes to the fresh waste (Fig. 5a). However, inoculum and fresh waste layers were distinguishable from each other (Fig.

5b). For MI, the microbial community composition grouped generally between the LI waste and LI inoculum layers; these results suggest that thorough mixing is likely beneficial. Interestingly, the NI microbial communities generally clustered with the LI waste layer and MI, indicating that after 192 days, leachate delivered generally the same types of bacteria to the fresh waste as the solid waste inoculum. However, NI communities grouped closer to the LI fresh waste layer than the inoculum layer, consistent with the observed benefits of inoculation with digested waste. These results are in agreement with previous research indicating that surface-attached bacteria are important for hydrolysis, particularly for cellulose (Lu et al., 2008; Wang et al., 2010). In one of the few studies to look at inoculation methods, El-Mashad et al. (2006) demonstrated that adding inoculum throughout the reactor height compared to leaving previously digested waste at the bottom of reactors increased methane generation. Thus, enhanced delivery methods (e.g., mixing) may increase hydrolysis performance further. Future research is needed to develop enhanced inoculation approaches suitable for full-scale implementation in dry digestion technologies.

3.4.3. Identification of inhibitor-tolerant microbial phylotypes

Bacterial-targeted 16S rRNA gene amplicon sequencing results revealed distinct phylotypes present in inoculum, leachate, and fresh waste. The highest species diversity was observed in the inoculum and one leachate sample (Shannon values of 4.7 and 3.37, respectively), whereas the lowest species diversity was observed in the other leachate sample (1.0) (Table S2). The majority (>95%) of microorganisms in all samples were identified beyond the domain level (Fig. 6).

Interestingly, the microbial community in fresh waste was dominated by anaerobes, as well as microorganisms associated with food waste. At the order level, *Lactobacillales* accounted

for over 89% of the microorganisms in the fresh waste, although its presence was less than 1 and 7% abundance in the leachate and inoculum, respectively. Within this order, 4 main genera (*Leuconostoc*, *Lactococcus*, *Weissella*, and *Lactobacillus*) were identified (Fig. 6), all of which are acid-tolerant anaerobes largely involved in the fermentation of dairy products and vegetables. The dominance of these anaerobes was somewhat unexpected and could reflect the particular waste utilized in this study or storage method of food waste prior to collection.

Although microbial communities in the leachate and inoculum were similar, differences observed suggest complimentary capabilities. *Bacteroidales* accounted for the majority of the organisms in the leachate (94 and 66%) and comprised 37 and 42% abundance in the two inoculum samples (order-level analysis not shown). *Bacteroides* was the dominant genus in leachate and comprised over 25% of the solid waste inoculum. *Bacteroides* are known to be cellulolytic and carbohydrate-utilizing (Wang et al., 2010). The particular species (*Bacteroides coprosuis*) dominating the genus *Bacteroides* observed in our reactors was first isolated in 2005 from a swine-manure storage pit (Whitehead et al., 2005) and has been documented since then in digesters treating municipal sludge and waste from cattle farms. Similar to the *cel48* gene-targeted T-RFLP data, results indicated that hydrolyzing bacteria in the form of *Bacteroides* (which contain the *cel48* gene) are present in the leachate. Based on these observations, *Bacteroides* are likely ammonia and salinity tolerant and contribute to improved performance under these conditions. Order *Clostridiales*, which includes *Family XI*, are bacteria well known for their cellulolytic capabilities (Wang et al., 2010), and comprised 40% of the inoculum communities, but were present at very low levels in the leachate (1 and 5%) and fresh waste (<2%). This observation is consistent with previous studies indicating *Clostridia* are solids-associated (Lu et al., 2008; Wang et al., 2010) and may help explain observed performance

improvements when solid inoculum was provided. Yard waste accounted for just over 10% of the total mass added to reactors and is a main source of cellulose in the system, and this feedstock may have been hydrolyzed more completely when *Clostridia* were provided.

4. Conclusions

The challenges associated with inhibitory ammonia and salinity concentrations encountered in AD systems can be overcome via hydrolysis seeding methods. Inoculum should be established by maintaining high levels of digested waste (~40-60% by mass) in hydrolysis reactors to seed successive batches at start-up or when salinity and ammonia are observed to be elevating. After a lag period, desired hydrolyzers accumulate in recirculated leachate. Thus, leachate recirculation combined with digested-waste inoculum (~10% by mass) is recommended for improved performance. Optimized hydrolysis may reduce capital and operating costs and ultimately increase the economic viability of dry AD systems that incorporate leachate recycle.

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Figure Captions

Fig 1. Reactor configuration for determining performance at start-up (a). Plan view of LBR depicting spacing of leachate distribution ports (b). Reactor configuration for determining performance over time (c). Configurations include leach bed reactors (LBRs), leachate storage tank (LST), and a fixed film (FF)

reactor. Arrows depict leachate flow path. LBR schematic depicting layered inoculum and fresh waste (d).

Fig. 2. VS reduction as a function of percentage of inoculum at start-up (a) and over time (b). At start-up (a), reactors were seeded with acclimated (▲) and unacclimated (■) inoculum operating under elevated ammonia and salinity. Three batches (starting on days 0, 16, and 32) were run for each percentage. Over time (b), reactors were seeded with unacclimated inoculum under low ammonia (●) to demonstrate optimal reactor performance without inhibition or acclimated inoculum under elevated ammonia and salinity (◆). Multiple data points at each percentage demonstrate results for successive batches (a and b). Data points and error bars represent average performance and standard deviation for the set of triplicate reactors for a single batch (b only). Days of operation are indicated at the top of the figure.

Fig. 3. Long-term performance data for multi-stage reactors operating under elevated ammonia and salinity (~3.5 g TAN/L and 45 mS/cm) at a retention time of 16 days. (a) VFA concentrations and (b) methane yield are shown for a range of acclimated inoculum percentages over time. Inoculum percentages are noted at the top of each graph section. Vertical lines indicate the end of each batch and start of next batch. 2 batches were run for 60%, 20% and 0% and 3 batches were run for 40% and 10%. Additional batches were run when the first two batches did not produce similar results.

Fig. 4. Comparison of quantities of bacterial 16S rRNA genes added to leach beds via acclimated inoculum and leachate recirculation throughout a 16-day batch period. The assay utilized targets Bacteria and avoids methanogen detection. Leachate and inoculum analyzed were collected on day 192. Thus, for all of the inoculum percentages (10-60%), bacterial 16S rRNA gene quantities were estimated based on a single sample to eliminate the impact of changes over time. The amount of fresh waste added to each reactor remained constant over time, and the amount of inoculum was adjusted accordingly. Error bars correspond to standard deviations for qPCR reactions run on DNA extracted from quintuplicate samples.

701

702 Fig. 5. MDS plots based on (a) 16S rRNA gene-targeted, (b) enlarged section of plot (a), (c) *hydA* gene-
703 targeted, and (d) *cel48* gene-targeted T-RFLP analysis.

704

705 Fig. 6. Relative genus-level abundance of 16S rRNA bacterial-targeted OTUs based on Illumina MiSeq
706 sequencing. Higher taxonomic levels are given when genus-level identification was not possible. Label
707 “Other Bacteria” indicates grouped phylotypes that individually represented less than 5% of the relative
708 abundance. Two representative samples each of inoculum, leachate, and fresh waste were chosen for
709 analysis. Leachate and inoculum analyzed were collected on day 192.

710

Fig. 1

Fig. 2

Fig. 3

Fig. 4

× Fresh waste + Original inoculum

Leachate over time:

- * Day 16 (60%, batch 1)
- * Day 32 (60%, batch 2)
- * Day 48 (40%, batch 1)
- * Day 80 (20%, batch 1)
- * Day 112 (10%, batch 1)
- * Day 192 (0%, batch 2)

Waste after a 5-day digestion period operated with leachate from day 192:

- ▲ LI: Inoculum layer
- ▲ LI: Fresh waste layer
- ◆ MI: Fresh waste mixed with inoculum
- NI: Fresh waste (without inoculum)

Fig. 5

Fig. 6