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An ASABE Meeting Presentation

Paper Number: 1111519

## Differing Effects of Glycerin on Anaerobic Co-digestion of Mixed Substrates in Bench-Scale Assays and Sub Pilot-Scale Reactors

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Written for presentation at the  
**2011 ASABE Annual International Meeting**  
Sponsored by ASABE  
Gault House  
Louisville, Kentucky  
August 7 – 10, 2011

**Abstract.** *Bench-scale methods such as Biochemical Methane Potential (BMP) assays and Anaerobic Toxicity Assays (ATAs) are useful tools in evaluating potential feedstocks for anaerobic digestion. The BMP method provides a preliminary indication of substrate biodegradability and methane production, while the ATAs provide an indication of substrate toxicity to anaerobic microbial consortia. Previous research using small (<20 L) reactors indicated that co-digestion of manures with small amounts of glycerin (ca. 1 – 2 %) can double methane production, but toxicity can result if glycerin exceeds 2% (volumetric basis). This paper investigated the relationship between bench-scale methods (BMPs and ATAs) and sub pilot-scale digester results, using glycerin as a test substrate mixed with a baseline feedstock (beef manure, corn processing wastewater, lagoon liquid, and short-fiber cardboard). The batch-fed, stirred ATAs indicated that glycerin was toxic to methane production at all inclusion levels. The batch-fed, stirred BMPs indicated no significant difference between methane production in the 0.0% to 4.0% addition levels; however at 8.0% addition, methane production tripled. The continuously fed, non-stirred, plug-flow sub pilot-scale reactors indicated toxicity effects in the 2.0% and 4.0% glycerin mixtures and no difference from the control in the 1.0% glycerin mixture. These results demonstrate the variations in scale performance of glycerin as a co-substrate and identify some serious challenges in extrapolating bench-scale assays to large-scale performance of mixed-waste anaerobic digestion systems.*

**Keywords.** Anaerobic Digestion, Biochemical Methane Potential (BMP), Anaerobic Toxicity Assays (ATAs), Glycerin, Co-digestion

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## Introduction

Co-digestion of animal manure with industrial wastewaters or other sources of biodegradable materials for increased energy production has become popular in recent years (Braun and Wellinger, 2003). Substrates of choice are generally high in chemical oxygen demand (COD), low in toxicity, and low in transportation costs. The rise in production of biodiesel in the upper Midwestern US has led to a glut of glycerin (also known as glycerol) in the region, because glycerin is a byproduct of biodiesel production, making it an attractive potential co-substrate since it is readily digestible and easily stored for long periods of time (Robra et al., 2010). The use of glycerin as a co-substrate and its effects on multiple scales of anaerobic digesters has not been widely reported. Instances that have reported on the use of glycerin found that there is a limiting concentration level (Wohlgemut, 2008; Fountoulakis et al., 2010).

The addition of glycerin to hog manure to boost biogas and methane production was studied by Wohlgemut (2008) to determine the ideal ratio of glycerin to hog manure. Four bench-scale completely stirred tank reactors (CSTRs) with a working volume of 3.5 L, and a hydraulic retention time of 17.5 d were employed. The addition of 1% glycerin doubled the methane and biogas production compared to hog manure without glycerin. At 2% glycerin inclusion, methane and biogas production was the greatest, although a 45-d stabilization period was necessary. The addition of 4% glycerin causes an overloading of COD and failure of the digester (Wohlgemut, 2008). Fountoulakis et al. (2010) focused on the feasibility of co-digesting crude glycerin with sewage sludge in a wastewater treatment plant in both batch and continuous experiments at 35°C. They found that glycerin increased biogas yields if the concentration did not exceed 1% (v/v). Above this concentration, organic overloading was thought to cause inhibition of methanogens due to the rapid degradation of glycerin. The addition of long-chain fatty acids have been reported as anaerobic digestion inhibitors, since they cause a lag period in the production of methane from acetate; however, some pretreatment can help reduce chemical oxygen demand and reduce the inhibitory effects (Hanaki et al., 1981). Siles et al. (2010) studied the co-digestion of pretreated glycerin with wastewater in batch laboratory-scale reactors at 35°C. Siles et al. (2010) only reported on experimental results of a 15% glycerin – 85% wastewater mixture where they found nearly 100% anaerobic biodegradability to be possible after pre-treatment by acidification and electrocoagulation. They acidified the glycerin with phosphoric acid, and centrifuged the acidified material to recover KOH contamination. In doing so COD of the glycerin was reduced and the possibility of a KOH toxicity problem was eliminated (Ma and Hansen, 2002). Robra et al. (2010) evaluated biogas production on mixtures of 5%, 10%, and 15% glycerin by weight co-digested with cattle slurry. This experiment was carried out in 3 L semi continuous CSTR digesters operated in the mesophilic range. Results show an increase of 9.5%, 14.3%, and 14.6% methane contents respectively for the treatments of 5% glycerin, 10% glycerin, and 15% glycerin compared to a control of cattle slurry without glycerin addition. However, only the 5% and 10% glycerin treatments had statistically higher total biogas yields (normalized per gram volatile solids) compared to the control of cattle slurry only. A failure in the heating system during the 6<sup>th</sup> week of operation along with high COD, methanol, and KOH concentrations caused the 15% glycerin mixture to have reduced methane production from which it could not recover (Robra et al., 2010). A similar experiment performed by Chen et al. (2008) used CSTR digesters operated at 35°C using mixtures of 100% glycerin, 60% glycerin/40% cattle manure, 45% glycerin/55% cattle manure, and 100% cattle manure on volatile solids (VS) basis. The result was an increase in biogas and methane yields as well as reduction in effluent VS due to greater treatment efficiency for increasing glycerin addition to dairy manure (Chen et al., 2008).

Each of the preceding article results shows a lack of consistency in establishing the correct ratio for glycerin addition as a co-substrate. Our work attempts to use ATAs and BMPs as tools for estimating the methane production of three sub pilot-scale reactors subjected to different glycerin inclusion amounts (1%, 2%, and 4% by volume). The ATAs were developed to evaluate potential substrate toxicity at a bench-scale prior to inclusion in a larger-scale anaerobic system. It was hypothesized that a glycerin ATA would provide information regarding a cutoff point to which glycerin can be added without overloading and causing methane suppression. This information could then be combined with BMP data for different glycerin and co-substrate mixtures to predict the performance of the sub pilot-scale reactors.

## **Materials and Methods**

### ***Substrates***

Manure was obtained directly from confined concrete beef cattle feedlot pens (open and covered) in eastern Iowa, from a facility where corn stover was the primary bedding material. At the time of collection, the manure's age was estimated at 2 – 3 d, and the manure was selected from areas with minimal bedding mixed in. We also collected wet mill corn processing wastewater and crude glycerin from a soybean & animal lard biodiesel manufacturing facility; both were collected within 1 d of delivery to the farm. Cardboard fibers too short for production for a cardboard box manufacturing facility were collected within 5 d of delivery to the farm. Lagoon liquid was collected using a dipper on the side opposite to the influent pipe for maximum lagoon treatment effects. The lagoon received beef manure feedlot runoff water and separated digester effluent. All samples were stored at 4°C and were analyzed within one week of collection. Sell et al. (2010) developed a mixture from these substrates to meet criteria including the use of all available manure, keeping total solids below 15% to facilitate pumping, maintaining pH between 6.5 and 8.2 for microbial ecology, providing high COD concentrations to maximize methane production, and achieving low ammonia to avoid toxicity (Speece, 1996).

### ***Laboratory Methods***

Substrates and mixtures were characterized for total solids (TS), volatile solids (VS), ammonia, alkalinity, and pH by the Iowa State University Agricultural Waste Management Laboratory. The TS and VS concentrations were measured using standard methods 2540 B and 2540 E, respectively. The pH measurements were taken with an Accumet Basic AB15 Plus pH meter and Accumet 13-620-285 pH probe. The chemical oxygen demand (COD) values were measured using Hach DR/890 Colorimeter Procedures Manual, Method 8000 and vials for COD 0-1500 ppm. Ammonia concentrations were measured using standard methods 4500-NH<sub>3</sub>-B Preliminary Distillation Step and 4500-NH<sub>3</sub>-C Titrimetric Method with 0.1-N HCl as the titrant instead of sulfuric acid. Alkalinity was measured using standard methods 2320 B with 0.1-N HCl as the titrant (Standard Methods, 1995).

### ***BMPs***

A BMP assay was performed in triplicate for each of the individual substrates and mixtures using a modified version of the International Standard ISO 11734:1995(E) per Moody et al. (2011b). Laboratory TS, VS, and COD results were used to calculate the sample size needed for a 250-mL BMP assay bottle. Sample sizes were calculated with a target of 125 mL CH<sub>4</sub> produced during a 30-d period, assuming 70% of COD converted to CH<sub>4</sub>, and 395 mL CH<sub>4</sub>/g COD reduced (Speece, 1996). This approach yielded average daily biogas volumes that were in a readily measurable range. The BMP reactors were seeded with an inoculum from a 60-L,

mesophilic (35°C), continuously stirred anaerobic reactor that was fed a mixture of high-protein dog food and nutrient medium (Moody et al. 2011b). The BMP reactors were also seeded with nutrient medium containing supplemental inorganic nutrients and alkalinity (Speece, 1996). Inoculum was added for a 2:1 mass ratio between substrate and inoculum VS. Assay bottles were purged with 70% nitrogen and 30% carbon dioxide gas at  $\sim 0.5 \text{ L min}^{-1}$  for 5 min. Bottles were then capped with septa and zip tied, and incubated at 35°C on an orbital shaker at 150 rpm. Biogas production was measured daily by inserting a glass syringe into the septum and allowing the biogas pressure to displace the wetted barrel of the syringe. The volume was recorded, and the biogas was injected into an infrared gas analyzer (NDIR-CH<sub>4</sub> Gasanalyzer, University Kiel, Germany) to obtain the methane content (Bishop et al., 2009). A blank that included the inoculum source but no substrate was run so that each BMP could be corrected for the methane created by the inoculum source.

### **ATAs**

The ATA methodology used at the Iowa State University Agricultural Waste Management Laboratory (ISU AWML) was a modified version of the method performed by Owen et al. (1979) and the International Standard ISO 13641-1 (2003) per Moody et al. (2011a). Aliquots of anaerobic inoculum and an easily degraded standard feedstock were assayed alone (for a fed control) and in combination with a range of eight potential toxicant inclusion rates. The inoculum source was the same as noted in the BMP method. Once materials were combined in the serum bottles, each bottle was purged with a 70% nitrogen and 30% carbon dioxide gas at  $\sim 0.5 \text{ L min}^{-1}$  for 5 min. Bottles were then capped with septa and zip tied, and incubated at 35°C on an orbital shaker at 150 rpm. Biogas production was measured every 24 h over for up to 5 d or until gas production ceased by inserting a glass syringe into the septum and allowing the biogas pressure to displace the wetted barrel of the syringe. The volume was recorded, and the biogas was injected into an infrared gas analyzer (NDIR-CH<sub>4</sub> Gasanalyzer, University Kiel, Germany) to obtain the methane content (Bishop et al., 2009). Results were used to calculate the percent inhibition of methane production for each substrate inclusion rate. Results are reported on a cumulative methane production over a 5 d period or until methane production has ceased as well as on an inclusion verse inhibition basis. In the inclusion verse inhibition display a negative inhibition percentage indicates that a substrate is non-toxic and a positive inhibition indicates signs of toxicity.

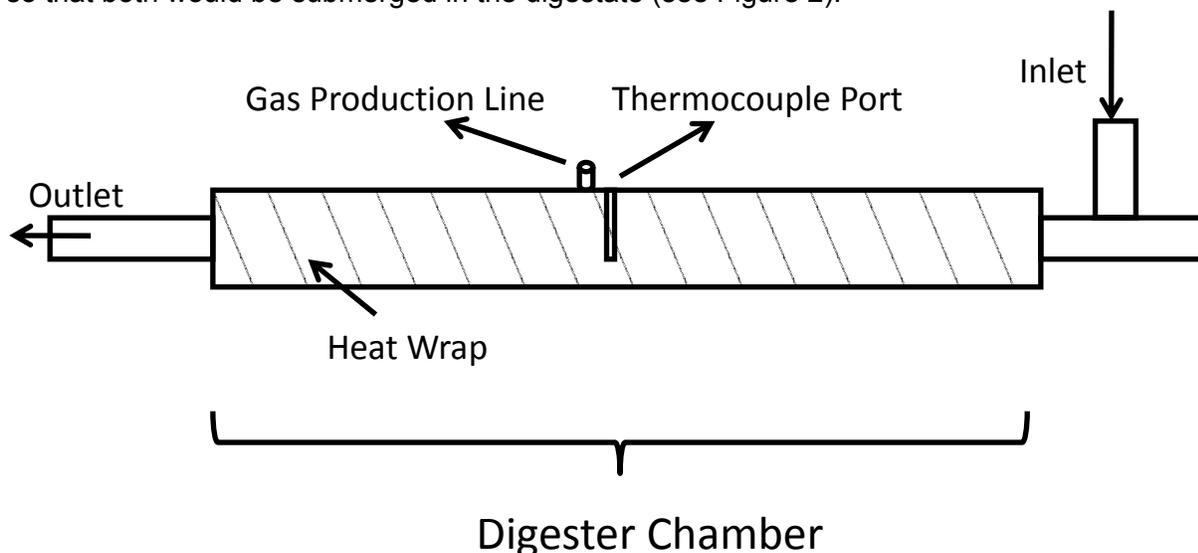
### **Sub Pilot-Scale Reactors**

Sub pilot-scale anaerobic digestion reactors were constructed out of 19.1-mm thick high density polyethylene (HDPE) piping with an inside diameter of 28.5cm. The HDPE pipes were cut to a length of 2.59 m and circular HDPE flanges were extrusion welded on the ends. Schedule 80 polyvinyl chloride (PVC) fittings were attached as shown in Figure 1.



**Figure 1. Photo of sub pilot-scale anaerobic digesters. Two of three sub pilot-scale 100-L, plug-flow anaerobic digesters. Reactors are aligned with flow counter to each other in this picture. Flow enters at stand pipes and exits through other side. Heat trace is wrapped around each reactor and covered with plastic bubble wrap insulation with a foil backing. Not shown is continuous temperature control is via a PC running LabView and continuous biogas monitoring via inverted tipping-bucket gas meters.**

Self-regulating heater cable (Nelson Heat Trace; HLT15-J; Tulsa, Oklahoma) was wrapped around the exterior of each digestion tube and connected to a 120 V wall outlet. Plastic bubble wrap insulation with a foil backing was wrapped around the pipe to reduce heat losses from the reactor. Two type-T thermocouples were placed in the reactor at the axial center, one at the radial cross sectional center of the pipe and the other about 50.8 mm from the internal surface so that both would be submerged in the digestate (see Figure 2).



**Figure 2. Preliminary design drawing (side view) for sub pilot-scale anaerobic digesters. Side view design drawing for sub pilot-scale anaerobic digesters. This diagram depicts the polyvinyl chloride (PVC) inlet and outlets, high density polyethylene (HDPE) digester chamber, copper thermocouple port, plastic gas production port, and heat trace.**

The temperature was collected and managed using LabView software (National Instruments) through a personal measurement device (PMD) connected to a PC. The program was set up in

a manner to control the temperature of each reactor at 35° C. A 6.35-mm gas port was installed on top of the pipe at the axial center of the digester body and was connected to an inverted tipping-bucket gas meter submerged in water. Each sub pilot-scale digester had a calibrated tipping-bucket gas meter that recorded gas production amounts using a magnetic reed switch via the LabView program. Methane content was determined using 1-L Tedlar bag samples that were measured using an infrared gas analyzer (NDIR-CH<sub>4</sub> Gasanalyzer, University Kiel, Germany). Prior to experimentation with glycerin as a potential toxicant, each digester was stabilized for 5 hydraulic retention times HRTs on the 22% beef manure, 16% corn processing wastewater, 14% short-fiber cardboard, and 48% lagoon liquid mixture as described by Sell et al. (2011). Each digester was manually fed in a semi-batch mode (twice per week) so that a 21-d HRT was maintained. All mixture components excluding glycerin were mixed every two weeks and stored at 4°C until ready for feeding. Glycerin was stored in sealed containers held at room temperature (22°C) and heated to ~35°C prior to mixing with feedstock using a microwave oven. This was done to increase viscosity and solubility for stirring prior to batch feeding.

## Results

Individual substrate characteristics results are shown in

**Table 1. Liquid samples were generally consistent, while solid materials had high variations in some measured variables from week to week (e.g., 15 – 30% TS in manure samples). Subsample results listed in**

Table 1 reflect an average of stockpiles, and we used representative samples for the ATA and BMP assays.

**Table 1. Characteristics of Selected Substrates.**

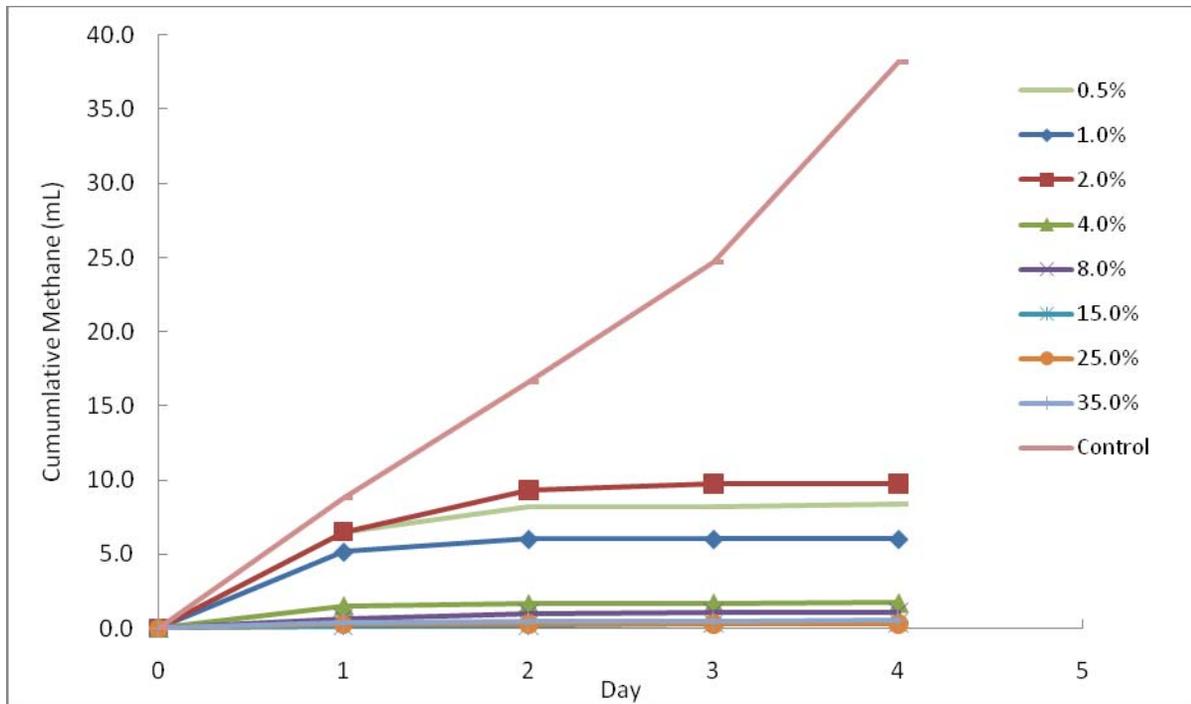
Substrate	TS (%)	VS (%)	pH	COD (mg/L or mg/g)	Ammonia (mg NH <sub>3</sub> -N/L)	Alkalinity (mg CaCO <sub>3</sub> /L)	BMP (mL CH <sub>4</sub> /g substrate)
<u>Off-Site Co-Substrates</u>							
Corn Processing Wastewater	8.3(0.05)	7.6 (0.05)	4.02	107,600(4,500) <sup>*</sup>	260(10)	0	20.2(3.2)
Short-Fiber Cardboard Waste	49.0(0.32)	39.4(0.19)	-	406(61)	400(80)	7,900(370)	82.0(6.2)
Glycerin	49.7(0.11)	43.6(0.11)	- <sup>1</sup>	>1,000,000	- <sup>2</sup>	- <sup>1</sup>	23.6(8.8)
<u>On-Site Materials</u>							
Lagoon Liquid	1.3(0.04)	0.9(0.03)	7.06	22,500(1,250) <sup>*</sup>	2,900(200)	8,560(400)	3.2(0.3)
Raw Manure	17.0(0.50)	14.0(0.81)	6.60	156(28)	1,980(280)	6,000(330)	14.2(2.6)

<sup>\*</sup>COD reported in mg/L. Values in parenthesis are standard deviations.

<sup>1</sup>pH and Alkalinity could not be accurately measure since glycerin stuck to pH probe and skewed readings.

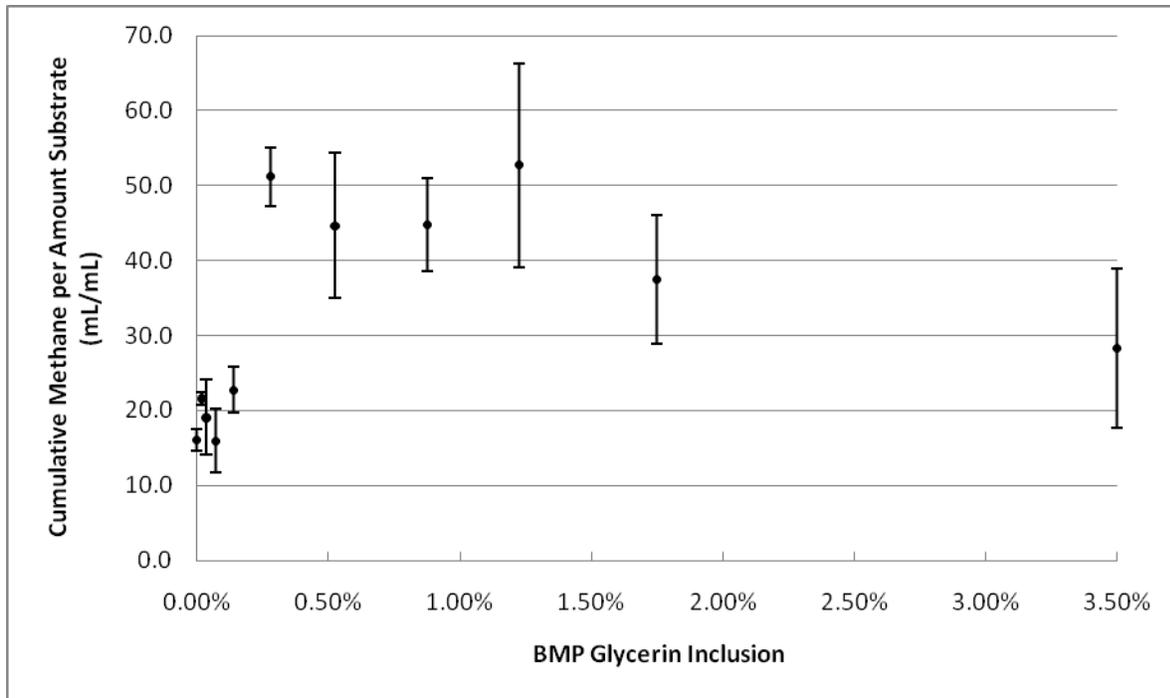
<sup>2</sup>Ammonia could not be measured on glycerin due to clogging and sticking of the distiller.

Figure 3 shows the results of the ATA performed on glycerin. It was expected that low glycerin inclusion rates would not cause overloading and would mimic or perhaps exceed the performance of the control. However, at all inclusion rates down to 0.5%, glycerin appeared to suppress methane production. The most likely cause was rapid hydrolysis and acidogenesis occurring from the glycerin and standard feedstock (sugar) which, in turn, dropped pH lower than the methanogens could overcome due to low alkalinity.



**Figure 3. Glycerin ATA.** This ATA was performed at various glycerin inclusion rates as noted in the legend. The control did not contain glycerin and represents the minimum slope for zero methane suppression.

Since the ATA did not provide any information how glycerin would perform when mixed with the baseline feedstock (48% lagoon liquid, 22% beef manure, 16% corn processing waste water, and 14% short fiber cardboard), a series of BMPs were performed with the mixed feedstock plus glycerin (see Figure 4). Glycerin was mixed into the baseline feedstock at inclusion percentages of 0.5%, 1%, 2%, 4%, 8%, 15%, 25%, and 35%. For calculation and comparison purposes 7 mL of each mixture was placed into each 200 mL BMP, thus reducing each glycerin BMP inclusion percentage to 0.0175%, 0.035%, 0.07%, 0.14%, 0.28%, 0.525%, 0.875%, 1.225%. For comparison BMPs containing only the 7 mL baseline feedstock, 7 mL of glycerin (3.5% BMP glycerin inclusion rate), and 3.5 mL glycerin/3.5 mL deionized water (1.75% BMP glycerin inclusion rate) were also performed.



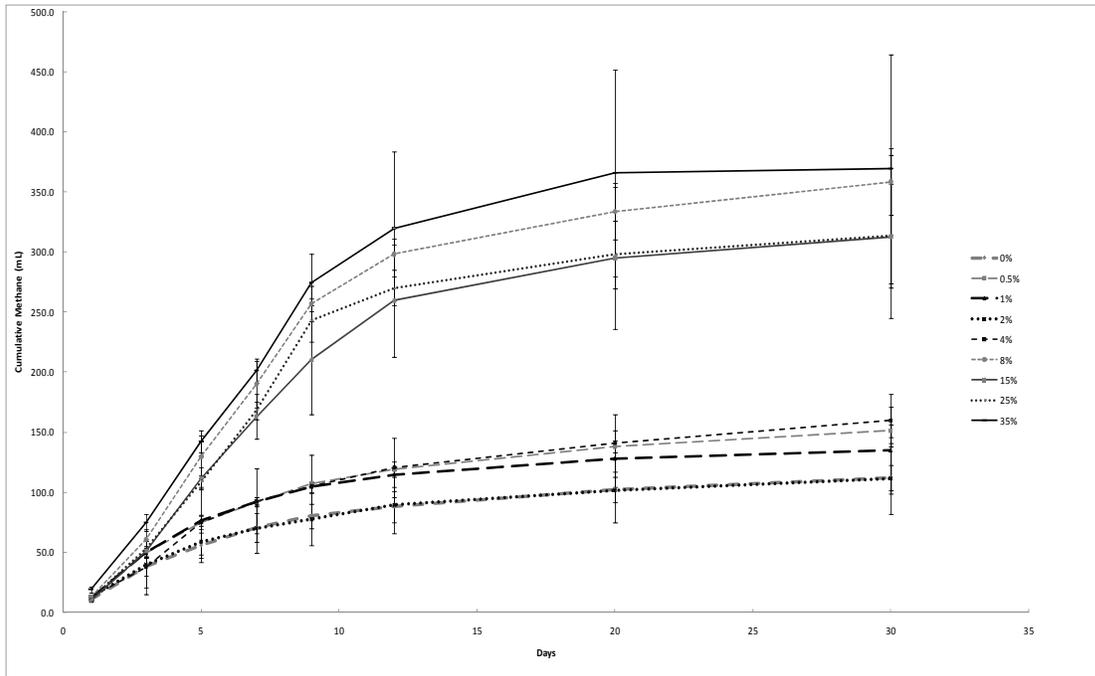
**Figure 4. BMP baseline feedstock and glycerin inclusion effects on methane production. This plot compares the BMP glycerin inclusion percentage with the cumulative methane produced per amount of substrate loaded on a mL of methane per mL of substrate basis. Error bars depict one standard deviation of the mean.**

The BMP results indicate that there were not significant differences for the addition of glycerin to the addition of the baseline feedstock for glycerin mixture inclusion rates of 0.5% to 4% (BMP inclusion rates of 0.0175% to 0.14%). However, upon increasing the glycerin mixture inclusion rate to 8% (0.28% BMP inclusion rate), the methane production more than tripled. The alkalinity provided by the baseline mixture appears to allow for higher methane production as seen by the lower results for the 3.5 mL glycerin/3.5 mL deionized water (1.75% BMP glycerin inclusion rate) and 7 mL glycerin mixtures (3.5% BMP glycerin inclusion rate).

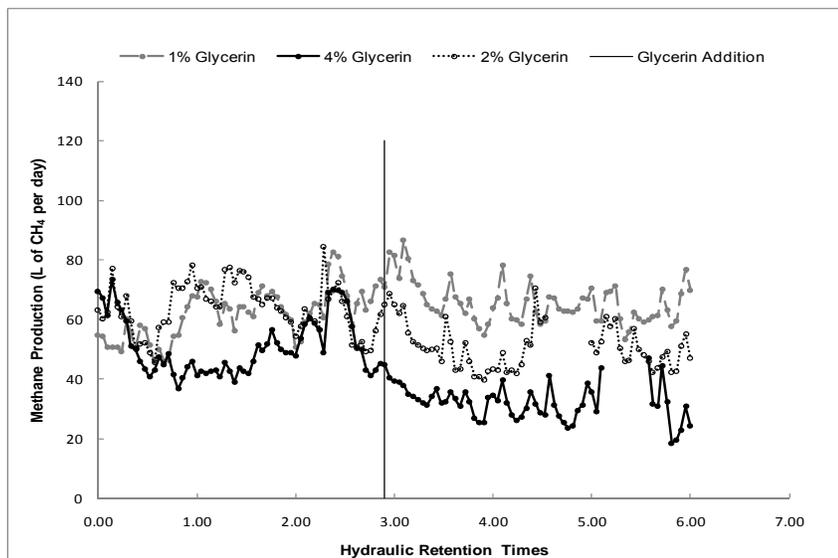
Figure 5 shows the cumulative daily methane production for each mixture of baseline feedstock and glycerin. Since 7 mL of each mixture was used in each BMP, the comparison of cumulative methane can be made without a correction factor. The first 10 d show rapid methane production for glycerin mixture inclusion rates between 8% and 35% (0.28% to 1.225% BMP inclusion rates) and the methane production is significantly different than the control (baseline feedstock) after the first 5 d. Since the glycerin mixture inclusion rates of 0.5% to 4% (BMP inclusion rates of 0.0175% to 0.14%) do not significantly differ than the control (baseline feedstock), there is a breakpoint between the 4% and 8% glycerin mixture inclusion rates that cause such drastic changes in methane production. The most likely cause is a balance between the amount of carbon or chemical oxygen demand loaded and the alkalinity present to withstand rapid hydrolysis and acidogenesis such that the methanogenic activity was not suppressed. Since these results do not take into account the act of continuous feeding, the sub pilot-scale reactors were performed as a means of comparison.

The daily methane production results for three sub pilot-scale reactors are shown in Figure 6. Results are shown in L of methane produced per d for three hydraulic retention times prior to the addition of glycerin and for three hydraulic retention times after the addition of glycerin. Prior

to the addition of glycerin, sub pilot-scale reactors were operated on the baseline feedstock for over seven hydraulic retention times as noted by Sell et al. (2011). The pre glycerin addition methane production was somewhat sporadic but after the addition of glycerin, each digester separated. At 4% glycerin inclusion, the largest drop in methane production was noticed.

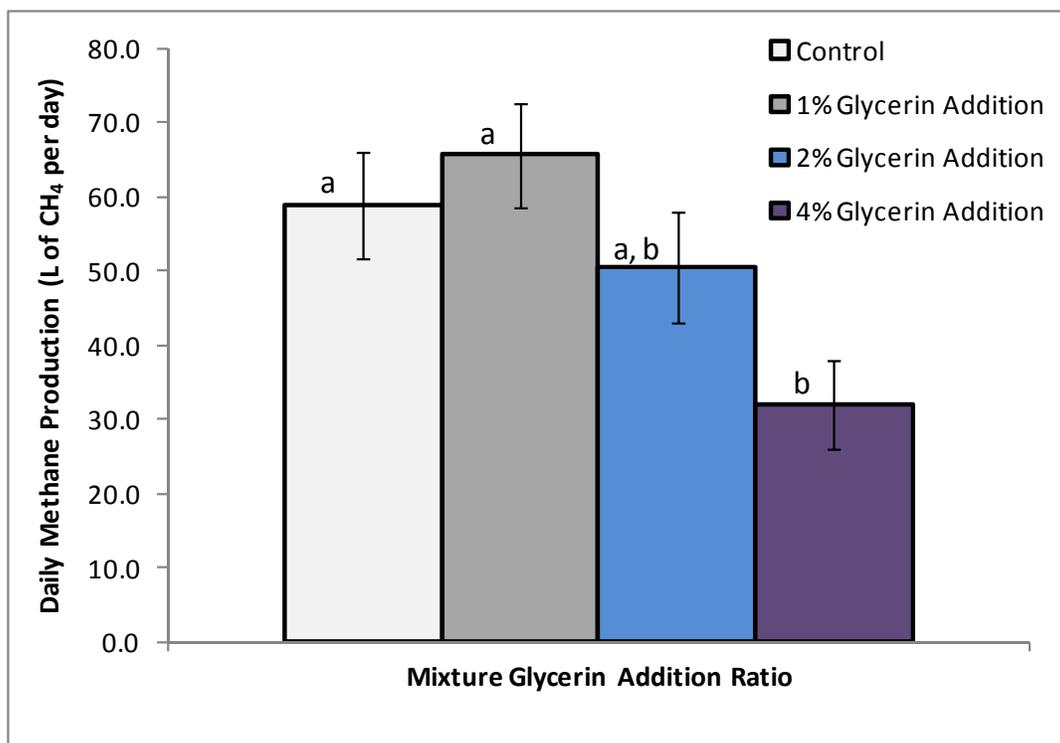


**Figure 5. Cumulative daily methane production for glycerin BMPs. Daily methane production for BMP mixtures containing 7mL of baseline feedstock mixed with various percentages of glycerin as noted in the legend. Error bars represent one standard deviation of the mean.**



**Figure 6. Sub pilot-scale daily methane production for glycerin mixtures. Daily methane production results are shown for three hydraulic retention times prior to glycerin addition and three hydraulic retention times with glycerin addition. One hydraulic retention time is equal to 21 d.**

Since the information in Figure 6 can be somewhat cloudy in interpreting the effect of glycerin addition on methane production, a bar graph depicting the average control (baseline feedstock) versus each average glycerin methane production is shown below in Figure 7. This graph shows that there were no significant changes during glycerin addition at 1% and 2%; however, at 4% the reduction in methane production is quite significant. There is a visible trend towards lower methane production with an increase in glycerin inclusion percentage.



**Figure 7. Bar graph of average glycerin methane production compared to control. This glycerin addition bars in this graph were formed using the average daily methane production for three hydraulic retention times of glycerin mixture operation. The control (or baseline feedstock) bar was formed using the average methane production for of all three sub pilot-scale reactors during the three hydraulic retention times prior to glycerin addition. Error bars represent one standard deviation of the mean. Similar letters represent treatments not significantly different from each other at a p-value = 0.05.**

Although the sub pilot-scale results do not explicitly match those of the BMP or ATA, they do exhibit some similarities. For instance, there does appear to be a toxicity effect from the overloading of glycerin as noted in the sub pilot-scale reactor loaded at 4% glycerin. There also seem to be little no significant changes in low glycerin addition amounts most likely due to the high alkalinity and buffering capacity of the baseline feedstock. It should be noted that the plug flow nature of the sub pilot-scale reactors was susceptible to short circuiting and perhaps settling of solids and more dense glycerin, resulting in poor contact between the glycerin feedstock and the entire microbial population in the digester.

## Conclusion

The use of ATAs and BMPs for selection of AD co-substrates and mixtures is a very critical first analysis tool. However, pilot scale studies are very beneficial in analyzing long term performance without the high risk of full-scale failure especially when selecting critical points of substrate addition. This is very important in the digestion of glycerin since it can double or even

triple methane production when combined in the right ratio but can be toxic if not combine with proper alkalinity and buffering capacity. This paper demonstrated an ATA that was performed with glycerin inclusion rates of 0.5%, 1.0%, 2.0%, 4.0%, 8.0%, 15%, 25%, and 35% by volume. A set of BMPs was also performed where a baseline mixture was combined with glycerin such that glycerin was 0.0%, 0.5%, 1.0%, 2.0%, 4.0%, 8.0%, 15%, 25%, and 35% of the combined mixture by volume. Control BMPs of 100% glycerin and 50% glycerin/50% DI water by volume were also performed. Three 100-L sub pilot-scale anaerobic digesters were operated at a 21-d hydraulic retention time (HRT) and were each fed in a semi-continuous manner twice weekly (6 loadings per HRT). Each digester was fed a combination of the mixture selected in paper one with a different amount of glycerin (1%, 2%, 4% by volume). The results of the batch-fed, stirred ATA indicate that glycerin was toxic to methane production at all inclusion levels. The batch-fed, stirred BMP indicated that there was no significant difference between methane production of the 0.0%, 0.5%, 1.0%, 2.0%, and 4.0% mixture combinations; however at 8.0% a triple in methane production was noticed. The continuously fed, non-stirred, plug-flow sub pilot-scale reactors indicated toxicity effects in the 2.0% and 4.0% glycerin mixtures and no difference from the control in the 1.0% glycerin mixture. These results demonstrate the variations in scale performance of glycerin as a co-substrate and identify some serious challenges in extrapolating bench-scale assays to large-scale performance of mixed-waste anaerobic digestion systems. Further study of mixing effects on glycerin inclusion rates is needed to indentify correct loading rates.

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