UNIVERSIDAD AUTÓNOMA CHAPINGO

POSGRADO EN INGENIERÍA AGRÍCOLA Y USO INTEGRAL DEL AGUA

"RESILIENCE EVALUATION TO OVERLOADING FEED DISTURBANCE IN THERMOPHILIC ANAEROBIC DIGESTION"

THESIS

A thesis submitted to Ingeniería Agrícola y Uso Integral del Agua at Universidad Autónoma Chapingo in partial fulfillment of the requirements for the degree of:

MAESTRO EN INGENIERÍA AGRÍCOLA Y USO INTEGRAL DEL AGUA

By

MARÍA DEL CARMEN GONZÁLEZ RANGEL

Chapingo, Texcoco, Estado de México, Oct 2014

1

RESILIENCE EVALUATION TO OVERLOADING FEED DISTURBANCE IN THERMOPHILIC ANAEROBIC DIGESTION

Thesis by Maria del Carmen Gonzalez Rangel under advisor committee direction, approved and accepted as partial requirement for the degree of:

MAESTRO EN INGENIERÍA AGRÍCOLA Y USO INTEGRAL DEL AGUA

DIRECTOR: DR. TEODORO ESPINOSA SOLARES

CO-DIRECTOR: PH.D. DAVID H. HUBER

ADVISOR: DR. GUADALUPE HERNÁNDEZ EUGENIO

ADVISOR: DR. NAGAMANI BALAGURUSAMY

AKNOWLEDGEMENTS

To Consejo Nacional de Ciencia y Tecnología (CONACYT) by economic supporting to realize the master degree studies. The support for project number 167896.

Thank to Universidad Autónoma Chapingo and Ingeniería Agrícola y Uso Integral del Agua to give me the opportunity and support to develop of this work.

I would like to thank my advisors Dr. Teodoro Espinosa Solares and Dr. David Huber for believe in my abilities and desire to work and give the opportunity to be part of their research team in México and West Virginia, USA.

I would like thanking to the rest of my committee for support and guiding Dr. Guadalupe Hernández Eugenio and Dr. Nagamani Balagurusamy.

Thank my family for give the opportunity to take my own decisions and always trust in me. Thank them for teaching me to fight and achieve my dreams. And thanks to my fiancé for encouragement and stay by my side.

My words of thanks to all my friends and classmates and everyone who was present during my research work.

Last, but by no means least, I thank to God for the entire blessing received.

DEDICATION

I would like to dedicate this thesis to my family, my father Antonio González, my mother Ma. de la Paz Rangel and my nine brothers: Ramón, Antonio, Juany, Greg, Cukis, Leo, Claudia, Miguel and Rosy. They are my biggest motivation to follow ahead, thanks them for the love and support not only now, all my life.

I would like also dedicate this work to my fiancé Jefferson U. Pérez for be patient and loving always, for believe in me and sharing dreams, laughs and love. I love you.

Thanks to all my friends and everyone who help me to my dreams come true.

BIOGRAPHICAL DATA

María del Carmen González Rangel was born on April 20th of 1988 in Querétaro, México, but she lived in Guanajuato, México. Son of Ma. de la Paz Rangel Vizcaya and Antonio González Gallegos. On July of 1994 she joined to elementary education in "Alvaro Obregón" school and finish in 2000. During 2000 and 2003 she attended secondary school in "Escuela Secundaria Técnica No. 19." On July 2003 she moved to Estado de México for high school at Universidad Autónoma Chapingo. At the same school she studied she's carrier on Ingeniería Agroindustrial ending in 2010. The next two years (2010-2012) she was working at "Rancho La Hondonada SPR de RL de IC" in Colón, Querétaro. In July 2012 she is accepted in "Ingeniería Agrícola y Uso Integral del Agua" master at Universidad Autónoma Chapingo.

TABLE OF CONTENTS

AKNOWLEDGEMENTS ii

DEDICATION iii

LIST OF FIGURES vi

LIST OF TABLES vii

ABSTRACT viii

INTRODUCTION 1

RATIONALE 9

OBJECTIVES 10

METHODS 11

- **Experimental strategy** 11
- **History of the digesters** 12
- **Laboratory digesters** 12
- **Physicochemical analysis of digester performance** 15
- **Molecular analysis: PCR and pyrosequencing** 17

RESULTS 20

- **Resilience evaluation** 27

CONCLUSIONS 37

RECOMENDATIONS 38

REFERENCES 39

ANNEXES 44

LIST OF FIGURES

Figure 1. Anaerobic digestion and microbial interactions source: Almeida et al. (2011). 4

Figure 2. Scheme of microbial changes presents when a disturbance take place and properties corresponding to each disturbance case, Resilience, Resistance and Functional redundancy (Allison and Martiny, 2008). 6

Figure 3. Experimental strategy for evaluating resilience in thermophilic anaerobic digesters. 11

Figure 4. Feeding strategy for overload pulse disturbance. Microbial diversity sampling are indicated by arrows 13

Figure 5. Design of pyro sequencing primers (454 Life Science Corp. and 2011). 19

Figure 6. Chemical responses of anaerobic digester to feed disturbances; Reactor D2 was used as control, D1 and D3 have been previously disturbed with stillage and glucose; D4 and D5 have never changed their feed scheme. 22

Figure 7. COD removed in percentage for each reactor.....23

Figure 8. Specific methanogenic activity profiles, using relation between methane produced and COD (Chemical Oxygen Demand) fed. 24

Figure 9. Principal components analysis for physicochemical performance related to the second pulse, 79.8% of variation is explained by two components. 27

Figure 10. Volatile fatty acids profiles of five digesters reported as equivalent-electron per liter (ee/L). 30

Figure 11. Principal components analysis based on stability parameters from the two pulses. The first component explains the 71.9% (declivity, resistance, stability to Propionate) and second component explained 16% (resilience and stability to acetate).....31

Figure 12. Bacterial composition by order, RDP classifier tool was used for classification with 80% confidence for five digesters (D2, D1, D3, D4 D5) during days -49 (T1), 48 (T2) and 55 (T3) of experimentation. 34

Figure 13. Principal Coordinate Analysis (PCoA) by unweighted UniFrac . Two components explain 29% of the variability. 36

Figure 14. Principal Coordinate Analysis (PCoA) with weighted UniFrac. Two components explain 67% or variability. 36

LIST OF TABLES

Table 1. Anaerobic digestion experiments at West Virginia State University, using poultry litter as main substrate. 8

Table 2. Digesters performance prior to disturbances. 20

Table 3. Chemical characteristics of the feed used in experiments. 20

Table 4. Biochemical data used to perform principal component analysis (PCA). Data correspond to second pulse. Labels represent digesters (D1, D2, D3, D4, and D5) and time before and after disturbances (day). 25

Table 5. Resistance, reactivity and declivity presented by five reactors during feed overloading. 28

Table 6. Resilience and stability parameters for acetate and propionate during feed overloading. .. 29

Table 7. Total number of sequences for each reactor after quality filtering. 32

ABSTRACT

RESILIENCE EVALUATION TO OVERLOADING FEED DISTURBANCE IN THERMOPHILIC ANAEROBIC DIGESTION

Anaerobic digestion is a biological process that involves different groups of microorganisms that interact in equilibrium to degrade organic matter. It has been used as effective technology in residual treatment for the recovering energy implied. The stability of process can be disrupted by changes in composition feed, overloading rate, hydraulic retention time, and it is characterize by accumulation of intermediate products as result of overgrowth and inhibition between groups of microbial community. Resilience is known as a recovery process after disturbance and it can has multiple routs. This study aims at knowing the resilience of anaerobic digestion system after receiving overloading feed pulses of different magnitude. Five anaerobic digesters of 10 liters of work volume and 20 days of HRT were used, feeding with poultry litter and located at West Virginia State University. One digester was used as control and the other four were disturbed increasing the feed concentration in terms of chemical oxygen demand (COD). Changes were monitored by volatile acids (VA) accumulation, chemical oxygen demand COD, nitrogen-ammonia, and methane content. Additionally, DNA samples were taken to analyze changes in microbial structure caused by disturbance. For DNA samples analysis, 454-pyro sequencing was used.

The results observed were accumulation of VAs, increase of COD, methane showed a stimulus after the stronger pulse. Volatile fatty acids were accumulated as

consequence of overloading. Microbial structure change as response to disturbance, *Clostridia* class was a representative class to increase the percentage when the maximum overloading feed pulse take place. The most representative orders were *MBA08, SHA-98, Clostridiales, BSA2B-08 and OPB-54* from *Clostridia* Class. Followed by *Thermotogales, Bacteriodales, and Synergistales.*

INTRODUCTION

Anaerobic digestion (AD) is a biochemical process represented by consortia of microorganisms that degrade organic matter, taken long carbon chain and transform in smaller compounds; this process is done under oxygen-free conditions and result in renewable energy resource (Chen et al., 2012, Kundu et al., 2014). Biogas production and effluent represent the economic advantages to develop this kind of technology.

Considered an effective treatment in residual handle (Shin et al., 2011), different substrates can be subject to treatment. Organic residuals are an important biomass sources and contribute significatively to the environment pollution (Chen et al., 2012). It converts organic residual as perfect candidate for anaerobic treatment. The efficiency of process depends on physical variables such as: temperature, organic loading rate, hydraulic retention time (HRT), solid concentration, and substrate composition. AD is a process, which involves a complex microbial structure and the interaction among several microorganisms. These relationships define the flow of intermediate products and dependent on chemical composition and operational conditions of the process.

Anaerobic digestion takes place in different environments such as wetlands, animal and human tract, rice fields, activated sludge, and lakes sediments (Hamberger et al., 2008, Bolzonella et al., 2005). Anaerobic digestion process presents advantages as contribution to reduction of pollution through dismissing methane emissions. It is an alternative to fossil fuels, pathogens and weed reduction, mass reduction and stabilization (Madsen et al., 2011, Luste and Luostarinen, 2010). AD has become an

environmental key technology for producing energy and fertilizer friendly to the Earth. It uses biomass as bioenergy source of low impact and it is able to use several substrates, principally organic residuals. It has been widely used for the economic and environmental benefits that it represents.

Animal and agricultural residuals are a source of environment pollution and represent a public health risk and contaminate drinking water sources. Food industries, at the same manner generate a large amount of residuals, which have high levels of organic matter. All previous and other residuals are subject to be treated by anaerobic digestion. Several papers have reported on residual treatment using AD, with cow manure (Kaparaju et al., 2008, Neves et al., 2009, Sutaryo et al., 2012, Cavinato et al., 2010, Yue et al., 2013, Comino et al., 2009), poultry and chicken residuals (Zhang et al., 2011, Salminen and Rintala, 2002, Bujoczek et al., 2000, Sharma et al., 2013), agro-industrial and agricultural residuals (Álvarez et al., 2010, Nges et al., 2012), municipal residuals (Hartmann and Ahring, 2005, Macias-Corral et al., 2008), and co-digestion with materials from diverse source.

Fluctuation in organic loading rates and changes in residual composition are frequently encountered in the anaerobic digestion of animal residuals (Chen et al., 2012). Recently studies on anaerobic digestion process have shown that the overloading feed leads a disruption on the system which is characterized by the accumulation of the organic acids and alcohols(McMahon et al., 2004). The presence of high levels of volatile fatty acids (VFAs) affects the biogas yield; specifically propionic acid has a harmful impact on methanogens (Rétfalvi et al., 2011). Methanogesis is considered as the limitation step under perturbation conditions (Chen et al., 2012).

Anaerobic digestion process is susceptible to environmental changes that can disrupt the stability into the reactor. Resilience is a quantitative response to disturbance and is the reciprocal to return time to the steady state (Neubert and Caswell, 1997, Hashsham et al., 2000). The responses to changes are characterized by an accumulation of organic acids; therefore volatile fatty acids evaluation is a good indicator of resilience.

In the anaerobic digestion process first, the complex polymers are broken by hydrolytic bacteria and transform in monomers and long-chain fatty acids. Fermentative bacteria are responsible to produce volatile fatty acids (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate) and alcohols (methanol and ethanol) from monomers (Ahring, 2003). Using acetate, acetoclastic methanogen archaea convert it into methane. On the other hand, hydrogenotrophic methanogen archaea take hydrogen and carbon dioxide to produce methane. The remaining volatile fatty acids and alcohols through hydrogen-producers bacteria are reduce to acetate (see Figure 1). An alternative pathway to acetate degradation involves acetate oxidizing bacteria (pathway 5 in Figure 1) and hydrogenotrophic methanogens archaea (pathway 7); it is a syntrophic acetate oxidation. This relationship is characterized by exchange of metabolites, but in slow concentrations and requires and reverse electron transfer (Rivera-Salvador et al., 2014).

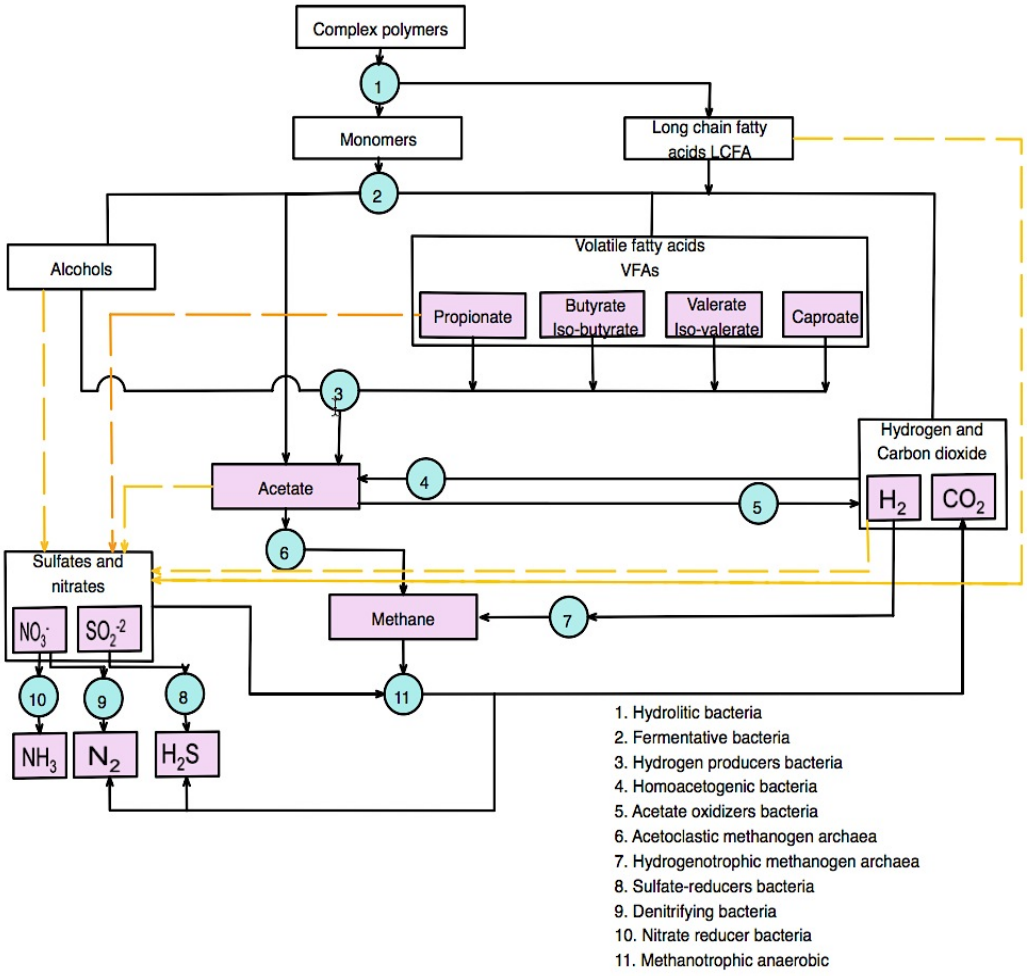


Figure 1. Anaerobic digestion and microbial interactions source: Almeida et al. (2011).

Several works are oriented to optimize the AD process. For example, research on different routs that the microorganisms follow to degrade substrates (Hashsham et al., 2000); effects of heating strategies to increase the biogas production (Espinosa-Solares et al., 2009); mixing effect in anaerobic co-digestion and probe different overloading rates (Stroot et al., 2001). Other research groups have reported the relationship of microbial communities in AD. For example, Shade et al. (2012) it has been shown that there is a close influence of physicochemical parameters on community structure

(Shade et al., 2012, McMahon et al., 2004). Shen et al. (2013) reported innovative techniques to explore composition, structure and correlations in microbial community.

The anaerobic digester system requires an integrated group of specialized microorganisms that function as ecological community. Environmental microbiology provides accumulation of biological information and contextual environment parameters, it can clear up on the environmental conditions, temporal and spatial distribution, and natural fluctuations on the system for explain patterns shed by all information (Hughes and Bohannan, 2004, Ramette, 2007). Microbial ecology intended to explain qualitative and quantitatively the responses of microbial diversity to environment changes, this changes are known as disturbances(McMahon et al., 2004, Shade et al., 2012). High-throughput molecular technologies have been developed for DNA analysis. DNA sequences are being accumulated at an unprecedented rate and technologies as pyro sequencing, Denaturing gradient gel electrophoresis and 16S rRNA clone libraries are used to extract DNA sequences and aided with advanced software's and databases analyze the obtained information (Shen et al., 2013). Microbial diversity usually examines diversity of operational taxonomic unit (OTU) and in 16S rRNA genes is assumed that the diversity of this gene is correlated with the overall diversity of bacteria. An important advantage of sequences is because it is the unique way to have robust estimates about diversity of complex communities; this method includes organisms that had not be cultivated or isolated in laboratory (Dunbar et al., 2000).

Disturbances are defined as causal events that alter the community environment or directly the community, affecting the ecosystem processes (Shade et al., 2012, Allison and Martiny, 2008). Several studies had developed techniques to assessing the effect of

disturbances and modeling. Those, after knowing the growth rates, effect of phenotypic change on genotypic fitness and lead to design strategies for abate the negative aspects and/or promoting the benefits of these disturbances (Hodgson et al., 2006, Rozdilsky et al., 2004, Neubert et al., 2002, Aikio, 2004).

General disturbances are classified in two kinds as follows: pulse and press disturbance. The first one is characterized as short-term and discrete. After a pulse disturbance is expected that community return to its original state. On the other hand, press disturbances are continues and long-term, this kind can change definitively the structure and dynamic of the community, leading the community to envelope to an alternative stable state (Shade et al., 2012). When a disturbance takes place it is possible to evaluate the ecological stability by resilience, resistance, and functional redundancy (Allison and Martiny, 2008).

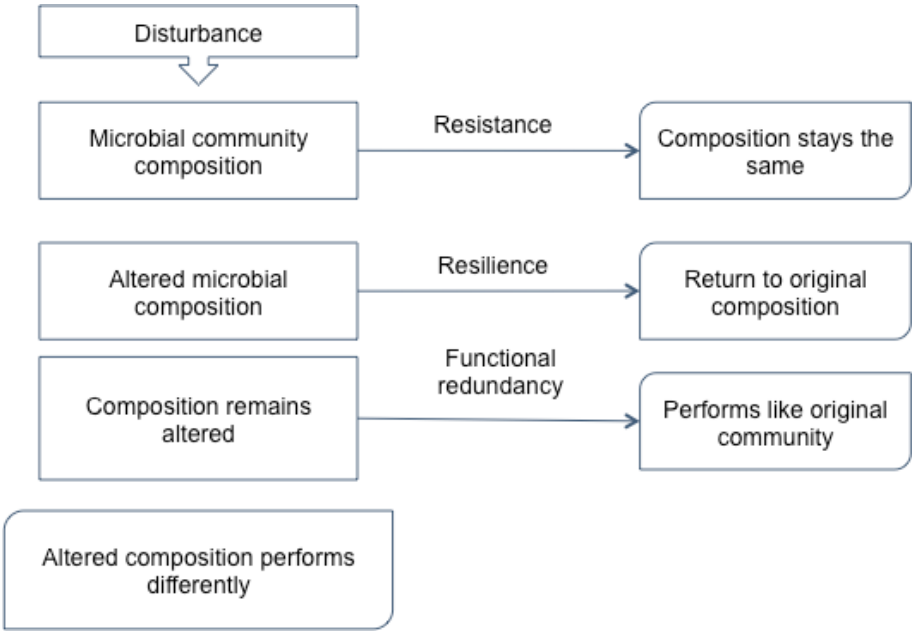


Figure 2. Scheme of microbial changes presents when a disturbance take place and properties corresponding to each disturbance case, Resilience, Resistance and Functional redundancy (Allison and Martiny, 2008).

Our research group in West Virginia State University has carried out previous researches on chicken litter and thermophilic anaerobic digestion. Optimization methods, a variety of control parameters as temperature and heating and energy economy of the system presented COD of 50,148 to 52,000 mg L^{-1} COD, 56.4 to 65.1 methane percentage and specific methanogenic activity (SMA) 0.252–0.311 m^3 $kgCOD^{-1}$ day^{-1} (Espinosa-Solares et al., 2010, Espinosa-Solares et al., 2009, Valle-Guadarrama et al., 2011); effects of feed frequencies (Bombardiere et al., 2007) showed similar performance with 55.9 methane percentage and 0.207 m^3 $kgCOD^{-1}$ day^{-1} of SMA. Smith et al. (2014) reported microbial structure of bacteria and archaea. It suggest that metabolic properties, stable performance, and rather properties of poultry litter as only source of feed indicate that this microbial community possesses properties that may characterized this type of anaerobic digesters. Rivera-Salvador et al. (2014) evaluated the hydrogenotrophic methanogens predominance and propose this pathway as principal in control of acetate levels in this reactors. The works are presented in Table 1.

Table 1. Anaerobic digestion experiments at West Virginia State University, using poultry litter as main substrate.

Reference	Objective	Digester	COD fed	VA effluent (mg/L)	CH_4 %	HTR (Days)	SMA (m^3 $kgVS^{-1}$ day^{-1})	SMA (m^3 $kgCOD^{-1}$ day^{-1})
(Espinosa-Solares et al., 2006)	To evaluate the performance of an anaerobic process, using a mass and energy balance and chemical characterization	Pilot-plant 40 m^3	44,366 mg L^{-1}	3546 to 6735	55.3 ± 0.6	10	0.281 m^3 $kgVS^{-1}$ day^{-1}	0.232 (m^3 kg COD^{-1} day^{-1})
(Bombardiere et al., 2007)	To test the effect of different feed frequencies on performance and stability	Pilot-plant 40 m^3			55.9 ± 0.6	29.5		0.207 m^3 $kgCOD^{-1}$ day^{-1}
(Espinosa-Solares et al., 2009)	To evaluate whether heating strategy in a thermophilic anaerobic pilot plant, can modify power consumption and whether these changes are reflected in the biogas production.	Pilot-plant 40 m^3	50,148 mg L^{-1}		61	32 to 37	0.330 to 0.451 m^3 $kgVS^{-1}$ day^{-1}	0.252 to 0.311 m^3 $kgCOD^{-1}$ day^{-1}
(Espinosa-Solares et al., 2010)	To determine the effect of temperature on methane production.	Pilot-plant 40 m^3	52,000 mg L^{-1}		56.4 - 65.1	40		
(Valle-Guadarrama et al., 2011)	To develop a model able to predict the temperature variations, under different experimental conditions.	Pilot-plant 40 m^3	50,148 mg L^{-1}		61	32 to 37	0.330 to 0.451 m^3 $kgVS^{-1}$ day^{-1}	0.252 to 0.311 m^3 $kgCOD^{-1}$ day^{-1}
(Sharma et al., 2013)	To test whether a thermophilic, could accommodate thin stillage during co-digestion, and to test the limits of thin stillage as co-substrate	Lab-scale D1, D2, and D3	48,000 mg L^{-1} by 24 days	4600 to 5100	62.28 ± 3.38	15		0.408 m^3kg COD^{-1} day^{-1}
(Smith et al., 2014)	To analyze the ecological structure of the bacterial and archaeal community of this unique digester	Pilot-plant 40 m^3	61,451 mg L^{-1}	2160	57.7 ± 2.2	29.5		0.34 m^3 $kgCOD^{-1}$ day^{-1}
(Rivera-Salvador et al., 2014)	To incorporate bacterial acetate oxidation into ADM1 as the main acetate degradation process.	Lab-scale D1, D2, and D3	25,000 mg L^{-1} 0.66L d^{-1}		62.28 ± 3.38	15		

RATIONALE

Ecosystem behavior is difficult to predict to external disturbances. Microbial populations are an important actor in the maintenance of equilibrium and stability in ecosystems. In fact, knowledge that enables predictions responses to disturbances could be the key for designing strategies to changes support and facilitate recovery process. Changes in the diversity and dynamics of microorganisms to disturbances may provide tools for understanding interactions within microbial communities (Shade et al., 2012, Shin et al., 2011).

Understanding the dynamics that follow populations in response to changes in their environment, leads us to the study of the properties that characterize these communities such as resilience, resistance and redundancy. In ecology, resilience is recognized as a property that allows individuals to adapt to the unpredictability of ecosystems (Gunderson, 2000). During resilience two aspects has to be considered, the steepness of slope and sides of the curve because this characteristics define the return time to the steady state of the system (Beisner et al., 2003). The accumulation of major soluble products was utilized in response to the perturbation, isoforms of butyric and valeric acids can remain in the medium for long time (Raposo et al., 2008).

This study aims to measure the resilience of a system in steady state after receiving a pulse overload of feed and to describe the changes that occur in the structure of the microbial community during the recovery period.

OBJECTIVES

The overall objective is to evaluate the resilience of thermophilic anaerobic digesters to a disturbance caused by overloading feed.

The specific objectives of this study are:

1. To compare responses and recovery capability of anaerobic reactors to two overloading pulses, with different magnitudes.
2. To determine resilience, and resistance in the reactors as a response to overload disturbance pulses.
3. To describe structural changes in the microbial community before and during the resilience period and to observe the response of reactors, which have been subjects of different treatments previous their stable state.

METHODS

Experimental strategy

Figure 3 presents the graphical description of the experiments carried out. Reactors with different histories were disturbed twice with different levels. The performance of them as well as the microbial population was monitored. These behaviors were analyzed using resilience evaluation and statistical analysis. The details of this are presented in the following lines.

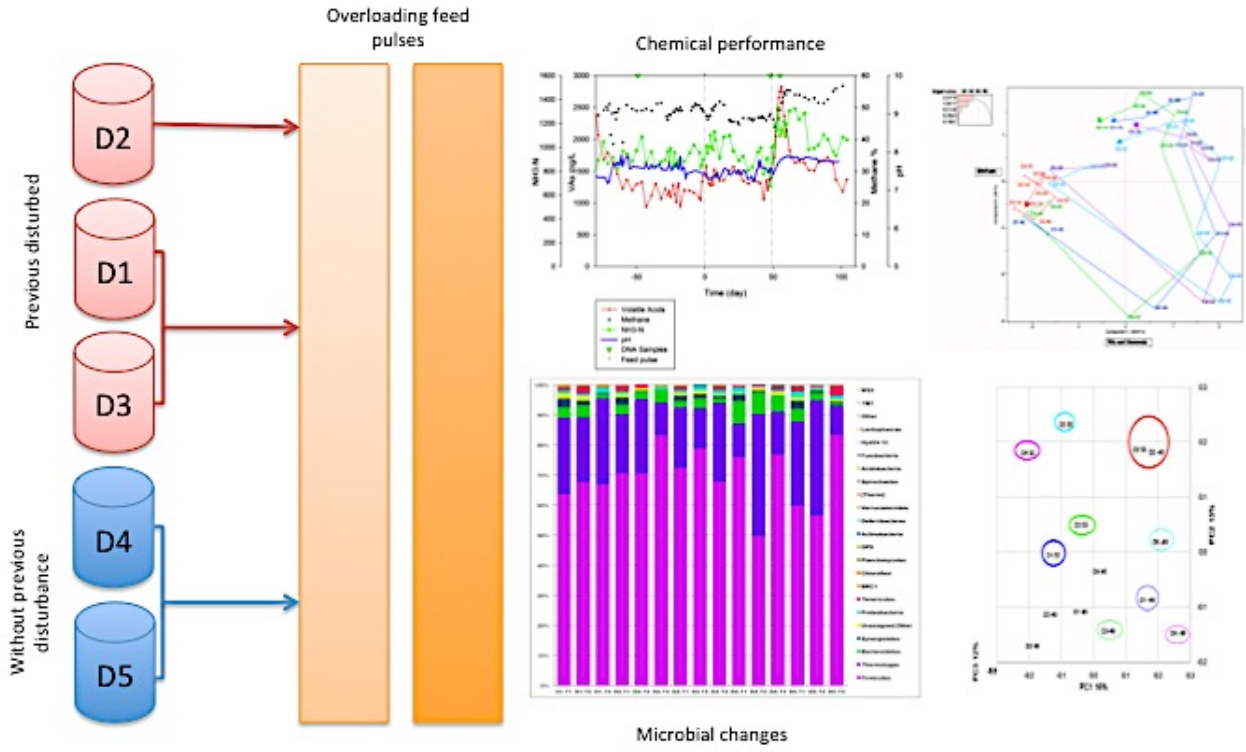


Figure 3. Experimental strategy for evaluating resilience in thermophilic anaerobic digesters.

History of the digesters

Since 2001, a pilot plant anaerobic digester at West Virginia State University has been operated at steady state and under thermophilic conditions (56 ˚C). The digester is a 40m^3 continuous stirred tank reactor (CSTR) and it is fed with diluted poultry litter (Espinosa-Solares et al., 2006). The laboratory scale digesters were inoculated from the pilot plant. Digesters D1, D2, and D3 were started in June 2008, they have received different treatments since then. First, the digesters were stabilized with poultry litter and then D1 and D3 were subjected to a co-digestion experiment with thin stillage Sharma et al. (2013). After this treatment, the digesters were returned to steady state operation with 100% poultry litter. Digesters D1, D2, and D3 were then used by Montenegro (2012) to evaluate response of digesters to a glucose pulse disturbance. Digesters D4 and D5 were started in August 2011 and they have only received poultry litter substrate. They have remained in steady state for almost three years.

Laboratory digesters

The experimental reactors were located at The Environmental Microbiology Laboratory at West Virginia State University, WV, USA. Five laboratory-scale digesters were operated at a thermophilic temperature (55.5±0.5˚C) and fed with poultry litter. The working volume was 10 liters with 3 liters of headspace. The hydraulic retention time (HRT) was 20 days. Each reactor was a cylindrical vessel with a round bottom and a glass jacket for heating with circulated water using a pump (VWR model 1104). Each

reactor had a thermocouple for monitoring temperature, pH (Cole Parmer), a magnetic pump (Iwaki CO., LTD model MD-6L) for recirculation and a peristaltic pump (Gorman-Rupp Industries, model 3600-006 S 115V) for feeding. The biogas production was measured with a made tipping flow meter that use volume displacement and a position sensor. The recirculation pumps were programmed at intervals of two hours and feed pumps for each hour. The experimental set up is shown in Figures 3 and 4. Pulse disturbances were applied to four digesters (D1, D3, D4, and D5), the fifth digester was used as control (D2); all of them were in similar conditions. Deviation from steady state was measured during the pulse and during the recovery period. The pulse disturbance consisted of increasing the concentration of the feed in terms of chemical oxygen demand (COD) as is showed in Figure 4.

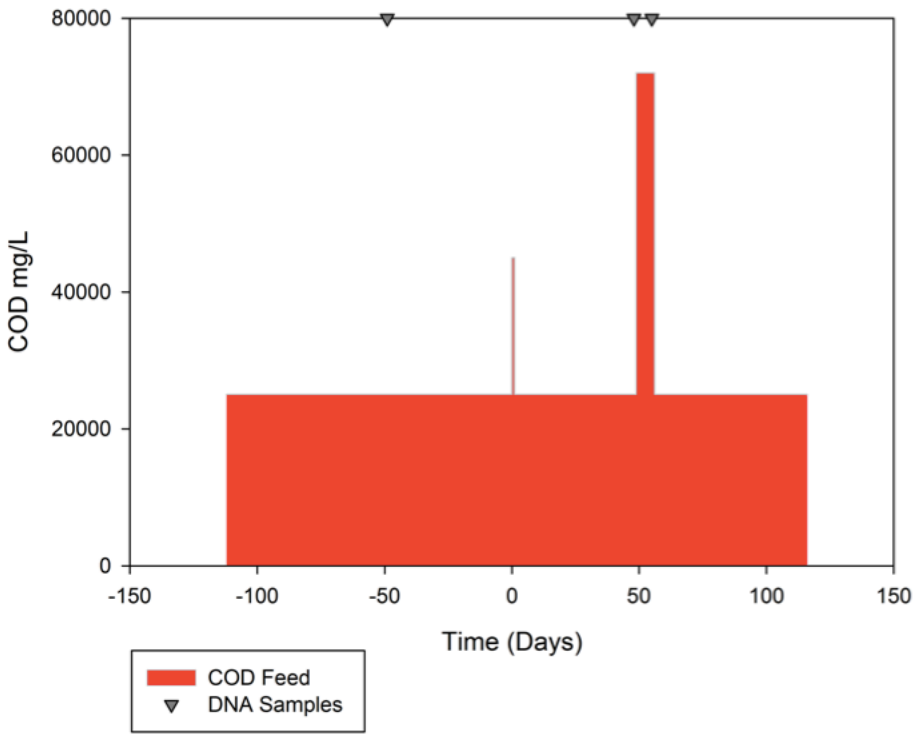


Figure 4. Feeding strategy for overload pulse disturbance. Microbial diversity sampling are indicated by arrows

The poultry litter feed was obtained from West Virginia and Virginia poultry farms. The feed was prepared as follows: poultry litter was mixed with water during two days and then was filtered and pressed to obtain a concentrated solution. The COD of the feed was analyzed and then diluted to reach the desired concentration around 25,000 mg L^{-1} COD. Total solids (TS) were 2.2 ± 0.07%, volatile acids (VAs) were 2000 ± 260 mg/L (as acetic acid), Nitrogen-ammonia was 568 ± 54 mg/L, and pH was 6.5 ± 0.2. Feed was prepared in batches of 90 L and stored at 4˚C until it was used.

All digesters exhibited quasi-stable operation with consistent pH, methane yield, ammonia and volatile acids (VAs) levels before the initial pulse disturbance. Each digester received 500 mL poultry litter feed that was distributed as 24 fractions (one each hour). For the first pulse disturbance, four digesters received a pulse of poultry litter feed with a concentration of 45,000 mg L^{-1} of COD for a period of 24 hours. The control digester was maintained with the normal feed (25,000 mg L^{-1} of COD). After the pulse, samples were taken every day during the first 7 days and then twice per week for two HTR periods.

The second test of resilience was conducted with a larger pulse of feed that could cause a COD overloading. The dynamic was different: the pulse was maintained for six days until significant change in volatile acids was observed. Four reactors were fed with poultry litter concentrated to 72,000 mg L^{-1} and the control digester was fed with the regular feed (Figure 4). The performance of the digesters was observed during two HRTs.

Physicochemical analysis of digester performance

COD, nitrogen-ammonia and volatile acids (VA) analysis were performed according to methods 8000, 8196, and 10031 in the HACH Water Analysis commercial kits (HACH, 2013). Biogas was sampled using gas syringes. Methane content was measured using a gas chromatograph equipped with a thermal conductivity detector (TCD), column HP-PLOT/Q (length 30m, diameter 0.530mm, and film 40μm) (model 7890A GC System, Agilent Technologies). Helium was used as the carrier gas. 100μl samples were injected with the gas tight syringe. Volatile fatty acids (VFAs) in the effluent samples were quantified using the same gas chromatograph equipped with a flame ionization detector (FID), column HP-FFAP (length 30m, diameter 0.320mm, and film 0.25μm). The amounts of all soluble intermediate VFAs were normalized to the maximum value by expressing the levels of compounds in terms of the available electron equivalents (ee), as reported by Hashsham et al. (2000).

To measure quantitatively the functional stability, we adapted the method whereby the amplification envelope of key intermediate products was measured in response to a perturbation methodology (Hashsham et al., 2000). Changes in the VFA profile were followed as the response to the perturbation. The amounts of all soluble intermediate VFA were normalized by expressing the levels of compounds in terms of the available electron equivalents (ee), as reported in the literature (Hashsham et al., 2000). Taking into account that resilience was considered as a quantitative characteristic of the system after perturbation (Neubert and Caswell, 1997), which was defined as the

reciprocal of return time, we introduce the term resilience, as a measure of how rapidly a system in steady state returns after a perturbation. For the calculus, resilience was considered as the reciprocal of the apparent-return time. The apparent-return time for each intermediate product was considered as the time required to go from the minimal value of a specific compound in the steady state condition to the minimum value of that compound in the return to steady state. Maximum-reactivity was another parameter considered to evaluate the stability of the system. It was considered as the maximal instantaneous rate at which perturbations can be amplified or the maximum slope of the rising limb of the envelope (Hashsham et al., 2000, Neubert and Caswell, 1997).

Declivity was previously defined as the average slope of the declining limb of the envelope (Hashsham et al., 2000). This was also calculated in order to compare the net rates of utilization of the soluble compounds. However, we report here a maximum-declivity as the maximum slope of the declining limb of the envelope, as an indicator of the maximum capacity of the system to deal with intermediate compounds.

An additional parameter that was considered for the stability analysis was the sum of the first moments of all envelopes of each compound (Hashsham et al., 2000). Equation 1 shows the calculus for the sum of first moments, while Equation 2 indicates the contribution in percentage of each *ith* compound to the moment.

$$M = \sum_{0}^{i} A_i \cdot t_i \tag{1}$$

$$M_i = \frac{A_i \cdot t_i \cdot 100}{\sum_{0}^{i} A_i \cdot t_i} \tag{2}$$

Where *Ai* is the area under the envelope of the *ith* compound and it is the moment arm for the envelope of the *ith* compound given by the distance from the center of gravity of the envelope to the perturbation axis. Equation 2 indirectly incorporates all the parameters of stability, including the effect of the shape of the amplification envelope. Like resistance, a higher numerical moment of area denotes lower system stability (Hashsham et al., 2000).

The apparent return time was taken in first pulse 11, 8, 18, and 21 days for digesters D1, D3, D4 and D5 respectively. For second pulse the days were 17, 16, 16 and 17 days respectively. In this work the stability was considered as the reciprocal of the sum of the moments, as it is presented in Equation (3) for all acids.

$$S = \frac{1}{M} \tag{3}$$

Molecular analysis: PCR and pyrosequencing

Samples of effluent were kept on ice in 35 mL tubes prior to DNA extraction. Samples were centrifuged at 10,000 rpm for 30 minutes using a refrigerated centrifuge (Sorvall® RC-5B, Du Pont Instruments). Pellets were collected under sterile conditions in 1.5 mL tubes and stored at -80°C for DNA extraction. DNA extraction was done using the PowerSoil® DNA isolation kit (MO BIO Laboratories, Inc., CA). Agarose gel electrophoresis was used to observe the quality and quantity of DNA extractions. A 0.8 percent agarose gel in 1X TAE buffer was run at 85 V for 35 minutes for all samples. Additionally, DNA concentration and quality were measured with a Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Inc., MA).

Bacteria 16S rRNA genes were amplified by polymerase chain reaction (PCR) targeting specifically the hyper variable V4 region. The forward primer corresponded to *E. coli* position 563 to 577 and the reverse primer corresponds to position 785 to 802. A set of twelve primers was used where each set had a different multiplex identifier (MID). The forward primer was 5'-AYTGGGYDTAAAGNG-3', and the reverse primers were R1, 5'-TACNVGGGTATCTAATCC-3', R3, 5'-TACCRGGGTHTCTAATCC-3', and R5, 5'-CTACDSRGGTMTCTAATC-3'. The mix of three reverse primers were in a molar ratio of 12(R1): 6(R3): 2(R5) according to Sul et al. (2011). Each primer had sequencing adaptor A or B, a key sequence, MID tag, and a sequence corresponding to the V4 region of 16S rRNA genes (Figure 2). The PCR mixtured contained 1.0 µL of each primer, 2.5 µL FastStart 10X Buffer #2, 0.5 µL dNTP mix (10 mM each), 1.25 units of FastStart High Fidelity Polymerase (Roche Applied Science), and 15-20 ng of DNA template in a total volume of 25 µL. PCR was done in a thermal cycler (model 2720, Applied Biosystems). The PCR conditions were as follows: incubation temperature at 94˚C for 2 minutes, followed by 30 cycles of 94˚C 45 seconds; 57˚C 45 seconds, and 72˚C 1 minute. A final extension of 4 minutes at 72 ˚C was done after the 30 cycles. Each sample was done in triplicate and run at the same time. PCR products were evaluated with agarose gel electrophoresis at 110 volts for 35 min in 1X TAE buffer. Amplicon bands were excised from the gel and purified by QIAquick gel extraction kit (Qiagen, MD). Amplicon extraction was confirmed by 0.8% agarose gel in 1X TAE Buffer for 35 min at 110 volts.

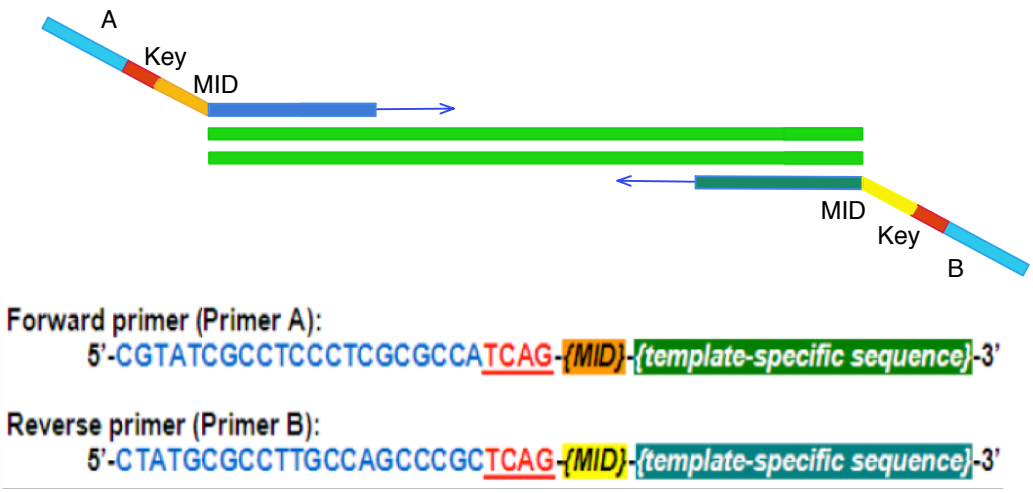


Figure 5. Design of pyro sequencing primers (454 Life Science Corp. and 2011).

Protocols used for amplicon library preparation, emulsion PCR and pyro sequencing were those of the manufacturer (Roche Applied Science, IN). Sequencing was done using the 454 GS Junior system (Roche Applied Science, IN) and processed by the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). The sequences generated from different samples were separated using their nucleotide barcode and filtered with a minimum score of 25 and the standard parameters of QIIME. Sequences were clustered as Operational Taxonomic Units (OTUs) with similarity of 97%, which represents the species level. OTUs were analyzed using the Ribosomal Database Project (RDP) Naïve Bayesian Classifier (Wang et al., 2007).

RESULTS

Prior to the overload feed pulses the replicate performance was monitored. The average of VA level, pH, methane percentage, Ammonia concentration, and COD removed are presented in Table 2. Also the chemical characteristics of regular feed and the feed used for two pulses are showed in Table 3. Values presented are the mean and standard deviation (Mean ± SD).

Table 2. Digesters performance prior to disturbances.

Digesters	VA (mg L^{-1})*	pH *	Methane (%) *	Ammonia (mg L^{-1})*	COD removed (%)*
D2 (control)	1537 ±466	7.3 ±0.1	48.0 ±1.9	956 ±474	55.3 ±27.6
D1	1317 ±342	7.5 ±0.5	48.5 ±2.8	911 ±450	56.8 ±28.2
D3	1288 ±242	7.4 ±0.1	46.1 ±9.6	969 ±480	54.6 ±27.4
D4	1404 ±433	7.5 ±0.4	49.8 ±3.3	964 ±477	54.6 ±27.2
D5	1371 ±321	7.5 ±0.1	51.9 ±2.1	943 ±467	57.2 ±28.3

*Values represent mean ± standard deviation

Table 3. Chemical characteristics of the feed used in experiments.

Parameter	Regular feed*	First pulse	Second pulse
TS (%)	2.2 ±0.1	4.5	6.6
COD (mg/L)	24,777 ±229	45,416	71,958
VA (mg/L)	2,012 ±260	3,263	5,549
pH	6.5 ±0.2	5.5	5.3
NH_3-N (mg/L)	568 ±55	894	

*Values represent mean ± standard deviation

Figure 6 shows the performance of the reactors under overload feed pulses as well as the control. Values of pH indicate the adequate condition for AD, in disturbed reactors a

variation was observed. However, the value of pH was still suitable for acidogenic and methanogen microorganisms development. Volatile acids were stables starting at -50 days to before day 0 (zero); the digesters seem to have similar performance. At first pulse, small changes were observed, however biogas production was reduced as it can be seen in Figure 6. For the second pulse, there are three different behaviors. This can be attributed to the previous history of the digesters. First, control digester, which was not disturbed; digesters D1 and D3 whom had received previous treatments before this research (stillage co-digestion and glucose pulses), and the last one for digesters D4 and D5 that had never been disturbed before.

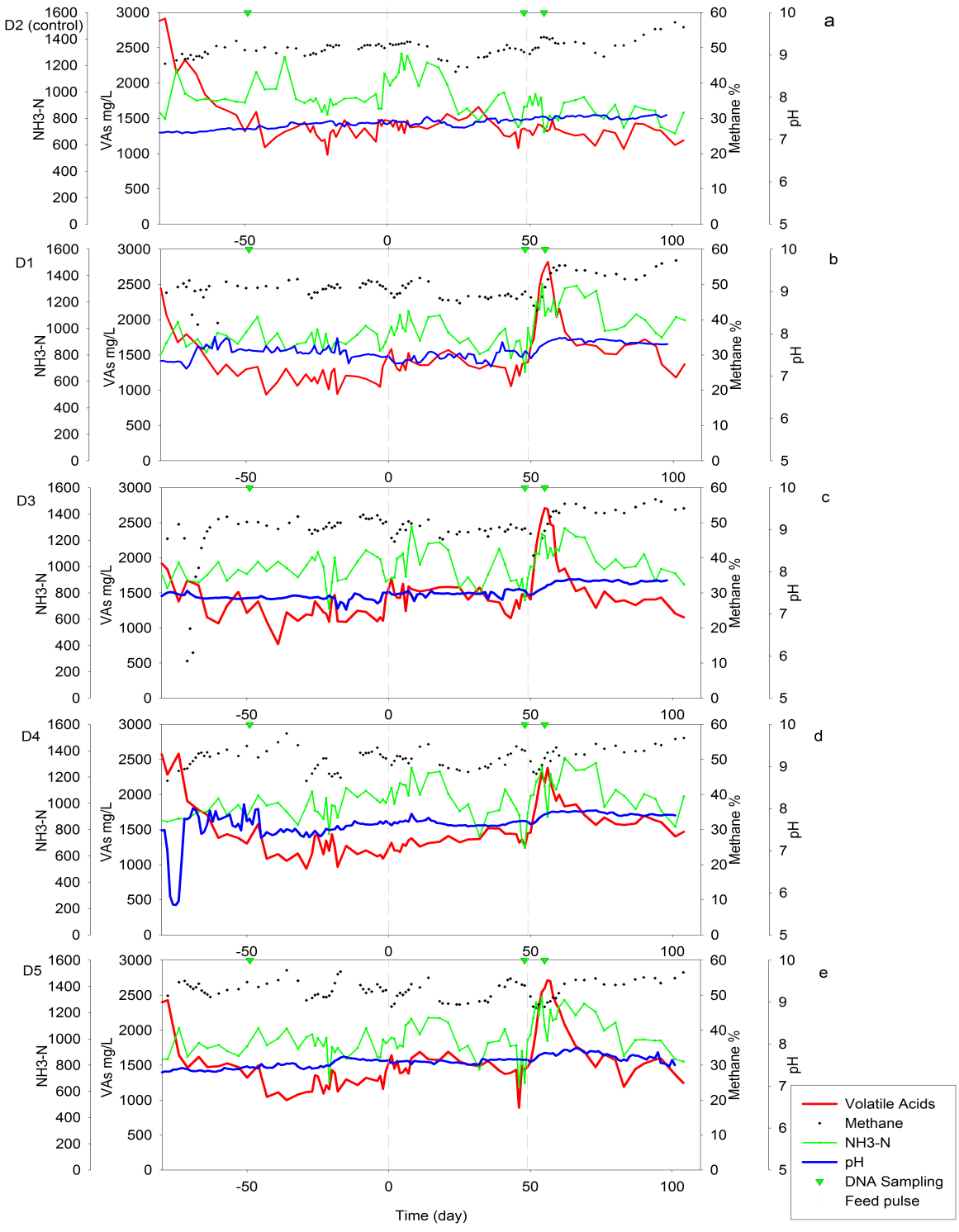


Figure 6. Chemical responses of anaerobic digester to feed disturbances; Reactor D2 was used as control, D1 and D3 have been previously disturbed with stillage and glucose; D4 and D5 have never changed their feed scheme.

COD remove is presented in Figure 7. During the first pulse is showing a small response, the change was easily adsorbed by the system. In second pulse, the change for COD remove was clearly stronger than the first, the same response for four reactors was an increase of COD remove during overloading feed pulse and decreasing immediately when the pulse was stopped. Figure 8 display the specific methanogenic activity (SMA) according methane produced and COD fed, where it is appreciate the treatments separation among control, previous disturbed and without previous treatment. SMA respect to COD removed is showed in annex C.

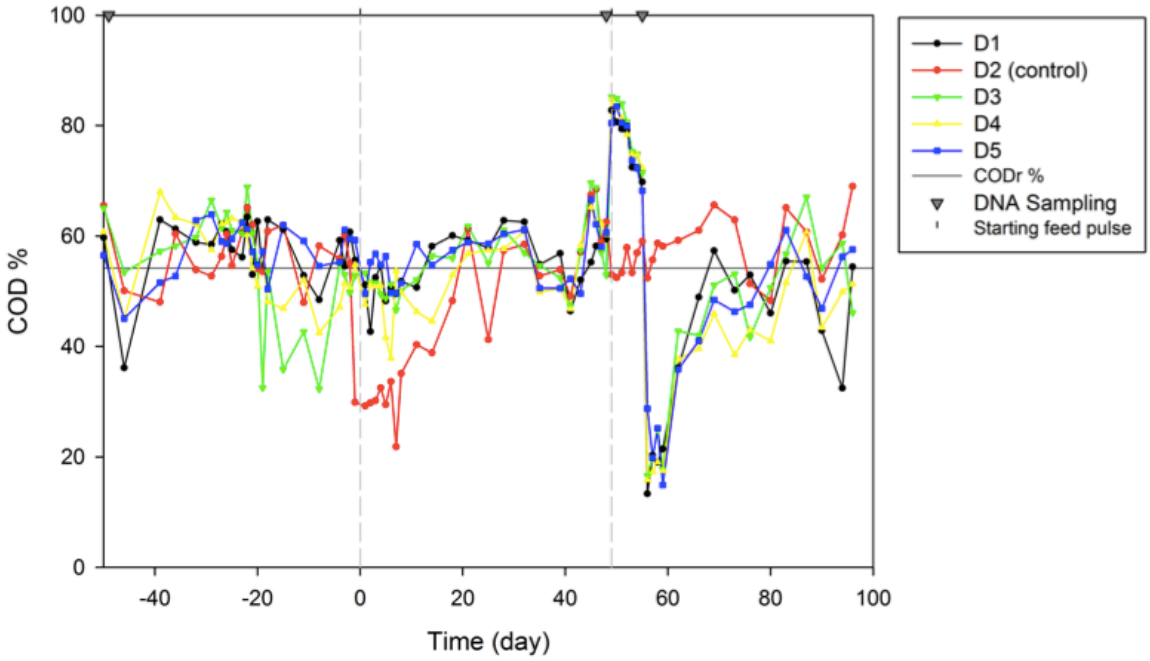


Figure 7. COD removed in percentage for each reactor

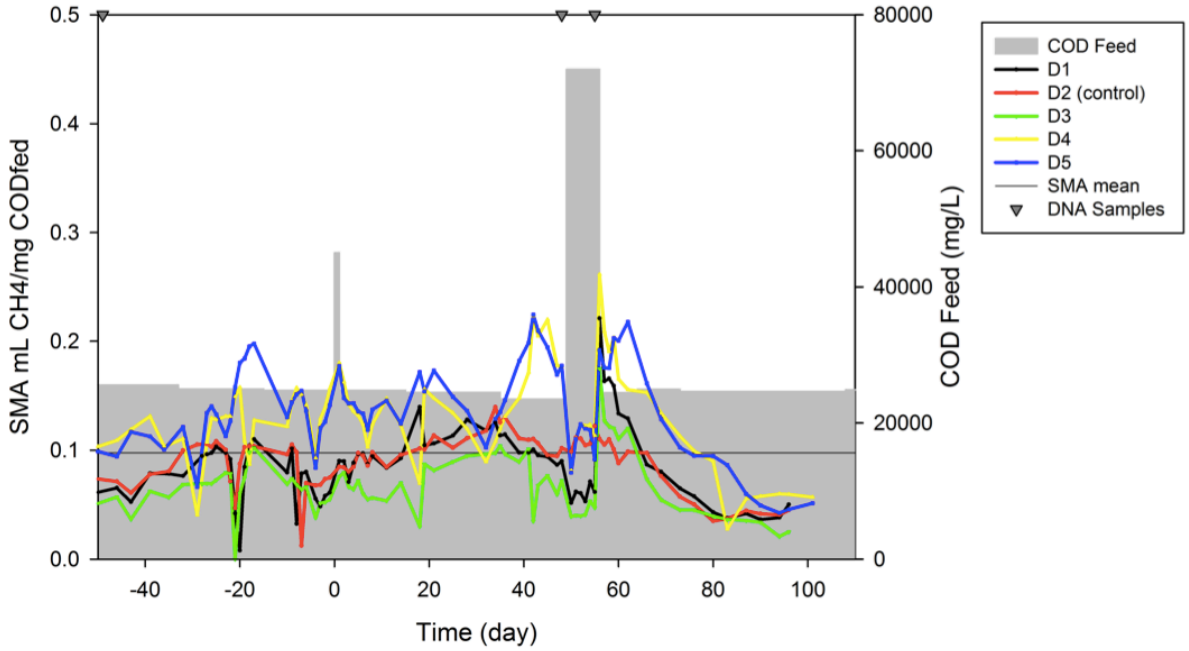


Figure 8. Specific methanogenic activity profiles, using relation between methane produced and COD (Chemical Oxygen Demand) fed.

Biochemical and physicochemical data were taken to analyze performance of second pulse (Table 4). The data ware taken before, during, and after overload feed to see the evolution of response. Results of the principal component analysis applied to these data are shown in Figure 9. First component explain the 46.8% of variability in horizontal axis and correspond to variables volatile acids (VAs) NH_3-N concentration; the second component represent 33% of variability and it is attributed to methane produced in vertical axis. Three routes are distinguish in the analysis, disturbed reactors are separated to the control, but also four reactors are grouped in two manners corresponding to previous treatment and without previous treatment. However, disturbed reactors are converging to the same area. Disturbances are characterized by accumulation of volatile acids, derived to the chain disruption in microorganism's equilibrium. First component explain the high movement in performance of disturbed digesters, which is give by accumulate intermediate products. Recovery is explained by methane production, the

disturbance by overloading feed affect negatively the methane production, but it immediately after shows an increase of methane concentration. It suggests that overloading feed pulse was a stimulus in biogas production. Community composition can be used to optimize the digestion process and maximize methane yield (Traversi et al., 2012). De Vrieze et al. (2013), found that the pulse feeding pattern leads to a higher degree of bacterial dynamics, which can be correlated to a higher tolerance to high levels of ammonium and organic overloading in anaerobic digestion, it means that the overloading feed pulses give to the reactors resistance to changes and increase biogas production (Yue et al., 2013).

Table 4. Biochemical data used to perform principal component analysis (PCA). Data correspond to second pulse. Labels represent digesters (D1, D2, D3, D4, and D5) and time before and after disturbances (day).

Digester	Label	VA ($mg \cdot L^{-1}$)	NH_3-N ($mg \cdot L^{-1}$)	pH	Methane %	Biogas ($mL \cdot day^{-1}$)
D1	D1-39	1327	962	7.57	46.88	2450
	D1-41	1318	998	7.60	46.85	2450
	D1-45	1355	903	7.56	45.95	2400
	D1-48	1381	673	7.59	47.98	2200
	D1-52	2042	1202	7.57	42.63	5100
	D1-55	2748	1097	7.77	49.22	4500
	D1-59	2036	1092	7.87	54.00	3600
	D1-62	1825	1304	7.91	55.35	2600
	D1-66	1632	1324	7.87	54.00	2000
	D1-69	1655	1233	7.78	53.98	1800
	D1-76	1519	982	7.87	52.47	1450
D2	D2-39	1365	978	7.38	49.10	2650
	D2-41	1255	996	7.46	49.45	2600
	D2-45	1307	768	7.48	49.77	2250
	D2-48	1359	886	7.47	48.78	2450
	D2-52	1326	984	7.53	49.95	2600
	D2-55	1361	696	7.54	53.05	2700
	D2-59	1346	805	7.56	51.34	2400
	D2-62	1301	917	7.51	51.32	2350
	D2-66	1255	937	7.54	51.63	2350
	D2-69	1275	961	7.53	51.25	1850
	D2-76	1334	798	7.58	47.49	1300
D3	D3-39	1364	1135	7.38	48.70	2150

Digester	Label	VA ($mg \cdot L^{-1}$)	NH_3-N ($mg \cdot L^{-1}$)	pH	Methane %	Biogas ($mL \cdot day^{-1}$)
	D3-41	1204	1015	7.58	47.51	2500
	D3-45	1401	899	7.58	48.54	1850
	D3-48	1446	745	7.55	48.35	1750
	D3-52	2176	1103	7.52	41.32	3450
	D3-55	2709	1231	7.63	47.70	3550
	D3-59	1975	1136	7.79	53.36	2750
	D3-62	1847	1291	7.79	55.39	2650
	D3-66	1523	1228	7.82	55.37	1650
	D3-69	1576	1222	7.78	54.25	1250
	D3-76	1522	979	7.81	52.75	1050
D4	D4-39	1514	948	7.59	50.33	3450
	D4-41	1441	1107	7.62	48.49	4150
	D4-45	1434	957	7.69	53.75	4800
	D4-48	1263	663	7.70	52.51	3950
	D4-52	1881	1169	7.70	45.91	9700
	D4-55	2163	1130	7.86	50.41	8100
	D4-59	1912	1105	7.92	53.40	4650
	D4-62	1832	1345	7.93	50.02	3800
	D4-66	1861	1252	7.94	51.52	3700
	D4-69	1709	1258	7.93	51.43	3250
	D4-76	1674	973	7.90	53.06	2300
D5	D5-39	1423	985	7.64	52.74	4050
	D5-41	1373	1074	7.62	52.38	4450
	D5-45	1433	951	7.64	53.73	4250
	D5-48	1428	665	7.63	52.77	3950
	D5-52	1997	1278	7.66	46.40	9600
	D5-55	2598	1101	7.79	46.67	7050
	D5-59	2381	1153	7.72	49.14	5050
	D5-62	2085	1296	7.87	53.23	5000
	D5-66	1771	1180	7.91	54.38	3700
	D5-69	1695	1269	7.80	54.30	2950
	D5-76	1656	1064	7.73	53.07	2200

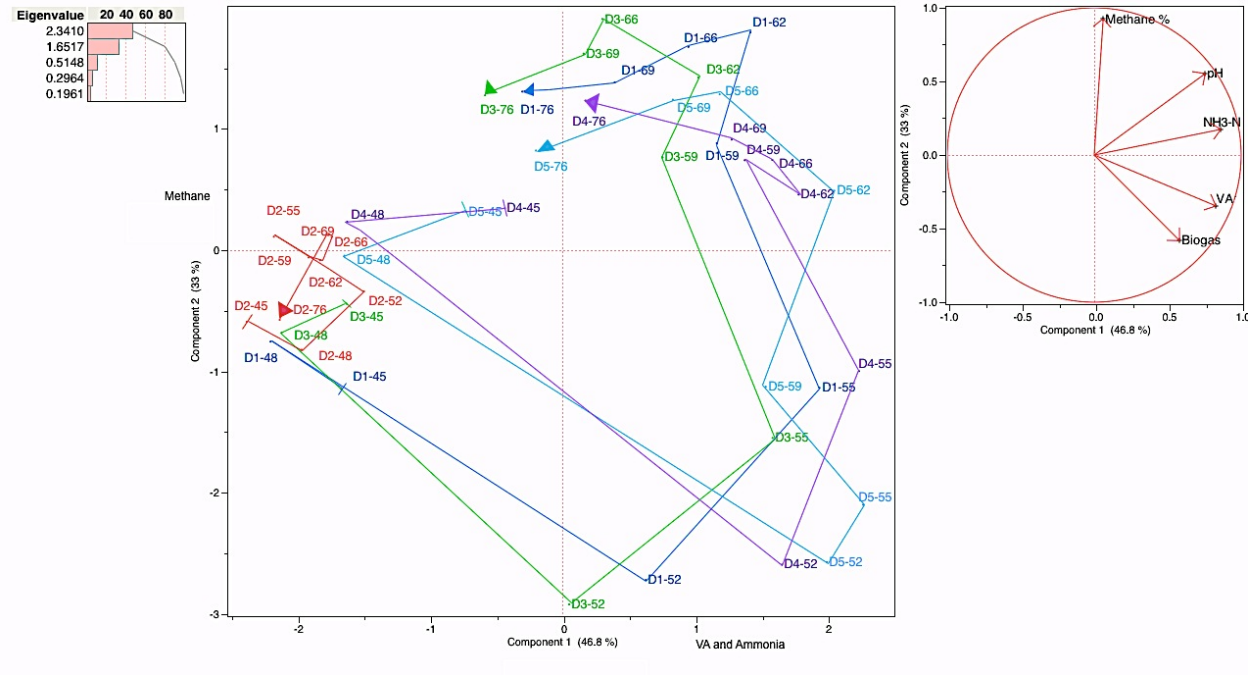


Figure 9. Principal components analysis for physicochemical performance related to the second pulse, 79.8% of variation is explained by two components.

Another possible explanation is related to the fact that these types of microorganisms could be adapted to assimilate more substrate. For example, Espinosa-Solares et al. (2006), worked with a 40 m^3 digester, reported that the maximum COD fed was of 44,366 mg•L^{-1}, meanwhile another studies report near of 50,000 mg•L^{-1} (Espinosa-Solares et al., 2010, Espinosa-Solares et al., 2009, Sharma et al., 2013, Smith et al., 2014, Valle-Guadarrama et al., 2011) and resulting in maximum levels of VA as 6,735 mg•L^{-1} in pilot plant and 4,600-5100 in lab-scale digesters.

Resilience evaluation

With purpose to evaluate resilience in Figure 10 are presented the volatile fatty acids (VFAs) profiles corresponded to each reactor. The first pulse cause the increase in acetate principally the influence of this pulse was small compared to the second pulse. At second pulse acetate, propionate and others important VFAs are presents. It seems

that the second pulse causes “instability” to the system, which recovers after releasing the disturbance. VFAs were measured and standardized in terms of the available equivalent-electrons. The accumulation of intermediate products were simultaneous as response to the overload feed pulses, it means that the parallel process of substrate is predominant in this systems (Hashsham et al., 2000). VFAs profiles were used to determined system attributes, as were reactivity, declivity, and resistance (Table 5); resilience and stability (Table 6).

Table 5. Resistance, reactivity and declivity presented by five reactors during feed overloading.

Parameter	Reactor	Pulse 1		Pulse 2	
		Acetate	Propionate	Acetate	Propionate
Resistance (Maximum concentration presented ee L^{-1})	D2	57.7	25.5	62.5	25.5
	D1	53.4	12.4	132.1	49.1
	D3	56.4	20.3	133.2	60.1
	D4	57.7	13.7	74.6	31.6
	D5	43.1	18.1	79.2	66.5
Maximum Reactivity (ee L^{-1})	D2	28.8	12.6	24.6	5.7
	D1	21.5	6.2	32.6	30.7
	D3	39.6	5.0	59.4	37.6
	D4	19.3	5.5	29.6	20.1
	D5	29.8	6.4	40.3	51.3
Maximum Declivity (ee L^{-1})	D2	-3.2	-3.7	-55.9	-16.9
	D1	-16.9	-1.1	-144.0	-77.2
	D3	-12.9	-2.7	-130.8	-102.0
	D4	-2.7	-0.7	-55.6	-55.7
	D5	-5.3	6.4	-84.1	-110.7

Table 6. Resilience and stability parameters for acetate and propionate during feed overloading.

Parameters	Reactors	Pulse 1		Pulse 2	
		Acetate	Propionate	Acetate	Propionate
Resilience (d^{-1})	D2				
	D1	0.091	0.167	0.059	0.059
	D3	0.125	0.125	0.063	0.043
	D4	0.056	0.167	0.063	0.071
	D5	0.048	0.200	0.059	0.038
Stability ($L \bullet d^{2} \bullet ee^{-1}$)	D2	4.51E-04	3.28E-03	3.02E-05	1.19E-04
	D1	3.20E-04	3.89E-03	1.75E-05	6.03E-05
	D3	3.99E-04	2.71E-03	1.90E-05	4.60E-05
	D4	1.37E-04	6.10E-03	2.27E-05	1.14E-04
	D5	9.60E-05	6.74E-03	1.74E-05	3.78E-05

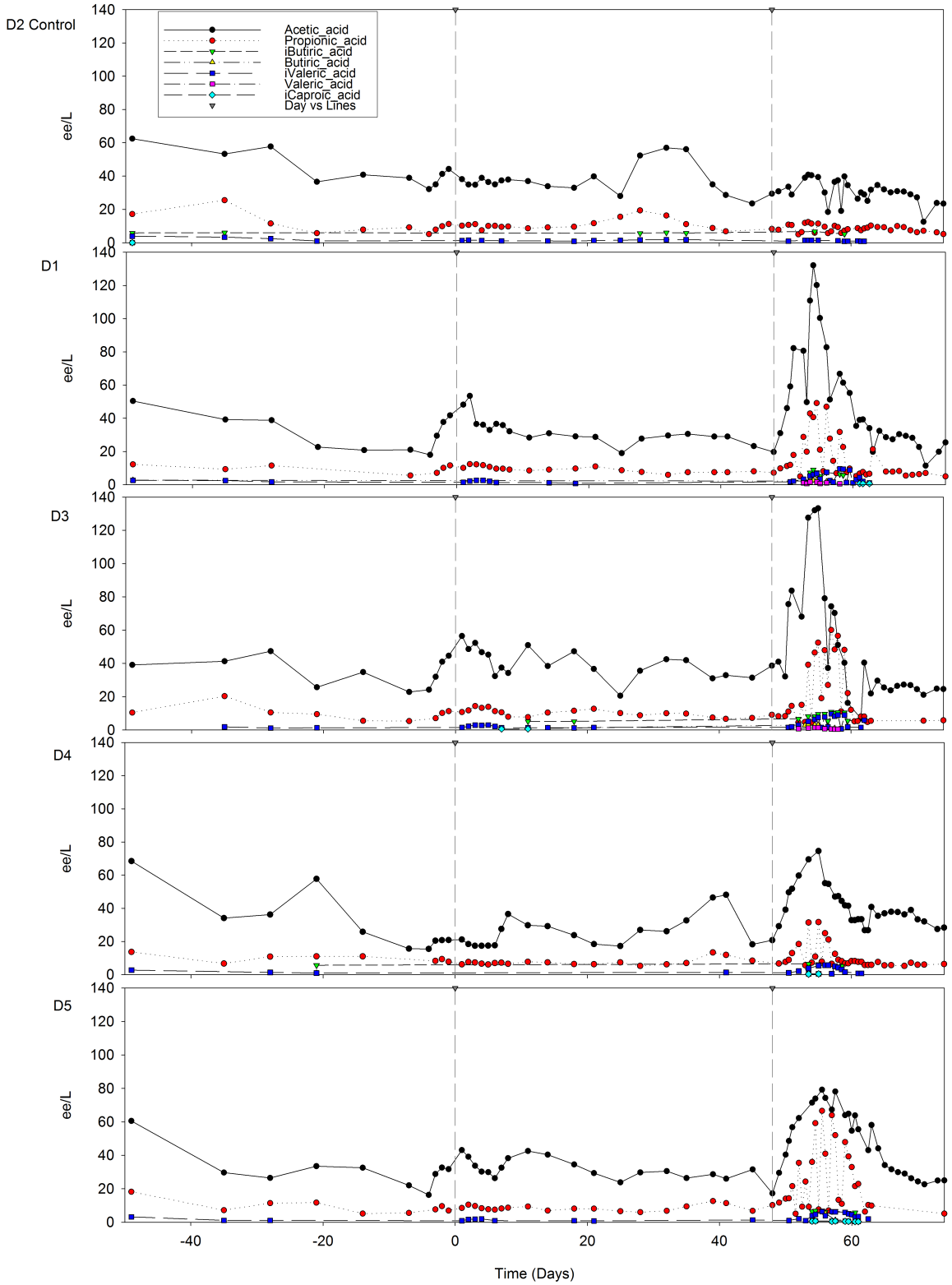


Figure 10. Volatile fatty acids profiles of five digesters reported as equivalent-electron per liter (ee/L).

Taken data analysis from short pulse and strong pulse for each reactor in treatment, Principal Components Analysis was run in aims to observe the most representative components that affect the performance. In Figure 11 shows that results. It is important to stress that the Tables 5 and 6 were used for that analysis. The results show difference between two pulses, which were indicated with blue circles. Points D1, D3, D4 and D5 represent the first pulse and points D1', D3', D4' and D5' second and stronger pulse. The first component explains 71.9% and resistance, declivity to acetate and propionate, and stability to propionate represents it. The second component explains 16% of variation and was represented by resilience and stability to acetate. In the superior right quadrant it can observe reactors D1 and D3, which were digesters with previous treatment; and behind of them were digesters D4 and D5 without previous treatment, the separation is attributable to resilience to acetate and stability. The second pulse differentiates all reactors, resulting D3' as reactor with higher resilience to acetate; D4' as the lower sensitive to changes and according data is also more resilient to propionate.

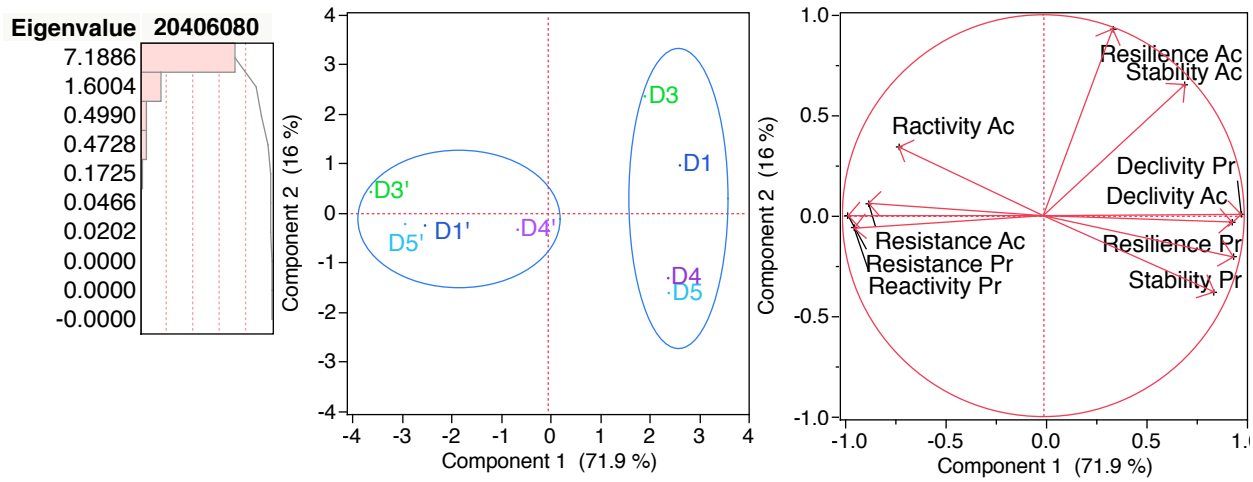


Figure 11. Principal components analysis based on stability parameters from the two pulses. The first component explains the 71.9% (declivity, resistance, stability to Propionate) and second component explained 16% (resilience and stability to acetate).

Develop of technology are continuously expand knowledge on microbial diversity and structure. 454-pyro sequencing produce millions of high-quality DNA sequences. Three time points were taken for 454 pyro sequencing of 16S rRNA genes of bacteria from the five digesters. Negative number for day represents the period before disturbances begin and during this period were taken samples to represent a steady state of system. Second point was taken before second disturbance and the third when maximum feed overload taken place. The objective was to observe the evolution of microorganism when their environment was changing. The resulting sequences from three samples were processed using the QIIME platform (Caporaso et al., 2010) and were obtained 76,174 sequences high quality (Table 7).

Table 7. Total number of sequences for each reactor after quality filtering.

Time after first disturbance	Number of sequences				
	D2	D1	D3	D4	D5
-49	5752	7315	17200	5313	5030
48	135	7538	265	364	992
55	280	294	1064	735	23897

RPD Classifier Tool was used by taxonomic classification of bacterial community, with default settings. All samples were classified in 23 phyla. The most abundant were *Firmicutes* (69.6%), *Thermotogae* (22.1%), *Bacteroidetes* (3.6%), *Synergistetes* (1.3%), *Proteobacteria* (1.2%), *Tenericutes* (1.0%), and unassigned (0.8%) see Figure 15 in annexes. During disturbance the significative changes were observed with major impact in the biggest groups. *Firmicutes* decrease from 67.61% to 66.93% on D1, from 78.81% to 67.70% for D3; however in digesters D4 and D5 values dropped from 49.76% to 76.84% and from 56.52 to 83.30% respectively. *Firmicutes* are syntrophic bacteria, which can degrade volatile fatty acids; the degradation

produces H_2 and then this is degraded by hydrogenotrophic methanogens (Riviere et al., 2009). *Thermotogae* increase for digesters D1 and D3 (21.50% to 28.53% and 13.43% to 23.20% respectively) and decrease for digesters D4 and D5 (40.43% to 14.25% and 38.3% to 9.78%, respectively). Others phyla as *Bacterioidetes*, *Synergistetes* decreased for all reactors; they intervene in protein degradation and amino acid fermentation, producing acetate (Vanwonterghem et al., 2014). *Proteobacteria* was also present as important phyla and had an increase during the maximum concentration of feed overloading.

Respect to classes, the most abundant was *Clostridia*, *Thermotogae*, *Bacteroidia* and *Synergistia* of 35 classes (Figure 16 annexes). These groups are reported as the most abundant bacteria in thermophilic digesters (Leven et al., 2007) and has been reported that these groups are fully influenced biogas production (Yue et al., 2013). The *Clostridia* class represents the organotrophs microorganisms that use organic compounds as energy source, hydrolytic bacteria and are correlated with higher methane production (Sundberg et al., 2013, Smith et al., 2014, Vanwonterghem et al., 2014). *Clostridia* have a proportion for each reactor of 28.53%, 26.20%, 14.25% and 9.78% for digesters D1, D3, D4 and D5 respectively. *Betaproteobacteria* appears when the maximum overloading feed takes place and are considered as consumers of acetate, propionate and butyrate.

The main orders were *MBA08, SHA-98, Clostridiales, BSA2B-08 and OPB-54* from *Clostridia* Class. Followed by *Thermotogales, Bacteriodales, and Synergistales* (Figure 12). MBA08 is increase during the maximum overload, suggesting a link with recovery performance during disturbances. The same behavior was observed in other members of *Clostridia* suggesting a response to degradation of matter demanded.

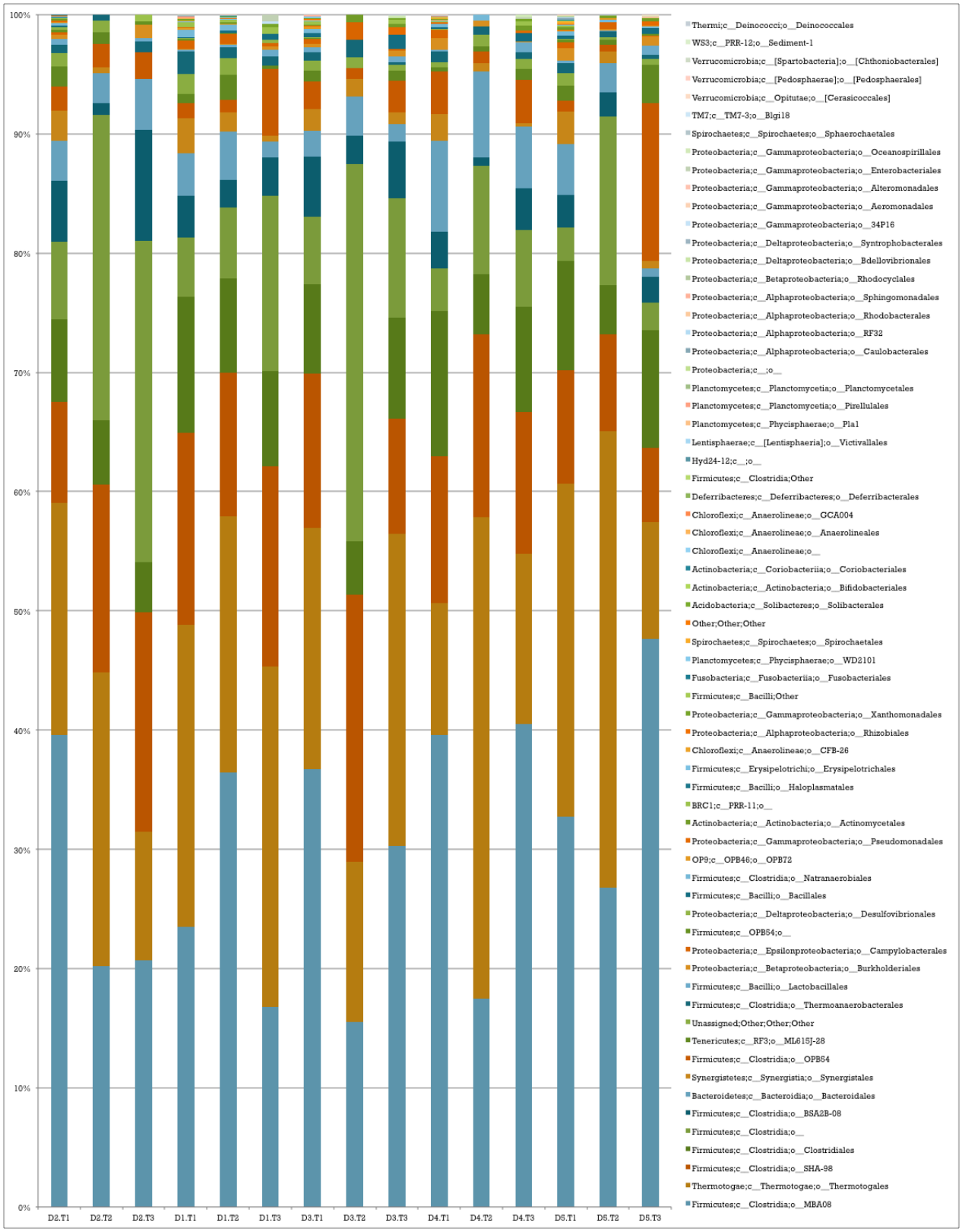


Figure 12. Bacterial composition by order, RDP classifier tool was used for classification with 80% confidence for five digesters (D2, D1, D3, D4 D5) during days -49 (T1), 48 (T2) and 55 (T3) of experimentation.

UniFrac is a β-diversity measure that uses phylogenetic information to compare environmental samples (Lozupone et al., 2011) and it is usable for identify factor that explain the differences around microbial communities and is supported by multivariate statistical tools as principal coordinates analysis (PCoA). UniFrac measures and compare the differences between two or more collection of samples and it is also implemented in QIIME (Caporaso et al., 2010). Unweighted UniFrac is suited to detect differences in presence or absence of lineages of bacteria in different communities. Weighted UniFract is well suited to detecting differences in abundance even when overall groups of microorganisms that are present in each sample remain the same (Lozupone et al., 2007).

Figure 11 shows unweughted UniFrac analysis. Three components were able to explain 41% of variability with PC1 16%, PC2 13% and PC3 12%. On day -49 of evaluation period all the reactors are located in the positive area of PC1. However on the maximum overloading feed in second pulse they show a separation in three different routes that correspond to digesters D1 and D3 who had been received previous disturbances (Sharma et al., 2013), digesters D4 and D5 that never before received any treatment and control showing any displacement. Figure 12 shows weighted UniFrac analysis, which takes into account information on relative abundance. The results obtained do not show tight clustering, however the explanation of variability is 78% composed by PC1 37%, PC2 30%, and PC3 11%.

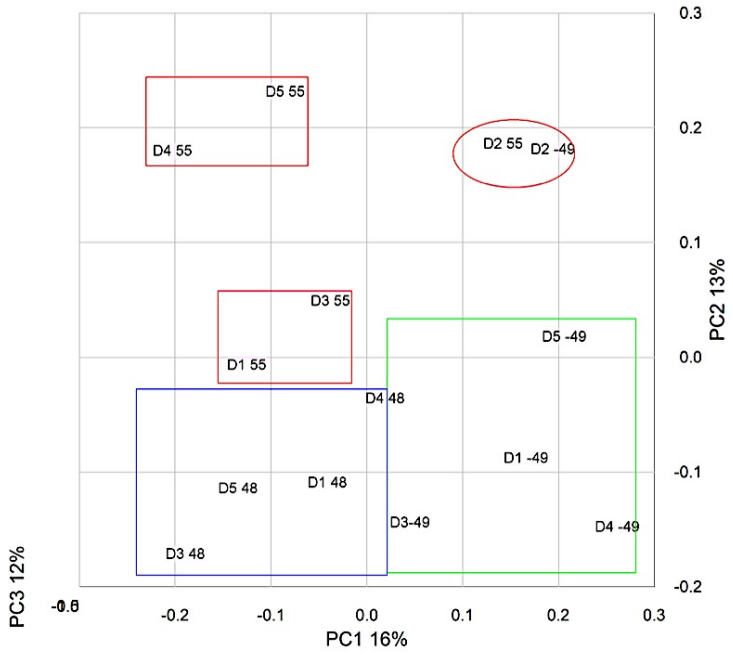


Figure 13. Principal Coordinate Analysis (PCoA) by unweighted UniFrac . Two components explain 29% of the variability.

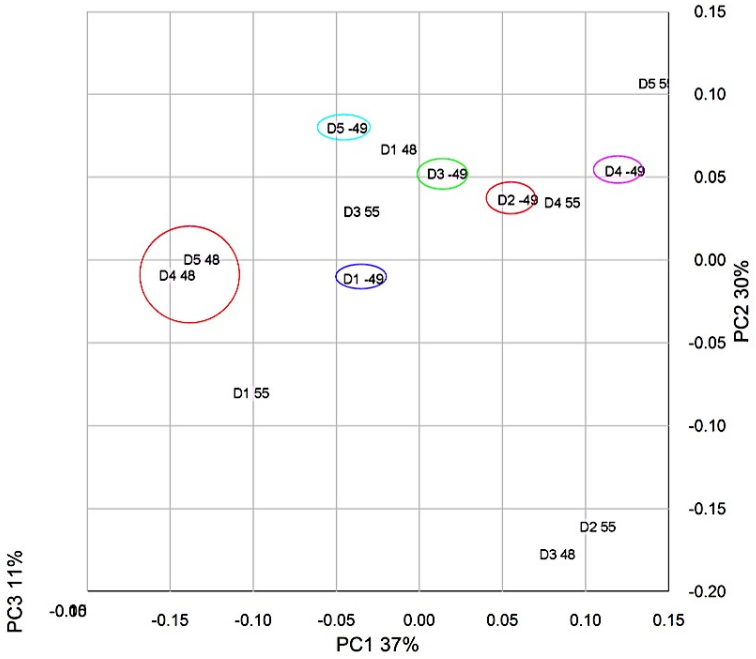


Figure 14. Principal Coordinate Analysis (PCoA) with weighted UniFrac. Two components explain 67% or variability.

CONCLUSIONS

Overloading feed pulses were applied to anaerobic digestion reactors. This study shows that the magnitude of the disturbance was proportional to the magnitude of the response. At first pulse the anaerobic digesters were recovered easily and continues with regular operation. The changes were mainly due to acetate increase..Second pulse was strong and longer, as response digesters perform change in terms of evident volatile fatty acids accumulation, COD accumulation, reduction of methane percentage, and rise propionate accumulation. However, thermophilic anaerobic digesters are resilient to overloading feed disturbances when the concentration feed increases almost 3 times their regular feed, concentration of six days of feeding. Resilience and resistance were calculated in terms of equivalent-electrons and can only take values between 0 and 1. The digester D3 showed easy recovery as higher both resistance and resilience. It can be attributed to the fact that controlled disturbances contribute to easily recovering. Based on VFAs profiles observed in the disturbed reactors, it is possible to indicate that parallel processing take place. This can be attributed to microbial capability to degrade different VFAs independently after releasing the disturbance. Parallel processing is related with a higher functional stability. After disturbance pulse, a significant increase in hydrolytic groups was observed, emphasizing in members of *Clostridia* class, which is related to hydrolytic performance increases. It can contribute to the rise of VFAs when a disturbance takes place.

RECOMENDATIONS

Is recommended analysis of more DNA samples to see the evolution during disturbance and recovery period. Repeat the pyro sequencing of days 48 and 55 in aims to obtain a higher number of sequences, because the number was small compared with the first sample sequenced. A stronger overloading feed pulse is necessary to confirm the results showed at this work and complemented it. Another DNA samples are necessary to sequencing for evaluate the changes during recovery of second pulse to observe which microorganisms react to degrade the cumulative intermediate products.

REFERENCES

454 LIFE SCIENCE CORP. & 2011. Sequencing System Guidelines for Amplicon Experimental Design.

AHRING, B. 2003. Perspectives for Anaerobic Digestion. *In:* AHRING, B., ANGELIDAKI, I., DE MACARIO, E. C., GAVALA, H. N., HOFMAN-BANG, J., MACARIO, A. J. L., ELFERINK, S. J. W. H. O., RASKIN, L., STAMS, A. J. M., WESTERMANN, P. & ZHENG, D. (eds.) *Biomethanation I.* Springer Berlin Heidelberg.

AIKIO, S. 2004. The contribution of direct and indirect flows to the resilience of element cycles. *Acta Oecologica,* 26**,** 129-135.

ALLISON, S. D. & MARTINY, J. B. 2008. Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences,* 105**,** 11512-11519.

ALMEIDA, A., NAFARRETE-RIVERA, E., ALVARADO, A., CERVANTES-OVALLE, A., LUEVANOS, M. P. E. & BALAGURUSAMY, N. 2011. Expresión genética en la digestión anaerobia: un paso adelante en la comprensión de las interacciones tróficas de esta biotecnología. *Revista Científica de la Universidad Autónoma de Coahuila,* 3**,** 14-34.

ÁLVAREZ, J. A., OTERO, L. & LEMA, J. M. 2010. A methodology for optimising feed composition for anaerobic co-digestion of agro-industrial wastes. *Bioresource Technology,* 101**,** 1153-1158.

BEISNER, B. E., HAYDON, D. T. & CUDDINGTON, K. 2003. Alternative stable states in ecology. *Frontiers in Ecology and the Environment,* 1**,** 376-382.

BOLZONELLA, D., PAVAN, P., BATTISTONI, P. & CECCHI, F. 2005. Mesophilic anaerobic digestion of waste activated sludge: influence of the solid retention time in the wastewater treatment process. *Process Biochemistry,* 40**,** 1453-1460.

BOMBARDIERE, J., ESPINOSA-SOLARES, T., DOMASCHKO, M. & CHATFIELD, M. 2007. Thermophilic anaerobic digester performance under different feed-loading frequency. *Appl Biochem Biotechnol,* 137-140**,** 765-75.

BUJOCZEK, G., OLESZKIEWICZ, J., SPARLING, R. & CENKOWSKI, S. 2000. High Solid Anaerobic Digestion of Chicken Manure. *Journal of Agricultural Engineering Research,* 76**,** 51-60.

CAPORASO, J. G., KUCZYNSKI, J., STOMBAUGH, J., BITTINGER, K., BUSHMAN, F. D., COSTELLO, E. K., FIERER, N., PENA, A. G., GOODRICH, J. K. & GORDON, J. I. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods,* 7**,** 335-336.

CAVINATO, C., FATONE, F., BOLZONELLA, D. & PAVAN, P. 2010. Thermophilic anaerobic co-digestion of cattle manure with agro-wastes and energy crops: Comparison of pilot and full scale experiences. *Bioresource Technology,* 101**,** 545-550.

CHEN, ZAMUDIO CAÑAS, E., ZHANG, Y., ZHU, Z. & HE, Q. 2012. Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. *Journal of applied microbiology,* 113**,** 1371-1379.

COMINO, E., ROSSO, M. & RIGGIO, V. 2009. Development of a pilot scale anaerobic digester for biogas production from cow manure and whey mix. *Bioresource Technology,* 100**,** 5072-5078.

DE VRIEZE, J., VERSTRAETE, W. & BOON, N. 2013. Repeated pulse feeding induces functional stability in anaerobic digestion. *Microb Biotechnol,* 6**,** 414-24.

DUNBAR, J., TICKNOR, L. O. & KUSKE, C. R. 2000. Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. *Appl Environ Microbiol,* 66**,** 2943-50.

ESPINOSA-SOLARES, T., BOMBARDIERE, J., CHATFIELD, M., DOMASCHKO, M., EASTER, M., STAFFORD, D. A., CASTILLO-ANGELES, S. & CASTELLANOS-HERNANDEZ, N. 2006. Macroscopic mass and energy balance of a pilot plant anaerobic bioreactor operated under thermophilic conditions. *Appl Biochem Biotechnol,* 129-132**,** 959-68.

ESPINOSA-SOLARES, T., DOMASCHKO, M., ROBLES-MARTÍNEZ, F., DURÁN-PÁRAMO, E., HERNÁNDEZ-EUGENIO, G. & BOMBARDIERE, J. 2010. *Short-term effects of temperature changes in a pilot plant for the production of biogas from poultry litter.*, Universidad Juárez Autónoma de Tabasco.

ESPINOSA-SOLARES, T., VALLE-GUADARRAMA, S., BOMBARDIERE, J., DOMASCHKO, M. & EASTER, M. 2009. Effect of heating strategy on power consumption and performance of a pilot plant anaerobic digester. *Appl Biochem Biotechnol,* 156**,** 35-44.

GUNDERSON, L. H. 2000. Ecological resilience - in theory and application. *Annual Review of Ecology and Systematics,* 31**,** 425-439.

HAMBERGER, A., HORN, M. A., DUMONT, M. G., MURRELL, J. C. & DRAKE, H. L. 2008. Anaerobic Consumers of Monosaccharides in a Moderately Acidic Fen. *Applied and Environmental Microbiology,* 74**,** 3112-3120.

HARTMANN, H. & AHRING, B. K. 2005. Anaerobic digestion of the organic fraction of municipal solid waste: Influence of co-digestion with manure. *Water Research,* 39**,** 1543-1552.

HASHSHAM, S. A., FERNANDEZ, A. S., DOLLHOPF, S. L., DAZZO, F. B., HICKEY, R. F., TIEDJE, J. M. & CRIDDLE, C. S. 2000. Parallel Processing of Substrate Correlates with Greater Functional Stability in Methanogenic Bioreactor Communities Perturbed by Glucose. *Applied and Environmental Microbiology,* 66**,** 4050-4057.

HODGSON, D., TOWNLEY, S. & MCCARTHY, D. 2006. Robustness: predicting the effects of life history perturbations on stage-structured population dynamics. *Theoretical population biology,* 70**,** 214-224.

HUGHES, J. & BOHANNAN, B. J. 2004. Application of ecological diversity statistics in microbial ecology. *Molecular microbial ecology manual,* 2**,** 1321-1344.

KAPARAJU, P., BUENDIA, I., ELLEGAARD, L. & ANGELIDAKIA, I. 2008. Effects of mixing on methane production during thermophilic anaerobic digestion of manure: Lab-scale and pilot-scale studies. *Bioresource Technology,* 99**,** 4919-4928.

KUNDU, K., BERGMANN, I., KLOCKE, M., SHARMA, S. & SREEKRISHNAN, T. R. 2014. Impact of abrupt temperature increase on the performance of an anaerobic hybrid bioreactor and its intrinsic microbial community. *Bioresource Technology,* 168**,** 72-79.

LEVEN, L., ERIKSSON, A. R. & SCHNURER, A. 2007. Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. *FEMS Microbiol Ecol,* 59**,** 683-93.

LOZUPONE, C., LLADSER, M. E., KNIGHTS, D., STOMBAUGH, J. & KNIGHT, R. 2011. UniFrac: an effective distance metric for microbial community comparison. *ISME J,* 5**,** 169-72.

LOZUPONE, C. A., HAMADY, M., KELLEY, S. T. & KNIGHT, R. 2007. Quantitative and qualitative beta diversity measures lead to different insights into factors that structure microbial communities. *Appl Environ Microbiol,* 73**,** 1576-85.

LUSTE, S. & LUOSTARINEN, S. 2010. Anaerobic co-digestion of meat-processing by-products and sewage sludge – Effect of hygienization and organic loading rate. *Bioresource Technology,* 101**,** 2657-2664.

MACIAS-CORRAL, M., SAMANI, Z., HANSON, A., SMITH, G., FUNK, P., YU, H. & LONGWORTH, J. 2008. Anaerobic digestion of municipal solid waste and agricultural waste and the effect of co-digestion with dairy cow manure. *Bioresource Technology,* 99**,** 8288-8293.

MADSEN, M., HOLM-NIELSEN, J. B. & ESBENSEN, K. H. 2011. Monitoring of anaerobic digestion processes: A review perspective. *Renewable and Sustainable Energy Reviews,* 15**,** 3141-3155.

MCMAHON, K. D., ZHENG, D., STAMS, A. J. M., MACKIE, R. I. & RASKIN, L. 2004. Microbial population dynamics during start-up and overload conditions of anaerobic digesters treating municipal solid waste and sewage sludge. *Biotechnology and Bioengineering,* 87**,** 823-834.

NEUBERT, M. G. & CASWELL, H. 1997. Alternatives to resilience for measuring the responses of ecological systems to perturbations. *Ecology,* 78**,** 653-665.

NEUBERT, M. G., CASWELL, H. & MURRAY, J. 2002. Transient dynamics and pattern formation: reactivity is necessary for Turing instabilities. *Mathematical biosciences,* 175**,** 1-11.

NEVES, L., OLIVEIRA, R. & ALVES, M. M. 2009. Co-digestion of cow manure, food waste and intermittent input of fat. *Bioresource Technology,* 100**,** 1957-1962.

NGES, I. A., ESCOBAR, F., FU, X. & BJÖRNSSON, L. 2012. Benefits of supplementing an industrial waste anaerobic digester with energy crops for increased biogas production. *Waste Management,* 32**,** 53-59.

RAMETTE, A. 2007. Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol,* 62**,** 142-60.

RAPOSO, F., BORJA, R., RINCON, B. & JIMENEZ, A. M. 2008. Assessment of process control parameters in the biochemical methane potential of sunflower oil cake. *Biomass and Bioenergy,* 32**,** 1235-1244.

RÉTFALVI, T., TUKACS-HÁJOS, A., ALBERT, L. & MAROSVÖLGYI, B. 2011. Laboratory scale examination of the effects of overloading on the anaerobic digestion by glycerol. *Bioresource technology,* 102**,** 5270-5275.

RIVERA-SALVADOR, V., LÓPEZ-CRUZ, I. L., ESPINOSA-SOLARES, T., ARANDA-BARRADAS, J. S., HUBER, D. H., SHARMA, D. & TOLEDO, J. U. 2014. Application of Anaerobic Digestion Model No. 1 to describe the syntrophic acetate oxidation of poultry litter in thermophilic anaerobic digestion. *Bioresource Technology,* 167**,** 495-502.

RIVIERE, D., DESVIGNES, V., PELLETIER, E., CHAUSSONNERIE, S., GUERMAZI, S., WEISSENBACH, J., LI, T., CAMACHO, P. & SGHIR, A. 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. *ISME J,* 3**,** 700-14.

ROZDILSKY, I. D., STONE, L. & SOLOW, A. 2004. The effects of interaction compartments on stability for competitive systems. *Journal of theoretical biology,* 227**,** 277-282.

SALMINEN, E. & RINTALA, J. 2002. Anaerobic digestion of organic solid poultry slaughterhouse waste – a review. *Bioresource Technology,* 83**,** 13-26.

SHADE, A., PETER, H., ALLISON, S. D., BAHO, D. L., BERGA, M., BURGMANN, H., HUBER, D. H., LANGENHEDER, S., LENNON, J. T., MARTINY, J. B., MATULICH, K. L., SCHMIDT, T. M. & HANDELSMAN, J. 2012. Fundamentals of microbial community resistance and resilience. *Front Microbiol,* 3**,** 417.

SHARMA, D., ESPINOSA-SOLARES, T. & HUBER, D. H. 2013. Thermophilic anaerobic co-digestion of poultry litter and thin stillage. *Bioresource Technology,* 136**,** 251-256.

SHEN, P., ZHANG, J., ZHANG, J., JIANG, C., TANG, X., LI, J., ZHANG, M. & WU, B. 2013. Changes in microbial community structure in two anaerobic systems to treat bagasse spraying wastewater with and without addition of molasses alcohol wastewater. *Bioresource Technology,* 131**,** 333-340.

SHIN, S. G., YOO, S., HWANG, K., SONG, M., KIM, W., HAN, G. & HWANG, S. 2011. Dynamics of transitional acidogenic community along with methanogenic population during anaerobic digestion of swine wastewater. *Process Biochemistry,* 46**,** 1607-1613.

SMITH, A. M., SHARMA, D., LAPPIN-SCOTT, H., BURTON, S. & HUBER, D. H. 2014. Microbial community structure of a pilot-scale thermophilic anaerobic digester treating poultry litter. *Appl Microbiol Biotechnol,* 98**,** 2321-34.

STROOT, P. G., MCMAHON, K. D., MACKIE, R. I. & RASKIN, L. 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions--I. Digester performance. *Water Res,* 35**,** 1804-16.

SUL, W. J., COLE, J. R., JESUS, E. D. C., WANG, Q., FARRIS, R. J., FISH, J. A. & TIEDJE, J. M. 2011. Bacterial community comparisons by taxonomy-supervised analysis independent of sequence alignment and clustering. *Proceedings of the National Academy of Sciences,* 108**,** 14637-14642.

SUNDBERG, C., AL-SOUD, W. A., LARSSON, M., ALM, E., YEKTA, S. S., SVENSSON, B. H., SORENSEN, S. J. & KARLSSON, A. 2013. 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. *FEMS Microbiol Ecol,* 85**,** 612-26.

SUTARYO, S., WARD, A. J. & MØLLER, H. B. 2012. Thermophilic anaerobic co-digestion of separated solids from acidified dairy cow manure. *Bioresource Technology,* 114**,** 195-200.

TRAVERSI, D., VILLA, S., LORENZI, E., DEGAN, R. & GILLI, G. 2012. Application of a real-time qPCR method to measure the methanogen concentration during anaerobic digestion as an indicator of biogas production capacity. *Journal of Environmental Management,* 111**,** 173-177.

VALLE-GUADARRAMA, S., ESPINOSA-SOLARES, T., LOPEZ-CRUZ, I. L. & DOMASCHKO, M. 2011. Modeling temperature variations in a pilot plant thermophilic anaerobic digester. *Bioprocess Biosyst Eng,* 34**,** 459-70.

VANWONTERGHEM, I., JENSEN, P. D., DENNIS, P. G., HUGENHOLTZ, P., RABAEY, K. & TYSON, G. W. 2014. Deterministic processes guide long-term synchronised population dynamics in replicate anaerobic digesters. *ISME J,* 8**,** 2015-28.

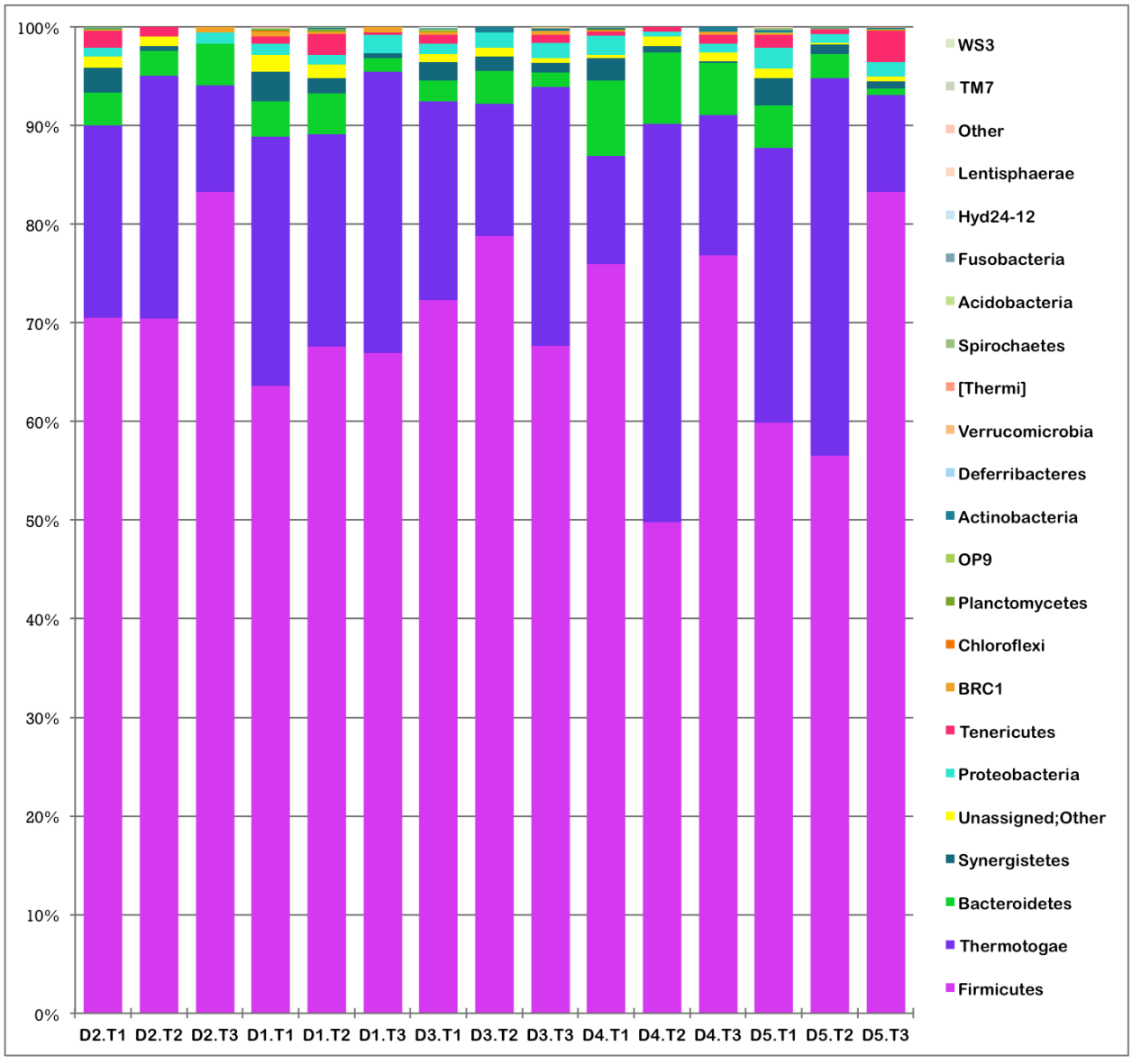
WANG, Q., GARRITY, G. M., TIEDJE, J. M. & COLE, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol,* 73**,** 5261-7.

YUE, Z., CHEN, R., YANG, F., MACLELLAN, J., MARSH, T., LIU, Y. & LIAO, W. 2013. Effects of dairy manure and corn stover co-digestion on anaerobic microbes and corresponding digestion performance. *Bioresource Technology,* 128**,** 65-71.

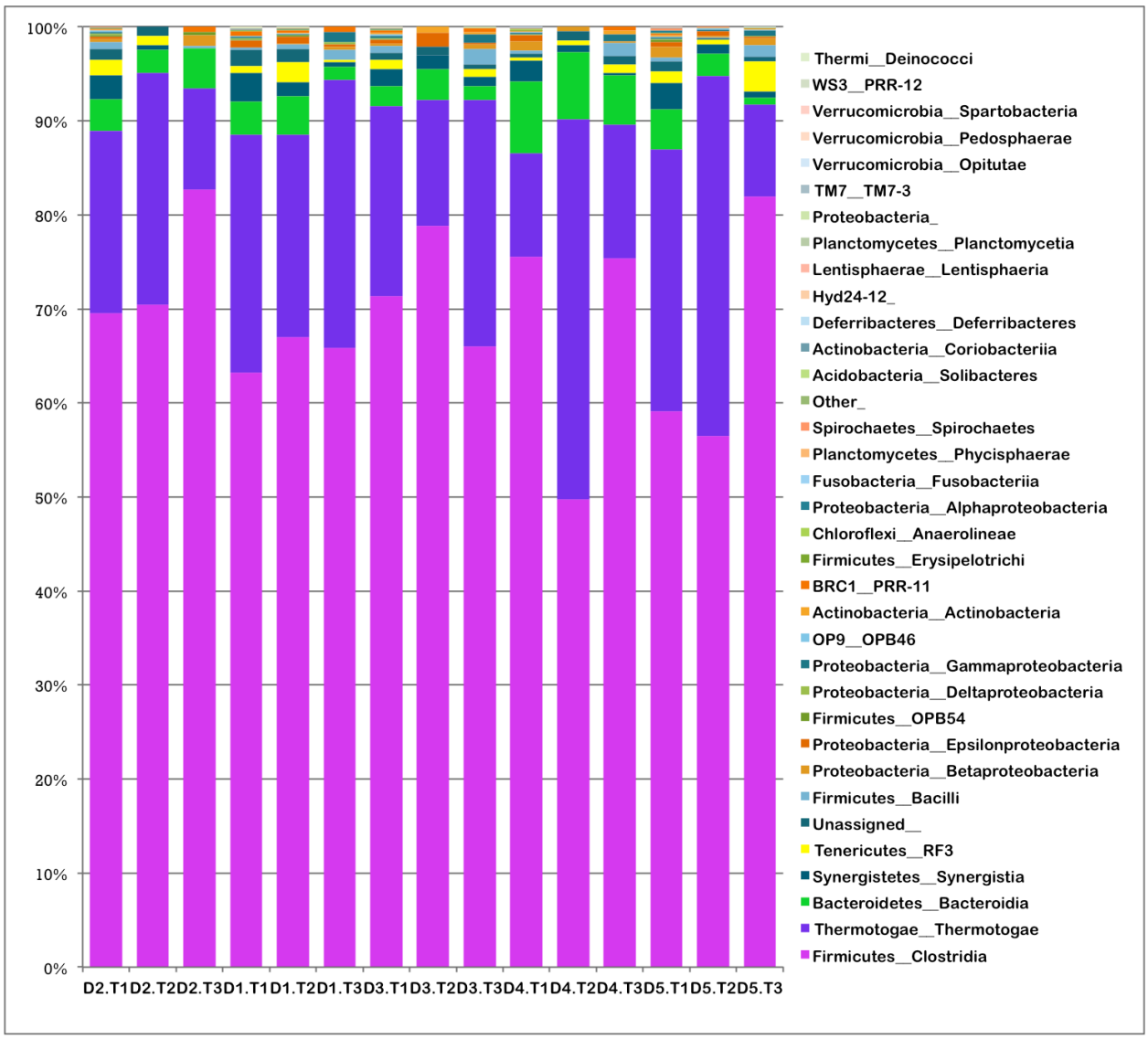
ZHANG, Y., ZAMUDIO CAÑAS, E. M., ZHU, Z., LINVILLE, J. L., CHEN, S. & HE, Q. 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. *Bioresource Technology,* 102**,** 779-785.

ANNEXES

Annex A. Bacteria community grouped by phyla for five digesters (D1, D2, D3, D4, and D5), three sampling points are observed for each reactor on days -49, 48 and 55 corresponding to T1, T2 and T3 respectively.



Annex B. Bacterial composition by classes for five digesters (D1, D2, D3, D4 and D5) during days -49 (T1), 48 (T2), and 55 (T3) using RDP classifier tool with 80% of confidence.



Annex C. Specific methanogenic activity (SMA) profiles calculated using chemical oxygen demand removed.