Enhancement of Modeling Phased Anaerobic Digestion Systems through Investigation of Their Microbial Ecology and Biological Activity

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Anaerobic digestion (AD) is widely used in wastewater treatment plants for stabilisation of primary and waste activated sludges. Increasingly energy prices as well as stringent environmental and public health regulations ensure the ongoing popularity of anaerobic digestion. Reduction of volatile solids, methane production and pathogen reduction are the major objectives of anaerobic digestion. Phased anaerobic digestion is a promising technology that may allow improved volatile solids destruction and methane gas production.

In AD models, microbially-mediated processes are described by functionally-grouped microorganisms. Ignoring the presence of functionally-different species in the separate phases may influence the output of AD modeling. The objective of this research was to thoroughly investigate the kinetics of hydrolysis, acetogenesis (i.e., propionate oxidation) and methanogenesis (i.e., acetoclastic) in phased anaerobic digestion systems. Using a denaturing gradient gel electrophoresis (DGGE) technique, bacterial and archaeal communities were compared to complement kinetics studies.

Four phased digesters including Mesophilic-Mesophilic, Thermophilic-Mesophilic, Thermophilic-Thermophilic and Mesophilic-Thermophilic were employed to investigate the influence of phase separation and temperature on the microbial activity of the digestion systems. Two more digesters were used as control, one at mesophilic 35 ^{0}C (C1) and one at thermophilic 55 ^{0}C (C2) temperatures. The HRTs in the first-phase, second-phase and single-phase digesters were approximately 3.5, 14, and 17 days, respectively. All the digesters were fed a mixture of primary and secondary sludges. Following achievement of steady-state in the digesters, a series of batch experiments were conducted off-line to study the impact of the digester conditions on the kinetics of above-mentioned processes. A Monod-type equation was used to study the kinetics of acetoclastic methanogens and POB in the digesters, while a first-order model was used for the investigation of hydrolysis kinetics.

Application of an elevated temperature (55 ^{0}C) in the first-phase was found to be effective in enhancing solubilisation of particulate organics. This improvement was more significant for nitrogen-containing material (28%) as compared to the PCOD removal (5%) when the M1 and T1 digesters were compared. Among all the configurations, the highest PCOD removal was achieved in the T1T2 system ($p_{value}<0.05$). In contrast to the solubilisation efficiencies, the mesophilic digesters (C1, M1M2 and T1M3) outperformed the thermophilic digesters (C2, T1T2 and M1T3) in COD removal. The highest

COD removal was obtained in the T1M3 digestion system, indicating a COD removal efficiency of 50.7±2.1%.

The DGGE fingerprints from digesters demonstrated that digester parameters (i.e., phase separation and temperature) influenced the structure of the bacterial and archaeal communities. This resulted in distinct clustering of DGGE profiles from the 1st-phase digesters as compared to the 2nd-phase digesters and from the mesophilic digesters as compared to the thermophilic ones.

Based on the bio-kinetic parameters estimated for the various digesters and analysis of the confidence regions of the kinetic sets (k_{max} and K_s), the batch experiment studies revealed that the kinetic characteristics of the acetoclastic methanogens and POB developed in the heavily loaded digesters (M1 and T1) were different from those species developed in the remaining mesophilic digesters (M2, M3 and C1). As with the results from the mesophilic digesters, a similar observation was made for the thermophilic digesters. The species of acetoclastic methanogens and POB within the T1 digester had greater k_{max} and K_s values in comparison to the values of the T3 and C2 digesters. However, the bio-kinetic parameters of the T2 digester showed a confidence region that overlapped with both the T1 and T3 digesters. The acetate and propionate concentrations in the digesters supported these results. The acetate and propionate concentrations in the M1 digesters were, respectively, 338±48 and 219±17 mgCOD/L, while those of the M2, M3 and C1 digesters were less than 60 mg/L as COD. The acetate and propionate concentrations were, respectively, 872±38 and 1220±66 in T1 digester, whereas their concentrations ranged 140-184 and 209-309 mg/L as COD in the T2, T3 and C2 digesters. In addition, the DGGE results displayed further evidence on the differing microbial community in the 1st- and 2nd-phase digesters.

Two first-order hydrolysis models (single- and dual-pathway) were employed to study the hydrolysis process in the phased and single-stage digesters. The results demonstrated that the dual-pathway hydrolysis model better fit the particulate COD solubilisation as compared to the single-pathway model. The slowly ($F_{0,s}$) and rapidly ($F_{0,r}$) hydrolysable fractions of the raw sludge were 36% and 25%, respectively. A comparison of the estimated coefficients for the mesophilic digesters revealed that the hydrolysis coefficients (both $K_{hyd,s}$ and $K_{hyd,r}$) of the M1 digester were greater than those of the M2 and M3 digesters. In the thermophilic digesters it was observed that the $K_{hyd,r}$ value of the T1 digester differed from those of the T2, T3 and C2 digesters; whereas, the hydrolysis rate of slowly hydrolysable matter (i.e., $K_{hyd,s}$) did not differ significantly among these digesters. The influence of the facultative bacteria, that originated from the WAS fraction of the raw sludge, and/or the presence of hydrolytic biomass with different enzymatic systems may have contributed to the different hydrolysis rates in the M1 and T1

digesters from the corresponding mesophilic (i.e, M2 and M3) and thermophilic (i.e., T2 and T3) 2nd-phase digesters.

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To

My mother and the loving memory of my father

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Nomenclature

Alk	Alkalinity
AD	Anaerobic Digestion
ACN	Acetonitrile
BMP	bichemical Methane Potential
COD	Chemical Oxygen Demand
DGGE	Dentarring Gradient Gel Electrophoresis
HRT	Hydraulic Retension Time
mL	Mili-liter
MSA	Methanesulfonic Acid
PCR	Polymerase Chain Reaction
PCOD	Particulate Chemical Oxygen Demand
POB	Propionate Oxidizing Bacteria
PS	Primary Sludge
SCOD	Soluble Chemical Oxygen Demand
SD	Standard Deviation
STKN	Soluble Total Kjeldahl Nitrogen
TCOD	Total Chemical Oxygen Demand
TKN	Total Kjeldahl Nitrogen
TTKN	Total Total Kjeldahl Nitrogen
TPAD	Temperature Phased Anaerobic Digestion
TS	Total Solids
TSS	Total Suspended Solids
VS	Volatile Solids
VSS	Volatile Suspended Solids
VFA	Volatile Fatty Acids
WAS	Waste Activated Sludge

F	Biodegradable Particulate COD
F_0	Initial Biodegradable Particulate COD
$F_{0,s}$	Initial Slowly Biodegradable Particulate COD
$F_{0,r}$	Initial Rapidly Biodegradable Particulate COD

J	Objective Function
k_{max}	Specific Maximum Substrate Utilization Rate Constant
K	Specific Solubilisation Rate Constant
K_s	Half-saturation Constant
K_{hyd}	Hydrolysis First-Order Constant
$K_{hyd,s}$	Hydrolysis First-Order Constant for Slowly Biodegradable Particulate COD
$K_{hyd,r}$	Hydrolysis First-Order Constant for Rapidly Biodegradable Particulate COD
n	Number of Measurements
p	Number of Parameters
S	Substrate
S_0	Initial Substrate Concentration
t	Time
T	Temperature
X_0	Initial Biomass Concentration
Y	Yield Coefficient
μ_{max}	Specific Maximum Biomass Growth Rate Constant
θ	Temperature Correction Parameter
θ	Parameter
σ^2	Mean Square Fitting Error

Chapter 1
Introduction

1.1 Statement of Problem

An inherent by-product of wastewater treatment operations is a highly putrescible material which is produced from the removal of settleable organics (primary sludge) and/or bacterial cells (waste activated sludge). Handling and processing of sludge is a complex and costly operation in wastewater treatment facilities (Metcalf and Eddy, 2003). Anaerobic digestion is the most widely used process for stabilisation and treatment of sludge. Because of its low energy requirements, reduction of CO_2 emission, possibility for energy recovery and production of a safe to handle residue, anaerobic digestion is a highly sustainable process (Ramirez et al., 2009).

Traditionally, mesophilic single-stage digesters have been employed in wastewater treatment facilities for sludge digestion. However, use of advanced digestion technologies is increasingly taken into account to enhance sludge stabilisation and production of pathogen free biosolids. Some examples of these technologies include two-phase (Ghosh, 1987) and temperature phased anaerobic digestion (Han et al., 1997). Several studies have shown better volatile solids reduction in phased anaerobic digestion systems as compared to single-stage digesters (Zahller et al., 2007; Gioannis et al., 2008). Phased digestion systems have been evaluated to determine the optimum operating conditions in terms of HRT, temperature, organic loading, configuration and pH in each phase. Most of the studies have focused on comparing the performance of phased digestion systems with that of single-stage digesters through the modification of the above-mentioned parameters. Previous studies have indicated improved treatment performance in terms of biogas production and volatile solids reduction for phased digestion systems (Skiadas et al., 2005; Watts et al., 2006; Bolzonella et al., 2007; Nges and Liu, 2009). However, fewer attempts have been made to look into underlying mechanisms resulting in improved performance of phased digestion systems. An improved understanding of the phenomena taking place in each stage of the phased digestion systems would be valuable in process optimization and design of these systems.

Anaerobic digestion is a complex process in which several groups of microorganisms with different growth rates and sensitivity to environmental conditions are involved. Gujer and Zehnder (1983) suggested a six-step reaction scheme for degradation of particulate organic matter. However, more steps may be considered for anaerobic conversion processes such as homoacetogenesis in which acetate may be

oxidized or produced depending on H_2 partial pressure (Kotsyurbenko, 2005; Gavala and Lyberatos, 2001). Among these processes, 5 major groups of microorganisms may be recognized including hydrolytic bacteria that convert macromolecules organics into soluble polymers and monomers; acidogenic bacteria which convert monomers into volatile fatty acids (VFA); acetogens that produce acetate and H_2 from VFA; and finally acetoclastic and hydrogenotrophic methanogens that generate methane from either acetate or H_2 (Sotemann et al., 2005).

Mathematical models are useful tools for comprehensive study of microbial ecology and major events taking place in anaerobic digestion processes (Kalyuzhnyi et al., 1998). In many anaerobic digestion models such as ADM1 (Batstone et al., 2002), microorganisms as an integral part of the models are grouped according to their function in the anaerobic degradation of organic matter. In addition, it is assumed that the species within each group have the same characteristics. ADM1 is a mechanistic model and has been widely used for modeling and simulation of anaerobic digestion of different wastes (Feng et al., 2006; Schoen et al., 2009). Even though this model has successfully been employed to simulate the behaviour of phased anaerobic digesters (Blumensaat and Keller, 2005; Parker, 2005), it still needs to be optimized for robust modeling of these systems. The simplistic assumption of lack of diversity in the characteristics of the species within the microbial groups may lead to over- or under-estimation of the model output when compared to observed data. Each of the above-mentioned microbial groups involves a variety of microorganisms performing a similar task. However, their characteristics (such as toxicity level tolerance, growth rates, tolerance to environmental changes) would not necessarily be the same.

Due to the limitations of culture-based techniques, the knowledge about anaerobic digestion microbiology has historicallybeen limited (Amann et al., 1995). Advances in molecular microbiological techniques offer the opportunity to analyze and understand the microbial ecology of anaerobic digestion. Molecular methods have demonstrated new levels of diversity within the microbial populations present in anaerobic digesters (Zumstein et al., 2000). In this regard, microbial ecology and functional diversity of methanogens have been studied more than the other groups of organisms that are active in anaerobic digestion (i.e, acidogens, acetogens). There is strong evidence available indicating the existence of two different sub-groups of acetoclastic methanogens in anaerobic digesters. Several studies have demonstrated the dominance of the slow-growing *Methanosaeta sp.* at low concentration of acetate (or long HRTs), while proliferation of the fast-growing *Methanosarcina sp.* at high levels of acetate (or short HRTs) (Raskin et al., 1995; McHugh et al., 2003; Vavalin et al., 2008; Karakat et al., 2010). Similar to the observations made for methanogens, recent studies indicate the presence of different sub-groups of

propionate oxidizing bacteria (POB). The species of the genera *Syntrophobacter* and *Smithella sp.* LR were reported to dominate at low concentrations (0.5-2.5 mM) of propionate or low dilution rates (0.01-0.08 d^{-1}); while, the species of *Pelotomaculum* and *Smithella sp.* SR were highly active at high propionate levels (>15mM) or high dilution rates (0.3 d^{-1}) (Liu et al., 1999; Shigematsu et al., 2006; Ariesyady et al., 2007).

It is well documented that the diversity of microbial species in aerobic or anaerobic treatment processes is influenced by the composition of influent substrate, environmental conditions such as pH and temperature and operational conditions such as HRT, feeding pattern, substrate concentration and organic loading (Yuan and Blackall, 2002; Wilderer et al., 2002). Phased anaerobic digestion systems are typically operated at differing organic load, pH, temperature and HRT in each phase. Hence, it would be likely that kinetically-different species within a similar functional group would dominate in separate phases. This may lead to inconsistency between predicting values by a model and experimental results. In addition, design, evaluation and optimization of a phased anaerobic digestion system will be unrealistic.

To this point, no attempts have been made to comprehensively investigate the microbial ecology of various configurations of phased digestion systems. The kinetics of hydrolysis, propionate oxidizing bacteria and acetoclastic methanogens have not been evaluated in each stage of the phased digestion systems. Information on the bacterial and archaeal structures of each stage is not available. In addition, the fate of bacterial and archaeal biomass which are present in either the influent raw sludge or the first-stage effluent in subsequent digesters have not been established. The use of Arrhenius-type relationships for temperature correction of biokinetic parameters has been used in ADM1 (Batstone et al., 2002) however they have not been assessed for phased digestion systems operated at different temperatures in the separate phases.

1.2 Objectives

The primary objective of this research was to improve the modeling of phased anaerobic digestion systems. The achievement of the primary objective relied on the accomplishment of the following secondary objectives.

1. To compare the efficiency of organic and protein-containing matter removal in phased and single-stage digesters

2. To identify the structures of the bacterial and archaeal communities using a PCR-based DGGE finger printing technique for comparison with kinetic studies results

3. To determine biological activity of acetoclastic methanogens through estimation of their bio-kinetic parameters using off-line batch experiments

4. To determine biological activity of propionate oxidizing bacteria through estimation of their bio-kinetic parameters using off-line batch experiments

5. To assess hydrolysis rate through estimation of hydrolysis kinetic parameters using off-line batch experiments

6. To evaluate the feasibility of applying Arrhenius-type relationships in phased anaerobic digestion systems for temperature-correction of the parameters estimated in the specific objectives 3-5

Chapter 2
Literature Review

2.1 Anaerobic digestion modeling

Anaerobic digestion has been used as the most common method of sludge digestion and is an effective way for production of an energy-rich biogas (CH_4). In spite of the wide spread application of sewage sludge anaerobic digestion, its design, operation and maintenance is still largely based on experience and empirical designs (Sotemann et al., 2005). Substantial advances have been made in understanding the fundamentals of anaerobic digestion, resulting in successful implementation of numerous full-scale sewage sludge digesters.

Mathematical modelling of anaerobic digestion of sewage sludge is of great interest because modelling and simulating a system is much faster and less expensive in comparison with performing laboratory experiments. Also, modelling provides a valuable tool for the comprehensive study of microbial ecology and gives expression of conceptual ideas to explain the main phenomena of interest occurring within the anaerobic digestion process (Sotemann et al., 2005). Using modelling, the parameters that significantly influence the process can be identified and hence this can give guidance for the establishment of design criteria. It also helps to find possible causes for system malfunction and to recommend remedial measures (Kalyuzhnyi et al., 1998). Despite the application of anaerobic digestion modelling and intensive research, the microbial ecology of sewage sludge digesters remains largely a black-box. This is largely due to the complex dynamics of biological, chemical and physical sub-systems with inter-relationships among them.

Depending on the purpose of model application, anaerobic models can be divided into two categories: simplified and complex models. The early models (Graef and Andrews, 1974; Hill and Barth, 1977) and the models that are developed to control and monitor anaerobic digesters (Bernard et al., 2001; Mata-Alvarez, 1987) have considered two key microbial populations of acidogens and methanogens. In more complicated and detailed models, several additional microbial populations and substrates have been included (Batstone et al., 2002; Batstone et al., 1997; Costello et al., 1991). The models describing with detail all the known processes of anaerobic digestion are generally difficult to use for process control, however, they are appropriate for identification and better understanding of the major mechanisms occurring in anaerobic digestion and for study of the microbial ecology of digesters.

Considerable variations in conceptual schemes of the biological processes of anaerobic digestion of sewage sludge can be found in the literature, ranging from a three-step reaction (hydrolysis, acidogenesis and methanogenesis) (Graef and Andrews, 1974) to the six-step reaction scheme recommended by Gujer and Zehnder (1983). In the later scheme, three main components of organic matter (proteins, carbohydrates and lipids) are separately hydrolyzed by extracellular enzymes into soluble end products (Figure 2.1). These hydrolysis products are small enough to be transported across the cell membranes of microorganisms. These soluble products are fermented or anaerobically oxidized to short chain fatty acids, alcohol, CO_2, H_2 and ammonia. The intermediate products such as propionate, butyrate, valerate, etc. which are produced in hydrolysis are converted to acetate, H_2 and CO_2 via a process called acetogenesis. Finally, methanogenesis occurs through two separate processes, that is, cleavage of acetate called acetoclastic methanogenesis, and reduction of hydrogen with CO_2 called hydrogenotrophic methanogenesis. This biological reaction scheme can be simplified for modelling of sewage sludge digestion due to the following reasons (Sotemann et al., 2005).

- Due to the difficulty in analysing sewage sludge for its main components, for simplification, hydrolysis of sewage sludge can be considered a single process in which biodegradable particulate organic matter are solubilized to microbially-usable and fermentable material. This is reasonable because in acidogenesis three organic groups of hydrolysis (simple sugars, amino acids and long chain fatty acids) are finally fermented to essentially similar end products, namely volatile fatty acids. Thus, recognition of three separate hydrolysis products and anaerobic oxidation of long chain fatty acids are not needed (Figure 2.1).

- Products of acidogenesis are influenced by H_2 partial pressure in anaerobic digestion systems. Under low H_2 partial pressure conditions acetate, CO_2 and H_2 are generated, while at high partial pressure of H_2, acetate together with other higher volatile fatty acids, CO_2 and H_2 are produced. Due to generation of low concentrations of butyrate and higher short chain fatty acids from sewage sludge digestion, only propionate is considered as the only short chain fatty acid in this reaction scheme (Figure 2.2).

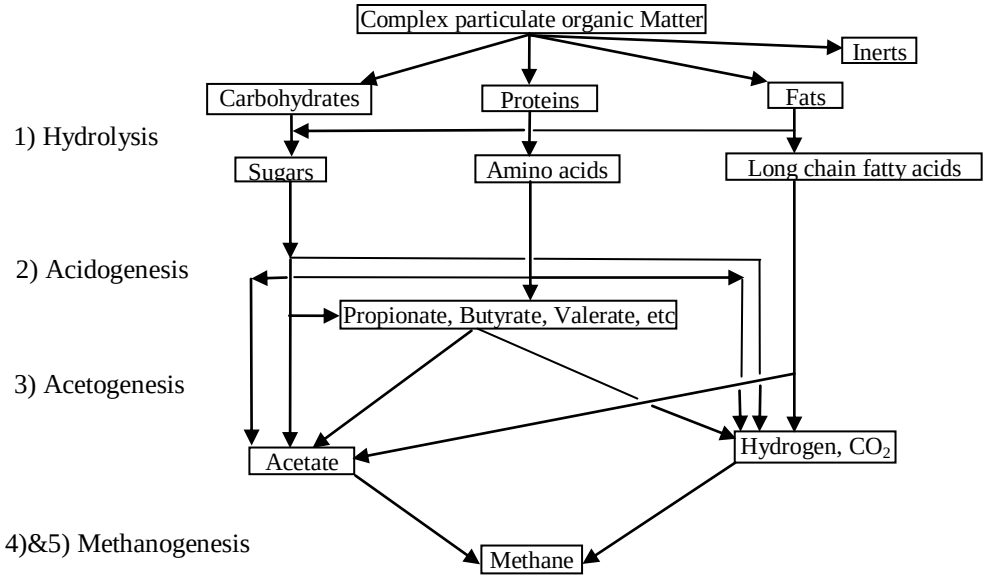


Figure 2.1. Anaerobic digestion of complex organic matter such as sewage sludge proposed by Gujer and Zehnder (1983)

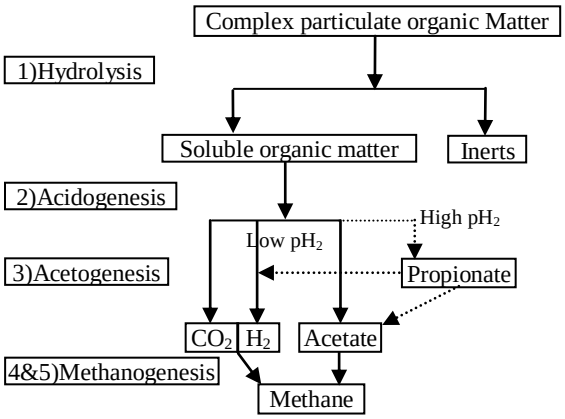


Figure 2.2. Anaerobic digestion of sewage sludge proposed by Sotemann et al. (2005)

Using the revised reaction scheme, the hydrolysis process and three biological processes (acidogenesis, acetogenesis and methanogenesis) that are mediated by the four microbial populations and their corresponding kinetic models are discussed below.

2.1.1. Biological processes and microbial groups of anaerobic digestion model

2.1.1.1 Hydrolysis

The hydrolysis process is an extracellular and partly non-biological process during which complex particulate organic matter is converted to readily biodegradable matter which can pass the cell membrane. This facilitates modelling of the complex hydrolysis step by considering a lumped kinetic for all steps of lysis, non-enzymatic decay, phase separation and physical breakdown (e.g. shearing). During the hydrolysis step inert particulate and soluble material are generated.

Hydrolysis and solubilization of particulate organic matter is influenced by several parameters such as: pH, hydrolytic bacteria concentration, adsorption of enzymes to particles, particle size, temperature, etc, and as a result, it is often difficult to model the hydrolysis step by reliable kinetics. First order kinetics, which are dependent only on the biodegradable substrate concentration, are typically assumed for the hydrolysis step (Pavlostathis and Giraldo-Gomez, 1991; Gujer and Zehnder, 1983; Eastman and Ferguson, 1981):

$$r_{Hyd} = K_h[S_{bp}] \qquad (2.1)$$

where, r_{Hyd} is hydrolysis rate; K_h is first order hydrolysis kinetic rate constant; and (S_{bp}) is biodegradable particulate organic matter concentration.

However, as mentioned above at least the hydrolytic bacteria concentration plays an important role that should be included in the kinetic model. Thus, a number of kinetic models have been used to describe the hydrolysis process considering the hydrolytic or acidogenic bacteria concentration. Eliosov and Argaman (1995) included the biomass concentration in the first order kinetic model.

$$r_{Hyd} = K_h[S_{bp}][X_{acid}] \qquad (2.2)$$

where, (X_{acid}) is the active biomass of acidogenic bacteria.

Vavalin et al. (1996) proposed Contois kinetics for modelling of hydrolysis of activated sludge. Besides acidogenic concentration, they considered the availability of substrate for biomass in their model.

$$r_{Hyd} = \frac{K_{\max,Hyd}\dfrac{[S_{bp}]}{[X_{acid}]}}{K_{s,Hyd} + \dfrac{[S_{bp}]}{[X_{acid}]}}[X_{acid}] \qquad (2.3)$$

where, $K_{max,Hyd}$ is maximum specific hydrolysis rate constant; and $K_{s,Hyd}$ is half saturation constant for hydrolysis.

Comparing a number of hydrolysis kinetic models, Vavalin et al. (1996) and Sotemann et al. (2005) found that a first order model was as effective as or only slightly poorer than the more complex surface-related models such as Contois. Thus, using simple first order kinetics, which have been supported by experimental observations, is reasonable for modelling hydrolysis process (Batstone et al., 2002).

The hydrolysis constant of the biodegradable particulate COD at 35 ^{0}C and a pH range of 5.14 to 6.67 has been reported to be in the range 0.11-0.20 d^{-1} (Eastman and Ferguson, 1981). Siegrist et al. (2002) reported disintegration rate constants of 0.25 and 0.40 d^{-1} for mesophilic (35 ^{0}C) and thermophilic (55 ^{0}C) anaerobic digesters fed with primary sludge, respectively. As can be seen from the reported hydrolysis rate constants, pH and temperature can considerably influence the rate constant.

Most data concerning hydrolysis kinetic constants have been reported for single-phase digesters. Regarding phased digesters, the different conditions of the first and second reactors have not been taken into account. The temperature dependence of the hydrolysis process is typically addressed by an Arrhenius-type relationship (Batstone et al., 2002). Hydrolysis process may be a rate-limiting step in sewage sludge anaerobic digestion and does not reach completion within the normal range of the hydraulic retention time (Sotemann et al., 2005). There is a lack of data regarding hydrolysis kinetics for phased digestion systems. In temperature phased reactors, due to differences in temperature between phases or different pH values in two-phase reactors with similar temperature, the hydrolysis rate may be different in each phase. Moreover, in primary sludge-fed phased digesters, due to the different nature of the substrate entering the first and the second reactor, a different hydrolysis rate may be applied because the substrate entering the second reactor would be slowly hydrolysable matter constituted of microbial biomass.

2.1.1.2 Fermentation and Acidogenesis

Acidogenesis is a microbial process in which reduced organic matter such as VFA are produced without an additional electron donor or acceptor (Gujer and Zender, 1983). During the acidogenesis step the solubilized and monomeric organic matter is mainly converted to VFA and alcohols by a diverse group of bacteria and to a lesser extent by protozoa, fungi and yeasts (Toerien and Hattingh, 1969; Gavala et al., 2003). A large variety of acidogens have been isolated with *Clostridium sp.*, *Streptococcus sp.* and

Bacillus sp. as the prevalent species (Holmes and Freischel, 1987). Interspecies hydrogen transfer (a symbiosis between hydrogen producing organisms and hydrogenotrophic methanogens), pH, HRT and previous acclimation of the inoculum have been reported to influence the production pathway of acidogenesis (Harper and Pohland, 1986; Gavala et al., 2003).

As described in the modified reaction scheme, the acidogenesis process is typically modelled according to the partial pressure of hydrogen. The soluble fermentable organic matter produced via the hydrolysis process is utilized by acidogenic organisms and acetate, H_2 and CO_2 are produced. Under high partial pressure of H_2, the acidogenic reaction produces propionate in addition to the products mentioned. This process is mostly modelled using Monod-type kinetics (Costello et al., 1991; Siegrist et al., 1993; Sotemann et al., 2005) including inhibition functions due to H_2 partial pressure and pH. Under conditions of low H_2 partial pressure, the substrate utilization rate may be formulated as equation 2.4.

$$r_{su,acid} = k_{\max,acid} \frac{[S_F]}{K_{s,acid} + [S_F]} X_{acid} \frac{K_{I,H2}}{K_{I,H_2} + [H_2]} I_{pH} \qquad (2.4)$$

where, $r_{su,acid}$ is readily fermentable substrate (S_F) utilization rate by acidogenic organisms (S_F is measured as ($S_{\text{-bio-COD}}$ − VFA); $S_{\text{-bio-COD}}$ is soluble biodegradable chemical oxygen demand); $k_{max,acid}$ is max. substrate utilization rate by acidogenic organisms; (S_F) is soluble fermentable substrate; $K_{s,acid}$ is half-saturation of acidogenic organisms; X_{acid} is biomass of acidogenic organisms; $K_{I,H2}$ is hydrogen ion inhibition constant; and I_{pH} is pH inhibition function.

For the production of propionic acid under high partial pressure of H_2, the substrate utilization rate by the acidogens can be formulated as equation 2.5.

$$r_{su,acid} = k_{\max,acid} \frac{[S_{bs}]}{K_{s,acid} + [S_{bs}]} X_{acid} \left\{ \frac{[H_2]}{K_{I,H_2} + [H_2]} \right\} I_{pH} \qquad (2.5)$$

Generally, a temperature correction correlation has been employed to model temperature effects in single-phase digesters and only one set of bio-kinetic parameters is used for mesophilic and thermophilic conditions. This assumption has been recommended for modelling of anaerobic phased digesters (Siegrist et al., 2002; Blumensaat and Keller, 2005). Application of current anaerobic digestion models to two-phase digesters (Siegrist et al., 2002) showed a deviation in predicting concentrations of VFA, especially propionate, under different HRT. However, in addition to temperature other parameters such as substrate concentration and pH may influence the microbial groups in the separate stage of phased digestion systesm. It is well-documented that different environmental conditions can stimulate the growth

of different groups of organisms with distinct values of kinetic constant (Heyes and Hall, 1981; Zoetemeyer et al., 1982a). Although with available molecular probes the presence of a new microbial group of acidogens cannot be distinguished, kinetic investigations using mathematical models enable us to study whether a different group of acidogens dominates under different conditions of each reactor of phased digesters. If this holds true, it would be required to use different kinetic constants for acidogens in each reactor.

As in all microbially-mediated reactions, the performance of treatment system always will depend on the quantity of viable and active cells and a well-balanced ecology. During modelling of a two-phase digester, Batstone et al. (2000) reported an inconsistency between gas flows and reactor load. Simulation of gas flows with high loading was reported to be close to experimental values, while the model under-predicted performance during low load periods. On the basis of a mass balance on carbon and nitrogen over the reactor, they attributed the higher gas flows to the entrance and degradation of upstream reactor biomass. On the other hand, having compared experimental data with those obtained through simulation of temperature-phased anaerobic digesters, Parker (2005) found an under-prediction of VFA concentration in the effluent of the second reactor. It was noted that further investigation is needed because assuming that the biomass leaving the upstream digesters to remain active in a different temperature may result in over-prediction of biomass activity in the downstream digesters. Therefore, to improve modelling of phased digesters, it is of interest to know the fate of the acidogenic biomass within the downstream reactors of anaerobic sewage sludge digesters.

2.1.1.3 Acetogenesis

Within the diverse group of anaerobic acidogens, acetate-forming bacteria are referred to as acetogens (Gavala et al., 2003). Two mechanisms are suggested for acetogenesis: acetogenic hydrogenation and acetogenic dehydrogenation. In the former, CO_2 and H_2 are converted to acetate by homoacetogenic bacteria. In anaerobic digestion, acetogenic dehydrogenation is the main mechanism during which VFAs are oxidized anaerobically to acetate by OPRA. In acetogenic dehydrogenation, short chain fatty acids are anaerobically oxidized mainly to acetate, resulting in the reduction of protons and formation of hydrogen. OPRA can only use protons as an electron acceptor. High concentrations of hydrogen inhibit the process as these oxidations became endergonic. Moreover, due to the reduced energy yield to OPRA, they cannot dispose of their electrons through forming reduced products (Dolfing, 1988). Some known mesophilic obligate proton reducing acetogens are presented in Table 2.1.

Table 2-1. Currently known mesophilic obligate proton reducing acetogens (OPRA) (Dolfing, 1988)

Organisms	Co-culture with	Source	Substrate
Syntrophobacter wolinii	*Desulfovibrio G11, or M. hungatei JF1 + D. G11*	Sludge Digester	Propionic acid
Syntrophomonas wolfei	*Desulfovibrio G11, M. hungatei JF1*	Sludge digester, rumen Aquatic sediments	Monocarboxylic saturated fatty acids
Syntrophus buswellii	*Desulfovibrio G11, M. hungatei JF1 + D. G11*	Sludge Digester, Aquatic sediments	Benzoic acid
Clostridium bryantii	*Desulfovibrio E70, M. hungatei M1h*	Sludge Digester, Marine sediments	Monocarboxylic saturated fatty acids
Strain Gra I val	*Desulfovibrio E70*	Aquatic sediments	Isovaleric acid

Most digestion models consider butyric acid as an acidogenesis product in anaerobic digestion of organic matter (Costello et al., 1991; Angelidaki et al., 1999; Batstone et al., 2002). However, due to low concentrations of butyrate in sewage sludge digestion (Sotemann et al., 2005; Siegrist et al., 2002), only propionate has typically been employed in models of the acetogenesis process. As with acidogens, Monod-type kinetics are often used to describe propionate conversion by acetogens. Since this reaction is thermodynamically feasible only at a low partial pressure of H_2, a non-competitive function is often incorporated into the Monod-type equation. Also, pH can influence acetogenic organisms at low and high values, thus a pH inhibition is considered in Monod equation.

$$r_{su,pro} = k_{\max,pro} \frac{[S_{pro}]}{K_{s,pro} + [S_{pro}]} X_{pro} \left\{ 1 - \frac{[H_2]}{K_{I,H_2} + [H_2]} \right\} I_{pH} \qquad (2.6)$$

where, $r_{su,pro}$ is propionate utilization rate; $k_{max,pro}$ is max. substrate utilization rate by propionate-utilizing organisms; (S_{pro}) is propionate concentration; $K_{s,pro}$ is half-saturation of propionate-utilizing organisms; X_{pro} is biomass of propionate-utilizing organisms; $K_{I,H2}$ is hydrogen ion inhibition constant; and I_{pH} is pH inhibition function.

In phased anaerobic digestion, the reactors are separated to provide enhanced environments for acidogens and methanogens. Due to the different environmental (e.g., pH and temperature) and operational (e.g., HRT) conditions, it is likely that a different population of propionate- utilizing organisms with distinct kinetics will proliferate in each phase (Heyes and Hall, 1981). This assumption could be further supported using molecular techniques to investigate the microbial communities of each reactor. Moreover, using quantitative molecular tools, it may be possible to study the relative quantity and activity of this significant microbial group in the anaerobic digestion process.

2.1.1.4 Methanogenesis

Methanogenesis is the final step in most anaerobic environments (Zinder, 1993). Methanogens can use a limited number of simple compounds composed mostly of single- or two-carbon substrates. The most versatility has been observed in some strains of *Methanosarcina* which are able to use seven substrates (Zinder, 1993). Methanogens are classified into 5 orders within the kingdom Archaeobacteria, including *Methanobacteriales*; *Methanococcales*; *Methanomicrobiales*; *Methanosarcinales*; and *Methanopyrales*. According to the metabolic pathways they can be categorized into three groups including: acetoclastic methanogens; methylotrophic methanogens; and hydrogenotrophic methanogens (Boone et al., 1993). Currently, there are probes available for identification of the first four orders which might be present in sewage sludge digesters (Raskin et al., 1994a).

Most methane produced in anaerobic digesters comes from acetate cleavage by acetoclastic methanogens (Ferry, 1993; Chynoweth and Mah, 1971; Smith and Mah, 1966). However, due to the lower growth rate of acetoclastic methanogens as compared to hydrogenotrophic methanogens, the methane produced in anaerobic digesters having low residence times may not be dominated by the acetoclastic pathway (Ferry, 1993; Zinder and Koch, 1984). In addition to the acetoclastic and hydrogen-utilizing methanogens, the acetate-oxidizing organisms have mostly been reported in thermophilic anaerobic reactors (Zinder and Koch, 1984; Peterson and Ahring, 1991; Griffin et al., 1998). However, literature information is scarce and needs further investigations to reveal under which circumstances (e.g., what HRT and acetate concentration) this pathway may be employed by methanogens in anaerobic digesters.

Only two genera of acetoclastic methanogens are known to use acetate: *Methanosarcina* and *Methanosaeta*. The former generally grows faster and can use several substrates (Ferry, 1993). *Methanosaeta sp.* is dominant under long HRTs that are typically employed for operation of anaerobic digesters. The lower saturation constants (K_s) favour *Methanosaeta sp.* at higher hydraulic retention times (Zinder et al., 1984).

The study of the ecology of methanogens is easier as compared to other microbial groups involved in anaerobic digestion (e.g., fermentative and acetogenic organisms), due to fewer species of this microbial group and well-defined reactions. Several studies have been performed on the variations in acetoclastic methanogens (Zinder et al., 1984; Chartrain and Zeikus, 1986; Raskin et al., 1994a) according to operational conditions (e.g., HRT or organic loading) and configuration (e.g., suspended- vs. attached-growth) of anaerobic digesters.

Generally, two sub-processes are considered for modelling of the methane production process, namely, acetoclastic and hydrogenotrophic methanogenesis. Acetoclastic methanogenesis is the process whereby acetic acid is converted to methane and CO_2 and growth of acetoclastic methanogens takes place. As with acidogenesis and acetogenesis, the acetate utilization rate is typically modelled using a Monod-type equation. Methanogens are fastidiouse organisms and are very sensitive to pH changes. Thus, inhibition functions are often employed in the Monod-type equation. The pH inhibition function for the hydrogenotrophic methanogens is less severe than that for the acetoclastic methanogens because they need to regulate the level of H_2 and allow the generation of methane at low pH values (Costello et al., 1991). Acetate and H_2 utilization rates are modelled using equations 2.7 and 2.8, respectively.

$$r_{su,ac} = k_{\max,ac} \frac{[S_{ac}]}{K_{s,ac} + [S_{ac}]} X_{ac} I_{pH} \qquad (2.7)$$

$$r_{su,h_2} = k_{\max,h_2} \frac{[S_{h2}]}{K_{s,ac} + [S_{h2}]} X_{h2} I_{pH} \qquad (2.8)$$

where, $r_{su,ac}$ and $r_{su,h2}$ is acetate and hydrogen utilization rates; $k_{max,ac}$ and $k_{max,h2}$ is Max. acetate and hydrogen utilization rates constants; S_{ac} and S_{h2} are acetate and hydrogen concentrations; $K_{s,ac}$ and $K_{s,h2}$ are half-saturation constants for acetate and hydrogen; X_{ac} and X_{h2} are biomass concentration of acetoclastic and hydrogenotrophic methanogens.

As mentioned above, the main methane production pathway in sewage sludge digesters is acetate cleavage which is performed by two major acetoclastic methanogens, namely, *Methanosarcina* and *Methanosaeta*. There is sufficient information in the literature that shows that depending on acetate concentrations in sewage sludge digesters, one of these species will dominate in a digester (Zinder et al., 1984; Chartrain and Zeikus, 1986; McMahon et al., 2004). This suggests that it may be desireable to use two distinct kinetic constants for modelling of acetate utilization at low and high concentrations of acetate. Due to lower growth rate of *Methanosaeta sp.*, lower biomass that is predictable by using equation 2.7 is produced in anaerobic digesters. There are quantitative molecular techniques such as FISH and quantitative real-time PCR which can be used to determine the relative concentration of acetoclastic methanogens using specific-group probes (Raskin et al., 1994a). Simultaneous use of modelling and molecular techniques may help to study the validity of the results predicted by modeling.

2.2 Phase Separation

The advantages of phase separation have been addressed as early as 1958 by Babbit and Baumann (1958). They reported that the negative effects of the products of the acidogenesis process, which can have inhibitory effects on the progress of the later processes, can be solved by using two or more separate stages. Therefore, the environment in the first reactor would favour acidogens, while the second reactor can be controlled to enhance the proliferation of methanogens, which would receive the products of the first phase. The physiology, nutrient requirements, metabolic characteristics and sensitivity to environmental factors are greatly different for acid formers and methane formers (Pohland and Ghosh, 1971). Based on these differences, Pohland and Ghosh (1971) suggested two-phase anaerobic digesters.

Phase separation in two-phase digesters is based on the difference in the specific growth rates of acidogens and methanogens. For example, the maximum specific growth rates reported for acidogenic bacteria are 1-3 d^{-1}, while those of methanogenic organisms are 0.3-0.5 d^{-1} (Henze et al., 1995). Ghosh and Pohland (1974) reported elimination of the methanogenic organisms from an acid-phase digester at HRTs of 11-14 h, using glucose as a feed substrate. The maximum specific growth rate for acidogens and methanogens were 1.25 and 0.14 h^{-1}, respectively. Kisaalita et al. (1989) found that HRTs of 30, 6.66 and 2.5 h were the critical HRTs for acetoclastic methanogens, hydrogenotrophic methanogens and acidogenic bacteria, respectively. The literature reveals that a wide range of HRT have been employed in the first and second reactors of two-phase digestion (Table 2.2).

The temperature-phased anaerobic digestion (TPAD) process is also a phased digestion system and typically consists of a thermophilic first phase digester operated in series with a mesophilic second-phase digester (Schmit and Ellis, 2001; Han and Dague, 1997). The main difference between the two-phase and temperature-phased processes is that methanogenesis is often inhibited in the first phase of a two-phase system; while in temperature-phased systems the thermophilic first stage often maintains methanogenesis. A large amount of VFA and other reduced compounds have been reported to be produced in the first phase of each system. In the first phase of temperature-phased systems a significant portion of the generated VFA and other reduced compounds are converted to methane and carbon dioxide and the remaining is converted in the second phase, while in two-phase anaerobic digestion this conversion is fulfilled in the methane reactor due to the low pH and short HRT kept in the first phase. Typical HRT values used in temperature-phased digesters are presented in Table 2.2.

Several researchers have reported better performance and improvements in operational stability in two-phase anaerobic digesters (Pohland and Ghosh, 1971; Ghosh 1978; Cohen et al., 1979; Massay and Pohland, 1978). Phased digestion allows enhanced metabolic and kinetic capabilities for acidogenic and methanogenic organisms due to the presence of optimum environmental (such as temperature, pH) and operational (such as HRT, organic loading rates) conditions for each group. For instance, using the most probable number (MPN) method, Zhang and Noike (1991) reported an equal number of acidogenic (10^8-10^9 MPN/mL) and acetogenic (10^8-10^{10} MPN/mL) bacteria in single- and two-phase anaerobic digesters. However, due to higher specific VFA production rates (mg COD-VFA/gVSS/d) in the first reactor of a two-phase digester as compared to a single-phase sustem, they concluded that phase separation could encourage the acidogens to grow at their maximum capacity. Cohen et al. (1980) also reported a higher maximum specific sludge load (g COD/g biomass/day) for a two-phase system than that for a single-phase digester. In addition, they found that although overloaded two-phase digesters produced several fatty acids, they were immediately converted after cessation of the glucose supply. In the single-phase reactor, the accumulation of acetic and propionic acids were observed and interruption of glucose supply resulted only in acetate turnover, while propionate lingered in the system. It seems that in the two-phase digestion, the OPRA and hydrogenotrophic methanogens allowed the system to recover following glucose cessation, while in the single-phase digester, the high hydrogen concentration did not let OPRA resume their activities; thus, propionate remained in the system, while acetoclastic methanogens could reduce the acetate level.

Most research on two-phase digestion has been focused on the configuration, operational strategies and amenability of feed substrate to phased digestion (Ghosh, 1987; Fox and Pohland, 1994; Han et al., 1997). Regarding modelling of two-phase digester, one set of kinetic constants is usually used to model the biological processes taking place in each reactor (Batstone et al., 2000); and in temperature phased digesters, changes in the rates of biological processes due to temperature increase are typically corrected using the Arrhenius equation (Siegrist et al., 2002; Batstone et al., 2002).

$$K_2 = K_1 \phi^{T_1 - T_2} \qquad (2.9)$$

where, K_2 is rate constant at temperature T_2; K_1 is rate constant at temperature T_1; and Φ is:

$$\phi = e^{\frac{-E_a}{RT_1T_2}} \qquad (2.10)$$

where, E_a is activation energy; R is universal gas constant; and T is temperature; Φ for biological treatment processes ranges 1 to 1.1 (Metcalf and Eddy, 2003).

Table 2-2. Hydraulic retention times (HRT) reported in first (R1) and second (R2) reactors of phased anaerobic digestion

Substrate	T (^{0}C)		Configuration	HRT in R1(day)	HRT in R 2(day)	Reference
	R1	R2				
Two-phase digestion						
Glucose	37	37	CMR[$]	0.47-1.2	6.47	Ghosh et al., 1975
PS[*]+WAS[**]	35	35	CMR	2	13	Ghosh, 1987
	55	55				
	35	35		2	13	
	35	55				
	55	55				
	35	35		0.9	2.1	
	55	55				
PS+WAS	37	50	Hybrid[&]	2	8	Chang et al., 1989
WAS	38	36	CMR	3.1	9.1	Ghosh et al., 1995
PS+WAS	35	35	CMR	2-2.7	10	Bhattacharya et al., 1996
Soft drink waste	35	35	Hybrid[&&]	2.2	5.2	Ghosh et al., 1985
Starch	35	35	CMR	0.35-2	1.4-8	Zhang and Noike, 1991
Dairy wastewater	34	34	Hybrid[&&]	0.5	1.5	Ince and Ince, 2000
Temperature-phased digestion						
PS	55	35	CMR	3.5	6.7-10	Han and Dague, 1997
PS+WAS	37	62	CMR	1	14	Cheunbarn and Pagilla, 2000
	62	37				
PS+WAS	55	35	CMR	7.4	12.6	Vandenburgh and Ellis, 2001
PS+ODMSW[***]	55	35	CMR	5	10	Schmit and Ellis, 2001
Dog food	55	55	Batch	2	18	Speece et al., 2005
	35	35				

[*] Primary sludge; [**] Waste activated sludge; [***] Organic fraction of municipal solid waste; $ Completely-mixed reactor; [&] Completely-mixed reactor and fixed-film reactor; [&&] completely-mixed reactor and up-flow anaerobic filter

As already mentioned about propionate-utilizing acetogens and acetoclastic methanogens, dominance of a group of the organisms with distinct biokinetic parameters may occur under different operational conditions. Also, assumption of degradation of hydrolytic-acidogenic biomass or, in contrast, survival of these organisms in downstream reactors may result in errors when predicting methane gas production or VFA concentrations. Finally, the influence of phased digestion on the biodegradability of digesting sludge and the rate of hydrolysis processes, which may have different characteristics in the acid- and methane-phase, need further study.

2.3 Factors affecting microbial consortia in anaerobic sewage sludge digesters

Several factors that can influence the microbial groups in anaerobic digesters have been addressed in the literature. Hydraulic retention time, temperature, pH, H_2 partial pressure in digesters, the source of inoculum, substrate composition and organic loading are the factors that may influence the microbial ecology of sewage sludge digesters. As mentioned above, in anaerobic modelling, the effect of pH and H_2 concentration, which can have inhibitory effects on some of the anaerobic microbial groups and the thermodynamics of the reactions, are typically taken into account by incorporation of inhibition functions in models (Siegrist et al., 2002; Batstone et al., 2002). The selection of inoculum containing large numbers of methanogens and acetogens (Stroot et al., 2001; McMahon et al., 2001), which have low growth rates, has been reported to reduce the start-up period of digesters, but in the long-term had no impact on the microbial ecology of digesters since the microbial species proliferate depending on the feed composition and operational conditions of the digester (Fongastitkul and Mavinic, 1994; Harper and Pohland, 1986); growth rates of the organisms present, resilience to any toxic/inhibitor products in the influent or within the digester and operational changes applied to the digester (Delbes et al., 2001; Briones and Raskin, 2003). Of the operational parameters, HRT and temperature are discussed in more detail since they may have more influence the microbial ecology and dominance of microbial species in the first and second reactors of phased digesters.

2.3.1 Hydraulic retention time (HRT)

Hydraulic retention time is one of the vital parameters impacting on population dynamics in anaerobic sludge digesters. In addition, kinetic parameters due to proliferation of new species and wash-out phenomena are also influenced by HRT (Zhang and Noike, 1991). This parameter has been used to separate acid and methane phases in digesters. Several studies have examined the effect of HRT on the kinetics of each phase, digester performance and to a limited extent the microbial ecology of the sludge digesters.

HRT may influence the dominant species in anaerobic sludge digesters. Organisms with low generation times (high specific growth rates) and high saturation constants will be dominant at low HRTs, while those with low specific growth rates and low saturation constants would proliferate at long HRTs. For example, it has been reported that *Methanosarcina sp.* grow rapidly with low K_S and proliferate at low HRTs, while *Methanosaeta sp.* (formerly *Methanothrix sp.*) predominate at long HRTs due to their slower growth rates and high K_S (Zinder et al., 1984; Chartrain and Zeikus, 1986). As regards to propionate oxidizing bacteria, a similar conclusion was reported by Heyes and Hall (1983). They found

two distinct groups of propionate oxidizing acetogens with different kinetic properties. Those with a higher specific growth rate of 0.05 h^{-1} and K_S of 330 mg/L were dominant in a digester with a relatively low HRT (8.2 days), while the other sub-group with a specific growth rate of 0.0054 h^{-1} and K_S of 11 mg/L proliferated at a higher HRT (14.5 days). They found that the slow growing propionate oxidizers were more sensitive to pH extremes than the fast growing ones.

Using the most probable number (MPN) method, Zhang and Noike (1991) studied the effect of a wide range of HRT values (1.5 to 245 h) on the microbial population levels of anaerobic digesters fed with starch as the sole carbon and energy source. They found that HRT had a considerable effect on the population levels of methanogens, homoacetogens and sulphate reducing bacteria, but they did not observe a significant effect on the population levels of hydrolytic bacteria and acetogens. A comparative study about the population levels in single- and two-phase anaerobic digester showed a considerably higher population of hydrogenotrophic methanogen concentrations in the two-phase digester (Zhang and Noike, 1991). The same study showed that acetoclastic methanogens were 2-10 times higher in the second phase of the two-phase digester as compared to the single-phase system.

Scully et al. (2005) used a molecular technique (terminal restriction fragment length polymorphism, t-RFLP) to study the microbial ecology of an expanded granule sludge bed reactor following an HRT decrease from 48 to 24 h. Bacterial and archaeal diversity decreased, although high levels of *Methanococcus-like* and *Methanobacterium-like* (H_2-utilizing) organisms were detected in the reactor. Several researchers have reported that the major route of methane production in imbalanced digesters is hydrogenotrophic methanogenesis (Delbes et al., 2001; Scully et al., 2005; Schnurer et al., 1999).

HRT is one of the main and controllable operational parameters that can influence the dominance of different microbial species, the kinds of intermediates produced by different microbial groups present and the rate of hydrolysis, fermentation and gasification reactions in digesters. Several studies have been performed to investigate the effect of HRT on anaerobic reactor performance, acidogenic products and hydrolysis and acidogenesis rates (Kisaalita et al., 1989; Puchajda and Oleszkiewicz, 2006; Ghosh et al., 1975). A few studies have been reported using traditional microbiological methods such as the most probable number (MPN) viable counting method, to examine the microbial population levels in two-phase reactors (Zhang and Noike, 1991; Ince and Ince, 2000). Due to the intrinsic limitations of traditional microbiological methods, precise investigation of microbial diversity was not possible, while new molecular biological techniques permit study of the diversity and structure of microorganisms without the

need to culture them first. Molecular biological tools as reliable techniques can be employed to study the contribution of microbial ecology to the performance of anaerobic digesters under various HRTs. In addition, it is of interest to know the fate of microorganisms present in the influent feed within acidogenic and methanogenic reactors. Ribosomal RNA hybridization techniques can produce information not only about the identity of microorganisms, but also about their relative activity and abundance in phased digesters (Molin and Givskov, 1999). On the other hand, the abundance of each microbial group can be predicted by anaerobic models. Comparing the results obtained by semi-quantitative molecular techniques such as FISH and real-time PCR with those predicted could enhance the validity of models.

In spite of the important contribution of HRT to the microbial structure of anaerobic reactors and as a consequence to microbially-mediated reactions such as methane production, investigations of the microbial ecology and the fate of microorganisms through phased completely-mixed digesters fed with sewage sludge is lacking.

2.3.2 Temperature

Temperature is one of the most important factors affecting the microbial activity within anaerobic digesters. The rates of anaerobic reactions such as acidification or methane production are temperature-dependent. Higher temperatures have been reported to result in higher hydrolysis, fermentative and methanogenesis rates (Gerardi, 2003). Most methanogens are generally believed to be active in two narrow temperature ranges including mesophilic (30-35 ^{0}C) and thermophilic (50-60 ^{0}C). Acidogenesis has been reported to proceed at reasonable rates at temperatures as low as 21 ^{0}C or lower, whereas, methane production rates at temperatures below 30 ^{0}C have been found to be relatively slow (Gerardi, 2003).

Operation at a thermophilic temperature has some advantages over conventional mesophilic anaerobic sludge digesters including increased pathogen destruction and higher hydrolysis, acidogenesis and methanogenesis rates. However, a higher sensitivity to organic loading and temperature changes, increased concentrations of VFA in the reactor effluent and reduced conversion of propionate to acetate have been reported in thermophilic sludge digesters (Han et al., 1997; Ghosh, 1987; Han and Dague, 1997).

Information in the literature shows the influence of temperature on the microbial ecology of anaerobic digesters. Pender et al. (2004) observed that temperature elevation caused a change in the methanogenic population from a *Methanosaeta sp.* dominated community during mesophilic operation

(37 ^{0}C) to *Methanosarcina sp.* dominance. A similar result has been reported where *Methanosaeta sp.* decreased following a temperature shift from mesophilic temperature (37 ^{0}C) to thermophilic temperature (55 ^{0}C) (Ahring, 1995). In general, methanogenic diversity has been reported to be greater in reactors operating under mesophilic conditions (Karakashev et al., 2005; Sekiguchi et al., 1999; Pender et al., 2004). Pender et al. (2004) reported a substantial increase of *Methanomicrobiales*, upon temperature increase from mesophilic to thermophilic conditions.

Anaerobic digestion of sludge and methane production at municipal wastewater treatment plants is mostly performed in the mesophilic range. At industrial wastewater treatment plants, thermophilic operation is sometimes preferred (Gerardi, 2003) due to limited space and the need for higher reaction rates in the anaerobic digesters. As regards to psychrophilic anaerobic digesters, although preliminary lab-scale studies showed the feasibility of using psychrophilic anaerobic reactors to treat low-strength wastewaters, their application is very limited (Lettinga et al., 2001). Currently, there is little information on the microbial populations of psychrophilic anaerobic reactors. It has been reported that under psychrophilic conditions, as with mesophilic conditions, methanogenesis was mainly achieved through acetate cleavage by acetoclastic methanogens, especially *Methanosaeta sp.* which was present in higher numbers in comparison to hydrogenotrophic methanogens. Also, substantial diversity of methanogenic populations has been reported (McHugh et al., 2003).

Besides the microbial ecology, biological activity of organisms may also be influenced by temperature. Characterization of biomass in anaerobic reactors in terms of biological activity (expressed as utilization rate of substrates or methane production) can show the response of various metabolic groups due to a change of environmental factors such as temperature.

There is controversy regarding the survival and acclimatization of mesophilic species at thermophilc conditions or shift in microbial population in phased anaerobic digesters. Vandenburgh and Ellis (2002) performed a specific methanogenic activity test on both mesophilic (35 ^{0}C) and thermophilic (55 ^{0}C) biomasses of a temperature-phased digester. The methanogenic activity of the thermophilic biomass was as high as 0.2 and 1 g CH_4/g VSS/d when the serum bottles were incubated at 35 and 55 ^{0}C, respectively. For the mesophilic biomass, the specific methane production rate at the mesophilic temperature was 0.4 and at the thermophilic temperature was 1 g CH_4/g VSS/d. As the specific methane production rates were almost similar at 35 ^{0}C and 55 ^{0}C for the biomass taken from thermophilic and mesophilic reactors they concluded that there were not necessarily a variation in microbial populations from mesophilic to thermophilic reactor. A similar conclusion was drawn by Song et al. (2004),

indicating survival and activity of mesophilic organisms in a thermophilic digester. However, no microbiological examinations were performed in both cases. On the other hand, a number of investigations performed using molecular techniques show variations in the diversity of microbial consortia after temperature changes (Delbes et al., 2000; McMahon et al., 2001; Pender et al., 2004; McHugh et al., 2003).

Historically, it has been believed that temperature can influence biochemical reactions and generally a temperature correction correlation has been used for modelling its effect on biological reactions in anaerobic phased digesters. Development of molecular techniques during recent years has enabled researchers to have a better understanding about the microbial ecology of mesophilic and thermophilic anaerobic digesters. As mentioned earlier, literature information is scarce about the microbial ecology of phased anaerobic sludge digestion. Therefore, it is proposed to investigate the microbial ecology of sewage sludge under temperature gradients (from mesophilic to thermophilic and vice versa) and under the same temperature (mesophilic-mesophilic and thermophilic-thermophilic) but different environments (acid-phase and methane-phase). Besides the microbial ecology, it is proposed to generate knowledge about the biological activity, expressed as either specific methane production rate (mL CH_4/g VSS/d) or specific substrate utilization rate, of particular microbial groups under the above-mentioned conditions. The results obtained through activity tests can be further confirmed by molecular techniques through identification of whether the same microbial groups survive under new conditions or a succession occurs in microbial communities.

2.4 Molecular techniques for study of microbial ecology

A number of molecular techniques that are appropriate for analysis of microbial ecology are available and these have been widely used for studying the microbial communities of natural and engineered systems. Wagner et al. (1993) have reported that culture-based methods for studying microbial ecology only reveal approximately 10% of the total populations. The culture-independent methods, can generally be put into two groups, include polymerase chain reaction (PCR)-based and hybridization methods.

2.4.1 PCR-based methods

In these methods, total DNA or RNA is extracted from microbial communities. Then, a pair of primers (forward and reverse primers) is used in combination with polymerase chain reactions to amplify the target DNA fragment from a whole community sample. After amplification by PCR, the fragment can

be used in several post-amplification methods (Figure 2.3). This group of molecular biological methods does not necessarily need pre-requisite knowledge of the microbial community and is used to determine the microbial community profile or structure (Dabert et al., 2002).

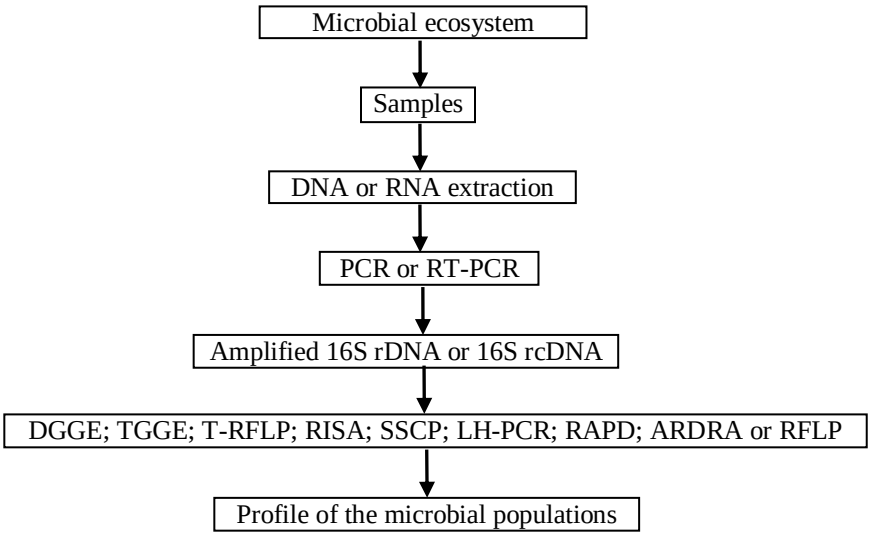


Figure 2.3. DNA fingerprinting techniques applied on environmental samples

As depicted in Figure 2.3, there are several PCR-based molecular fingerprinting techniques used for studying the microbial communities of environmental samples. A brief description of each method is provided below. Advantages and limitations of PCR-based techniques are summarized in Table 2.3. Based on the information provided, three methods are proposed for both quantitative and qualitative study of phased anaerobic sewage sludge digesters.

Amplified Ribosomal DNA Restriction Analysis (ARDRA) or restriction fragment length polymorphism (RFLP) involves digesting the 16S rRNA gene sequence amplified by PCR. For 16S rRNA digestion, different restriction enzymes are employed. Then, gel electrophoresis is used to separate the restricted fragments. McHugh et al. (2003) used this technique to study the diversity of methanogens in a range of full- and lab-scale anaerobic bioreactors.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) is based on similar principles as ARDRA analysis; however, labelled-primers are used in the PCR reaction. This makes this method able to detect only the terminal fragments. Each terminal restriction fragment (t-RFs) is separated according to its size in base pairs and intensity of fluorescence (Liu et al., 1997).

Denaturing Gradient Gel Electrophoresis (DGGE) separates PCR-amplified ribosomal DNA based on its GC-content within a gradient denaturing gel. Due to the use of a GC-clamp in the PCR primers, the DNA is not completely denatured. Separation is based on the decreased electrophoretic mobility of the partially melted double-stranded DNA molecule. A variant of DGGE is called Temperature Gradient Gel Electrophoresis (TGGE) in which the gradient is based on the temperature rather a chemical (Muyzer et al., 1993).

Length Heterogeneity PCR (LH-PCR) is sometimes called Amplicon Length Heterogeneity (ALH) and is a molecular technique similar to T-RFLP, separating amplified community ribosomal DNA according only to the size. As with T-RFLP, the relative abundance of PCR amplicons originating from different genes are determined from the fluorescence emission of labeled PCR primers. However, no endonuclease enzyme is used in this method (Suzuki et al., 1998).

Ribosomal Intergenic Spacer Analysis (RISA) is a molecular method in which the space between small subunit and large subunit ribosomal DNA (16S rDNA-23S rDNA) is amplified. This region has a high degree of sequence and length variability among species (Fisher and Triplett, 1999), enabling a more detailed taxonomic identification and distinguishing between closely-related species.

Single-Stranded Conformation Polymorphism (SSCP) separates single DNA fragments on the basis of variations in their secondary structure due to their sequence variation. Fragments of similar length but different sequence can be separated on a gel (Zumstein et al. 2000). Due to the lack of a database for SSCP peaks, this technique should be used with other methods such as clone libraries (Speigelman et al., 2005) to compare the unique peaks obtained from the clones with those from the total microbial community and then to use sequencing of the corresponding clones.

Randomly Amplified Polymorphism DNA (RAPD) uses a single short PCR primer to amplify random segments of DNA. Thus, no knowledge is required about the DNA sequence of the target organism(s) unlike other sequence-dependent methods such as t-RFLP or ARDRA that target 16S or 23S-rDNA.

The real-time PCR method can monitor the concentration of PCR products throughout the amplification cycles using a fluorescent reagent. This reagent binds with the PCR product at the end of each cycle. The fluorescence intensity emitted during this process shows the PCR product concentration at each cycle. The cycle at which the fluorescence emission intensity rises above background noise is

referred to as the threshold cycle. The threshold cycle is determined at the exponential phase of the PCR reaction and is inversely proportional to the copy number of the target sequence. A standard curve is constructed with serial dilutions of known concentrations of plasmid DNA or other sources and determination of the threshold cycle for each concentration. The copy number of the target sequence of sample can be quantified by comparing the threshold cycle with the standard curve (Zhang and Fang, 2006).

2.4.2 PCR-independent molecular techniques

The second group of molecular techniques is categorized as probe hybridization methods. As shown in Figure 2.4, two methods include Fluorescence In Situ Hybridization (FISH) and dot-blot or membrane hybridization (Dabert et al., 2002). In these methods, short, fluorescently labelled oligonucleotide probes generally of known sequences are hybridized to complementary nucleic acid sequences, usually 16S rRNA.

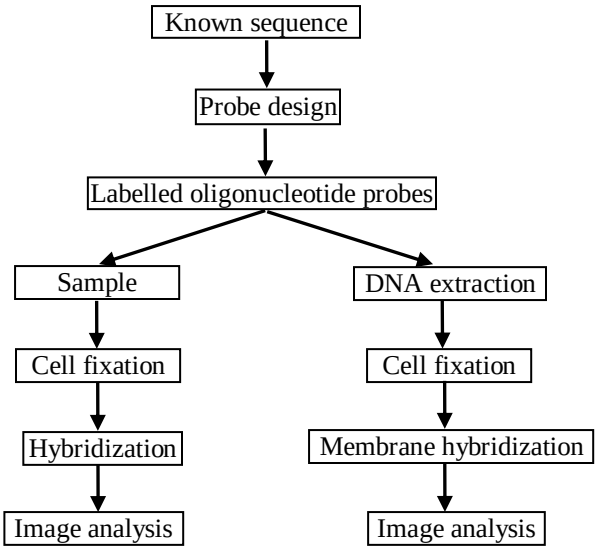


Figure 2.4. Process of hybridization methods

Fluorescence in situ hybridization is a method in which a cell is made fluorescent by labelling it with a specific nucleic acid probe. This nucleic probe is tagged to a fluorescent dye. If the targeted cells (organisms) have known sequences, molecular probes can be used to study the microbial populations present in man-made or natural environments. In FISH, 16S rRNA molecules of a single organism or a

group of organisms are specifically targeted by labelled oligonucleotide probes. A fluorescently-labelled oligonucleotide probe is applied to the fixed target cells during hybridization. Epifluorescence microscopy, confocal laser scanning microscopy (CLSM) or flow cytometry are used to observe the targeted cells (Amann, 1990 a,b). The results are usually expressed as the proportion of a microorganisms' rRNA that is detected by a specific probe to the RNA of total microbial population detected by a universal probe. Oligonucleotide probes are mostly employed for the following three purposes (Speigelmann et al. 2005).

i. to study the presence/absence of various taxonomic groups in the community

ii. to quantify the relative abundance of specific taxa

iii. to determine the spatial distribution of species or groups of interest

Through the last decade, significant progress has been made in designing probes for FISH. Available probes can be used to estimate the abundance of all organisms as well as all archaea present in a digester using a universal probe and archaeal-domain probe, respectively. These probes are designed to target the 16S rRNA of virtually all known organisms and archaea. Usually, the abundance of archaea is expressed relative to total organisms present. Also, the relative abundance of order, family, genus and species ribosomal RNA can be determined through comparing the results obtained by group-specific probes to that obtained by family- or order-specific probes. Currently available probes can be used to determine the relative abundance of some species of acetogens and many archaeal family and many major genus and species that are involved in anaerobic sludge digesters. As a consequence, the major trophic groups in phased reactors and their activity can be studied through using FISH. Also, it is possible to identify which group of these organisms, specifically on the genus and species level, is dominant in each reactor in comparison to the total and family-specific ribosomal RNA identified using universal probe and family-specific probe, respectively.

The second category of hybridization methods is dot-blot hybridization. The principles for membrane or dot-blot hybridization are similar to FISH, except that total nucleic acids from a sample are initially extracted. Then, the denatured nucleic acids are fixed on a membrane for hybridization with selected probes (Raskin et al., 1994a). This method cannot determine the in situ activity of organisms because the DNA is extracted from the sample.

The limitations and advantages of the various DNA profiling methods are provide in Table 2.3. Based on the information provided in this table, T-RFLP and DGGE are superior for studying the

population dynamics in sludge digestion systems due to the good databases that are available. Although T-RFLP analysis depends on the appropriate selection of the endonuclease enzymes, it can show both the microbial community structure and abundance of each isolate because the intensity of the fluorescent primers can be measured in this method. However, its dependence on enzyme specificity may preclude the detection of new species within the microbial community. On the other hand, DGGE can give a better phylogenetic resolution because the bands can be excised for further analysis by sequencing (Collins et al., 2006). The limitation of hybridization methods for investigation of anaerobic sludge digestion is that few studies have been done on the hydrolytic, acidogenic and acetogenic organisms; as a result, limited probes are available for studying the microorganisms involved in these steps of sludge digesters. This may be attributed to the diverse microbial groups present in these steps or limited information for probe design; thus, there are few studies of the organisms present in hydrolytic-fermentative and acetogenic steps of the anaerobic reactors using FISH and hybridization techniques (Cirne et al., 2007; Hansen et al., 1999).

Table 2-3. Advantages and limitations of the PCR-based molecular biological techniques (Dabert et al., 2002; Speigelman et al., 2005; Collins et al., 2006)

Method	Limitation	Advantage
ARDRA	➢ optimization in each experiment is required ➢ various enzymes produce different fragments ➢ selection of an appropriate enzyme is necessary ➢ species diversity cannot be determined precisely as ARDRA is enzyme-dependent	➢ separates rDNA genes according to differences in both size and sequence ➢ rapid and cost-effective ➢ if used on formerly cloned sequences, it is a screening method to minimize sequencing of clone libraries
T-RFLP	➢ as with ARDRA, selection of an appropriate enzyme is critical	➢ more precise than ARDRA ➢ automated sequencing for size selection of the digested amplicons
LH-PCR	➢ since size is the determining factor, overlapping of the different amplicons with the same size is more probable comparing to other methods ➢ abundance of organisms cannot be determined due to large fluorescent fragments produced	➢ simple and rapid
SSCP	➢ double-stranded amplified DNA fragments are needed to be denatured (single-strand) ➢ high rate of re-annealing single strands of DNA leads to more than one band ➢ no database for SSCP bands	➢ simple procedure compared to DGGE due to no need for GC-clamp or gradient gels
RAPD	➢ reproducibility of the banding pattern is dependent on equipment or reagents employed ➢ Many amplicons are produced, especially in complex communities ➢ sequencing for further recognition of available taxonomic groups cannot be done	➢ rapid, simple and cost-effective ➢ no sequence knowledge is required
DGGE	➢ optimization for gradient gel and electrophoresis duration in each experiment is required ➢ DNA fragments typically less than 500 bp are analyzed ➢ less than 500 bp limits the amount of sequence for further identification ➢ large quantity of DNA should be extracted from sample for effective resolution	➢ co-migration of the DNA fragments with the same size is less of problem; thus, Higher resolution can be obtained ➢ the bands can easily be transferred to hybridization membranes or used for sequencing
RISA	➢ intergenic spacer (the space between 16S rRNA and 23S rRNA) database is not as large as that of 16S	➢ no need for gradient gels; long electrophoresis time; or restriction enzymes ➢ automated version is available ARISA

2.5 Activity test

Microbial activity tests can be used for determining the activity (i.e., kinetics) of key microbial groups to monitor the different phases of the anaerobic digestion process. The experimental methods to determine the activity of fermentative organisms, propionate-utilizing synthrophs, and hydrogenotrophic and acetoclastic methanogens are similar to biochemical methane potential (BMP) tests that are often used to determine the biodegradability of anaerobic sludges.

Two different methods can be considered in sludge activity measurements, depending on the information required. They include either an overall measurement (or methanogenic activity) giving information about the whole degradative activity; or group-specific activity measurements of each of the main groups present in the different stages of digestion. The overall measurement can be used to select an appropriate inoculum for a sludge digester, while the measurement of the activity of different microbial groups involved is a valuable tool to monitor reactor performance and to determine the rate-limiting step of the process because changes in the concentrations of intermediates are indirect indications of changes in activity of different trophic groups (Soto et al., 1993).

The measurement of methane production can be employed for monitoring substrates that are directly converted to methane such as acetate, hydrogen and formate. As regards other substrates which are converted to methane through several steps it is recommended to measure the turn-over of the substrate itself to avoid the potential interference of methanogens or other groups of organisms (Sorensen and Ahring, 1993). There are some factors that may impact the results of activity tests, including initial substrate concentration, the background level of substrate in the biomass, the amount of inoculum in the serum bottles and the intensity of shaking where phase transfer limitation of gaseous substrate from the headspace to the liquid is important (Coates et al., 1996).

The initial substrate concentration and the amount of inoculum are of significant importance in setting up activity tests. Soto et al. (1993) recommended that the initial substrate concentration should be higher than the half-saturation constant of a particular microbial group in order to avoid the substrate from being limiting. The concentration of the substrate being tested should be well below the saturation constant (K_s) values in control bottles and above K_s values in test bottles. Therefore, in the case of high VFA concentrations in an inoculum, dilution is required to lower their concentrations below the K_s values. The saturation constants that have been reported for different trophic groups are provided in Table 2.4.

For a reliable activity test, the methane production should be dependent on the amount of sludge over a range of sludge concentrations. It has been reported that there exists a linear relationship between amount of sludge and methane production rate (Dolfing and Bloemen, 1985; Soto et al., 1993).

Table 2-4. Half-saturation constants (K_s) of some microbial groups involved in anaerobic digesters (Sorensen and Ahring, 1993)

Substrate	Organism or System	K_s values
Acetate	*Methanosarcina sp.*	3-5 mM
	Mathanosaeta sp.	0.46-1.2 mM
	Thermophilic acetoclastic methanogens	0.4-0.8 mM
Propionate	Mesophilic sludge	0.04-0.19 mM
	Continuous enrichment culture	0.28-8.28 mM
Butyrate	Butyrate degraders	0.065-0.076 mM
Hydrogen	*Methanobacterium sp.*	0.011-0.015 atm
	Mesophilic sludge	0.105 atm
Hydrogen	Hydrogenotrophic methanogens	0.0025-0.013 mM

Activity tests alone (Dolfing and Bloemen, 1985; Sorensen and Ahring, 1993; Soto et al., 1993) or in combination with FISH and (Hansen et al., 1999; O'Reilly and Colleran, 2006) have been used for studying the activity of acidogenic organisms, butyrate- and propionate-utilizing acetogens, sulphate reducing bacteria and hydrogenotrophic and acetoclastic methanogens of anaerobic digesters. The combined techniques can be used to understand the impact of phased digestion on the various trophic groups involved in anaerobic digestion.

2.6 Information gaps

Several studies have been performed to investigate kinetics, microbial ecology, modelling and process optimization of anaerobic digesters. Most of this work has been focused on single-phase digesters. In anaerobic digestion processes, as indicated in previous sections, several microbial groups are involved to convert organic matter to a final product of methane gas. Based on the different characteristics of microbial groups involved, Ghosh and Pohland (1971) developed a two-phase digestion process in order to optimize the process by providing optimal conditions for the two major groups of organisms (acidogens and methanogens) to allow them to reach their maximum physiological capacity. Since then, several studies have been carried out to optimize phased anaerobic digestion process.

However, it was found that literature information regarding phased digesters is scarce with respect to the microbial ecology, biological activity and sludge biodegradability (i.e., hydrolysis). Further studies on these subjects can improve modelling of the phased anaerobic digesters.

- Due to the limitations of traditional culture-based microbial methods, less work has been performed to determine the microbial ecology and fate of microorganisms that is present in feed sludges in downstream reactors. In wastewater treatment plants, sludge digesters are fed with sludges that are generated from upstream processes such as organic carbon and nutrient removal systems in which different aerobic, facultative and anaerobic organisms are present. It is not clear whether these organisms die or acclimatize to their new environment and contribute to the digestion process. Likewise, the fate of hydrolytic-acidogenic organisms is not obvious after entering the methane phase. This subject can be investigated using recently developed molecular techniques. The use of DGGE can reveal the microbial communities present at each phase and in parallel can give qualitative information on the variations of microbial consortia within each phase. Identification of microorganisms can be achieved using post PCR methods such as cloning and sequencing to determine genetic sequences of organisms. Then, they can be identified by comparing their sequences with the GeneBank database (Maidak et al., 1994).

- Anaerobic digestion models are mainly based on microbially-mediated reactions which are performed by several groups of organisms. The biological activity of key microbial groups may be influenced in phased digestion due to different conditions (pH, temperature, HRT) provided in each reactor. A combined use of activity tests with anaerobic models can give information about the kinetics and functional diversity of the microbial groups which are active in each phase. For modelling of phased digesters it is important to know which fraction of the key microbial group is biologically active in each phase. For instance, assuming that the upstream acidogenic biomass remains totally active or decay in methanogenic reactor could result in either under- or over-prediction of either the VFA concentrations in the effluent of the second reactor (Parker, 2005) or the methane production (Batstone et al., 1997). Knowing the biologically active fraction of the key microbial groups can give a better understanding of the activity of the microbial groups in each phase, leading to improved modelling and simulation of phased anaerobic digesters.

- As stated in the literature review, different operating conditions may lead to the dominance of sub-groups with different kinetic properties. This has been reported about propionate-oxidizing organisms and methanogens (Heyes and Hall, 1983; Zinder et al., 1984). A Succession of sub-groups could result in a deviation in simulation results, if a single set of kinetic parameters is employed for modeling of

anaerobic digesters (Siegrist et al., 2002; Batstone et al., 2002). For instance, Siegrist et al. (2002) found an inconsistency at the change from medium (8.7 d) to short (3.5 d) HRT between experimental propionate concentrations and those of simulation results. Besides kinetic studies, this problem can be investigated using the molecular techniques which are able to target particular microbial groups. In addition, the relative abundance of the microbial groups, which is influenced by their kinetic parameters, can be measured using semi-quantitative molecular techniques such as FISH and real-time PCR. Comparing the relative abundance of these microbial groups with those obtained from anaerobic models can reveal whether the anaerobic model is able to predict the abundance of the key microbial groups in a different operational condition.

- There is lack of data whether the existence of different conditions in each reactor of two-phase digesters influences the sludge biodegradability and hydrolysis rates. In temperature phased reactors, due to a different temperature in the second phase or a different pH in two-phase reactors with similar temperature, the hydrolysis rate can be different in each phase. The temperature effect may be addressed using a temperature correction correlation for modelling of single-phase anaerobic digesters (Batstone et al., 2002). However, in two-phase anaerobic digesters, the pH effect may also need to be considered for the hydrolysis process. Moreover, in primary sludge-fed phased digesters, due to a different nature of the substrate entering the first and the second reactor, a different hydrolysis rate may be employed because the substrate entering the second reactor mainly consists of microbial biomass.

Chapter 3
A Comprehensive Comparative Study on Phased and Single-Stage Anaerobic Digestion

3.1 Introduction

Sewage sludge is commonly treated using the anaerobic digestion process. In general, reduction of solids and chemical oxygen demand (COD) content, methane production and pathogen reduction are the major objectives of anaerobic digestion. However, the degradability of the sludge is often a limiting factor and if increased, would make the digestion process a more cost effective technology. Due to the accumulation of inert material present in the raw wastewater as well as inert biomass decay products, the sludge degradability is reduced in the wastewater treatment systems with a long sludge age (Gossett and Belser, 1982). Even though new technologies such as sonication and microwaving have been reported to increase sludge degradability (Eskicioglu et al., 2007b; Kianmehr, 2010), high sludge age systems are typically used in small-scale wastewater treatment plants where limited options are economically applicable for degradability improvement (Barr et al., 2008). Alternatively, phased anaerobic digestion systems, which comprise of two or three reactors in series, may be employed to improve degradability and biogas production (Ge et al., 2011).

Various configurations of anaerobic digestion such as mesophilic single-stage digestion, thermophilic single-stage digestion and temperature-phased anaerobic digestion (TPAD) have been studied. The TPAD process involves digesters operating at thermophilic and mesophilic temperatures in series. Even though either thermophilic or mesophilic digesters may be first in the sequence, the most common configuration has the thermophilic digester ahead of the mesophilic one. Fundamental reactor theory indicates a better performance of anaerobic digesters in terms of solids destruction when the digesters are operated in series rather than as single-stage digesters (Pohland and Ghosh, 1971). In phased digestion systems, two main groups of microorganisms with kinetically different characteristics, namely acidogens and methanogens, are allowed to dominate in each phase depending on the hydraulic retention time (HRT) selected for each phase. The fast growing acidogens dominate in the first-phase with a short HRT and methanogens in the second-phase with a longer HRT (Pohland and Ghosh, 1971). Optimization of hydrolysis and acidogenesis reactions, methane production, sludge degradability and pathogen reduction are cited as the main advantage for phased digestion systems (Han and Dague, 1997; Oles et al., 1997). The first-phase digester in the TPAD system is typically operated with an HRT of 2-5 days, while

the second phase digester is operated at HRTs of more than 7 days (Han et al., 1997; Dichtl, 1997, Watts et al., 2006). Traditionally, digesters are operated at 30-38° C for mesophilic conditions and 50-60°C for thermophilic (Dague et al., 1996). However, higher temperatures have been employed in recent years to improve the hydrolysis process and sludge degradability (Ge et al., 2010; Watts et al., 2006).

Several researchers have reported improved VS removal efficiency and operational stability in phased digestion systems (Ghosh, 1987, Bhattacharya et al., 1996; Han and Dague, 1997; Vandenburgh and Ellis, 2002; Skiadas et al., 2005; Watts et al., 2006; Nges and Liu, 2009). The majority of the research has focused on comparisons of phased digestions systems with the mesophilic or thermophilic single-stage digesters. Also, most of the studies have investigated the effect of different temperatures and HRTs of the 1st-phase digester on overall performance of the digestion system. However, direct parallel comparisons of the various temperature combinations of phased anaerobic digestion have rarely been completed (Ge et al., 2010).

The performance of the anaerobic digestion process is influenced by the microbial community structure (McMahon et al., 2004). Research has been done and verified the use of PCR-dependent and –independent techniques for the study of the microbial ecology of single-phase and hybrid digesters fed with simple (i.e., glucose) and complex organic matter (sewage sludge) under various operational conditions and during start-up (McMahon et al., 2001; Griffin et al., 1998; Sekiguchi et al., 1999; Delbes et al., 2000; Ueno et al., 2006; Pender et al., 2004; Leung and Topp, 2001; Sousa et al., 2007). The type of organisms and their relative population levels in digester biomass have been found to depend on substrate availability and operational conditions. Limited studies have been done to investigate the microbial community structure of phased anaerobic digesters which will be exposed to differing substrate levels and operating conditions in each phase. There is little information to indicate whether the species within the microbial groups differ between the phases. Also, there is limited information on the fate of organisms transferring between phases having different temperatures. This is of importance for process control and modeling of phased anaerobic digestion systems since interspecies competetion may lead to the dominance of kinetically-different microorganisms within a functional group in each phase.

Consequently, the objective of this study was to comprehensively compare phased anaerobic digestion systems of various temperature combinations with single-stage anaerobic digesters, operating with the same feed at similar hydraulic retention times (HRTs). Also, a direct comparison was made between mesophilic-mesophilic, thermophilic-mesophilic, mesophilic–thermophilic and thermophilic–

thermophilic phased anaerobic digestion systems. The microbial structure of the phased and single-stage digesters was investigated using a PCR-based denaturing gradient gel electrophoresis (DGGE) technique.

3.2 Methodology

3.2.1 Design of anaerobic digesters

For the purpose of the experiments, 8 bench-scale digesters of differing volumes were made using heat-resistance PVC pipe to facilitate operation at selected hydraulic retention times (HRT). The details of the digesters dimensions are given in Appendix C. Two water-baths, each holding four of the digesters at a target temperature of either 35 ^{0}C or 55 ^{0}C were also constructed. The temperature of the water-baths was kept constant using two separate heaters that were controlled by temperature controllers. To ensure that the correct temperature was maintained, thermometers were inserted through the ports on top of the digesters (two for mesophilic and two for thermophilic) to monitor the temperature inside the digesters. Two externally-mounted variable speed motors were used to mix the contents of the digesters at a speed of approximately 60-70 rpm. The generated biogas for each of the digesters was measured by an individual low flow gas meter that was made at the University of Waterloo. Tedlar® gas sampling bags were connected between the gas exit ports and the gas meters to buffer pressure during sludge feeding and wasting. The digesters were automatically fed through the feed ports at the top of the digesters, using timer-controlled pumps. The digested sludge was wasted by gravity through a port located close to the bottom of each digester. A tube was extended from the outlet to the liquid level of each digester and then the height of the tube was adjusted by trial and error depending on the pressure required to replace water in the relevant gas meters. An image of the digesters and gas meters is presented in Figure 3-1.

3.2.2 Start-up and operation of digesters

After design and assembly, the thermophilic and mesophilic digesters were seeded from a mesophilic anaerobic digester at the Waterloo Wastewater Treatment Plant (WWTP). A total of 21 L of digested sludge was taken to fill two thirds of the digester volumes. The following day, the digesters were fed with a mix of primary and waste activated sludges (PS + WAS) that was taken from the same treatment plant. The feed sludge was collected weekly from the Waterloo WWTP and stored in a carboy that was located inside a fridge with a temperature of 4 ^{0}C to minimize sludge degradation over the week (Appendic C: Table C-5). The temperature of the digesters was then rapidly adjusted to the target temperatures (i.e., 35 ^{0}C for mesophilic digesters and 55 ^{0}C for thermophilic digesters). Sludge was not added to the digesters for a period of 2-3 days and the biogas composition and effluent pH were

monitored to ensure active methanogens were present in all the digesters. The target volume of feed sludge was added to the digesters when the percentage of methane in the biogas steadily increased in concentration.

Once the digesters were operating at their target loadings they were fed three times a day using timer-controlled pumps. Overall, three peristaltic pumps were used including one pump with four heads for transferring partially digested sludge from the first-phase digesters to the second-phase digesters; one pump with two heads for feeding raw sludge to the first-phase digesters; and one pump with two heads to feed the single-stage digesters. The timers were set in a way that the second-phase digesters were fed first by transferring the partially digested sludge from the first-phase digesters. Then, raw sludge was fed to the first-phase and single-stage digesters. Consequently, a direct comparison among the various digestion systems was made possible by using a similar feed sludge.

The digesters were initially run for 45 days and it was found that the mixing was not sufficient as was indicated by occasional clogging of the outlet and inconsistent results. Therefore, the mixer of each digester was replaced. Following the replacement, the volatile solids and total chemical oxygen demand (TCOD) removal of the digesters increased significantly. The digesters were run for another 60 days to achieve steady-state conditions while monitoring their performance through analysis of conventional parameters. In this study, the stability of digesters was assessed based on less than 10% and 5% variation, respectively, in effluent COD concentration and CH_4 composition in 5 consecutive sampling (Bhattachrya et al., 1996). Following the achievement of steady-state, sampling of the digesters continued for almost a year to compare the performance of the various digestion combinations.

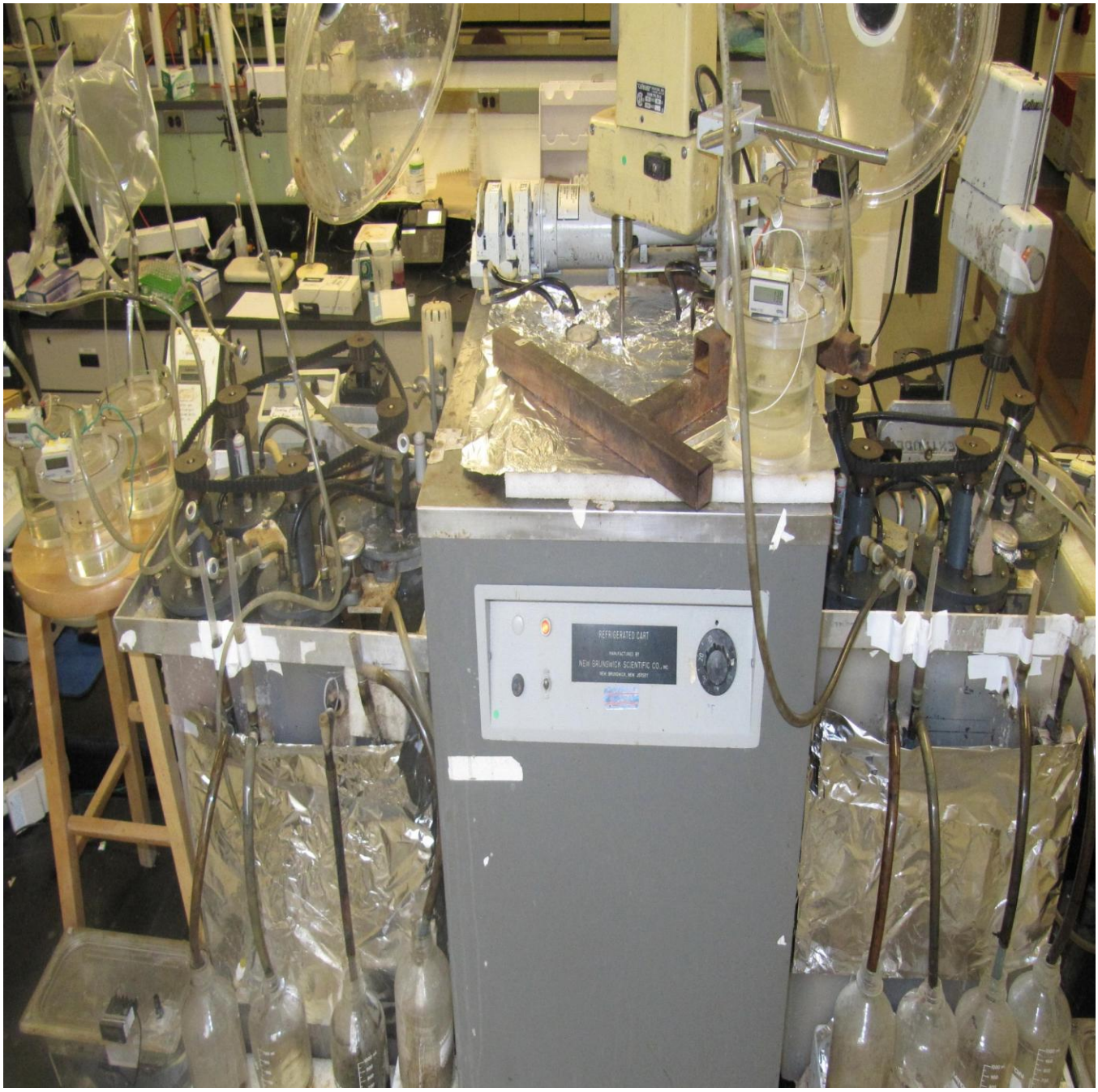


Figure 3.1. Image showing the digesters within the water-baths

3.2.3 Digester Operating Conditions

Four combinations of temperature in phased anaerobic digestion were investigated. A thermophilic temperature of 55 ^{0}C and a mesophilic temperature of 35 ^{0}C were chosen to run the digesters. The combinations included:

- Mesophilic-Mesophilic (M1M2)
- Thermophilic-Mesophilic (T1M3)
- Thermophilic-Thermophilic (T1T2)
- Mesophilic-Thermophilic (M1T3)

Six lab-scale completely-mixed digesters were employed in total for phased operation. Two smaller digesters with working volumes of 2.25 L were used as the first-phase digesters, one was operated at the mesophilic temperature and the other one at the thermophilic temperature. The partially digested sludge of the mesophilic first-phase was fed to separate mesophilic and thermophilic second-phase digesters, each having a working volume of 4.2 L. This operation resulted in the mesophilic-mesophilic and mesophilic-thermophilic combinations. Accordingly, the partially digested sludge of the thermophilic first-phase was fed to separate mesophilic and thermophilic second-phase digesters that also had volumes of 4.2 L. This operation resulted in the thermophilic-mesophilic and thermophilic-thermophilic combinations.

In addition, two lab-scale completely-mixed digesters with working volumes of 5.3 L were designed as mesophilic and thermophilic single-stage digesters. These single-stage digesters were considered as control digesters because single-stage operation is the most common anaerobic sludge digestion process in wastewater treatment plants. The first-phase, second-phase and single-stage digesters were operated at HRTs of 3, 14 and 17 days by controlling the feed and waste flows. Figure 3-2 schematically shows the various combinations of the digesters studied.

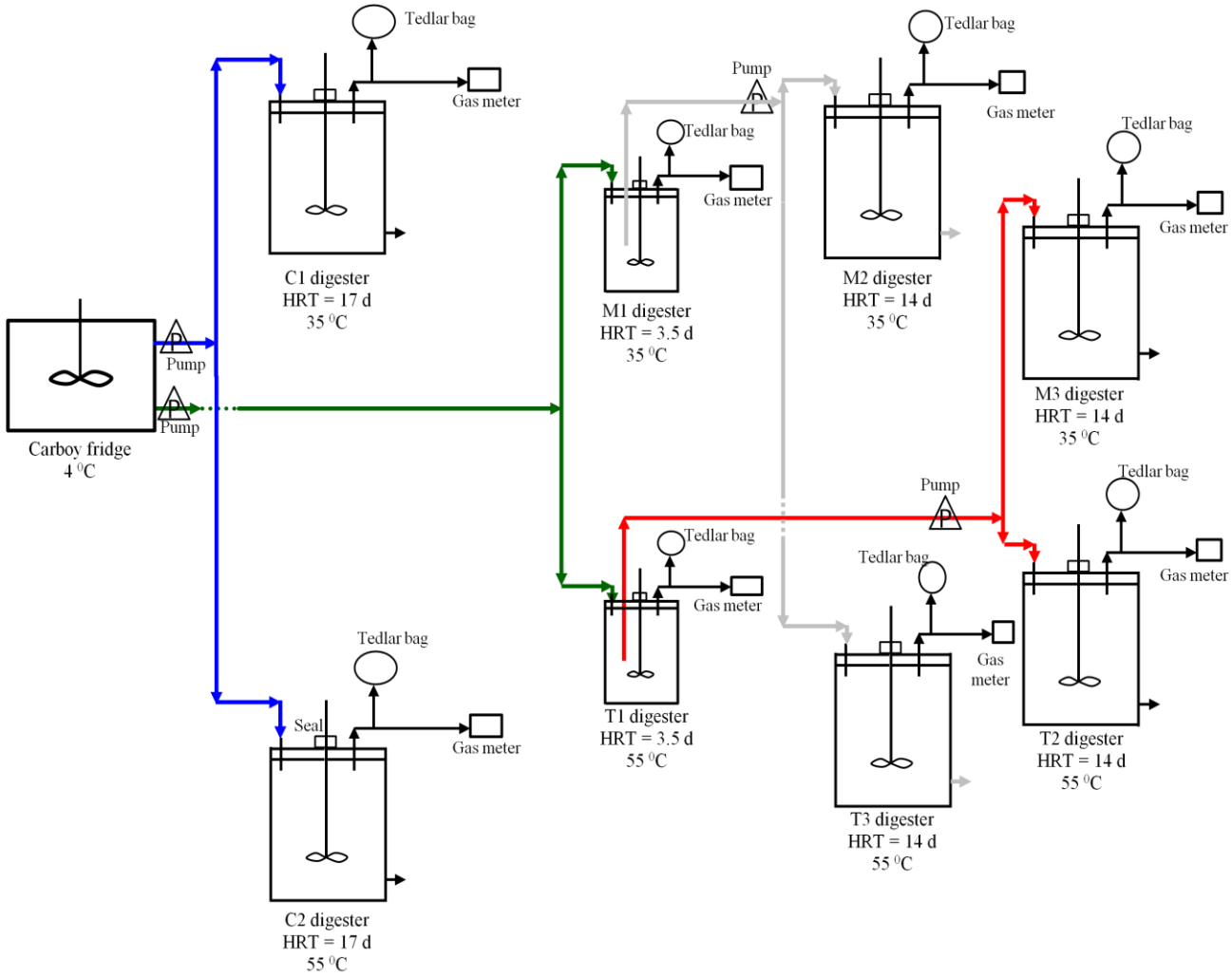


Figure 3.2. Schematic diagrams showing the codes and configurations of the phased and single-stage digesters

3.2.4 Sampling plan

3.2.4.1 Sampling frequency

The sampling frequencies of the analytical parameters within the start-up and steady-state periods are summarized in Table 3-1. The digesters were operated for more than 3 HRTs before steady-state data collection began. Then, the digesters were operated for almost a year to collect sufficient data. To minimize the influence of the feed sludge on the first phase digester effluent samples they were collected just before the pumps started feeding.

3.2.4.2 Analytical methods

As shown in Table 3-1, conventional parameters were analyzed for the feed and digested sludges to monitor the performance of the digesters in terms of solids reduction, carbonaceous and proteinaceous organic matter degradation and biogas production. The complete analyses were important from two viewpoints: 1) to compare the performance of the various digesters; and 2) to establish the steady-state and stability conditions of the digesters as inputs to the associated batch experiments. The procedures employed to analyze the parameters listed in Table 3-1 are subsequently explained.

Table 3-1. Summary of conventional parameters analyzed for bench-scale digesters

<table>
<tr><th rowspan="2">Parameters</th><th rowspan="2">Feed Sludge</th><th colspan="3">Start-up</th><th colspan="3">Steady-state</th></tr>
<tr><th>1st-phase</th><th>2nd-phase</th><th>Single-stage</th><th>1st-phase</th><th>2nd-phase</th><th>Single-stage</th></tr>
<tr><td>Total Solids (TS)</td><td colspan="4" rowspan="2">Once per week</td><td colspan="3" rowspan="8">Once per week</td></tr>
<tr><td>Volatile Solids (VS)</td></tr>
<tr><td>Total Suspended Solids (TSS)</td><td>NA*</td><td>NA</td><td>NA</td><td>NA</td></tr>
<tr><td>Volatile Suspended Solids (VSS)</td><td>NA</td><td>NA</td><td>NA</td><td>NA</td></tr>
<tr><td>Total Chemical Oxygen Demand (TCOD)</td><td colspan="4" rowspan="2">Once per week</td></tr>
<tr><td>Soluble Chemical Oxygen Demand (SCOD)</td></tr>
<tr><td>Ammonium-Nitrogen (NH_3)</td><td>NA</td><td>NA</td><td>NA</td><td>NA</td></tr>
<tr><td>Total Kjeldahl Nitrogen (TTKN)</td><td>NA</td><td>NA</td><td>NA</td><td>NA</td></tr>
<tr><td>Soluble Kjeldahl Nitrogen (STKN)</td><td>NA</td><td>NA</td><td>NA</td><td>NA</td></tr>
<tr><td>Volatile Fatty Acids (VFAs)</td><td>NA</td><td>NA</td><td>NA</td><td>NA</td><td colspan="3">Six times within the last 3 weeks</td></tr>
<tr><td>Total Alkalinity</td><td colspan="4">Once per week</td><td colspan="3" rowspan="2">Once per week</td></tr>
<tr><td>pH</td><td colspan="4">Twice per week</td></tr>
<tr><td>Temperature</td><td colspan="4">Daily</td><td colspan="3">Daily</td></tr>
<tr><td>Methane/Carbon Dioxide (CH_4/CO_2)</td><td colspan="4">Twice per week</td><td colspan="3">Once/Twice per week</td></tr>
<tr><td>Biogas</td><td colspan="4">Daily</td><td colspan="3">Daily</td></tr>
</table>

* Not Available;

A) Solids

The measurement of solids in this research followed procedures from Standard Methods 2540 B, D, and E for total, total suspended, and volatile solids, respectively (APHA, 1992). Sewage sludge contains both inorganic (TS) and organic matter (VS) and each fraction can further be divided into soluble and particulate matter (TSS/VSS). A combustion step was used to measure the organic and inorganic

fractions and a glass fibre filter (typically with a pore size of 1.5 µm) was used to measure the particulate and soluble fractions.

The TS/VS concentrations of the sludge were measured by transferring a known volume of a well-mixed sample into a weighed aluminum dish. The dish containing the sample was placed in an oven at ~ 105 ^{0}C overnight to evaporate the water content. The weight difference between the empty dish and the dish with sample represented the total solids. For VS measurement, the dried residue was then placed in a furnace at ~ 550 ^{0}C for about an hour to combust the organic fraction. After cooling the dish was cooled and weighed to measure the VS.

For measurement of TSS and VSS, the samples were first filtered through a Whatman glass fibre filter (pore size 1.5 µm) and placed in a weighed aluminum dish. Then, a similar procedure was used as mentioned-above.

B) Chemical Oxygen Demand

COD provides a measure of the carbonaceous material of sludge. The performance of digesters in terms of the degradation of organic matter due to microbially-mediated reactions can be assessed from COD data. The total COD can be divided into various fractions. If a sample was filtered through a Whatman glass fibre filter of 1.5 µm pore size and the filtrate was analyzed for COD, the fraction is called soluble COD (SCOD). However, since a pore size of 1.5 µm is not small enough to remove colloidal matter, an improved measure of the truly soluble COD can be determined by filtering a sample through a filter of 0.45 µm after addition of a coagulant (Mamais et al., 1993). This fraction is referred to as the flocculated filtered COD (ffCOD) throughout this document.

COD fractionations can further be extended into biodegradable and non-biodegradable fractions. Under anaerobic conditions, the biodegradable fraction of COD can be determined by performing a long-term batch test, called biochemical methane potential (BMP).

The COD analysis in this research was performed according to a method modified from Standard Method 5220 D, Closed reflux, colorimetric method (APHA, 1992) with potassium dichromate and concentrated sulfuric acid as the oxidants. For determination of TCOD, a known volume of the sample was transferred by a pipette with a modified tip to a beaker, homogenized and diluted. The homogenized sample was put in COD vials for digestion in a heating block. Regarding SCOD and ffCOD, samples were centrifuged and then initially filtered via a Whatman glass fibre filter (1.5 µm pore size). A portion of the filtrate was then diluted and used for SCOD measurement. The ffCOD was determined by passing

the filtrate through a filter with a pore size of 0.45 µm after colloidal matter was flocculated through addition of 0.4 ml of a 0.15 M $Al_2(SO4)_3$. 6H2O solution to a 15 mL filtered (1.5 µm) sample.

C) TKN and Ammonium-nitrogen

During anaerobic digestion, the organic fraction of the nitrogen-containing material is degraded and nitrogen is released in the form of ammonium and/or ammonia depending on the pH of the environment. A typical technique for measuring the sum of organic and ammonia nitrogen is TKN in which amino nitrogen (i.e., organic fraction) of sludge is converted to ammonium.

As described for COD, TKN can also be divided into total and soluble fractions. For measuring the soluble TKN, the samples were passed through a filter of 1.5 µm pore size. After transferring 1 mL of the filtrate of each sample to the TKN tubes, 0.5 mL of digestion solution was added and the tubes were digested for 2.5 hr at 180 ^{0}C and then 3.5 hr at 380 ^{0}C. For total TKN, the samples were homogenized and 1 mL of each sample was added to the tubes and digested as mentioned earlier. The digested samples of both soluble and total TKN were diluted and analyzed using a BRAN LUEBBE ammonia analyzer. The digestion method used in this research was developed at the Environment Canada Wastewater Technology Center (Burlington, Canada) and modified in the lab. The TKN digestion solution was prepared by dissolving 40 gram of K_2SO_4 and 2 mL of selenium oxychloride (97%) in 250 mL of concentrated sulfuric acid. The volume was brought to 500 mL with de-ionized water. Ammonium reacts with phenate to produce a blue compound, allowing an automatic colorimetric analysis at 660 nm.

D) Volatile Fatty Acids (VFAs)

Short chain fatty acids were determined using a Dionex liquid chromatography (LC) instrument. The method was optimized in the lab to measure acetic, propionic, butyric, n-valeric and iso-valeric acids. The IC was equipped with an autosampler, an advanced gradient pump, a short sample loop 25 µL and an Acclaim OA column (5 µm 120^o, 4.0×250 mm). To protect the analytical column, a guard column (OnGuard II, Dionex) was installed. The detection system consisted of a variable wavelength UV detector.

A gradient procedure was employed using acetonitrile (ACN) (purity, 99.9%) and methanesulfonic acid (MSA) (2.5 mM) as eluents. The pH of the 2.5 mM MSA was adjusted to ~2.75 using 50% NaOH, if required. The eluents were degassed with helium before use. The flow rate was set at 0.45 mL/min. The gradient was as follows:

1- 0-12 min: 100% MSA

2- 12- 20 min: 82% MSA and 18% ACN

3- 20-40 min: 82% MSA and 18% ACN

4- 40 min: 100% MSA

5- 40-60 min: 100% MSA

Acetic and propionic acids are highly water soluble, thus, no ACN was used for the first 12 min to minimize the appearance of disturbing peaks.

Samples were centrifuged and passed through a 0.45 µm syringe filter. Their pH was adjusted to ~ 2.75 by adding MSA. Samples were put in the Dionex vials and mounted on the autosampler. The concentrations of VFAs were calculated based on a calibration performed with a 10 mM VFA mix standard solution (Sigma-Aldrich Canada Ltd, Oakville, ON).

E) pH

The pH is usually measured in anaerobic sludge digesters as a measure of process stability. A 25 mL sample was used to measure pH values using a pH meter (Orion model 710A, with Ag/AgCl as reference electrode). Calibration with standard pH solutions was conducted before analysis of the samples. The pH values were determined immediately after sample collection.

F) Biogas volume and composition

The flow of biogas produced in each digester was recorded on-line using eight individual low flow gas-meters (Figure 3-3). The biogas produced in anaerobic digestion is mainly composed of CH_4 and CO_2. Hence, the biogas was analyzed for CH_4 and CO_2 by a gas chromatograph. A 1 mL volume was taken from the headspace of digesters and analyzed by a SRI 310C gas chromatograph (GC) equipped with a thermal conductivity detector (TCD) and a 6'×1/8"OD Porapak column (80/100 mesh). The temperatures of the injector and column were 30°C.

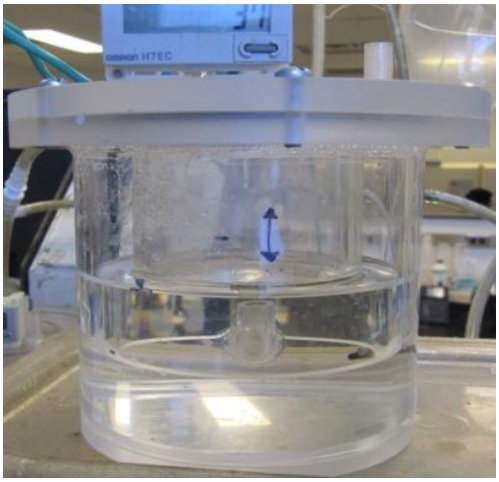


Figure 3.3. Image of a low-flow gas meter used in this research

3.2.4.3 Molecular biology analysis

Molecular techniques were employed to assist with understanding the microbial ecology of the anaerobic digestion processes. Accordingly, denaturing gradient gel electrophoresis (DGGE) as a PCR-dependent fingerprinting technique was employed for analysis of the bacterial and archaeal communities in the phased and single-stage digesters to investigate the impact of phase separation on the microbial populations.

3.2.4.3.1 DNA extraction

Samples taken from the various digesters were kept at -80 ^{o}C until further analysis. DNA was extracted from the digested samples using a FastDNA Spin kit for soil (MP Biomedicals, OH, USA). The DNA extraction from 0.5 g of sludge sample was done following the instructions provided by the manufacturer. The final DNA sample was eluted in 100 µl of DES (DNase/Pyrogen-Free Water). The extracted DNA that contains the genomic DNA of the microorganisms of the sludge samples was kept at -20 ^{0}C until PCR. After DNA extraction, the quality of the DNA was assessed on a 1% agarose gel that was stained with ethidium bromide and imaged and quantified against a DNA ladder with an AlphaImager® HP Imaging System.

3.2.4.3.2 PCR of bacterial 16S rRNA genes for denaturing gradient gel electrophoresis (DGGE)

Polymerase chain reaction is a technique that is employed to amplify the number of copies of a specific region of DNA. The 16S rRNA genes of the extracted DNA from the sludge samples were amplified using PCR with two different primer sets. One primer set, 338f and 518r, amplified a product of

236 bp in length (Muyzer et al., 1993). The 338f primer complements a DNA region conserved among members of the domain Bacteria while the reverse primer, 518r, is based on a universally conserved DNA region. The PCR mixture used for amplification of the bacterial sequences contained 1.5 µL bovine serum albumin (BSA) (10 µg/mL), 2.5 µL Thermopol buffer (10X), 0.05 µL deoxyribonucleotide triphosphate (dNTPs; 100 µM), 0.05 µL forward primer 341f-GC (100 µM), 0.05 reverse primer 518r (100 µM) and 0.25 µL *Taq* DNA polymerase. A volume of 1 µL of sludge sample DNA (1-10 ng DNA) was added to the PCR mixture and the final volume was brought to 25 µL with sterile water. Together with the DNA samples extracted from sludge samples, the DNA of *Sinorhizobium meliloti* (in a separate tube) was used as a positive control in PCR amplification. PCR amplification was carried out on a thermal cycler with the following protocol: initial denaturation at 95 ^{0}C for 5 min, 30 cycles of denaturation at 95 ^{0}C for 1 min, annealing at 55 ^{0}C for 1 min, and extension at 72 ^{0}C for 1 min. Final extension at 72 ^{0}C for 7 min. Amplification of the correct length target sequence of the DNA was assessed by electrophoresis as explained in Section 3.3.6.1.

3.2.4.3.3 PCR of archaeal 16S rRNA genes for denaturing gradient gel electrophoresis (DGGE)

Archaeal 16S rRNA sequences were amplified by nested PCR. Initially a primer set consisting of 109f as forward and 958r as reverse primers were employed for a first amplification (Delong, 1992; Grobkopf et al., 1998). The PCR mixture used for amplification contained 1.5 µL of bovine serum albumin (BSA) (10 µg/mL), 2.5 µL Thermopol buffer (10X), 0.2 µL deoxyribonucleotide triphosphate (dNTPs; 100 µM), 0.125 µL forward primer 109f (100 µM), 0.125 reverse primer 958r (100 µM) and 0.125 µL *Taq* DNA polymerase. A volume of 1 µL of sludge sample DNA (1-10 ng) was added to the PCR mixture and the final volume was brought to 25 µL with sterile water. Along with DNA of the sludge samples, the DNA of *Methanosacrcina barkeri* was used as a positive control in PCR amplification. The PCR program for the first amplification was as follows: initial denaturation at 95 ^{0}C for 5 min, 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 45 ^{0}C for 1 min, and extension at 72 ^{0}C for 1 min. The final extension was conducted at 72 ^{0}C for 7 min.

For the second amplification, the PCR mixture contained 1.5 µL bovine serum albumin (BSA) (10 µg/mL), 2.5 µL Thermopol buffer (10X), 0.2 µL deoxyribonucleotide triphosphate (dNTPs; 100 µM), 0.125 µL (SA1f-GC + SA2f-GC) as forward primer (100 µM), 0.125 PARCH 519r as reverse primer (100 µM) and 0.125 µL *Taq* DNA polymerase (Ovreas et al., 1997; Nicol et al., 2003). A volume of 1 µL of the PCR product obtained in the first round was used as a template and added to the PCR mixture. The final volume of the mixture was brought to 25 µL by adding 19.4 µL of sterile water. The PCR program

for the second round was as follows: initial denaturation at 95 ^{0}C for 5 min, 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing at 53.5 ^{0}C for 1 min, and extension at 72 ^{0}C for 1 min and a final extension at 72 ^{0}C for 7 min. The presence of PCR products for each round was assessed by running products reaction aliquots on a 1% agarose gel.

3.2.4.3.4 Denaturing gradient gel electrophoresis (DGGE)

DGGE is a gel-based technique, which can be used to separate and identify dominant members of the microbial community based on PCR-amplified DNA sequences. DGGE was performed using a DGGEK-2001-110 system (C.B.S. Scientific Inc., California, USA) according to a previously published protocol (Green et al. 2010). A volume of 5 µl of PCR product was loaded into one of the lanes of the DGGE polyacrylamide gel in 1X TAE. The polyacrylamide gels had a denaturing gradient of 30-70% (where 100% denaturant gradient contains 21 g of urea and 20 mL of formamide in a final volume of 50 mL) for bacterial and archaeal PCR products. The gels were polymerized by adding 1% ammonium persulfate (APS) and 0.1% TEMED. The electrophoresis was run at 60°C for 14 h at 85 V. After electrophoresis, the gels were stained for 45 min with SYBR® Green I nucleic acid gel stain (Invitrogen, Ontario, Canada) and rinsed in water. The gels were scanned using a Typhoon® 9400 Variable Mode Imager system (GE Healthcare Lifesciences, Quebec, Canada). Bands were cut from the gel and were sequenced by Beckman Coulter Genomics (ABI PRISM 3730xl). DGGE band sequences were manually edited to correct base miscalls, and primer sequences were removed prior to analysis. The Band sequences were manually edited for quality and compared to the ribosomal database project to assess sequence similarity to known reference organisms (Maidak et al., 2001).

3.3 Results and Discussion

3.3.1 Feed characterization

The raw sludge was regularly analyzed within each week to observe sludge degradation over the week. Table A.1 (Appendix A) shows the concentrations of TCOD and SCOD and the percentage change of these parameters within one week storage. Overall the concentration of raw sludge TCOD reduced, on average, 5% while that of SCOD increased about 10% on average. It was observed that the storage influenced solubilisation more than COD reduction. Thus, overall, it was inferred that one-week storage did not result in severe degradation of raw sludge.

The results of the analysis on the feed sludge (a mixture of primary and waste activated sludges) are shown in Table 3.2. The Waterloo WWTP employs co-thickening of primary and secondary sludges

and hence the sludge employed in this study reflected the overall sludge generation practices at the plant. The raw sludge samples that were obtained over the period of a year showed fluctuations in characteristics as evidenced by the relatively large standard deviations. This is typical for raw sludge as the influent wastewater characteristics change seasonally. Primary sludge (PS) has higher solids contents than that of waste activated sludge (WAS) that typically ranges 5-9% and 0.8-1.2%, respectively (Metcalf and Eddy, 2003). The feed sludge solids presented in Table 3.2 were considered to be typical of mixed sludges. The fraction of soluble COD out of TCOD (SCOD/TCOD) was somewhat high. This was likely due to the storage of raw sludge that resulted in some solubilisation over the week. The fractions of TKN/TCOD as well as TKN/VS were within typical ranges. TKN was comprised of a 16.6% soluble fraction of which 34% was ammonium-nitrogen.

Table 3-2. Feed sludge characteristicse*

Parameter	Unit	Mixed sludge**
pH	-	6.36 ± 0.90
TS	g/L	34.11 ± 6.9
VS	g/L	27.20 ± 5.0
VS/TS	-	0.79
TCOD	g/L	47.5 ± 6.50
SCOD	g/L	5.14 ± 2.22
SCOD/TCOD	-	0.10
TCOD/VS	-	1.74
TKN	g/L	1.99 ± 0.16
Soluble TKN	g/L	0.33 ± 0.09
Ammonia	g/L	0.11 ± 0.06
TKN/VS	-	0.07
sTKN/TKN	-	16.6
Ammonia/TKN	-	0.05
Alkalinity	g/L as $CaCO_3$	1.1± 0.3

*Based on approximately 48 samples;

**Average ± deviation

3.3.2 COD mass balance over the various digesters

The influent COD to a digester is partly converted to methane gas and the rest remains in effluent. The mass balance for the digesters was calculated based on the average values of the influent CODin, effluent CODout and average methane production from each digester. The methane was expressed as COD equivalence (COD_{CH4}) at standard temperature and pressure (0 ^{0}C and 1 atm). Good mass balance

closure indicates good agreement between these measurements. Equation 3.1 was used to check for lack of mass balance closure for the digesters.

$$COD\ balance\ (\%) = (\ COD_{in} - COD_{out}\ - COD_{CH4}) \times \frac{100}{COD_{in}} \qquad (3.1)$$

The deviations in mass balance for the mesophilic single-stage digester (C1), mesophilic 1st-phase digester (M1), mesophilic-mesophilic (M1M2) digestion system and thermophilic-mesophilic (T1M3) digestion system were, respectively, -0.48, 3.16, 5.45 and 4.04% and for the thermophilic single-stage digester (C2), thermophilic 1st-phase digester (T1), thermophilic-thermophilic (T1T2) digestion system and mesophilic-thermophilic (M1T3) digestion system was, respectively, -0.50, 4.79, 0.93 and 2.27%. The positive values of mass balance results were likely due to a lack of precision in the methane gas measurement. Use of more precise gas meters such as bubble counter gas meters equipped with a laser detector may help to reduce methane measurement errors. In addition, gas leaks from the digesters may have added to the error. Determination of TCOD of samples also contributed to the inaccuracy in mass balance as the preparation of a completely homogenized sample is difficult for sludge samples. In general, achievement of less than 10% error in lack of mass balance on the various digesters was acceptable and allowed reliable comparisons of the digesters.

The deviations in mass balance for the mesophilic single-stage digester (C1), mesophilic 1st-phase digester (M1), mesophilic-mesophilic (M1M2) digestion system and thermophilic-mesophilic (T1M3) digestion system were, respectively, -0.48, 3.16, 5.45 and 4.04% and for the thermophilic single-stage digester (C2), thermophilic 1st-phase digester (T1), thermophilic-thermophilic (T1T2) digestion system and mesophilic-thermophilic (M1T3) digestion system was, respectively, -0.50, 4.79, 0.93 and 2.27%. The positive values of mass balance results were likely due to a lack of precision in the methane gas measurement. Use of more precise gas meters such as bubble counter gas meters equipped with a laser detector may help to reduce methane measurement errors. In addition, gas leaks from the digesters may have added to the error. Determination of TCOD of samples also contributed to the inaccuracy in mass balance as the preparation of a completely homogenized sample is difficult for sludge samples. In general, achievement of less than 10% error in lack of mass balance on the various digesters was acceptable and allowed reliable comparisons of the digesters.

3.3.3 Alkalinity and pH

One of the most important factors for efficient operation of an anaerobic digester is pH. In anaerobic digestion pH is controlled by the different acid-base equilibria including carbonic, phosphoric, sulphuric, volatile fatty acids and ammonia (Pohland, 1969). At typical operational pH of digesters (6-8), the main chemical system controlling pH is the carbon dioxide-bicarbonate system (McCarty, 1964). Besides pH, alkalinity should also be taken into consideration as a process control parameter. It is well known that the alkalinity in an anaerobic process can be influenced by degradation of nitrogenous compounds, reduction of sulphate, release of phosphate and generation of volatile fatty acids (Capri and Marais, 1975). Accordingly, pH and alkalinity of the various digestion systems were monitored in the course of the study to ensure the microbially-mediated processes were not influenced severely.

The results of pH measurement for the mesophilic and thermophilic digesters are presented in Figure 3.4. The pH ranged between 7.5 and 8 in the mesophilic digesters of which M1digester showed the lowest value (7.55). Compared to the mesophilic digesters, the pH values were slightly higher in the thermophilic digesters. Higher pH values were consistent with the greater alkalinity and ammonium-nitrogen release obtained for the thermophilic digesters compared to those of the mesophilic ones. In addition to NH_4-N, the higher pH values of the thermophilic digesters were likely due to the operation of these digesters at the elevated temperature. Overall, it was concluded that pH did not cause inhibition of the microbial activities in general and methanogenic activities in particular as the pH values were within the pH optima cited for acetogens and methanogens (Williams and Crawford, 1985).

The alkalinity values (Figure 3.5) were consistent with the pH measurements. Higher production of NH_4-N due to the degradation of nitrogen-containing matter caused the pH and alkalinity values to be greater in the M1M2 and T1M3 digestion systems than that of the C1 digesters. On the other hand, higher production of fatty acids in the M1 digester resulted in both lower pH and alkalinity compared to the values measured for the C1 digester and M1M2 and T1M3 systems (Figure 3.6). Comparison of alkalinity in the thermophilic digesters demonstrated that the T1T2 and M1T3 digestion systems had higher alkalinity than that of the C2 digester. Overall the T1T2 system showed maximum alkalinity that agreed with the results obtained for the ammonium-nitrogen release, while the lowest alkalinity was observed in the T1 digester that is most likely related to the higher VFA production (Figure 3.7) compared to the C2 digester and T1T2 and M1T3 digestion systems.

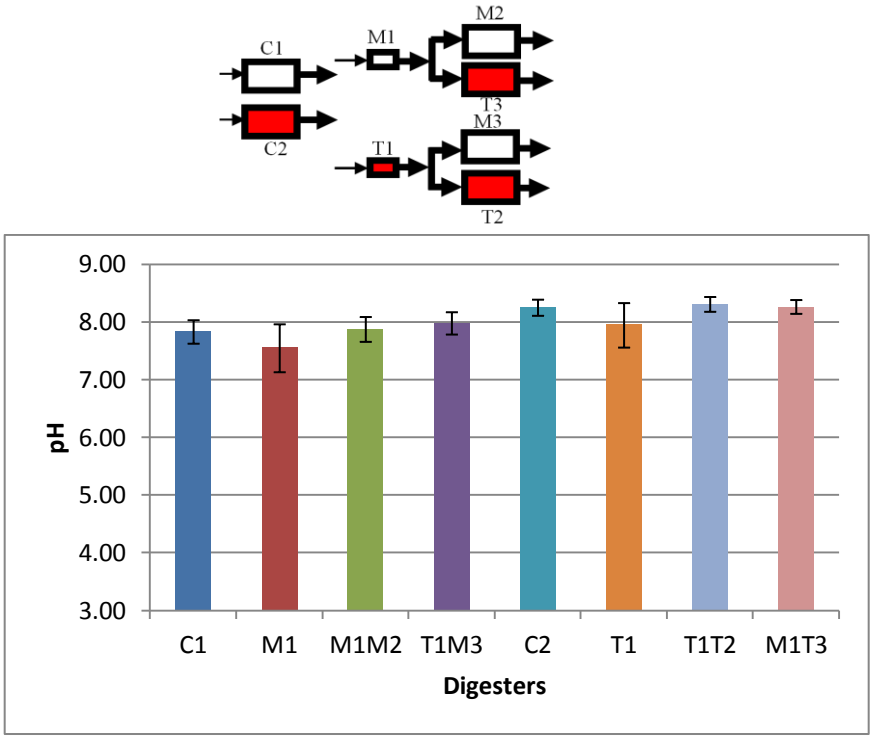


Figure 3.4. Average pH measurements in the phased and single-stage anaerobic digesters (Average±SD)

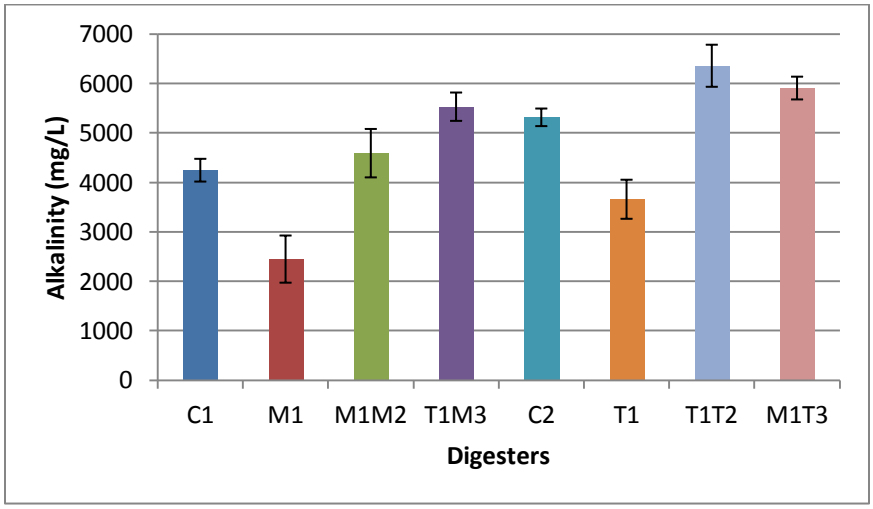


Figure 3.5. Average alkalinity concentrations (as CaCO3) in the phased and single-stage anaerobic digesters (Average±SD)

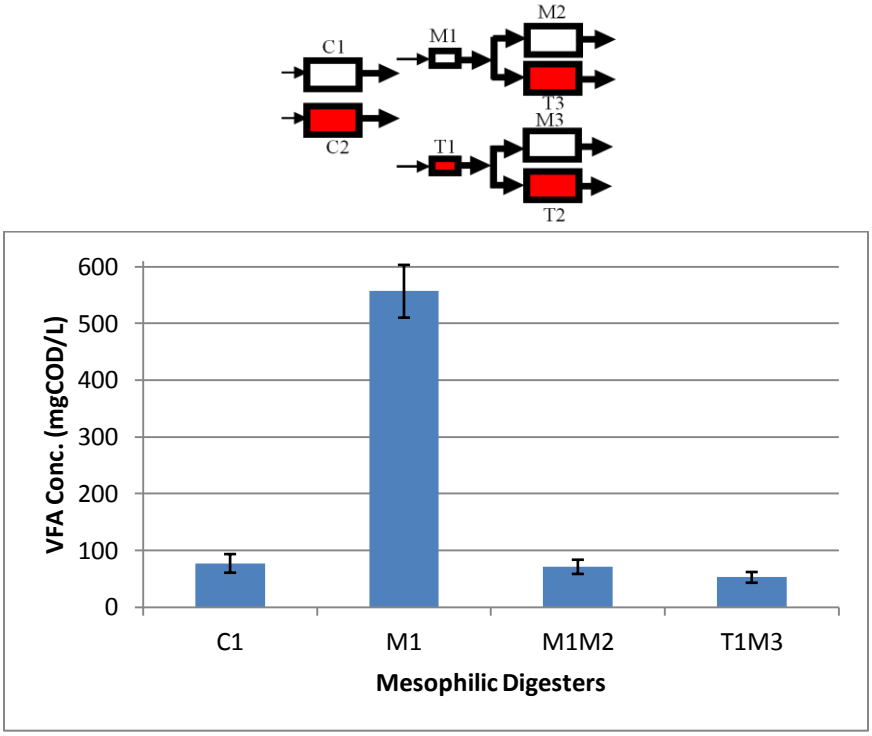


Figure 3.6. Average VFAs concentrations (as mgCOD/L) in the mesophilic digester (Average±SD)

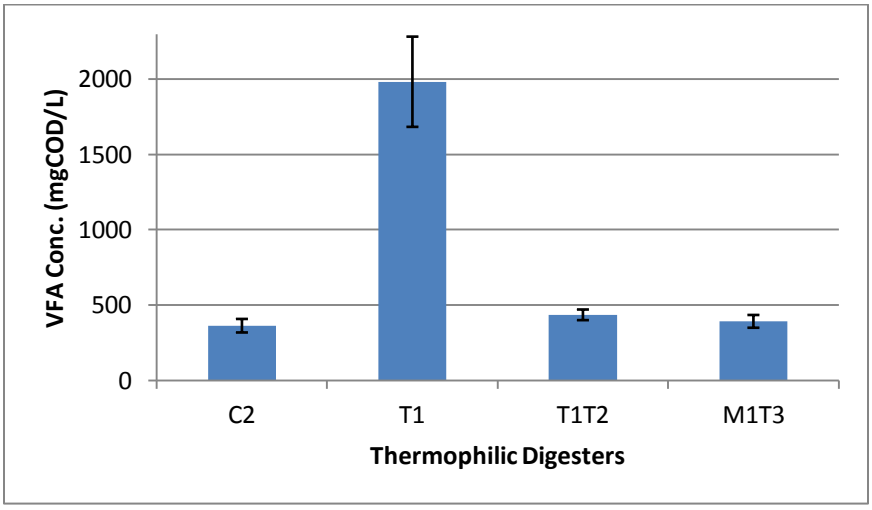


Figure 3.7. Average VFAs concentrations (as mgCOD/L) in the thermophilic digester (Average±SD)

3.3.4 Solubilization of particulate fraction of sludge

The anaerobic digestion process comprises of hydrolysis, acidogenesis, acetogenesis and methanogenesis. Hydrolysis of biodegradable particulates in sludge is a slow process and is typically considered as the rate limiting step (Eastman and Ferguson, 1981; Gavala et al., 2003). The rate of solubilisation is a function of pH, temperature, microbial biomass, digester configuration, particle size and concentration of degradable particulates (Gavala et al., 2003).

As these parameters typically differ in phased anaerobic digestion systems, organic particulates removal and solubilisation rates were determined to evaluate the influence of phase separation and temperature on them. The PCOD removal efficiencies of the phased digestion systems were evaluated and compared to determine the effect of various temperature configurations on these parameters. The performance of the various digestion systems was compared in terms of PCOD removal to those of the single-stage digesters. Then, solubilisation rate constants were calculated and compared to further evaluate the effect of temperature and operating conditions on the kinetics of hydrolysis in the digesters. In this section, only PCOD removals are presented and for comparison the VSS removal efficiencies are given in Appendix A.

3.3.4.1 PCOD removal in digesters

The average values and associated standard deviations (SD) of the PCOD removals in the mesophilic phased digestion systems and the mesophilic single-stage digester are presented in Figure 3.8. The removal efficiencies for PCOD in the M1M2 and T1M3 systems were 2.4 and 5.3 percentage points greater than that of the C1 digester respectively. A statistical analysis (ANOVA PostHoc test, α=0.05) confirmed this difference (Table 3.3). The results show that in the M1M2 system, phase separation was effective in achieving higher PCOD removal. This confirms that improved environments may be provided for hydrolytic microorganisms in each phase through phase separation. The application of a 1st-phase with an elevated temperature followed by a mesophilic digester (T1M3) further enhanced PCOD removal. These results were consistent with the previous studies that showed a better performance of mesophilic-mesophilic and thermophilic-mesophilic systems in VSS destruction as compared to a mesophilic single-stage digester (Zahller et al., 2007; Watts et al., 2006; Coelho et al., 2011).

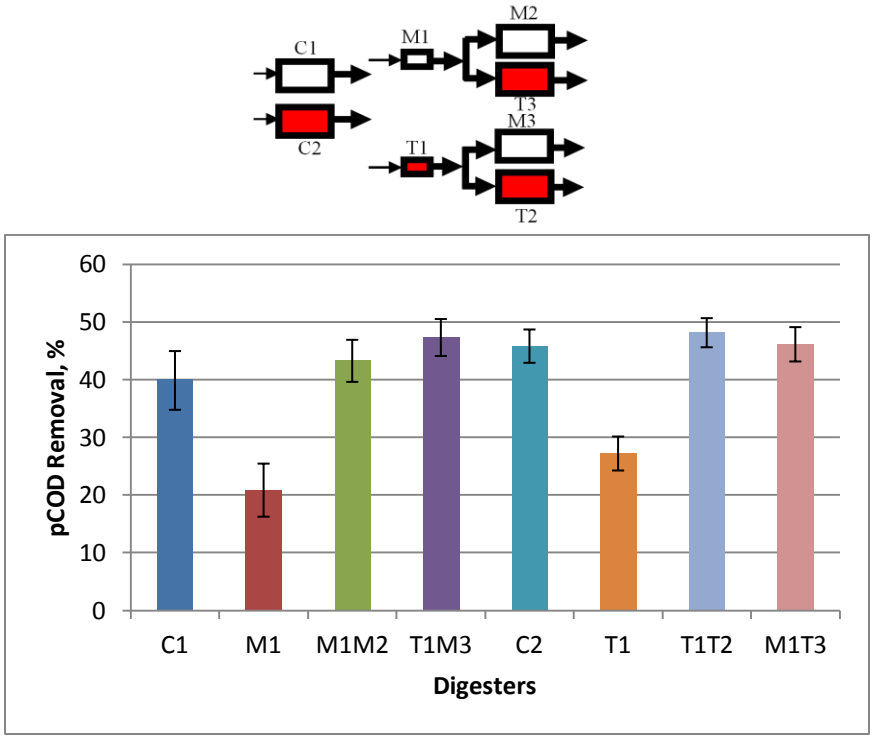


Figure 3.8. Particulate COD Removal in Digestion Systems (AVERAGE ± SD)

With regards to the thermophilic digesters, the PCOD removal results indicated 2.5% higher PCOD removal in the T1T2 system as compared to the C2 digester, while the removal efficiency of the M1T3 system was comparable to that of the C2 digester. These results indicated that operation of the phased digestion systems at similar temperatures (55-55 ^{0}C) improved the removal of particulate matter, while the operation of digesters at mesophilic-thermophilic temperatures did not appear to offer any additional benefits.

Table 3-3. Statistical analysis for comparison of PCOD removal (ANOVA Tukey test, α=0.05)*

System	C1	M1	M1M2	T1M3	C2	T1	M1T3	T1T2
C1		XX	XX	XX	XX	XX	XX	XX
M1			XX	XX	XX	XX	XX	XX
M1M2				XX	XX	XX	XX	XX
T1M3					**X**	XX	**X**	XX
C2						XX	**X**	XX
T1							XX	XX
M1T3								XX

*XX significant; X not significant

3.3.4.2 Phased digestion systems

An objective of this study was to comprehensively compare the performance of the various configurations of phased digestion systems. In this section, removal of PCOD in the various digestion systems were compared to determine if any of the configurations was more effective.

A comparison of the PCOD removal efficiencies in the phased digestion systems showed that the T1T2 system had the maximum removal of 58.2% (Figure 3.8). The results of an ANOVA Tukey test for statistical analysis of the PCOD removal efficiencies are presented in Table 3.3. Based on PCOD removals, the T1M3, T1T2 and M1T3 systems were significantly different from the M1M2 system. The relative improvements for the T1M3, T1T2 and M1T3 digestion systems ranged between 6.5%, 11.3% and 5.3%, respectively. This demonstrated that the elevated temperature of 55 ^{0}C was effective in improving the PCOD removal in the digestion systems that included at least one thermophilic digester. The effect of temperature could be directly observed when comparing the PCOD removal results in the M1 and T1 digesters. Removal of PCOD in the T1 digester was, on average, 5% higher than that of the M1 digester. The enhanced PCOD removal was confirmed by statistical analysis (3.3).

3.3.4.3 Solubilisation rates

The mass flow of PCOD in the influent and effluent of the digester was used to calculate the specific hydrolysis rate (K) in the digesters (Equation 3.2). As the determination of the active biomass is difficult, the removed mass of PCOD was normalized by the mass of VS within the digester (Schmit and Ellis, 2001; Coelho et al., 2011).

$$K = \frac{(Mass\ Flow\ of\ pCOD_{in}) - (Mass\ Flow\ of\ pCOD_{e})}{Mass\ of\ VS} \qquad (3.2)$$

The specific solubilisation rate constants for the various phased and single-stage digesters are presented in Table 3.4. This was conducted to assess the effect of differing conditions (temperature and organic loading) on hydrolysis kinetics. As already mentioned (Gavala et al., 2003), the hydrolysis kinetics would be influenced by the conditions of the digesters (temperature, pH and concentration of particulate matter), comparisons were made among the digesters operated at similar conditions. Subsequently, the impact of temperature on the hydrolysis kinetics was compared.

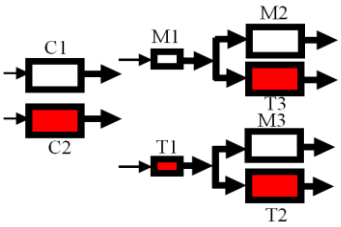


Table 3-4. Solubilisation rate constants for the various anaerobic sludge digesters (standard deviations in brackets)

System	Solubilisation rate constant* (gPCOD/gVS.d)
Mesophilic	
C1	0.064 (0.009)
M1	0.134 (0.018)
M2	0.045 (0.010)
M3	0.039 (0.008)
Thermophilic	
C2	0.090 (0.015)
T1	0.184 (0.022)
T2	0.042 (0.008)
T3	0.057 (0.018)

3.3.4.4 Comparison of solubilisation rates

The solubilisation rate constants calculated in this research were comparable with the values reported by previous researchers (Schmit and Ellis, 2003; Coelho et al., 2011). A comparison of the solubilisation rate constants in the 2nd-stage of the phased digestion systems with the corresponding 1st-phase digesters at a similar temperature (M1 vs. M2 and M3; T1 vs. T2 and T3) showed that the solubilisation rates were lower in the 2nd-phase digesters. At mesophilic temperatures the value for K in the M1 digester was 3 and 3.4 times higher than those of the M2 and M3 digesters. A similar observation was made for the thermophilic digesters, with higher K value in the T1 digester as compared to those values calculated for the T2 and T3 digesters. The K value of the T1 digester was 4.3 and 3.2 times greater than those of the T2 and T3 digesters.

These results indicate that both M1 and T1 had greater hydrolysis rates compared to the rates calculated for the 2nd-phase digesters at the same temperature. It was possible that disruption and disintegration of biomass originated from the WAS of the raw sludge resulted in release of some hydrolytic enzymes in the first-digester, resulting in improved solubilisation rates in these digesters.

Also, underestimation of hydrolysis rates would be expected using Equation 3.2. because VS includes both the soluble and particulate organic matter, consequently it would be expected that this

equation would underestimate the solubilisation rates in these digesters. This change would likely have minimal effect on the control and second stage digesters as there would be minimal accumulation of soluble organics. However, in the first stage digester this accumulation might be significant. Hence, it would be more appropriate to use VSS as an indicator of biomass in Equation 3.2.

3.3.4.5 Temperature effect on solubilisation rate

The effect of temperature was evaluated through a comparison of the hydrolysis rates in the various digesters. A comparison of the solubilisation rates of the 1st-phase digesters showed that the specific solubilisation rate constant (K) of the T1 digester was higher (1.37 times) than the value calculated for the M1 digester. A similar effect of temperature was observed in the single-stage digesters. The K value for the C2 digester was 1.4 times greater compared to that of the C1 digester. This observation agreed with the results reported elsewhere (Coelho et al., 2011). As the operating conditions (except temperature) of the digesters compared with each other (i.e., T1 vs. M1 and C2 vs. C1) were similar, it can be inferred that because of the higher temperature, thermophilic sludge had greater intrinsic reaction rates compared to the mesophilic one. In addition, it can be observed that improved solubilisation rate in the T1 digester lead to the production of the higher volatile fatty acids (Figures 3.6 and 3.7) and methane compared to that of the M1 digester (discussed later).

Based on the specific solubilisation rates, it can be inferred that both phase separation and temperature improved solubilisation in the phased digestion systems, although the temperature was more effective. Therefore, operation of the 1st-phase digester at a thermophilic temperature of 55 ^{0}C was much more effective in improving the solubilisation rate of a mixed sludge compared to application of the mesophilic temperature of 35 ^{0}C. Either of the T1T2 or T1M3 configurations could be employed to increase solubilisation rate. However, the operation of the 2nd-phase (T2) at a thermophilic temperature did not appear to offer a significant increase of the solubilisation rate over the mesophilic 2nd-phase (M3). This was observed by comparison of the specific solubilisation rates that were only 5.5% in the T2 digester compared to the M3 digester. As explained earlier, even though solubilisation is of significant importance in sludge digestion the selection of the best configuration should not be based solely on the solubilisation rate. The operation costs of running two digesters at thermophilic temperature as well as the effluent quality should be taken into account.

3.3.4.6 Degradation of nitrogen-bearing material

During solubilisation of particulate compounds, organic nitrogen is hydrolyzed to form polypeptides and amino acids which are in turn fermented to VFA and ammonium-nitrogen (Pavlostathis and Giraldo-Gomez, 1991). The products of hydrolysis and fermentation of nitrogen-bearing organics can be assessed by measuring changes in soluble TKN that comprises biodegradable organic nitrogen, ammonium-nitrogen and inert soluble nitrogen. Even though hydrolysis is better described by measurement of soluble TKN, measurement of NH_4-N may also be indicative of solubilisation of nitrogen-bearing organics because the fermentation process typically proceeds at a higher rate than the hydrolysis process (Vavalin et al., 2008). Therefore, soluble TKN (sTKN) and NH_4-N were measured in the various digestion systems to provide additional information on the solubilisation of nitrogen-bearing organics. Measurement of these parameters helped to determine the effect of phase separation and temperature on the hydrolysis and fermentation of nitrogen-containing material. The results of total TKN (TTKN), sTKN and NH_4-N for the various digesters are presented in Table 3.5.

The solubilisation of nitrogen-bearing matter was investigated by measuring soluble TKN in the mesophilic digesters of C1, M1M2 and T1M3 (Table 3.5). Figure 3.9 shows the relative increase (ratio) of soluble TKN in the mesophilic digesters due to solubilisation of protein-containing matter of the feed sludge. Soluble TKN concentrations increased in all the digesters in comparison to the sTKN concentration in the feed sludge, indicating the hydrolysis of nitrogen-bearing matter in the digesters. This increase was greatest in the T1M3 digestion system that showed a 3.3 fold increase compared to the sTKN of the feed sludge.

A comparison of the extent of protein-containing matter solubilisation in the M1M2 and T1M3 digestion systems with that of the C1 digester demonstrated that soluble TKN in the phased digestion systems (both M1M2 and T1M3) were higher by 10% and 28% compared to the C1 digester. A similar pattern was found for the NH_4-N release in the mesophilic digesters. Both M1M2 and T1M3 digestion systems improved ammonium release by 9% and 44% as compared to the average value obtained for the C1 digester. These results were analyzed statistically to determine whether the differences were significant. The statistical analysis confirmed these improvements (Table 3.6 and 3.7). Based on these results it appeared that both solubilisation of nitrogen-bearing and ammonium release were influenced by phase separation at the mesophilic digestion systems; however, as with the solubilisation of organic matter that was presented in the previous section, this enhancement was much greater when a 1st-phase digester (T1) with an elevated temperature of 55 ^{0}C was included in the digestion system.

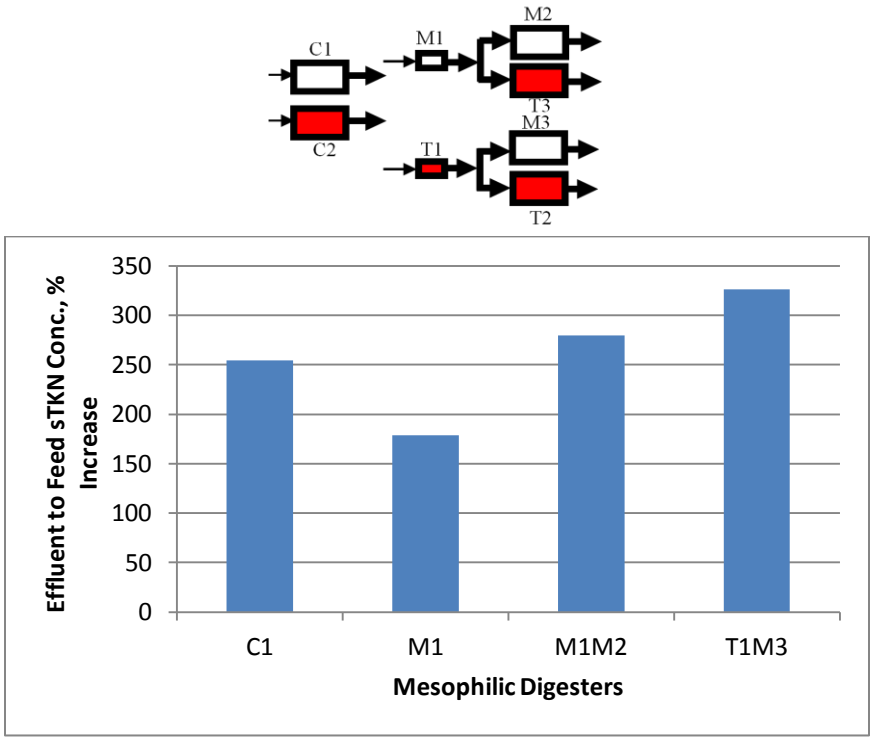


Figure 3.9. Increase of sTKN concentration in digesters relative to the sTKN of the feed sludge

Table 3-5. Nitrogen fractions in the various digesters

Digester	Total TKN (mg/L)	Soluble TKN (mg/L)	Ammonium-Nitrogen (mg/L)
Feed			
	1988±162	335±95	110±61
Mesophilic digesters			
Mesophilic Single-stage	1816 ± 200	850 ± 66	593 ± 61
Mesophilic 1st-phase	1723 ± 248	597 ± 88	394 ± 46
Mesophilic-Mesophilic	1831 ± 226	934 ± 58	647 ± 34
Thermophilic-Mesophilic	1851 ± 164	1090 ± 88	856 ± 36
Thermophilic digesters			
Thermophilic Single-stage	1820 ± 195	1031 ± 86	848 ± 27
Thermophilic 1st-phase	1747 ± 243	765 ± 68	511 ± 53
Mesophilic-Thermophilic	1858 ± 199	1168 ± 110	881 ± 34
Thermophilic-Thermophilic	1865 ± 177	1231 ± 82	923 ± 47

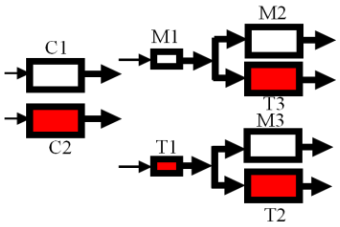


Table 3-6. Statistical analysis of comparison of sTKN solubilisation within various digesters (ANOVA Tukey test, α=0.05)*

System	C1	M1	M1M2	T1M3	C2	T1	M1T3	T1T2
C1		XX	XX	XX	XX	XX	XX	XX
M1			XX	XX	XX	XX	XX	XX
M1M2				XX	XX	XX	XX	XX
T1M3					X	XX	X	XX
C2						XX	XX	XX
T1							XX	XX
M1T3								X

*XX significant; X not significant

Table 3-7. Statistical analysis for comparison of NH4-N release within various digesters (ANOVA Tukey test, α=0.05)*

System	C1	M1	M1M2	T1M3	C2	T1	M1T3	T1T2
C1		XX	XX	XX	XX	XX	XX	XX
M1			XX	XX	XX	XX	XX	XX
M1M2				XX	XX	XX	XX	XX
T1M3					X	XX	X	XX
C2						XX	X	XX
T1							XX	XX
M1T3								X

*XX significant; X not significant

The results of solubilisation of nitrogen-bearing substances in the thermophilic digesters (C2, T1T2 and M1T3) are presented in Table 3.5. As with the mesophilic digesters, the sTKN concentration was higher in the digesters in comparison to the sTKN concentration of the feed sludge (Figure 3.10), indicating the hydrolysis of particulate nitrogen-bearing within the digesters. The highest increase of sTKN concentration was observed in the T1T2 digestion system.

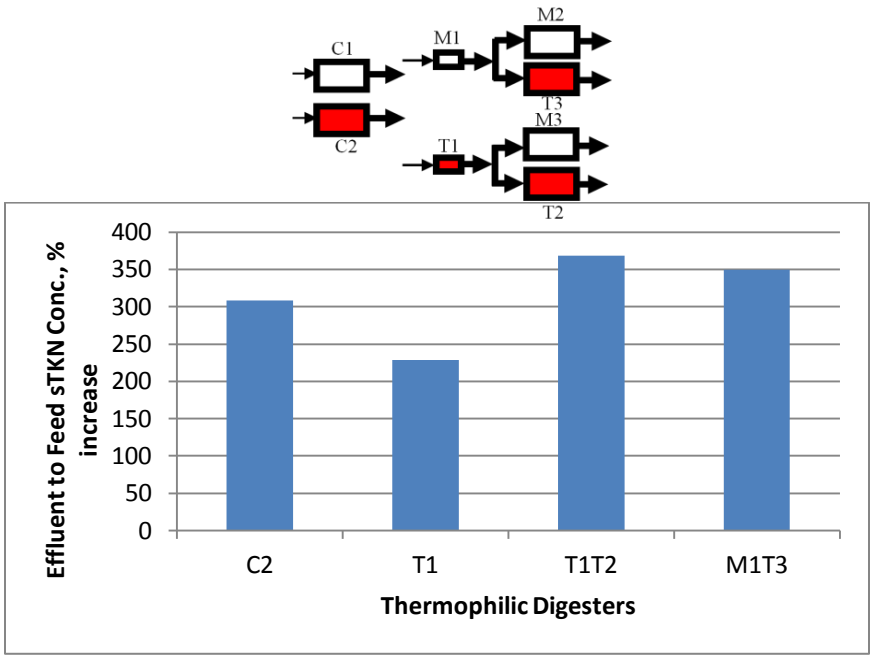


Figure 3.10. Increase of sTKN concentration in the digesters relative to the sTKN of the feed sludge

The statistical analysis results are presented in Tables 3.6 and 3.7. The solubilisation of particulate nitrogen-bearing to sTKN was statistically different for both digestion systems of T1T2 and M1T3, while NH_4-N release was only different in the T1T2 digestion system when the results were compared against the C2 digester (ANOVA PostHoc test, α=0.05). The average sTKN concentrations in the T1T2 and M1T3 effluents were 19% and 13% higher than that of the C2 digester. NH_4-N concentration in the T1T2 was 9% greater as compared to the average value of the C2 digester.

The results observed for the NH_4-N release in the thermophilic digesters were not consistent with those of soluble TKN. This suggests that the T1T2 and M1T3 digestion configurations were effective in solubilisation of particulate nitrogen-containing matter while fermentation of soluble organic nitrogen was only higher in the T1T2 digestion configuration. The deamination of amino acids and subsequent conversion to VFAs is the most common pathway in anaerobic fermentation. However, some amino acids such as leucine, proline and phenylalanine are resistant to fermentation under high partial pressures of hydrogen (Fox and Pohland, 1994). Thus, effective H_2 removal by hydrogenotrophic methanogens makes the conversion of these amino acids more favourable. Hanaki et al. (1987) and Breure et al. (1986b) reported a reduction in amino acids removal when hydrogen partial pressure was increased. On the other hand, activity of microorganisms involved in anaerobic digestion generally increases with temperature increases. Ahring et al. (2001) observed an enhanced activity of hydrogen consuming methanogens through increasing the digester temperature to 65 ^{0}C. Therefore, it can be concluded that the

operation of both digesters at identical temperatures of 55 ^{0}C (i.e., T1T2) enhanced the H_2 removal through increased activity of hydrogen consuming methanogens that consequently resulted in enhanced deamination of amino acids, while this improvement was not optimal in the C2 digester.

3.3.4.7 Phased digestion systems

The phased digestion systems were compared to determine which configuration was more effective in enhancing the solubilisation of nitrogen-containing matter of sludge. Similar to the solubilisation results obtained for PCOD, the lowest solubilisation of nitrogen-containing matter occurred in the M1M2 where both digesters kept at mesophilic temperature. The sTKN concentrations with the corresponding standard deviations (SD) were 934±58, 1090±88, 1168±110 and 1231±82 mg/L in the M1M2, T1M3, M1T3 and T11T2 digestion systems, respectively. Similarly, the lowest NH_4-N release was achieved in the M1M2 digestion system. The corresponding NH_4-N concentrations (±SD) were 647±34, 856±36, 881±34 and 923±47in the M1M2, T1M3, M1T3 and T1T2 digestion systems, respectively. With the exception of the M1T3 system, the sTKN and NH_4-N values in the T1T2 digestion system were significantly different from the other configurations. On the other hand, the M1M2 digestion system showed lowest solubilisation of nitrogen-containing matter among the phased systems. These results showed that the synergistic effect of temperature and phase separation resulted in better solubilisation of nitrogen-containing material. A comparison of the results obtained for solubilisation of nitrogen-containing matter and NH_4-N release in the various digestion systems demonstrated that these data were in general consistent with the solubilisation data of PCOD. These results agreed with previous studies that have reported the degradation of nitrogen-containing organic compounds was greater under thermophilic than mesophilic conditions (Sanchez et al., 2000; Song et al., 2004; Ge et al., 2011).

3.3.5 Removal of organic matter

Following hydrolysis and solubilisation of sludge particles, methanogenesis is the main pathway for removal of organic matter from sludge in the form of methane and CO_2. One of the objectives of anaerobic digestion is reduction of the organic content of raw sludge to meet the requirements for sludge land application, beneficial use or disposal. In addition, optimization of biogas production is of interest as the methane can be used as a source of energy. In this section, the COD reduction and methane production measured for the various digestion systems were compared. For comparison, VS destruction results and methane production rate are given in Appendix A.

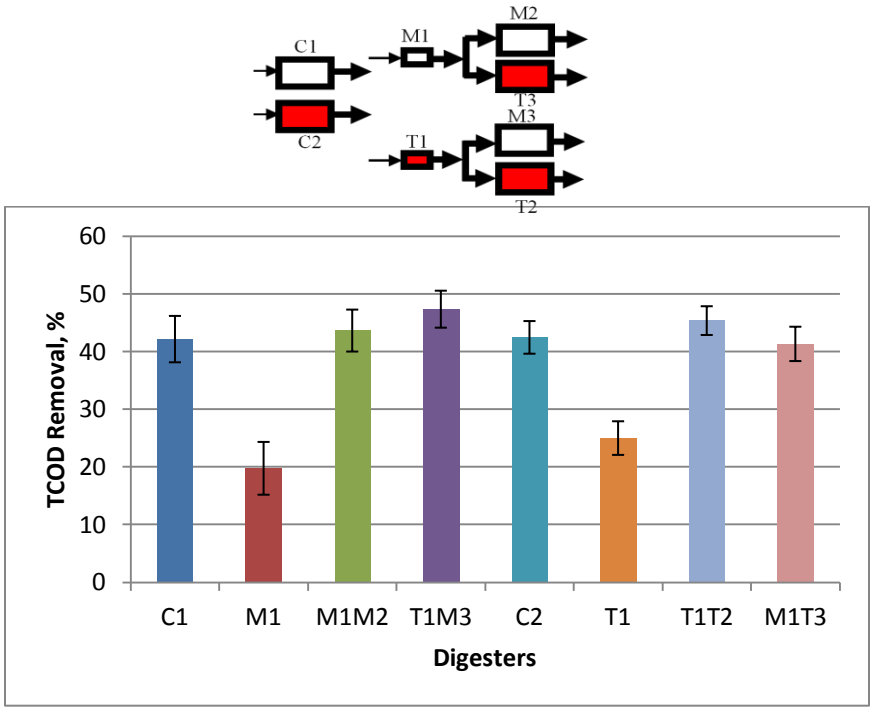


Figure 3.11. Total COD removal in Digestion Systems (AVERAGE ± SD)

Removal efficiencies of total COD for all the mesophilic and thermophilic digesters are presented in Figure 3.11. From Figure 3.11, it can be seen that both the M1M2 and T1M3 digestion systems resulted in a greater COD destruction as compared to the value obtained for the mesophilic single-stage digester (C1). ANOVA PostHoc test (α=0.05) results (Table 3.8) showed both digestion systems were statistically different from the C1 digester.

Table 3-8. Statistical analysis for comparison of COD removal in various digesters (ANOVA Tukey test, α=0.05)*

System	C1	M1	M1M2	T1M3	C2	T1	M1T3	T1T2
C1		XX	XX	XX	XX	XX	XX	XX
M1			XX	XX	XX	XX	XX	XX
M1M2				XX	XX	XX	XX	XX
T1M3					XX	XX	XX	XX
C2						XX	**X**	XX
T1							XX	XX
M1T3								XX

*XX significant; X not significant

Average methane yields were calculated based on methane production adjusted to standard temperature and pressure (STP) and the mass of VS fed to the systems (Table 3.9). As can be seen from

Table 3.9, the average methane yields in both phased digestion systems were higher than that of the mesophilic single-stage digester. The methane yield improved 25% and 47% in the M1M2 and T1M3 systems, respectively. As discussed earlier, the mesophilic phased systems achieved greater COD destruction efficiencies. Given a negligible growth yield for anaerobic microorganisms, the higher COD removal in the phased systems resulted in the generation of more methane. This in turn increased the methane yield for the phased digestion systems. Overall, good consistency was found between COD and methane results for the mesophilic digesters. These results indicate that phase separation had a positive effect on sludge digestion. Since the major COD removal pathway in anaerobic digestion is CH_4 generation, these results demonstrated that activity of methanogens was enhanced through phase separation. The results obtained in this study are consistent with those reported elsewhere (Coelho et al., 2011; Zahller et al., 2007).

With regard to the thermophilic digesters, the COD removal was greatest in the T1T2 system (45.3%), indicating 3 and 4 percentage points higher COD removal than the C2 digester and M1T3 system, respectively. As presented in Table 3.8, this difference was statistically significant from both C2 digester and M1T3 system, while no difference was found between the C2 digester and M1T3 digestion system.

Table 3.9 shows the average methane yields and the relevant standard deviations (SD) for the thermophilic digesters. As can be seen from Table 3.9, the average methane yields in both phased digestion systems were higher than that of the C1 digester and improved 10% and 4% in the T1T2 and M1T3 systems, respectively. Direct comparisons of thermophilic single-stage digesters with the T1T2 digestion systems have rarely been reported. Coelho et al., (2011) reported improved biogas production by operation of digesters in series at thermophilic temperatures as compared to a thermophilic single-stage digester when the digesters were fed with either microwaved or non-microwaved sludge.

For the thermophilic digesters, it can be observed that only the T1T2 digestion system outperformed the C2 digester. Thus, it can be concluded that the operation of phased digestion systems with a similar temperature in both digesters (T1T2) was better reflected the advantageous of phase separation.

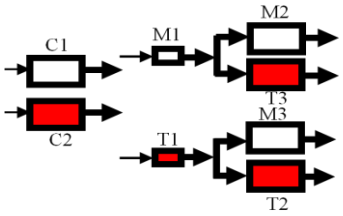


Table 3-9. Methane yields for the various digestion systems (standard deviations in brackets)

System	Methane yield, L/g VS_{added}		
	1st-phase	**2nd-phase**	**Overall**
Mesophilic digesters			
C1	-	-	0.235 (0.05)
M1M2	0.077 (0.02)	0.182 (0.03)	0.271 (0.04)
T1M3	0.102 (0.02)	0.217 (0.05)	0.324 (0.05)
Thermophilic digesters			
C2	-		0.252 (0.04)
T1T2	0.102 (0.02)	0.183 (0.02)	0.277 (0.03)
M1T3	0.077 (0.02)	0.176 (0.02)	0.263 (0.02)

3.3.5.1 Phased digestion systems

The COD removals in the T1M3, M1M2, T1T2 and M1T3 digestion systems were 50.73, 47.6, 43.3 and 39.9%, respectively. A statistical analysis showed that they were all statistically different from each other (Table 3.8). A comparison of the COD removal efficiencies in the phased digestion systems revealed that the lowest removal occurred in the M1T3 system. Therefore, the relative improvement in organic content of sludge was evaluated according to the M1T3 digestion system. The T1T2, M1M2 and T1M3 digestion systems demonstrated 8.5%, 19% and 27% higher organics removal relative to the M1T3 system.

Overall the thermophilic digestion systems had lower organic removal efficiencies than those of the mesophilic digestion systems. The higher soluble COD level in the effluent of thermophilic digesters has widely been reported (van Lier, 1996; Kim et al., 2002; Kim et al., 2004; Song et al., 2004). Even though thermophilic digestion demonstrated increased hydrolysis rates, the conversion of their products did not improve similarly and was likely attributable to the higher half saturation coefficients (Ks) of the methanogenic microorganisms (Discussed in Chapters 4 and 5). Use of a second stage mesophilic digester resulted in improved COD removal due to the greater affinity (lower Ks values) of mesophilic methanogens.

Based on the measured methane production and VS fed to the systems, the methane yields were calculated as 0.263, 0.270, 0.276 and 0.324, and L CH4 per g VS_{added} for the M1T3, M1M2, T1T2 and M1T3 digestion systems, respectively. These methane yields were within the range of values reported in literature (Coelho et al., 2011; Schmit and Ellis, 2010; Ghosh, 1987). Similar to the COD removal efficiencies, a comparison of the digestion systems demonstrated that the methane yield was lower in the M1T3 system in comparison to the values obtained for the other digestion systems.

Overall, based on the COD and methane yield results it appeared that the T1M3 digestion system showed better performance for digestion of sludge as compared to the remaining phased digestion systems. As stated by earlier researchers (Watts et al., 2006; Ge et al., 2010), in the temperature phased anaerobic digestion systems, a digester with a short HRT that follows by a mesophilic digester typically functions as a pre-treatment unit that is effective in either modification of the feed sludge through disintegration process or enhancement of hydrolysis process. Thus, it can be concluded that in addition to phase separation that improved methane production (comparison of the M1M2 vs. C1), inclusion of a thermophilic digester appeared to enhance pre-methanogenesis and methanogenic steps that overall resulted in higher COD and VS removals in the T1M3 digestion system. Therefore, in addition to phase separation, the elevated temperature (55 ^{0}C) in the 1st-phase of the T1M3 system probably enhanced solubilisation and gas production reactions that ultimately resulted in the higher VS destruction. These results were supported by the results presented in the following chapters regarding the bio-kinetic investigations on the hydrolysis, acetogenesis (i.e., propionate oxidation) and acetoclastic methanogenesis within the various digesters. The T1M3 digestion systems benefited from a high hydrolysis rate due to an elevated temperature as well as a more efficient hydrolytic biomass in the 1st-phase compared to the mesophilic 1st-phase. In addition, this system (i.e., T1M3) benefited from the presence of more kinetically efficient species (due to the lower K_s values) of the propionate oxidizing bacteria and acetoclastic methanogens in the 2nd-phase compared to the biomass of the thermophilic 2nd-phase digesters (Discussed in Chapters 4 and 5). The lower K_s values of the mesophilic biomass appeared to make them more capable in removing soluble COD (which is mainly in the form of VFA) in the effluent in comparison to their thermophilic counterparts.

3.3.6 Analysis of microbial populations

Recently, culture-independent molecular techniques, especially those based on 16S rRNA gene sequences, have been used to investigate the diversity and structure of microbial populations in engineered sludge treatment systems (Sousa et al., 2007; Collins et al., 2006; Ariesyady et al., 2007; Shin

et al., 2010; McMahon et al., 2004). The microbial population composition in anaerobic digesters depends on the operational conditions applied to the digesters (McHugh et al., 2003). On the other hand, the overall performance of anaerobic digesters is influenced by the structure of the microbial population. Therefore, a polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) fingerprinting technique was used to study the impact of phase separation on the structure of Bacteria and Archaea domains that developed in each phase. The microbial populations of the single-stage digesters (C1 and C2) were also investigated as controls for comparison purposes.

3.3.6.1 Amplification of extracted DNA of sample

The extracted DNA of the samples taken from the various digesters was amplified in a single polymerase chain reaction (PCR) amplification for the domain Bacteria and a nested PCR method for the domain Archaea to obtain sufficient archaeal PCR product. Ideally, electrophoresis of PCR products results in a single strong band of correct size, as determined by comparison with the ladder run on the same gel. Gel electrophoresis of Bacteria and Archaea PCR products demonstrated that the size of DNA of the samples were the same as that of the positive control samples, indicating a successful amplification of the target sequences for these domains (Figures in Appendix B).

3.3.6.2 Microbial community structure as determined by DGGE

The DGGE technique, together with the sequencing of DGGE bands, can be employed to analyze and identify microbial community structure of environmental samples (Muyzer et al., 1993; Sousa et al., 2007) and hence can be useful for comparing the community structure of different treatment systems (Liu et al., 2002; Kim et al., 2011). Consequently, this technique was used to compare the structure of the bacterial and archaeal populations developed within each phase of the various digestion systems. Two samples were taken from the digesters on 23 August 2010 and 22 October 2010. Analysis of the samples taken on different dates are shown as duplicate patterns in Figures 3.12 and 3.13. From the examination of the DGGE profile for duplicate samples from each digester, the results demonstrated that a diverse community of Bacteria and Archaea were associated with all digesters. The numbers on Figures 3.12 and 3.13 show the bands that were excised and sequenced successfully, while the stars show the bands that could not be sequenced successfully.

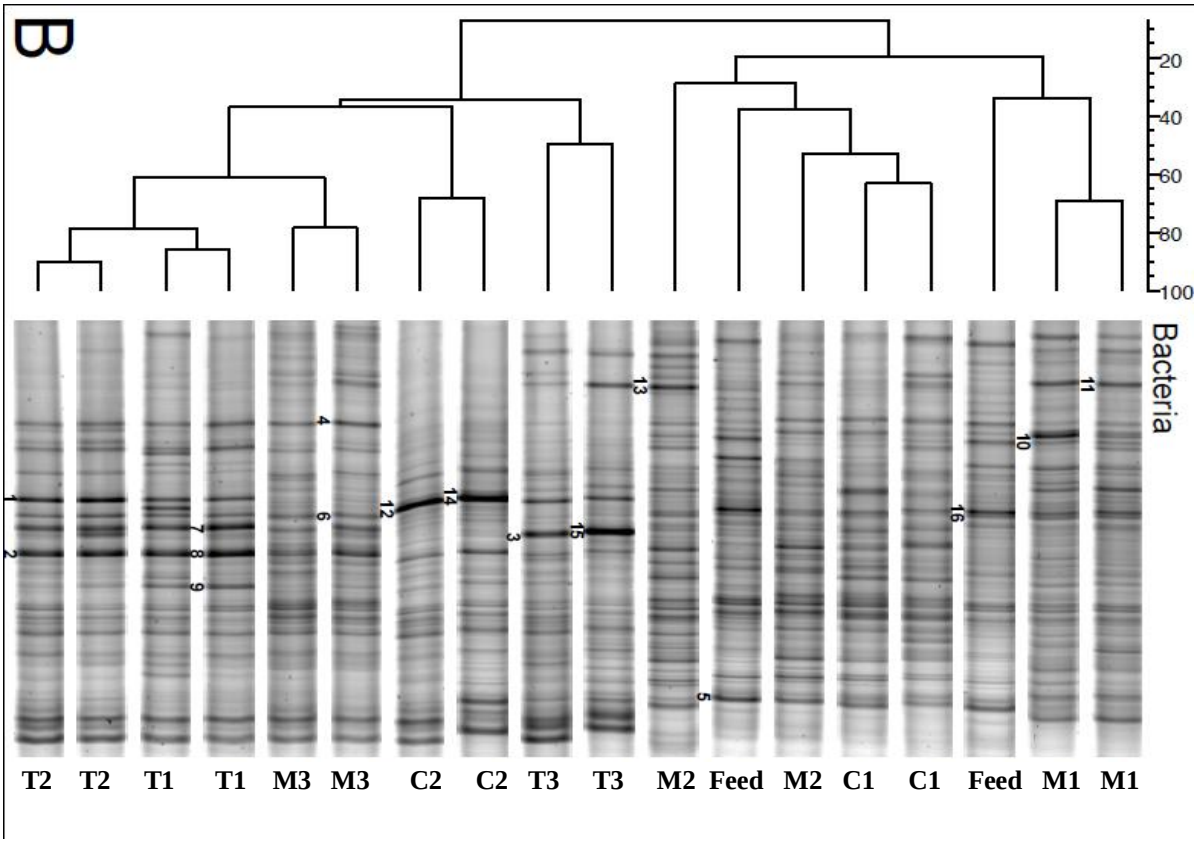


Figure 3.12. Bacterial community DGGE profile

The bacterial DGGE profiles for the mesophilic and thermophilic digesters are presented in Figure 3.12. It can be observed that the number of DGEE bands of the thermophilic bacterial community was relatively less than those of the mesophilic digesters. Overall, it was found that temperature was more effective than phase separation in changing the bacterial community in the digesters.

Despite having similar bacterial community DGGE profiles, the M3 digester, which was fed by the T1, exhibited dominant bands (i.e., darker bands) in different positions on the DGGE profile when compared to the profiles of the remaining mesophilic digesters (M1 and M2). Similarly, the T3 digester, which was fed by the M1, exhibited dominant bands at different positions on the DGGE profile as compared to the profiles of the T1 and T2 digesters. Even though not robustly quantitative (Akarsubasi et al., 2006), the notable differences in band intensity of the M3 and T3 digester, which were reproducible within replicate samples, would suggest that the 2nd phase digesters that were fed from the 1st-phase digester with different temperatures, displayed relatively different dominant bands on the DGGE profile, while the DGGE profiles for the T1 vs. T2 and M1 vs. M2 digesters displayed relatively similar dominant band patterns.

The organisms represented by the bands of the DGGE profile were identified by sequencing of the dominant bands that were excised from the gel and re-amplified. The affiliations of the sequenced bands were determined by comparison with the Ribosomal Database Project (RDP-II) (Table 3.10). RDP is an organized database that has ribosome-related data, analysis services and associated computer programs.

A total of eight bands were determined including bands 5 and 16 of the feed sludge; 9, 10 and 11 of the M1 digester; 13 of the M2 digester; and 4 and 6 of the M3 digester (Figure 3.12). Bands 5 and 16 were most closely related to the genera *Thermonospora* (or *Thermomonospora*) and *Trichococcus*. The genus *Thermonospora* has been isolated from composted horse manure and includes both aerobic and anaerobic species that mostly are involved in degradation of lignocellulosic material (McCarthy, 1987; Zhang et al., 1998). The *Trichococcus* species are fermentative and facultative anaerobes and are psychrotolerant mesophiles (Pikuta et al., 2006). Bands 4 and 11 of M1 digester and 13 of M3 digester showed very low sequence similarity with genus *Lutaonella*. Members of the genus *Lutaonella* are both aerobes and facultative anaerobes and grow at 20-55 ^{0}C. They are reported to ferment amino acids and some organic acids (Arun et al., 2009). Band 10 was related to the genus *Klaistella* which the role of the genus was unclear. Band 6 of the M3 digester was associated with the genus *Akkermansia*. The Akkermansia species is strictly anaerobic and utilizes protein with an optimum growth at 37 ^{0}C (Derrien et al., 2004). Band 9 of T1 digester was related to the genus *Acetivibrio*. The members of the genus *Acetivibrio* are mesophilic fermentative bacteria which are able to degrade carbohydrates (Tanaka et al., 1991).

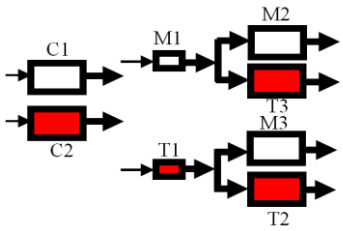


Table 3-10. Taxonomic classifications of sequenced 16S rRNA gene DGGE bands from bacterial fingerprints according to the Ribosomal Database Project (RDP-II)a. ARC and BAC denote DGGE bands sequenced from the archaeal and bacterial analyses, respectively (see Figure 3-12 and 3-13).

	DGGE band	Length (bp)	Phylum	Class	Order	Family	Genus
ARC	1	88	Euryarchaeota (95)	Methanobacteria (90)	Methanobacteriales (90)	Methanobacteriaceae (90)	Methanobrevibacter (76)
	2	97	Euryarchaeota (96)	Methanobacteria (95)	Methanobacteriales (95)	Methanobacteriaceae (95)	Methanothermobacter (95)
BAC	1-T2	136	Thermotogae (72)	Thermotogae(72)	Thermotogales (72)	Thermotogaceae (72)	Petrotoga (52)
	2-T2	150	Proteobacteria (98)	Betaproteobacteria (98)	Burkholderiales (98)	Burkholderiaceae (98)	Ralstonia (98)
	3-T3	149	Bacteroidetes (36)	Sphingobacteria (18)	Sphingobacteriales (18)	Sphingobacteriaceae (6)	Nubsella (6)
	4-M3	144	Bacteroidetes (96)	Flavobacteria (61)	Flavobacteriales (61)	Flavobacteriaceae (54)	Lutaonella (5)
	5-Feed	122	Actinobacteria (92)	Actinobacteria (92)	Actinomycetales (54)	Thermomonosporaceae (44)	Thermonospora (15)
	6-M3	142	Verrucomicrobia (57)	Verrucomicrobiae (45)	Verrucomicrobiales (45)	Verrucomicrobiaceae (45)	Akkermansia (18)
	7-T1	120	Thermotogae (99)	Thermotogae(99)	Thermotogales (99)	Thermotogaceae (99)	Fervidobacterium (99)
	8-T1	123	Thermotogae (98)	Thermotogae(98)	Thermotogales (98)	Thermotogaceae (98)	Fervidobacterium (98)
	9-T1	128	Firmicutes (59)	Clostridia (46)	Clostridiales (39)	Ruminococcaceae (28)	Acetivibrio (24)
	10-M1	134	Bacteroidetes (78)	Flavobacteria (34)	Flavobacteriales (34)	Flavobacteriaceae (30)	Klaistella (15)
	11-M1	131	Bacteroidetes (98)	Flavobacteria (77)	Flavobacteriales (77)	Flavobacteriaceae (77)	Lutaonella (34)
	12-C2	139	Thermotogae (89)	Thermotogae(89)	Thermotogales (89)	Thermotogaceae (89)	Petrotoga (70)
	13-M2	145	Bacteroidetes (100)	Flavobacteria (77)	Flavobacteriales (77)	Flavobacteriaceae (77)	Lutaonella (39)
	14-C2	140	Thermotogae (88)	Thermotogae(88)	Thermotogales (88)	Thermotogaceae (88)	Petrotoga (65)
	15-T3	152	Bacteroidetes (30)	Sphingobacteria (16)	Sphingobacteriales (16)	Sphingobacteriaceae (5)	Nubsella (5)
	16-Feed	147	Firmicutes (96)	Bacilli (92)	Lactobacillales (90)	Carnobacteriaceae (90)	Trichococcus (84)

[a] Classification conducted June and July 2011 using the naïve Bayesian Classifier vs 2.2, March 2010. The confidence of classifications is shown in brackets.

With regards to the thermophilic digesters, a total of eight bands were sequenced including bands 7 and 8 from T1; 3 and 15 of the T2 digester; 1 and 2 of the T3 digester; and 12 and 14 of the C2 digester (Figure 3.12). Bands 1, 12 and 14 were most closely related to the genera *Petrotoga*. The genus *Petrotoga* has been isolated mostly from hot deep petroleum reservoirs (Magot et al., 2000). Members of *Petrotoga* are strictly anaerobic and moderately thermophilic to thermophilic fermentative heterotrophs which use elemental sulphur as the terminal electron acceptor and grow at temperatures in the range 45-65 ^{0}C (Miranda-Tello et al., 2007). Bands 2 and 15 of the T2 and T3 digesters showed sequence similarity with the genus *Ralstonia*. Members of the genus *Ralstonia* are facultative. Some species of the genus *Ralstonia* have been isolated from activated sludge (Coenye et al., 2003). Band 3 was related to the recently identified genus *Nubsella* which has been reported to be a strictly aerobic bacterium (Asker et al., 2008). However, as indicated in Table 3.10 the confidence of the classification was very low (5%). Thus, this band may be associated with a different unidentified bacterium. Bands 7 and 8 of the T1 digester were associated with the genus *Fervidobacterium*. The members of *Fervidobacterium* are strictly anaerobic thermophilic bacteria and have the ability to use many simple and complex carbohydrates such as xylose, cellobiose and starch as growth substrates (Patel et al., 1985).

The archaeal 16S rRNA gene fragments are presented in Figure 3.13. Comaprison of the archaeal and bacterial DGGE profiles showed that bacterial community in the digesters were more diverse than that of the archaeal community. In contrast to the results found for the bacterial community, it was found that phase sepraration was also effective in influencing the archaeal community in addition to temperature.

From the archaeal DGGE profile it can be observed that some bands of the feed sludge appeared within the samples from the mesophilic digesters of M1, M2, M3 and C1. As can be observed from Figure 3.13, the relative number of DGGE bands increased in the digesters compared to the number of bands displayed for the feed sludge. It can also be observed that new bands appeared in the mesophilic digesters. This indicated that methanogens, which did not originate from the raw sludge, developed in the digesters. On the other hand, temperature was found to influence the raw sludge community. As can be observed from Figure 3.13, most of the DGGE bands of the raw sludge disappeared or showed very low intensity in the samples taken from the thermophilic digesters of the T1, T2, T3 and C2 digesters. This indicated that the methanogens of the raw sludge could not survive or remain active at 55 ^{0}C. Thus, most of the archaeal community of the thermophilic digesters were developed exclusively within the thermophilic conditions and did not originate from the feed sludge.

Amongst the mesophilic digesters, the only digester that displayed a significantly different DGGE profile was the M3 digester. Similarly, the DGGE profile of the T3 digester, which was fed by the M1, was different from those of the T1 and T2 digesters. A comparison of the M1 and M2 DGGE profiles revealed that the relative number of bands significantly increased in the M2 digesters. In contrast to the observations made for the M1 and M2 digesters, the DGGE profile of the phased digester with similar temperature (i.e., T1 and T2) showed a relatively similar number of bands. These results would suggest that the methanogenic biomass which transferred from the T1 digester might have remained active in the T2 digester.

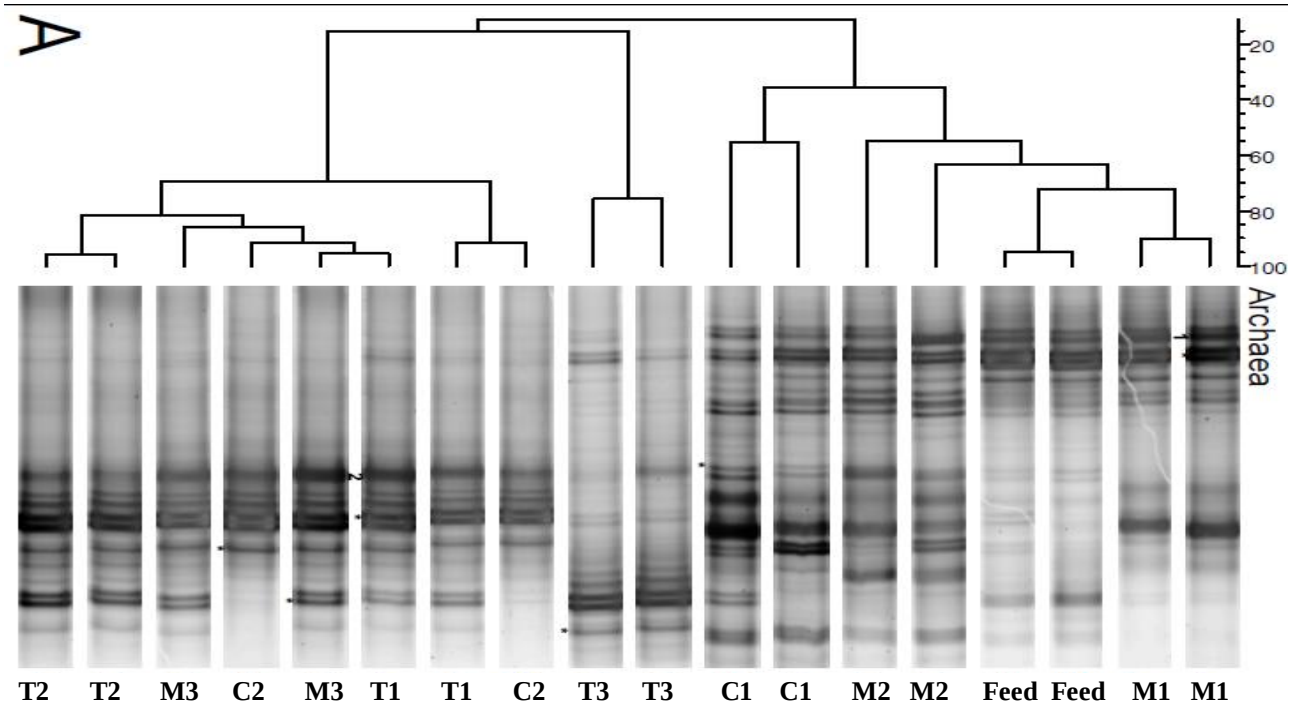


Figure 3.13. Archaeal community DGGE profile

Among the excised bands of the archaeal DGGE profile (Table 3.10), band 1 of the A1 digester and band 2 of the T2 digester were successfully sequenced (Figure 3.13). Band 1 was affiliated with the genus Methanobrevibacter. The members of Methanobrevibacter belong to the group of hydrogenotrophic methanogens that utilize H_2 and CO_2 to produce methane (Lin et al., 1997). Band 2 was affiliated with the genus Methanothermobacter. The members of Methanothermobacter belong to the hydrogenotrophic methanogens that utilize H_2 and CO_2 to produce methane (Sawayama et al., 2006).

3.3.6.3 Microbial community and operational conditions of the digesters

An investigation of the microbial ecology of phased anaerobic digesters may be complimentary to the modeling efforts as it gives a better understanding of the structure of the microorganisms as well as the dominant species in each phase (Merkel et al., 1999; Rittmann, 2002). A considerable number of studies have been carried out to investigate the microbial ecology of sequencing batch anaerobic reactors, single-stage anaerobic digesters and granular sludge systems (Angenent et al., 2002; Sekiguchi et al., 1999; Liu et al., 2002). Thus far, few studies have been performed on the microbial community structure of phased anaerobic digesters (Raskin et al., 1995) and hence a comparative study of several digestion systems with differing configurations was addressed in this research.

Microbial groups will be subjected to different types and loadings of substrates in the phased anaerobic digesters due to the differing operational conditions in each phase. As an example, as shown in Figures 3.6 and 3.7, it can be seen that the VFA concentrations in the 1^{st}- and 2^{nd}-digesters varied significantly. This difference likely resulted in the dominance of certain genera and species of bacteria and archaea in each phase. Despite the limitations associated with quantitative analysis of DGGE profiles, the differences in the relative number and intensity of DGGE bands could be attributed to the dominance of different bacterial and archaeal species in each phase. The influence of substrate composition and concentration on microbial population of biological treatment systems has been widely reported by various researchers (Akarsubasi et al., 2005; Shin et al., 2010; Sousa et al., 2007; Krakat et al., 2011). However, it should be noted that physiologically inactive organisms is also detected by DGGE and hence use of complementary molecular techniques such as microautoradiography combined with fluorescence in situ hybridization (MAR-FISH) may give more information on ecophysiological roles of bacteria and archaea in the phased digestion systems. This is of highly importance for acidogens and POB because of less studies on the functionally dominant species in the phased idgestion systems.

In addition to phase separation, temperature was another significant determinant affecting the bacterial and archaeal community structures in the digestion systems (Figures 3.12 and 3.13). Analysis of the bacterial and archaea DGGE profiles revealed that the number of archaeal DGGE bands was reduced in the thermophilic digesters; however, due to the diversity of the domain Bacteria it was difficult to evaluate the phase and temperature influence on the bacterial community in the separate phase. Very interestingly, it was found that archaeal community of the 2^{nd}-phase digesters (i.e., M3 and T3) that had different temperature from their 1^{st}-phase counterparts (i.e., M1 and T1) were influenced by temperature and phase sepration. Conversely, the DGGE profiles of the 2^{nd}-phase digesters (i.e., M2 and T2) that had

similar temperature to their 1st-phase counterparts (i.e., M1 and T1) indicated the methanogens from the M1 and T1 digesters retained activity presence in the M2 and T2 digesters. This would appear to have influenced the bio-kinetic characteristics of the methanogens in the corresponding 2nd-phase digesters. This will be discussed in greater detail in Chapters 4 and 5.

3.4 Conclusion

In this chapter, the various configurations of phased anaerobic digestion systems were compared with each other and versus the mesophilic (C1) and thermophilic (C2) single-stage digesters. For comprehensive comparison, several parameters were analyzed and discussed in detail, including:

- the efficiencies of solubilisation through measurement and calculation of VSS and PCOD removal, hydrolysis rate constants and release of NH_4-N;
- the reduction of organic content of sludge through calculation of VS and COD removals;
- methane gas production;
- alkalinity, VFA and pH;
- microbial community structure.

Based on multi-parameter comparison of the digestion systems, the following conclusions were made.

1. The solubilisation rate in the T1 digester was significantly higher than that of the M1 digester. Interestingly, when solubilisation of organic material in terms of PCOD removal was compared with solubilisation of the nitrogen-containing matter (sTKN production) in the T1 and M1 digesters it was found that the solubilisation of nitrogen-bearing matter was enhanced significantly (28%) as compared to the solubilisation of PCOD which was 5%.

2. A multi-parameter comparison (i.e., PCOD removal, sTKN increase, NH_4-N release and solubilisation rate constants) showed that lowest solubilisation efficiency consistently obtained in the C1 digester and this was followed by the M1M2 digestion system. On the other hand, the T1T2 digestion system, which showed the highest solubilisation efficiency, was statistically different from the T1M3 digestion and C2 digester; whereas, no difference was found between the T1T2 and M1T3 digestion systems.

3. The influence of phase separation on the solubilisation rate was determined by comparing the M1M2 digestion system with the the C1 digester and the T1T2 digestion system with the C2 digester. The parameters measured demonstrated the phase separation was effective in the solubilisation improvement.

4. In general, the mesophilic digesters (C1, M1M2 and T1M3) outperformed the thermophilic digesters (C2, T1T2 and M1T3) in COD removal. The M1T3 digestion system and C2 digester consistently showed the lowest COD removal. The highest COD removal was obtained in the T1M3 digestion system, indicating a removal efficiency of 50.7±2.1%

5. A study of the microbial community structure using a DGGE fingerprinting technique revealed that both phase separation and temperature influenced the structures of bacterial and archaeal communities in the phased digestion systems.

6. DGGE analysis of archaeal community from the mesophilic digesters (i.e., M1, M2 and M3) showed some similar bands in the profiles of the M1 and M2 digesters, while DGGE patternof the M3 digester were different from the M1 digester. Similar to this observation, the DGGE profiles of the T1 and T2 digesters were relatively similar, while that of the T3 digester was different.

7. Based on overall results, the T1M3 digestion system appeared to be the best configuration in terms of process performance. The T1M3 system showed the highest COD removals and methane production. With the exception of solubilisation efficiency of the T1T2 digestion system, the T1M3 system showed comparable solubilisation efficiency to that of the C2 and M1T3 systems.

Chapter 4
Impact of Phased Digestion on Microbial Populations and Kinetics of Methanogens during Sludge Digestion

4.1 Introduction

Anaerobic digestion is a technology that exhibits several advantages such as low sludge production, high organic loading and energy generation. Anaerobic digestion is a process in which sequential reactions of hydrolysis, acidogenesis, acetogenesis and methanogenesis are performed by consortia of microorganisms, resulting in the production of methane and carbon dioxide. Operation of anaerobic digesters in series with either identical or different temperatures has been reported to have several advantages compared to conventional single-stage digestion process (Pohland and Ghosh, 1971; Siegrist et al., 1993; Han and Dague, 1997). In phased digestion systems, solubilisation and acid production processes are completely or partially separated from acetogenesis and methanogenesis reactions such that the former reactions become dominant in the first phase and the latter in the second phase.

Modeling of anaerobic digestion processes goes back to the early 1970s. Modeling is a valuable tool for better understanding the complex microbial activity and biological reactions occurring within an anaerobic digester. For modeling of single-stage digesters, most models describe the anaerobic digestion process in a continuous-flow completely-mixed reactor in which biological degradation reactions are described by functionally-grouped microorganisms. With the exception of the hydrolysis processes, Monod kinetics have typically been used to describe the rate of substrate consumption for each process (Angelidaki et al., 1999; Siegrist et al., 2002; Batstone et al., 2002). In this regard, modeling of phased anaerobic digestion systems has typically been based on the same modeling concepts as those employed for single-phase digesters, implementing a single set of kinetic parameters which were adjusted for temperature (Siegrist et al., 2002; Bluemensaat and Keller, 2005).

To this point modeling of phased anaerobic digestion systems has not considered the potential for development of microbial communities (i.e. methanogenic species) that have differing growth characteristics in the separate phases. Acetoclastic methanogenesis typically accounts for 65% of methane production and can be mediated by two methanogen species (*Methanosarcina sp.* and *Methanosaeta sp.*). The literature indicates that depending on acetate concentrations, one of the species will proliferate in a digester (Zinder et al., 1984a; Chartrain and Zeikus, 1986; McMahon et al., 2004).

Hence, it might be expected that the kinetic parameters for acetoclastic methanogens will differ between the first and second stages of phased digesters due to differences in acetate availability. Accurate prediction of the growth-kinetics of methanogens is important, particularly for the modeling of phased anaerobic digestion processes where kinetic limitations might influence performance.

The aim of the present study was to investigate the activity of methanogens through the estimation of their kinetic parameters (k_{max} and K_s) in various temperature configurations of phased digestion systems. In this regard, the influence of phase separation on the dominance of the methanogenic population in each phase of the digesters was studied. For comparison, the Monod bio-kinetic parameters were estimated in mesophilic and thermophilic single-stage digesters. Hence, the feasibility of applying Arrhenius-type relationships for adjustment of the coefficients for modeling of digesters with different temperatures was investigated.

4.2 Approach

4.2.1 Batch experiments

This section introduces the experiments carried out to study the influence of phase separation on the kinetics and activity of acetoclastic methanogens. The method employed in the batch experiments was similar to that used for biochemical methane potential (BMP) assays with some modifications. The BMP assay is usually used to determine the methane production of an organic substance. The test is a relatively simple and reliable procedure that has been used for estimation of bio-kinetic coefficients for different organic matters (Donoso-Bravo et al., 2009; Donoso-Bravo et al., 2011; Raposo et al., 2011).

4.2.1.1 Batch experiments on acetate degradation

The literature indicates the existence of two sub-groups of acetoclastic methanogens with different growth characteristics where one sub-group becomes dominant at low acetate concentrations and the other sub-group dominates at high levels of acetate. To investigate whether the operation of anaerobic digesters in series induced the dominant acetoclastic methanogens to be different in each phase, a series of batch experiments were conducted.

The biomass developed in the source digesters was used as an inoculum for the batch experiments. De-ionized water was placed in Wheaton glass bottles, purged with nitrogen gas and kept at either 35 ^{0}C or 55 ^{0}C. The active biomass was taken from each digester and transferred into the bottles that were pre-filled with nitrogen gas and diluted using de-ionized water that was already prepared. The

dilution was made according to preliminary studies and based on the COD and VSS concentrations in the source digesters to achieve approximately equal amounts of active biomass in the batch experiments. The bottles with the diluted inoculum were kept at the same temperature as the source digesters from which the inoculum originated. Two 1000 mL volumes of mineral solution were prepared according to Angelidaki et al. (1990) and one was kept at 35 ^{0}C and the other one at 55 ^{0}C to minimize temperature shock.

The batch experiments were conducted in 500 mL serum bottles. Each bottle was filled with 250 mL of diluted inoculum and 50 mL of mineral solution. Sodium acetate was added to each bottle as the sole carbon and energy source. The final concentration of acetate was approximately 1000 mg/L as COD in the bottles inoculated with the biomass taken from the mesophilic digesters and 1350 mg/L in the bottles inoculated with the biomass taken from the thermophilic digesters. Extra serum bottles were prepared as controls without acetate and with the addition of 250 mL de-ionized water to monitor the degradation of biomass that may result in methane production. The serum bottles were sealed by butyl rubber stoppers and aluminum caps. Before sealing the bottles, 5 mL was taken from each bottle to measure the COD and VSS of the mixture at the beginning of the test. All the serum bottles were purged with nitrogen gas to remove oxygen. To measure the initial acetate concentration, 2 mL of liquid sample was taken by a syringe with a #18G needle and then the bottles were incubated at the same temperature as the source digester from which the inoculum was taken. The evolution of acetate, TCOD, VSS and sCOD was monitored by taking 3 mL liquid samples over the course of the experiments. A fraction of the samples was centrifuged, filtered by 0.45 µm syringe filters and kept at -20 ^{0}C until being analyzed by liquid chromatography for acetate. The remaining portion was used to determine the COD fractions. The biogas generated was monitored at the same time the liquid samples were taken. The methane content was determined whenever the biogas was measured. A 1 mL gas sample was taken from the head space of the serum bottles and analyzed by gas chromatography. The experiments were carried out in triplicate and an extra bottle with no acetate was prepared to monitor the biomass degradation.

4.2.1.2 Modeling of Batch Experiments and Parameter Estimation

Mathematical models are becoming more popular in design and process control of biological treatment systems. The models consist of four components including: input or independent variable; output or dependent variables; parameters and constants; and differential equations (models). Selection of appropriate equations does not result in reliable output if the parameters of the model are not estimated

accurately. Therefore, an important part of modeling is parameter estimation. The estimated parameters should enable the model to predict the experimental results reliably.

4.2.1.2.1 Model Selection for Parameter Estimation

Many authors have estimated the parameters of Monod kinetics by linearization of the model. The well-known expressions are Lineweaver-Burk and Eadie-Hoftsee that transform the Monod-type kinetics into linear form. These expressions have been employed for estimation of the maximum specific growth rate (μ_{max}) and the half-saturation coefficient (K_s). However, linearization techniques magnify uncertainty in estimation of the bio-kinetic parameters. Even though use of the linearized form is simple, it violates an important assumption of regression which is that the independent variable is error free. In addition, when experimental data are transformed (e.g., 1/S), the measurement errors are also transformed and this results in a change of the error structure. Thus, even though normal assumptions may hold true for the original data, it may not be assumed for the transformed data. Consequently, the normal distribution of errors that is an inherent assumption for linear regression may not be valid anymore because linearization changes the error distribution (Dochain and Vanrollegham, 2001).

A more desirable alternative is to solve the differential or integral forms of the Monod model through application of non-linear regression analysis. These forms of the Monod model have been used for modeling of microbially-mediated processes (Merkel et al., 1996; Knightes and Peters, 2000; Nielsen et al., 2008). Differential and integrated forms of Monod equation have been solved for either substrate or biomass and substrate together to estimate kinetic parameters such as the maximum substrate utilization rate coefficient (k_{max}) and the half-saturation constant (K_s). The application of the integral form of the Monod equation is advantageous over other forms as the bio-kinetic parameters (k_{max} and K_s) may be determined using a single substrate depletion curve. Several experiments should be conducted at different initial substrate concentrations if the differential form of the Monod equation is to be used for parameter estimation. In addition, fitting the integrated form to the substrate utilization data allows initial substrate (S_0) and initial biomass (X_0) concentrations to be treated as extra parameters.

In the integrated form of the Monod equation, the endogenous biomass decay is assumed to be negligible and biomass production is related to the substrate utilization through stoichiometry (Equation 4-1). As shown by Andrews (1984) and Ong (1983), this is a reasonable assumption for batch experiments employing a high substrate concentration initially.

$$X = X_0 + Y(S_0 - S) \quad (4\text{-}1)$$

Substituting Equation (4-1) into the substrate utilization rate (Equation 4-2) for a batch process results in Equation 4-3.

$$\frac{dS}{dt} = -k_{max}\frac{S}{K_s+S}X \quad (4\text{-}2)$$

$$\frac{dS}{dt} = -k_{max}\frac{S}{K_s+S}(X_0 + Y(S_0 - S)) \quad (4\text{-}3)$$

This equation may be integrated to give the integral form of the Monod equation (Equation 4-4).

$$\frac{K_s Y + X_0 + YS_0}{(X_0 + YS_0)Y} * \ln\frac{X_o + Y(S - S_0)}{X_0} - \frac{K_s}{(X_0 + YS_0)} * \ln(\frac{S}{S_0}) = k_{max}t \quad (4\text{-}4)$$

The parameters of the Monod kinetics (k_{max} and K_s) for acetoclastic methanogens were estimated by fitting the integral form of Monod model to the substrate consumption data obtained in the batch experiments. The acetate data of the triplicate bottles were pooled and the model was fit to the data simultaneously. The yield coefficient in the model was chosen from the literature and was assumed to be the same for mesophilic and thermophilic temperatures (Siegrist et al., 2002; Bluemensaat and Keller, 2005). The initial concentration of methanogens (X_0) was unknown and was estimated in the parameter estimation exercise.

4.2.1.3 Estimation of Parameters

Parameter estimation consists of finding the optimum values of the parameters of a selected model using measured data (Petersen et al., 2003b). The optimum value means the parameter values which give the best fit to the measured data. Initially, the model structure, the initial concentrations of the components and experimental data are needed (Petersen et al., 2003b). As described in Figure 4-1, initial estimates of the parameters and initial conditions are specified. Then, the parameter estimation routine is started. When the objective function reaches a minimum with certain accuracy, it is terminated. Many numerical techniques for parameter estimation of linear and non-linear models have been developed and a brief description of some techniques can be found in Dochain and Vanrolleghem (2001).

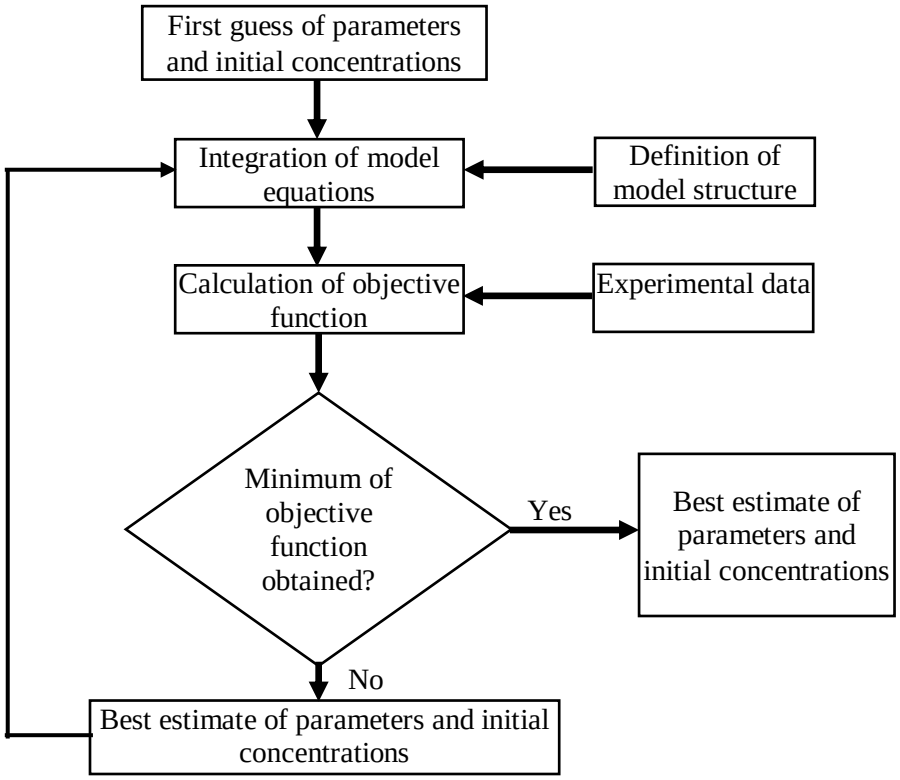


Figure 4.1. Parameter estimation procedure (Dochain and Vanrolleghem, 2001)

4.2.1.3.1 Objective Functions for Parameter Estimation

Objective functions are employed in optimization techniques to make the parameter estimation procedure quantitative through optimizing or minimizing the value of the function (Dochain and Vanrolleghem, 2001). Several objective functions are available for parameter estimation and include maximum likelihood, least-square, Bayesian and robust estimation approaches. The selection of an objective function should be based on the characteristics of errors.

The general least square regression is derived from the maximum likelihood technique by assuming that the observations have normally distributed errors and a constant variance. Robinson (1985) pointed out that the maximum likelihood method is more advantageous to a least square estimation, however, the measurement errors covariance matrix should be known a priori or estimated along with the parameters and this makes its implementation more difficult (Knightes and Peters; 2000; Bard, 1974).

The least-square technique has been widely used for estimation of Monod kinetic parameters (Simkins and Alexander, 1984; Saez and Rittmann, 1992; Merkel et al., 1996). Depending on the error

structure, either an absolute or relative least square regression may be used for parameter estimation. In the absolute least square method, the sum of squares of residuals between the experimental and model predicted values are minimized for the dependent variable (Draper and Smith, 1981).

$$F = \sum_{i=1}^{n}(y_i - y_i^*)^2 \qquad (4\text{-}5)$$

where,

y_i is observed values;

y_i*is model predicted values;

n is the number of observations.

It is usually assumed that the residuals are independent and identically distributed normally (iidN). Even though independence of residuals may be valid in many experiments, the variance of the residuals is non-constant and it increases as the value of the dependent variable increases (Saez and Rittmann, 1992). Thus, more weight is given to the higher values in fitting the model to the data. A possible solution to this problem is to normalize the equation (4-5) through dividing the residuals by either measured or predicted values (Dochain and Vanrolleghem, 2001; Saez and Rittmann, 1992).

$$F' = \sum_{i=1}^{n}\left(\frac{y_i - y_i^*}{y_i}\right)^2 \qquad (4\text{-}6)$$

Based on an analysis of the residuals and goodness-of-fit by using either a least square or a weighted least square function in the parameter estimation procedure, a weighted least square (Equation 4-6) was used as an objective function in this research. As the substrate data varied an order of magnitude or more during the batch experiments, the weighted least square was found to better predict the experimental data.

4.2.1.3.2 Importance of Initial Conditions

The asymptotic nature of the Monod equation makes the estimation of its parameters complicated. Robinson and Tiedje (1983) carried out a sensitivity analysis to investigate the impact of the S_0/Ks ratio on the correlation among Monod parameters (i.e., k_{max}, K_s and Y). They found a strong correlation among these parameters at low (0.02) and high (50) ratios of S_0/K_s. However, when the ratio increased to 4, the correlation was negligible and the parameters could be estimated uniquely. Using a sensitivity analysis on the parameters of the Monod equation, Ellis et al. (1996) recommended that the

initial substrate concentration (S_0) to half-saturation constant (K_s) ratio should be at least 1 or higher if a unique estimate of k_{max} and K_s is intended.

Another factor of importance in the estimation of Monod parameters is the initial substrate to initial biomass concentrations (S_0/X_0). Simkins and Alexander (1985) found significant differences in the estimated values of the Monod parameters (μ_{max} and K_s) in treatments that differed in the initial (S_0/X_0) ratios. They also noted that the yield coefficient (Y) varied with different (S_0/X_0) ratios. However, the authors declared that this finding could not be true as the yield coefficients increased at lower (S_0/X_0). Based on their sensitivity analysis on the Monod integrated equation, a high correlation was reported for Y, μmax and Ks. Consequently, they concluded any difference in either of these parameters (μ_{max} and K_s) would change the yield coefficient (Y).

Even though Simkins and Alexander (1985) found that the yield coefficient unrealistically changed amongst the various treatments with differing (S_0/X_0) ratios, it is usually recommended to consider it as a priori known. The value of the yield coefficient is independent of the growth rate and may be estimated using a metabolic relation (Van Loosdrecht and Heijnen, 2002; Beun et al., 2000a).

Liu and Zachara (2001) studied the influence of initial experimental conditions (i.e., S_0/K_s and $X_0/(K_s\ Y)$) on the correlation of Monod model parameters. The authors found that a high correlation between the Monod parameters (μ_{max} and K_s) resulted in large standard errors for the estimated parameters. They, however, indicated that at an initial S_0/K_s ratio of 5 or more, the uncertainty of the estimates reduced under no growth conditions.

As recommended by previous researchers through sensitivity analysis of the Monod equation (Robinson and Tiedje, 1983; Liu and Zachara, 2001), the initial substrate concentration (S_0) was selected to be higher than the expected half-saturation constant (K_s) to minimize the effect of correlation of Monod kinetic coefficients during parameter estimation.

4.2.1.3.3 Kinetics of acetoclastic methanogenesis

As mentioned previously, the parameters of the Monod kinetics (k_{max} and K_s) as well as the biomass concentration were estimated using an integrated form (Equation 4-4) of the kinetics for a batch process that assumed negligible cell growth. This calibration process was carried out using a nonlinear optimization (fminsearch in Matlab), through a minimization of the normalized sum of squares between the model and measured outputs (Equation 4-7). Various initial values of the model parameters were tested to ensure that global minima were obtained rather than local minima.

$$J = \sum_{i=1}^{n} \left(\frac{S_i - \hat{S}_i}{S_i}\right)^2 \qquad (4\text{-}7)$$

The uncertainties for the estimated optimum parameters of the Monod model were evaluated by an approximate method described for numerical modeling applications (Draper and Smith, 1985; Smith et al., 1998). The mean square error (MSE) for each parameter was calculated from the mean square fitting error and the sensitivity function. The mean square fitting error was calculated using Equation (4-8).

$$\sigma^2 = \frac{1}{n-p} \sum_{i=1}^{n} \left(\frac{S_i - \hat{S}_i}{S_i}\right)^2 \qquad (4\text{-}8)$$

where, n is the number of measurements and p is the number of parameters being estimated.

The sensitivity function of a model is derived by calculating the first derivative of the dependent variable with respect to each parameter. The sensitivity function was determined by calculating approximate first derivatives through perturbing one parameter at a time by a small step and fixing the other ones to evaluate the model predicted values (Equation 4-9).

$$\frac{\partial \hat{S}_i}{\partial t} \approx \frac{\hat{S}_i(\hat{\theta}+\Delta\theta) - \hat{S}_i(\hat{\theta})}{\Delta\theta} \qquad (4\text{-}9)$$

where

$\theta\hat{}$ is the optimum estimate of θ;

$\theta\hat{}+\Delta\theta$ is a nearby value of θ;

$(S_{\_i})(\theta\hat{}+\Delta\theta)$ is the model predicted concentrations after perturbation of parameter; and

$(S_{\_i})(\theta\hat{})$ is the model predicted concentrations at optimum parameter estimates.

It should be pointed out that even though the model-predicted values were not compared to the observed values in these calculations, the two sets of model-predicted values (before and after perturbation) were determined at the points corresponding to the observation values. Therefore, if the substrate concentrations were observed at experimental points that are sensitive to the model parameters, high accuracy of parameter values can be expected. The mean square error (MSE) of each parameter was calculated from equation (4-10).

$$MSE = \frac{\sigma^2}{\sum_{i=1}^{n}(\frac{\partial \widehat{S_i}}{\partial \theta})^2} \quad (4\text{-}10)$$

The square root of MSE is the standard error from which the approximate 95% confidence interval (CI95%) for each parameter (θ) was calculated (Equation 4-11).

$$\hat{\theta} \mp 2\sqrt{MSE} \quad (4\text{-}11)$$

If more than one fitting parameter is to be estimated, the sensitivity functions are represented by a square matrix (M) as shown in equation (4-12) for a case with two parameters. The diagonal elements of the matrix are the sensitivity coefficients for each parameter.

$$M = \begin{bmatrix} \sum_{i=1}^{n}(\frac{\partial \widehat{S_i}}{\partial \theta_1})^2 & \sum_{i=1}^{n}(\left(\frac{\partial \widehat{S_i}}{\partial \theta_1}\right)\left(\frac{\partial \widehat{S_i}}{\partial \theta_2}\right)) \\ \sum_{i=1}^{n}(\left(\frac{\partial \widehat{S_i}}{\partial \theta_1}\right)\left(\frac{\partial \widehat{S_i}}{\partial \theta_2}\right)) & \sum_{i=1}^{n}(\frac{\partial \widehat{S_i}}{\partial \theta_2})^2 \end{bmatrix} \quad (4\text{-}12)$$

The standard error for individual parameters was calculated by multiplying the inverse of the diagonal elements of the matrix, M, by the measurement variance (σ^2) (Equation 4-13).

$$V = \sigma^2 M^{-1} \quad (4\text{-}13)$$

where, V is mean square error matrix; and M^{-1} is the inverse of the matrix.

The 95% confidence interval for one parameter, θ_1, can then be computed by equation (4-14).

$$\widehat{\theta_1} \mp 2\sqrt{V_{11}} \quad (4\text{-}14)$$

Even though, the confidence interval of individual parameters is informative, it does not indicate the joint variability of all parameters to be estimated. The joint confidence region is an ellipse for the case with two parameters. As the integral Monod model does not have an analytical solution, the confidence region for the two parameters is the locus of values for the parameters which satisfy the inequality (Equation 4-15).

$$\frac{1}{\sigma^2}(M_{11}(\theta_1 - \hat{\theta}_1)^2 + M_{22}(\theta_2 - \hat{\theta}_2)^2 + +2M_{12}(\theta_1 - \hat{\theta}_1)\ \ (\theta_2 - \hat{\theta}_2)\ \ \leq Z \qquad (4\text{-}15)$$

where, θ_1 and θ_2 are the optimum estimates of the parameters. The value of Z is calculated based on the F distribution and is a function of the numbers of measurements (n), parameters (p) and the selected confidence interval probability (Equation 4-16):

$$Z = p.F(p, n-p, 1-\alpha) \qquad (4\text{-}16)$$

where, F(p, n-p, 1-α) is taken from an F-distribution table, (n-p) is the degree of freedom, and 1-α is the fractional probability.

4.2.1.4 Residual analysis

The residuals were analyzed to check the validity of the assumptions of the least square technique. The assumptions include normal distribution of residuals with a mean of zero and a constant variance. A normal probability plot of residuals was used to check the normal distribution of residuals. If the plot is a straight line, then it indicates that the residuals come from a normal distribution (Montgomery, 2001). In addition, this plot helps to find any outliers in data.

The second important assumption of regression is that the data points have the same precision and consequently the residuals have a constant variance. Analysis of residuals by plotting the residuals versus predicted values was used to check if the errors were independent and had a constant variance. A random scatter of residuals versus the predicted values verifies this assumption.

4.3 Results and Discussion

4.3.1 Performance of Source Digesters

The bench scale digesters that provided inoculum for the batch kinetic studies were characterized with respect to conventional parameters such as COD destruction and volatile fatty acid accumulation (Chapter 3). Briefly, a comparison of the COD removal among the mesophilic digesters (M1M2, T1M3 and C1) showed that the M1M2 (43.6%) and T1M3 (47.3%) digestion systems achieved greater COD removal compared to the removal efficiency (42.1%) obtained for the C1 digester (ANOVA PostHoc test, α=0.05).

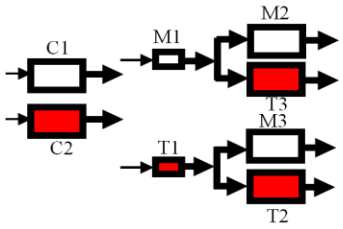


Table 4-1. Measured acetate concentration in source digesters

Digester	Acetate Concentration, mgCOD/L		Digester	Acetate Concentration, mgCOD/L	
	Mean	SD*		Mean	SD
C1	53.6	13.1	C2	139	30.6
M1	338.0	48.0	T1	872.7	83.8
M2	42.7	8.4	T2	125.2	17.1
M3	33.7	10.0	T3	184.5	28.4

*Standard Deviation

Among the thermophilic digesters (T1T2, M1T3 and C2 digesters), only the T1T2 digestion system (45.3%) improved COD removal performance, whereas the COD removal efficiencies in the M1T3 digestion system (41.3%) and C2 digester (42.4%) were comparable (ANOVA PostHoc test, α=0.05). Moreover, the mesophilic digesters overall outperformed the thermophilic digesters in COD removal. The heavily loaded 1st-phase (M1 and T1) digesters of the phased digestion systems had considerably reduced COD removals as compared to the overall efficiency of the systems. Consequently, it was anticipated that there would be considerable accumulation of soluble COD, especially volatile fatty acids, in these digesters. Table 4.1 presents a summary of the acetate concentrations that were measured in each digester. From Table 4.1 it can be observed that the 1st-phase of the phased digestion systems had significantly higher acetate concentration as compared to the other digesters (i.e. the single-stage digesters and the 2nd-phases of the phased digestion systems). Statistical analysis confirmed this difference, using an ANOVA PostHoc test (α=0.05).

4.3.2 VSS and pH of Batch Experiments

The value of pH influences the relative concentrations of free acids and bases. An increased concentration of free acids and bases can result in inhibition of microbially-mediated anaerobic processes, especially methanogenesis (Batstone et al., 2002). Thus, to determine if any severe changes in pH occurred, the pH was measured at the end of the experiments to ensure the methanogenesis could proceed without any inhibition. As shown in Figure 4.2, the pH values ranged from 7.36 to 8.41 which were

within the pH optima cited for methanogens (Williams and Crawford, 1985). The pH of all the bottles kept at 55 ^{0}C was higher than the corresponding bottles kept at 35 ^{0}C. This was most likely due to the higher release of CO_2 to the bottle headspace at the elevated temperature. Overall, it was concluded that pH did not cause inhibition of the methanogenic activities and was not a concern in the parameter estimation exercise.

The ratio of substrate to biomass is an important factor that can influence the estimation of Monod kinetic parameters (Simkins and Alexander, 1985). Accordingly, a relative biomass density was used in the parameter estimation exercise. In addition to affecting parameter estimation, the substrate to biomass ratio may influence the Monod parameter correlation (Simkins and Alexander, 1985; Liu and Zachara, 2001). Therefore, based on the VSS concentrations of the digesters, the samples were diluted to have approximately an equal amount of biomass in the bottles of similar temperature. This ensured that the substrate to biomass ratio was approximately equal in the bottles of the same temperature. Also, the concentrations of volatile suspended solids were determined at the start and end of the batch tests to determine if there were any significant changes in the biomass through the tests.

The average concentrations of VSS together with their standard deviations (SD) for the various batch experiments are presented in Figures 4.3 and 4.4 for the mesophilic and thermophilic tests respectively. An ANOVA PostHoc test (α=0.05) was conducted to compare the VSS concentrations present in the bottles at similar temperatures (either mesophilic or thermophilic). No significant difference was found among the VSS concentrations in the bottles of either the mesophilic or the thermophilic temperature. Therefore, the initial substrate to biomass (S_0/X_0) ratio was approximately similar in the bottles of similar temperature, making the estimated parameters more comparable. As can be seen from Figures 4.3 and 4.4, VSS concentrations remained approximately the same in the bottles in either the mesophilic or the thermophilic batch experiments. Statistical analysis showed no significant difference in VSS concentrations between the beginning and end of the experiments (paired t-test, α=0.05).

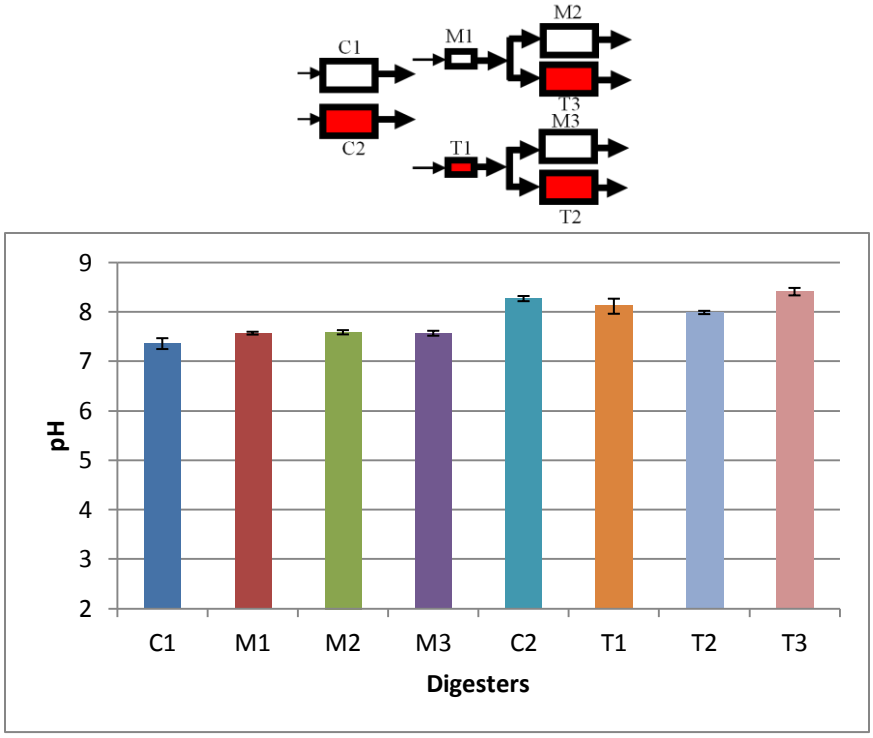


Figure 4.2. Average pH values in batch experiments

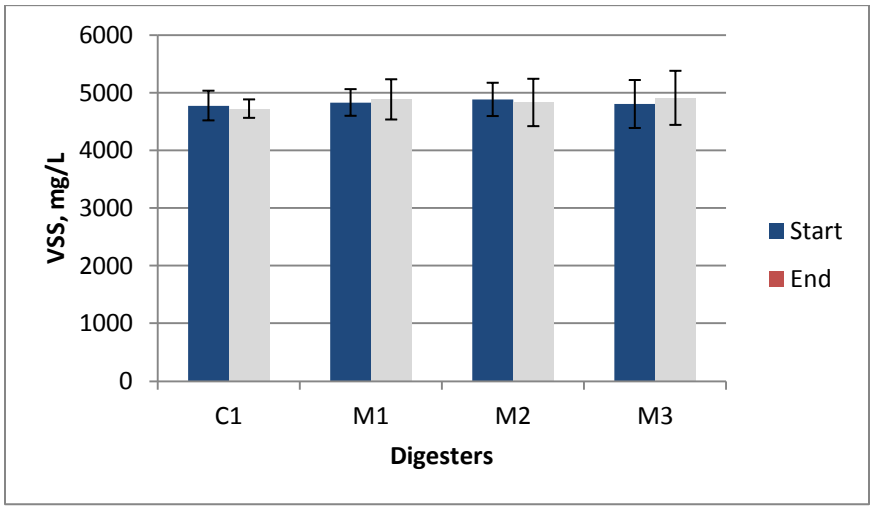


Figure 4.3. Average VSS concentrations in mesophilic batch experiments (Average ± SD); S=Start of experiment; E=End of experiment.

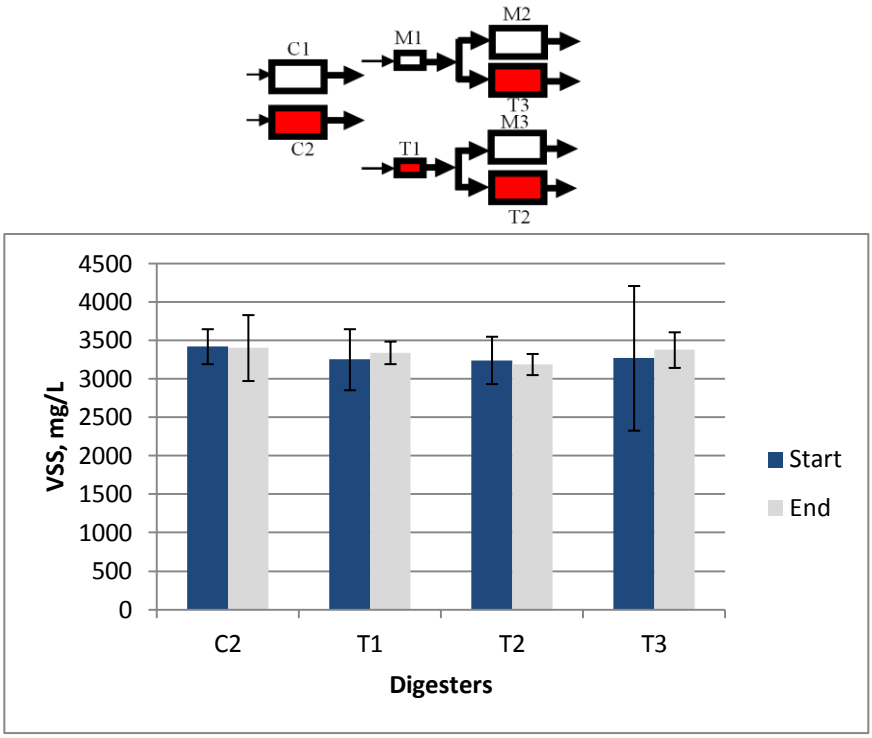


Figure 4.4. Average VSS concentrations in thermophilic batch experiments (Average ± SD); S=Start of experiment; E=End of experiment.

4.3.3 COD evolution in batch experiments

The COD of the bottles was characterized for total and soluble fractions. The total COD (TCOD) was used to determine the reliability of the test through establishment of a mass balance on the batch experiments. In anaerobic digestion, methane production is the major pathway for the removal of COD. Similar to the procedure used for the digesters, the lack of mass balance was calculated from Equation (4-18). TCOD in the beginning and end of the batch tests were measured. Cumulative methane gas production monitored and recorded. The CH4-COD was calculated for STP. Hence, the lack of COD balance was examined in the test bottles using Equation 4.18).

$$COD\ balance\ (\%) = (\ COD_{in} - COD_{out}\ - COD_{CH4}) \times \frac{100}{COD_{in}} \qquad (4\text{-}18)$$

In addition to TCOD, soluble COD (SCOD) of the contents was measured in the bottles and particulate COD (PCOD) was computed by subtracting TCOD from SCOD. The PCOD calculations were employed to assess whether there was any significant decay of biomass during the experiments. At high COD concentration, significant decay can make the parameter estimation inaccurate as high decay

products may undergo hydrolysis and acidogenesis and add to the net substrate concentration of interest (i.e., in this case acetate) (Robinson and Characklis, 1984). However, these processes are not included in the integrated Monod model used in this research for estimation of bio-kinetic parameters.

Overall very low deviation was observed in mass balance of the bottles, indicating a good agreement between COD and methane gas measurements. Even though the acetate concentration was directly used in the parameter estimation exercise, acetate is a major precursor for production of methane (Jeris and McCarty, 1965) that indicates the activity of methanogens within the bottles. Thus, a good mass balance on the bottles indicated the reliability of the data was used in parameter estimation. The values calculated for lack of mass balance for the batch experiments inoculated from the C1, M1, M2 and M3 digesters were -0.5, -0.2, -1 and -0.8%, respectively. These values for the thermophilic batch experiments which were seeded from the C2, T1, T2 and T3 digesters were 2.4, 2.7, 3 and 2%, respectively.

The average total COD of triplicate bottles for the mesophilic and thermophilic batch tests are shown in Figures 4.5 and 4.6. The general trend of COD was similar at both temperatures, indicating reduction of COD during the experiments that then appeared as methane gas. TCOD removal in the mesophilic bottles ranged 13-15% and for the thermophilic bottles was 10-13%. In addition, the rates of TCOD decline were determined in both the mesophilic and thermophilic bottles. The TCOD reduction rates were 0.45, 0.52, 0.46 and 0.44 g/L.d in the C1, M1, M2 and M3 bottles, respectively. The decline rates for the thermophilic bottles of C2, T1, T2 and T3 were calculated as 0.63, 0.71, 0.66 and 0.60 g/L.d. The results indicated that the TCOD reductions rate under the thermophilic condition was, on average, 1.4 times greater than that of the mesophilic bottles. Overall these results demonstrated that thermophilic temperature increased biomass activity compared to the values obtained for the mesophilic condition.

The removal of PCOD was calculated for the mesophilic and thermophilic bottles. It was found that 3-5% of the PCOD was removed in the mesophilic bottles. Compared to the mesophilic batch tests, relatively higher PCOD removals were observed for the thermophilic batch tests. The values ranged 6-10% for the thermophilic bottles. These results indicated that a negligible fraction of the inocula added to the bottles underwent decomposition that would slightly influence the net propionate concentration in the bottles.

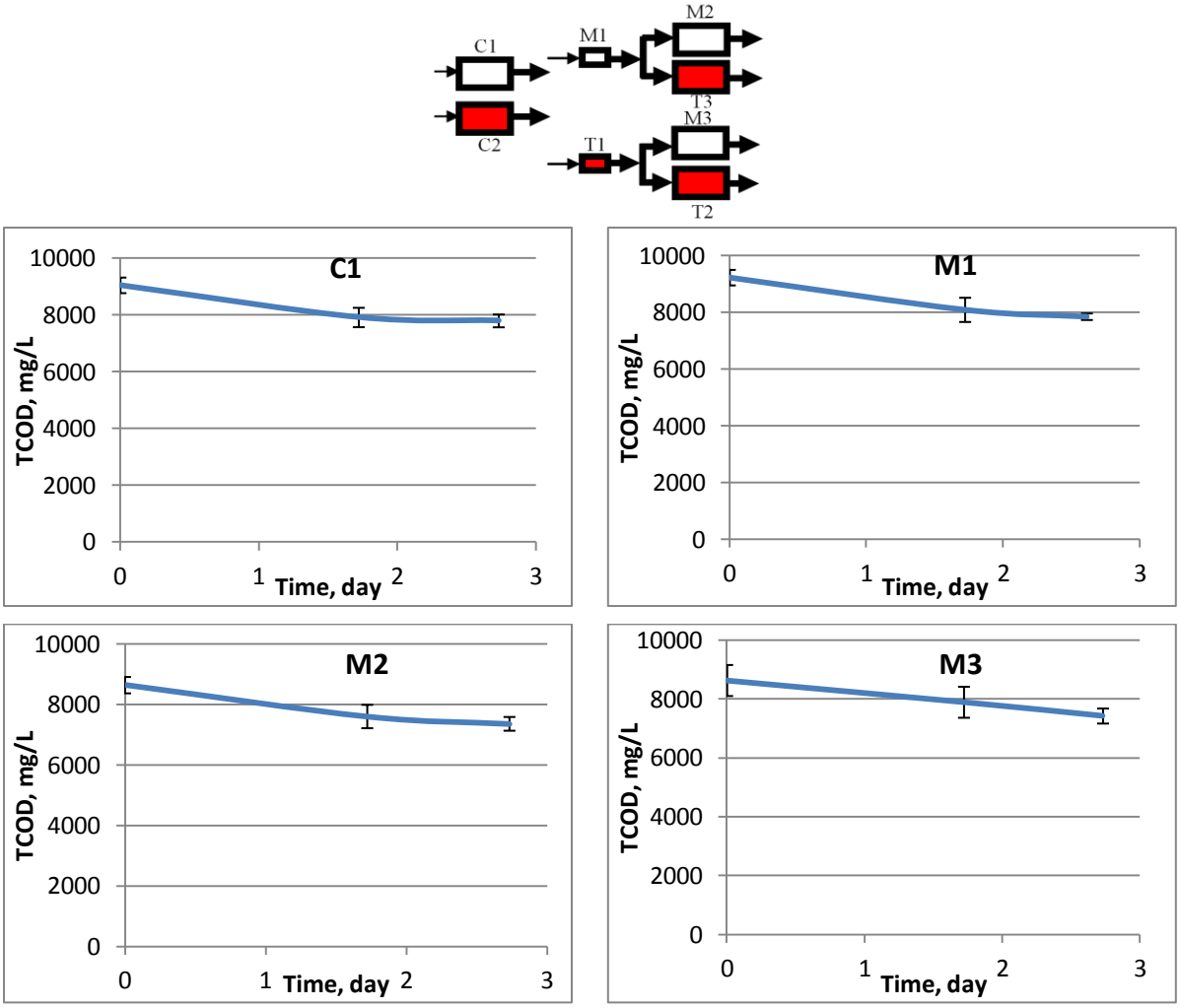


Figure 4.5. Total COD values in the mesophilic batch experiments.

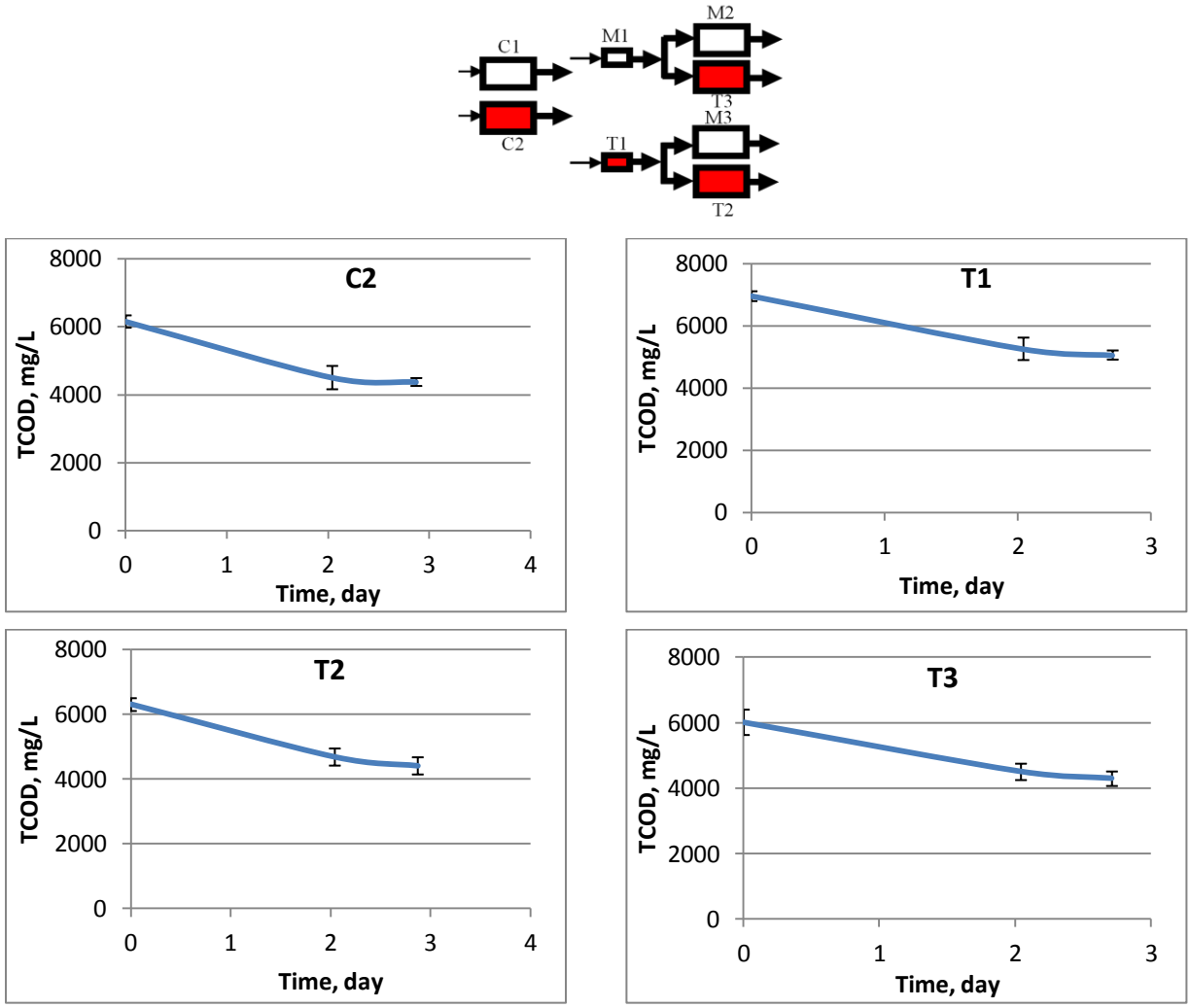


Figure 4.6. Total COD values in the thermophilic batch experiments.

The average soluble COD concentrations of the triplicate bottles for the mesophilic and thermophilic batch tests are presented in Figures 4.7 and 4.8. The SCOD measured at the beginning of the test was higher than the theoretical COD equivalent of acetate added to the bottles. The values of the acetate to soluble COD ratio for the mesophilic bottles were in the range of 70-80% and for the thermophilic bottles were in the range of 75-82% at the beginning of the test. At the end of the test, this ratio was significantly reduced to 0.5-2% and 4-7% for the mesophilic and thermophilic bottles, respectively. These results indicated the SCOD reduction was mainly due to the acetate degradation in the test bottles.

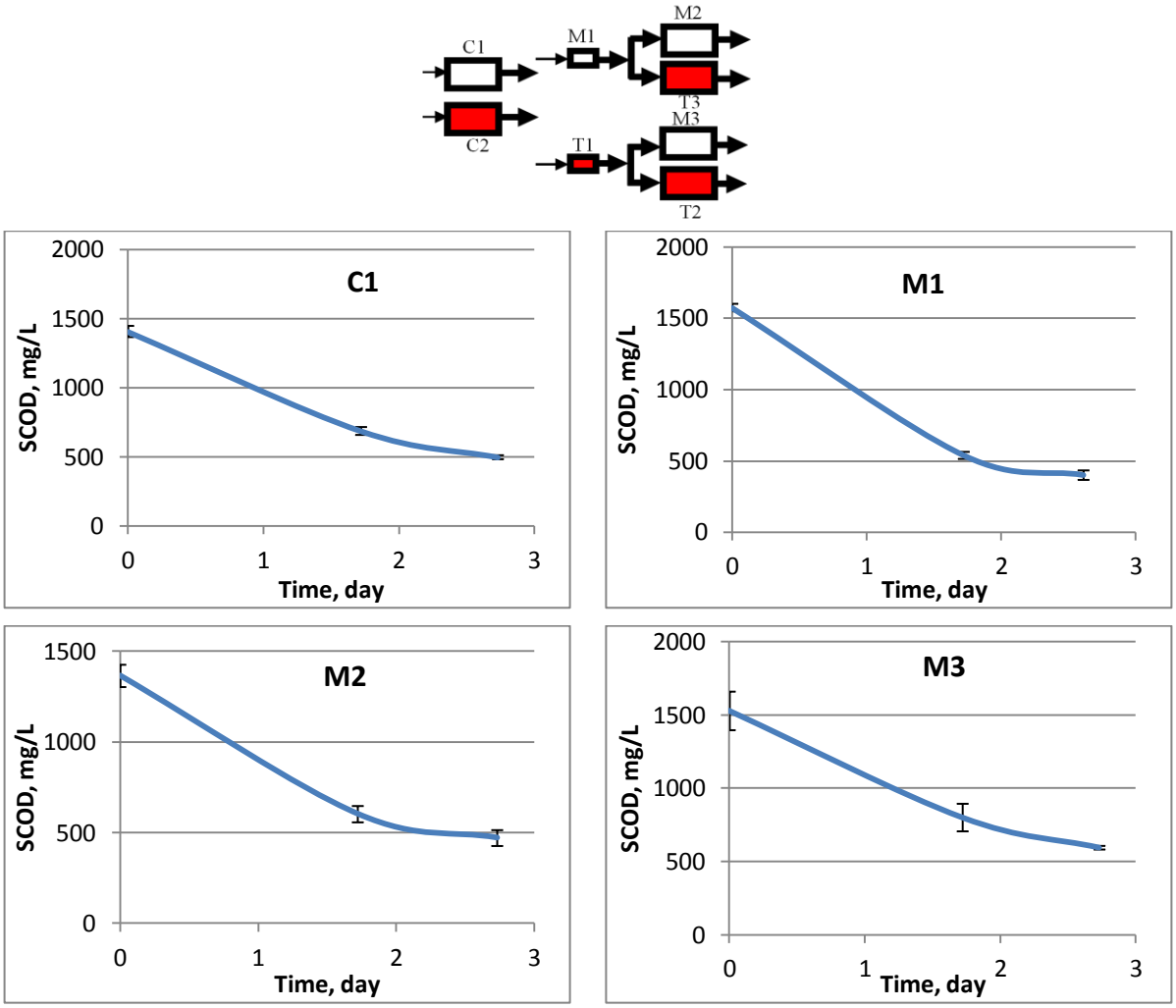


Figure 4.7. Soluble COD values in the mesophilic batch experiments.

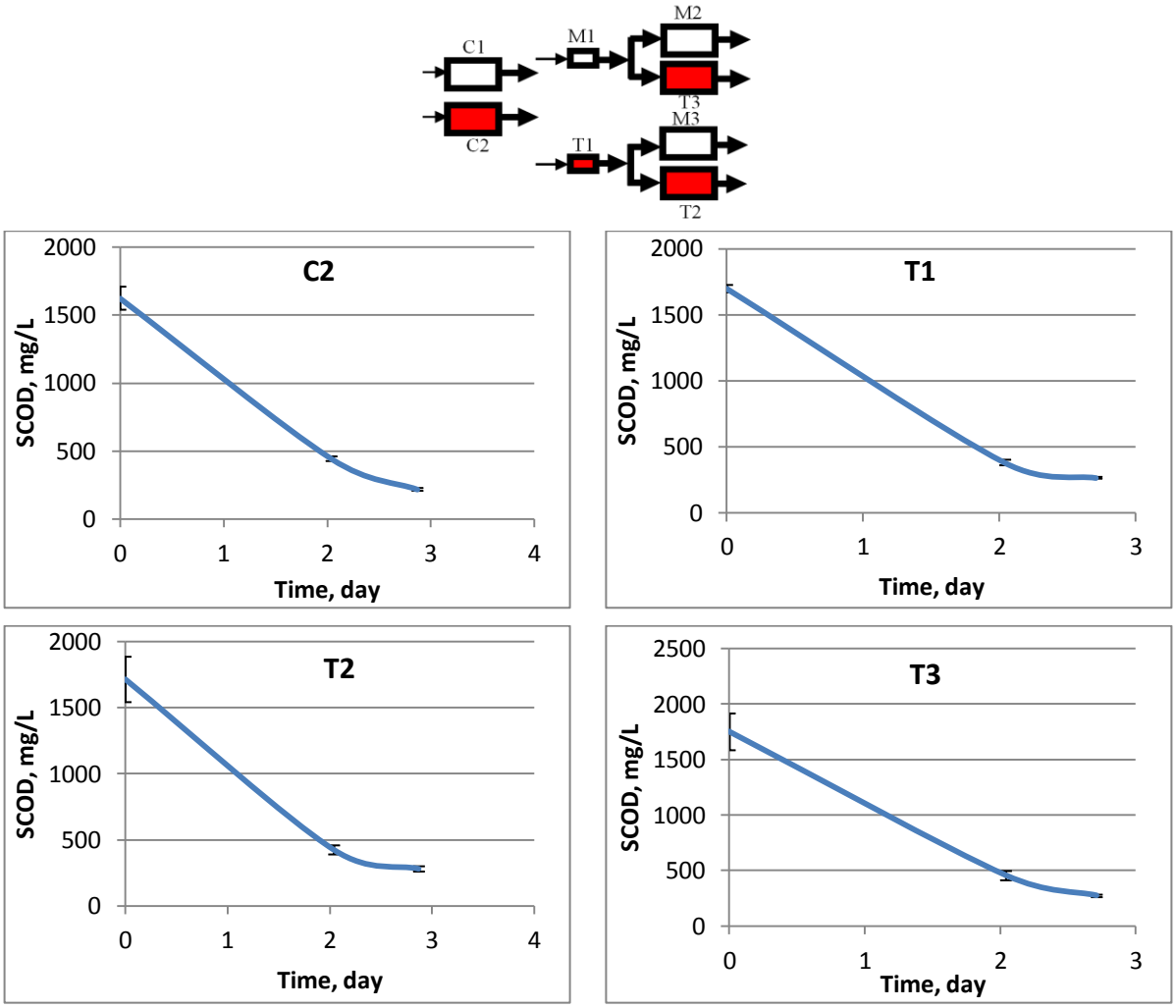


Figure 4.8. Soluble COD values in the thermophilic batch experiments.

4.3.4 Parameter Estimation

4.3.4.1 Preliminary experiments to determine optimum S_0/X_0

The selection of an optimum initial substrate (S_0) to biomass (X_0) ratio is important not only for identifiability of Monod kinetic parameters (Grady et al., 1969; Liu and Zachara, 2001), but also for reducing the influence of substrate production due to the degradation of biomass under conditions of high inoculum quantities in the test bottles. Under anaerobic conditions, decay of biomass results in materials that can be converted to VFAs such as propionate and acetate through acidogenic and acetogenic

processes. This can make the estimation of Monod kinetic parameters, especially Ks, imprecise if a simple model such as the integrated Monod model is to be used because the model does not include biomass degradation (Robinson and Characklis, 1984; Flotats et al., 2003; Nielsen et al., 2008).

The results of preliminary experiments for the estimation of Monod kinetic parameters (K_{max} and K_s) in the M2 and T1 digesters are presented in Figures 4.9 and 4.10. The acetate concentration in the mesophilic and thermophilic bottles were approximately 1250 and 1600 mg/L as COD. The biomass from the 1st-stage digesters was diluted to have equal amount of biomass for all the bottles at similar temperature. The total COD of the mesophilic and thermophilic bottles were, on average, 16.5 and 14 g/L. The required macro- and micro-nutrients were also added to the bottles as explained earlier.

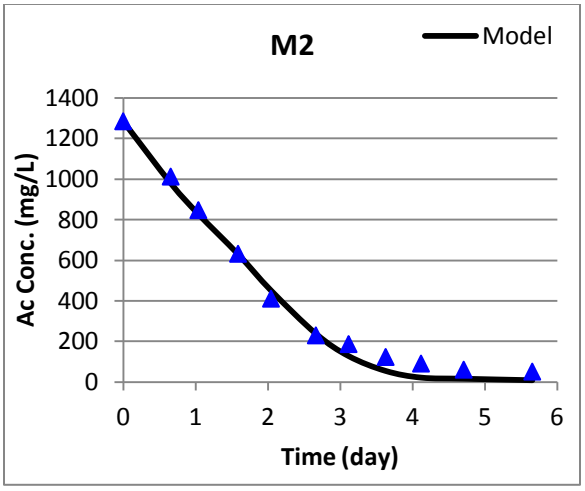


Figure 4.9. Results of preliminary model fit with experimental data: M2 digester

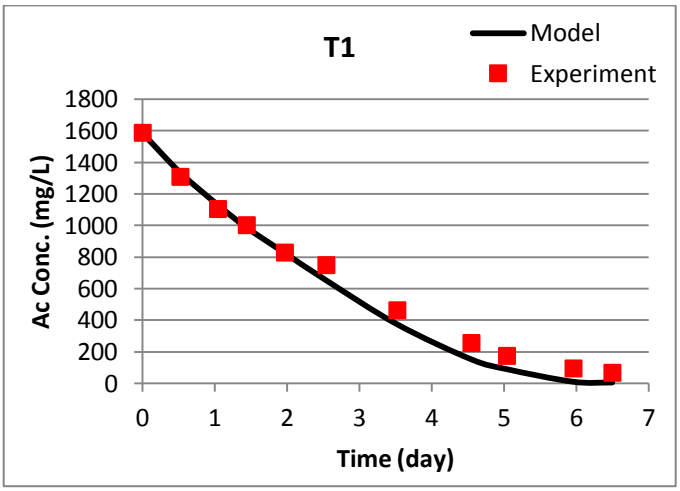


Figure 4.10. Results of preliminary model fit with experimental data: T1 digester

From about day 3 in the mesophilic digester (M2) and day 2.5 in the thermophilic digester (T1), the model deviated from the experimental data. It would appear that the acetate concentration was not consumed at the same rate predicted by the model. This was likely due to the degradation of inoculum that resulted in generation of some acetate in the test bottles (Nielsen et al., 2008). Based on these results, the batch experiments were modified through shortening the duration of the experiments (about 3 days) and further dilution of the inoculum to reduce the influence of biomass degradation on acetate concentration. The total COD in the batch experiments was reduced to approximately 9 and 6 g/L in the mesophilic and thermophilic bottles, respectively.

4.3.5 Residual analysis

Prior to analyzing the results of the parameter estimation, it was important to determine if the assumptions of the least squares approach were reasonably satisfied. The least square assumptions, especially error distribution, were analyzed to establish the subsequent tests of significance and calculation of confidence intervals for comparison of parameter estimates were valid (Marshall et al., 1959). The assumptions include selection of an appropriate model, normal distribution of errors, constant variance or errors and independency of errors from one another (Montgomery, 2001).

Graphical representation of errors (the difference between observed and predicted values) is a useful tool to check the normal distribution of errors. Figures 4.11 and 4.12 illustrate the results of testing the normal distribution of errors for the mesophilic and thermophilic batch experiments. A linear line was obtained for the errors of the acetate concentrations of all the batch tests, indicating a normal distribution of the residuals.

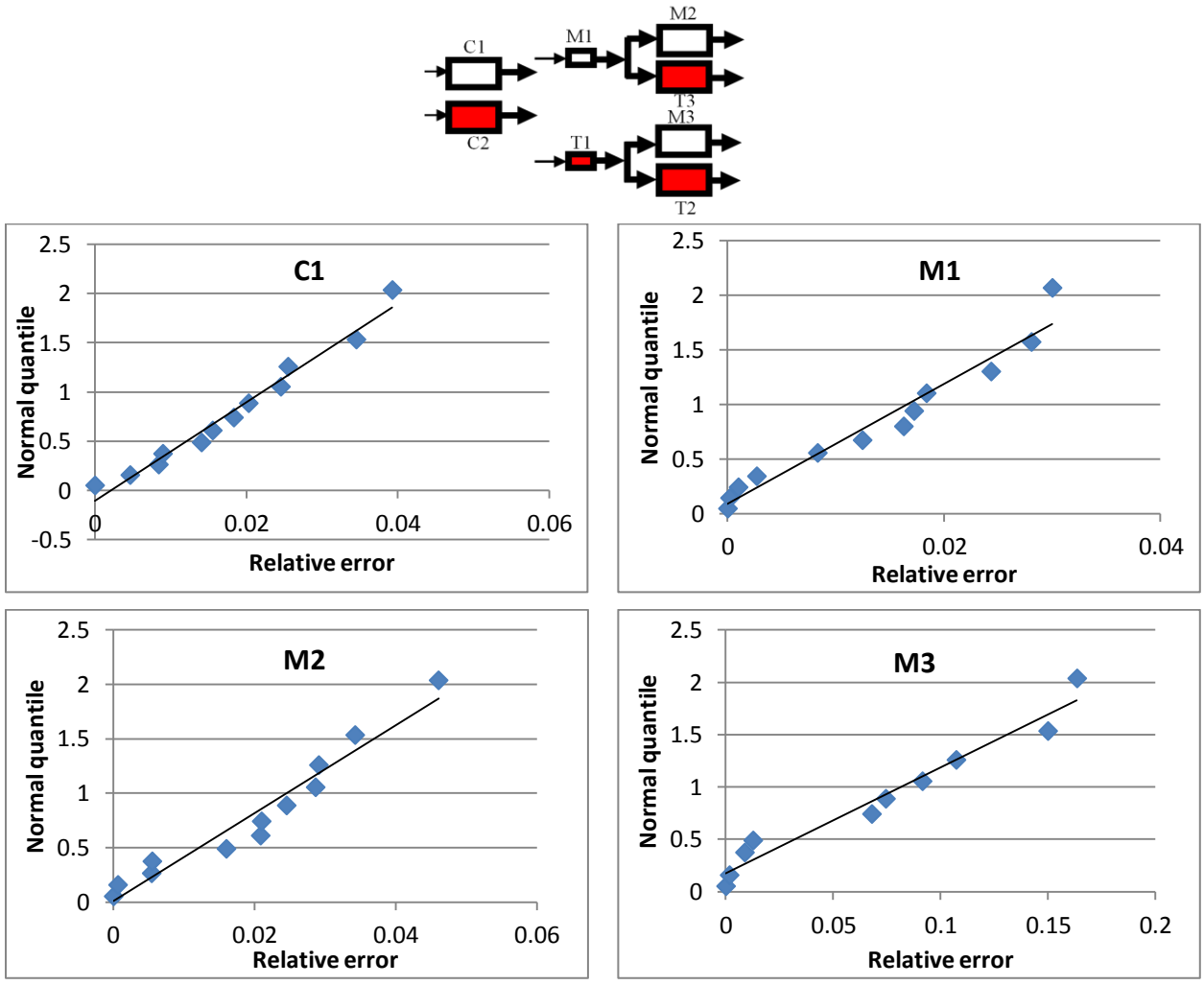


Figure 4.11. Half-normal quantile-quantile plot of errors for acetate data of the mesophilic batch experiments

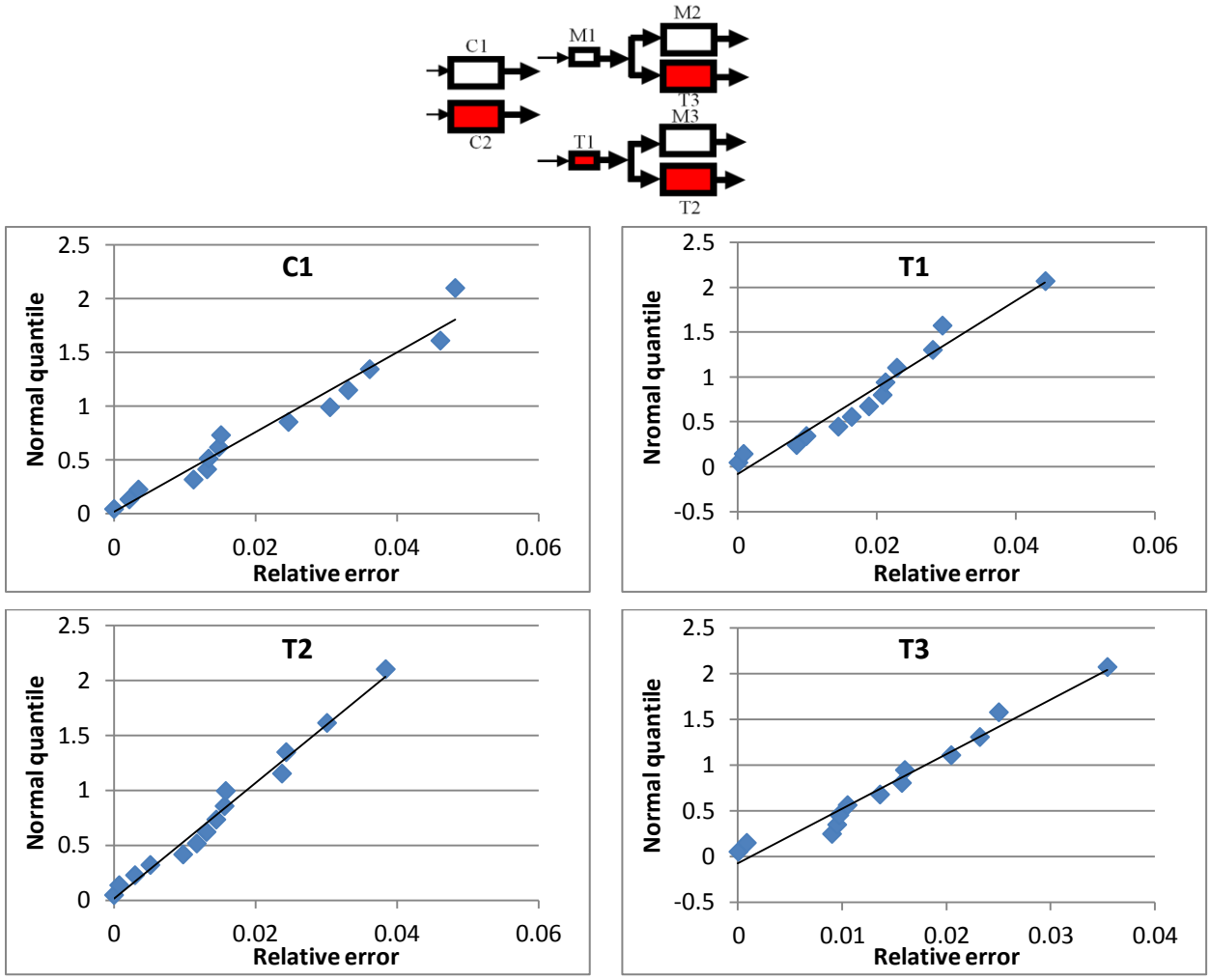


Figure 4.12. Half-normal quantile-quantile plot of errors for acetate data of the thermophilic batch experiments

The homoscedasticity assumption of residuals is important in parameter estimation as significance tests and the confidence intervals of the estimated parameters are not valid in cases with unequal variances for residuals. A simple method to check for homoscedasticity is to plot the residuals versus model predicted values. A randomly scattered pattern of residuals indicates the errors have a constant variance (Mongomery, 2001). Accordingly, the errors of the acetate concentrations were plotted versus the model predicted values for the mesophilic and thermophilic batch experiments to check the randomness of errors (Figures 4.13 and 4.14). The scattered pattern of the residuals demonstrated that the errors of the acetate data in the batch experiments had approximately constant variance.

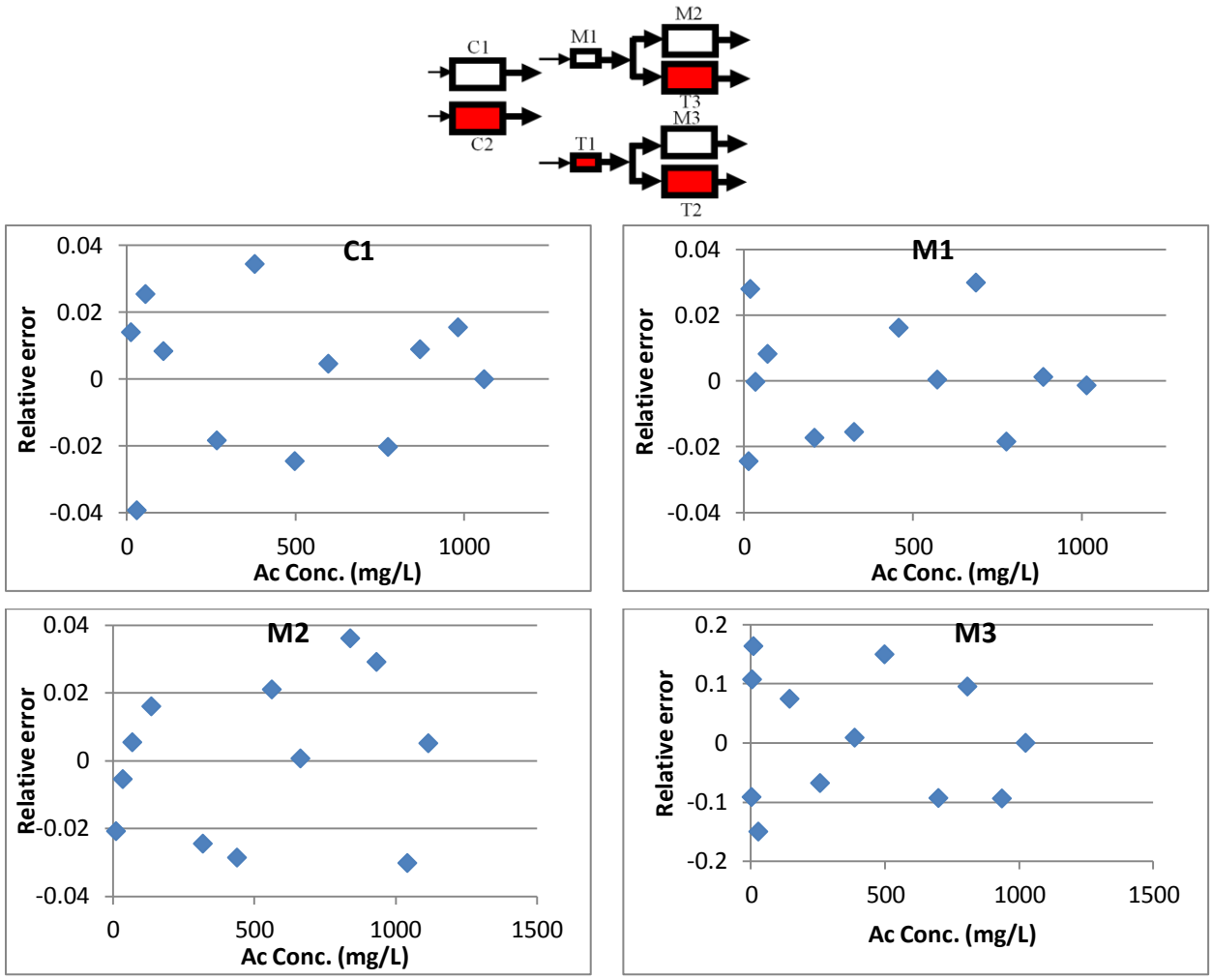


Figure 4.13. Residual (error) vs. predicted acetate concentration for the mesophilic batch experiments

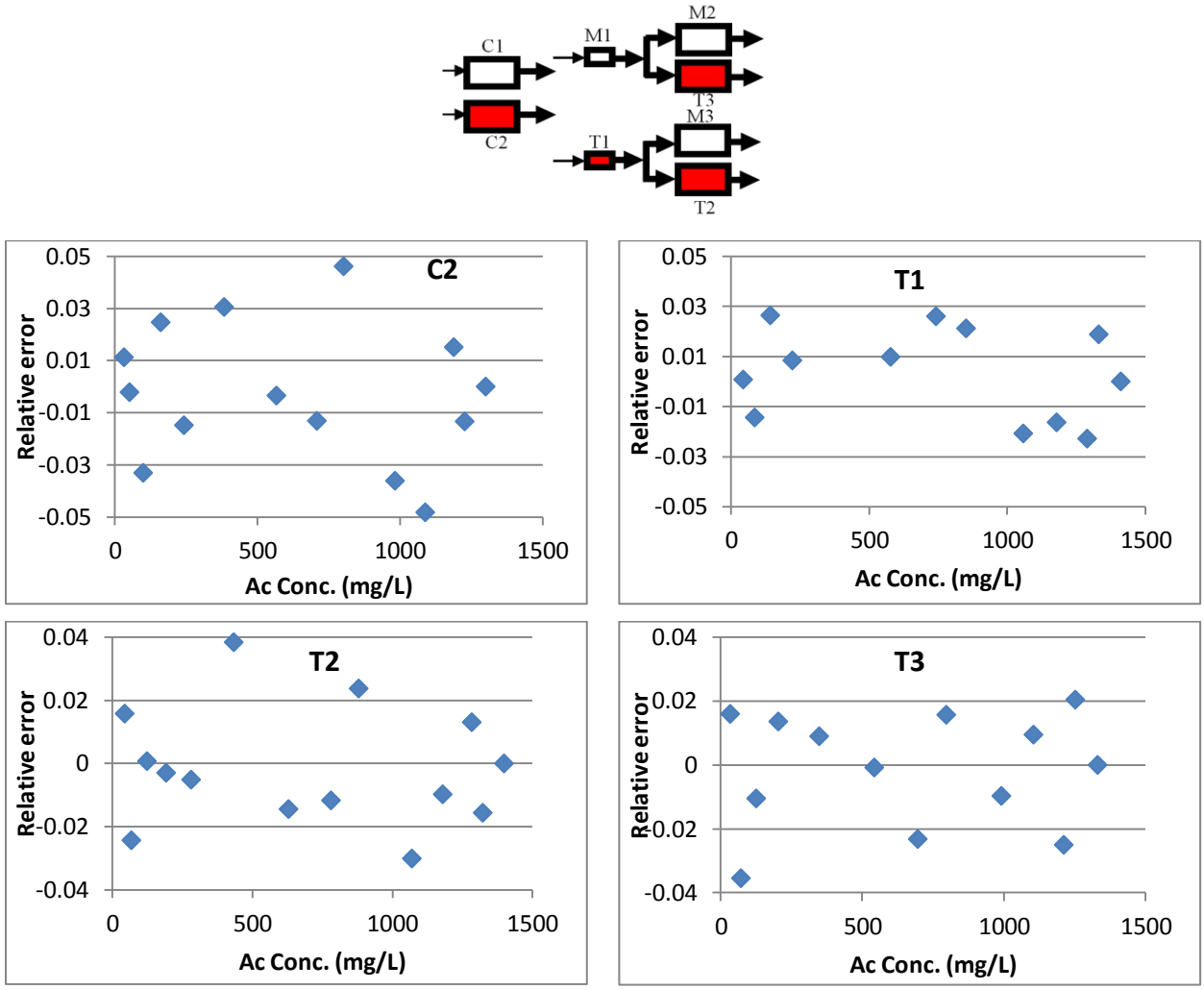


Figure 4.14. Residual (error) vs. predicted acetate concentration for the thermophilic batch experiments

4.3.6 Estimation of parameters of Monod kinetics

Anaerobic digestion models have become a useful tool for understanding the underlying reactions that are being carried out by the complex mixture of microorganisms in this process. However, the values of parameters should be estimated accurately to improve the models prediction (Flotats et al., 2003). Methanogenesis is the last step of anaerobic process that has been extensively studied by researchers (Jeong et al., 2005; Demirel and Scherer, 2008). However, investigation of methanogenesis in the phased anaerobic digestion is still scarce. In this section, the impact of phase separation on the kinetics of the acetoclastic methanogens was studied.

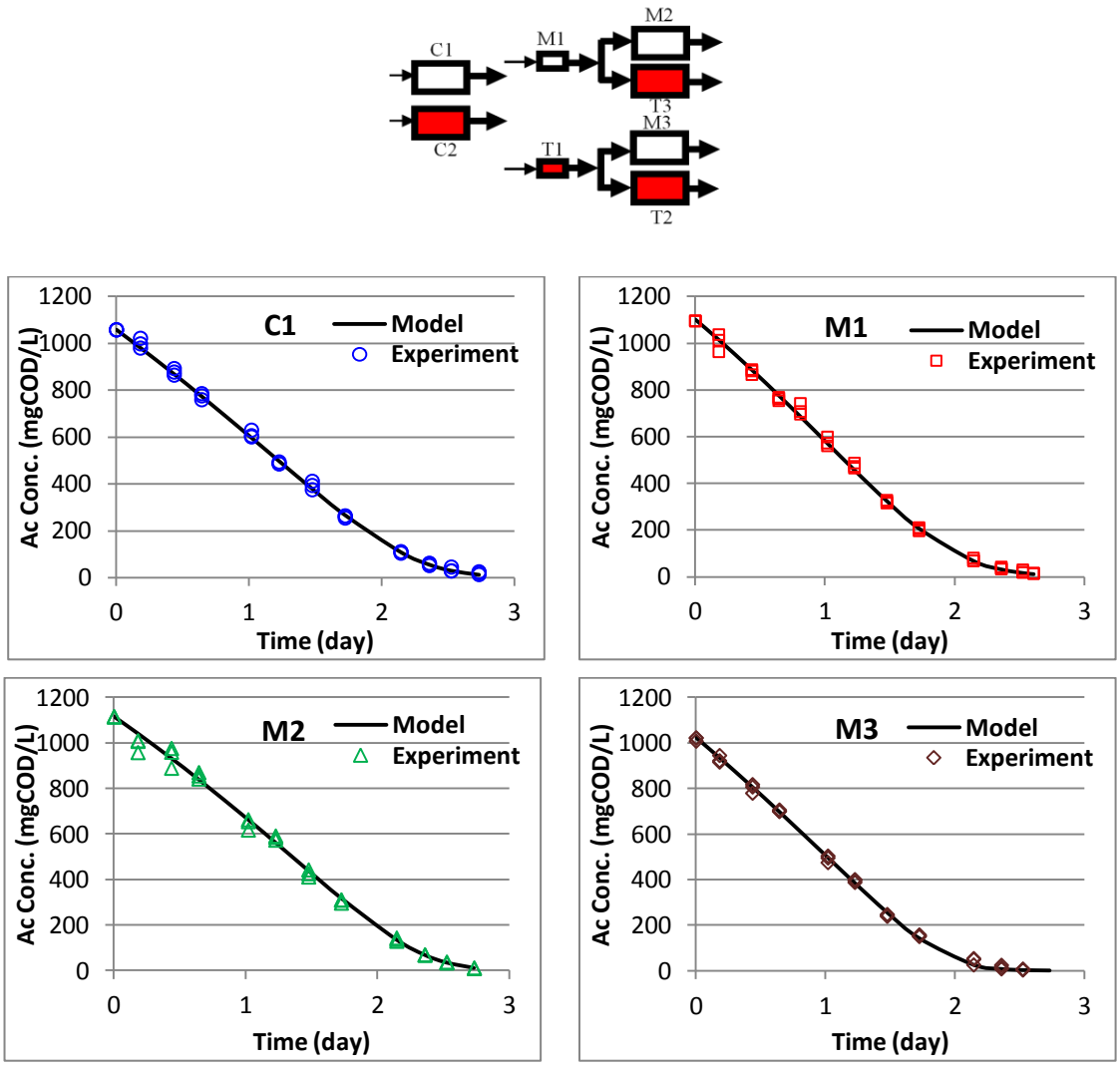


Figure 4.15. Results of the model fit with acetate experimental data: mesophilic batch tests

The Monod coefficients (k_{max} and K_s) and the concentration of acetoclastic organisms (X_o) in the batch tests were estimated by fitting Equation (4-4) to the measured acetate concentrations versus time. Figures 4.15 and 4.16 show the acetate concentrations over time and the fitted Equation (4-4) for the mesophilic and thermophilic batch experiments. Overall, it can be observed that the calibrated model achieved a good fit of the data.

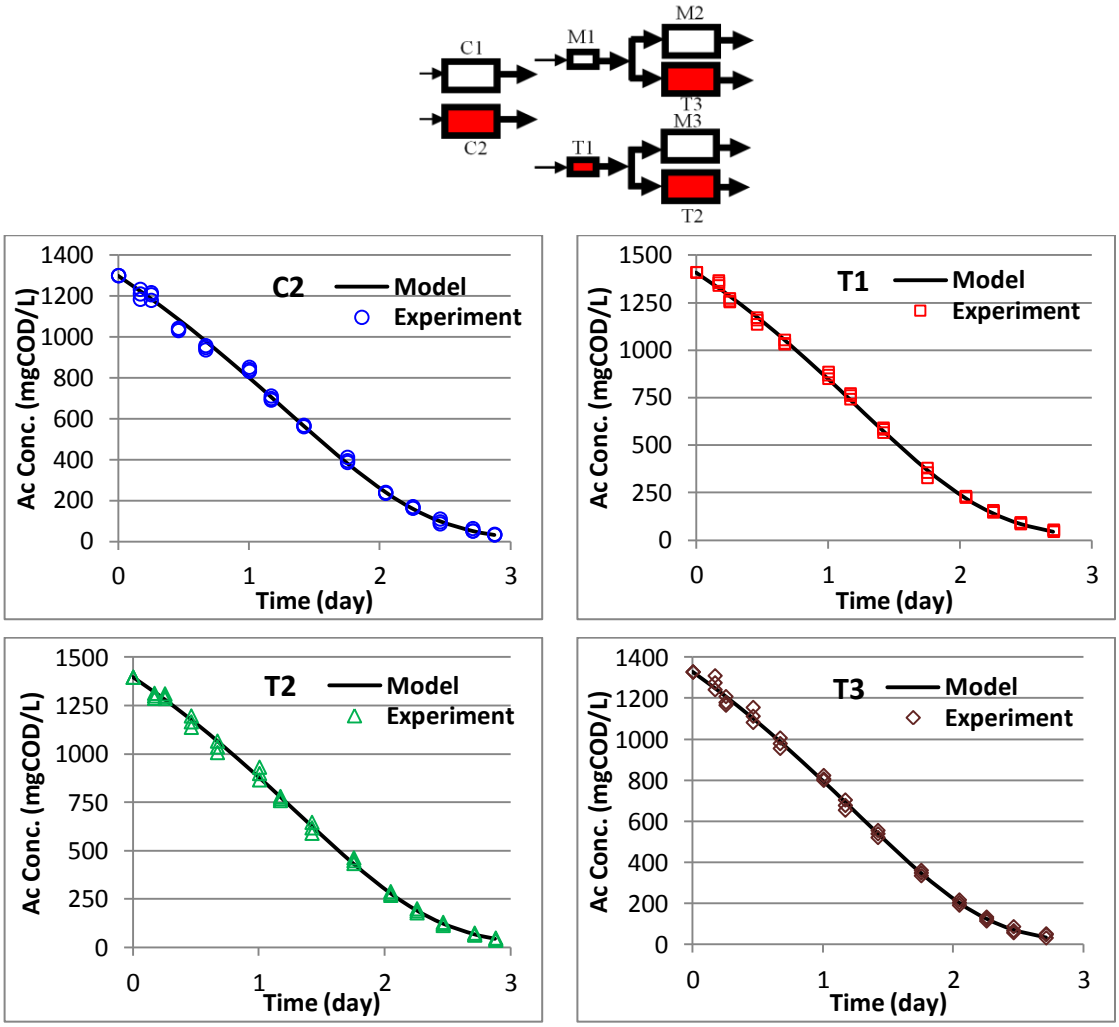


Figure 4.16. Results of model fit with experimental acetate data: thermophilic batch tests

The estimated kinetic parameters (k_{max} and K_s) and linear uncorrelated estimates of the 95% confidence intervals (CI) of these parameters for the single-stage and phased digestion systems are presented in Tables 4.2, 5.3 and 4.4, respectively. Both mesophilic and thermophilic digesters showed larger relative errors in K_s as compared to the values obtained for k_{max} (relative error was calculated as the ratio of standard error of the parameter to the value of the parameter). High uncertainties in estimation of K_s values were also reported by Kalfas et al. (2006) who studied mesophilic and thermophilic anaerobic digesters treating olive pulp. Large confidence intervals related to the estimated kinetic coefficients, especially for K_s, may be attributed to the limited number of samples collected from the batch experiments.

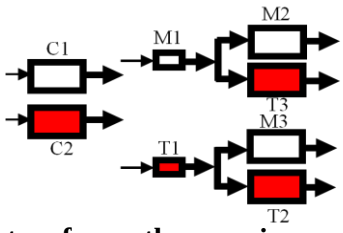


Table 4-2. Estimated parameters for methanogenic populations of single stage digesters

Parameter		C1		C2	
	Unit	Best fit value	CI*	Best fit value	CI
k_{max}	d^{-1}	7.4	6.5-8.3	15.2	13.8-16.6
K_S	mgCOD/L	155	120-190	379	268-489
X_0**	mgCOD/L	649	504-795	400	306-493

*95% linear uncorrelated Confidence Interval; **initial biomass concentration

Table 4-3. Estimated parameters for methanogenic populations of mesophilic phased digesters

Parameter		C1		C2	
	Unit	Best fit value	CI*	Best fit value	CI
k_{max}	d^{-1}	7.4	6.5-8.3	15.2	13.8-16.6
K_S	mgCOD/L	155	120-190	379	268-489
X_0**	mgCOD/L	649	504-795	400	306-493

Table 4-4. Estimated parameters for methanogenic populations of thermophilic phased digesters

Parameter		C1		C2	
	Unit	Best fit value	CI*	Best fit value	CI
k_{max}	d^{-1}	7.4	6.5-8.3	15.2	13.8-16.6
K_S	mgCOD/L	155	120-190	379	268-489
X_0**	mgCOD/L	649	504-795	400	306-493

In general, the accuracy of parameter estimation can be increased through increasing the number of measurements (Guisasola et al., 2006). Moreover, based on the sensitivity analysis of the Monod equation with regards to k_{max} and K_s, previous researchers demonstrated that k_{max} is more easily estimated and always has lower relative error than Ks estimates (Dochain and Vanrolleghem, 2001; Guisasola et al., 2006).

The parameter values that were estimated in this study were compared against literature values to validate the approach which was employed (Table 4.5). From Table 4.5 it can be observed that the

parameters estimated in this study were within the range of the literature values. Hence, when considered together with the previously described statistical assessment of the quality of parameter fits, it was concluded that the approach employed for parameter estimation was valid.

Table 4-5. Summary of kinetic parameters for acetoclastic methanogens from literature

System	Temp. (^{0}C)	HRT (d)	K_s mgCOD/L	k_{max} d^{-1}	Reference
Two-phase*	35	7.2	40	13	Siegrist et al., 2002
	55	6.2	300	52	Siegrist et al., 2002
Single	38	20	160	13	Feng et al., 2006
Single	35	20	310	9.99	Kalfas et al., 2006
	55	20	630	---	Kalfas et al., 2006
Single	35	---	150	8	Batstone et al., 2002
	55	---	300	16	Batstone et al., 2002
Two-phase	35	20	150	9	Blumensaat and Keller, 2005
	55	2-4	400	25	Blumensaat and Keller, 2005

*Parameter coefficients for thermophilic digester were calculated

The 95% confidence regions for the k_{max} and K_s values for the mesophilic digesters are presented in Figure 4.17, with error bars showing the linear uncorrelated error estimates. The regions for the parameters are "true" confidence spaces which are considered preferable to the linear confidence intervals (Batstone et al., 2003). Based on a comparison of the confidence regions for the bio-kinetic parameters (k_{max} and K_s) estimated for the mesophilic digesters, it was found that the parameter set for the M1 digester was different from the ones obtained for the M2, M3 and C1 digesters. These results indicated that the methanogenic culture in the M1 digester was functionally different from that of the other digesters that were all operated at a lower loading rate. These results agree with the evidence presented in the literature indicating the presence of biokinetically-different acetoclastic methanogens in sludge digesters. The presence of different acetoclastic methanogens in mesophilic digesters has been addressed previously in many studies (Raskin et al., 1995; McHugh et al., 2003; Conklin et al., 2006; Vavalin et al., 2008; Karakat et al., 2010). Two genera of acetoclastic methanogens, *Methanosaeta sp.* with a low k_{max} and K_s that dominate at low concentrations of acetate (less than 2mM), and *Methanosarcina sp.* with a high k_{max} and K_s that proliferate at high concentrations of acetate have been reported (Gujer and Zehnder, 1983). In addition, a study by Raskin et al., (1995), reported that the dominant methanogenic population in the 2nd-phase of a two phase digestion system was different from those in the 1st-phase digester.

The acetate concentrations in the 1st- and 2nd-stage of the phased digestion systems further support the presence of fast growing *Methanosarcina spp.* in the 1st-stage and slow growing *Methanosaeta spp.* in the 2nd-stage digesters (Table 4.1). *Methanosarcina spp.* generally have greater specific growth rates (μ_{max}) than *Methanosaeta spp.* (Min and Zinder, 1989), so this allows them to be more competitive at high concentration of acetate. In addition, the low HRT of the 1st-phase digesters was challenging for the slow growing methanogens to be kept in these digesters. On the other hand, at low concentrations of acetate, competition is based on K_s values and *Methanosaeta spp.* with higher affinities (lower K_s) would dominate in the digesters.

The 95% confidence regions for k_{max} and K_s values for the thermophilic digesters are presented in Figure 4.18, with error bars showing the linear uncorrelated error estimates. A similar approach was used to compare the parameter sets obtained for the thermophilic acetate utilization in the thermophilic digesters. It was found that the confidence regions of the T2, T3 and C2 digesters overlapped but were different from the T1 digester. As with the results from the mesophilic digesters, it would appear that the first phase digester of the thermophilic digesters had a methanogenic biomass that was functionally different from that of the lower loaded digesters. Similar to the presence of two kinetically-different genera of mesophilic acetoclastic methanogens, previous studies have indicated the existence of thermophilic genera of *Methanosarcina* and *Methanosaeta* in anaerobic environments depending on the acetate concentration. For example, Zinder et al. (1984) reported the proliferation of *Methanosarcina spp.* during the start-up of a thermophilic anaerobic digester with acetate concentrations of 2-5 mM (120-300 mg/L); whereas, *Methanosaeta spp.* dominated the system when acetate concentrations were 0.3 to 1.5 mM (30-90 mg/L). Similar results have been reported by several other researchers as (Touzel et al., 1985; Zinder et al., 1987; Min and Zinder, 1989).

The higher acetate concentration in the T1 digester compared to the T2 and T3 digesters supported the bio-kinetic parameters estimated for the thermophilic digesters (Table 4.1). As explained for the mesophilic digester, the slow-growing methanogens with high affinities for acetate (low K_s) would be favoured under low acetate concentrations (i.e., the T2 and T3 digesters); while the fast growing methanogens with higher μmax would dominate at the T1 digester with high concentrations of acetate.

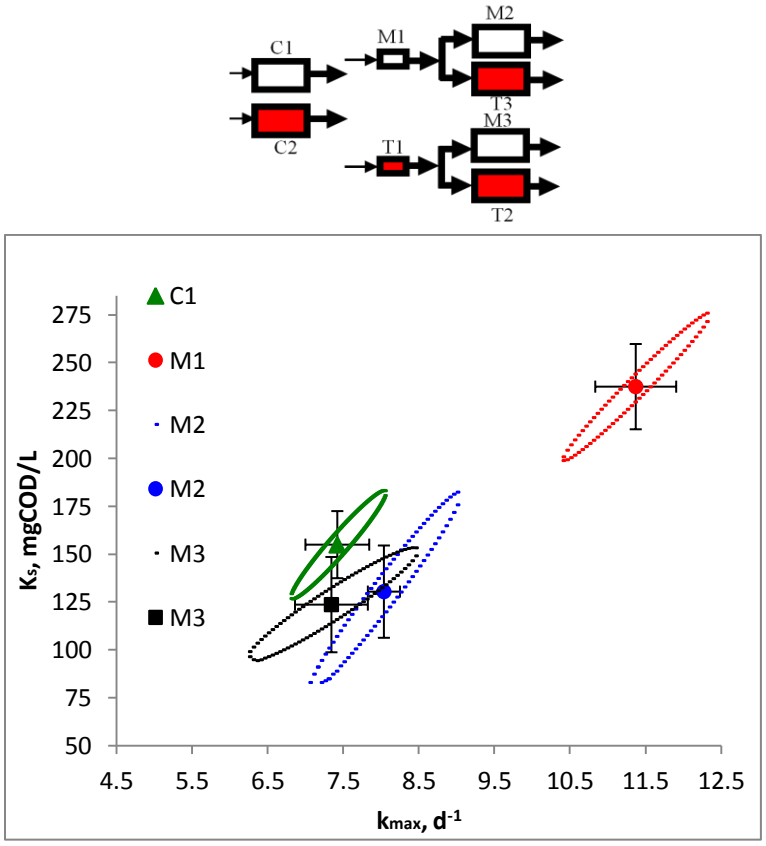


Figure 4.17. 95% confidence regions for methanogen kinetics in mesophilic digesters

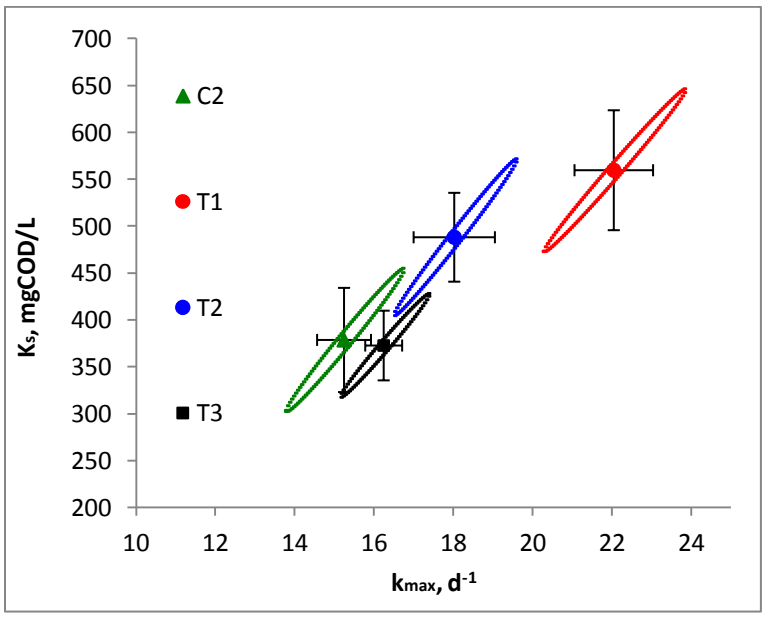


Figure 4.18. 95% confidence regions for methanogens in thermophilic digesters

On the basis of the higher affinity of mesophilic acetoclastic methanogens for acetate as reflected in their lower Ks values, it is recommended that a mesophilic digester should be employed after either thermophilic or mesophilic heavily loaded 1st-phase digesters to achieve reasonably low concentrations of acetate in the final effluent. Achieving very low concentrations of acetate with thermophilic digesters (either phased or single-stage) would be challenging due to the lower affinity of the thermophile methanogens in the completely-mixed anaerobic digesters.

4.3.7 Effect of temperature on bio-kinetic parameters

Temperature can influence bio-kinetic coefficients of biochemical reactions, resulting in increased rates of degradation of organic matter in the anaerobic digestion process. In modeling of anaerobic digesters in general and temperature phased anaerobic digestion systems in particular, an Arrhenius-type relationship (Equation 4-19) has typically been employed for describing the temperature dependency of kinetic parameters (Siegrist et al., 2002; Batstone et al., 2002; Efstathiou et al., 2003).

$$K_2 = K_1 * EXP(\theta * (T2 - T1)) \qquad (4\text{-}19)$$

Where, K_2 is coefficient at target temperature; K_1 is known coefficient; θ temperature coefficient; T temperature.

In this study the Monod parameters of the mesophilic digesters were compared with those of the thermophilic digesters to assess the impact of temperature on the rate constants and to evaluate the application of an Arrhenius-type equation for modeling of phased digestion systems. A comparison of the bio-kinetic parameters obtained for the mesophilic and thermophilic conditions showed that both the k_{max} and K_s values at the thermophilic temperature were greater than those of the mesophilic digesters. This observation generally agreed with the literature, reflecting greater values of kinetic coefficients of acetoclastic methanogens at elevated temperatures (Kenealy and Zeikus, 1982; Zinder et al., 1984; Kalfas et al., 2006).

As it was previously demonstrated that biokinetics of the first phase digesters (i.e., M1 and T1) were significantly different from the other configurations, the constants from this pairing of digesters were compared. The values from Tables 4.2-4.4 were employed to calculate ratios of the thermophilic parameters to the mesophilic ones and it was found that the values of k_{max} and K_s for the T1 digester were 1.93 and 2.35 times greater than the values obtained for the M1 digester. A similar analysis of the remaining digesters (T2, T3 and C2) exhibited a similar influence of temperature on the kinetic parameters. The values of k_{max} and K_s were, on average, 2.21 and 3.03 times higher in the thermophilic

digesters than the mesophilic ones. Accordingly, the temperature correction coefficients (θ) calculated for k_{max} and K_s for the first-phase digesters (T1 vs. M1) were, respectively, 0.0331±0.0002 and 0.0428±0.003 which, with the exception of temperature coefficient for K_s, the values were close to the temperature correction coefficient (0.0346) suggested for ADM1 (Batstone et al., 2002). The temperature correction coefficients for the other digesters were calculated as 0.0387±0.0005 for k_{max} and 0.0554±0.004 for K_s. A comparison of these values with the value suggested for ADM1 showed that only the temperature coefficient for K_s was significantly different from that of ADM1. Thus, the application of the ADM1 value did not lead to significantly different k_{max} values as compared to the values estimated in this study. As such, it may be concluded that the application of Arrhenius-like equations for temperature correction of k_{max} values would be applicable in digesters with relatively similar organic loading or residence times; however, as the k_{max} and K_s values are typically highly correlated use of Arrhenius-type relationships should be employed with caution.

4.3.8 Initial concentrations of acetoclastic methanogens

The values of initial biomass concentration (X_0) that were estimated from the batch tests were corrected for dilution to calculate the values in the source digesters. The concentrations of acetoclastic methanogens that were estimated from the parameter estimation exercise are reported in Tables 4.2-4.4. From these tables it can be seen that the concentrations present in the first phase of the phased digestion systems (M1 and T1) were consistently less than those observed in the second phase of the phased systems and the single digester systems. However, based on the analysis of the calculated confidence intervals for the biomass concentrations, this difference was only significant in the mesophilic digesters. The reduced concentrations of acetoclastic methanogens in the first phase digesters were consistent with the elevated volatile fatty acid concentrations in the source digesters. It is apparent that the relatively short hydraulic residence time in the digesters (3.5 days) limited the establishment of the methanogen populations in these digesters. In addition, the concentrations of acetoclastic methanogens in the thermophilic digesters were in general lower than the corresponding thermophilic digesters. This was likely due to the higher decay rates reported for acetoclastic methanogens (Siegrist et al., 2002).

It has been suggested that methanogens roughly comprise 1-10% of total biomass (based on COD) in typical anaerobic sludge digesters (Munch et al., 1999a; Efstathiou et al., 2003). Based on the total COD of the source digesters and estimated acetoclastic methanogen populations, overall the methanogen fractions were within the range reported. This fraction for the M1 digester was 1.02% and for the rest of the mesophilic digesters ranged between 2.5-2.9%. The methanogen biomass fractions for the

T2, T3 and C2 digesters were, on average, 1.4%; whereas, this value for the T1 digester was calculated as 0.69% which was lower than the range reported.

It should be pointed out that the concentration of the methanogen population estimated in this study were approximate in nature and should be considered as a relative estimates because a high correlation (> 80%) was found between the Monod kinetic parameters, especially k_{max}, and initial biomass concentration. However, as the correlation existed in the parameter estimation process of all the batch experiments, the estimated initial biomass concentrations should relatively reflect the methanogen populations in the source digesters.

4.3.9 Comparison of specific growth rates

An analysis of the k_{max} and K_s values collectively revealed that in all cases the magnitude of the parameters varied together (i.e. k_{max} increased when K_s increased) and a high correlation existed between these parameters. Many authors have reported a high correlation between k_{max} and K_s in their investigations (Merkel et al., 1996; Batstone et al., 2003; Batstone et al., 2004; Kalfas et al., 2006). Both k_{max} and K_s interact in the calculation of the specific growth rate of methanogens and the extent of the interaction depends upon substrate concentration. Hence, to better assess the importance of the differences between the sets of parameter values on the specific growth rate of organisms in the various digesters operated in this study the mean values of the estimated parameter pairs were employed to estimate specific growth rates over a range of acetate concentrations (Equation 4-20). The mesophilic and thermophilic responses are presented in Figures 4.19 and 4.20 respectively.

$$\mu = Y k_{max} \frac{S}{K_s + S} \qquad (4\text{-}20)$$

Where, μ is specific growth rate; Y is yield coefficient assumed as 0.035 (Siegrist et al., 2002).

From Figure 4.19 it can be seen that under mesophilic conditions, when the acetate concentration was greater than 200-250 mg/L as COD, the specific growth rate in the M1 digester was distinctly greater than those of the other digesters (i.e., M2 and M3). The measured acetate concentrations in the source digester (M1) were in the range of 338±48 mg/L (Table 1) and hence the differing bio-kinetic parameters of the biomass coincided with the elevated substrate concentrations. This supported the hypothesis of a shift in the composition of the acetoclastic methanogens at the elevated concentrations of acetate in the heavily loaded M1 digester. The second-phase of the mesophilic-mesophilic digester (M2 digester) also demonstrated a higher specific growth rate than those of the M3 and C1 digesters, although these bio-kinetic parameters had been found to be not statistically different. These findings were consistent with the

relatively lower acetate concentrations in the M2, M3 and C1 digesters that were less than 60 mg/L (Table 4.1).

Figure 4.20 illustrates that under thermophilic conditions the dependence of the specific growth rates of the acetoclastic methanogens on acetate concentrations followed a similar trend to that of the mesophilic digesters. Once again the specific growth rate for the biomass from the T1 digester was higher than those of the other digesters when the acetate concentration exceeded 500 mg/L and this was consistent with the higher acetate concentrations in the source digester (Table 4.1). The measured acetate concentrations in the effluents of the other thermophilic digesters (T2, T3 and C2), were not significantly different (ANOVA PostHoc, α=0.05). Accordingly, it was inferred that a different methanogenic biomass would not be expected in these digesters.

As described above, an interesting observation was found when the specific growth rate was calculated under different acetate concentrations using the estimated kinetic parameters (k_{max} and K_s) for the second-phases of the phased digesters (i.e., M2 and T2) operated at a similar temperature (M1M2 and T1T2 digestion systems). When acetate concentrations exceeded about 250 mg/L and 750 mg/L in the M2 and T2 digesters, respectively, the specific growth rates differed from the other mesophilic (M3 and C1) and thermophilic digesters (T3 and C2). Although these concentrations were greater than the values measured in the digesters, it would be inferred that continuous transfer of fast-growing acetoclastic methanogens from the first-phases of similar temperatures contributed to a relatively different values of the kinetic parameters. However, this inference needs to be further investigated to distinguish the relative abundance of the fast- and slow-growing methanogens through application of semi-quantitative molecular techniques.

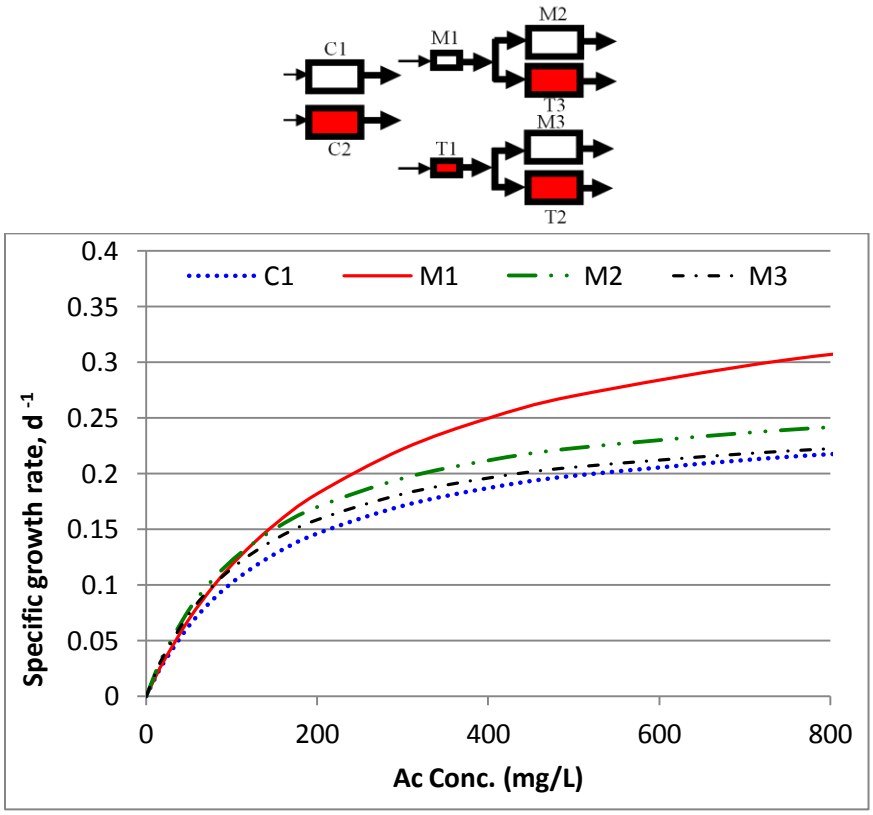


Figure 4.19. Specific growth rate vs. acetate concentration based on the kinetic parameters estimated for mesophilic digesters.

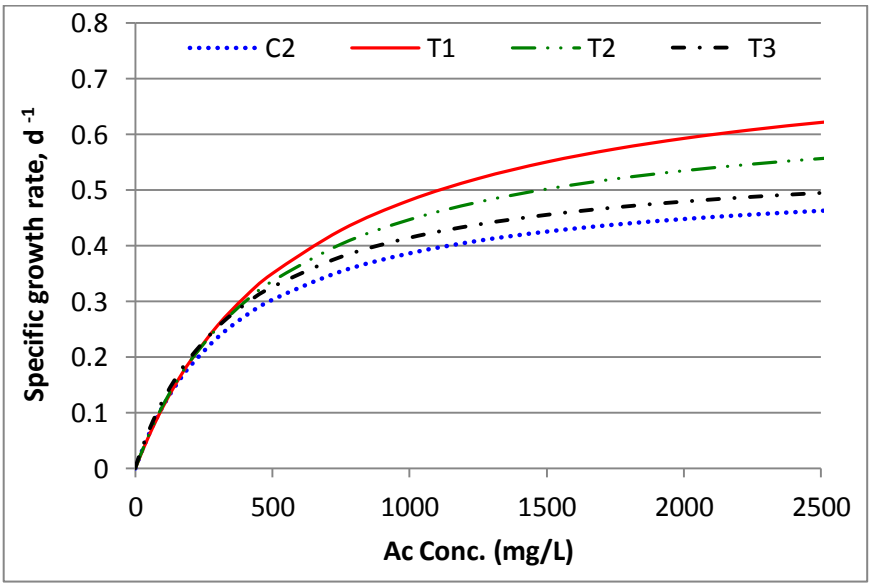


Figure 4.20. Specific growth rate vs. acetate concentration based on the kinetic parameters estimated for thermophilic digesters.

4.3.10 Simulation of methane in batch experiments

During the batch experiments, methane gas was measured in the bottles in addition to the measurement of acetate concentrations. In order to verify the estimated bio-kinetic parameters, the methane data was simulated as a second response using the estimated parameters. The Aquasim software was used for the simulation and a simple model was employed including uptake of acetate and production of methane (Figure 4.21). Methane gas as one of the main end products of anaerobic digestion process is produced through conversion of acetate by acetoclastic methanogens as well as H_2 utilization by hydrogenotrophic methanogens. However, the former pathway is the major pathway and typically constitutes 70% of methane produced (Jeris and McCarty, 1965; Smith and Mah, 1966; Gujer and Zehnder, 1983) and was used for simulation of methane production in the batch tests.

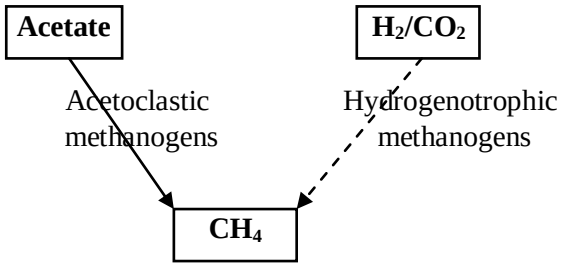


Figure 4.21. Simple model showing methane production from acetate and H_2

The process rate and the corresponding stoichiometry for the acetoclastic methanogenesis process are given in Table 4.6. A Monod-type model was used to describe the methanogenesis process. The temperature-correction for the Henry's Law constant was implemented according to the ADM1 manual (Batstone et al., 2002). The pH measured in the bottles was between 7 and 8, and hence no pH inhibition term was implemented in the rate equation.

Table 4-6. Stoichiometry and kinetic expression for acetoclastic methanogenesis

Process	S_{ac}	S_{CH4}	S_{IN}	X_{ac}	rate
Acetate uptake	-1	$1-Y_{ac}$	$-N_{bio}(Y_{ac})$	Y_{ac}	$k_{max}X_{ac}\frac{S_{ac}}{K_{s,ac}+S_{ac}}I_{IN,lim}$

S_{ac}, S_{CH4}, S_{IN} and X_{ac} represent acetate, methane, inorganic nitrogen and biomass, respectively. N_{bio} and Y_{ac} are nitrogen content of biomass and yield coefficient for acetoclastic methanogens, respectively. During acetoclastic methanogenesis, acetate is utilized partly for biomass production and cell maintenance (Y_{ac}) and the rest is converted to methane gas ($1-Y_{ac}$).

The simulation results for acetate utilization and methane production are presented in Figure 4.22 for the mesophilic bottles and Figure 4.23 for the thermophilic bottles. Both responses were found to be well fit by the Monod-type kinetics. In addition to methane concentration, methane mass in the bottles was used in the simulation exercise. Initial trials to fit the experimental and model data resulted in consistent underestimation of methane production in the bottles. Then, the methane produced in the bottles with acetate was subtracted from the values obtained for the control bottles (without acetate). This resulted in improved simulation of methane data. The lack of good fit was likely due to the decay products generated during the batch experiments and resulted in extra acetate and/or H_2 as extra source for methane generation. However, the produced acetate due to the biomass decay did not appear in the acetate data as the model simulated the acetate data very well. PCOD data indicated some degradation of biomass within the bottles. Some hydrogen production may be expected from biomass degradation. Hence, a fraction of methane gas may be routed from the activity of hydrogenotrophic methanogens. Although negligible, it may result in deviation of methane production by the model. Thus, implementation of acetoclastic and hydrogenotrophic methanogens in a combined model would improve methane prediction. However, the kinetics of the hydrogenotrophic methanogens were not addressed in this study.

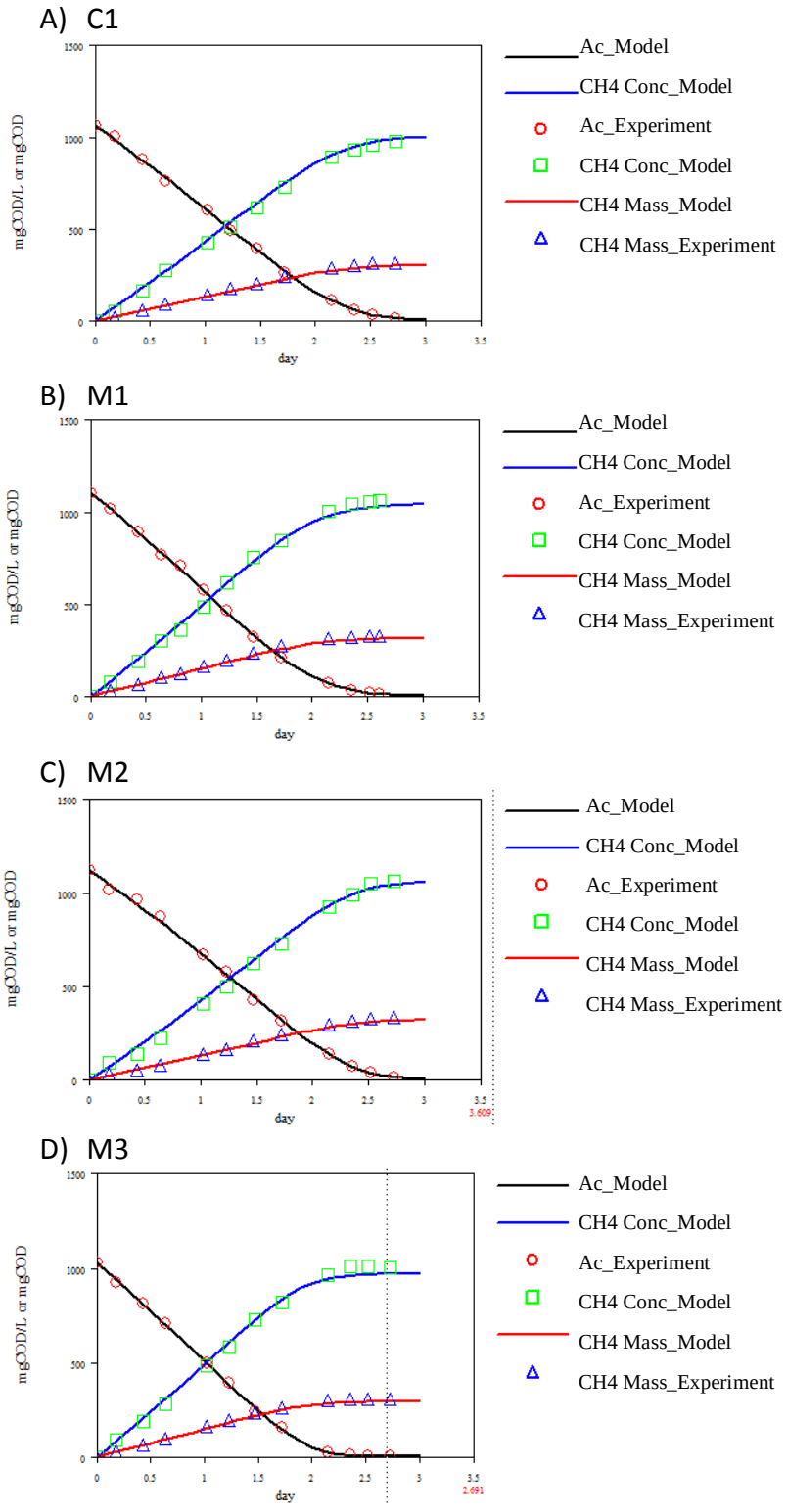


Figure 4.22. Simulation results for acetate degradation in mesophilic batch experiments

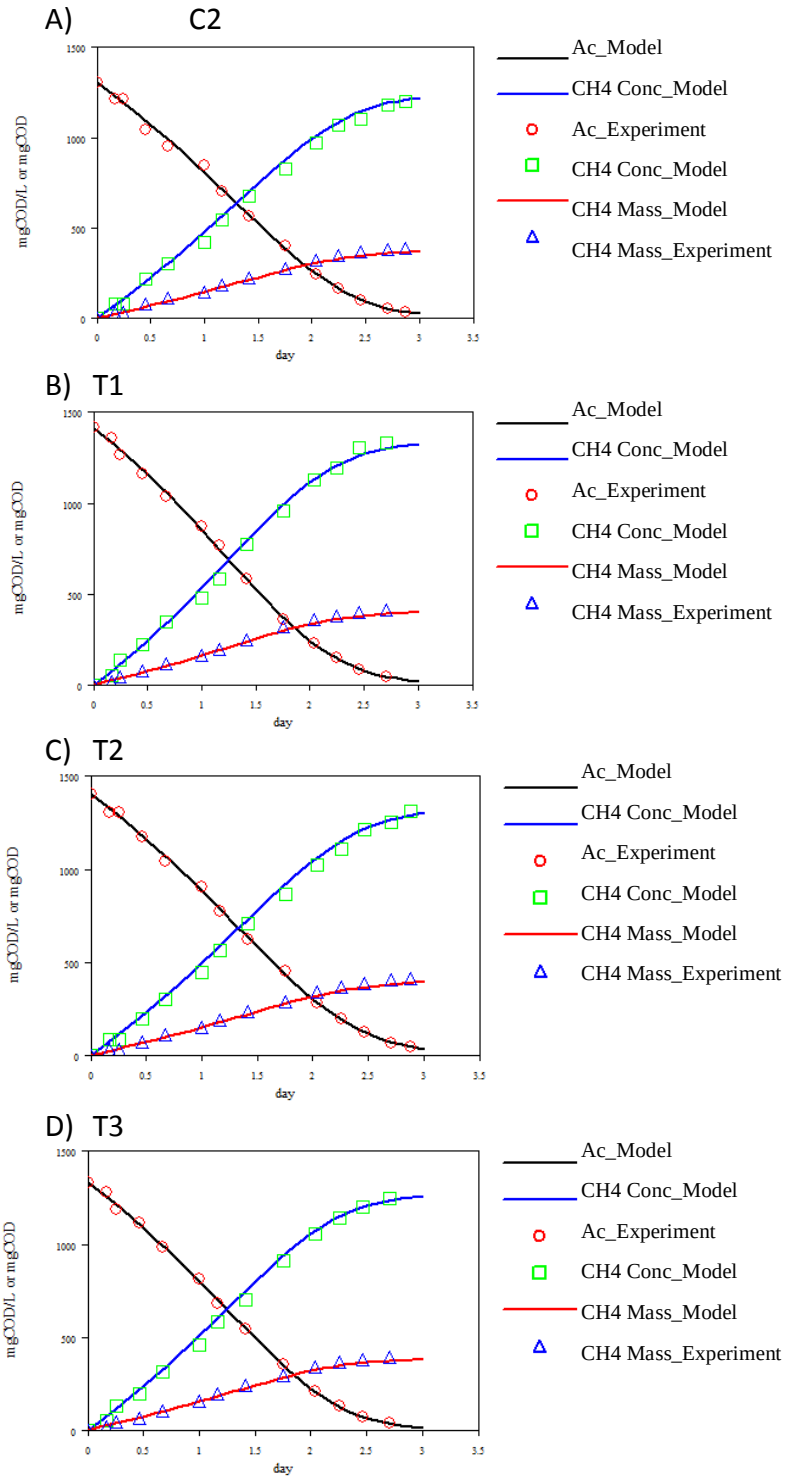


Figure 4.23. Simulation results for acetate degradation in thermophilic batch experiments

4.4 Conclusions

Based on the results of this study, the following conclusions were drawn:

The operation of phased anaerobic digestion systems with a short HRT (3.5 days) followed by longer HRT digesters (14 days) influenced the bio-kinetic parameters of acetoclastic methanogens in each phase. The short HRT and heavily loaded first-phase digesters with either thermophilic or mesophilic temperatures encouraged the development of fast-growing methanogens with high k_{max} (11.37 and 22.04 d^{-1} for mesophilic and thermophilic digesters) and K_s (237.6 and 559.7 mgCOD/L for mesophilic and thermophilic digesters) values. On the other hand, longer HRTs digesters (second-phases and single-stage) resulted in significantly lower bio-kinetic coefficients of k_{max} (on average 7.6 and 16.6 d^{-1} for mesophilic and thermophilic digesters) and K_s (136 and 413 mgCOD/L for mesophilic and thermophilic digesters). These results indicated the dominance of a different methanogenic population in each phase depending on the organic loading.

The results of this study indicate that two distinct species of acetoclastic methanogens should be employed for modeling phased anaerobic digesters operated in a different HRT. The shift between methanogen populations with acetate concentration (and/or organic loading rates) could be implemented through the application of two different sets of kinetic parameters for the two species.

An analysis of the bio-kinetic parameters estimated for the mesophilic and thermophilic temperatures demonstrated a relationship between the bio-kinetic parameters of the acetoclastic methanogens and temperature. Therefore, in case of a differing temperature in each phase, a temperature-correction relationship such as Arrhenius-type equation would be applied for the temperature dependency of parameters when the digesters were operated with similar organic loading or HRT.

On the basis of the higher affinity of mesophile acetoclastic methanogens for acetate as reflected in their lower Ks values, it is recommended that a mesophilic digester should be employed after either thermophilic or mesophilic heavily loaded 1^{st}-phase digesters to achieve reasonably low concentrations of acetate in the final effluent. Achieving very low concentrations of acetate with thermophilic digesters (either phased or single-stage) would be challenging due to the lower affinity of the thermophile methanogens in the completely-mixed anaerobic digesters.

Chapter 5
Characterization of Propionate Utilizing Bacteria in Phased Anaerobic Sludge Digestion Systems

5.1 Introduction

Anaerobic digestion of sewage sludge proceeds through a series of microbially-mediated reactions, involving several groups of microorganisms. Following hydrolysis and solubilisation of particulate matter, complex volatile fatty acids are formed, which are in turn converted to acetate and hydrogen as the main methanogenic substrates. Among the volatile fatty acids, propionate is an important intermediate in the anaerobic digestion process (Kaspar and Wuhrmann, 1978a; de Bok et al., 2004). Wiegant et al. (1986) reported propionate as the major VFA in the effluent of an anaerobic digestion system treating vinasse waste, comprising up to 35% and 66% of the COD at 35 and 55 ^{0}C, respectively.

Propionate degradation is very sensitive to operating conditions due to its highly endergonic reaction. Several researchers have indicated that following a perturbation to anaerobic digesters, acetate and propionate accumulated to higher levels as compared to the other VFAs measured; however, it was propionate which degraded more slowly and took longer to return to the level before disturbance (Nielsen et al., 2007; Gallert and Winter, 2008).

In contrast to methanogens, less attention has been paid to the ecology and functional diversity of propionate oxidizing bacteria (POB) involved in the anaerobic digestion of sludge. Among the limited studies on POB, almost all of them have focused on either granular sludge systems (Harmsen et al., 1996; Sekiguchi et al., 1999; Heppner et al., 1992) or single-stage anaerobic digesters (Ariesyady et al., 2007; McMahon et al., 2004; Nielsen et al., 2008) with very long residence times (SRT >20-d).

Phased anaerobic digestion is a promising technology for the treatment of wastewater sludges. The phases may be operated at either identical (e.g., mesophilic-mesophilic) or different (e.g., thermophilic-mesophilic) temperatures. Typically a short residence time (< 5-d) with high organic loading is employed for the 1st- phase, primarily for hydrolysis and acidogenesis of particulate organic matter (Dichtl, 1997; Schmit and Ellis, 2001); while, a longer residence time second phase (>10-d) is provided to achieve further hydrolysis, acidogenesis, and methanogenesis (Han and Dague, 1997). It has been

hypothesized that the differences in loading might result in the selection of bio-kinetically-different species of the microorganisms (especially acetogens and methanogens) in each phase (Ghosh, 1987).

Some researchers have reported differences in the dominance of POB depending on the propionate concentrations in digesters (Heyes and Hall, 1983; Lueders et al., 2004b; Ariesyady et al., 2007; Shigematsu et al., 2006). The prevalence of biokinetically-different species of POB is important in modeling of phased anaerobic digesters as kinetic limitations can impact their performance. To this point modeling of phased anaerobic digestion systems has not considered the potential for development of POB species that have differing growth characteristics in the separate phases. In addition, modeling of phased anaerobic digesters with different temperatures in each phase is usually performed using one set of bio-kinetic parameters for one phase and correcting the parameters for the other phase by implementing temperature correction equations (Siegrist et al., 2002). This may be applicable when both digesters are operating at long HRTs; however simulation accuracy may be reduced when this approach is employed for phased digesters with different operational conditions (e.g., HRT or organic loading) in each phase.

The aim of the present study was to investigate the activity of propionate oxidizing bacteria (POB) through the estimation of their kinetic parameters (k_{max} and K_s) in various temperature configurations of phased anaerobic digestion systems. In this regard, the influence of phase separation on the dominance of the POB population in each phase of the anaerobic digesters was studied. For comparison, the Monod bio-kinetic parameters were estimated in mesophilic and thermophilic single-stage digesters. In addition, the application of Arrhenius-type relationships for adjustment of the coefficients for modeling of digesters with different temperatures was investigated.

5.2 Approach

5.2.1 Batch experiments on acetate degradation

This section introduces the experiments carried out to study the influence of phase separation on the kinetics and activity of propionate oxidizing bacteria (POB). The batch experiments were relatively similar to the experiments explained in Chapter 5 with some modifications. Therefore, only the preparation of the bottle tests is briefly explained below.

The biomass developed in the source digesters was used as an inoculum for the batch experiments. Following achievement of steady-state in the digesters, a series of batch experiments were conducted off-line to study the impact of the digester conditions on the kinetic characteristics of the propionate oxidizing bacteria (POB). Active biomass was taken from the digesters and diluted to

minimize the background level of propionate and was then used as inoculum in the batch experiments. Sodium propionate was added as the sole carbon and energy source in the batch tests.

Three 500-mL serum bottles for each digester were dosed with sodium propionate to a final concentration of about 1200 mg/L as COD for the bottles kept at the mesophilic temperature and about 1900 mg/L as COD for the bottles kept at the thermophilic temperature. Mineral medium was supplemented to the bottles as described by Angelidaki et al. (1990). Each bottle was fed with 250 mL of diluted inoculum and 50 mL of mineral solution. All the bottles were purged with nitrogen gas and kept at the same temperature from which the inoculum originated. The serum bottles were sealed by butyl rubber stoppers and aluminum caps.

Before sealing the bottles, 5 mL was taken from each bottle to measure the COD and VSS of the mixture at the beginning of the test to verify that all of the bottles at a given temperature received similar amounts of biomass. The pH and VSS concentration of the bottles were also measured at the end of the tests. To measure the initial propionate concentration, 2 mL of liquid sample was taken by a syringe with a #18G needle. The evolution of propionate, TCOD, VSS and sCOD were monitored by taking 3 mL liquid samples over the course of the experiments. A fraction of the samples was centrifuged, filtered by 0.45 µm syringe filters and kept at -20 ^{0}C until analysis was conducted by liquid chromatography for propionate and acetate concentrations. The remaining portion was used to determine the COD fractions. In addition, the biogas generated was monitored at the same time the liquid samples were taken. The methane content was determined whenever the biogas was measured. A 1 mL gas sample was taken from the head space of the serum bottles and analyzed by gas chromatography. The experiments were carried out in triplicate and an extra bottle with no propionate was prepared to monitor the biomass degradation.

5.2.2 Modeling of Batch Experiments and Parameter Estimation

The parameter estimation exercise, model selection, kinetic model and residual analysis were similar to those conducted for acetoclastic methanogens (Chapter 4).The data sets describing propionate concentrations in the three replicate bottles were pooled and one set of biokinetic parameters was estimated in the calibration exercise.

5.3 Results and Discussion

5.3.1 Performance of source digesters

The composition of the contents of the source digesters employed in this study varied considerably due to the differing HRTs and temperatures. Characterization of the digester contents provided insight into the conditions that resulted in the establishment of the cultures that were kinetically assessed in the batch tests. The concentrations of propionate and acetate in the digesters (Table 5.1) were considered to be particularly important in this regard in that propionate is the substrate for POBs while acetate is one of the main by-products of propionate oxidation. Based on the stoichiometry provided in the ADM 1 manual (Batstone et al., 2002), 55% of the propionate is converted to acetate.

From Table 5.1 it can be observed that the propionate concentrations were greater in M1 and T1 as compared to the other relevant mesophilic and thermophilic digesters, whereas the remaining digesters at the same temperature (either mesophilic or thermophilic) had similar concentrations. Statistical analysis confirmed these observations using an ANOVA PostHoc test (α=0.05). The greater concentrations of propionate in the M1 and T1 source digesters would encourage the dominance of POB that are more competitive under conditions of high substrate availability. It can also be observed that the concentrations of acetate were higher in the first phase digesters. As shown in Chapter 4 it was found that the methanogenic cultures in these reactors had elevated values of both k_{max} and K_s and these bio-kinetics led to elevated acetate concentrations.

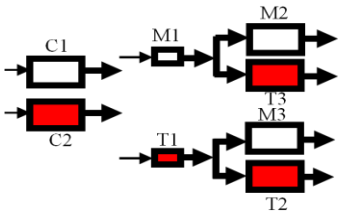


Table 5-1. Propionate and acetate concentrations in source digesters

Digester	Propionate Concentration, mgCOD/L		Acetate Concentration, mgCOD/L	
	Mean	SD*	Mean	SD*
C1	23.7	5.8	53.6	13.1
M1	219	16.9	338	48.1
M2	28.5	13.5	42.7	8.4
M3	19.0	6.9	34.0	10.0
C2	223.3	27.5	139.0	30.6
T1	1220	66.6	872.7	83.8
T2	309.4	40.6	125.2	17.2
T3	206.8	32.1	184.5	28.4

*Standard Deviation;

5.3.2 VSS and pH in Batch Experiments

Since pH influences the growth rates of propionate oxidizing bacteria (POB), an inhibition term is typically implemented in modeling of the acetogenesis process (Batstone et al., 2002; Siegrist et al., 2002). Therefore, the pH of the bottles was measured at the end of the experiments to determine whether the pH might have affected propionate degradation. As shown in Figure 5.1, the pH values were in the range 7.0-8.4 which were within the pH optima reported for propionate degraders (Chen et al., 2005; Boone and Xun, 1987). Overall it was concluded that pH should not have had any inhibiting effects on the growth rates of the propionate oxidizing bacteria in the bottles.

As discussed in Chapter 4, the substrate to biomass ratio can influence the results of parameter estimation with Monod kinetics as it may cause a high correlation between parameters (Simkins and Alexander, 1985; Liu and Zachara, 2001). The substrate to biomass values suggested in the literature were taken into account in preparation of the bottles. In addition, based on the VSS concentrations of the source digesters, the inocula taken from the source digesters were diluted to obtain an approximately equal amount of biomass in the bottles of similar temperature. This would ensure that the substrate to biomass ratio to be approximately equal in the bottles of the same temperature. In addition, the concentrations of volatile suspended solids were determined at the start and end of the batch tests to assess if there were any significant changes in the biomass.

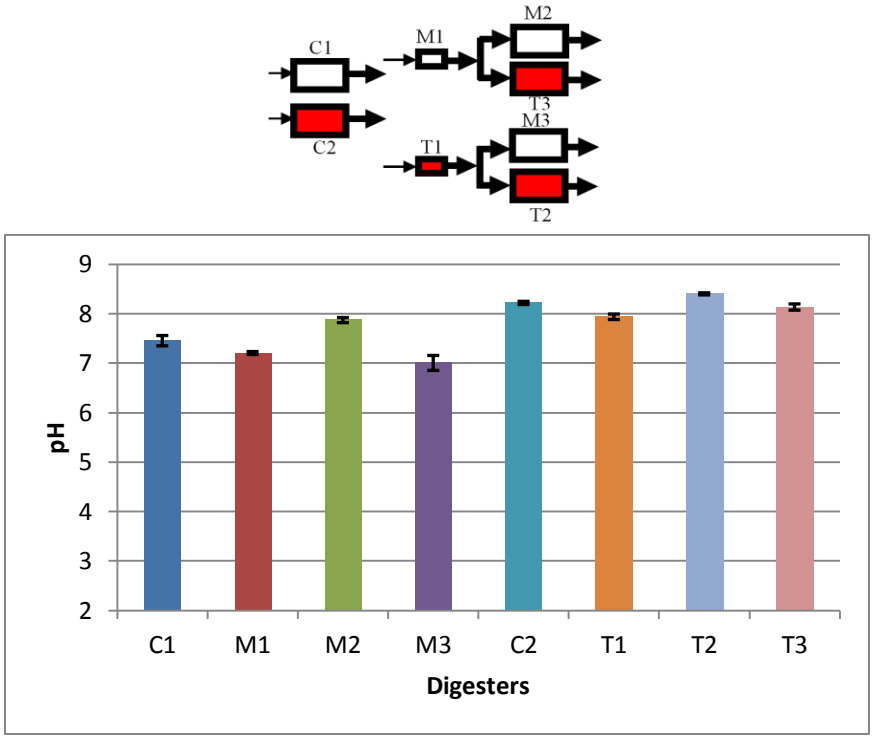


Figure 5.1. Average pH values in batch experiments on POB kinetics

The average concentrations of VSS, together with their standard deviations (SD) for the various batch experiments, are presented in Figures 5.2 and 5.3 for the mesophilic and thermophilic batch tests, respectively. An ANOVA PostHoc test (α=0.05) on the bottles at the same temperature revealed no significant differences among the VSS concentrations of either the mesophilic or the thermophilic bottles, indicating equal introduction of inoculum from the original digesters to the bottles. This made the parameter estimation more comparable.

To determine whether the VSS concentration changed significantly through the batch experiments, the VSS results were analyzed statistically using a paired-t test (α=0.05). The VSS concentrations remained approximately the same through the mesophilic batch experiments. A similar analysis on the thermophilic batch tests demonstrated that with the exception of the bottle inoculated with the biomass from the C2 digester, the VSS concentration did not change significantly in the thermophilic bottles. Overall the changes in VSS were modest and hence it was assumed that biomass was constant for the purposes of modelling.

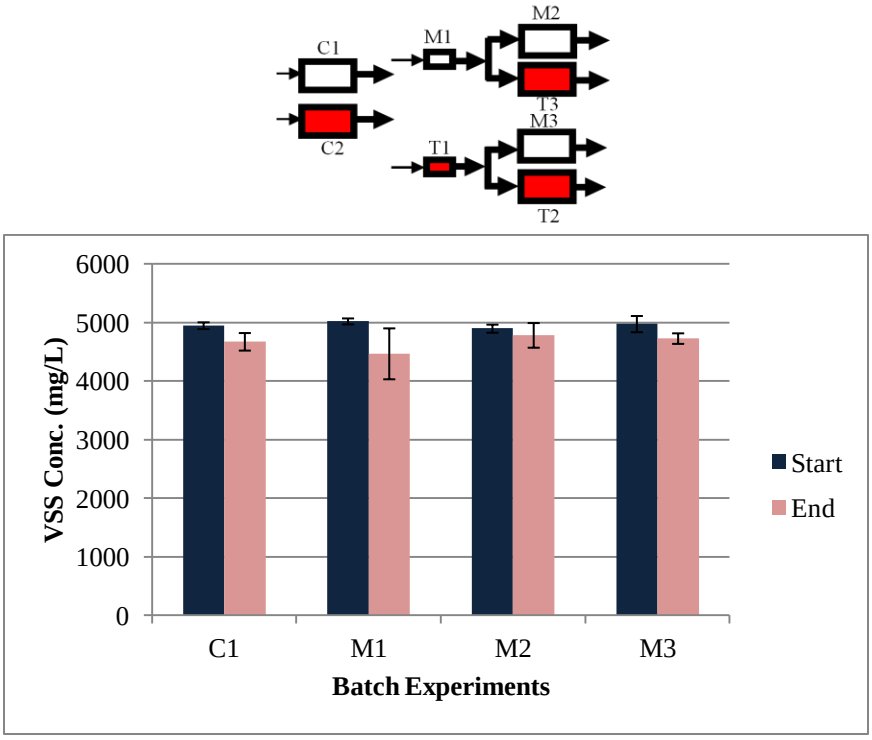


Figure 5.2. Average VSS concentrations in mesophilic propionate-fed bottle tests (Average ± SD); S=Start of experiment; E=End of experiment.

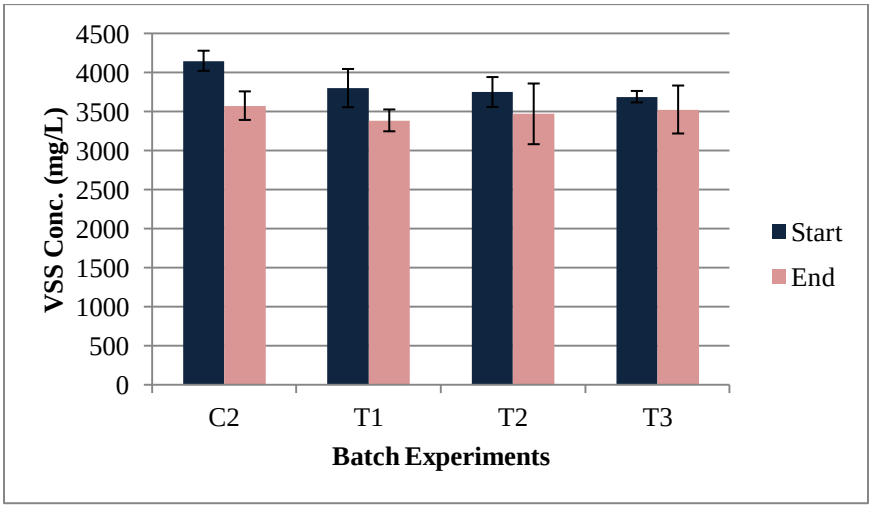


Figure 5.3. Average VSS concentrations in thermophilic propionate-fed bottle tests (Average ± SD); S=Start of experiment; E=End of experiment.

5.3.3 COD evolution in the batch experiments

The COD of the bottles was characterized with respect to total and soluble fractions. The total COD (TCOD) may be used to determine the reliability of the test through establishment of a mass balance on the batch experiments. In anaerobic digestion, methane production is the major pathway for the removal of COD. Therefore, measurement of the methane produced along with the determination of TCOD at the beginning and end of the batch experiment was employed to establish a mass balance for each bottle (Equation 5.1).

$$COD\ balance\ (\%) = (\ COD_{in} - COD_{out}\ - COD_{CH4}) \times \frac{100}{COD_{in}} \qquad (5.1)$$

Where CODin and CODout are mass of total COD at the beginning and end of the test; and COD-CH_4 is the mass of COD equivalent of methane gas.

In addition to TCOD, the soluble COD (SCOD) of the bottles contents was monitored and hence the particulate COD (PCOD) was calculated by subtracting the SCOD from the TCOD values. Assessment of the PCOD contents of the bottles was used to assess whether there was any significant decay of the inoculum during the experiments. At high COD concentration, significant decay of biomass can make the parameter estimation inaccurate as high decay products may undergo hydrolysis and acidogenesis and add to the net substrate concentration of interest (i.e., in this case propionate) (Robinson and Characklis, 1984).

Overall a reasonable deviation (less than 5%) was calculated for the mass balance of the bottles, indicating measurements were reliable. Even though, the propionate concentration was directly used in the parameter estimation exercise, a reasonable mass balance over the bottles demonstrated that POB and methanogens were active and the processes proceeded well in the batch experiments. Thus, a good mass balance on the bottles indicated the reliability of the data to be used in parameter estimation. The values calculated for lack of mass balance for the batch experiments inoculated from the C1, M1, M2 and M3 digesters were 0.81, -1.50, 0.55 and -1.42%, respectively. These values for the thermophilic batch experiments which were seeded from the C2, T1, T2 and T3 digesters were 2.40, 2.80, 1.33 and 4.56%, respectively.

The average total COD of the triplicate bottles for the mesophilic and thermophilic batch tests are shown in Figures 5.4 and 5.5. The general trend of COD was similar at both temperatures, indicating reduction of COD during the experiments and this COD appeared as methane gas. TCOD removal in the mesophilic bottles ranged from 12-13%, while that for the thermophilic bottles was 25-28%. Therefore, an average of

14% higher TCOD removal was observed in the thermophilic bottles. The elevated temperature of 55 ^{0}C appeared to enhace the conversion rate of propionate to acetate and acetate to CH_4. In addition, the rates of TCOD decline were determined in both the mesophilic and thermophilic bottles. The rates of COD reduction in the C1, M1, M2 and M3 bottles were 0.48, 0.52, 0.44, 0.45 g/L.d, respectively. The decline rates for the thermophilic bottles of C2, T1, T2 and T3 were calculated as 0.77, 0.86, 0.79 and 0.74 g/L.d. A comparison based on the average values of the COD decline rates revealed that the rates under the thermophilic condition were 1.6 times greater than that of the mesophilic bottles. Overall these results indicated the increased activity of biomass under the thermophilic conditions.

Examination of the PCOD of the contents of the bottles showed a reduction of 3-5% for the mesophilic bottles and 4-7% for the thermophilic ones. These values indicated that a negligible fraction of the inocula added to the bottles underwent decomposition that would influence the net propionate concentration in the bottles.

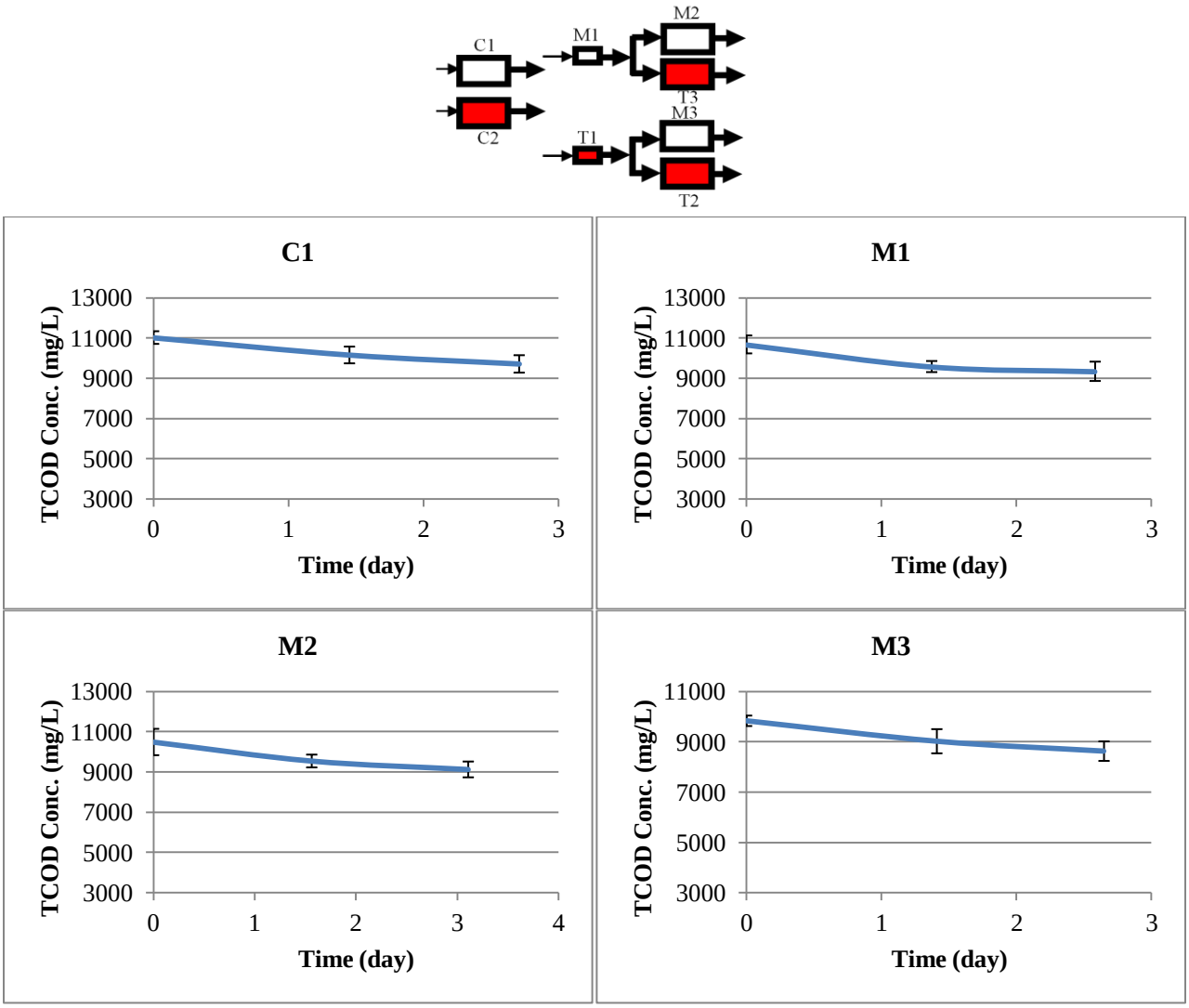


Figure 5.4. Total COD values in mesophilic batch experiments on POB kinetics

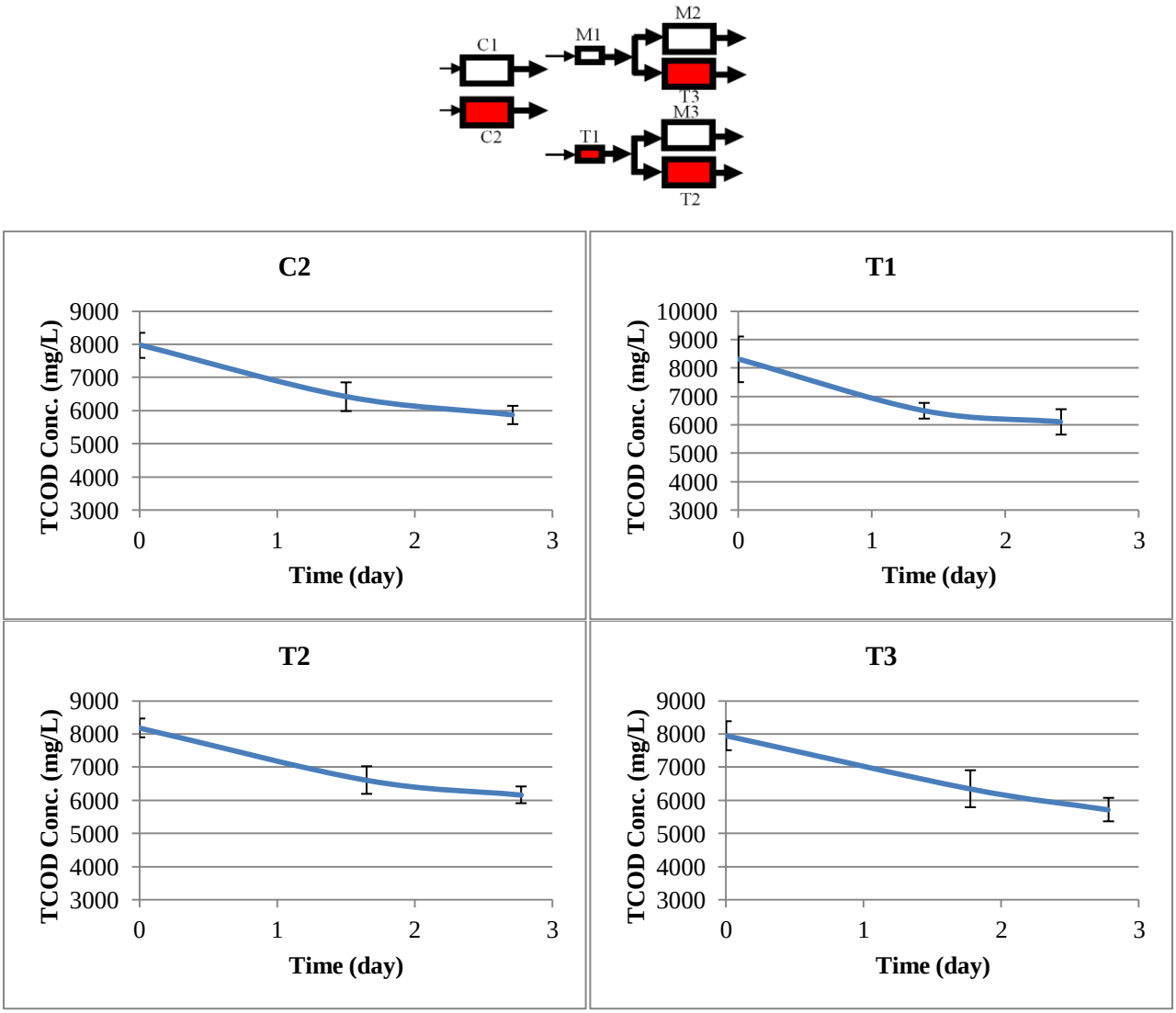


Figure 5.5. Total COD values in thermophilic batch experiments on POB kinetics

The average soluble COD concentrations of the triplicate bottles for the mesophilic and thermophilic batch tests are presented in Figures 5.6 and 5.7 respectively. The SCOD concentrations were higher in the batch experiments when compared to the COD equivalent of the propionate added to the bottles. The values of the propionate COD to SCOD ratio were in the range 76-90% for the mesophilic bottles and in the range of 80-88% for the thermophilic bottles at the beginning of the test, while, at the end of the test, this ratio was significantly reduced to 3.5-14% and 10-13% for the mesophilic and thermophilic bottles, respectively. These results indicate that propionate was the major component of SCOD at the beginning of the test and its degradation in the test bottles was the main pathway in reduction of SCOD.

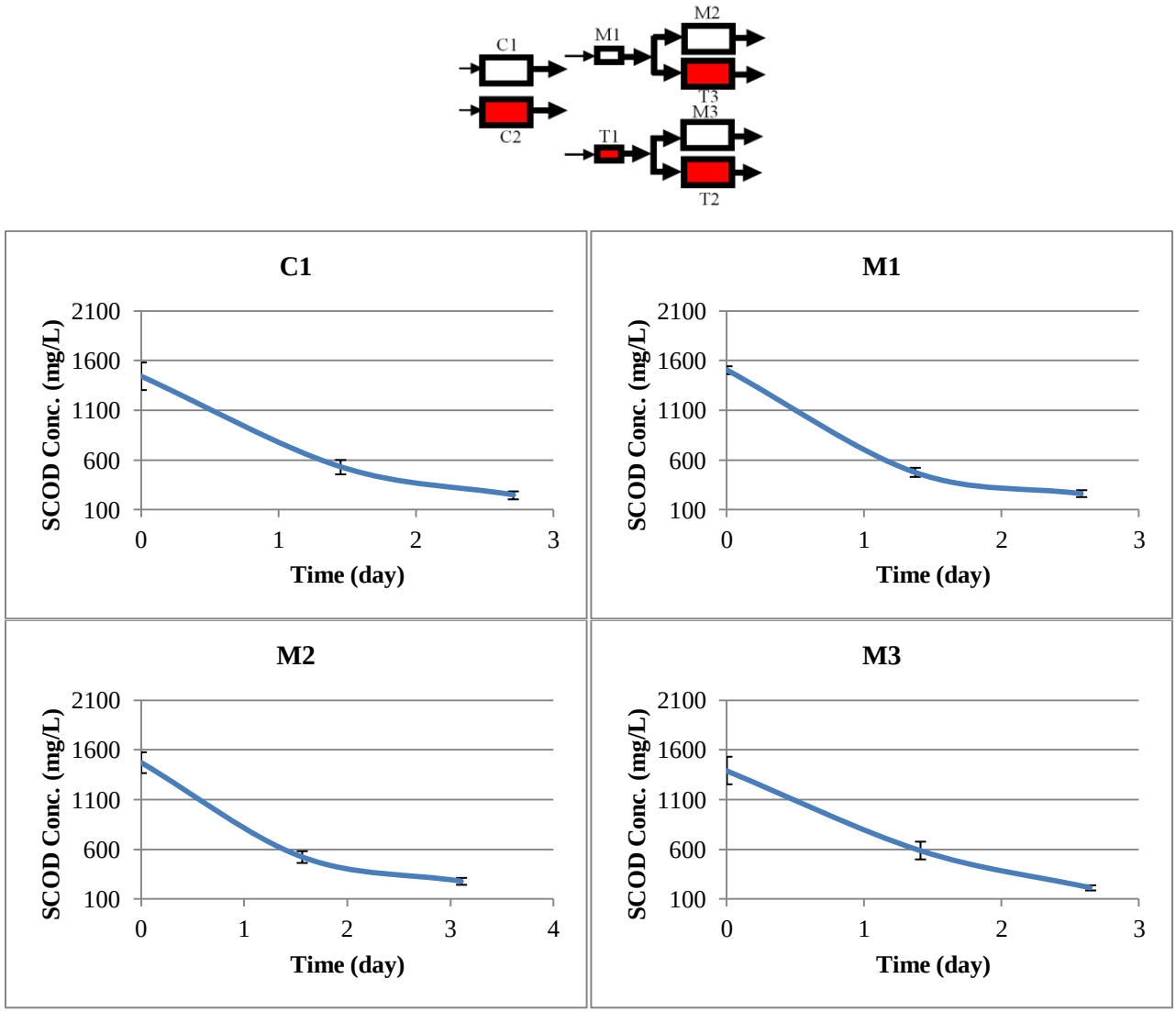


Figure 5.6. Soluble COD values in mesophilic batch experiments (on POB kinetics)

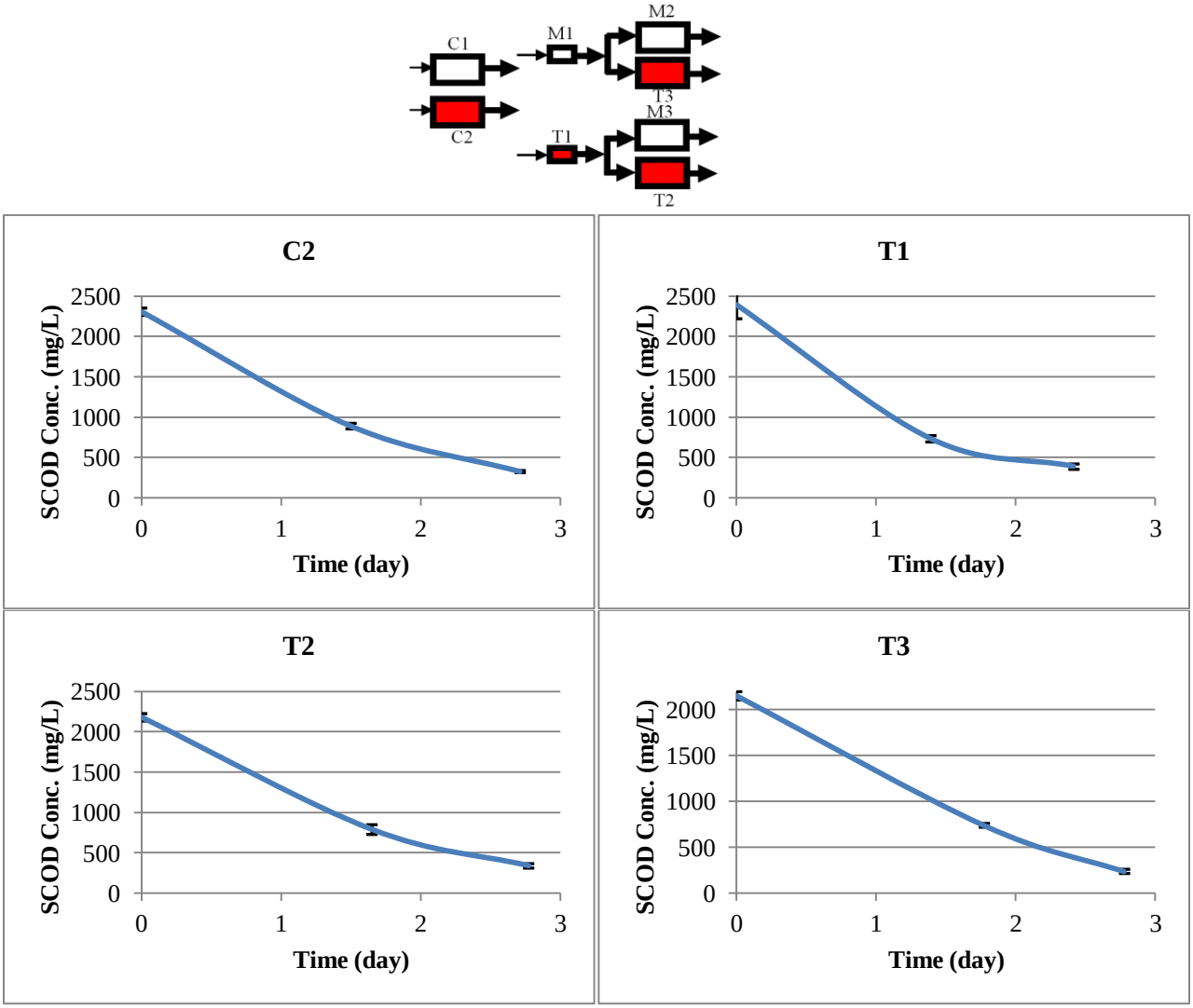


Figure 5.7. Soluble COD values in thermophilic batch experiments (on POB kinetics)

5.3.4 Assessment of Product Inhibition

Propionate degradation can be influenced by high concentrations of hydrogen and to a lesser extent by acetate (Pind et al., 2003). These compounds must be removed to allow efficient propionate metabolism. Acetate and methane were measured in the course of the batch experiments as indicators of the potential for inhibition of propionate degradation due to accumulation of by-products. The cumulative methane production and acetate concentrations measured in the bottles inoculated from the mesophilic digesters are presented in Figure 5.8. As can be observed from Fig. 5.8, propionate degradation resulted in an increase in acetate concentrations and subsequently methane production. Interestingly, this increase

was more apparent in the 2nd- phase digestion systems (M2 and M3) and the mesophilic single-stage digester (C1) and lasted for less than one day. However, the concentrations of acetate in the bottles were not at levels that have been reported to inhibit propionate degradation (Fukuzaki et al., 1990; Mosche and Jordening, 1999). The continuous production of methane showed that the methanogens were active and were not severely influenced by the elevated propionate concentrations (Aguilar et al., 1995; Mosche and Jordening, 1999). The increase of acetate was likely due to the time required by methanogens to acclimatize to the environment and the propionate levels within the batch tests. The reduced time required for acclimation of the biomass from M1 was likely due to the elevated acetate and propionate levels in the original digester that were higher than the other mesophilic digesters (Table 5.1). Based on these results it was concluded that the propionate degradation was not seriously stressed by product inhibition.

The cumulative methane production and acetate concentrations (expressed as COD) observed in the bottles inoculated with biomass from the thermophilic digesters are shown in Figure 5.9. As with the mesophilic batch experiments, acetate initially accumulated and then was degraded. Similarly, methane production rapidly increased after day 1 of the experiments. Acetate accumulated to a higher extent as compared to the levels observed in the mesophilic experiments. This was more apparent for the bottles inoculated with the biomass from the T3 digester (Fig. 5.9b). This was likely due to the higher propionate concentrations that were used in the thermophilic experiments. Despite the slight accumulation of acetate in the bottles, the following acetate degradation as well as the methane production demonstrated that propionate metabolism was not seriously influenced by its end-products. The continuous production of acetate through propionate degradation revealed that H_2 was also likely removed efficiently and hence did not limit propionate oxidation.

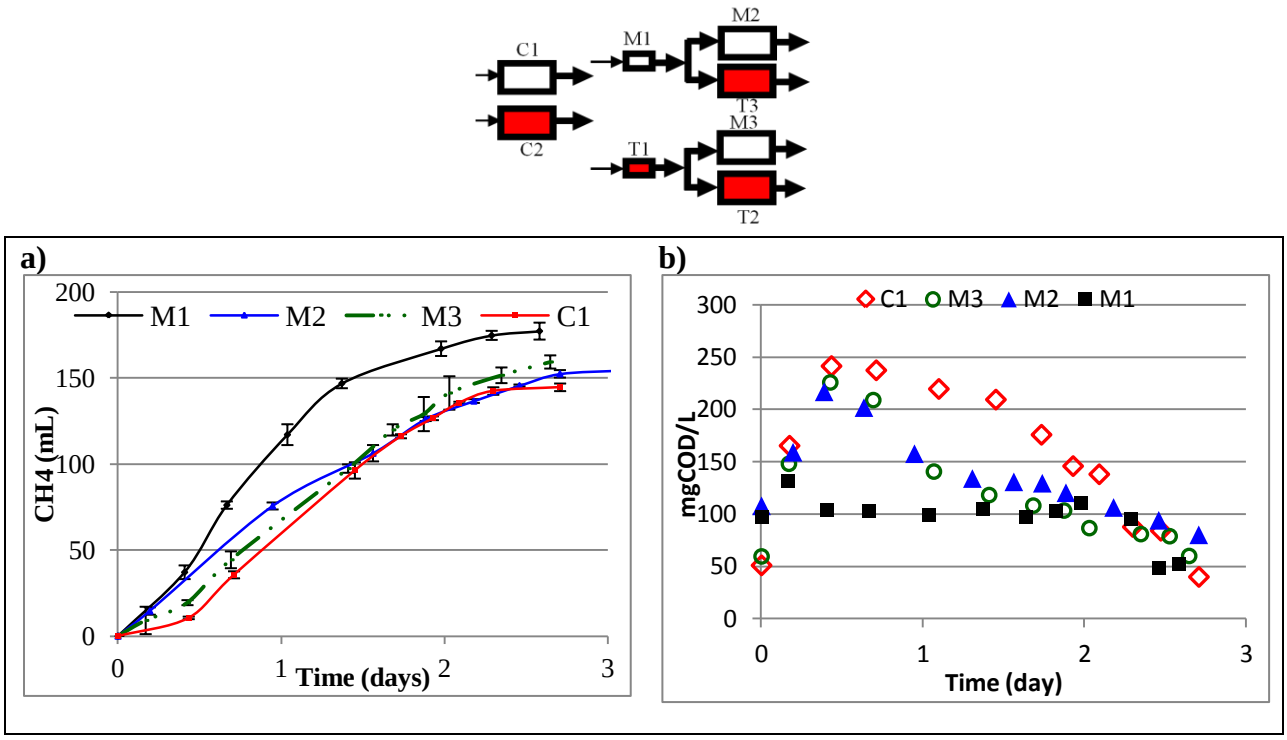


Figure 5.8. a) Cumulative methane production and b) Acetate concentrations in mesophilic bottles

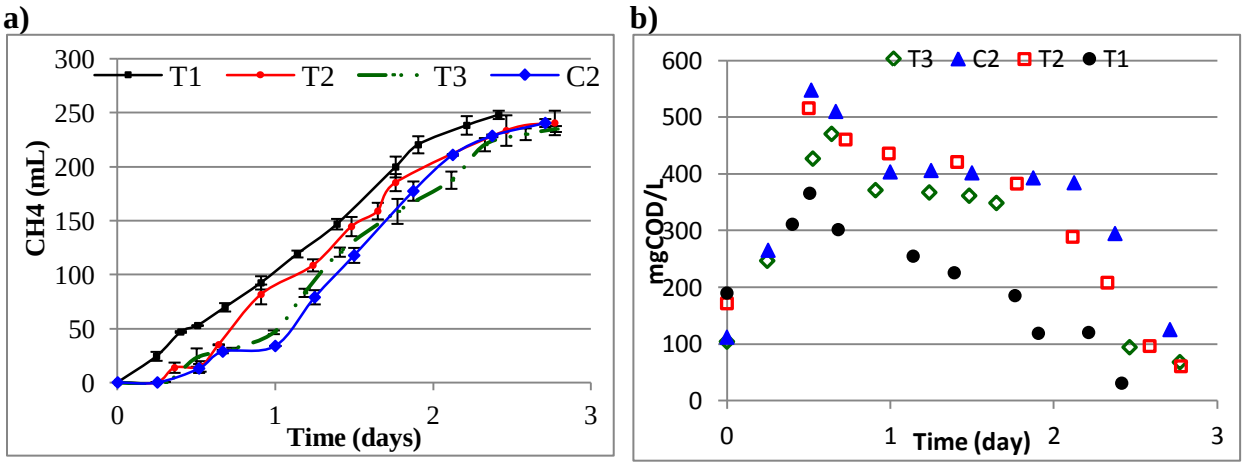


Figure 5.9. a) Cumulative methane production and b) Acetate concentration in thermophilic bottles

5.3.5 Parameter Estimation

5.3.5.1 Residual analysis

Prior to analyzing the results of the parameter estimation, the assumptions associated with the least squares approach were evaluated. This verification allowed for tests of significance and calculation of confidence intervals to be used for comparisons of the parameter estimates (Marshall et al., 1959). The assumptions include selection of an appropriate model, normal distribution of errors, constant variance of errors and independence of errors from one another (Montgomery, 2001).

The residuals (i.e., the difference between the observed and predicted data) were examined for normal distribution through establishment of a normal probability plot. Figures 5.10 and 5.11 represent the results of testing the normal distribution of errors for the mesophilic and thermophilic batch experiments. A normal probability plot of errors is linear if the residuals have normally distributed errors (Montgomery, 2001). In all cases a straight line was obtained for the residuals of the propionate concentrations, indicating normal distributions of the residuals.

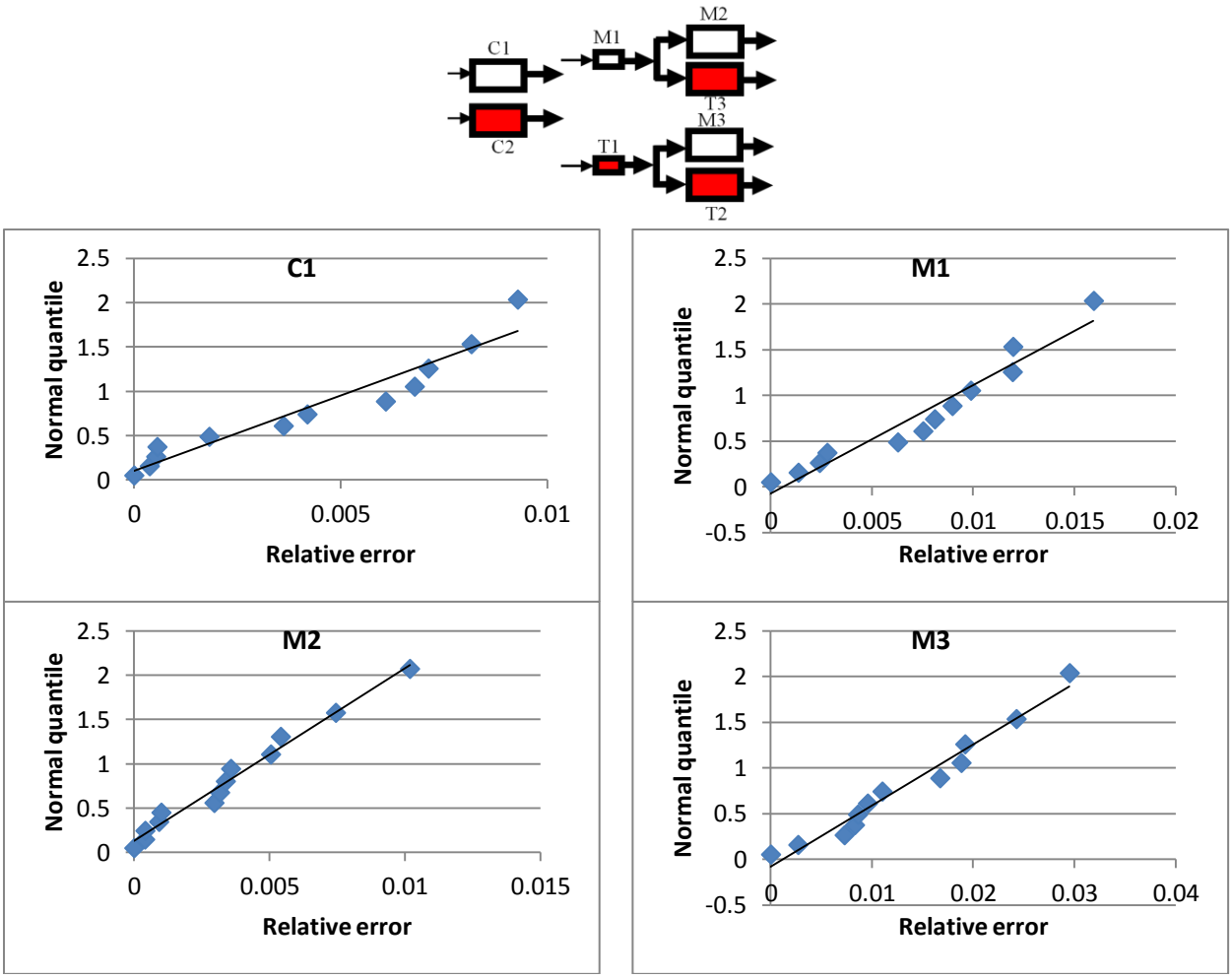


Figure 5.10. Half-normal quantile-quantile plots of errors for propionate data of the mesophilic batch experiments

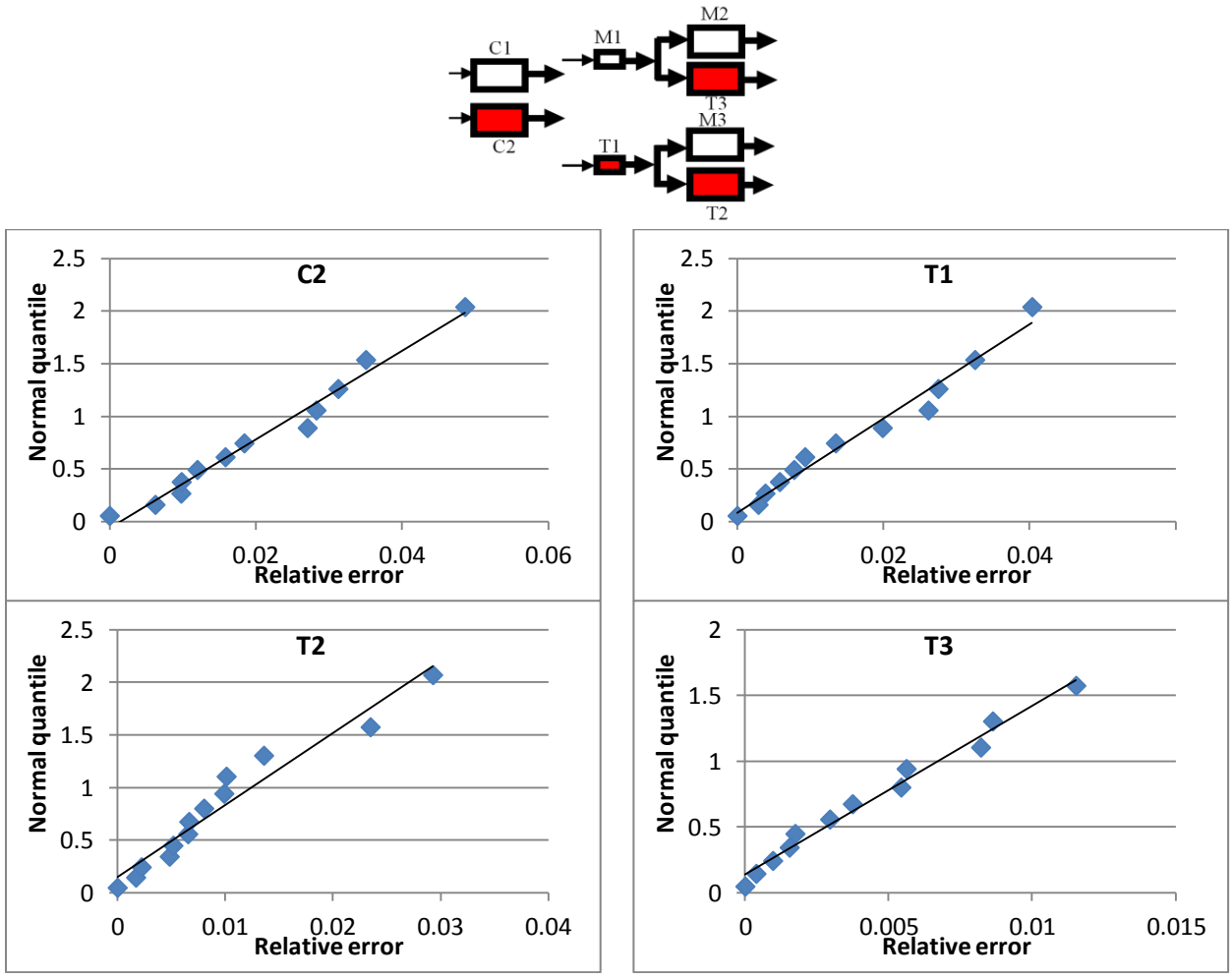


Figure 5.11. Half-normal quantile-quantile plots of errors for propionate data of the thermophilic batch experiments

The homoscedasticity assumption of residuals is important in parameter estimation as significance tests and the confidence intervals of the estimated parameters are not valid in cases with unequal variances of residuals. A simple method to check for homoscedasticity is to plot the residuals versus model predicted values. A scattered pattern of residuals indicates the errors have a constant variance (Mongomery, 2001). Accordingly, the errors of the propionate concentrations were plotted versus the model predicted values for the mesophilic and thermophilic batch experiments to check the randomness of errors (Figures 5.12 and 5.13). The scattered pattern of the residuals demonstrated that the errors of the acetate data in the batch experiments had approximately constant variances.

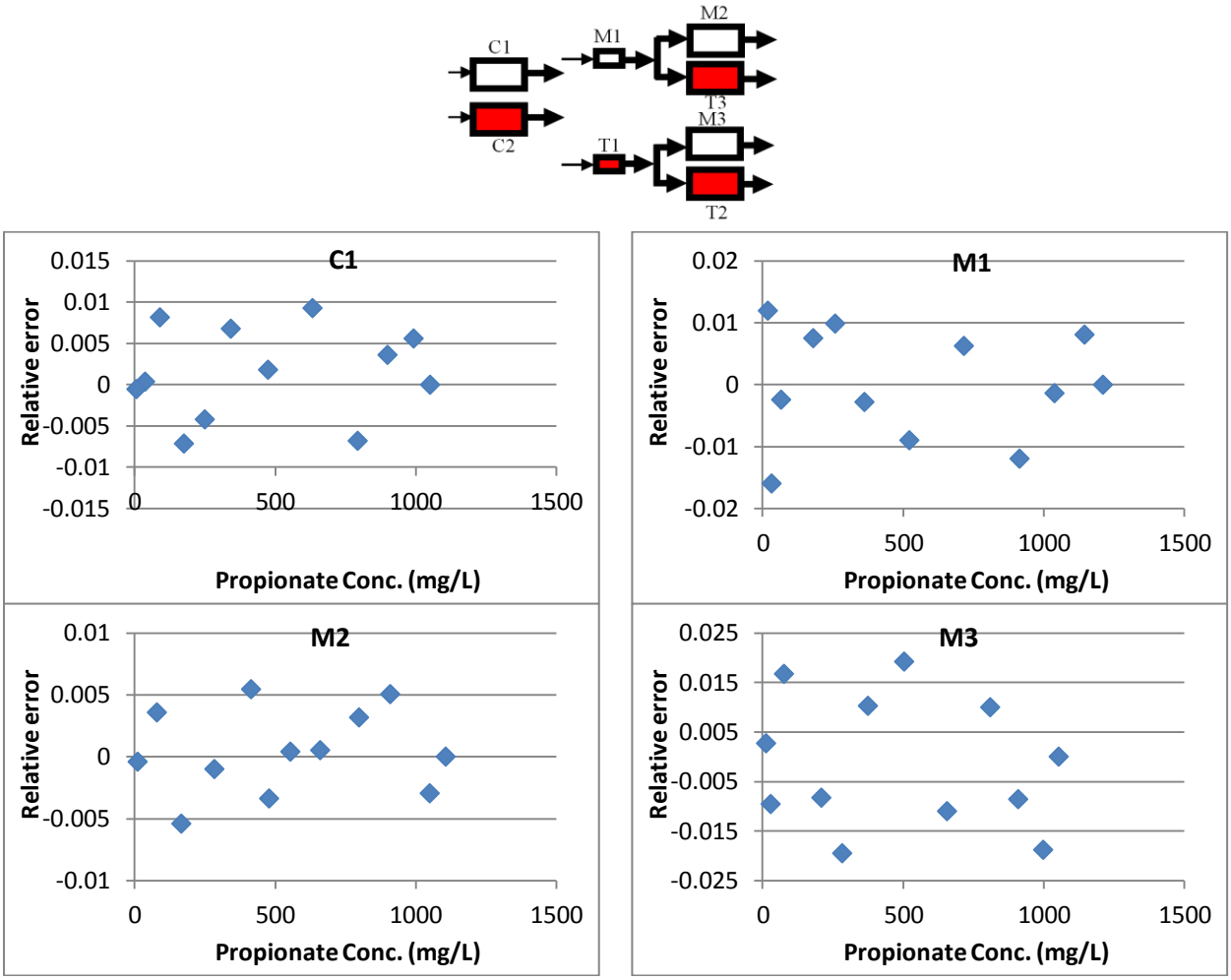


Figure 5.12. Residuals (errors) vs. predicted propionate concentrations for the mesophilic batch experiments (on POB kinetics)

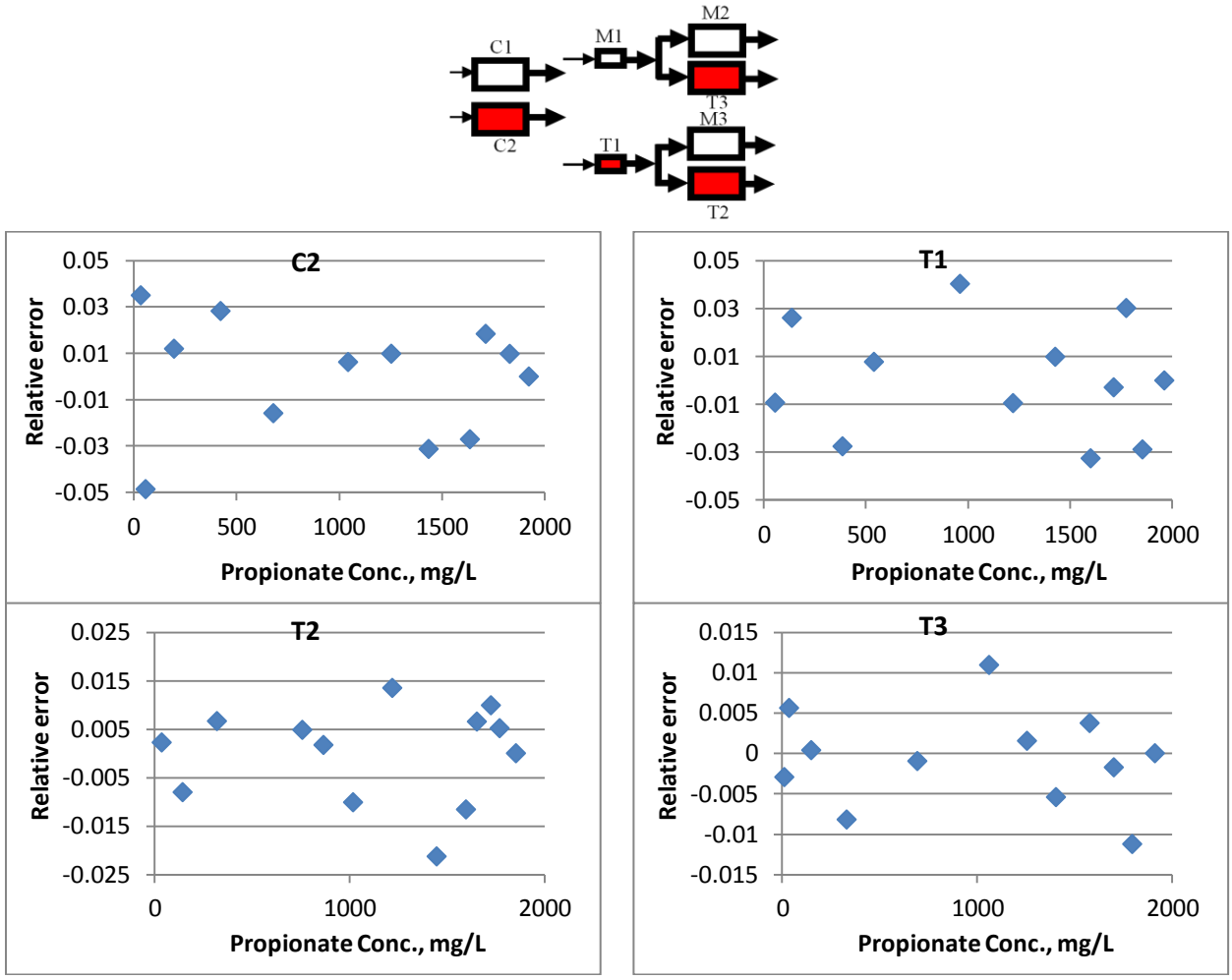


Figure 5.13. Residuals (errors) vs. predicted propionate concentrations for the thermophilic batch experiments (on POB kinetics)

5.3.5.2 Estimation of parameters of Monod Kinetics

Propionate is an important intermediate in anaerobic digestion process. In comparison to studies on the acetate behaviour in anaerobic digesters, fewer investigations have been carried out on the kinetics of propionate degradation. Among the limited works (Heyes and Hall, 1983; Nielsen et al., 2008), propionate degradation kinetics in phased digestion systems have not been reported. Thus, in this section the characteristics of propionate oxidizing bacteria (POB) were kinetically studied in the phased anaerobic digestion systems.

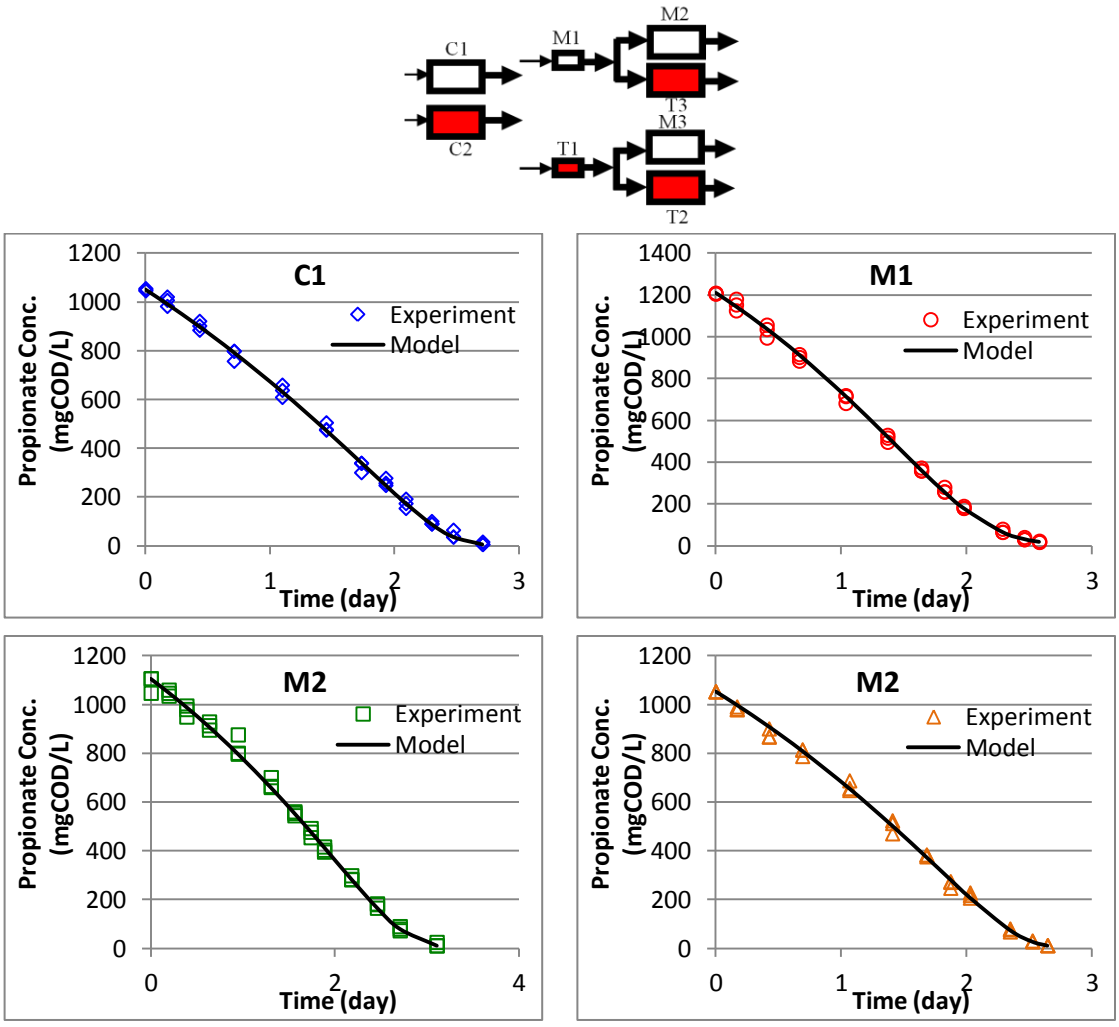


Figure 5.14. Results of the model fit with propionate experimental data: mesophilic batch tests

The Monod coefficients (k_{max} and K_s) for POB and the concentration of propionate degrading organisms (X_o) in the batch tests were estimated by fitting the integrated Monod equation (Equation 4-4: Chapter 4) to the measured propionate concentrations versus time. Figures 5.14 and 5.15 show the propionate concentration over time and the fitted model for the mesophilic and thermophilic batch experiments. Examination of these Figures exhibits that the calibrated model reasonably fit the propionate degradation data.

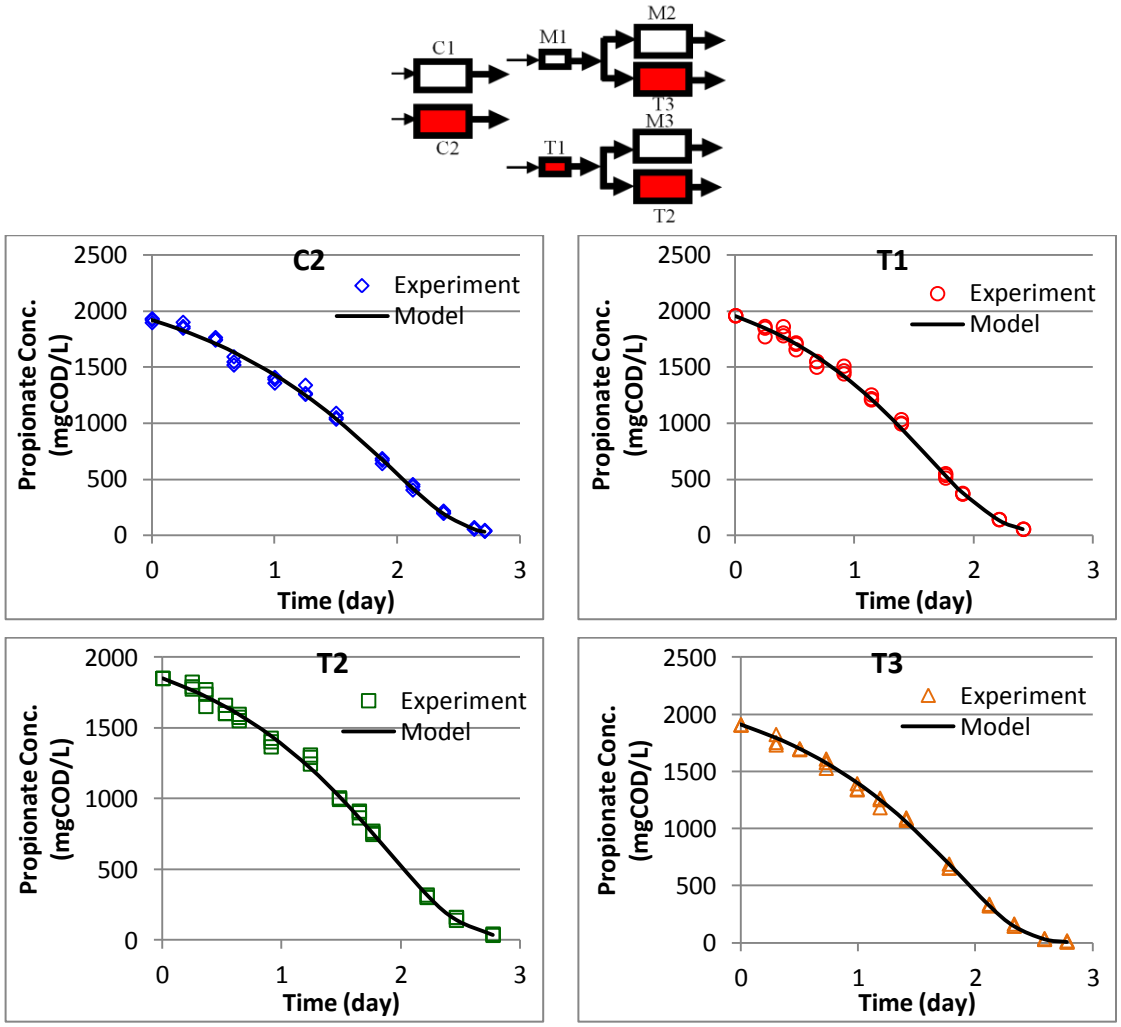


Figure 5.15. Results of the model fit with propionate experimental data: thermophilic batch tests

The estimated kinetic parameters (k_{max} and K_s), the initial concentrations of POBs and the associated linear uncorrelated approximate 95% confidence intervals (CI) of these parameters for the single-stage and phased digestion systems are presented in Tables 5.2, 5.3 and 5.4, respectively. Calculation of relative errors (the ratio of the standard deviation to parameter value) revealed that in all tests there was a larger relative error in K_s values as compared to the values obtained for k_{max} values. High uncertainties in the estimates of K_s values were also reported by Kalfas et al. (2006) when mesophilic and thermophilic anaerobic digesters treating olive pulp were studied.

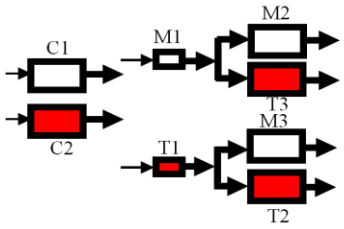


Table 5-2. Estimated parameters for POB populations of single stage digesters

Parameter		C1		C2	
	Unit	Best fit value	CI*	Best fit value	CI
k_{max}	d^{-1}	6.2	6.1-6.3	17.1	16.8-17.4
K_S	mgCOD/L	69	40-97	281	210-352
X_0**	mgCOD/L	285	264-305	112	95-128

*95% linear uncorrelated confidence Interval; **initial biomass concentration

Table 5-3. Estimated parameters for POB populations of mesophilic phased digesters

Parameter		M1		M2		M3	
	Unit	Best fit value	CI	Best fit value	CI	Best fit value	CI
k_{max}	d^{-1}	11.0	9.3-12.7	6.8	6.0-7.7	7.0	6.1-7.7
K_S	mgCOD/L	210	186-235	99	60-139	75	39-111
X_0	mgCOD/L	136	103-168	296	169-424	318	301-335

Table 5-4. Estimated parameters for POB populations of thermophilic phased digesters

Parameter		T1		T2		T3	
	Unit	Best fit value	CI	Best fit value	CI	Best fit value	CI
k_{max}	d^{-1}	23.6	21.9-25.4	20.3	19-21.6	18.2	17.6-18.8
K_S	mgCOD/L	504	435-572	398	337-459	282	239-324
X_0	mgCOD/L	102	82-123	110	98-121	108	91-126

Large confidence intervals related to the estimated kinetic coefficients, especially for K_s, may also be attributed to the limited number of samples collected from the batch experiments. In general, the accuracy of parameter estimation can be increased through increasing the number of measurements (Guisasola et al., 2006). Moreover, based on the sensitivity analysis of the Monod equation with regards to k_{max} and K_s, previous researchers have demonstrated that k_{max} is more easily estimated and has lower relative error than K_s estimates (Merkel et al., 1996; Dochain and Vanrolleghem, 2001; Guisasola et al.,

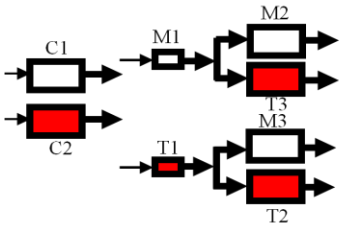
2006). Hence, the results of this study were consistent with the results of similar studies that have been reported in the literature.

The parameter values that were estimated in this study were compared against literature values to validate the approach which was employed (Table 5.5). From Table 5.5 it can be observed that the parameters estimated in this study were within the range of the literature values. Hence, when considered together with the previously described statistical assessment of the quality of parameter fit, it was concluded that the approach employed for parameter estimation was valid.

Table 5-5. Kinetic parameters of anaerobic propionate degradation reported in literature

System	Temp. (^{0}C)	HRT (d)	K_s mgCOD/L	k_{max} d^{-1}	Reference
Single	35	7.2	20	11	Siegrist et al., 2002
	55	6.2	150	33	Siegrist et al., 2002
Single	35	14	16.5	3.7	Heyes and Hall, 1983
	35	8.2	495	34	Heyes and Hall, 1983
Single	35	20	60	3.5	Kalfas et al., 2006
	55	20	225	37.8	Kalfas et al., 2006
Single	35	---	100	13	Batstone et al., 2002
	55	---	300	20	Batstone et al., 2002
Two-phase	35	20	200	9	Blumensaat and Keller, 2005
	55	2-4	400	16	Blumensaat and Keller, 2005

The 95% confidence regions for the k_{max} and K_s values for the mesophilic digesters are presented in Figure 5.16, with error bars showing the linear uncorrelated error estimates. The regions for the parameters are "true" confidence spaces which are considered preferable to the linear confidence intervals (Batstone et al., 2003). Based on a comparison of the confidence regions for the bio-kinetic parameters (kmax and Ks) estimated for the mesophilic digesters, it was found that the parameter set for the M1 digester was different from the ones obtained for the M2, M3 and C1 digesters. Based on these results, it was concluded that the mesophilic propionate degrading organisms in the first stage of the phased digesters were functionally different from those of the other mesophilic digesters (i.e., M2, M3 and C1) that were all operated at a lower organic loading.



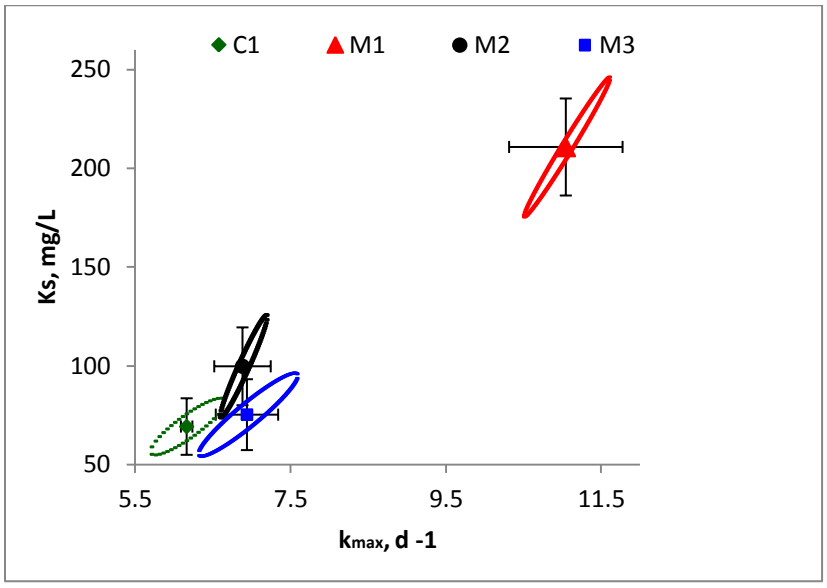


Figure 5.16. 95% confidence regions for POB in mesophilic digesters

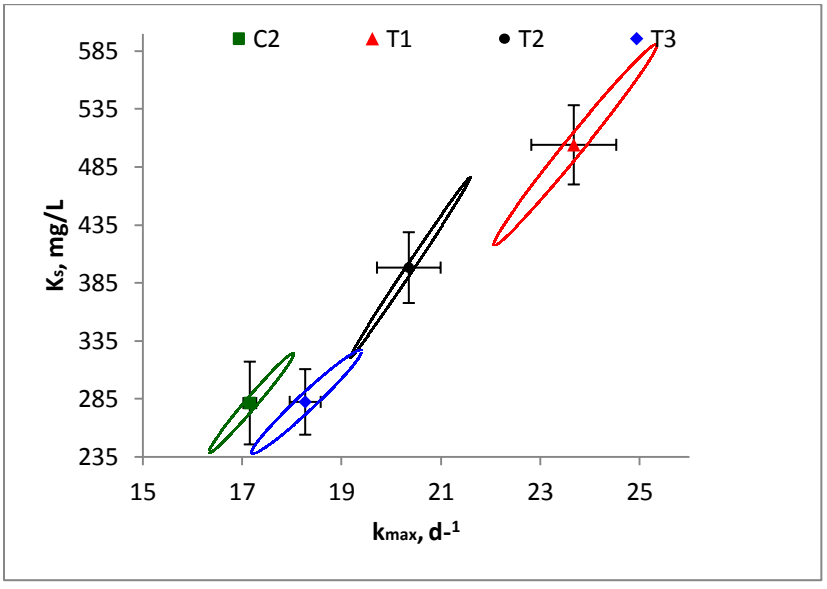


Figure 5.17. 95% confidence regions for POB of the thermophilic digesters

Even though much less investigation (compared to methanogens) has been made regarding the microbial ecology and functional diversity of POB in sludge digesters, there is evidence in the literature indicating the presence of POB with varying growth rates (Liu et al., 1999; Shigematsu et al., 2006; Ariesyady et al., 2007). The species of the genera *Syntrophobacter* and *Smithella sp.* LR were reported to dominate at low concentrations (0.5-2.5 mM) of propionate or low dilution rates (0.01-0.08 d-1); while, the species of *Pelotomaculum* and *Smithella sp.* SR were highly active at high propionate levels (>15mM) or high dilution rates (0.3 d-1). Also, Heyes and Hall (1983) reported the presence of two distinct subgroups with different growth kinetics depending on the HRT of the digesters. From these reports, it can be concluded that *Pelotomaculum* and *Smithella sp.* SR, which have greater growth rates, would be dominant in the 1st-phase digester with a high concentration of propionate (Table 5.1), while those of *Syntrophobacter* and *Smithella sp.* LR would proliferate in the 2nd-phase digesters that had lower propionate levels (Table 5.1). At low concentrations of propionate, organisms with higher affinities for propionate (low K_s) would have higher chance to be dominant in the digesters.

The 95% confidence regions for k_{max} and K_s values for the thermophilic digesters are presented in Figure 5.17, with error bars showing the linear uncorrelated error estimates. Using a similar approach to that employed for the mesophilic digesters, it was found that the confidence regions of the T3 and C2 digesters overlapped but were different from the T1 digester. The bio-kinetic parameters of the T2 digester showed a confidence region that overlapped with both the T1 and T3 digesters. This indicated mixed kinetic characteristics of POB within the T2 digester, reflecting the presence of both slow and fast-growing POB. This will be further discussed in section 5.3.6.

As with the results from the mesophilic digesters, it would appear that the first phase thermophilic digester had a propionate degrading biomass that was functionally different from that of the lower loaded digesters. Relative to the known species of mesophilic POB, fewer thermophilic POB have been isolated and studied so far. All the studies performed regarding the functional diversity of POB have been limited to mesophilic digesters (Liu et al., 1999; Shigematsu et al., 2006; Ariesyady et al., 2007). Hence, comparison of the bio-kinetic values estimated in this study with literature values was not possible. The results of this study suggested the presence of functionally-different thermophilic POB in the each stage of phased digestion systems. This is the first known report of this type of diversity of thermophilic POB.

In general, the results of the parameter estimation reveal that the higher estimated values of k_{max} and K_s for both the thermophilic and mesophilic 1st-phase digesters were consistent with the propionate concentrations in the source digesters (Table 5.1). The fast growing POB could proliferate in the low

HRT (3 days) digesters, while those POB with lower growth rates would not be retained in the digesters. On the other hand, the lower K_s (higher affinity) values of single-stage and 2nd-phase digesters (both mesophilic and thermophilic) in comparison to the K_s values of the corresponding 1st-phase digesters caused the slow growing POB to be dominant in these digester with lower propionate concentrations.

Interestingly, it can be seen from Tables 5.2-5.4 that all the thermophilic digesters showed high K_s values compared to the corresponding digesters kept at a mesophilic temperature. The reduced affinity (high K_s) of the POB present in the thermophilic digesters explains the higher propionate concentrations in the effluent of these digesters. The elevated effluent concentrations of propionate are typically a problem for thermophilic anaerobic digesters. On the other hand, the C1, M2 and M3 digesters had low propionate concentrations that were in agreement with the low K_s (high affinity) values estimated for these digesters. This is important for practical applications as it demonstrates that a mesophilic 2nd-phase digester should be employed for effective removal of propionate in phased anaerobic digestion systems.

5.3.5.3 Effect of temperature on bio-kinetic parameters

One of the important factors affecting the growth of microorganisms is temperature. Its influence on microbial growth rates is described by various models. In modeling of biological wastewater and sludge treatment processes, an Arrhenius-type Equation (5.2) is typically used to implement the effect of temperature (Dochain and Vanrolleghem, 2001). Equation 2 has been applied to various parameters such as maximum specific growth rate (μmax), saturation constant (K_s), hydrolysis rate and inhibition constant (Hashimoto, 1982; Angelidaki et al; 1993; Siegrist et al., 2002).

$$K_2 = K_1 * EXP(\theta * (T2 - T1)) \quad (5.2)$$

Where, K_2 is coefficient at target temperature; K_1 is known coefficient; θ is temperature correction coefficient; T is temperature.

In this study, the Monod parameters of the mesophilic digesters were compared with those of the thermophilic digesters to evaluate the influence of temperature on the rate constants. Thus, the application of an Arrhenius-type relationship for modeling of phased digestion systems was also evaluated. As it was previously demonstrated that the POB kinetic behavior of the first phase digesters was significantly different from the other configurations, the constants (i.e., k_{max} and K_s) from this pairing (i.e., mesophilic vs. thermophilic) of digesters were compared. The values from Tables 5.2-5.4 were employed to calculate

the ratios of the thermophilic parameters to the mesophilic ones and it was found that the values for k_{max} and K_s for T1 were 2.1 and 2.4 times higher than the values obtained for the M1.

A similar analysis was conducted for the remaining digesters (thermophilic vs. mesophilic single-stage and second-phases) and it was found that, in contrast to the 1st-phase digesters, the influence of temperature on the kinetic parameters was much higher. The values of k_{max} and K_s were, on average, 2.8 and 4.0 times greater in the thermophilic digesters than the mesophilic ones. Accordingly, the values of θ calculated for k_{max} and K_s for the first-phase digesters were, respectively, 0.0381±0.0035 and 0.0436±0.0011. Excluding the values of the T2 digester in calculations, these coefficients for the lower loaded digesters were 0.0488±0.0023 and 0.0621±0.0085 for k_{max} and K_s, respectively. The θ values suggested for ADM1 are 0.02 for k_{max} and 0.055 for K_s (Batstone et al., 2002). As can be seen, the θ values for k_{max} were significantly higher than the value of the suggested for ADM1, while those values for K_s were close to the θ of the ADM1. The higher temperature dependence may be attributed to the employment of the phased digestion system in this study; whereas, the ADM1 values were suggested for general application to anaerobic digestion processes such as single-stage digesters. In addition, the values in ADM1 were not based upon directly measured kinetic values and this may explain the difference between the θ values calculated in this study with those of ADM1. These results suggest that the relationship between POB kinetic parameters and temperature differs between the slow and fast growing organisms. Thus, the application of Arrhenius-like equations for temperature correction of kinetic coefficients should be applied with caution in modeling of propionate degradation in these systems.

5.3.5.4 Initial concentration of propionate oxidizing bacteria

The initial biomass concentrations (X_0) that were estimated from the batch tests were corrected for dilution to calculate the values in the source digesters. As with the methanogen populations (Chapter 4), the concentrations of the POB population estimated in this study should be considered approximate estimates because of the high correlation (> 80%) that was found between the Monod kinetic parameters, especially k_{max}, and initial biomass concentration. However, as the correlation existed in the parameter estimation process of all the batch experiments, the estimated initial biomass concentrations should relatively reflect the POB populations in the source digesters.

The concentrations of POB that were estimated from the parameter estimation exercise are reported in Tables 5.2-5.4. As can be seen from these tables, the POB concentrations present in the mesophilic digestion systems were consistently greater than those observed in the thermophilic ones. This

result is in agreement with the higher endogenous decay rates reported for thermophilic temperatures (Siegrist et al., 2002).

The POB concentrations in the M1digester were consistently lower than those of the other mesophilic digesters (i.e., M2, M3 and C1). The mean estimated concentration of POB biomass in T1 was also found to be less than those of the other thermophilic digestion systems (i.e., T2, T3 and C2), however this difference was not statistically significant due to the high uncertainty in the estimated biomass concentrations in the thermophilic systems. The relatively short residence times in the first phase digesters appeared to limit the yield of POBs in these units. In addition, since acidogenesis mainly happens in the 1^{st}-phase digesters in which the H_2 concentration would be relatively higher compared to that of the corresponding 2^{nd}-phase digesters, this would limit the yield of POB in these digesters. The elevated residual propionate concentrations that were observed in the source digesters support this conclusion as there was clearly residual substrate available for additional growth.

The POB fraction (biomass COD to total COD of digesters) for the M1 digester was 0.40% and for the rest of the mesophilic digesters ranged between 1.10-1.35%. The methanogen biomass fractions for the T2, T3 and C2 digesters were, on average, 0.42%; whereas, this value for the T1 digester was calculated as 0.27% which was lower than the range of reported values. Jeong et al. (2005), who simulated the methane production in a batch test, estimated POB biomass as 49 COD mg/L in bottles with 4250 mg/L VSS. Rodrigues et al. (2006) reported a POB biomass of 89 COD mg/L in a chemostat digester with 10 g/L glucose as feed. Based on the total COD of the source digesters and estimated POB biomass, overall the POB fractions were within the range reported. Therefore, considering a much higher VSS and use of a complex feed in the source digesters, a higher concentration of POB in the various digesters of this study would appear to be reasonable compared to the values reported in the literature.

5.3.6 Comparison of Specific Growth Rates

A review of the k_{max} and K_s values collectively revealed that in all cases the magnitude of the parameters varied together (i.e. k_{max} increased when K_s increased), indicating a high correlation between these parameters. Many authors have reported the existence of a high correlation between k_{max} and K_s in previous studies (Dochain and Vanrolleghem, 2001; Batstone et al., 2003; Flotats et al., 2003; Kalfas et al., 2006). The calculation of the specific growth rates for POB is influenced by the interaction of these parameters and the extent of the interaction depends upon substrate concentration. As such, to better assess the importance of the differences between the sets of parameter values on the specific growth rate of POB in the various digesters operated in this study the mean values of the estimated parameter pairs

were employed to estimate specific growth rates over a range of propionate concentrations (Equation 5.3). The mesophilic and thermophilic responses are presented in Figures 5.18 and 5.19, respectively.

$$\mu = Yk_{max}\frac{S}{K_s+S} \quad (5.3)$$

Where, μ is specific growth rate; Y is yield coefficient selected as 0.05 COD/COD (Siegrist et al., 2002).

From Figure 5.18 it can be seen that under mesophilic conditions, when the propionate concentration was more than 150-200 mg /L, the specific growth rate of the POBs from the M1 digester was distinctly greater than those of the other digesters. The measured propionate concentrations in the source digester were in the range of 200-250 mg/L (Table 5.1) and consequently the differing bio-kinetic parameters of the biomass coincided with the elevated substrate concentrations. This supported the hypothesis of a shift in the POB population at the elevated concentrations of propionate in the heavily loaded digester (M1). As illustrated in Figure 5.18, the specific growth rates of the POBs from the other mesophilic digesters were approximately the same. Due to the relatively long HRTs (14 days in M2 and M3; and 17 days for C1) and consequently lower organic loading rates, a biokinetically-similar POB was developed in these digesters. This agreed with the much lower propionate concentrations in these digesters that were less than 30 mg/L.

Figure 5.19 illustrates that under thermophilic conditions, with the exception of the T2 POBs, the dependence of the specific growth rates of the POBs on propionate concentrations followed a similar trend to that of the mesophilic digesters. The specific growth rate for the POB from the T1 digester was higher than those of the other digesters when the propionate concentration exceeded 400-450 mg/L and this was consistent with the significantly higher propionate concentration in the source digester (Table 1). However, even though the propionate concentration in T2 did not differ significantly (ANOVA Post Hoc, α=0.05) from that of the C2 digester, the specific growth rate of POB biomass developed in this digester was higher and it was closer to the specific growth rate calculated for the T1 digester (Figure 5.19).This may have been due to two reasons: (a) high uncertainties in estimation of k_{max} and K_s values for this digester that prevented the values for the T2 digester from being discriminated from those of the other digesters; and (b) the higher specific growth rate in the T2 digester when the propionate concentration exceeded 500 mg/L might be attributed to the continuous transfer of fast-growing POB from the T1 digester, contributing to relatively different values of the kinetic parameters.

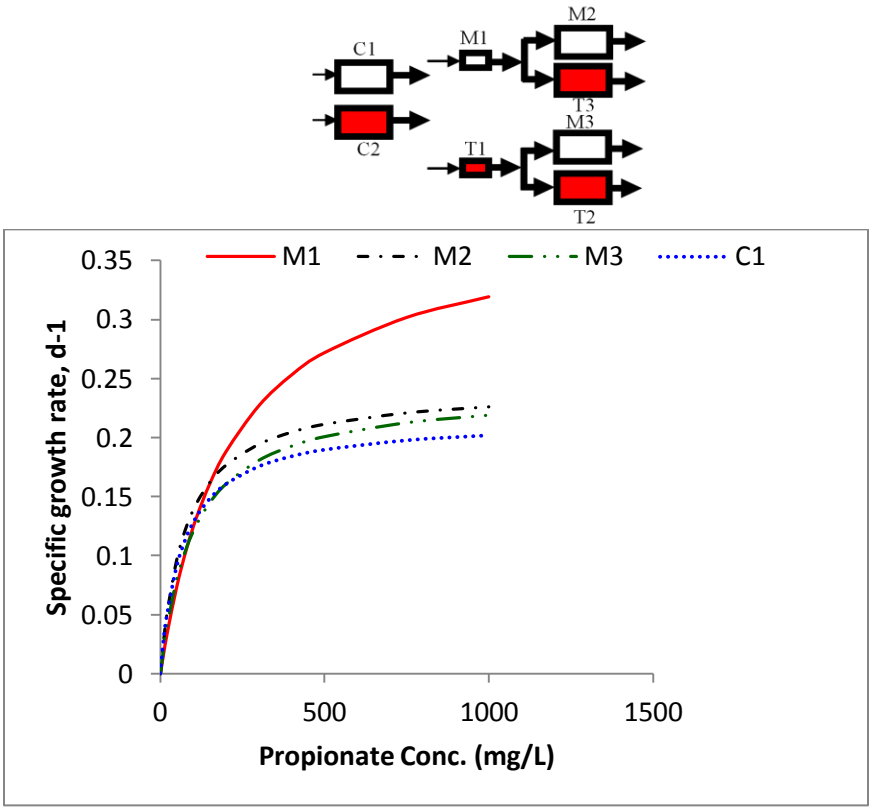


Figure 5.18. Specific growth rate vs. propionate concentration based on the kinetic parameters estimated for mesophilic digesters.

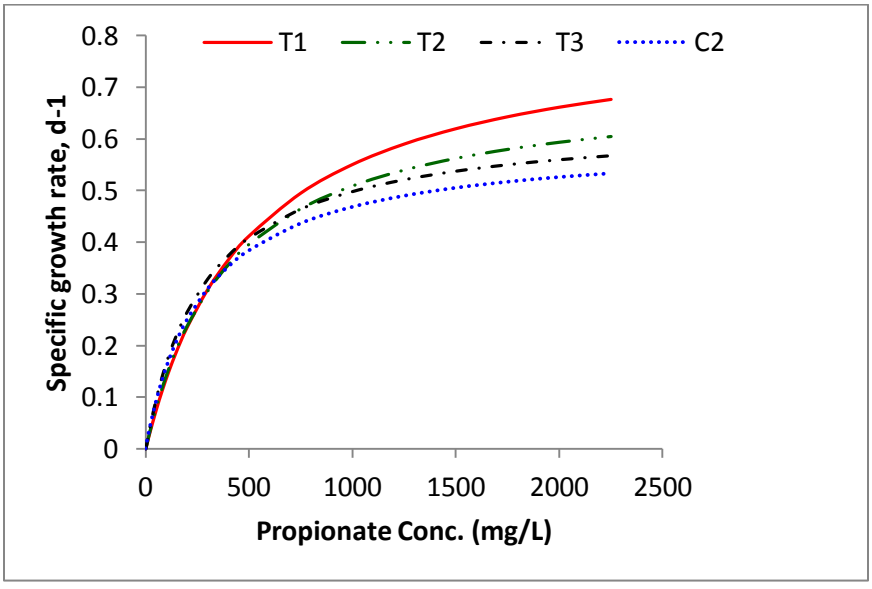


Figure 5.19. Specific growth rate vs. propionate concentration based on the kinetic parameters estimted for thermophilic digesters.

It would appear that the propionate oxidizing bacteria were being inactivated when biomass was transferred from the 1st-phase digesters with a different temperature. Therefore, the POB developed in the M3 and T3 digesters were grown in these digesters with no contribution from the 1st-phase digesters. On the other hand, the digestion systems with identical temperature in both phases demonstrated a different response to the biomass transfer from the 1st-phase digesters. It seems that the POB developed in both M1 and T1 digesters were able to survive in the corresponding 2nd-phase digesters although the contribution to the total biomass of POB would be limited due to the different affinities for propionate. These results were consistent with the specific growth rates discussed for the POB of the various digesters. As depicted in Figures 5.18 and 5.19, when the propionate concentrations increased, the specific growth rates of POB of the M2 and T2 digesters responded differently (i.e., increased more rapidly) compared to the 2nd-phase of the phased systems with different temperatures in each phase (i.e., M3 and T3). Due to the diversity in domain bacteria, the DGGE analysis did not lead to distinguishable profiles among the digesters. Application of other molecular methods such as fluorescence in situ hybridization or quantitative PCR could provide more information. However, application of these methods depends on the availability of probes to target the organism of interest.

5.3.7 Simulation of propionate and acetate evolution in batch experiments

During the batch experiments, acetate as a by-product of propionate degradation was measured in the bottles in addition to the measurement of propionate concentrations. In order to verify the estimated bio-kinetic parameters for POB, the acetate and propionate data was simulated using the parameters estimated in this study. The Aquasim software was used for the simulation and a simple model was employed including uptake of propionate and acetate (Figure 5.20). Under anaerobic conditions, propionate is degraded by propionate oxidizing bacteria to acetate and hydrogen. These products are then removed by acetoclastic and hydrogenotrophic methanogens. Hydrogen production was not measured in this study and therefore only the acetate responses were used in the simulations.

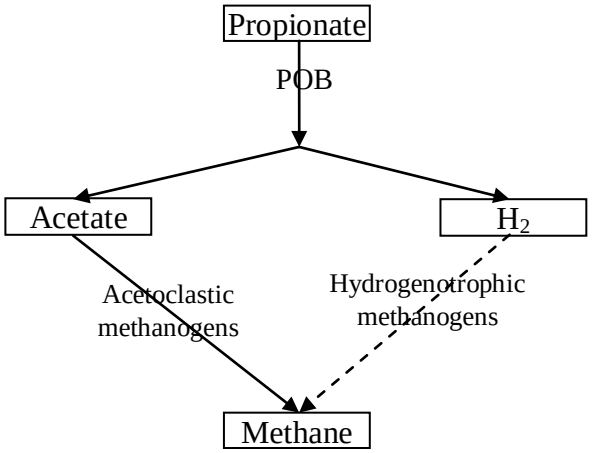


Figure 5.20. Schematic diagram of main pathways of propionate degradation

The process rates and the corresponding stoichiometry for the propionate degradation and acetoclastic methanogenesis processes are presented in Table 5.6. A Monod-type model was used to describe the propionate oxidation process. The temperature-correction for Henry's gas transfer constant was implemented according to the ADM1 manual (Batstone et al., 2002). The pH measured in the bottles was between 7 and 8.5, and hence no pH inhibition term was implemented in the rate equation. As previously explained, H_2 concentration was not believed to have inhibited propionate oxidation in the bottles and hence it was not considered in the simulation exercise.

Table 5-6. Stoichiometry and kinetic expression for POB and acetoclastic methanogenesis

Process	S_{pro}	S_{ac}	S_{H2}	S_{CH4}	X_{pro}	X_{ac}	rate
Propionate uptake	-1	(1- Y_{pro})0.57	(1- Y_{pro})0.43		Y_{pro}		$k_{max,pro}X_{pro}\frac{S_{pro}}{K_{s,pro}+S_{pro}}$
Acetate uptake		-1		1-Y_{ac}		Y_{ac}	$k_{max,ac}X_{ac}\frac{S_{ac}}{K_{s,ac}+S_{ac}}$

S_{pro}, S_{ac}, S_{H2}, S_{CH4}, X_{pro} and X_{ac} represent propionate, acetate, hydrogen, methane, POB biomass and acetoclastic methanogens biomass, respectively. Y_{pro} and Y_{ac} are yield coefficients for POB and acetoclastic methanogens, respectively. During propionate oxidation, propionate is utilized partly for biomass production and cell maintenance (Ypro) and the rest is converted to acetate (1- Y_{pro})0.57 and hydrogen (1- Y_{pro})0.43 (Batstone et al., 2002).

The simulation results for propionate utilization and acetate production are presented in Figure 5.21 for the mesophilic bottles and Figure 5.22 for the thermophilic bottles. The propionate degradation

response was reasonably fit using the Monod-type kinetics; while, the observed acetate concentrations demonstrated some deviation from the model in the early stage of simulation. However, overall the Monod-type kinetics was able to adequately simulate the acetate behaviour in the batch experiments. The deviation between the experimental data and model prediction of acetate was likely due to either inhibition by propionate (substrate inhibition) (Mosche and Jordening, 1999) or adaptation required by methanogens. Propionate inhibition of acetate utilization may occur at very high concentrations of propionate (50 mM or higher) (Mosche and Jordening, 1999; Pind et al., 2003); however, the propionate concentration in the batch experiments was much lower than this level. Hence, it would appear that the accumulation of acetate was not due to propionate inhibition of methanogens. It would appear to be more likely due to the time required by methanogens to acclimate to the bottle conditions. This can be further verified by examination of the simulation results obtained for the bottles inoculated from the 1st-phase digesters (M1 and T1). As can be observed in Figures 5.21B and 5.22B, the deviation was almost negligible and the model satisfactorily simulated the acetate concentration profile in the M1 and T1 bottles. As presented in Table 5.1, it can be seen that the propionate concentrations in both the M1 and T1 digesters were greater than the remaining digesters at the same temperature. Thus, it would appear that the biomass within the 1^{st}-phase digesters that had been previously exposed to higher concentrations of propionate adapted more readily to the relatively high concentrations (i.e., compared to the level in the source digesters) added to the bottles. Conklin (2004) reported that the fast-growing *Methanosarcina sp.* were more resistant to organic shocking loads as compared to the slow growing *Methanosaeta sp.* Thus, the biomass in the M1 and T1 bottles were acclimatized faster and started acetate consumption earlier.

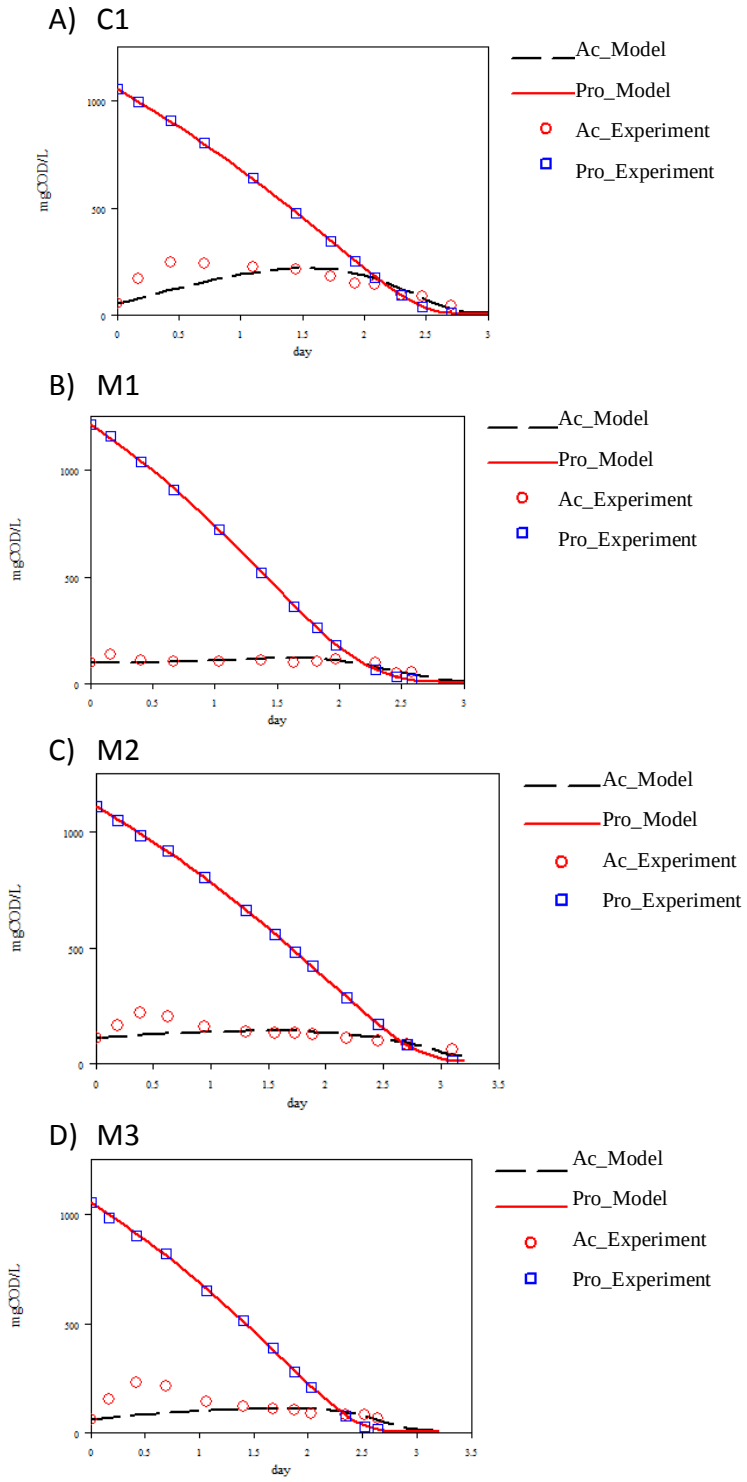


Figure 5.21. Comparison of propionate and acetate degradation between the experimental and simulated data in the mesophilic batch tests

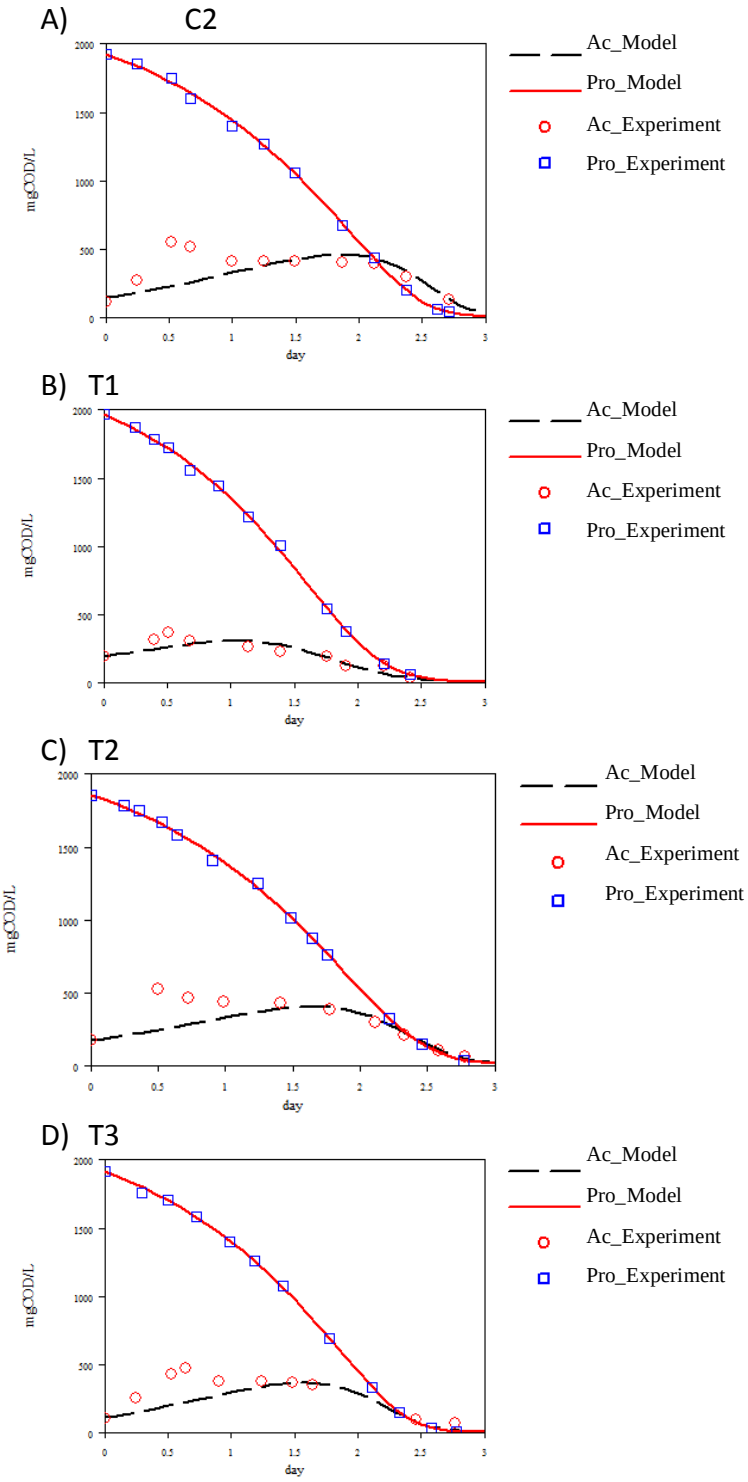


Figure 5.22. Comparison of propionate and acetate degradation between the experimental and simulated data in the thermophilic batch tests

5.4 Conclusions

The following conclusions were drawn from this study:

A kinetic assessment of POB in phased anaerobic digesters revealed that the operational conditions influenced the dominance of POB species developed in each phase. Fast-growing species with maximum specific growth rates of 11.0 d^{-1} and 23.7 d^{-1} were developed in the heavily loaded mesophilic and thermophilic 1^{st}-phase digesters, respectively; while those of the mesophilic and thermophilic 2^{nd}-phase digesters were, on average, 6.7 d^{-1} and 18.6 d^{-1}, respectively. This is the first known report of differentiation in kinetics of POBs under thermophilic temperatures.

Application of two or more sets of bio-kinetic parameters depending on the propionate level in each phase would result in the improvement of modeling the acetogenesis step of anaerobic digestion.

The relationship between bio-kinetic coefficients and temperature was found to be inconsistent for the phased digestion systems that were operated at different temperatures (i.e., thermophilic-mesophilic or mesophilic-thermophilic). Therefore, the use of Arrhenius-like equations for temperature correction of kinetic coefficients in modeling of POBs in such systems should be used cautiously.

Based on the low affinity of thermophilic species of POB for propionate, a thermophilic digester should be followed by a mesophilic digester to achieve a low level of propionate in the effluent.

Chapter 6

Assessment of Hydrolysis Kinetic Coefficients in Phased Anaerobic Sludge Digestion Systems

6.1 Introduction

Phased anaerobic digestion is a promising technology for the treatment of wastewater sludges. Phased digestion systems have been evaluated to determine the optimum operating conditions in terms of HRT, temperature, organic loading, configuration and pH in each phase. Most of the studies have focused on comparing the performance of phased digestion systems with that of single-stage digesters through the modification of the above-mentioned parameters and have indicated improved treatment performance for the phased digestion systems (Han et al., 1997; Skiadas et al., 2005; Watts et al., 2006; Bolzonella et al., 2007; Nges and Liu, 2009). However, underlying mechanisms that lead to better enhanced performance of the digestion systems have been investigated less often.

Anaerobic digestion models may be used for efficient design and operation of anaerobic sludge digestion as well as for the investigation of underlying processes (Lyberatos and Skiadas, 1999). Anaerobic digestion models are complex as they incorporate different biochemical and physico-chemical reactions including disintegration of particulate solids, hydrolysis of macromolecules, acidogenesis of soluble organic matter, acetogenesis of long and short chain fatty acids and finally methanogenesis from acetic acid and hydrogen (Tomei et al., 2009). Hydrolysis is usually the first step of anaerobic digestion that may or may not be the rate-limiting step, depending on the characteristics of the feed sludge. Several kinetic models have been suggested for modeling of hydrolysis process; however, this process is often expressed by a first-order relationship under constant pH and temperature (Batstone et al., 2002). The simplification of the hydrolysis process may lead to inaccuracy in describing the hydrolysis of particulate matter and hence it was recently suggested to use more elaborate models to consider the readily and slowly degradable fractions of the sludge (Yasui et al., 2008; Ramirez et al., 2009).

Modeling of phased anaerobic digestion has been reported less frequently than other configurations (Siegrist et al., 2002; Blumensaat and Keller, 2005). Parker (2005) examined several configurations of phased anaerobic digestion systems using ADM1 (Batstone et al., 2002). The results of this study showed some deviations (i.e., over- or under-prediction) in VFA production under low and/or high HRTs. In addition to the reasons cited in Parker (2005), the operating conditions of phased anaerobic digestion systems may lead to different hydrolysis rates in each phase, which are typically operated at different organic load, temperature and HRT.

Even though hydrolysis is an important step in anaerobic digestion and may be a rate limiting step, it is still a less defined process in anaerobic digestion modeling (Vavalin et al., 2008). Hydrolysis is a complex process and may be influenced by several parameters including particle size, pH, diffusion and adsorption of enzymes to particles, particulate concentration, biomass concentration, temperature and acclimation and origin of biomass (Vavalin et al., 2008; Gavala et al., 2003; Gavala and Lyberatos, 2001). Despite much research, the impact of staging on hydrolysis kinetics has not been investigated, although this would be useful in understanding the underlying mechanisms resulting in performance improvement in phased digestion systems. In addition, estimation of reliable kinetic coefficients for hydrolysis is essential for accurate modeling and design of phased anaerobic digestion systems because it may influence the overall system performance (Lee et al., 2009).

The aim of the present study was to investigate the hydrolysis kinetics in various configurations of phased and single-stage anaerobic sludge digesters. In this regard, the influence of phase separation on the kinetic coefficients of hydrolysis models was studied. In addition, the applicability of a temperature correction relationship to hydrolysis kinetic parameters in phased digestion systems with different temperatures was assessed.

6.2 Approach

This section describes the methodology used to assess the hydrolysis kinetics in the phased anaerobic digestion systems. Based on sensitivity analyses that have been conducted on the kinetic parameters in the anaerobic digestion model (ADM1) which have been reported in previous studies (Lee et al., 2009; Blumensaat and Keller, 2005), the hydrolysis kinetic parameter has been cited as an important parameter in optimizing modeling. A first-order function with regard to concentration of biodegradable particulate matter has been mostly used for modeling of hydrolysis processes (Eastman and Ferguson, 1981; Ge et al., 2011). Therefore, the impact of phase separation on hydrolysis kinetics was

investigated in this section. Biochemical methane potential tests that were used to estimate the biodegradable fraction of the feed sludge will be presented and then the batch experiments and parameter estimation of hydrolysis kinetics will be described.

6.2.1 Biochemical Methane Potential (BMP) Assay

The biochemical methane potential test is used to estimate the degradability of organic matter under anaerobic conditions. The BMP test may be defined as the anaerobic equivalent of the biochemical oxygen demand (BOD) test that is used to evaluate biodegradability under aerobic conditions. In this study these tests were conducted in 500 mL serum bottles. The digested sludge from the mesophilic single-stage digester was used as an inoculum. The raw sludge (mix of PS+WAS) was taken from the Waterloo WWTP on the same days that the assays were set up. A mineral solution was prepared according to Angelidaki et al. (1990) to supply the essential nutrients and buffering capacity. A sludge volume of 250 mL was placed in the serum bottles and 50 mL of each of the inoculum and mineral solution were then added. Control bottles were prepared similarly but instead of the raw sludge 250 mL of de-ionized water was added to evaluate the inoculum contribution to the methane production. The bottles were sealed with septum and aluminum caps and kept in an incubator at 35 ^{0}C. Before placing the bottles in the incubator, 5 mL of liquid sample was taken to measure total and soluble COD. The tests were carried out for approximately 70 days. The biogas volume and composition were analyzed daily in the first 10 days of the test and then periodically for the remainder of the tests. The biogas composition was analyzed for CH_4 and CO_2 by injecting 1 mL of head space gas into a gas chromatograph. Since sludge characteristics vary over time in wastewater treatment plants, the experiments were performed at 3 different times and each time in triplicate. The biodegradable fraction was calculated using Equation 6.1. In calculation of the biodegradable fraction of raw sludge, the methane and COD values of the bottles (with raw sludge) were subtracted from those of the control bottles (without raw sludge) and hence the net values of methane and COD were used in Equation 6.1.

$$i_{biodeg} = \frac{V_{CH4}}{350 * M_{COD}} \qquad (6.1)$$

where,

ibiodeg = biodegradable fraction of sludge;

V_{CH4} = volume of total cumulative methane generated;

M_{COD} = mass of COD introduced to the bottle;

350 = volume of methane generation (mL) per g COD at STP;

6.2.2 Hydrolysis Batch Experiments

This section introduces the experiments that were carried out to study the influence of phase separation on the kinetics of hydrolysis of the particulate fraction of the sludge. The batch experiments were relatively similar to the BMP assays with some modifications.

6.2.2.1 Batch Experiments on Hydrolysis

The kinetic parameters for hydrolysis (first-order kinetic constant) were estimated from serum bottle batch tests that employed biomass that was obtained from the source digesters under study. The experiments were carried out in 500 mL serum bottles sealed with butyl rubber stoppers and aluminum crimps. A known amount of digesting sludge as inoculum was taken from each digester and immediately transferred to the Wheaton bottles that were pre-filled with nitrogen gas to minimize oxygen exposure. De-ionized water that was purged with nitrogen gas and kept at the same temperature from which the biomass was taken was employed to dilute the inoculum. Fresh mixed primary and waste activated sludge taken from the Waterloo WWTP was kept in a water-bath with a temperature of 35-40 ^{0}C during the experiment set-up to minimize temperature shock to the biomass. A mineral solution containing nutrients, buffer, trace metals and vitamins was prepared according to Angelidaki et al. (1990). The experiments were carried out in triplicate. Each serum bottle was fed with the mixed sludge (250 mL), mineral solution (50 mL) and inoculum (50 mL), resulting in a total volume of 350 mL. Two more serum bottles were prepared as controls by adding 50 mL inoculum, 250 mL de-ionized water and 50 mL mineral solution to monitor the biomass degradation. The head space of the serum bottles was purged with nitrogen gas to remove oxygen. Finally, 3 mL of bromo-ethane sulfonate (BES) was added to each serum bottle to inhibit the activity of methanogens. This allowed for measurement of the cumulative production of VFAs. Since acidogenesis is not typically a rate-limiting step (Eastman and Ferguson, 1981), the inhibition of methanogenesis blocked utilization of hydrolysis and acidogenesis products. All the serum bottles were stored in an incubator at a temperature of either 35 or 55 ^{0}C depending on the temperature of the source digesters from which the inoculum was taken. Before placing the serum bottles in the incubator, a 3 mL liquid sample was taken by a syringe with a #18G needle to measure TCOD and ffCOD and pH. All the soluble COD measurements were carried out using a flocculated filtered COD (ffCOD) method that represents the true soluble COD within the samples. A similar measurement was performed for samples that were taken regularly during the experiments.

The biodegradable particulate of sludge is typically used to estimate the hydrolysis rate constant. The soluble COD measured at the end of the BMP test (~ 70 days) was assumed to be the inert soluble ($SCOD_{I,e}$) (Melcer et al., 2003). The particulate inert COD at the end of the test ($PCOD_{I,e}$) was calculated from Equation 6.2.

$$PCOD_{I,e} = TCOD_e - SCOD_{I,e} \quad (6.2)$$

where $TCOD_e$ is total COD at the end of the test. Assuming that inert PCOD would be the same as effluent in the course of the batch experiments, the particulate COD ($PCOD_t$) at each time step was calculated from Equation 6.3.

$$PCOD_t = TCOD_t - SCOD_t \quad (6.3)$$

Accordingly, the biodegradable PCOD (bPCOD) at each time step was calculated from Equation 6.4.

$$bPCOD_t = PCOD_t - PCOD_{I,e} \quad (6.4)$$

The COD values of the control bottles were subtracted from those of the sample bottles to exclude the COD of the inoculum. Therefore, the COD data used in the parameter estimation exercise of hydrolysis kinetics contained only raw sludge COD.

6.2.2.2 Hydrolysis Kinetics

Hydrolysis rate constants (K_{hyd}) for the various digesters were determined by fitting a first-order model (Equation 6.6) to the biodegradable particulate COD (F) profile obtained from the batch experiments. Alternatively, a dual-pathway first-order model (Equation 6.11) was fit to the biodegradable pCOD (F) data to evaluate which model better described the data. The single-pathway model was:

$$\frac{dF}{dt} = -K_{hyd}.F \quad (6.5)$$

Integration of equation 7-5 yields:

$$\frac{F}{F_0} = EXP(-K_{hyd}.t) \quad (6.6)$$

The dual-pathway model for hydrolysis was:

$$\frac{dF_s}{dt} = -K_{hyd,s}.F_s \quad (6.7)$$

$$\frac{dF_r}{dt} = -K_{hyd,r}.F_r \quad (6.8)$$

Given the sum of Fs and Fr is F, integration of Equations 6.7 and 6.8 yields:

$$F = F_{0,s}.EXP(-K_{hyd,s}.t) + F_{0,r}.EXP(-K_{hyd,r}.t) \quad (6.9)$$

$$F_0 = F_{0,s} + F_{0,r} \quad (6.10)$$

Equation (6.10) can be re-arranged to replace it in Equation (6.9) to yield Equation 6.11.

$$\frac{F_{0,s}}{F}EXP(-K_{hyd,s}.t) + \frac{F_0 - F_{0,s}}{F}EXP(-K_{hyd,r}.t) = 1 \quad (6.11)$$

After estimation of $F_{0,s}$ through parameter estimation exercise, then, $F_{0,r}$ can explicitly be determined using Equation (6.10).

where,

F = biodegradable particulate COD at time t, in mg/L;

F_0 = initial biodegradable particulate COD, in mg/L;

$F_{0,s}$ = initial slowly biodegradable particulate COD, in mg/L;

$F_{0,r}$ = initial rapidly biodegradable particulate COD, in mg/L;

K_{hyd} = first-order kinetic constant, in day;

$K_{hyd,s}$ = first-order kinetic constant for slowly biodegradable COD, in day;

$K_{hyd,r}$ = first-order kinetic constant for rapidly biodegradable COD, in day;

t = time, in day.

A nonlinear regression was performed using the nlinfit function of Matlab to estimate the first-order coefficients of the hydrolysis rate as well as the initial slowly and rapidly biodegradable organic matter through fitting Equation (6.11) to the biodegradable particulate COD degradation data. As the Matlab function returns the fitted parameters, residuals, estimated covariance matrix for the fitted parameters and mean squared error (MSE), the nlparci function was further used to find the 95% confidence interval for the estimated parameters.

6.3 Results and Discussion

6.3.1 Biochemical methane potential (BMP) test

The estimation of hydrolysis kinetic parameters required that the biodegradable fraction of particulate matter be known. The BMP assay is a batch test supplied with enough biomass and carried out

under controlled conditions (Owen et al., 1979) that can be used to measure the biodegradable fraction of organic matter under anaerobic conditions. Based on the method described in Kianmehr (2010), a series of BMP tests was carried out on sludge samples taken from the Waterloo wastewater treatment plant at three different dates (June, August and October 2010) to determine the biodegradable fraction of the sludge. Similar to the approach used in the previous chapters, the lack of mass balance was determined for each bottle to check the reliability of the results.

The cumulative methane production for one of the BMP tests is presented as an example in Figure 6.1, representing the methane production after correction for the inoculum methane production. The three replicate bottles (R1, R2 and R3) produced relatively similar methane gas, indicating a good reproducibility for the test. The methane production from the triplicate bottles was statistically analyzed using an ANOVA test (α=0.5) and no significant difference was found among the bottles (p_{value}>>0.05). The test was run for over 70 days and when the methane production was negligible it was stopped. As can be observed from Figure 6.1, most of the methane was produced within the first 30 days of the experiments and then the methane production continued at a slower rate.

Table 6.1 represents methane production and COD characterization for the three runs of the BMP tests for three different dates. The values for each run are the average and standard deviation (SD) of triplicate bottles. The raw sludge values indicate the net methane production and COD after subtraction of the values from the control (without raw sludge) bottles. The methane volume produced was calculated for STP conditions for mass balance calculations. In general, the mass balance deviations were less than 4% for the bottles and found to be within an acceptable range. Overall a reasonable agreement was found between the mass of COD at the beginning and end of the BMP tests and the methane data.

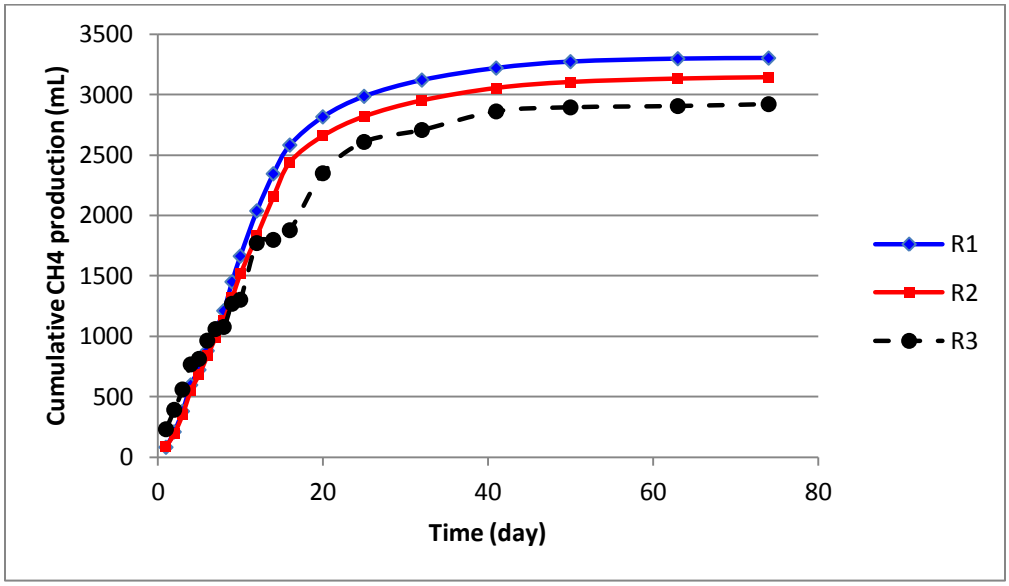


Figure 6.1. Cumulative methane production in typical BMP test

Table 6-1. COD characterization of the BMP tests

Parameters	Run 1		Run 2		Run 3	
	Raw sludge	Inoculum	Raw sludge	Inoculum	Raw sludge	Inoculum
CH_4, mL	3122±191	75±6	2657±119	83±5	2993±106	107±11
$TCOD_{in}$, g/L	41.8±1.7	2.6±0.05	36.2±1.2	2.7±0.07	39.5±1.4	3.2±0.09
$SCOD_{in}$, g/L	4.1±0.6	0.3±0.02	3.4±0.5	0.3±0.02	2.9±0.1	0.2±0.08
$PCOD_{in}$, g/L	37.7±1.5	2.4±0.04	32.7±1.3	2.4±0.09	36.5±1.4	3.0±0.1
$TCOD_{out}$, g/L	16.3±1.4	2.0±0.08	14.6±0.4	2.1±0.07	15.3±0.5	2.4±0.09
$SCOD_{out}$, g/L	2.7±0.8	0.13±0.02	2.6±0.5	0.17±0.06	1.9±0.3	0.1±0.03
$PCOD_{out}$, g/L	13.4±1.3	1.9±0.09	12.0±0.9	1.9±0.08	13.4±0.3	2.3±0.1
Mass						
CH_4, gCOD	8.9±0.5	0.2±0.01	7.6±0.3	0.24±0.01	8.5±0.3	0.3±0.03
$TCOD_{in}$, g	14.6±0.6	0.9±0.02	12.6±0.4	0.96±0.02	13.8±0.5	1.1±0.03
$TCOD_{out}$, g	5.7±0.5	0.7±0.03	5.1±0.16	0.72±0.02	5.4±0.2	0.8±0.03
Mass balance, %	-3 to 4	-2.5 to 1.4	-4 to 3	-1 to 1.7	- 2 to 1.3	-2 to 1
Biodegradability	0.62		0.59		0.61	

The biodegradable fraction of the sludge was determined using Equation 6.1. For calculation of biodegradability of raw sludge, the net values of the total influent COD and methane production (i.e., after subtraction from the influent COD of inoculum and methane production of the control bottles) were employed. The methane produced in each bottle was divided by 350 to obtain the mass of CH_4-COD at STP. Then, the CH_4-COD was divided by the total influent COD mass of each bottles to determine the biodegradable fraction of the raw sludge. The biodegradable fraction of the sludge from the three runs ranged 0.59-0.62 of the total sludge. The average biodegradable fraction (0.61) was found to be lower than the value reported for primary sludge (Parkin and Owen, 1986; Rameriz et al., 2011), while it was higher than the typical values reported for waste activated sludge (Parker, 2005). This was due to the use of a mixed primary sludge and WAS in the experiments.

6.3.2 Hydrolysis batch experiments: initial conditions

The initial conditions in the hydrolysis batch experiments are presented in Table 6.2. The inoculum from the source digesters was diluted to have approximately equal amounts of biomass in the bottles of similar temperature. Yasui et al., (2009) reported that the hydrolysis rate of the rapidly biodegradable fraction of a sludge was influenced by both the initial biomass and substrate concentrations, while the slowly biodegradable fraction of sludge was only affected by the initial concentration of sludge (as substrate). As such, the inocula from the digesters were diluted to make the estimated hydrolysis kinetic parameters, especially the dual-pathway model, more comparable. Before addition of the mixed primary and secondary sludges as substrate to the bottles, samples were taken from each bottle to ensure approximately equal amounts of inoculum were introduced to them. Statistical analysis of the biomass COD values in the bottles at similar temperature demonstrated no significant difference among the bottles (ANOVA PostHoc test, α=0.05). As presented in Table 6.1, the F/M (food to microorganism) ratios for the mesophilic bottles were approximately the same and were, on average, 2.56. A similar result was obtained for the thermophilic bottles with an F/M ratio of 2.37.

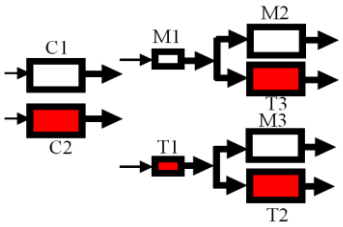


Table 6-2. Initial conditions of hydrolysis batch experiments

Bottle	Raw sludge		Inoculum		F/M ratio
	COD, mg/L		COD, mg/L		
	TCOD	ffCOD	TCOD	ffCOD	
Mesophilic					
C1	37847±922	3203±229	14339±427	215±16	2.64
M1	38751±1142	3484±147	15215±407	383±14	2.54
M2	39534±995	3318±120	15668±713	131±7	2.52
M3	38045±1079	3353±223	14718±258	128±9	2.58
Thermophilic					
C2	43088±752	4210±174	18484±500	313±12	2.33
T1	42778±670	4501±142	18685±417	387±25	2.29
T2	42543±1151	4611±271	18973±683	343±17	2.24
T3	43271±1065	4282±319	17684±733	320±18	2.44

6.3.3 pH of the hydrolysis batch experiments

Although buffer was added to the bottles, due to the inhibition of methanogenesis it was expected that the pH in the hydrolysis batch experiments would drop and might make the test condition incomparable to that of the source digesters. Therefore, pH was measured over the course of the batch experiments to determine how it changed. The resulting pH values over time for the mesophilic batch tests are shown in Figure 6.2. Overall, the pH values in all the bottles decreased over time. As methanogenesis was inhibited in the bottles, the hydrolysis and acidification of organic matter caused the pH within the bottles to drop from the beginning value of about 8. The final pH values for the mesophilic bottles ranged between 5.5 and 6.7. These values were somewhat different from the pH values observed in the source digesters (Figure 6.3), however, those of the M2, M3 and C1 bottles were still within the neutral range.

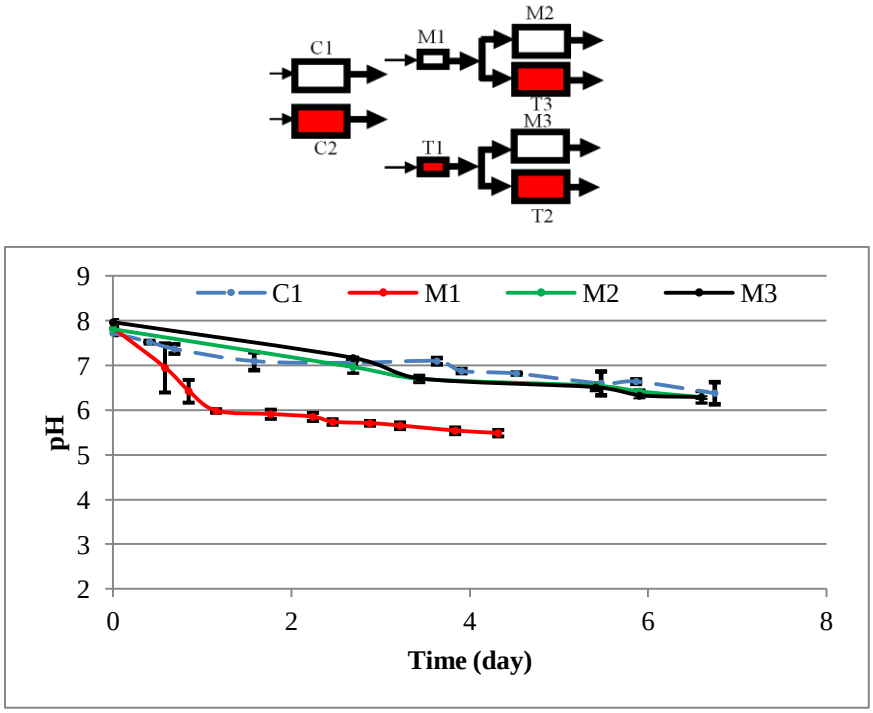


Figure 6.2. pH values over time in mesophilic batch tests on hydrolysis kinetics

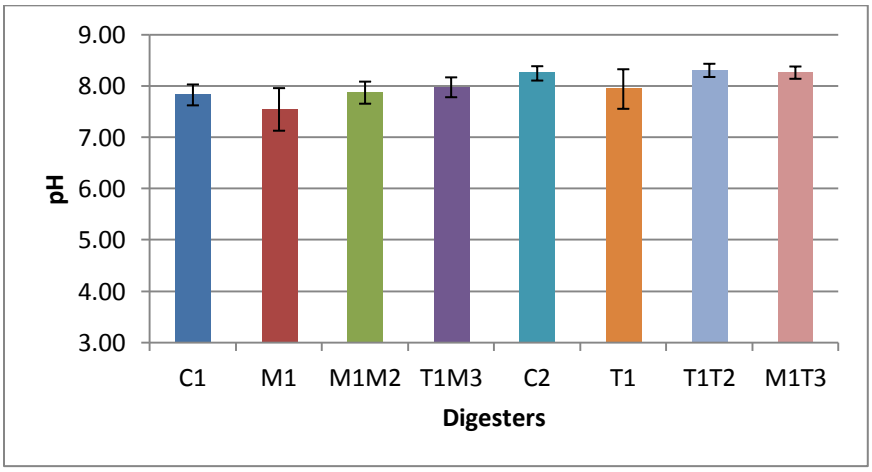


Figure 6.3. Average pH measurements in the phased and single-stage anaerobic digesters (Average±SD)

Ghosh (1987) has demonstrated that pH influences the rate of hydrolysis. However, there are recent publications showing that hydrolysis rate was improved under neutral pH or alkaline conditions (Gomec and Speece, 2003; Zhang et al., 2005; Ge et al., 2011). Therefore, the hydrolysis rate in the M1 bottle might have been influenced due to the relatively different pH in the test bottle with that of the source digester (M1).

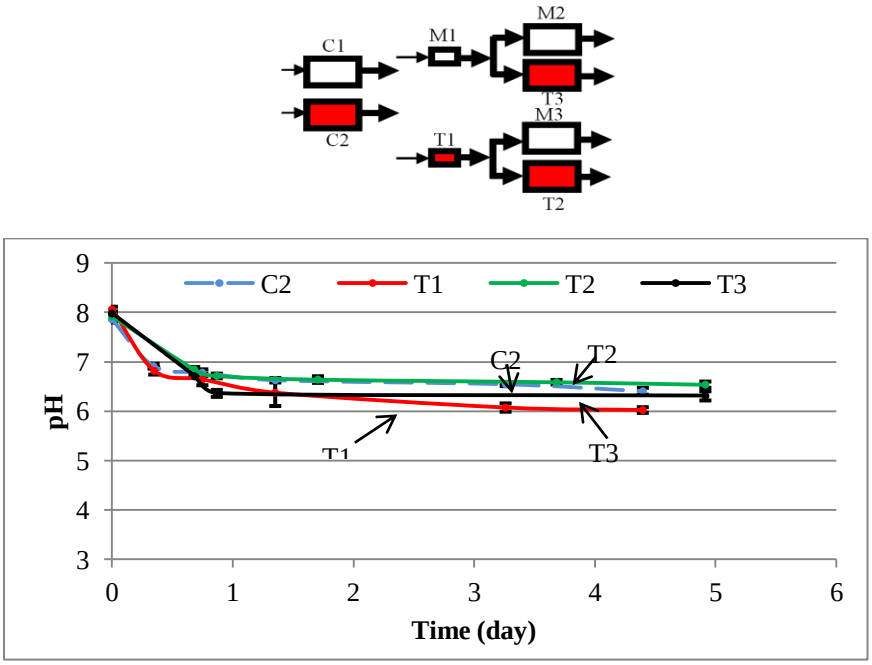


Figure 6.4. pH values over time in thermophilic batch tests on hydrolysis kinetics

The pH values of the thermophilic bottles are shown in Figure 6.3, showing a range of 6 to 6.5. Similar to the results found for the mesophilic bottles, the pH values in the thermophilic test bottles were also lower compared to the pH values of the thermophilic source digesters. As mentioned above, this was due to the inhibition of methanogenesis and accumulation of VFAs in the bottles. However, the pH of the thermophilic bottles was within the neutral pH range and hence it would not influence the estimation of hydrolysis parameters severely.

6.3.4 Residual analysis

The Matlab function employed (nlinfit) uses a nonlinear least square technique to find a minimum of the objective function, which in this study was the sum of square of errors, while seeking for the optimum estimates of the parameters. Therefore, it was necessary to check the assumptions of nonlinear regression. A similar approach to that described in the previous chapters was employed to assess the normal distribution and homoscedasticity of residuals. Since the dual hydrolysis model (Equation 6.7) was better described the degradation of the particulate matter in the test bottles (described in Section 6.3.5), therefore the residuals between this model and the experimental data were only employed in this section for analysis. The results of residual analyses for the normal distribution of errors are depicted in Figures 6.4 and 6.5 for the mesophilic and thermophilic batch experiments. Graphical representation of the residuals demonstrated a linear plot, indicating normal distribution of errors (Knightes and Peters, 2000; Marshall et al., 1995).

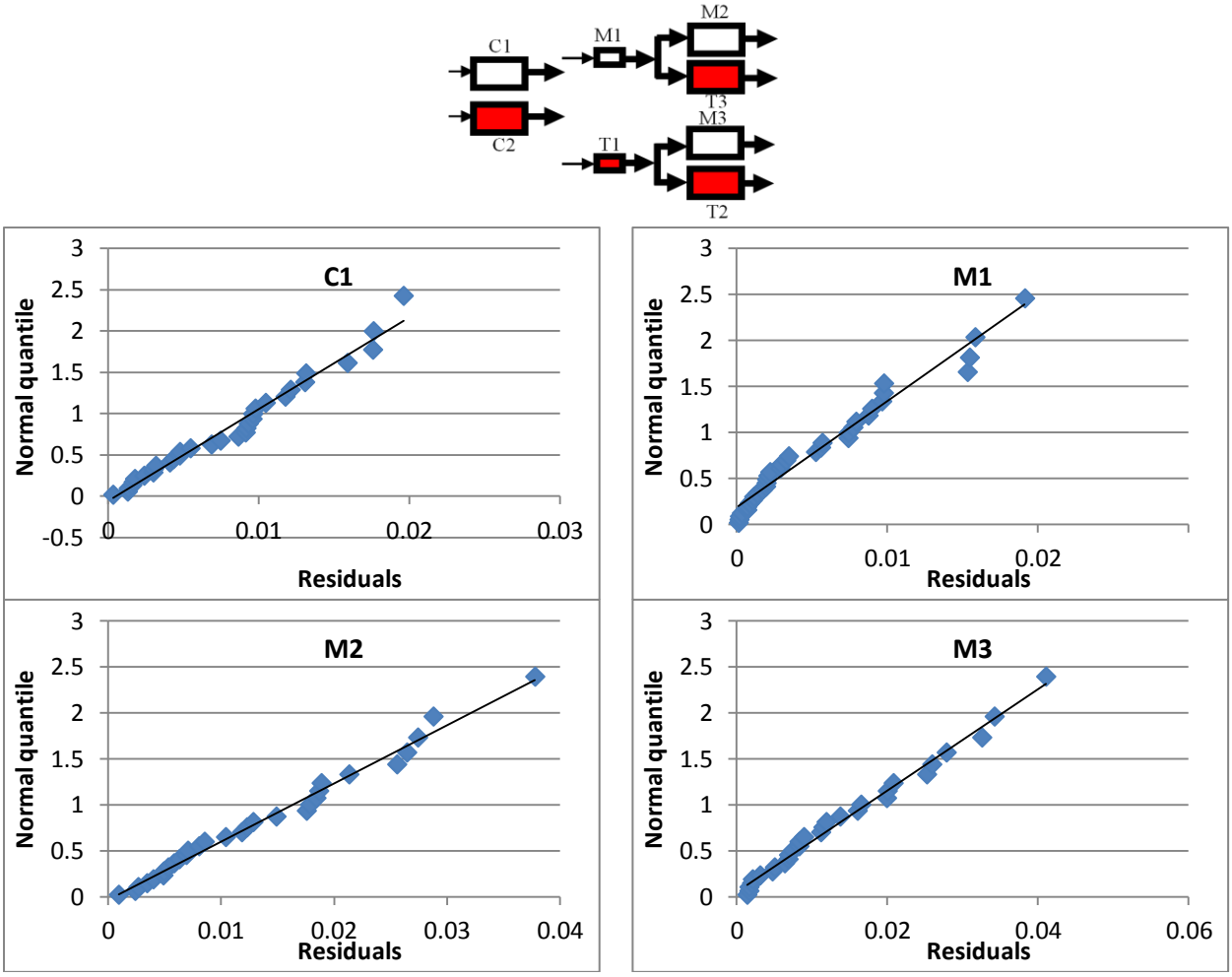


Figure 6.5. Normal probability plots of residuals for the mesophilic hydrolysis batch experiments

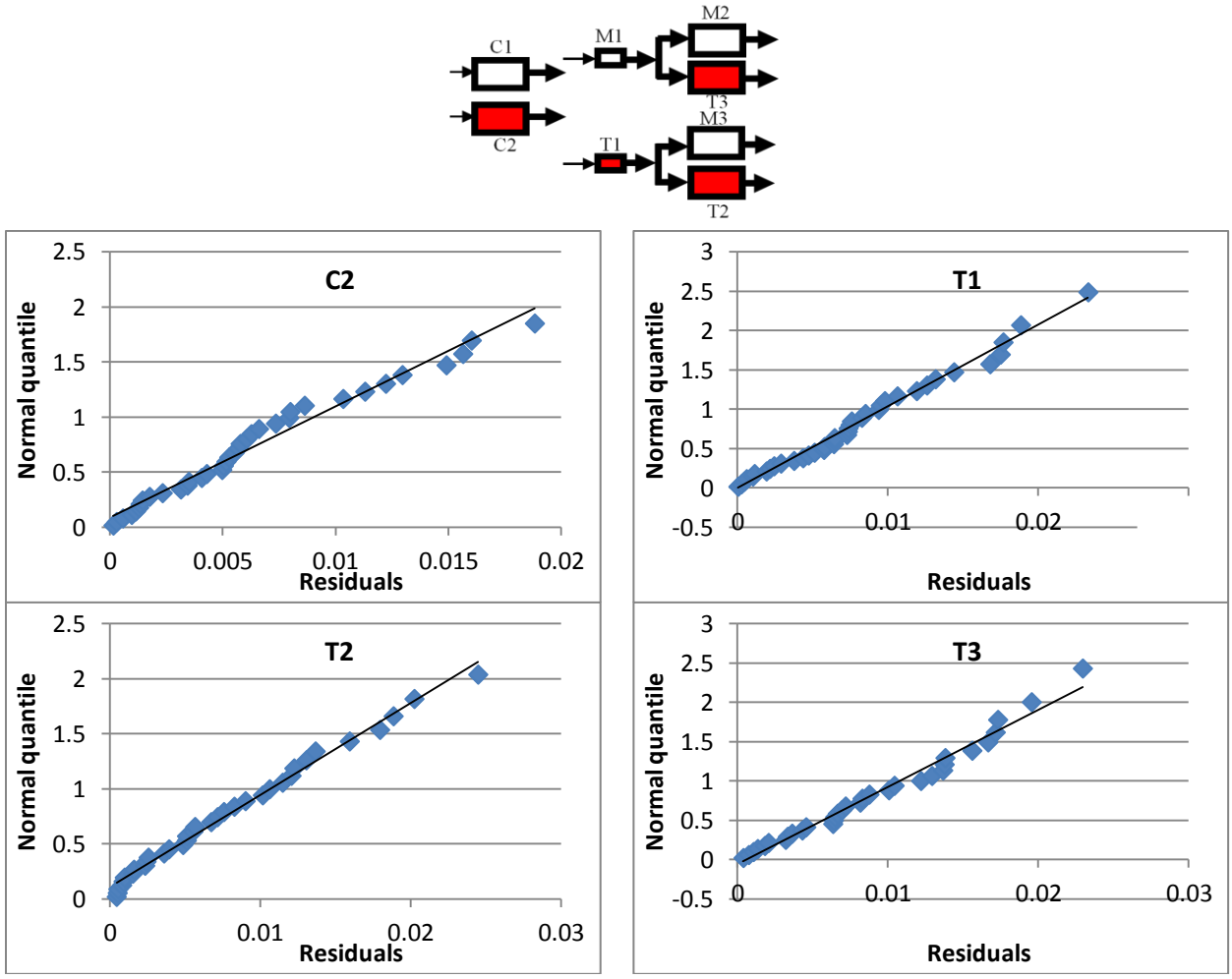


Figure 6.6. Normal probability plots of residuals for the thermophilic hydrolysis batch experiments

The residuals were depicted against the model predicted values to test their homoscedasticity (Dochain and Vanrolleghem, 2001). The results are given in Figures 6.6 and 6.7 for the mesophilic and thermophilic batch experiments. As can be observed from Figures 6.6 and 6.7, the residuals exhibited a trendless plot, indicating a constant variance for the residuals.

Based on the analysis of residuals characteristics, it was inferred that the assumptions of the nonlinear regression were met and further statistical comparisons of the estimated parameters from the different digesters was permissable.

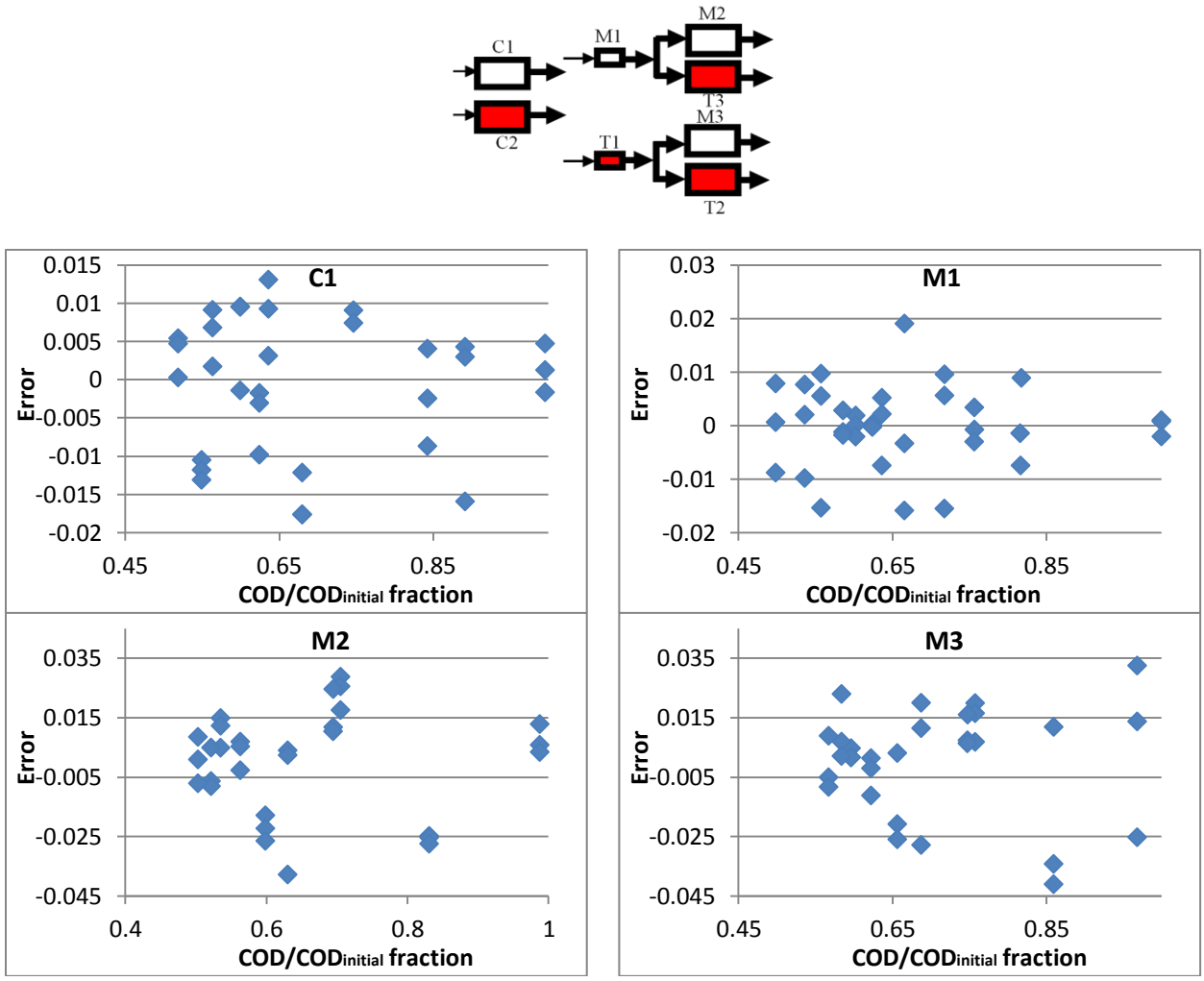


Figure 6.7. Residuals vs. model predicted values for the mesophilic hydrolysis batch tests

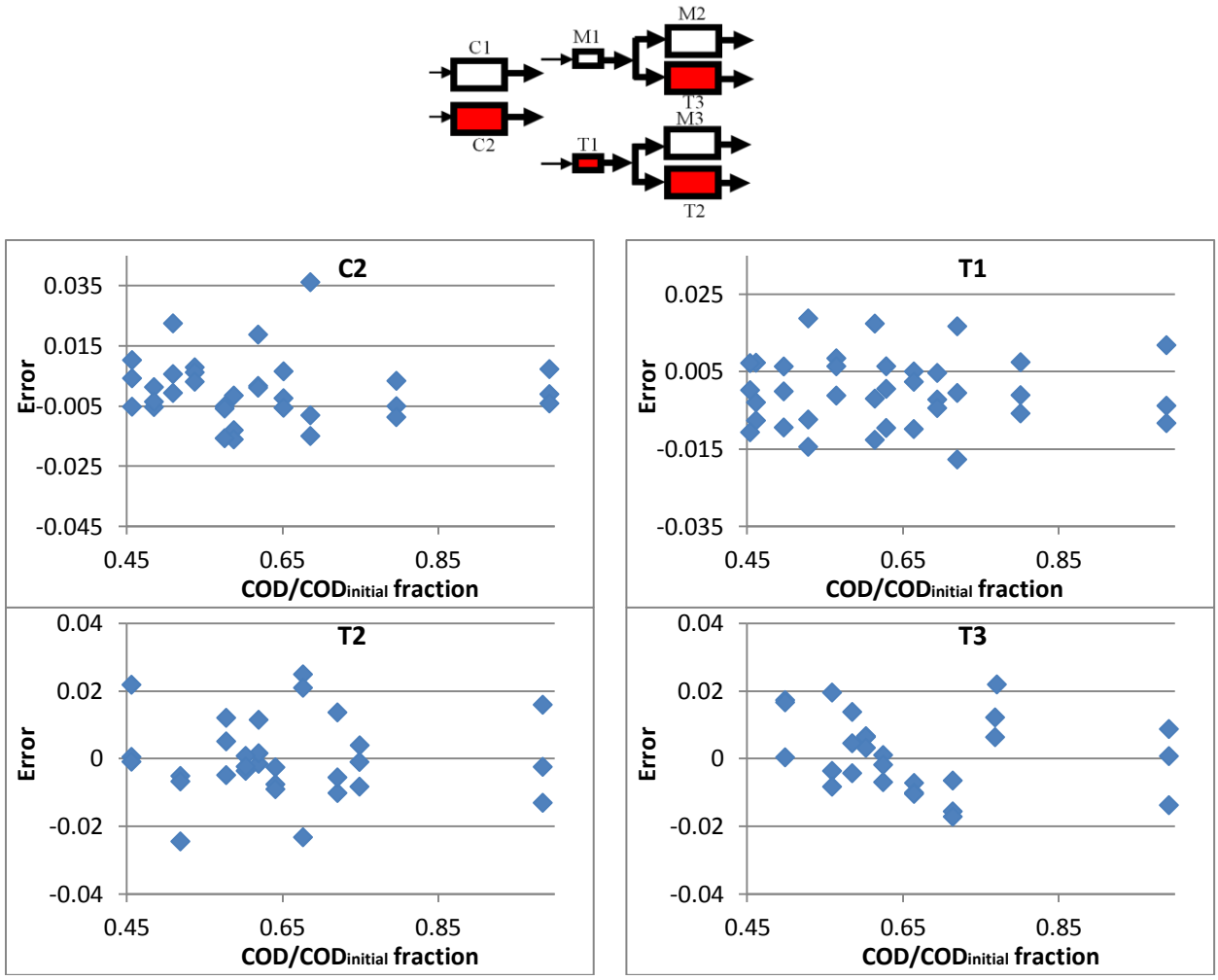


Figure 6.8. Residuals vs. model predicted values for the thermophilic hydrolysis batch tests

6.3.5 Model selection: Single-pathway hydrolysis vs. dual-pathway hydrolysis

Particulate degradation has mostly been implemented in anaerobic digestion models using first-order kinetics (Equation 6.4) with regards to the biodegradable particulate fraction of wastewater sludge (Batstone et al., 2002; Seigrist et al., 2002). For example the IWA anaerobic digestion model (ADM1, Batstone et al., 2002), which is a powerful tool for study of anaerobic digestion process, used simplified first-order kinetics for hydrolysis of particulate matter. However, some researchers have suggested employing a more complex hydrolysis model considering rapidly biodegradable and slowly biodegradable fractions of the particulate solids of sludge (Sollfrank and Gujer, 1991; Straub et al., 2006; Ramirez et al., 2009).

It has been shown that the nature of hydrolysable matter in the primary sludge (PS) and waste activated sludge (WAS) is different (Yasui et al., 2008). PS is mostly composed of slowly hydrolysable matter (X_s), whereas WAS is mainly heterotrophic biomass (X_H). The X_s fraction of PS and WAS is also different as the X_s of the former contains settleable solids, while that of the latter is mostly colloidal. These components have been reported to hydrolyze with different rates under anaerobic conditions (Yasui et al., 2008; Yasui et al., 2006). On the basis of this background, both hydrolysis models (Equations 6.6 and 6.14) were employed to examine which model best described the hydrolysis process in the phased digestion systems.

The results of fitting the single- and dual-pathway models to the solubilisation of biodegradable fraction of the observed particulate COD for the mesophilic and thermophilic batch experiments are presented in Figures 6.8 and 6.9. In both cases, the dual-pathway hydrolysis model better fit the particulate COD solubilisation as compared to the single-pathway model. For a given data set and fitting a nonlinear model, the goodness of fit may be measured using root mean square error (RMSE) of fitting exercise (Kemmer and Keller, 2010). On the basis of visual examination of the curves presented in Figures 6.8 and 6.9 and much smaller RMSE it was concluded that the dual hydrolysis model described the particulate COD solubilisation much better. The RMSE of the both models for the mesophilic and thermophilic batch experiments are shown in Table 6.3.

A better fit of the dual-pathway model was likely due to the presence of slowly and rapidly hydrolysable materials in the mixed sludge that underwent solubilisation at different rates. The single hydrolysis mechanism failed to fit the first part of the experimental data (less than 2 days) that would most probably indicate the hydrolysis of rapidly hydrolysable particulate matter fraction of the raw sludge (Yasui et al., 2008).

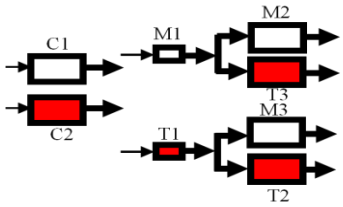


Table 6-3. Root mean square error (RMSE) of fitting dual and single hydrolysis models

Digesters	Fitting RMSE, (mg/L)	
	Dual	Single
Mesophilic		
C1	0.009	0.052
M1	0.007	0.068
M2	0.018	0.075
M3	0.018	0.065
Thermophilic		
C2	0.011	0.098
T1	0.009	0.081
T2	0.011	0.080
T3	0.012	0.077

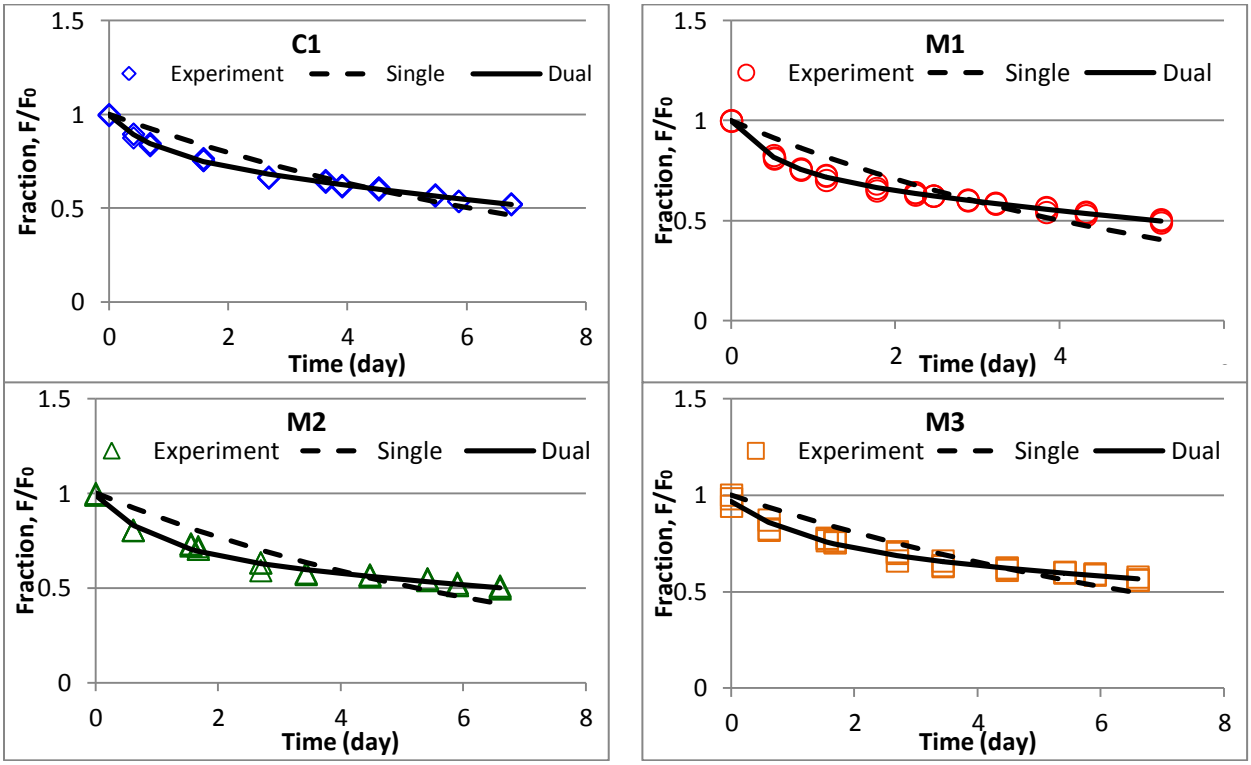


Figure 6.9. Fitting of single- and dual-pathway hydrolysis models to biodegradable particulate COD for the mesophilic batch experiments

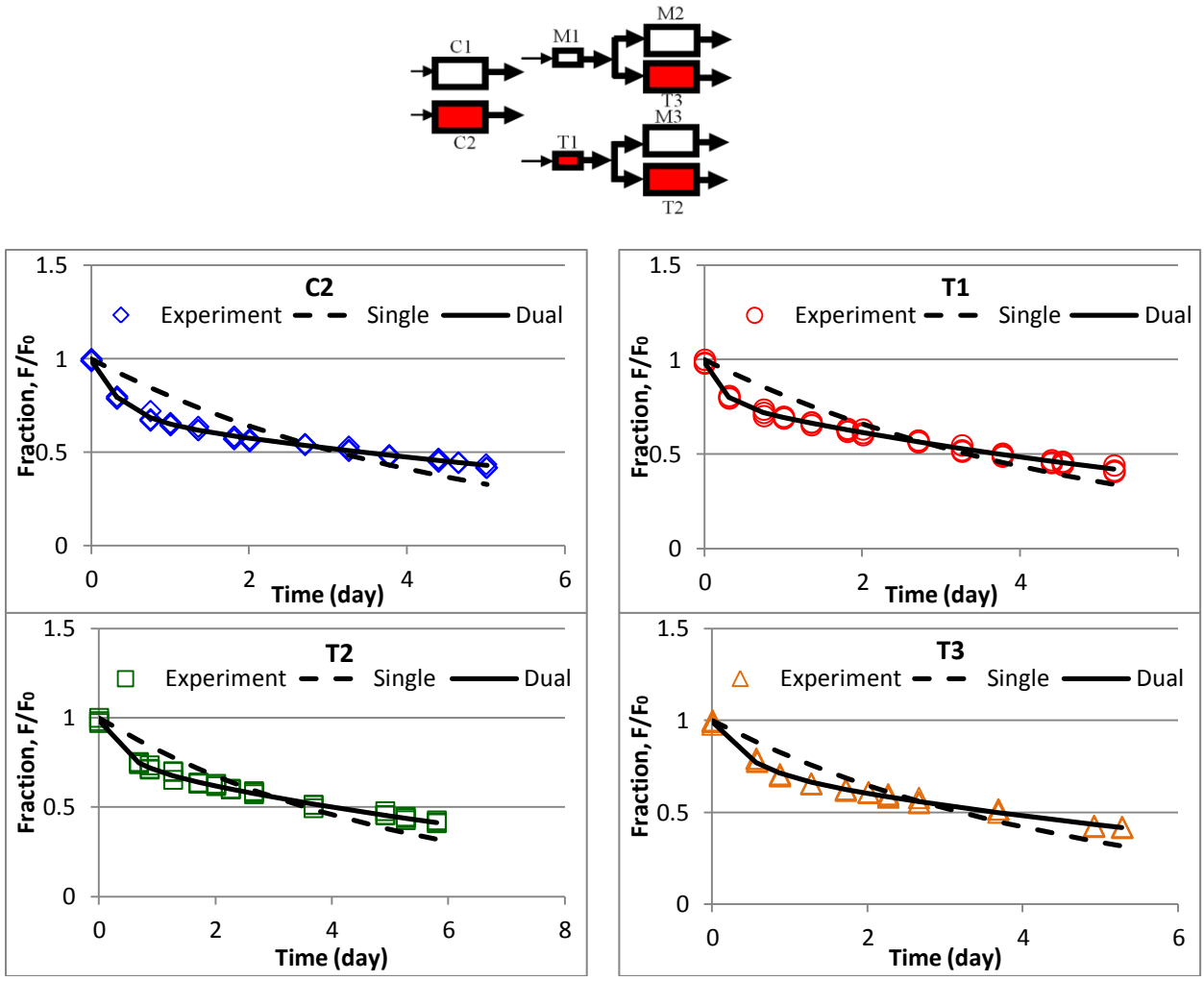


Figure 6.10. Fitting of single- and dual-pathway hydrolysis models to biodegradable particulate COD for the thermophilic batch experiments

6.3.6 Parameter estimation of hydrolysis kinetics

The rapidly hydrolysable ($F_{0,r}$) and slowly hydrolysable ($F_{0,s}$) fraction of COD were estimated in the parameter estimation exercise. Since the source of the raw sludge used in the batch experiments was the same (i.e., a mixed sludge taken from the Waterloo Treatment Plant), estimates determined for the slowly ($F_{0,s}$) and rapidly hydrolysable ($F_{0,r}$) matter were relatively comparable amongst the tests. The average values of $F_{0,s}$ and F0,r were 0.59±0.036 and 0.41±0.036, respectively. These $F_{0,s}$ and $F_{0,r}$ values were multiplied by the total biodegradable fraction (i.e, 0.61) to determine the fraction of these components in the raw sludge. The $F_{0,s}$ fraction of the raw sludge was 36% and that of the $F_{0,r}$ was 25%.

Values of 44 and 31% have been previously reported for the rapidly and slowly hydrolysable fractions of a primary sludge (Straub et al., 2006). The lower values of this study compared to those of Straub was likely due to the type of the sludge used in this research. As already mentioned, PS is a richer source of readily hydrolysable matter, while WAS contains more slowly hydrolysable material. In this regard, Yasui et al. (2006) who studied hydrolysis of WAS reported a value of 53% for the slowly and 20% for the rapidly hydrolysable particulates. Hence, it was concluded that the $F_{0,s}$ and $F_{0,r}$ values estimated by the dual hydrolysis model were reliable. Also, estimation of comparable $F_{0,s}$ and $F_{0,r}$ values for different test bottles supported the reliability of the estimation approach used.

The hydrolysis rate coefficients ($K_{hyd,r}$ and $K_{hyd,s}$) and the 95% confidence intervals estimated in this study for the various digesters are presented in Table 6.4. The coefficients were compared with values reported in the literature to determine the reliability of the parameter estimation approach. The hydrolysis coefficients for the rapidly hydrolysable (K_{hyd_r}) fraction of pCOD were within the range of values reported elsewhere (Orhon et al., 1998; Straub et al., 2006; Yasui et al., 2008). However, the $K_{hyd,s}$ values were lower than those reported in the literature. Depending on the characteristics (i.e., protein, carbohydrate and lipid, inert fractions) of the tested sludge, hydrolysis rate could be different. Thus, the lower $K_{hyd,s}$ values were likely due to the characteristics of the mixed sludge used in this study. In addition, the hydrolysis rate of lipids might have been slightly influenced due to the inhibition of methanogens in the test bottles (Vavalin et al., 2008). However, for the purpose of relative comparison of hydrolysis rate in the stages of the various phased digestion system this would not be major concern. Overall the values for K_{hyd_s} were within the values reported by Gujer and Zehnder (1983) who reviewed literature on the hydrolysis of proteins and lipids of various substrates. Therefore, considering that the results of this study were within the wide range of literature values it was inferred that the estimation technique applied was reliable.

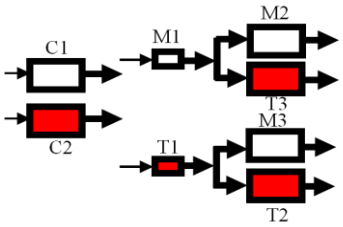


Table 6-4. Hydrolysis coefficients with 95% confidence intervals

Digesters	Rapidly biodegradable	Slowly biodegradable
	K_{hyd-r}, d^{-1}	K_{hyd-s}, d^{-1}
Mesophilic		
C1	1.38±0.33	0.075±0.007
M1	1.88±0.27	0.094±0.006
M2	1.06±0.42	0.061±0.018
M3	0.82±0.49	0.050±0.023
Thermophilic		
C2	2.76±0.42	0.121±0.008
T1	4.63±0.96	0.133±0.006
T2	2.55±0.68	0.118±0.009
T3	2.29±0.49	0.124±0.008

As it was shown that the residuals had a normal distribution with a constant variance, the ydrolysis rate coefficients for the 1st- and 2nd-phase digesters at the same temperature were compared on the basis of the 95% confidence intervals. If the confidence interval of the hydrolysis rate coefficients for the 2nd-phase digester overlapped with that of the 1st-phase digester, the coefficients were inferred as "not different" (Marshall et al., 1995; Montgomery, 2001). A comparison of the estimated coefficients for the mesophilic digesters revealed that the hydrolysis coefficients (both $K_{hyd,s}$ and $K_{hyd,r}$) of the M1 digester were greater than those of the M2 and M3 digesters. A similar approach was used to compare the hydrolysis coefficients of the thermophilic digesters and it was observed that the $K_{hyd,r}$ value of the T1 digester differed from those of the T2, T3 and C2 digesters; whereas, the hydrolysis rate of slowly hydrolysable matter (i.e., $K_{hyd,s}$) did not differ significantly among these digesters.

Given that some of the above-mentioned parameters such as pH, particle size, temperature and biomass to substrate concentrations were approximately similar in the batch test bottles incubated at either the mesophilic or thermophilic conditions, greater hydrolysis rates in the M1 (both $K_{hyd,s}$ and $K_{hyd,r}$) and T1 (only $K_{hyd,r}$) may be attributed to several reasons. The raw sludge fed to the source digesters through the study included a mixture of PS and WAS. A major fraction (55-75%) of WAS is composed of active

heterotrophic biomass (Parker et al., 2008). It was likely that part of this active biomass (i.e, mainly facultative bacteria) remained active under the anaerobic conditions of the 1st-phase digesters and contributed to the hydrolysis process. A comparison of the bacterial DGGE profile of the feed, M1 and T1 digesters further supported this explanation. From the DGGE profile, it can be observed that many of the bands of the feed appeared in the DGGE profile of the T1 and more apparently of the M1 digesters. Gioannis et al. (2008) reported an improved VS reduction (63%) for a phased anaerobic digester treated municipal solid waste through continuously adding WAS to the hydrolytic-acidogenic stage to improve hydrolysis process.

As already stated, the hydrolysis rate of substrate depends much on the origin and the previous acclimation of the anaerobic biomass (Gavala et al., 2003; Gavala and Lyberatos, 2001). It would be expected that major fraction of the particulate COD would be hydrolyzed and acidified in the 1st-phase digesters and only slowly hydrolysable particulates would remain for further hydrolysis in the 2nd-phased digesters. This may induce the hydrolytic-acidogenic biomass of each phase to develop different enzymatic systems. Due to the lower organic loading in the single-stage digesters (i.e., C1 and C2), it would appear that a hydrolytic biomass with lower enzymatic activity resulted in low hydrolysis rates. Therefore, the biomass taken from the 2^{nd}-phase digester and single-stage digesters might have required a longer time to shift the enzymatic system for hydrolyzing the fresh WAS and PS added to the bottles.

Many different hydrolysis kinetic models such as second-order kinetics, surface-related kinetics and Contois kinetics have been used to describe the hydrolysis process of anaerobic digestion systems (Vavalin et al., 2008). As such, it should be pointed out that due to the complexity of the hydrolysis mechanism, the use of first-order kinetics (either single or dual hydrolysis model) are relatively simplistic and application and/or development of more complex hydrolysis models may be required to completely describe the hydrolysis process of the phased digestion systems. Also, it is possible that particulate degradation in each phase may behave differently. Schober et al (1999) reported that zero-order kinetics better described the hydrolysis process in the first-phase of a two-stage system. Thus, two different hydrolysis kinetic models may be applied in each phase to model the hydrolysis process of a phased digestion system. It is however challenging in practice to parameterize the more complex models that are available due to the large number of parameters that need to be estimated.

6.3.7 Effect of temperature on hydrolysis kinetics

Hydrolysis is influenced by several parameters among which temperature has gained more attention (Ramirez et al., 2009; Mottet et al., 2009; Ge et al., 2011). Hydrolysis kinetics are affected by temperature and are typically corrected using an Arrhenius relationship in modeling of sludge digestion processes (Siegrist et al., 2002; Efstathiou et al., 2003). In this study, the influence of temperature on hydrolysis kinetics was evaluated by comparing the kinetic coefficients obtained from the mesophilic and thermophilic batch experiments. A comparison of the mesophilic digesters with the thermophilic ones revealed that both K_{hyd_s} and K_{hyd_r} values were consistently higher in the thermophilic digesters. This observation was in agreement with literature on hydrolysis constants of anaerobic digestion, indicating increased hydrolysis rate constants as temperature increases (Pavlostathis and Giraldo-Gomez, 1991).

A comparison of the K_{hyd_s} and K_{hyd_r} values of the thermophilic digesters with those of the mesophilic digesters demonstrated that overall the hydrolysis rates in the thermophilic digesters were greater than those of the mesophilic ones. Accordingly, an examination of the hydrolysis kinetic parameters revealed that the K_{hyd_s} and K_{hyd_r} values for the T2 digester were 1.5 and 2.5 times greater than those of the T1 digester; whereas, the kinetic coefficients of the thermophilic 2^{nd}-phase digesters were, on average, 2.4 and 2.6 times higher than the corresponding mesophilic 2^{nd}-phase digesters (i.e., M2 and M2). For better examination of temperature influence, the values of the temperature dependence constant (θ) of the Arrhenius equation were calculated to determine whether this equation may be used for modeling of the thermophilic digester based on the hydrolysis coefficients estimated for the mesophilic one and vice versa. The temperature dependence constants (along with their confidence intervals) for the 1^{st}-phase digesters (M1 vs. T1) were 0.020±0.002 and 0.045±0.003 for K_{hyd_s} and K_{hyd_r} values. Based on the average values of the hydrolysis kinetic coefficients, the θ values for the other digesters (C1, M2 and M3 vs. C2, T2 and T3) were calculated as 0.035±0.012 and 0.042±0.009 for K_{hyd_s} and K_{hyd_r} values. The θ values of the 1st-phase digesters for K_{hyd_s} were close to the value (0.024) reported by Siegrist et al. (2002). A similar assessment could not be made for the θ values of K_{hyd_r} because no value has been reported in literature. Examination of the confidence intervals of the θ values revealed that the temperature correction factor was significantly different for K_{hyd_s} values when the high rate and low rate systems were considered. Conversely, the θ values for $K_{hyd,r}$ were similar for the high and low rate systems. Accordingly, application of temperature-correction relationships for slowly biodegradable matter should be employed cautiously when modeling phased digestion systems if a dual-pathway model is to be used.

6.4 Conclusions

Based on the results of this study the following conclusions were drawn:

Application of a dual hydrolysis mechanism better described the hydrolysis of a mixed sludge (PS+WAS) in both the mesophilic and thermophilic batch experiments. A reasonably comparable prediction on the rapidly and slowly hydrolysable fractions for the bottles fed with the same raw sludge demonstrated the reliability of the model.

Statistical analysis of the hydrolysis kinetic coefficients (K_{hyd_s} and K_{hyd_r}) estimated for the mesophilic digesters showed that both K_{hyd_s} and K_{hyd_r} of the M1 digester were different from the values obtained for the mesophilic 2nd-stage digesters of M2 and M3. However, the hydrolysis coefficient K_{hyd_r} of the T1 digester was only significantly different from the K_{hyd_r} of the thermophilic 2^{nd}-phase digesters i.e., T2 and T3).

Estimation of different hydrolysis coefficients in the 1st-phase digesters (i.e, K_{hyd_s} and K_{hyd_r} in case of M1 and K_{hyd_r} in case of T2) using a dual hydrolysis model was likely due to influence of facultative bacteria originated from the WAS fraction of the raw sludge on the hydrolysis rates in these digesters. In addition, presence of hydrolytic biomass with different enzymatic system would be reason for the different hydrolysis rates in the M1 and T1 digesters from the corresponding mesophilic (i.e, M2 and M3) and thermophilic (i.e., T2 and T3) 2nd-phase digesters. However, it was argued that the dual hydrolysis model may be too simplistic to describe the degradation of particulate matter in the phased digestion systems.

Examination of temperature dependence factor (θ) of the hydrolysis coefficients (K_{hyd_s} and K_{hyd_r}) revealed that the θ values were only different for the hydrolysis coefficient K_{hyd_s}. This indicted the limited applicability of temperature dependence factor for modeling of phased anaerobic digestion systems.

Chapter 7
Conclusions and Recommendations

7.1 Conclusion

Four phased digestion systems M1M2, T1M3, T1T1 and M1T3 as well as two single-stage digesters C1 and C2 were employed to compare their efficiencies in terms of solubilisation of particulate organic matter and nitrogen-containing matter, COD removal and methane production. The biomass from the digesters was used in off-line batch experiments to investigate the kinetics of hydrolysis, propionate oxidation and methanogenesis in the digesters. Using a DGGE technique, the structures of the bacterial and archaeal communities of the digesters were examined to assess the influence of the condition of each digester on these communities and to enhance the interpretation of the kinetic studies. The specific conclusions drawn are summarized below.

Operation at an increased temperature (i.e., 55 ^{0}C) was found to be more effective in improving solubilisation of particulate organics and nitrogen-containing matter than phase separation. On the other hand, phase separation was effective in enhancing COD removal and methane production. Therefore, the T1M3 digestion system suggested as the best configuration which benefited from both a high temperature in the 1st-phase and a mesophilic 2nd-phase in which highly efficient (low K_s) POB and methanogens proliferated.

The kinetics of the acetoclastic methanogens were estimated by fitting an integrated form of the Monod equation to batch test data. This model reasonably fitted the acetate degradation over time. Based on the estimates of k_{max} and K_s for each digester, a confidence region was constructed. Examination of the confidence region of each digester revealed that the acetoclastic methanogens of the M1 digester did not overlap with those of the 2nd-phase digesters (M2 and M3) as well as that of the C1 digester. However, the confidence regions of the M1, M2 and C1 digesters were not statistically different (no overlaps). A similar examination of the confidence regions established for the thermophilic digesters showed that the confidence region of the T1 digester was different from those of the T3 and C2 (no overlaps), while it overlapped with the confidence region of the T2 digester. Similar to the observation made for the mesophilic digesters, the confidence regions of the remaining digesters (T2, T3 and C2) overlapped, indicating that the kinetic sets (k_{max} and K_s) of these digesters were not different. Based on these observations, it would appear that the acetoclastic methanogens developed in the heavily loaded digesters (i.e., M1 and T1) were kinetically different from the biomass which developed in the

corresponding mesophilic and thermophilic 2nd-phase and single-stage digesters (with the exception of T2 digester). The biomass of the T2 digester appeared to show a mixed kinetic characteristic, indicating that the acetoclastic methanogens originating from the T1 digester maintained some level of activity in the T2 digester.

The acetate concentrations measured in the source digesters supported these conclusions as the acetate levels in both M1 and T2 digesters were significantly higher than those of the corresponding mesophilic and thermophilic 2nd-phase and single-stage digesters. The DGGE results further supported these conclusions. The archaeal DGGE profile displayed relatively fewer bands for the M1 digester as compared to those of the M2 and M3 digesters. However, the DGGE profile for the thermophilic T1 digester was relatively similar with that of the T2 digester, while it was different from that of the T3 digester when the number and position of the bands was considered.

The thermophilic k_{max} and K_s values of the methanogens were greater than those of the mesophilic values. The values of k_{max} and K_s for the T1 digester were 1.93 and 2.35 times greater than the values obtained for the M1 digester. The k_{max} and K_s values of the remaining thermophilic digesters (T2, T3 and C2) were, on average, 2.21 and 3.03 times higher than those of the mesophilic ones. Based on the temperature correction coefficients (θ) calculated for k_{max} and K_s, it was observed that the θ value of k_{max} was comparable with that of the ADM1, while the θ values of K_s were significantly different. Therefore, it was concluded that the Arrhenius-like equations may be employed for temperature correction of k_{max} values in digesters with relatively similar organic loading or residence times; however, as the k_{max} and K_s values are typically highly correlated, application of Arrhenius-type relationships should be employed with caution.

The generated methane mass and concentration due to the acetate degradation within the test bottles was simulated using the estimated kinetic parameters for each digester. The model fitted the methane masses and concentrations reasonably.

Similar to the approach used for the estimation of the bio-kinetic parameters of acetoclastic methanogens, the kinetics of propionate oxidation was evaluated in the various digesters. The integrated Monod model fitted the propionate degradation data well. The k_{max} and K_s values estimated for the mesophilic digesters through the calibration exercise were examined based on their confidence regions plots. Similar to the observation made for the acetoclastic methanogens, it was found that the POB developed in the M1 digester were kinetically different from those of the M2, M3 and C1 digesters. On the other hand, the POB within the mesophilic 2nd-phase digesters and single-stage digester reflected

similar kinetic properties. With regard to the thermophilic digesters, the kinetic properties of the POB developed within the T1 digester were different from those of the T3 and C2 digesters. The POB within the T3 and C2 digesters were similar, while that of the T2 digester reflected a biomass with mixed kinetic properties of the T1 and T3 digesters. This observation was similar to the result found for the acetoclastic methanogens, supporting the assumption that the microorganisms (both acetoclastic methanogens and POB) originated from the T1 digester remained active in the T2 digester.

The k_{max} and K_s values obtained for the mesophilic and thermophilic digesters were consistent with the propionate concentrations observed in their effluents. The propionate concentrations were significantly higher in the M1 and T1 digesters as compared to the propionate levels observed in the corresponding mesophilic and thermophilic 2nd-phase and single-stage digesters. The high k_{max} values enabled the POB of the M1 and T1 digester to proliferate at the low HRT (3 days) digesters, while those POB with low K_s (higher affinity) values of single-stage and 2nd-phase digesters (both mesophilic and thermophilic) dominanted in these digester with lower propionate concentrations. In addition, all thermophilic digesters has higher K_s values as compared to the mesophilic ones. The reduced affinity (high K_s) of the thermophilic POB explains the elevated effluent concentrations of propionate which are typically reported for thermophilic anaerobic digesters.

Due to the diversity of bacterial species, the DGGE results could not be compared with the kinetic studies data.

The influence of temperature on the POB bio-kinetic parameters was assessed by comparing the k_{max} and K_s values of the thermophilic digesters with those of the mesophilic ones. It was found that the thermophilic k_{max} and K_s values of the T1 digester were 2.1 and 2.4 times higher than the values obtained for the M1 digester. The values of k_{max} and K_s of the remaining thermophilic digesters (i.e., T3 and C2) were, on average, 2.8 and 4.0 times greater in the thermophilic digesters than the mesophilic ones. The θ values estimated for k_{max} were significantly higher than the value suggested for ADM1, while those values for K_s were close to the θ of the ADM1. These results suggest that the relationship between POB kinetic parameters and temperature differs between the slow and fast growing organisms. Thus, the application of Arrhenius-like equations for temperature correction of kinetic coefficients should be applied with caution in modeling of propionate degradation in these systems.

The propionate degradation in the test bottles resulted in acetate production. Based on the k_{max} and K_s values estimated for the POB and acetoclastic methanogens, the acetate production within the

bottles was simulated using a Monod-type model. Overall the model predicted the acetate evolution in the bottles reasonably.

A dual pathway hydrolysis model appeared to fit the particulate COD degradation data better than the single hydrolysis model in both mesophilic and thermophilic batch experiments. The coefficients estimated with the dual pathway model for the M1 digester (both $K_{hyd,s}$ and $K_{hyd,r}$) were greater than those of the M2 and M3 digesters. It was also found that the $K_{hyd,r}$ value of the T1 digester was different from those of the T2, T3 and C2 digesters; whereas, the hydrolysis rate constant for slowly hydrolysable matter (i.e., $K_{hyd,s}$) did not differ significantly among these digesters.

The DGGE results supported a hypothesis that bacteria which originated from the WAS would improve the hydrolysis rates in the 1st-phase of the phased digestion systems (i.e., M1 and T1). The DGGE profile displayed that many of the bands of the feed (raw sludge) appeared in the DGGE profile of the T1 and M1 digesters.

A comparison of the mesophilic digesters with the thermophilic ones revealed that both K_{hyd_s} and K_{hyd_r} values were consistently higher in the thermophilic digesters. The K_{hyd_s} and K_{hyd_r} values for the T2 digester were 1.5 and 2.5 times greater than those of the T1 digester; whereas, the kinetic coefficients of the thermophilic 2nd-phase digesters were, on average, 2.4 and 2.6 times higher than the corresponding mesophilic 2nd-phase digesters (i.e., M2 and M2). The estimated θ values for K_{hyd_s} were significantly different when the high rate and low rate systems were considered. Conversely, the θ values for $K_{hyd,r}$ were similar for the high and low rate systems. Accordingly, application of temperature-correction relationships for slowly biodegradable matter should be employed cautiously when modeling phased digestion systems if a dual-pathway model is to be used.

7.2 Recommendations

The results of this research suggested a better understanding of microbial ecology of phased digestion systems can improve their modeling through considering the dominance of microbial sub-groups in separate phase. In this regard, further research on modeling concepts of phased anaerobic digestion systems are outlined below.

1. Operation of the 1st-stage of a phased digestion system at a thermophilic temperature was found effective in improving overall performance of the system. Application of higher temperatures in the 1st-phase may further enhance anaerobic digestion efficiency. Application of higher temperatures (55-70 ^{0}C)

in the 1st-stage and study their influence on hydrolysis, propionate oxidation and methanogenesis processes are recommended.

2. A dual hydrolysis model was used to study the hydrolysis mechanism in the 1^{st}- and 2^{nd}-phases. Although the model considers different rates for hydrolysis of slowly and rapidly fractions of sludge, it would appear that the application of the model with two first-order equations is still simplistic in describing hydrolysis process in anaerobic digestion in general and in phased digestion systems in particular. A thorough investigation of hydrolysis process using more complex models, especially the ones considering the biomass concentration is recommended. However, the increase of number of parameters and complexity should be taken into account.

3. Two microbial groups which are playing important roles in anaerobic digestion were not addressed in this research: acidogens and hydrogenotrophic methanogens. From a modeling viewpoint, it would be very interesting to investigate the activity of acidogens in the 1^{st}- and 2^{nd}-stages. In addition, due to the important role of hydrogenotrophic methanogens in maintaining low hydrogen pressure in digesters and allowing the activity of propionate oxidizing bacteria, it would be valuable to study the effects of staging on the activity of these organisms in each phase.

4. The results of this research on the kinetics of methanogenesis, propionate oxidation and hydrolysis suggested the implementation of two sets of bio-kinetic parameters for modeling of phased digestion systems. Application of two separate sets of bio-kinetic parameters for each stage of phased digestion system would be valuable to assess this concept in bench- or full-scale digestion systems.

5. Use of group-specific primers in DGGE analysis would reveal more information on the microbial ecology of phased anaerobic digestion systems.

Bibliography

Aguilar, Z.; Casas, C; Lema, J. M. (1995) Degradation of volatile fatty acids by differently enriched methano-genic cultures: kinetics and inhibition. Water Research, 29(2), 505-509.

Ahring, B. K., Ibrahim, a a, and Mladenovska, Z. (2001) Effect of temperature increase from 55 to 65 degrees C on performance and microbial population dynamics of an anaerobic reactor treating cattle manure. Water Research, 35(10), 2446-52.

Akarsubasi, A., Ince, O., Oz, N., Kirdar, B., and Ince, B. (2006) Evaluation of performance, acetoclastic methanogenic activity and archaeal composition of full-scale UASB reactors treating alcohol distillery wastewaters. Process Biochemistry, 41(1), 28-35.

Amann, R.I., Binder, B.J. Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990b) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology, 56: 1919-1925

Amann, R.I., Krumholz, L. and Stahl, D.A. (1990a) Fluorescent oligonucleotide probing of whole cells for determinative, phylogenetic and environmental studies in microbiology. Journal of Bacteriology, 172: 762-770

Amann, R.I., Ludwig, W., Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiology and Molecular Biology Reviews, 59(1): 143-169.

Andrews, G. F. (1984) Parameter estimation from batch culture data. Biotechnology and bioengineering, 26(7), 824-5.

Angelidaki, I., Ellegaard, L. and Ahring, B.K. (1993). A mathematical model for dynamic simulation of anaerobic digestion of complex substrates: focusing on ammonia inhibition. Biotechnology and Bioengineering, 42 (2), 159-166.

Angelidaki, I., Ellegaard, L. and Ahring, B.K. (1999) A comprehensive model of anaerobic bioconversion of complex substrate to biogas. Biotechnology and Bioengineering, 63: 363-372

Angelidaki, I., Petersen, SP, Ahring, B. (1990). Effects of lipids on thermophilic anaerobic digestion and reduction of lipid inhibition upon addition of bentonite. Applied Microbiology and Biotechnology. Vol. 33 (4), pag. 469-472.

Angenent, L. T., Zheng, D., Sung, S., and Raskin, L. (2011) Microbial Activity Structure Community in a Compartmentalized, Anaerobic Bioreactor. Water Environment Research, 74(5), 450-461.

APHA , (1998) Standard methods for the examination of water and wastewater, 20th ed., American Public Health Association, Washington, DC., USA

Ariesyady, H.D., Ito, T., Yoshiguchi, K. and Okabe, S. (2007) Phylogenetic and functional diversity of propionate-oxidizing bacteria in an anaerobic digester sludge. Applied microbiology and biotechnology 75(3): 673-83

Arun, A. B., Chen, W.M., Lai, W.A., Chou, J.H., Shen, F.T., Rekha, P. D., and Young, C.C. (2009) Lutaonella thermophila gen. nov., sp. nov., a moderately thermophilic member of the family Flavobacteriaceae isolated from a coastal hot spring. International journal of systematic and evolutionary microbiology, 59(8), 2069-73.

Asker, D., Beppu, T., and Ueda, K. (2008) Nubsella zeaxanthinifaciens gen. nov., sp. nov., a zeaxanthin-producing bacterium of the family Sphingobacteriaceae isolated from freshwater. International journal of systematic and evolutionary microbiology, 58(3), 601-6.

Babbit, H.E. and Baumann, E.R. (1958) Sewerage and sewage treatment. John Wiley and Sons, Inc., New York.

Bard, Y. (1974) Nonlinear parameter estimation. Academic Press, New York.

Barr, K. G., Solley, D. O., Starrenburg, D. J., and Lewis, R. G. (2008) Evaluation, selection and initial performance of a large scale centralised biosolids facility at Oxley Creek Water Reclamation Plant, Brisbane. Water science and technology, 57(10), 1579-86.

Batstone, D. J., Pind, P. F., and Angelidaki, I. (2003) Kinetics of Thermophilic Anaerobic Oxidation of Straight and Branched Chain Butyrate and Valerate. Biotechnology and Bioengineering, 84(2): 195-204.

Batstone, D. J., Torrijos, M., Ruiz, C., and Schmidt, J. E. (2004) Use of an anaerobic sequencing batch reactor for parameter estimation in modelling of anaerobic digestion. Water science and technology , 50(10), 295-303.

Batstone, D. J.; Keller, J.; Angelidaki, I.; Kalyuzhnyi, S. V.; Pavlostathis, S. G.; Rozzi, A.; Sanders, W. T .M.; Siegrist, H.; Vavilin, V. A. (2002) Anaerobic digestion model No. 1 (ADM1). IWA Task Group for Mathematical Modeling of Anaerobic Digestion Processes, IWA Publishing, London, UK.

Batstone, D.J., Keller, J., Newell, R.B. and Newland, M. (2000) Modelling anaerobic degradation of complex wastewater. I: model development. Bioresource Technology 75, 67-74 (2000).

Bernard, O., Hadj-Sadok, Z., Dochain, D., Genovesi, A and Steyer, J.P. (2001) Dynamical model development and parameter identification for an anaerobic wastewater treatment process. Biotechnology and bioengineering 75, 424-438.

Beun, J. J., Verhoef, E. V., Van Loosdrecht, M. C., and Heijnen, J. J. (2000) Stoichiometry and kinetics of poly-beta-hydroxybutyrate metabolism under denitrifying conditions in activated sludge cultures. Biotechnology and bioengineering, 68(5), 496-507.

Bhattacharya, S., Madura, R., Walling, D., and Farrel, J. (1996). Volatile solids reduction in two-phase and conventional anaerobic sludge digestion. Water Research, 30(5): 1041-1048.

Blumensaat, F.; Keller, J. (2005) Modeling of two-stage anaerobic digestion using the IWA anaerobic digestion model No. 1(ADM1). Water Research, 39(1), 171-183.

Bolzonella, D., Pavan, P., Zanette, M. and Cecchi, F. (2007) Two-Phase Anaerobic Digestion of Waste Activated Sludge: Effect of an Extreme Thermophilic Prefermentation. Industrial and Engineering Chemistry Research 46, 6650-6655.

Boone, D. R. and Xun, L. (1987) Effects of pH, Temperature, and Nutrients on Propionate Degradation by a Methanogenic Enrichment Culture. Applied and environmental microbiology, 53(7), 1589-92.

Boone, D.R., Whitman, W.B. and Rouviere, P. (1993) Diversity and taxonomy of methanogens. p. 35-80. In: J.G. Ferry (ed.) Methanogenesis: ecology, physiology, biochemistry and genetics, Chapman and Hall, New York.

Breure, A. M., Mooijman, K. A., and Andel, J. G. V. (1986) Protein degradation in anaerobic digestion : influence of volatile fatty acids and carbohydrates on hydrolysis and acidogenic fermentation of gelatin. Applied Microbiology Biotechnology,24(5): 426-431.

Briones, A. and Raskin, L. (2003) Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Current Opinion in Biotechnology, 14: 270-276

Capri, M.G. and Marais, G.v.R. (1973) pH Adjustment in anaerobic digestion. Water Research, 9(3): 307-313.

Chartrain, M and Zeikus, J.G. (1986) Microbial ecophysiology of whey biomethanation: characterization of bacterial trophic populations and prevalent species in continuous culture. Applied and Environmental Microbiology, 51(1): 188-196.

Chen, S., Liu, X., and Dong, X. (2005) Syntrophobacter sulfatireducens sp. nov., a novel syntrophic, propionate-oxidizing bacterium isolated from UASB reactors. International journal of systematic and evolutionary microbiology, 55(3), 1319-24.

Chynoweth, D.P. and Mah, R.A. (1971) Volatile acid formation in sludge digestion. Advances in Chemistry Series, 105: 41-54.

Coelho, N. M. G., Droste, R. L., and Kennedy, K. J. (2011) Evaluation of continuous mesophilic, thermophilic and temperature phased anaerobic digestion of microwaved activated sludge. Water Research, 45(9), 2822-34.

Coenye, T. (2003) Classification of Ralstonia pickettii-like isolates from the environment and clinical samples as Ralstonia insidiosa sp. nov. International Journal of Systematic and Evolutionary Microbiology, 53(4), 1075-1080.

Cohen, A., Breure, A.M., van Andel, J.G. and van Deursen, A. (1980) Influence of phase separation on the anaerobic digestion of glucose. I. Maximum COD-turnover rate during continuous operation. Water Research, 14: 1439-1448.

Cohen, A., Zoetemeyer, R.J., van Duerson, A. and van Andel, J.G. (1979) Anaerobic digestion of glucose with separated acid production and methane formation. Water Research, 13: 571-580.

Collins, G., Kavanagh, S., McHugh, S., Connaughton, S., Kearney, A., Rice, O., Carrigg, C., Scully, C., Bhreathnach, N., Mahony, T., Madden, P., Enright, A.M. and O'Flaherty, V. (2006) Accessing the black box of microbial diversity and ecophysiology: recent advances through polyphasic experiments. Journal of Environmental Science and Health Part A, 41: 897-922

Conklin, A., Stensel, H. D., and Ferguson, J. (2006) Growth Kinetics and Competition Between Methanosarcina and Methanosaeta in Mesophilic Anaerobic Digestion. Water Environment Research, 78(5), 486-496.

Costello, D.J., Greenfield, P.F. and Lee, P.L. Dynamic modelling of a single-stage high-rate anaerobic reactor—II. Model verification. Water Research 25, 859-871 (1991).

Dabert, P., Delgenes, J. P., Moletta, R. and Godon, J.J. (2002) Contribution of molecular microbiology to the study in water pollution removal of microbial community dynamics. Reviews in Environmental Science and Biotechnology, 1: 39-49

de Bok, F. A. M.; Plugge, C. M.; Stams, A. J. M. (2004) Interspecies electron transfer in methanogenic propionate degrading consortia. Water Research, 38(6), 1368-1375.

De Gioannis, G., Diaz, L.F., Muntoni, A and Pisanu, A. (2008) Two-phase anaerobic digestion within a solid waste/wastewater integrated management system. Waste Management, 28(10): 1801-1808.

Delbes, C., Moletta, R., and Godon, J.J. (2001) Bacterial and archaeal 16S rDNA and 16S rRNA dynamics during an acetate crisis in an anaerobic digester ecosystem. FEMS Microbiology Ecology, 35: 19–26.

DeLong, E. F. (1992) Archaea in coastal marine environments. Proceedings of the National Academy of Sciences of the United States of America, 89(12), 5685-9.

Demirel, B. and Scherer, P. (2008) The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane : a review. Reviews in Environmental Science and Biotechnology, 7(2): 173-190.

Derrien, M., Vaughan, E. E., Plugge, C. M., and de Vos, W. M. (2004) Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. International journal of systematic and evolutionary microbiology, 54(5), 1469-76.

Dichtl, N. (1997) Thermophilic and Mesophilic (Two-Stage) Anaerobic Digestion. Water and Environment Journal, 11(2): 98–104

Dochain, D. and Vanrolleghem, P.A. (2001) Dynamical modeling and estimation in wastewater treatment processes. IWA Publishing, London, UK

Dolfing, J. (1988) Acetogenesis. 417-468. In: A.J.B. Zenhnder (ed.), Biology of anaerobic organisms, John Wiley and Sons, Inc., New York

Dolfing, J. and Bloeman, W.G. (1985) Activity measurements as a tool to characterize the microbial composition of methanogenic environments. Journal of Microbiological Methods, 4(1): 1-12

Donoso-Bravo, a, Retamal, C., Carballa, M., Ruiz-Filippi, G., and Chamy, R. (2009) Influence of temperature on the hydrolysis, acidogenesis and methanogenesis in mesophilic anaerobic digestion: parameter identification and modeling application. Water Science and Technology, 60(1): 9-17.

Donoso-Bravo, A., García, G., Pérez-Elvira, S., and Fdz-Polanco, F. (2011) Initial rates technique as a procedure to predict the anaerobic digester operation. Biochemical Engineering Journal, 53(3): 275-280.

Draper, N. R. and Smith, H. (1981) Applied regression analysis, John Wiley and Sons, Inc., New York.

Duran, M. and Speece, R. E. (1997). Temperature-staged anaerobic processes. Environmental Technology, 18: 747-754

Eastman, J.A. and Ferguson, J.F. (1981) Solubilization organic phase of of carbon anaerobic particulate during the digestion acid. Journal of Water Pollution Control Federation 53(3): 352-366

Efstathiou, E., Mamais, D., Tsourtis, S., Tridimas, P., Supply, A. W., and Company, S. (2003) Mathematical modelling of primary sludge anaerobic hydrolysis. 8th International Conference on Environmental Science and Technology, Lemnos Island, Greece, September 8 - 10.

Ellis, T. G., Barbeau, D. S., Smets, B. F., and Grady, C. P. L. Jr. (1996) Respirometric technique for determination of extant kinetic parameters describing biodegradation. Water Environment Research, 68, 917-926.

Eskicioglu, C., Terzian, N., Kennedy, K. J., Droste, R. L., and Hamoda, M. (2007) Athermal microwave effects for enhancing digestibility of waste activated sludge. Water Research, 41(11), 2457-66.

Feng, Y., Behrendt, J., Wendland, C., and Otterpohl, R. (2006) Parameter analysis of the IWA Anaerobic Digestion Model No. 1 for the anaerobic digestion of blackwater with kitchen refuse. Water Science and Technology, 54(4): 139–147

Fernández, A., Huang, S., Seston, S., Xing, J., Hickey, R., Criddle, C., Tiedje, J., Huang, S., Seston, S., Xing, J., and Hickey, R. (1999) How Stable Is Stable? Function versus Community Composition. Applied and Environmental Microbiology, 65(8): 3697-3704

Ferry, J.G. (1993) Fermentation of acetate. p. 304-334. In: J.G. Ferry (ed.) Methanogenesis: ecology, physiology, biochemistry and genetics, Chapman and Hall, New York

Flotats, X., Ahring, B.K. and Angelidaki, I. (2003) Paramter identification of thermophilic anaerobic degradation of valerate. Applied Biochemistry and Biotechnology, 109(1-3): 47-62.

Fongastitkul, P. and Mavinic, D.S. (1994) A two-phase anaerobic digestion process: Concept, process failure and maximum system loading rate. Water Environment Research, 66(3): 243-254

Fox, P., Pohland, F.G. (1994) Anaerobic treatment applications and fundamentals: substrate specificity during phase separation. Water Environment Research. 66(5): 716-724.

Fukuzaki, S., Nishio, N., Shobayashi, M., and Nagai, S. (1990a) Inhibition of the fermentation of propionate to methane by hydrogen, acetate and propionate. Applied and Environmental Microbiology, 56(3), 719-723.

Gallert, C., and Winter J. (2008) Propionic acid accumulation and degradation during restart of a full-scale anaerobic biowaste digester, Bioresource Technology, 99 (1), 170-178.

Gavala, H., Yenal, U., Skiada, I., Westermann, P., and Ahring, B. (2003a) Mesophilic and thermophilic digestion of primary and secondary sludge. Effect of pre-treatment and elevated temperature. Water Research, 37(19): 4561-4572

Gavala, H.N. and Lyberatos, G. (2001) Influence of anaerobic culture acclimation on the degradation kinetics of various substrates. Biotechnology and Bioengineering 74(3): 181-95

Gavala, H.N., Angelidaki, I. and Ahring, B.K. (2003b) Kinetics and modeling of anaerobic digestion process. Advances in Biochemical Engineering/Biotechnology 81, 57-93

Ge, H., Jensen, P. D., and Batstone, D. J. (2010) Pre-treatment mechanisms during thermophilic-mesophilic temperature phased anaerobic digestion of primary sludge. Water Research, 44(1), 123-30.

Ge, H., Jensen, P. D., and Batstone, D. J. (2010) Temperature phased anaerobic digestion increases apparent hydrolysis rate for waste activated sludge. Water Research, 45(4), 1597-1606.

Ge, H., Jensen, P. D., and Batstone, D. J. (2011) Increased temperature in the thermophilic stage in temperature phased anaerobic digestion (TPAD) improves degradability of waste activated sludge. Journal of Hazardous Materials, 187(1-3), 355-361.

Gerardi, M.H. (2003) "The Microbiology of Anaerobic Digesters". John Wiley and Sons, Inc., USA.

Ghosh, S. (1987) Improved sludge gasification by two-phase anaerobic digestion. Journal of Environmental Engineering, 13(6): 1265-1284

Ghosh, S. (1991) Pilot scale demonstration of two-phase anaerobic digestion of activated sludge. Water Science and technology, 23: 1179-1188

Ghosh, S. and Pohland, F.G. (1974) Kinetics of substrate assimilation and product formation in anaerobic digestion. J. Water Pollution Control Federation, 46: 750–759.

Ghosh, S., Buoy, K., Dressel, L., Miller, T., Wilcox, G., and Loos, D. (1995) Pilot- and full-scale two-phase anaerobic digestion of municipal sludge. Water Environment Research, 67(2): 206-214

Gomec, C. Y. and Speece, R. E. (2003) Organic material solubilization of domestic primary sludge in anaerobic digestion at controlled pH. Water Science and Technology, 48(4), 195-8.

Gosset, J.M. and Belser, R.L. (1982) Anaerobic Digestion of Waste Activated Sludge. Journal of Environmental Engineering, 108: 1101-1120

Grady, C.P.L., Smets, B.F. and Barbeau, D.S. (1996) Variability in kinetic parameter estimates: A review of possible causes and a proposed terminology. Water Research, 30(3): 742–748.

Graef, S.P. and Andrews, J.F. (1974) Mathematical modeling and control of anaerobic digestion, AIChE Symposium Series, 136: 101-131.

Green, S.J., Leigh, M.B., Neufeld, J.D. (2010). Denaturing gradient gel electrophoresis (DGGE) for microbial community analysis. Hydrocarbon Microbiology. Timmis K (Ed.) Springer Berlin Heidelberg. pp. 4137-4158.

Griffin, M.E., McMahon, K.D., Mackie, R.I. and Raskin, L. (1998) Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnology and bioengineering 57(3), 342-55

Großkopf, R., Janssen, P. H., and Liesack, W. (1998) Diversity and Structure of the Methanogenic Community in Anoxic Rice Paddy Soil Microcosms as Examined by Cultivation and Direct 16S rRNA Gene Sequence Retrieval, Appl. Environ. Microbiol. , 64(3): 960-969

Guisasola, A., Baeza, J.A., Carrera, J., Sin, G., Vanrolleghem, P.A., and Lafuente, J. (2006) The influence of experimental data quality and quantity on parameter estimation accuracy: Andrews inhibition model as a case study. Trans IChemE, Part D, Education for Chemical Engineers, 1:139-145

Gujer, W. and Zehnder, A.J.B. (1983) Conversion processes in anaerobic digestion. Water Science and Technology, 15(8-9): 127-167

Han, Y. and Dague, R.R. (1997) Laboratory studies on the temperature-phased anaerobic digestion of domestic primary sludge. Water Environment Research, 69(6): 1139-1143

Han, Y., Shihwu, S. and Dague, R.R. (1997) Temperature-phased anaerobic digestion of wastewater sludges. Water Science and Technology, 36(6-7): 367-374

Hanaki, K., Matsuo, T., Nagase, M. and Tabata, Y. (1987) Evaluation of Effectiveness of Two-Phase Anaerobic Digestion Process Degrading Complex Substrate. Water Science and Technology, 19(1-2): 311–322.

Hansen, K., Ahring, B.K. and Raskin, L. (1999) Quantification of syntrophic fatty acid-β-oxidizing bacteria in a mesophilic biogas reactor by oligonucleotide probe hybridization. Applied and Environmental Microbiology, 65(11): 4767-4774

Harmsen H. J. M.; Akkermans, A. D. L.; Stams, A. J. M.; deVos, W. M. (1996) Population dynamics of propionate oxidizing bacteria under methanogenic and sulfidogenic conditions in anaerobic granular sludge. Applied and Environmental Microbiology, 62(6), 2163–2168.

Harper, S.R. and Pohland, F.G. (1985) Recent developments in hydrogen management during anaerobic biological wastewater treatment. Biotechnology and Bioengineering, 28(4): 585-602.

Hashimoto, A.G. (1982) Methane from cattle waste: Effects of temperature, hydraulic retention time, and influent substrate concentration on kinetic parameter (k). Biotechnology and Bioengineering, 24(9), 2039-52.

Henze, M., Harremoes, P., Jansen, J., and Arvin, E. (1995) Wastewater Treatment: Biological and Chemical Processes. Springer Verlag, Berlin.

Heppner, B., Zellner, G., and Diekmann, H. (1992) Start-up and operation of a propionate-degrading fluidized-bed reactor. Applied Microbiology and Biotechnology, 36(6), 810–816.

Heyes, R. H.; Hall, R. J. (1983) Kinetics of two sub-groups of propionate-using organisms in anaerobic digestion. Applied and Environmental Microbiology, 46(3), 710-715

Hill, D.T. and Barth, C.I. (1977) A dynamic model for simulation of animal waste digestion, J. Water Pollution Control Federation, 49 : 2129–2143.

Holmes, P. and Freischelt, M.R. (1978) H2-producing bacteria in digesting sewage sludge isolated on simple, defined media.. Microbiology 36(2), 394-395.

Huber, R., Woese, C. R., Langworthy, T. A., Kristjansson, J. K., and Stetter, K. O. (1989) Thermosipho africanus gen. nov., tepresents a new genus of Thermophilic eubacteria within the “Thermotogales”. Systematic and Applied Microbiology, 12(1): 32-37.

Ince, B.K. and Ince, O. (2000) Changes to bacterial community make-up in a two-phase anaerobic digestion system. Journal of Chemical technology and Biotechnology, 75: 500-508

Jeong, H.S., Suh, C.W., Lim, J.L., Lee, S.H., and Shin, H.S. (2005) Analysis and application of ADM1 for anaerobic methane production. Bioprocess and biosystems engineering, 27(2), 81-9.

Jeris, J.S. and McCarty, P.L. (1965) Biochemistry of methane fermentation using C14 tracers, Water Pollution Control Federation, 37(2): 178-192.

Kalfas H, Skiadas I, Gavala H, Stamatelatou K, Lyberatos G. (2006). Application of ADM1 for the simulation of anaerobic digestion of olive pulp under mesophilic and thermophilic conditions. Water Science and Technology, 54(4): 149-156

Kalyuzhnyi, S., Fedorovich, V., Lens, P., Hulshoff Pol, L. and Lettinga, G. Mathematical modelling as a tool to study population dynamics between sulfate reducing and methanogenic bacteria. Biodegradation 9, 187-99 (1998).

Karakashev, D., Batstone, D. and Angelidaki, I. (2005) Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Applied and Environmental Microbiology, 71(1): 331-338

Karakashev, D., Batstone, D.J., Trably, E. and Angelidaki, I. (2006) Acetate oxidation is the dominant methanogenic pathway from acetate in the absence of Methanosaetaceae. Applied and environmental microbiology 72, 5138-5141.

Kaspar, H. F.; Wuhrmann, K. (1978a). Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. Applied and Environmental Microbiology, 36(1), 1-7.

Kemmer, G. and Keller, S. (2010) Nonlinear least-squares data fitting in Excel spreadsheets. Nature Protocols, 5(2), 267-81.

Kenealy, W.R., and Zeikus, J.G. (1982) One-carbon metabolism in methanogens: evidence for synthesis of a two-carbon cellular intermediate and unification of catabolism and anabolism in Methanosarcina barkeri. J. Bacteriology, 151:932-941

Kianmehr, P. (2010) Characterization of Pretreatment Impacts on Properties of Waste Activated Sludge and Digestibility. PhD Thesis, University of Waterloo, Waterloo, Ontario, Canada

Kim, M., Ahn, Y.H., and Speece, R. (2002) Comparative process stability and efficiency of anaerobic digestion: mesophilic vs. thermophilic. Water Research, 36(17): 4369-4385

Kim, M., Bae, W., and Speece, R. E. (2004) Improved Anaerobic Process Efficiency Using Mesophilic and Thermophilic Elutriated Phased Treatment. Journal of Environmental Engineering, 130(9), 960-966.

Kisaalita, W.S., Lo, K.V. and Pinder, K.L. (1989) Influence of dilution rate on the acidogenic phase products distribution during two-phase lactose anaerobiosis. Biotechnology and Bioengineering, 34: 1235-1250

Knightes, C. D. and Peters, C. a (2000) Statistical analysis of nonlinear parameter estimation for Monod biodegradation kinetics using bivariate data. Biotechnology and Bioengineering, 69(2), 160-70.

Kotsyurbenko, O.R. (2005) Trophic interactions in the methanogenic microbial community of low-temperature terrestrial ecosystems. FEMS Microbiology Ecology 53(1), 3-13.

Krakat, N., Schmidt, S., and Scherer, P. (2010) Mesophilic Fermentation of Renewable Biomass : Does Hydraulic Retention Time Regulate Methanogen Diversity? Applied and Environmental Microbiology, 76(18): 6322-6326

Leadbetter, J. R. (1998) Methanobrevibacter filiformis sp. nov., a filamentous methanogen from termite hindguts. Archives of Microbiology, 169(4), 287-292.

Lee, M.Y., Suh, C.W., Ahn, Y.T., and Shin, H.S. (2009) Variation of ADM1 by using temperature-phased anaerobic digestion (TPAD) operation. Bioresource Technology, 100(11): 2816-2822.

Lettinga, G., Rebac, S. and Zeeman, G. (2001) Challenge of anaerobic psychrophilic anaerobic wastewater treatment. Trends in Biotechnology, 19: 363-370

Leung, K. and Topp, E. (2001) Bacterial community dynamics in liquid swine manure during storage: molecular analysis using DGGE/PCR of 16S rDNA. FEMS Microbiology Ecology, 38(2-3), 169-177.

Liu C. and Zachara, J.M. (2001) Uncertainties of Monod kinetic parameters nonlinearly estimated from batch experiments. Environmental Science and Technology 35(1):133-141

Liu, W.-T., Chan, O.-C., and Fang, H. H. P. (2002) Microbial community dynamics during start-up of acidogenic anaerobic reactors. Water Research, 36(13), 3203-3210.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment polymorphism of genes encoding 16S rRNA. Applied and Environmental Microbiology, 62: 4516-4522

Liu, Y., Balkwill, D.L., Aldrich, H.C., Drake, G.R. and Boone, D.R. (1999) Characterization of the anaerobic propionate-degrading syntrophs Smithella propionica gen. nov., sp. nov. and Syntrophobacter wolinii. International journal of systematic bacteriology 49(2): 545-56

Lueders, T.; Pommerenke, B.; Friedrich, M. W. (2004b) Stable-isotope probing of microorganisms thriving at thermodynamic limits: Syntrophic propionate oxidation in flooded soil. Applied and Environmental Microbiology, 70(10), 5778–5786.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Jr. C.T.P., Saxman, P.R., Farris, R.J. et al (2001). The RDP-II (Ribosomal Database Project). Nucleic Acids Res, 29: 173-174.

Maidak, B.L., Larsen, N., McCaughey, M.J., Overbeek, R., Olsen, G.J., Foge, K., Blandy, J. and Woese, C.R. (1994) The Ribosomal Database Project. Nucleic Acids Research, 22: 3485–3487

Marshall, P., Szikszai, T., Lemay, V., and Kozak, A. (1995) Testing the distributional assumptions of least squares linear regression. The Forestry Chronicle, 71(2), 213-218.

Massey, M.L. and Pohland, J.F. (1978) Phase separation of stabilization by kinetic controls. Journal of Water Pollution Control Federation, 50: 2204-2222

McCarthy, A.J. (1987) Lignocellulose-degrading actinomycetes. FEMS Microbiology Letters, 46(2), 145-163.

McHugh, S., Micheal, C., Mahony, T. and O'Flaherty, V. (2003) Methanigenic population structure in a variety of anaerobic bioreactors. FEMS Microbiology Letter, 219: 297-304

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(7): 823–834.

McMahon, K.D., Stroot, P.G., Mackie, R.I. and Raskin, L. (2001) Anaerobic co-digestion of municipal solid waste and biosolids under various mixing conditions-II: Microbial population dynamics. Water Research, 35(7): 1817-1827

Melcer, H., Dold, P.L., Jones, R. M., Bye, C.M. and Takacs, I. (2003) Methods for wastewater characterization in activated sludge modeling, WERF Report 99-WWF-3, IWA Publishing, London, and Water Environment Federation, Alexandria, VA.

Merkel, W., Schwarz, a, Fritz, S., Reuss, M., and Krauth, K. (1996) New strategies for estimating kinetic parameters in anaerobic wastewater treatment plants. Water Science and Technology, 34(5-6), 393-401.

Metcalf and Eddy (2003) "Wastewater engineering: treatment and reuse". 4th ed., McGraw-Hill, New York, USA.

Min, H. and Zinder, S. H. (1989) Kinetics of Acetate Utilization by Two Thermophilic Acetotrophic Methanogens: Methanosarcina sp. Strain CALS-1 and Methanothrix sp. Strain CALS-1. Applied and Environmental Microbiology, 55(2), 488-491.

Miranda-Tello, E., Fardeau, M.-L., Joulian, C., Magot, M., Thomas, P., Tholozan, J.L., and Ollivier, B. (2007) Petrotoga halophila sp. nov., a thermophilic, moderately halophilic, fermentative bacterium isolated from an offshore oil well in Congo. InternationalJournal of Systematic and Evolutionary Microbiology, 57(1): 40-4.

Moen, G., Stensel, H.D., Lepisto, R., and Fersudon, J.F. (2003) Effect of solids retention time on the performance of thermophilic and mesophilic digestion of combined municipal wastewater sludges. Water Environment Research, 75(6): 539-548

Moen, G., Stensel, H.D., Lepisto, R., and Fersudon, J.F. (2003) Effect of solids retention time on the performance of thermophilic and mesophilic digestion of combined municipal wastewater sludges. Water Environment Research, 75(6): 539-548

Molin, S. and Givskov, M. (1999) Application of molecular tools for in situ monitoring of bacterial growth activity. Environmental Microbiology, 1(5): 383-391

Montgomery, D. C. (2001) Design and analysis of experiments. 5th Edition, John Wiley and Sons, New York

Mosche, M.; Joerdening, H. (1999) Comparison of different models of substrate and product inhibition in anaerobic digestion. Water Research, 33(11), 2545-2554.

Mottet, a, Steyer, J. P., Déléris, S., Vedrenne, F., Chauzy, J., and Carrère, H. (2009) Kinetics of thermophilic batch anaerobic digestion of thermal hydrolysed waste activated sludge. Biochemical Engineering Journal, 46(2), 169-175.

Muyzer, G., De Wall, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial population by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. Applied and Environmental Microbiology, 59: 695–700.

Newton, C. (1999) Solids destruction in anaerobic digestion. M.S. Thesis, University of Washington, Seattle.

Nges, I.A. and Liu, J. (2009) Effects of anaerobic pre-treatment on the degradation of dewatered-sewage sludge. Renewable Energy 34(7): 1795-1800

Nicol, G.W., Glover, L.A., Prosser, J.I. (2003) The impact of grassland management on archaeal community structure in upland pasture rhizosphere soil. Environmental Microbiology, 5: 152–162.

Nielsen H.B., Uellendahl, H., Ahring, B.K. (2007) Regulation and optimization of the biogas process: Propionate as a key parameter. Biomass and Bioenergy, 31(11/12): 820-830.

Nielsen, H. B.; Maldenovska, Z.; Ahring, B. K. (2008) Kinetics of Propionate Conversion in Anaerobic Continuously Stirred Tank Reactors, Environmental Technology, 29(2): 151-160

O'Reilly, C. and Colleran, E. (2006) Effect of influent COD/SO42- ratios on mesophilic anaerobic reactor biomass populations: physico-chemical and microbiological properties. FEMS Microbial Ecology, 56: 141-153

Oles, J., Dichtl, N., and Niehoff, H. (1997) Full-scale experience of two-stage thermophilic-mesophilic sludge digestion. Water Science and Technology, 36(6-7): 449

Ong, S. L. (1983) Least-squares estimation of batch culture kinetic parameters. Biotechnology and Bioengineering, 25(10), 2347-58.

Orhon, D., Çokgör, E. U., and Sözen, S. (1998) Dual hydrolysis model of the slowly biodegradable substrate in activated. Biotechnology Techniques, 12(10), 737-741.

Ovreas L, Forney L, Daae FL, Torsvik V (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Applied and Environmental Microbiology, 63: 3367–3373.

Owen, W.F., Stuckey, D. C., Healy, J. B., Young, L. Y., and McCarty, P. L. (1979) Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Research, 13(6), 485-492.

Parker, W.J. Application of the ADM1 model to advanced anaerobic digestion. Bioresource Technology 96, 1832-42 (2005).

Parkin, G.F. and Owen, W.F. (1986) Fundamentals of Anaerobic Digestion of Wastewater Sludges Journal of Environmental Engineering. 112(5): 867-920

Patel, B. K. C., Morgan, H. W., Daniel, R. M., and Zealand, N. (1985). Fervidobacterium nodosum gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Archives of Microbiology, 141, 63-69.

Pavlostathis, S.G. and Giraldo-Gomez, E. (1991) Kinetics of anaerobic treatment: A critical review. Critical Reviews in Environmental Control, 21(5,6): 411-490

Pender, S., Toomey, M., Carton, M., Eardly, D., Patching, J.W., Colleran, E. and O'Flaherty, V. (2004) Long-term effect of operating temperature and sulphate addition on the methanogenic community structure of anaerobic hybrid reactors. Water Research, 38: 619-630

Petersen, B., Gernaey, K., Devisscher, M., Dochain, D., and Vanrolleghem, P. a (2003) A simplified method to assess structurally identifiable parameters in Monod-based activated sludge models. Water Research, 37(12): 2893-904.

Petersen, S.P. and Ahring, B.K. (1991) Acetate oxidation in a thermophilic anaerobic sewage-sludge digestor: the importance of non-aceticlastic methanogenesis from acetate. FEMS Microbiology Letters 86(2): 149-158.

Pikuta, E. V., Hoover, R. B., Bej, A. K., Marsic, D., Whitman, W. B., Krader, P. E., and Tang, J. (2006) Trichococcus patagoniensis sp. nov., a facultative anaerobe that grows at -5 degrees C, isolated from penguin guano in Chilean Patagonia. International journal of systematic and evolutionary microbiology, 56(9), 2055-62.

Pind, P. F., Angelidaki, I., and Ahring, B. K. (2003) Dynamics of the anaerobic process: effects of volatile fatty acids. Biotechnology and bioengineering, 82(7): 791-801.

Pohland, F.G. and Ghosh, S. (1971) Developments in anaerobic stabilization of organic wastes: The two-phase concept. Environmental Letters, 1(4): 255-266

Puchajda, B. and Oleszkiewicz, J. (2006) Thermophilic anaerobic acid digestion of biosolids: hydrolysis, acidification and optimization of retention time of acid digestion. Journal of Environmental Engineering and Science, 5: 187-195

Ramirez, I., Mottet, A., Carrère, Hélène, Déléris, Stéphane, Vedrenne, Fabien, and Steyer, J.P. (2009) Modified ADM1 disintegration/hydrolysis structures for modeling batch thermophilic anaerobic digestion of thermally pretreated waste activated sludge. Water Research, 43(14), 3479-3492.

Ramirez, I., Volcke, E.I.P., Rajinikanth, R. and Steyer, J.P. (2009) Modeling microbial diversity in anaerobic digestion through an extended ADM1 model. Water Research 43, 2787-800.

Raposo, F., Fernández-Cegrí, V., De la Rubia, M. a, Borja, R., Béline, F., Cavinato, C., Demirer, G., Fernández, B., Fernández-Polanco, M., Frigon, J. C., Ganesh, R., Kaparaju, P., Koubova, J., Méndez, R., Menin, G., Peene, a, Scherer, P., Torrijos, M., Uellendahl, H., Wierinck, I., and de Wilde, V. (2011) Biochemical methane potential (BMP) of solid organic substrates: evaluation of anaerobic biodegradability using data from an international interlaboratory study. Journal of Chemical Technology and Biotechnology, 86(8): 1088-1098.

Raskin, L., Poulsen, L.K., Noguera, D.R., Rittmann, B.E. and Stahl, D. A. (1994) Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. Applied and Environmental Microbiology 60: 1241-1248.

Raskin, L., Zheng, D., Griffin, M. E., Stroot, P. G., and Misra, P. (1995) Characterization of microbial communities in anaerobic bioreactors using molecular probes. Antonie Van Leeuwenhoek. 68 (4): 297-308.

Rittmann, B. (2002) The role of molecular methods in evaluating biological treatment processes. Water Environment Research, 74(5): 421-427.

Robinson, J. A. and Tiedje, J. M. (1983) Nonlinear estimation of Monod growth kinetic parameters from a single substrate depletion curve. Applied and Environmental Microbiology, 45(5): 1453-1458.

Robinson, J.A. (1985) Determining microbial kinetic parameters using nonlinear regression analysis: advantages and limitation in microbial ecology. Advances in Microbial Ecology, 8: 14–61.

Robinson, J.A. and Characklis, W.G. (1984) Simultaneous estimation of Vmax, Km, and the rate of endogenous substrate production (R) from substrate depletion data, 10(2): 165-178.

Rodriguez, J., Lema, J.M., van Loosdrecht, M.C.M. and Kleerebezem, R. (2006) Variable stoichiometry with thermodynamic control in ADM1. Water Science and Technology, 54(4): 101-110.

Saez, P.B. and Rittmann, B.E. (1992) Model parameter estimation using least squares. Water Research, 26(6): 789–796.

Sánchez, E., Borja, R., Weiland, P., Travieso, L., and Martín, A (2000) Effect of temperature and pH on the kinetics of methane production, organic nitrogen and phosphorus removal in the batch anaerobic digestion process of cattle manure. Bioprocess Engineering, 22(3): 247-252.

Sawayama, S., Tsukahara, K., and Yagishita, T. (2006) Phylogenetic description of immobilized methanogenic community using real-time PCR in a fixed-bed anaerobic digester. Bioresource Technology, 97(1): 69-76.

Schmit, K.H. and Ellis, T.G. (2001) Comparison of temperature-phased and two-phase anaerobic co-digestion of primary sludge and municipal solid waste. Water Environment Research, 73(3): 314-321

Schoen, M.A., Sperl, D., Gadermaier, M., Goberna, M., Franke-Whittle, I., Insam, H., Ablinger, J, Wett, B. (2009) Population dynamics at digester overload conditions. Bioresource Technology, 100(23): 5648-5655

Schonheit, P., Kristjansson, J.K., and Thauer, R.K. (1982) Kinetic mechanism for the ability of sulfate reducers to outcompete methanogens for acetate. Archives of Microbiology 132(3):285-288.

Scully, C., Collins, G., and O'Flaherty, V. (2005) Assessment of anaerobic wastewater treatment failure using terminal restriction fragment length polymorphism analysis. Journal of Applied Microbiology, 99: 1463–1471.

Sekiguchi, Y.; Kamagata, Y.; Nakamura, K.; Ohashi, A.; Harada, H. (1999) Fluorescence in situ hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Applied and Environmental Microbiology, 65(3), 1280–1288.

Shigematsu, T.; Era, S.; Mizuno, Y.; Ninomiya, K.; Kamegawa, Y.; Morimura, S.; Kida, K. (2006) Microbial community of a mesophilic propionate-degrading methanogenic consortium in chemostat cultivation analyzed based on 16S rRNA and acetate kinase genes. Applied and Environmental Microbiology, 72(2): 401–415.

Shin, S. G., Han, G., Lim, J., Lee, C., and Hwang, S. (2010) A comprehensive microbial insight into two-stage anaerobic digestion of food waste-recycling wastewater. Water Research, 44(17): 4838-4849.

Siegrist, H., Renggli, D. and Gujer, W. (1993) Mathematical modelling of anaerobic mesophilic sewage sludge treatment. Water Science and Technology, 27(2): 25-36.

Siegrist, H., Vogt, D., Garcia-Heras, J. and Gujer, W. (2002) Mathematical model for meso- and thermophilic anaerobic sewage sludge digestion. Environmental Science and Technology, 36(5): 1113-1123

Simkins, S., and Alexander, M. (1985) Nonlinear estimation of the parameters of Monod kinetics that best described mineralization of several substrate concentrations by dissimilar bacterial densities. Applied and Environmental Microbiology, 50(4): 816-824.

Simkins, S. and Alexander, M. (1984) Models for mineralization kinetics with the variable of substrate concentration and popular density. Applied and Environmental Microbiology, 47: 1299-1306

Skiadas, I.V., Gavala, H.N., Lu, J. and Ahring, B.K. (2005) Thermal pre-treatment of primary and secondary sludge at 70 degrees C prior to anaerobic digestion. Water Science and Technology, 52(1-2): 161-166

Smith, L.H., McCarty, P.L. and Kitanidis, P.K. (1998) Spreadsheet method for evaluation of biochemical reaction rate coefficients and their uncertainties by weighted nonlinear least-squares analysis of the integrated monod equation, Applied and Environmental Microbiology, 64(6): 2044-2050

Smith, P.H. and Mah, R.A. (1966). Kinetics of acetate metabolism during sludge digestion. Applied Environmental Microbiology 14 (3): 368-371.

Sollfrank, U. and Gujer, W. (1991) Characterisation of domestic wastewater for modelling of the activated sludge process. Water Science and Technology, 23(4-6): 1057–1066.

Song, Y.C., Kwon, S.J. and Woo, J.H. (2004) Mesophilic and thermophilic temperature co-phase anaerobic digestion with single-stage mesophilic and thermophilic digestion of sewage sludge. Water Research, 38(7): 1653-1662

Sorensen, A.H. and Ahring, B.K. (1993) Measurements of specific methanogenic activity of anaerobic digester biomass. Applied Microbiology and Biotechnology, 40: 427-431

Soto, M., Mendez, R. and Lema, J.M. (1993) Methanogenic and non-methanogenic activity tests: theoretical basis and experimental set up. Water Research, 27(8): 1361-1379

Sousa, D. Z., Smidt, H., Alves, M. M., and Stams, A. J. M. (2007) Syntrophomonas zehnderi sp. nov., an anaerobe that degrades long-chain fatty acids in co-culture with Methanobacterium formicicum. International Journal of Systematic and Evolutionary Microbiology, 57(3): 609-615.

Speece, R.E. (1996) Anaerobic biotechnology for industrial wastewaters. Archae Press, Nashville, Tennessee

Speigelman, D., Whissell, G. and Greer, C.W. (2005) A survey of the methods for the characterization of microbial consortia and communities. Canadian Journal of Microbiology, 51: 355-386

Steinle, P., Stucki, G., Stettler, R., and Hanselmann, K. W. (1998) Aerobic mineralization of 2,6-dichlorophenol by Ralstonia sp. strain RK1. Applied and environmental microbiology, 64(7): 2566-2571.

Straub, A. J., Conklin, A. S., Ferguson, J. F., and Stensel, H. D. (2006) Use of the ADM1 to investigate the effects of acetoclastic methanogen population dynamics on mesophilic digester stability. Water Science and Technology, 54(4): 59-66.

Stroot, P.G., McMahon, K.D., Mackie, R.I. and Raskin, L (2001) Anaerobic co-digestion of municipal solid waste and biosolids under various mixing conditions - I. performance data, Water Research, 35(7): 1804-1816

Tanaka, K., Nakamura, K., and Mikami, E. (1991) Fermentation of cinnamate by a mesophilic strict anaerobe Acetivibrio multivorans sp. nov. Archives of Microbiology, 155(2): 120-124.

Toerien, D.F. and Haltingh, W.H.J. (1969) Anaerobic digestion. I. The microbiology of anaerobic digestion. Water Research, 3: 385-416

Tomei, M. C., Braguglia, C. M., Cento, G., and Mininni, G. (2009) Modeling of Anaerobic Digestion of Sludge. Critical Reviews in Environmental Science and Technology, 39(12): 1003-1051.

Touzel, J. P., Petroff, D., Maestrojuan, G. M., Prensier, G., and Albagnac, G. (1988) Isolation and characterization of a thermophilic Methanobacterium able to use formate, the strain FTF. Archives of Microbiology, 149(4): 291 - 296.

Ueno,Y, Haruta,S, Ishii, M. and Igarashi, Y. (2001) Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Applied Microbiology and Biotechnology, 57(4): 555-562.

v. Münch, E., Keller, J., Lant, P., and Newell, R. (1999) Mathematical modelling of prefermenters—I. Model development and verification. Water Research, 33(12): 2757-2768.

van Lier, J. B. (1996) Limitations of thermophilic anaerobic wastewater treatment and the consequences for process design. Antonie van Leeuwenhoek, 69(1): 1-14.

van Loosdrecht, M. C. M. and Heijnen, J. J. (2002) Modelling of activated sludge processes with structured biomass. Water Science and Technology, 45(6): 13-23.

Vandenburgh, S.C. and Ellis, T.G. (2002) Effect of varying solid concentration and organic loading on the performance of temperature-phased anaerobic digestion process. Water Environment Research, 74(2): 142-148

Vavilin, V. A., Fernandez, B., Palatsi, J., and Flotats, X. (2008) Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview. Waste management 28(6): 939-51.

Vavilin, V. A., Rytov, S.V., and Lokshina, L.Y. (1996) A description of hydrolysis kinetics in anaerobic degradation of particulate organic matter. Bioresource Technology 56: 229-237.

Vavilin, V.A., Qu, X., Mazéas, L., Lemunier, M., Duquennoi,C., He, P., Bouchez, T. (2008) Methanosarcina as the dominant aceticlastic methanogens during mesophilic anaerobic digestion of putrescible waste. Antonie van Leeuwenhoek 94(4): 593-605.

Wagner, M., Amann, R., Lemmer, H. and Schleifer, K. H. (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Applied and Environmental Microbiology, 59: 1520–1525.

Watts, S., Hamilton, G., and Keller, J. (2006) Two-stage thermophilic-mesophilic anaerobic digestion of waste activated sludge from a biological nutrient removal plant. Water Science and Technology, 53(8): 149-157.

Wiegant, W. M.; Hennink, M.; Lettinga, G. (1986) Separation of the propionate degradation to improve the efficiency of thermophilic anaerobic treatment of acidified wastewaters. Water Research, 20(4): 517-524.

Wilderer, P.A., Bungartz, H.J., Lemmer, H., Wagner, M., Keller, J., Wuertz, S. (2002) Modern scientific methods and their potential in wastewater science and technology. Water Research 36(2): 370-393.

Williams, R. T. and Crawford, R. L. (1985) Methanogenic bacteria, including an Acid-tolerant strain, from peatlands. Applied and Environmental Microbiology, 50(6): 1542-1544.

Yasui, H., Goel, R., Li, Y. Y., and Noike, T. (2008) Modified ADM1 structure for modelling municipal primary sludge hydrolysis. Water Research, 42(1-2): 249-259.

Yasui, H., Sugimoto, M., Komatsu, K., Goel, R., Li, Y.Y., and Noike, T. (2006) An approach for substrate mapping between ASM and ADM1 for sludge digestion. Water Science and Technology, 54(4): 83-92.

Yuan, Z. and Blackall, L. L. (2002) Sludge population optimisation: a new dimension for the control of biological wastewater treatment systems. Water Research, 6(2): 482-90.

Zahller, J.D., Bucher, R.H., Ferguson, J.F., and Stensel, H.D. (2007) Performance and stability of two-stage anaerobic digestion. Water Environment Research, 79(5): 488-497

Zhang, B., Zhang, L.L., Zhang, S.C., Shi, H.Z., and Cai, W.M. (2010) The Influence of pH on Hydrolysis and Acidogenesis of Kitchen Wastes in Two-phase anaerobic digestion. Environmental Technology, 29: 329-339.

Zhang, T. and Fang, H.H.P. (2006) Application of real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Applied Microbiology and Biotechnology, 70: 281-289

Zhang, T.C. and Noike, T. (1991) Comparison of one-phase and two-phase anaerobic digestion processes in characteristics of substrate degradation and bacterial population levels. Water Science and Technology, 23(7-9): 1157-1166

Zhang, Z., Wang, Y., and Ruan, J. (1998) Reclassification of Thermomonospora and Microtetraspora. International journal of systematic bacteriology, 48(2): 411-22.

Zinder, S.H. (1993) Physiological ecology of methanogens. p. 128-206. In: J.G. Ferry (ed.) Methanogenesis: ecology, physiology, biochemistry and genetics, Chapman and Hall, New York

Zinder, S.H., and Koch, M. (1984) Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic co-culture. Archives of Microbiology. 138: 263–272

Zinder, S.H., Anguish, T., and Cardwell, S.C. (1984a) Effects of temperature on methanogenesis in a thermophilic (58°C) anaerobic digestor. Applied and Environmental Microbiololgy, 47:808-813

Zinder, S.H., Cardwell, S.C., Anguish, T., Lee, M. and Koch, M. (1984b) Methanogenesis in a thermophilic (58 0C) anaerobic digester: Methanothrix sp. as an important aceticlastic methanogen. Applied and Environmental Microbiology, 47: 796-807

Zoetemeyer, R.J., van den Heuvel, J.C. and Cohen, A. (1982a) pH influence on acidogenic dissimilation of glucose in an anaerobic digester. Water Research, 16(3): 303-311

Zumstein, E., Moletta, R. and Godon, J.J. (2000) Examination of two years community dynamics in an anaerobic bioreactor using fluorescence polymerase chain reaction (PCR) single-stranded conformation polymorphism analysis. Environmental Microbiology, 2(1): 69-78.

Appendix A

Additional information on sludge characteristics

Table A.1. Percent change of raw sludge COD over each week storage at 4 0C

Date	TCOD			SCOD		
	Mean mg/L	SD* mg/L	% Change	Mean mg/L	SD mg/L	% Change
1 March 2009	41630	636	5	4007	176	12
5 March 2009	39530	919		4545	106	
13 March 2009	41180	848	2	5032	53	22
15 March 2009	40330	353		6532	88	
3 April 2009	38186	715	8	4569	71	7
9 April 2009	35080	636		4936	37	
21 May 2009	35630	212	5	4720	216	8
26 May 2009	33629	777		5107	18	
18 June 2009	38930	721	6	6207	176	12
22 June 2009	36680	982		7107	116	
9 July 2009	37480	1258	3	3907	152	4
16 July 2009	3628	451		4082	159	
4 September 2009	39613	618	4	3282	194	8
9 September 2009	37880	1137		3561	44	
17 November 2009	39927	185	5	4675	32	9
22 November 2009	38066	997		5140	56	
2 December 2009	41317	185	7	3626	72	-1
6 December 2009	38232	556		3579	15	

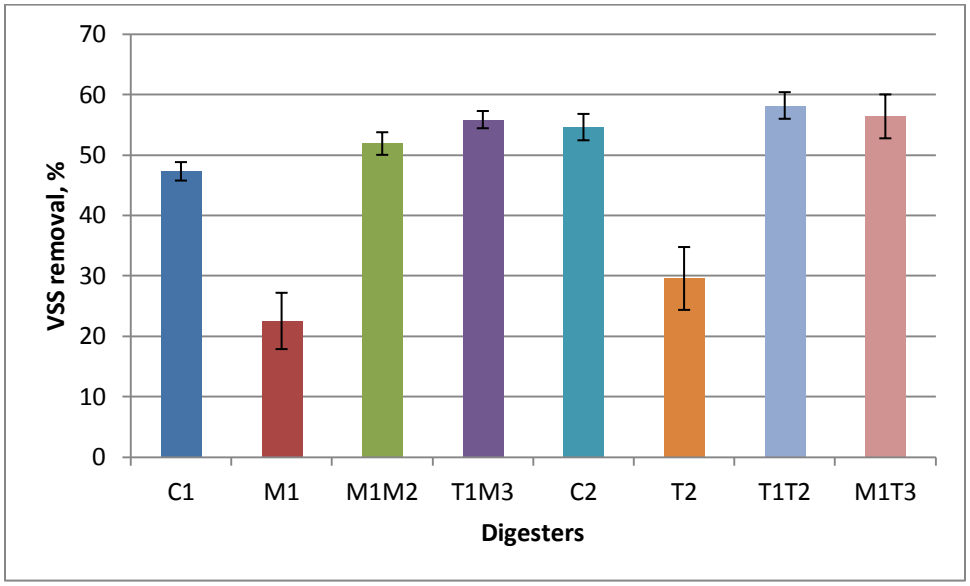


Figure A.1. Volatile Suspended Solids Reduction in Digestion Systems (AVERAGE ± SD)

Table A.2. Statistical analysis for comparison of VSS removals (ANOVA Tukey test, α=0.05)*

System	C1	M1	M1M2	T1M3	C2	T1	M1T3	T1T2
C1		XX	XX	XX	XX	XX	XX	XX
M1			XX	XX	XX	XX	XX	XX
M1M2				XX	XX	XX	XX	XX
T1M3					X	XX	X	XX
C2						XX	X	XX
T1							XX	XX
M1T3								X

*XX significant; X not significant

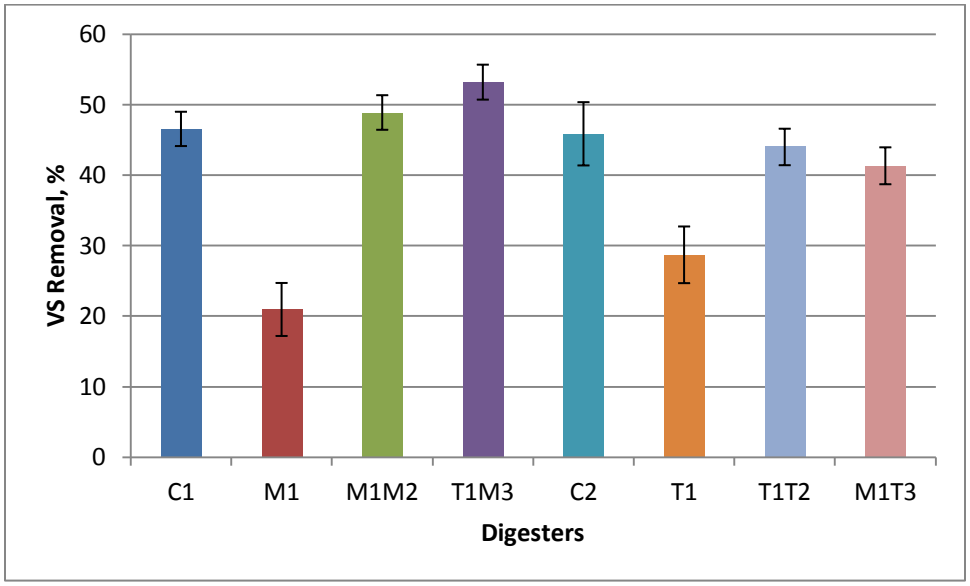


Figure A.2. Volatile Solids Reduction in Digestion Systems (AVERAGE ± SD)

Table A.3. Statistical analysis for comparison of VS reduction in various digesters (ANOVA Tukey test, α=0.05)*

System	C1	M1	M1M2	T1M3	C2	T1	M1T3	T1T2
C1		XX	XX	XX	X	XX	XX	XX
M1			XX	XX	XX	XX	XX	XX
M1M2				XX	XX	XX	XX	XX
T1M3					XX	XX	XX	XX
C2						XX	XX	X
T1							XX	XX
M1T3								XX

*XX significant; X not significant

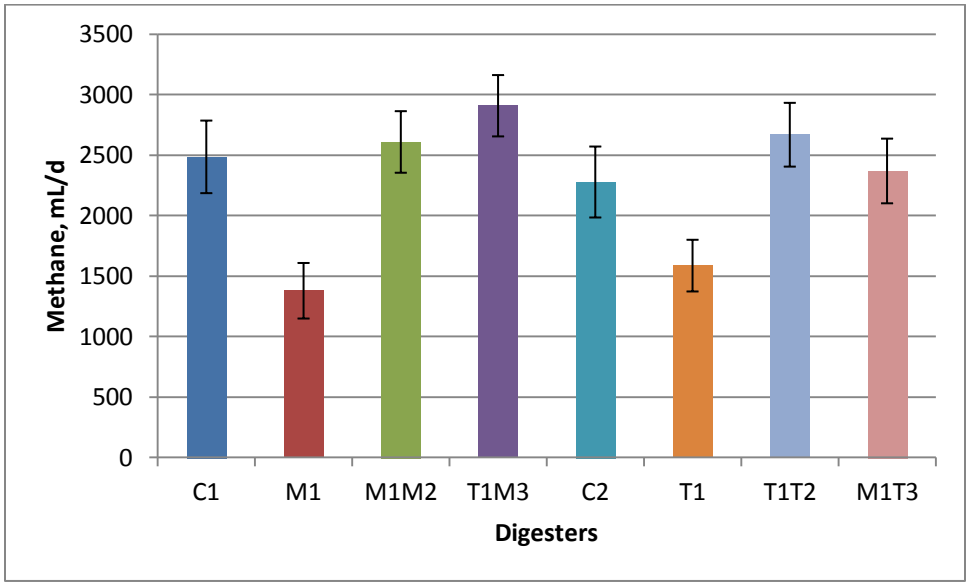


Figure A.3. Average and SD of methane gas production in digestion systems.

Table A.4. Statistical analysis for comparison of methane production in various digesters (ANOVA Tukey test, α=0.05)*

System	C1	M1	M1M2	T1M3	C2	T1	M1T3	T1T2
C1		XX	XX	XX	XX	XX	XX	XX
M1			XX	XX	XX	XX	XX	XX
M1M2				XX	XX	XX	XX	X
T1M3					XX	XX	XX	XX
C2						XX	X	XX
T1							XX	XX
M1T3								XX

*XX significant; X not significant

Appendix B

Electrophoresis of PCR products

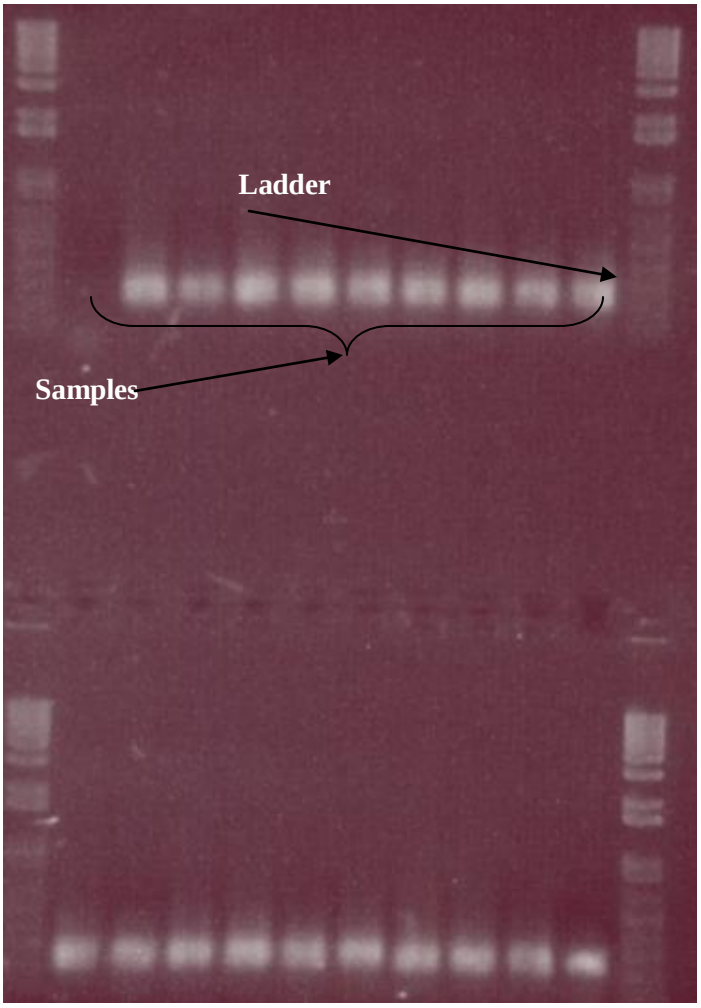


Figure B.1. Agarose gel electrophoresis of bacterial PCR products. Upper: Samples taken on 23 September 2010; Lowe: Sample taken on 22 October 2010.

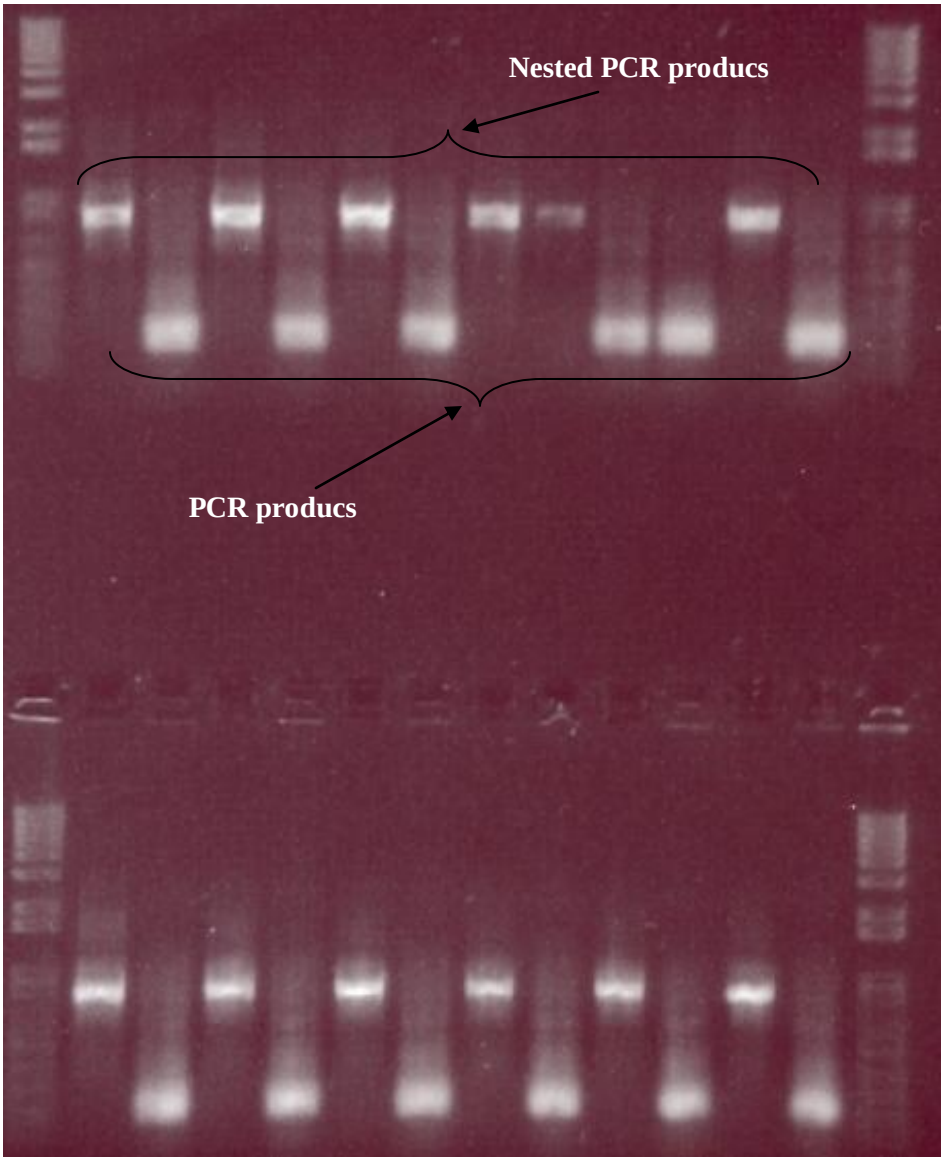
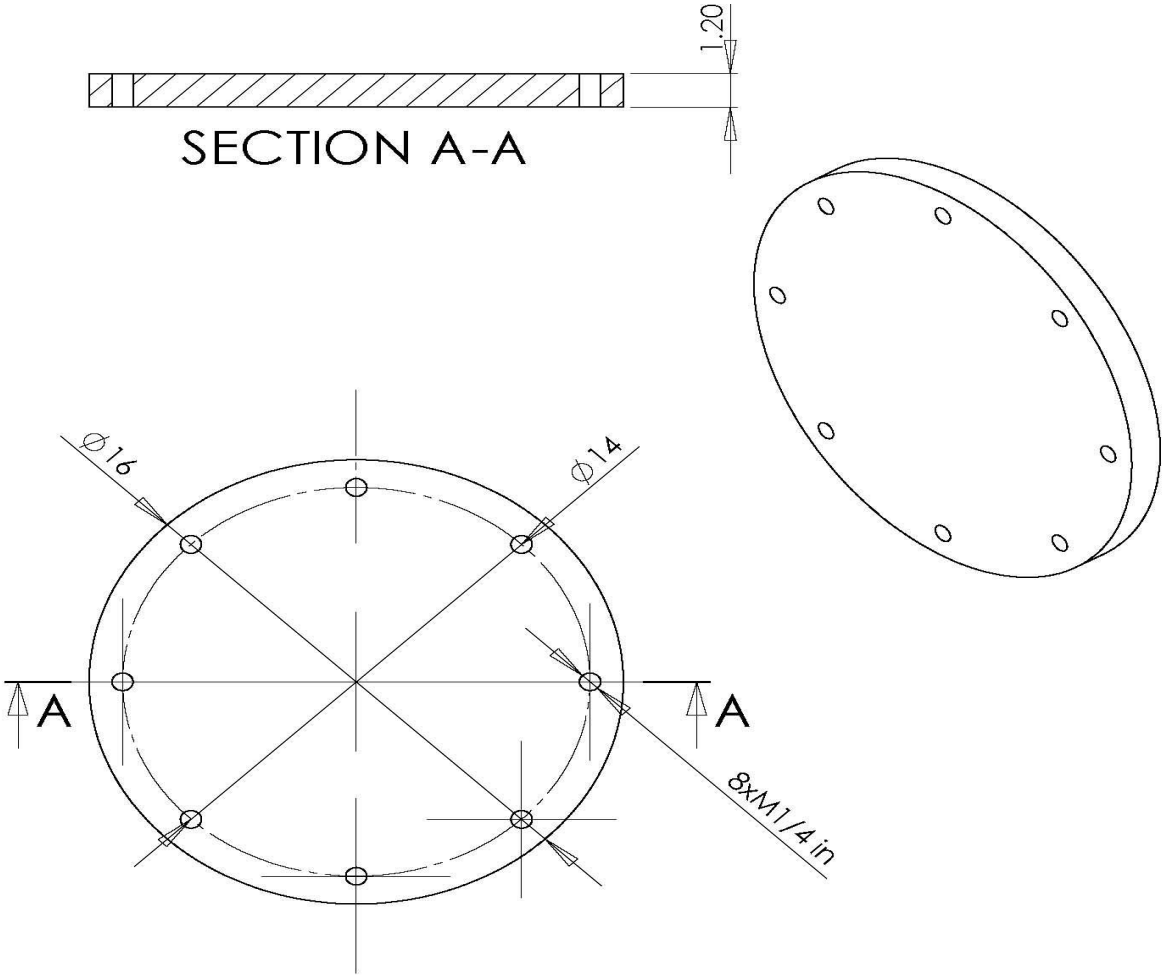


Figure B.2. Agarose gel electrophoresis of archaeal PCR products. Upper: Samples taken on 23 September 2010; Lowe: Sample taken on 22 October 2010.

Appendix C

Details of digesters dimensions



		DIMENSIONS ARE IN INCHES TOLERANCES: FRACTIONAL ± ANGULAR: MACH ± BEND ± TWO PLACE DECIMAL ± THREE PLACE DECIMAL ±		NAME	DATE
			DRAWN		
			CHECKED		
			ENG APPR.		
			MFG APPR.		
		MATERIAL PVC	Q.A.	4	
NEXT ASSY	USED ON	FINISH	COMMENTS:		
APPLICATION		DO NOT SCALE DRAWING	All Dimentions in cm		

SIZE	DWG. NO.	REV.
A	plate_b	
SCALE:5:1	WEIGHT:	SHEET 1 OF 1

Figure C.1. Bottom plate of the 1st-phase digesters

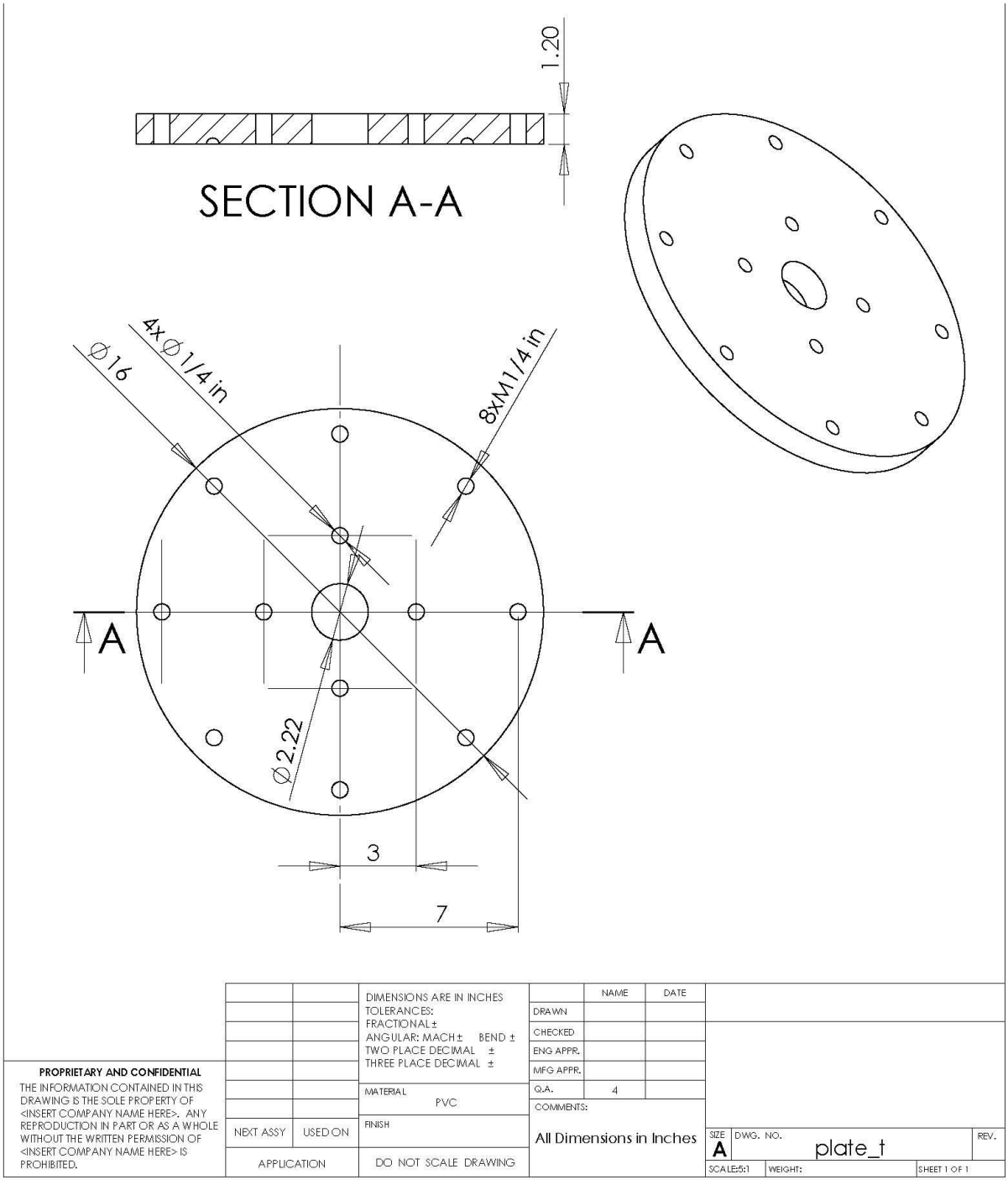
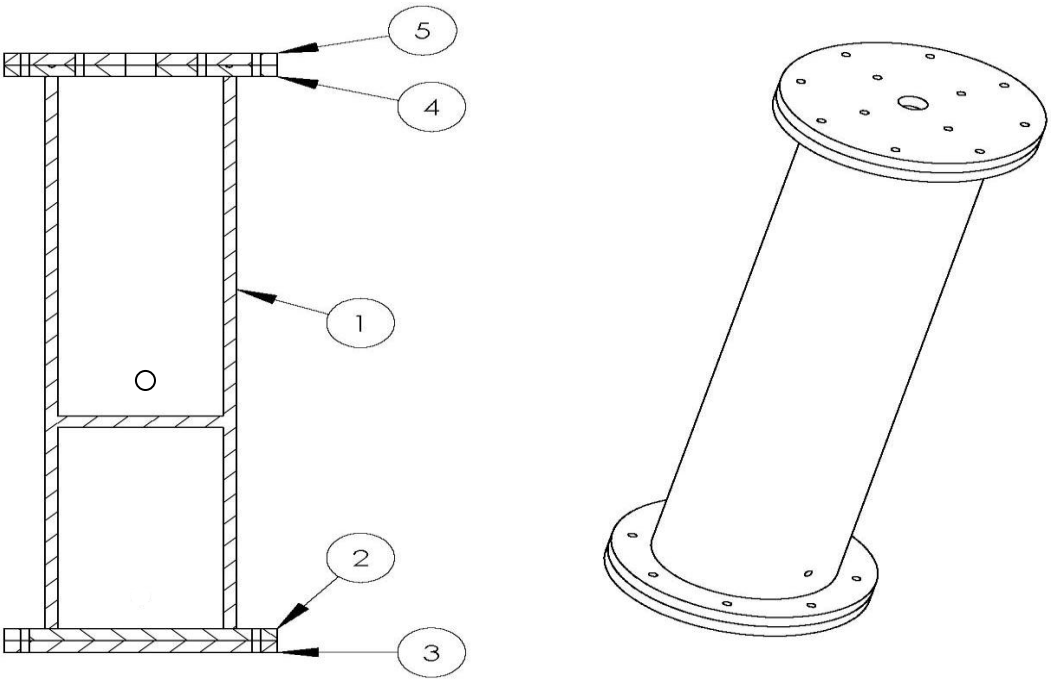
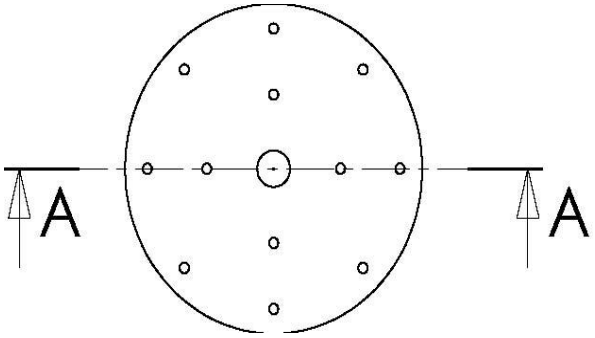


Figure C.2. Top plate of the 1st-phase digesters



SECTION AA



Part No	Part Name	Quantity
1	Tube	2
2	Plate_b	2
3	Plate_b_b	2
4	Plate_t	2
5	Plate_t_t	2

Figure C.3. Assemply parts of the 1st-phase digesters

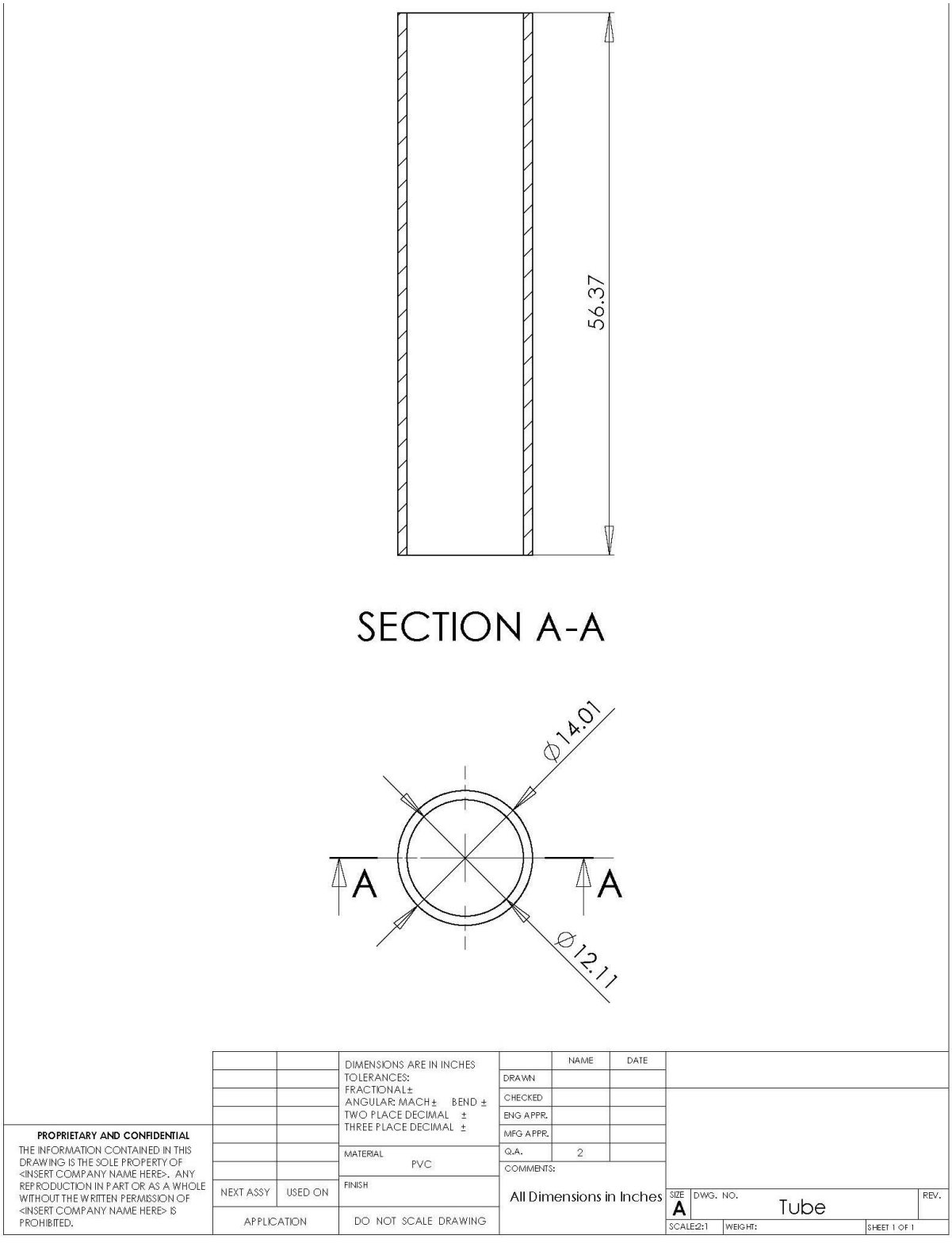


Figure C.4. Dimensions of the single-stage digesters

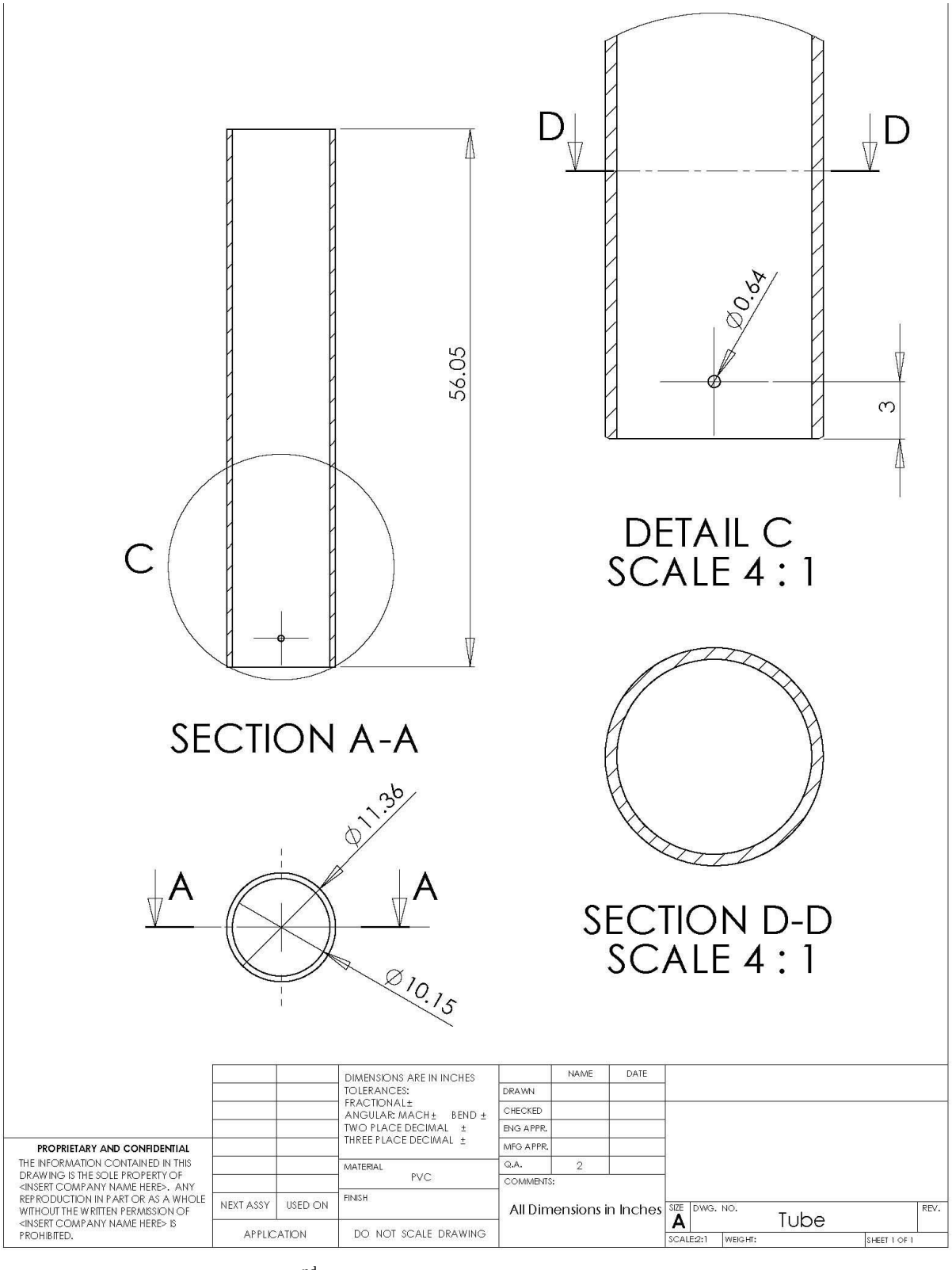


Figure C.5. Dimensions of the 2nd-phase digesters