Optimization of Anaerobic Digestion: Influence of Trace Elements on Methanization Processes

Vom Promotionsausschuss der
Technischen Universität Hamburg-Harburg
Zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften

genehmigte Dissertation

Von

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aus
Nigeria

2014

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Tag der mündlichen Prüfung: 11.04.2014

This doctorate thesis was carried out at:

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www.tuhh.de/aww

Printed with support from:

Deutschen Akademischen Austauschdienst (DAAD)

Kennedyallee 50

D-53175 Bonn

Germany

Tel: (0228) 882-0

Fax: (0228) 882-444

URL: www.daad.de

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URL: www.gfeu.org

ISBN 978-3-942768-12-2

Hamburger Berichte zur Siedlungswasserwirtschaft

Band 87

ACKNOWLEDGEMENT

My utmost regards and gratitude go to PD Dr.-Ing. habil Ina Körner for accepting my request for supervision and PhD research proposal, facilitating my visa to Germany, and helping to secure scholarship for me from the Deutscher Akademischer Austauschdienst (DAAD). I am also very thankful to her for funding my research and conference attendances, encouragement and management of the quality of my research. I am also very thankful to Prof. Bamidele Solomon and Dr. Christie Onyia (National Biotechnology Development Agency, Nigeria) for the encouragement to enrol for the PhD programme and for approving a study leave for this purpose.

I am indebted to, and pledge my unalloyed support and cooperation to the government of Germany for the DAAD scholarship, which has offered me the opportunity to build a solid hub for my future career. I am obliged to the Hamburg University of Technology (TUHH) for accepting my contributions to knowledge as worthy of a doctorate degree. I am very grateful to Prof. Dr.-Ing Ralf Otterpohl who accepted us in his institute and ensured sustainable continuity of my research when the journey took a turn, which could have been unfavourable. Susanne Eggers of the Institute of Water and Wastewater Management (AWW) was never tired of my many requests for analytical protocols. She and Binnur Kuscu (AWW) were always willing to help with my analyses. These ones, I strongly extol. I am also appreciative of all staff and students of the amazing AWW institute for their support.

I strongly laud Irene Richardt-Brauer of the Institute for Environmental Technology and Energy Economics (IUE) who happily did my countless analyses involving trace elements determination. I appreciate Dr. Kerstin Sahm and John Patrick (Technical Microbiology) for the microbial analyses of my samples. I compliment the following students: Sandra Martinez, Sakeena Quraishi, James Ezinma, Lanre Adebayo, Krisada Techamanoon, Maxwell Onoh and Jieyun Tang who worked with me as assistants, project or master thesis students. Their trust in my hypothesis and faithful conduct of the experiments and analyses strongly contributed to the success of my research. I also commend the Herrling Group and other companies in Germany for supplying the substrates and starting microbial culture for my investigations.

Finally, my eternal gratitude goes to the God of my heart.

ABSTRACT

Trace elements (TEs) supplementation to an anaerobic digester provides the necessary bio-catalysis required for process resilience against perturbations that induce decline in efficiency during methanization. The need for TEs supplementation is obvious from the poor TEs balance and low concentrations of nickel (Ni), cobalt (Co), selenium (Se) and molybdenum (Mo) in substrates commonly used for methanization. Co and Se are particularly limiting in methanization substrates. Complex substrates such as grease trap residues, blackwater and sludges have large reservoirs of TEs, but in rather unbalanced composition. Balance in TEs composition and concentrations are important and resulted in average of 50% increase in the methanization processes of substrate degradation and methane formation in mesophilic operation. Lower proportions of methanization enhancement were achieved in thermophilic condition.

Volatile fatty acids (VFAs) concentration determines the extent of TEs influence and the significance of TEs interactions during methanization. TEs supplementation based on VFAs concentration offers the most significant methanization enhancement. The TEs mixture that is optimized for different methanization phases also produced comparable enhancement, but with specific weaknesses. Generally, TEs supplementation enhanced microbial substrate affinity in thermophilic systems and microbial maximum substrate conversion rate in mesophilic systems. Significant change in microbial population due to TEs supplementation was not observed during methanization.

Bioavailability of TEs during methanization is influenced by TEs adsorption to digester solids. Ni and Co have about 40 - 60% bioavailability; and TEs mixture composition influenced the bioavailability of Ni and Co, but not Se and Mo. Se has about 60% bioavailability and Mo is about 60% bio-unavailable. The quality of methanization digestate regarding TEs content was not compromised due to TEs supplementation. Optimum supplementation range for Ni is below the legal limits set by both the European Directive 86/278/EEC and the German adoption of the European Directive for total Ni concentration in methanization sludge intended for use in soil amendment. There are no such limits for Co, Se and Mo in digestate but optimum concentrations of Ni, Co, Se and Mo for methanization, and their respective levels in digestate are in similar ranges.

Key words: *Trace elements, optimization, methanization, conversion rate, affinity, bioavailability and adsorption.*

SUMMARY

In anaerobic digestion (AD), even when pH, carbon-nitrogen ratio, substrate combination and loading rate are optimum, shortage or unavailability of the trace elements (TEs) nickel, cobalt, selenium and molybdenum, could result in decline in methanization efficiency. TEs are structural and catalytic components of the active sites of important enzymes associated with hydrogen, carbondioxide and acetate metabolism during AD. Investigations relating to the influence of TEs abound but inconsistencies in results are common. Inconsistencies in results could be due to various uninvestigated factors that influence the biochemistry of TEs during methanization. Reports on the relationships between TEs of AD importance and other factors of methanization such volatile fatty acids (VFA) concentration and temperature are lacking. There is also no report on the influence of TEs on hydrolysis, the mechanism of TEs enhancement during methanization and comparative supplementation approaches.

The absence of these reports creates a wide gap in knowledge and accounts for weak understanding of the issues surrounding effective TEs mixture formulation and supplementation, and the resultant TEs influences in AD. Consequently, this research investigated the sufficiency or otherwise of TEs in common AD substrates and determined whether TEs supplementation was necessary or not. It also appraised the possible supplementation approaches; explored the mechanisms of AD improvements due to TEs supplementation; estimated TEs bioavailability during AD; and investigated the change in microbial groups due to TEs supplementation.

The results of this research confirmed that thermophilic-, mesophilic methanization and different VFAs concentrations have optimum TEs requirements that differ in composition and concentration. In both mesophilic and thermophilic AD, supplementing TEs on the basis of digester VFAs concentration produced the most positive influence on methanization efficiency. However, a VFA-independent optimum TEs mixture that has similar influences as VFA-dependent TEs supplementation was derived statistically. This derived single dose enhanced methanization by increasing the maximum reaction rate of VFAs degradation at mesophilic methanization; and increasing substrate affinity at thermophilic methanization.

The results further confirmed that the mechanisms of TEs enhancement in AD vary with temperature; and also highlighted the differential bioavailability of TEs. Validation of the derived VFA-independent optimum TEs mixture and its variants in continuous AD investigations confirmed the methanization enhancements in the batch AD. The enhancements in the continuous AD resulted in > 50% increase in substrate loading rate without negative impact on process stability; and > 40% increase in CH_4 production. Microbial population studies showed no emergence of new population and confirmed the enhancement of the biocatalytic potentials of the resident bacteria and archaea population. Based on the findings of this research, proposition for the modification of AD plants for effective TEs dosing, as well as precautionary measures to maximize the positive influences of TEs supplementation were offered.

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ABBREVIATIONS

ACS	Acetyl coenzyme-A synthase
AD	Anaerobic digestion
ANOM	Analysis of mean
AP	Adaptation period
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BW	Blackwater
CD	Custom design
CI	Confidence interval
COD	Chemical oxygen demand
CODH	Carbon monoxide dehydrogenase
D	Desirability function
DGGE	Denaturing gel gradient electrophoresis
DIN EN	Deutsches institut fur normung (German insitute for standards)
DM	Dry matter
DoE	Design of experiment
DS	Digested sewage sludge
EEC	European economic community
F_{430}	Factor 430
FAAS	Flame atomic absorption spectroscopy
FDH	Formate dehydrogenase
FF	Full factorial
FMD	Formyl-methanofuran dehydrogenase
FOS/TAC	Acidity/alkalinity
FR	Formate reductase
GTR	Grease trap residue
H_4MPT	Tetrahydromethanopterin
HA	Hydrolysis and acidification
HAR	Hydrolysis and acidification rate
IA	Inverse affinity
LCFA	Long chain fatty acid
LDL	Lower decision limits
M/A	Methanogen and acetogen ratio
MCR	Methyl-CoM reductase
MEs	Metallo-enzymes
MeTr	Methyltransferase
MFR	Mixed fruit residue
MMC	Methylmalonyl CoA
MPB	Methane producing bacteria
MR	Methanofuran reductase
MRR	Maximum reaction rate
oDM	organic dry matter
OLR	Organic loading rate
ORP	Oxidation reduction potential

OTC-VFA_120	Optimum trace element configuration for VFA concentration between 100 and 120 mmol/L
OTC-VFA_DL	Optimum trace element configuration for VFA at different levels
R	Reactor
RPV	Relative prediction variance
RSM	Response surface methodology
Rt	Retention time
RW	Restaurant biowaste
[S]	Substrate concentration
SAO	Syntrophic acetate oxidation
SE	Sequential extraction
SF	Silage feedstock
SMs	Standard methods
SRB	Sulphate reducing bacteria
TAN	Total ammonia nitrogen
TC- compromise	Trace element configuration of the compromise setting
TC- control	Trace element configuration of the control reactor
TEs	Trace elements
T_m	Mesophilic temperature
T_t	Thermophilic temperature
TVFA	Total volatile fatty acid
UDL	Upper decision limits
VFA	Volatile fatty acid
V_{max}	maximum velocity
Δtime	change in time
ΔVFA	change in VFA concentration

CHAPTER 1 INTRODUCTION

Anaerobic digestion (AD) involves microbiological degradation of organic materials under anoxic conditions. It is also referred to as methanization because it involves substrate mineralization to biogas, which predominantly comprises methane (CH_4) and carbon-dioxide (CO_2), in a series of interdependent biochemical phases. Methanization offers the advantages of the recovery of CH_4, which is used as energy source in transportation, electricity generation and heating. The slurry from methanization is the digestate, and it is used for soil fertility enhancement. Methanization is also important for climate protection due to the use of renewable carbon for energy generation (Macias-Corral *et al.,* 2008).

Methanization is influenced by temperature, pH, substrate composition and other factors such as digester design and substrate particle size (Mata-Alvarez, 2003). One of the important challenges in methanization is to find an optimum balance between all the important factors and considerable information exist for this purpose. However, even when these factors are within optimum range, process instability, longer digestion period, poor CH_4 yield and eventual digester failure have been reported (Ahn and Foster, 2002; Chen *et al.,* 2008). A number of independent or overlapping causes might account for such unexpected digester failure, but most considered are inhibition to sensitive micro-organisms and imbalance in microbial nutrient.

AD is a biochemical process, and microbial activities such as growth rate and substrate utilization potentials play significant roles in process stability and efficiency (Gerardi, 2003). Changes in factors such as microbial population ratio in start-up inoculum, and nutrient-related factors such as availability of micro-nutrients have been known to influence the microbiology and biochemistry of AD (Hinken *et al.,* 2008). Proportions of macro-nutrients such as carbon (C), nitrogen (N) and sulphur (S) ratios have wide relevance in AD (Hansen, 1993). Relevance of micro-nutrients such as nickel (Ni), cobalt (Co), selenium (Se), molybdenum (Mo) and tungsten (W) to the biochemical efficiency of enzymatic and microbial processes is also documented (Climenhaga and Banks, 2008; Lo *et al.,* 2010).

In allied-disciplines, transition elements such as zinc (Zn), iron (Fe), Ni, Co, Se and Mo are reported to be constituents and activators of certain enzymes, which are generally referred to as metallo-enzymes (MEs) (Matsumoto, 2005; Ragsdale, 2006). The Critical enzymes of methanization are MEs (Kida *et al.,* 2001; Karrasch *et al.,*

1990). These authors reported that even when other factors are optimum, MEs have reduced activity or are non-functional when the activator elements are absent. The micro-nutrients are transition elements and are required in trace amount; therefore, they are referred to as trace elements (TEs). TEs of documented relevance in AD include Ni, Co, Se, Mo and W (Ahn and Foster, 2002); however, conflicting reports trail research on TEs composition and concentrations that are appropriate for AD. Specifically, knowledge is sparse on the factors that influence the requirements of TEs during AD, and their mechanisms of improving methanization.

Regardless of the lack of specific knowledge, general knowledge of the microbial requirements for transition elements such as Fe, Ni and Co form the basis for the commercial formulation of mixtures of TEs, and their use in AD. Generally these are mixtures with fixed TEs composition and concentrations. The problem with the use of such commercial TEs mixtures for the optimization of methanization is that different phases of methanization might have different TEs requirements due to the dominance of different micro-organisms. Moreover, the composition and concentrations of the TEs determine whether their influences are positive or negative during methanization (Lin, 1992). Knowledge of the influences of TEs in the different phases of AD, interactions between the TEs in mixture, and interaction between TEs and important factors of AD is important for reliable use of TEs in the optimization of AD.

Furthermore, considering that unused TEs remain in digestate, it is important that the optimum composition of TEs and ranges of their concentrations do not impair the use of digestate in agriculture. To fill the identified gaps in the knowledge of the use of TEs in AD, this study combines literature reviews, empirical- and statistical methods, to determine the influence of Ni, Co, Se and Mo in batch mesophilic and thermophilic AD. The factors that influence the requirements of these TEs are investigated, as well as the mechanisms by which the TEs enhance methanization. The last part of the investigation involves the validation of the optimum TEs composition and concentrations (configuration) in a continuous AD operation.

CHAPTER 2 ANAEROBIC DIGESTION

2.0 Chapter overview

Municipalities, industries and agriculture produce different waste types that could be used as substrates in AD. These substrates include biotonne, food residues from restaurants; process residues from agro-industries; silages from agricultural residues; and wastewater (Mata-Alvarez, 2003; Körner *et al.,* 2013). Other non-waste substrates include energy crops such as maize silage and sunflower.

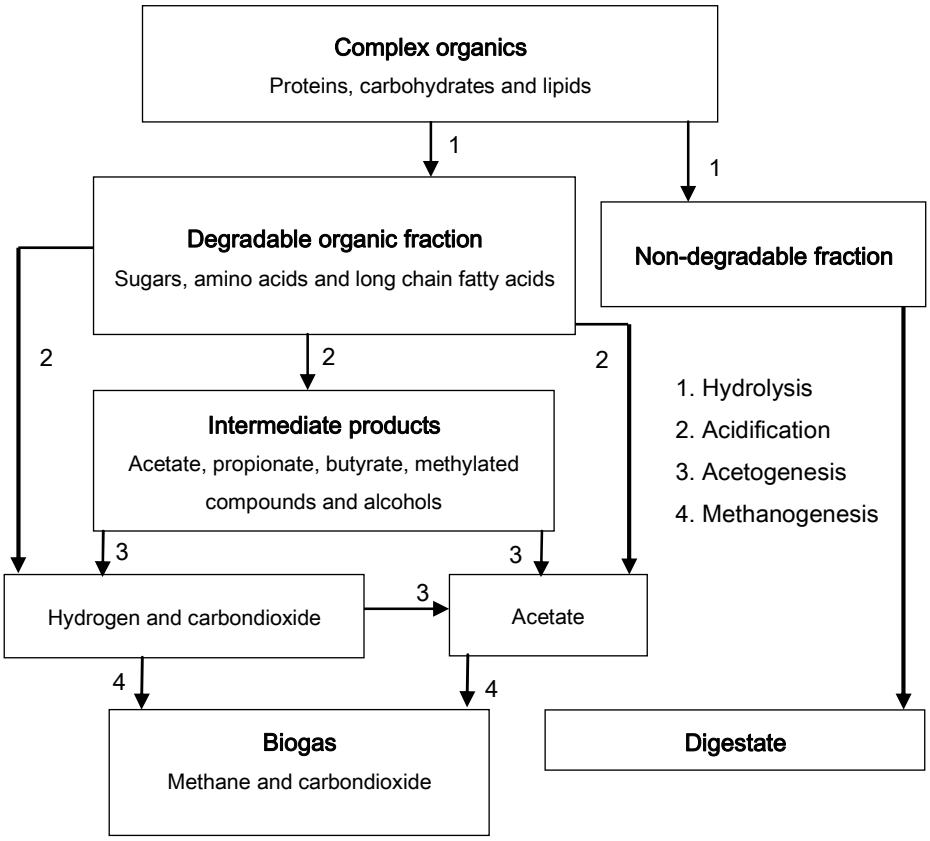


Figure 2.1 Fractions, phases and the intermediate products in the different phases of anaerobic digestion (modified from Mata-Alvarez, 2003; Cheng *et al.,* 2008)

Figure 2.1 shows the general fractions of AD substrates and the processes involved in their conversion to bioenergy through AD. AD substrates are complex organic compounds composed of degradable and non-degradable components. The Complex degradable organics are hydrolysed to simpler compounds such as sugars, amino acids and fatty acid of various carbon lengths. The simpler compounds are further converted to energy-rich biogas through interdependent microbial processes

(Hill and Holmberg, 1988; Schink and Stams, 2005). Biochemically non-degradable fractions remain as digester solids or digestate.

Application of AD for CH_4 and digestate generation from organic substrates is widespread but the scale of operation varies and depends on regional factors (Appels *et al.,* 2008; Banks and Zhang, 2012). In Europe, the driving forces for CH_4 and digestate generation from organic substrates are government renewable energy policies and economic incentives (Körner *et al.,* 2013). During stable methanization, CH_4 content of biogas could range between 50 -75% depending on whether carbohydrate- or lipid-rich substrates are used as substrates. Other constituents of biogas include CO_2 (25 – 50%); O_2 (< 1%); and N_2 < (1%). Traces of hydrogen sulphide gas (H_2S); hydrogen gas (H_2) and chlorinated hydrocarbons could also be present (Appels *et al.,* 2008).

The digestate from AD could be solid, but it is mostly slurry because wet AD is predominant. The digestate (slurry) is dewatered by solid-liquid separation; may be composted, and is mostly used in agriculture for soil fertility enhancement (Albuquerque *et al.,* 2012). Some important considerations for the use of digestate in agriculture are heavy metals content, nitrogen content and sanitary conditions.

2.1 Fundamentals of anaerobic digestion

Some general conditions apply to the methanization of substrates in AD, while other process conditions must be kept at very strict operational ranges. The relationship between the process conditions is always an important consideration for stable methanization. Some of these conditions are discussed next.

2.1.1 General operating conditions: Organic waste streams could be fed to AD digester in batch, continuous or semi-continuous mode. AD temperature could be mesophilic or thermophilic, and a variety of digester designs are utilizable (Appels *et al.,* 2008). Anaerobic condition with oxidation reduction potential (ORP) of < -200 mV is also an important requirement for acetogenesis and methanogenesis (Gerardi, 2003). For any substrate feeding mode, AD temperature or digester design, optimum methanization is only possible with the right range of pH; C, N and S ratio; appropriate substrate particle sizes and micro-nutrient (Karakashev *et al.,* 2005; Gustavsson *et al.,* 2010). This is because the concentrations of reduced products such as CH_4 and H_2S depend on optimality of the factors during AD.

2.1.2 Optimum operating conditions: Determining what ranges of the general factors that constitute optimum condition during methanization is a challenge. This is because AD substrates vary widely in composition, and different micro-organisms are associated with different substrate types, digester design and temperature (Gerardi, 2003). Hence, methanization conditions are optimized for the microbes that produce CH_4 or for the microbes that degrade the fatty acids to CH_4-producing intermediates (Chen *et al.,* 2008; Mata-Alvarez *et al.,* 2000). Some of the factors that need to be considered for optimum CH_4 production are discussed next.

a. Temperature: Temperature stimulates changes in the composition, growth rate and metabolism of the micro-organisms, and also affects substrate degradation rates, (Gerardi, 2003). Thermophilic temperature, T_t ($45°C \leq T_t \leq 60°C$) can speed-up slow and energy demanding reactions such as the degradation of volatile fatty acids (VFA) to acetate, CO_2 and H_2. Conversely, mesophilic temperature, T_m ($35°C \leq T_m \leq 45°C$) are required for reactions which are exergonic (energy yielding) e.g. hydrogen-dependent methanogenesis (Kleerebezem and Stams, 2000). Temperature variation ≥ 1°C/day in both mesophilic and thermophilic AD is detrimental to optimum methanization; but variations ≤ 0.6°C/day are tolerable (Man-Chang *et al.,* 2006; Ahn and Forster, 2002). Methanogens are the most sensitive to variations in temperature; and aceticlastic methanogens are more sensitive than hydrogenotrophs (Sri Bala Kameswari *et al.,* 2011; Chen *et al.,* 2008).

b. Process duration and substrate retention time: Depending on substrate type, loading rate and other process conditions, substrate retention in the digester could range between 5 and 60 days. 5 -10 days are recommended for microbial stabilization and substrate methanization in sewage sludge and bio-waste. Conversely, longer retention time is required for lipid-like substrates such as grease trap residue, which could begin CH_4 formation not earlier than 10 days (Appels *et al.,* 2008). Wetland (2008) recommended about 15 - 20 days of retention time in methanization of blackwater, and co-digestion of blackwater with kitchen waste. Long retention time allows for better establishment of microbial population and removal of organics (Turovskiy and Mathai, 2006; Rincon *et al.,* 2008).

c. VFA/Alkalinity and pH: The ratio of VFA to alkalinity during methanization is principally controlled by the concentrations of VFA and hydrogen carbonate (HCO_3^-) (Sri Bala Kameswari *et al.,* 2011). Ammonia (NH_3) and ammonium (NH_4^+) are pH-dependent and inter-convertible; and could also contribute to alkalinity (Körner, 2009). The ratio of VFA to alkalinity is related to the ratio of C to N since N content is

related to NH_3 formation and VFA derives from C content. Hills (1979) recommended a C/N of 25; and Zeshan and Visvanathan (2012) also suggested a ratio of 32 for stable methanization. Mata-Alvarez (2003) suggests that any ratio between 30 and 35 is optimum for maintaining optimum VFA/alkalinity. Appels *et al.* (2008) suggest that for stable pH during AD, a steady HCO_3^-/VFA molar ratio of 1.4 should be maintained. pH influences microbial rate of growth, metabolism and dominance, as well as composition of VFA. Appels *et al.* (2008) documented that acetate-propionate dominance is established at pH 8.0 and acetate-butyrate dominance prevails at pH < 8. The phases of methanization also vary in optimum pH; and Boe (2006) suggests a pH range of 4.0 to 8.5 for acidogenesis. Turovskiy and Mathai (2006) reported that methanogenesis is optimum within a narrower pH range of 6.5 to 7.4.

2.2 Phases of anaerobic digestion

AD proceeds in four biochemically interdependent phases as shown in Figure 2.1. Each phase generates intermediates products which become substrates for succeeding stages until the substrates are completely mineralized (Siriwongrungson *et al.,* 2007; De Bok *et al.,* 2001). AD phases include hydrolysis, acidogenesis, acetogenesis and methanogenesis, and these are discussed next.

2.2.1 Hydrolysis: Vivalin *et al.* (2008), and Rajagopal and Beline (2011) reported that substrate hydrolysis is the slowest phase in methanization; and Table 2.1 indicates the hydrolytic rates of different substrates. Substrate hydrolysis is essentially a solubilisation of macromolecules. High molecular weight compounds such as lipids, polysaccharides, proteins and nucleic acids are converted into soluble organics such as sugars, amino acids and fatty acids. Hydrolysis could be solely by physicochemical means, or aided by enzymes of hydrolytic bacteria (Burgess and Pletschkte, 2008). The physicochemical factors include substrate composition and pH; and biochemical factors include the concentrations of the hydrolytic enzymes and enzyme-substrate interactions (Higuchi *et al.,* 2005).

Table 2.1 Hydrolytic rate constants of different polymeric substances

Substrate	Hydrolysis and acidification rate constants (d^{-1})
Carbohydrates	0.025 - 0.200
Cellulose	0.040 - 0.130
Proteins	0.015 - 0.075
Lipids	0.005 - 0.010

(Christ *et al.,* 2000)

2.2.2 **Acidogenesis (Acidification):** Soluble organics are further split during acidogenesis to VFA molecules with carbon length between 1 and 5 (C1-5) by population of heterogeneous fermentative bacteria (acidogenic bacteria or acidogens). Other products of this phase include alcohols (KOH), H_2S, NH_3, and CO_2. Acidogenic bacteria are both obligate and facultative anaerobes (Bhattacharyya and Banerjee, 2007). Factors such as pH, interspecies H_2 transfer and age of the inoculum play significant roles in converting soluble organics into VFA during acidogenesis (Gerardi, 2003; Mata-Alvarez *et al.,* 2000).

2.2.3 Acetogenesis: In this phase, acetate producing bacteria (acetogens or acetogenic bacteria) degrade the VFA and KOH produced in acidogenesis to acetate, CO_2 and H_2 (Equations 2.2 and 2.3). The H_2 concentration must be kept at low concentration to avoid inhibition (Fukuzaki *et al.,* 1990). Therefore, most of the acetate is produced by syntrophic feeding relationship between VFA-oxidizing bacteria and H_2-utilizing micro-organisms (Liu *et al.,* 1999; Scholten and Conrad, 2000). Certain species of the *Clostridium and Pseudomonas* genera are capable of producing acetate as sole product from CO_2 and H_2 using the reductive acetyl-CoA pathway as shown in Equation 2.1. These species are called homoacetogens and the process is referred to as homoacetogenesis (Gerardi, 2003; Ragsdale and Pierce, 2008).

Equation 2.1	$4H_2 + 2HCO_3^- + H^+$	→	Acetate + $4H_2O$	ΔG_0 = -104.6 kJ/mol
Equation 2.2	Propionate + $3H_2O$	→	Acetate + HCO_3^- + H^+ + $3H_2$	ΔG_0 = +76.1 kJ/mol
Equation 2.3	Butyrate + $2H_2O$	→	2Acetate + H^+ + $2H_2$	ΔG_0 = +48.3 kJ/mol

2.2.4 Methanogenesis: Methanogenesis completes the mineralization of substrates to CO_2 and CH_4. Important substrates for methanogenesis include acetate, CO_2, H_2; and methylated compounds such as methanol, methylamine and dimethylsulphide. Other substrates include carbon-monoxide (CO) and formate ($HCOO^-$). About 65% to 70% of the CH_4 produced during methanization originates from acetate (Siegrist *et al.,* 1993). Methanogenesis complements acetogenesis by the utilization of acetogenic substrates such as H_2, thereby making VFA degradation energetically favorable and thermodynamically feasible (Schink, 1997; Schink and Stams, 2005).

Figure 2.2 summarizes the flow of the C intermediates between the different phases of methanization. Part of the energy generated from degrading the substrates is

retained in the C-H bond in CH_4, and is not available to the micro-organisms involved in AD. The retained energy is the important resource in CH_4. Retaining the energy of degradation of VFA in CH_4 is also responsible for lower energetics and slower degradation of substrates during AD when compared with aerobic digestion (Kleerebezem and Stams, 2000). The low energy available to the microbes makes the entire process sensitive; and methanogenesis is the most sensitive phase.

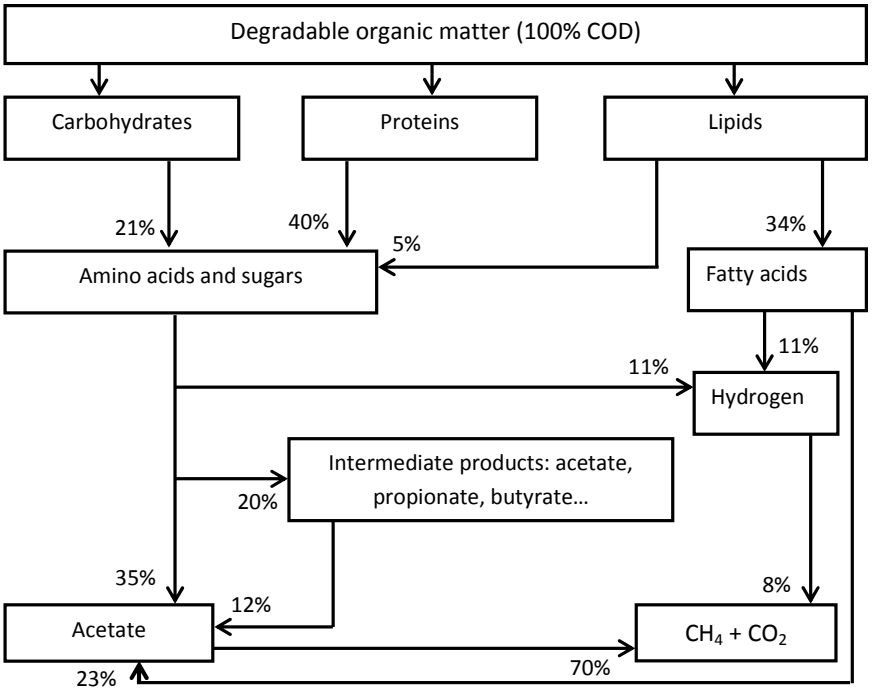


Figure 2.2 Bioconversion of substrates in anaerobic digestion with flow of substrate intermediates expressed as percent of chemical oxygen demand (COD) (Modified from Siegrist *et al.,* 1993).

2.3 Other important processes in AD

In addition to the basic processes associated with the AD phases that are discussed in sections 2.2.1 to 2.2.4, unwanted changes in the operating conditions of AD could result in side-reactions whose implications influence methanization. Some of these reactions are discussed next.

2.3.1 Sulphate reduction: Table 2.2 shows some of the sulphate (SO_4^{2-}) and CO_2 reduction reactions, and VFA oxidation in AD. H_2 is an intermediate product in the phases of acidogenesis and acetogenesis and can be used by micro-organisms to form CH_4 or H_2S. Liu *et al.* (1999) indicated that instead of the reduction of CO_2 to CH_4 by CH_4-producing bacteria (MPB), SO_4^{2-} or sulphite (SO_3^{2-}) reduction to H_2S by sulphate-reducing bacteria (SRB) could occur. Table 2.2 also indicates that SO_4^{2-}

reduction is more energy-yielding compared to CO_2 reduction because the energy that could be stored in CH_4 is released for metabolism (Schink, 1997). Furthermore, SO_4^{2-} reduction depletes methanogenic substrates, especially H_2 and acetate, and results in low CH_4 production (O'Flaherty *et al.,* 1999).

Table 2.2 Energetics of sulphate reduction and methanogenic reactions during utilization of methanization intermediates

Sulphate reduction			**ΔG_0 (kJ/mol substrate)**
$4H_2 + SO_4^{2-} + H^+$	→	$HS^- + 4H_2O$	-151.9
Acetate + SO_4^{2-}	→	$2HCO_3^- + HS^-$	-47.6
Propionate + $0.75SO_4^{2-}$	→	Acetate + $HCO_3^- + 0.75HS^- + 0.25H^+$	-37.7
Butyrate + $0.5SO_4^{2-}$	→	$2Acetate + 0.5HS^- + 0.5H^+$	-27.8
Lactate + $0.5SO_4^{2-}$	→	Acetate + $HCO_3^- + 0.5HS^- + 0.5H^+$	-80.0
Ethanol + $0.5SO_4^{2-}$	→	Acetate + $0.5HS^- + 0.5H + H_2O$	-66.4
Methanogenesis			**ΔG_0 (kJ/mol substrate)**
$4H_2 + HCO_3^- + H^+$	→	$CH_4 + 3H_2O$	-135.5
Acetate + H_2O	→	$CH_4 + HCO_3^-$	-32.3
Methanol $0.25H^+$	→	$0.75CH_4 + 0.25HCO_3^- + 0.25H_2O$	-79.9
Formate + H^+	→	$0.25CH_4 + 0.75CO_2 + 0.5H_2O$	-36.1

(Thauer *et al.,* 1977)

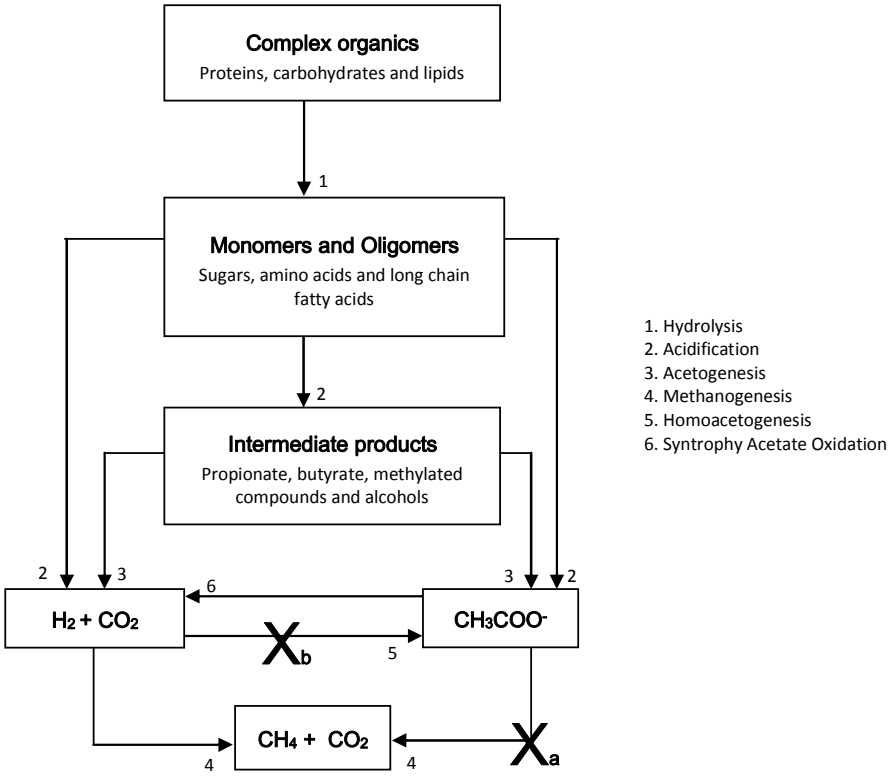


Figure 2.3 Phases of anaerobic digestion indicating blocked pathways during syntrophic acetate oxidation (**Blocked pathways: X_a**, aceticlastic methanogenesis; **X_b**, homoacetogenesis)

2.3.2 Syntrophic acetate oxidation (SAO): Figure 2.3 shows the scheme of syntrophic acetate oxidation. Acetate formation from VFA degradation by acetogens could exceed its utilization by methanogens for CH_4 formation. When this happens, acetate is oxidized to CO_2 and H_2 (Iranpour *et al.*, 2005). SAO could proceed alongside H_2-dependent methanogenesis. SAO dominates under NH_3 inhibition and at temperature above 60°C (Ho *et al.,* 2013). Hansen *et al.* (1999) and Karakashev *et al.* (2006) indicated that SAO occurs in the digestion of protein-rich substrates; and results in high concentrations of NH_3 and VFA due to the low efficiency of acetate-utilizing methanogens at thermophilic temperature.

CHAPTER 3 VOLATILE FATTY ACIDS DEGRADATION

3.0 Chapter overview

Volatile fatty acids (VFA) are produced during acidogenesis as degradation products of long chain fatty acids (LCFA). The carbon length of VFA could range between C1 and C5 (Chapter 2) and these include formate, acetate, propionate and butyrate (Figure 2.1). Others VFA encountered in AD include valerate and the iso-forms of butyrate and valerate. The most important VFA are acetate, propionate and butyrate because they are the regular intermediate products from the beta-oxidation of LCFA. Small amount of propionate is produced by beta-oxidation of LCFA with odd number of carbon atoms. Oxidative degradation of branched-chain amino acids such as valine, isoleucine, threonine and methionine also produce propionate (Rosenberg, 1983).

Butyrate is produced from degradation of LCFA with even number of carbon (Sousa *et al.,* 2007). It is also produced by carboxylation of propionate (Dolfing, 2013). Acetate is a common intermediate in the degradation of VFA and in homoacetogenesis. During methanization, VFA are activated and degraded to CH_4 and CO_2 by different micro-organisms that use different pathways. These processes and pathways are the focus of this chapter.

3.1 VFA activation

VFA metabolism to intermediates that are utilizable by CH_4-forming micro-organisms begins with activation. Activation requires biochemical energy and involves transfer of phosphate group (PO_3^{4-}) from adenosyl triphosphate (ATP) to the VFA to form phosphorylated VFA (acyl-phosphate) as shown in Equations 3.1.

Equation 3.1	VFA + ATP	→	Acyl-Pi
Equation 3.2	Acyl-Pi + SH-CoA	→	Acyl-CoA + ATP

According to Equation 3.2, the phosphorylated VFA further react with co-enzyme A (SH-CoA) to form an acyl-CoA (Bhattacharyya and Banerjee, 2007). The activated VFA enter the appropriate degradation pathways. A number of pathways are possible for VFA degradation; due to the importance of propionic acid in AD, the relevant pathways for propionate degradation in AD are discussed next.

3.2 Propionate degradation pathways

During propionate degradation, the 3-Carbon backbone is either decarboxylated to form 2-Carbon acetate or carboxylated to a 4-Carbon molecule that can undergo beta-oxidation. Bhattacharyya and Banerjee (2007) and Dolfing (2013) reported that nutrient availability, process conditions and microbial composition are the major determinants of the prevalent pathway for propionate degradation during methanization. Two important pathways that are commonly reported include the methyl-malonyl-CoA and butyryl-CoA pathways.

3.2.1 Methyl-malonyl CoA pathway: Complete degradation of propionate to CH_4 occurs mostly through the methylmalonyl-CoA (MMC) pathway. MMC involves the carboxylation of propionate to the 4-C succinate, which is beta oxidized to acetate as shown in Figure 3.1. MMC pathway is common to syntrophic relationship between VFA-oxidizing bacteria species of *Syntrophobacter*, including *S. pfennigii* and *S. fumaroxidans*, and H_2-utilizing methanogens of the orders *Methanobacteriales* and *Methanomicrobiales* (Li *et al.,* 2012; Gan *et al.,* 2012). An important determinant of the acetate-yielding intermediates in the MMC pathway is the concentration of Co. Succinate is produced and converted to acetate when Co is optimum, and hydroxypropionate is produced and converted to acetate in the absence of Co (Halarnkar and Blomquist, 1988; De Bok *et al.,* 2005).

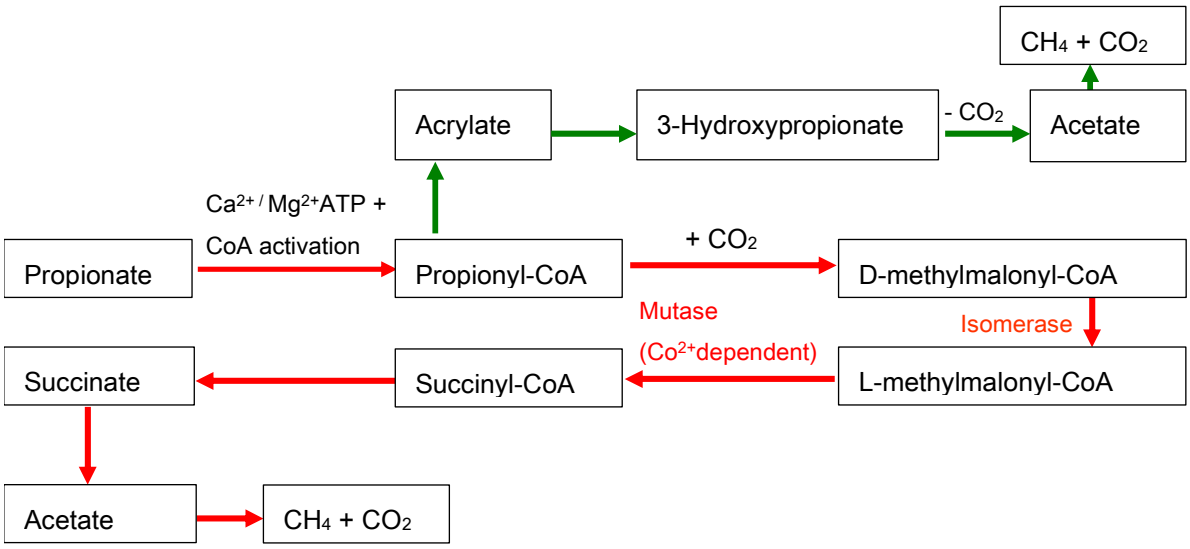


Figure 3.1 Propionate oxidation in the methyl-malonyl-CoA pathway: Red line- Cobalt-dependent; Green line- Cobalt-independent (modified from De Bok *et al.*, 2005; Halarnkar and Blomquist, 1988).

In Figure 3.1, Ca and Mg are associated with ATP-dependent activation of propionate. Ahn *et al*. (2006) confirms the optimality of 3 - 3.4 g/l Ca in propionic acid degradation. The addition of the CO_2 group to propionate molecule and its translocation between D and L methylmalonyl is carried out by a biotin-dependent carboxylase. Succinyl-CoA formation is carried out by a Co-dependent methyl-CoA mutase (Ferry, 1999; Huang *et al.,* 2010). MMC pathway involving succinate intermediate could be more energy saving than the alternative pathway involving hydroxypropionate (Scholten and Conrad, 2000).

3.2.2 Butyryl-CoA pathway: This pathway is independent of the concentration of Co. It is found in the syntrophic relationships between members of the genera *Smithella* and *Syntrophomonas,* and aceticlastic-methanogens of the genera *Methanosaeta and Methanosarcina.* It involves conversion of propionate to hydroxyl-butyryl-CoA, and β-oxidation of hydroxyl-butyryl-CoA to acetate (Liu *et al.,* 1999; Kleerebezem and Stams, 2000). There are conflicting reports on the methanization advantages of MMC and butyryl-CoA pathways in terms of energetics during propionate degradation (Dolfing, 2013; Kleerebezem and Stams, 2000). However, most authors believe that both pathways are energy-demanding, and could only be energy-yielding in syntrophic feeding relationships (Li *et al.,* 2012; Gan *et al*., 2012).

3.3 VFA accumulation and anaerobic digester failure

Accumulation of the intermediate products of the degradation of VFA inhibits further degradation. H_2, formate and acetate are the intermediates generated from VFA oxidation; and these must be removed. The accumulation of H_2 or formate in AD reactors inhibits acetate, propionate and butyrate oxidation; whereas the accumulation of H_2, formate, acetate or butyrate inhibits propionate oxidation (Fukuzaki *et al.,* 1990; Guven *et al.,* 2005; Li *et al.,* 2012). The removal of VFA degradation intermediates by methanogens during methanization controls VFA degradation rate and bio-energetics (Schink, 1997; Kleerebezem and Stams 2000). Furthermore, reactor acidification during methanization induces loss of HCO_3^- buffer as gaseous CO_2. This partly accounts for high CO_2 content in biogas during conditions of failed methanization (DEFRA, 2010).

3.3.1 Suppression of methanogens as cause for digester failure: Suppression of methanogens that utilize the intermediate products of VFA degradation results in VFA accumulation and digester failure. Suppression of methanogens could be due to presence of inhibitory substances or as a result of competition for H_2 by sulphate reducing bacteria (SRB). Methanogenic suppression is generally accompanied by

low pH --and decline in CH_4 content in biogas (Penning and Conrad, 2006; Tiantao *et al.,* 2010). Accumulation of propionic acid and to a lesser extent, the iso-forms of valeric and butyric acids, generally precede methanogenic suppression (DEFRA, 2010).

3.3.2 Dominance of unionized VFA species as cause for digester failure: According to Fukuzaki *et al.* (1990), digester failure is more often associated with conditions that induce accumulation of unionized forms of VFA, and the most important factor is pH. The author reported that pH ranges that keep the VFA in ionized state reduce the risk of digester failure in spite of relatively high VFA concentrations. Microbial competition is an important but secondary factor; and Erdirencelebi and Ozturk (2006) recommended a pH range of 7.8 to 8.2 for AD to maintain VFA in ionized forms and eliminate SRB competition. Methanization pH is particularly important for propionic acid because it is usually in the highest concentration when VFA accumulate. Li *et al.* (2012) indicated 7.0 to 8.5 as appropriate methanization pH range for keeping propionic acid concentration low and degradable. Accumulation of propionic acid is related to the following biochemical conditions:

- Similarity in the enzymes (Kinases) of propionate and acetate degradation, with acetate-Kinase having better kinetics (Ingram-Smith *et al.,* 2005); and
- Joint inhibition by butyric- and acetic acids, with butyric acid being about 2.4 times more inhibitory to propionic acid degradation than acetic acid (Amani *et al.,* 2011).

3.3.3 Monitoring VFA accumulation and digester failure: Different biochemical models have been proposed to manage the conditions that prompt digester acidification or failure due to VFA accumulation. An extensive review of the models is presented in the report by Lyberatos and Skiadas (1999). One agreement is common in all the models: VFA accumulation to 'critical levels', especially propionic and acetic acids, is an indication of failure. Consequently, for any digester, deviation from regularly observed VFA levels or established VFA degradation rates and CH_4 yields are the most reliable indicators that the micro-organisms are responding to inhibitory influences (Batstone *et al.,* 2000; Voss *et al.,* 2009; DEFRA, 2010). However, there is no consensus on VFA concentrations that constitute the 'critical levels' (Batstone *et al.,* 2000; Angelidaki and Ahring, 1993; Hill and Holmberg, 1988).

Table 3.1 shows some of the recommendations for the use of VFA concentration as indicator of stability during methanization. Ahring *et al.* (1995) suggested that VFA concentration ≥ 50 mmol/L is ideal for stable methanization; and ≥ 200 mmol/L could

induce instability in AD. Fatty acid/alkalinity (FOS/TAC) is also an important parameter for monitoring process stability during methanization. Voss *et al.* (2009) reported that FOS/TAC value of 0.15 – 0.45 indicate stability; whereas values above 0.6 are an indication of the onset of instability.

Table 3.1 Fatty acids concentrations as indicators of digestion process stability as given by various authors

Fatty acid indicator	Critical level	Stable level
[1]All fatty acids (mM)	≥ 200	≥ 50
[2]Acetate (mM)	> 13	-
[2]Propionate/acetate (mM)	< 1.4	-
[3]Iso- forms of C4-C6 (mM)	≥ 0.06 – 0.17	< 0.06
[1]Propionate and Valerate (mM)	≥ 100	-
[4]Fatty acid/alkalinity (FOS/TAC)	> 0.6	0.15 – 0.45

([1]Ahring *et al.,* 1995; [2]Hill *et al.,* 1987; [3]Hill and Holmberg 1988; [4]Voss *et al.,* 2009)

In practice, some of the most applied management strategies to avoid digester acidification during methanization, as documented by Appels *et al.* (2008) include:

- Establishing and maintaining optimum and steady substrate loading rate;
- Maintaining pH in optimum range and avoiding temperature fluctuations; and
- Co-digestion of substrates to provide nutrient balance.

3.4 Other factors that affect VFA degradation

Apart from pH and its relationship with VFA ionization and accumulation, factors that affect the growth of the desired microbial population are capable of impeding VFA utilization. These are discussed next.

3.4.1 Methanogen and acetogen (M/A) ratio: The M/A of the microbial population participating in syntrophic feeding relationship is an important parameter for VFA degradation. Considering that methanogens are slow growing anaerobes (Penning and Conrad, 2006), relatively high proportion of methanogens is required at the start-up of methanization compared to fast growing acetogens. Amani *et al.* (2011) indicated that utilization efficiency for propionic, butyric and acetic acids increased from 10 to almost 60% by increasing M/A from 1:1 to 2:1. However, at M/A of 3:1, the advantage for VFA degradation was lost, and the accompanying low pH resulted in the accumulation of acetate. The author concluded that at high M/A (3:1), there are less propionic- and butyric acid oxidizing bacteria than required to carry out

degradation of the accumulating VFA to acetate. Other authors also made similar observations (Liu *et al.,* 1999; Schmidt and Ahring, 1993).

3.4.2 Inoculum structure: VFA must be in contact with micro-organism or with microbial enzymes in the reactor in order to be degraded (Schmidt and Ahring, 1993). This requires that the diffusion distance (d) between the micro-organisms and the substrates must be short as illustrated in Figure 3.2. Short diffusion distance allows for rapid transfer of metabolites and increases degradation energetics and kinetics (Kleerebezem and Stams, 2000; Gerardi, 2003). Short diffusion distance exists between VFA and micro-organism when the micro-organisms are in clusters, form micro-niches or biofilms (Appels *et al.,* 2008); and is particularly important for H_2 and formate metabolism.

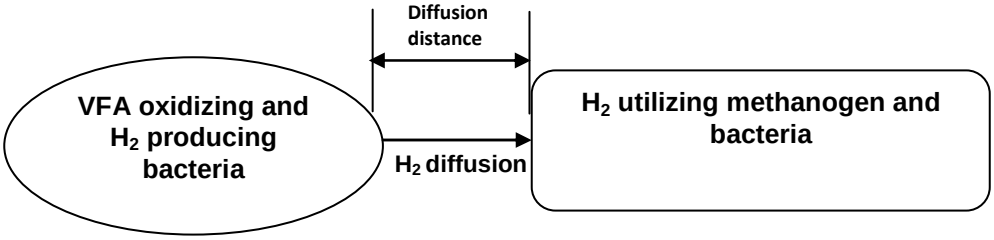


Figure 3.2 Schematic representation of metabolite sharing between micro-organisms during anaerobic digestion

Schmidt and Ahring (1993) reported that sewage inoculum with well-developed microbial clusters enhanced degradation of propionic acid by 30% and butyric acid by 20% due to shorter diffusion distance for the metabolites compared to sewage inoculum with dispersed granular structure. However, the influence of short diffusion distance appears true for H_2 and formate metabolism, but not for acetate. Schmidt and Ahring (1993) observed that sludge structure, and hence diffusion distance, had little or no effect on kinetics of acetate utilization during propionate degradation. This corroborates the findings of Fukuzaki *et al.* (1990) that acetate metabolism is rarely a limiting step in methanization under stable AD conditions.

3.4.3 Microbial competition for carbon sources: Most competition during methanization is between micro-organisms that use H_2 generated during VFA degradation to reduce CO_2 to CH_4 (MPB), and microbes that reduce SO_4^{2-} to H_2S (SRB). SRB possess stronger kinetic affinity for H_2 than MPB (Cabirol *et al.,* 2003; O'Flaherty *et al.,* 1999; Choi and Rim, 1991; Thauer *et al.,* 1977). MPB produce CH_4 (Equation 3.3a) and SRB produce H_2S (Equation 3.3b) with H_2 generated during VFA

degradation. The use of H_2 by SRB to produce H_2S is at the expense of methanogenesis and results in increased concentration of H_2S in biogas and toxicity of sulfide ion (HS^-) to MPB (Chen *et al.,* 2008).

Equation 3.3a	$4H_2 + HCO_3^- + H^+$	→	$CH_4 + 3H_2O$	$\Delta G_0 = -135.5$ kJ/mol
Equation 3.3b	$4H_2 + SO_4^{2-} + H^+$	→	$HS^- + 4H_2O$	$\Delta G_0 = -151.9$ kJ/mol

Table 3.2 indicates widely reported kinetic parameters for SRB and syntrophic acetogens (O' Flaherty *et al.,* 1998; Omil *et al*., 1996; and Colleran *et al*., 1995). Just like methanogens and acetogens, SRB also grow on propionate, butyrate and acetate (Colleran *et al.,* 1995). SRB have higher affinity and energetics (better degradation efficiency) for H_2 and propionate; and low affinity for acetate, butyrate and ethanol (Colleran and Pender, 2002; Overmeire *et al.,* 1994). Equation 3.4a and b show the bioenergetics advantage of the SRB (a) over acetogens (b) in propionate conversion to acetate. Consequently, SRB have tendency to dominate the microbial flora when SO_4^{2-} is abundant. However, SRB are not involved in hydrolysis of natural polymers such as starch, protein and lipids (Hansen, 1993).

Table 3.2 Kinetic parameters of sulphate reducing bacteria (SRB) and syntrophic acetogens for volatile fatty acids utilization

Kinetic Parameter	SRB	Syntrophic acetogens
K_s (half saturation constant)	23 mg/L	34 mg/L
μ_{max} (maximum growth rate)	0.15 d^{-1}	0.05 d^{-1}

(O' Flaherty *et al.,* 1998; Omil *et al.,* 1996; and Colleran *et al.,* 1995)

Equation 3.4a	Propionate + $0.75SO_4^{2-}$	→ acetate + HCO_3^- + $0.75HS^-$ + $0.25H^+$	$\Delta G_0 =$	-37.7 kJ/mol
Equation 3.4b	Propionate + 2H2O	→ acetate + CO_2 + $3H_2$	$\Delta G_0 =$	+76.1kj/mol

Sulphate reduction may increase VFA degradation rate (due to better kinetic properties of SRB), but it equally reduces CH_4 formation (due to competition for substrate between MPB and SRB). It could be an unwanted phenomenon when predominant. Therefore, identifying factors that enable SRB to out-compete other microbes of methanogenic significance is important for methanogenesis. Two factors have been widely reported and these are discussed next.

a. Chemical Oxygen Demand/SO_4^{2-} and SRB/MPB: Chemical Oxygen Demand (COD) and SO_4^{2-} ratio and the start-up ratio of SRB and MPB influence aceticlastic methanogenesis. Chen *et al.* (2008) documented that the influences of these ratios are substrate dependent as shown in Table 3.3.

Table 3.3 Chemical oxygen demand/sulphate ratio and substrate-dependent relations between sulphate reducing bacteria and methane producing bacteria

COD/SO_4^{2-}	Influence	Substrate
< 2.7	Suitable for sulphate reduction	Acetate
> 1.7	Suitable for aceticlastic methanogenesis	Acetate
< 2.7 > 1.7	Competition between sulphate reducers and aceticlastic methanogens, with transient loss in methanogenesis	Acetate
Between 3.0 and 5.7	Competition; relative dominance of methanogenesis	Butyrate and Ethanol

(Chen *et al.*, 2008)

b. Temperature: Operational AD temperature plays a significant role during H_2 utilization by SRB and MPB. Colleran and Pender (2002) reported that H_2-dependent reduction of SO_4^{2-} prevails over H_2-dependent CH_4 formation at 35°C - 37°C. Conversely, H_2-dependent CH_4 formation prevails over H_2-dependent SO_4^{2-} reduction at 55°C. This might suggests that processes involving MBP are reasonably optimized by temperature in thermophilic methanization, while mesophilic methanization requires optimization measures for MPB for enhanced H_2 utilization.

3.4.4 Inhibitory substances: A substrate or its intermediate is inhibitory when it affects growth of microbes, adversely changes the microbial population, kinetics or energetics of a particular phase with reference to the generation of a choice product (Chen, *et al.,* 2008). Table 3.4 shows some of the inhibitors in AD. NH_3, SO_4^{2-} and HS^- are mostly reported as dominant inhibitory intermediates products of substrate degradation that lead to reduction in the rate of CH_4 production and cause accumulation of VFA (Belmonte *et al.,* 2011; Cabirol *et al.,* 2003; Koster *et al.,* 1986). The problems of inhibition are discussed with emphasis on NH_3 since it is the most common due to imbalances in C/N.

NH_3 is produced from degradation of urea and protein-rich compounds (Kayhanian, 1999). NH_3 is protonated to ammonium ion (NH_4^+) in low pH solution and both species form the total ammonia nitrogen (TAN) (Körner, 2009; Krylova *et al.,* 1997). NH_3 is capable of diffusing across bacterial and archeal membranes and causing adjustment in intracellular proton balance (Liu and Sung, 2002). Other toxic

intracellular effects of NH_3 include interferences with process bioenergetics and enzyme inhibition (Belmonte *et al.,* 2011; Gallert *et al.*, 1998). Angelidaki and Ahring (1994) reported that toxicity of NH_3 causes digester instability due to the accompanying fluctuations in process pH. The ratio of NH_3/NH_4^+ is dependent on process temperature and pH; and TAN ≤ 5.0 g/L with NH_3 concentration of about 1.0 g/L (pH 7.9; 55°C) is toxic to both acetate-producing and utilizing micro-organisms (Borja *et al.,* 1996). Gallert and Winter (1997, 2008) reported that thermophilic flora are twice as tolerant to NH_3 inhibition as are mesophilic flora.

Hansen *et al.* (1999) indicated that higher temperature generally increases toxicity of NH_3. NH_3 toxicity can be attenuated. Krylova *et al.* (1997) reported that phosphorite ore contains Na^+, Ca^{2+} and Mg^{2+}, and that these ions are responsible for attenuation of NH_3 toxicity when phosphorite ore is added to anaerobic digesters. Metabolism of process intermediates such as methanol (≥ 0.5 mM) by some group of obligate NH_3-oxidizing bacteria that are capable of the oxidation of NH_3 to dinitrogen gas can also attenuate NH_3 toxicity (Güven *et al.,* 2005). Chen *et al.* (2008) and Borja *et al.* (1996) are of the opinion that inoculum acclimation to high NH_3 concentrations reduces toxicity of NH_3, but usually at the expense of CH_4 yield.

Other important inhibitors of VFA degradation include heavy metal ions such as Zn, Cr, Co, Ni, Cu and Mo and inhibitory light metals such as K, Mg, Na, and Ca (Feijoo *et al.,* 1995; Jin *et al.,* 1998). Depending on concentration, halogenated compounds and aldehydes also play significant inhibitory roles (McCue *et al,* 2003; Gonzales-Gil *et al.,* 2002). According to Frey and Hegeman (2007), the mechanisms of inhibition vary widely but are generally related to the following:

- Influx of ions into intracellular fluids;
- Disruption of reactive bridges of protein in microbial cells; and
- Replacement of natural occurring metals on MEs and co-factors thereby changing the biochemistry, affinity and catalytic properties of such enzymes and co-factors.

Blum and Speece (1991) reported hydrolysis as the least vulnerable to inhibition and suggested that vulnerability to inhibition in the different phases of AD could be considered in increasing order as follows: Hydrolysis → acidogenesis→ acetogenesis → methanogenesis.

Table 3.4 Common anaerobic digestion inhibitors and their associated properties

Inhibitor	Inhibitory IC_{50}	General beneficial concentration	Mechanism	Vulnerable population	Tolerant population
[1,2]Free NH_3 (Thermophilic)	1.7 - 14 g/L	< 200 mg/L	Adjustment of intracellular proton balance; increase in maintenance energy requirement; enzymatic inhibition	*Methanospirillium*	*Methanosarcinae Methanobacterium* (up to 10 g/l)
[3]SO_4^{2-}	COD/SO_4^{2-} < 1.7	COD/SO_4^{2-} above 2.7	-	Aceticlastic Methanogens	-
	COD/SO_4^{2-} of 0.5	-	-	Acetogens utilizing butyrate and ethanol	
[4]H_2S (pH 6.8 – 7.2)	50 – 400 mg/L	1 – 25 mg/L S	Diffuses into cells and deactivates protein; induces sulphide and disulphide linkages;	Methanogens and acetogens	Fermentative bacteria; Hydrolytic bacteria
[5,6]Total HS^- (pH 7.8 – 8.0; or above pH 7.2)	100 - 8000 mg/L	1 – 25 mg/L S	Interference with coenzyme-sulphur linkages and general sulphur assimilatory mechanism	SRB	-
[7]Aluminum (Al^{3+})	1000 mg/L	-	Inhibits iron and manganese uptake	Methanogens and acetogens	-
[8,9]Ca^{2+}	400 - 8000 mg/L	200 mg/L	Phosphates and carbonate precipitation; loss in buffer and nutrient; and decrease in activity of methanogens	Aceticlastic methanogens	-

(IC_{50} Concentration capable of causing 50% rate retardation)

Table 3.4 Cont'd Common anaerobic digestion inhibitors and their associated properties

Inhibitor	Inhibitory IC_{50}	General beneficial concentration	Mechanism	Vulnerable population	Tolerant population
[10]Mg^{2+}	400 mg/L	≈ 720 mg/L beneficial for *methanosarcinae* dominant reactor	Reduction in aceticlastic activities	Bacteria and archaea in general	-
K^+	[11]Above 1.0 M [12]0.15 - 0.74 M	Less than 400 mg/L	Extraction of TEs from cellular complexes	Acetoclastic methanogens	Acidogens
[8]Na^+	5.6 - 53 g/L	100 - 200 mg/L for mesophilic anaerobes; Up to 230 mg/L for mesophilic acetoclastic methanogens; 350 mg/L for H_2-utilizing methanogens	-	Thermophiles; methanogens; and SRB; *Methanosarcaea* most vulnerable	Mesophilic methanogens adapt, but rather slowly.
[8]Heavy metals	-	-	Binding with proteins and replacing natural metals of metallo-enzymes	Methanogens	Acidogens

(- not given; [1]Liu and Sung 2002; [2]Sung and Liu 2003; [3]Hansen, 1993; [4]O'Flaherty *et al.,* 1999; [5]Koster *et al.,* 1986; [6]Choi and Rim 1991; [7]Cabirol *et al.,* 2003; [8] Chen *et al.,* 2008; [9]Mouneimne *et al.,* 2003; [10]Schmidt and Ahring, 1993; [11]Ilangovan and Noyola, 1993)

CHAPTER 4 TRANSITION AND TRACE ELEMENTS

4.0 Chapter overview

In Table 3.4 of Chapter 3, certain elements were considered inhibitory in AD. Nonetheless, the biochemistry of acetate metabolism involves series of redox reactions dominated by enzymes whose activities are enhanced by specific elements (Zandvoort *et al.,* 2006; Gerardi, 2003). These elements are transitional, required in small concentrations and play catalytic roles in enzyme-controlled reactions. This section considers transition elements that are important in AD.

4.1 Transition elements and AD

Transition elements include thirty-eight elements of groups 3 to 12 of the periodic table; these elements are reactive, exhibit variable oxidation states due to easy loss of valence or reactive electrons, and are catalytic (Greenwood and Earnshaw, 1997). In biochemistry, some transition elements are referred to as trace elements (TEs) because they are required in very small concentration as co-factors of enzymes (Bisswanger, 2004). Some of the TEs that are relevant in AD include Co, Ni, W, Se and Mo (Pobeheim *et al.,* 2009; Zandvoort *et al.,* 2006). Other biologically important transition elements that are not TEs include Mg, Fe and Ca (Ahn *et al.,* 2006; Schmitz and Ahring, 1993). The major roles of TEs in bioconversion include:

- Formation of reactive complexes with amino acids at the active sites of enzymes (Garret and Grisham, 2005); and
- Provision of charged surfaces in enzymes for binding, activation, transformation and transfer of functional groups (Heikinheimo *et al.,* 2001; Thomas and Ward, 2005).

Enzymes that contain TEs depend on the activity of the elements for biocatalysis. The activity or otherwise of the TEs in the enzymes is a function of the prevailing oxidation state (Illanes, 2008; Bisswanger, 2004). Depending on the pH and type of reaction that is catalysed, TEs can exist in any of the oxidation states shown in Table 4.1 (Greenwood and Earnshaw, 1997). Therefore, in this review, the TEs are presented without their oxidation states, except in reactions with well documented active oxidation states.

Table 4.1 Oxidation states of some elements used in anaerobic digestion

Elements	Negative oxidation state	Positive oxidation state	Comment
Ni	-1	+1, +2, +3, +4	TEs
Co	-1	+1, +2, +3, +4, +5	
Se	-2	+1, +2, +4, +6	
Mo	-1, -2	+1, +2, +3, +4, +5, +6	
W	-1, -2	+1, +2, +3, +4, +5, +6	
Fe	-1, -2	+1, +2, +3, +4, +5, +6	Non-TEs
Zn		+1, +2	
Mg		+1, +2	
Cr	-1, -2	+1, +2, +3, +4, +5, +6	

(Greenwood and Earnshaw, 1997)

The biocatalytic roles of TEs in functional group transfer are important because the transfer rates for functional groups such as methyl, H^-, OH^-, PO_4^{3-}, amino acid residues and acyls (R-CO) define the efficiency of bioconversion processes (Ragsdale, 2008). Catalytic properties of TEs have been exploited in industrial catalysis, and are capable of optimizing biomethanization (Schmid *et al.,* 2001). Investigations involving the role of TEs in AD have recently assumed new momentum due to increasing knowledge of their roles in enzymes of AD (Gustavsson *et al.,* 2010; Stock and Rother, 2009; Ragsdale and Speece, 2008). However, wide differences exist in the reporting of the influences of TEs in AD, and this is due to varying sensitivities of the micro-organisms to concentrations of TEs (Fermoso *et al.,* 2010). Figure 4.1 illustrates an example of microbial sensitivity to TEs concentration.

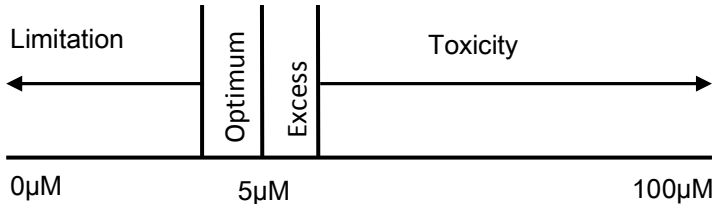


Figure 4.1 Boundary conditions for cobalt in anaerobic digestion
(Adapted from Fermoso *et al.,* 2010)

4.2 TEs and substrate degradation in AD

TEs have long been identified as important for the growth and metabolism of micro-organisms that are involved in AD (Burgess *et al.,* 1999; Kayhanian and Rich, 1995; Kirby *et al.,* 1981). Reports of the actual influences of TEs on both growth and metabolism during AD vary (Pobeheim *et al.,* 2009; Ishaq *et al.,* 2005; Hinken *et al.,*

2008). Process improvements ranging between 10 and 86% increase in biogas and CH_4 production in continuous AD operations have been reported (Zitomer *et al.,* 2008; Pobeheim *et al.,* 2009; Ishaq *et al.,* 2005; Gustavsson *et al.,* 2010). Some authors have also reported that TEs have no influence on AD (Hinken *et al.,* 2008; Jarvis *et al.,* 1997). Responses to TEs supplementation depend, among other factors, on:

- Whether digestion process is continuous or batch; and
- Whether or not the TEs content of the inoculum and substrate are sufficient for the metabolism of micro-organisms within the period of methanization.

Balance in composition and concentration of TEs is essential for AD; and AD operations that cause changes in these factors may induce instability in AD (Zandvoort *et al.,* 2006). For example, continuous feeding operation in AD, which is usually accompanied by loss in seeding sludge, may result in reduced CH_4 yield due to loss of TEs during digestate removal. This necessitates TEs supplementation even when other factors are kept optimum (Climenhaga and Banks, 2008). Furthermore, concentrations of certain TEs could also become limiting during AD when a digester is fed continuously with mono-substrates or a poor mixture of substrates for a long time (Lebuhn *et al.,* 2008; Clemens, 2007; Jarvis *et al.,* 1997). Stimulatory influences of TEs have been reported for AD of both mono-substrates and complex substrates.

Table 4.2 Results of metals supplementation during anaerobic digestion of silages

Substrate	Trace Elements	Improved parameter	Improvement (%)	Stimulatory concentration
[1]Maize silage	Ni Co Mo	Biogas production	35	11 mg/kg COD 9 mg /kg COD 7 mg/kg COD
[2]Maize silage	Ni Co	CH_4 yield	30 10	0.4 – 10.6µM 0.4 – 2.0µM
[3]Wheat silage	Ni Co Fe	Process stability; CH_4 production and VFA degradation rate	-	0.2 mg l^{-1} 0.5 mg l^{-1} 0.5 g l^{-1}
[4]Wheat silage	Co Se Mo	Process stability; CH_4 production and degradation rate	-	5.9 – 120 mg m^{-3} 79 - 790 mg m^{-3} 48 mg m^{-3}

(- not given; [1]Hinken *et al.,* 2008; [2]Pobeheim *et al.,* 2009; [3]Gustavsson *et al.,* 2010; [4]Demirel and Scherer, 2011)

4.2.1 Mono-substrates: Table 4.2 shows results of TEs supplementation by different authors during maize and wheat silage degradation. Silages of maize- and

wheat have low TEs concentration and balance, and increase in biogas and CH_4 production generally occurs when TEs are supplemented to these substrates during AD. Co deficiency is widely reported as most acute for mono-substrates (Demirel and Scherer, 2011; Hinken *et al.,* 2008; Lebuhn *et al.,* 2008). Co deficiency results in low biogas production and process instability, especially under thermophilic condition (Alger *et al.,* (2008). Hence, supplementation to alleviate deficiency may be necessary. TEs may be supplemented singly or in combination with other TEs during AD. Single or co-supplementation of TEs could influence the outcome due to synergy or antagonism arising from TEs interactions (Lin, 1992; Ahring and Westermann, 1985).

4.2.2 Complex substrates: Complex substrates such as organic fraction of municipal waste or co-digestion involving two or more mono substrates are reported to have better TEs composition (Banks and Zhang, 2012; Uemura, 2010; Kayhanian and Rich, 1995). Though complex substrates have better balance in TEs composition, some TEs could be limiting and require supplementation for improved CH_4 production. The stimulatory concentrations of TEs supplemented by different authors for improved CH_4 production during AD of complex substrate are shown in Table 4.3. Table 4.4a shows composition of biowaste from different sources. It also shows recommendations for optimum TEs concentrations and TEs content of methanogenic bacteria. Table 4.4b highlights variation in TEs composition of inoculum, which is an important consideration for determining TEs supplementation requirements.

Table 4.3 Reported stimulatory concentrations of trace elements during mesophilic degradation of complex substrates

Trace elements	(mg m^{-3})	Author
Cr	2.2 – 21.2	Lo *et al.,* 2010
Ni	801 – 5,362	
Co	148 – 580	
Mo	44 – 52.9	
Se	≤ 160	Zhang *et al.,* 2011
Co	≤ 220	
Se	≤ 8	Feng *et al.,* 2010
Co	≤ 60	
W	≤ 18	

Generally, reported positive influences of TEs in AD of complex substrates include the promotion of relatively more stable AD; increase in biogas and CH_4 yield; and attenuation of inhibitory influences of extreme concentrations of individual TEs

(Macias-Corral *et al.,* 2008; Callaghan *et al.,* 1999). Authors vary on optimum composition and concentration of TEs required for complex substrate methanization in order to achieve improved methanization (Lo *et al.,* 2010; Feng *et al.,* 2010). Supplementation requirements for TEs depend on TEs content of the inoculum and the substrate used for AD. Tables 4.4a and b show TEs concentrations in some AD substrates and digestates used as starting culture. Uemura, (2010); Takashima and Shimada (2004) reported that the minimum requirement for Ni, Co and Fe could be higher in thermophilic AD of complex substrates due to higher biological activity compared to mesophilic AD.

Table 4.4a Trace elements content of complex substrates and methanogens

Trace elements	Food waste[1]	Biobin waste[2]	Microbial nutrient requirement[3]	Micronutrients in acclimated methanogens[4]	Composition of methanogenic bacteria[5]
	mg/kg DM				
Cu	-	4 - 8	20.06	52.52	< 10 - 160
Ni	-	0.08 - 4	2.01	16.12	65 - 180
Co	0.06 ± 0.04	0.08 - 2.4	-	3.12	10 - 120
Mo	0.21 ± 0.09	0.08 - 4.8	1.67	-	10 - 70
Zn	-	25.60 - 71.2	53.49	52.56	50 - 630
Fe	44.83 ± 9.64	460 - 960	2674.29	382.72	720 - 2150
Se	0.19 ± 0.13	ND - 0.8	-	-	-
W	0.25 ± 0.01	ND - 0.8	-	-	-

(- not given; ND not detected; [1]Banks and Zhang, 2012; [2]Kayhanian and Rich, 1995; [3]Hinken *et al.,* 2008; [4]Zhang *et al.,* 2003; [5]Scherer *et al.,* 1983)

Table 4.4b Trace elements content of inoculum as reported by different authors

Trace elements	Sewage sludge[1]	Wastewater sludge[2]		Sewage sludge[3]
		Alcohol distillery	Paper mill	
	mg/kg^{-1}			
Mn	2.4	5.2	53.9	-
Cu	-	57	29.5	-
Ni	11.6	12.3	9.4	0.1 - 0.2
Co	1.7	2.2	13.7	3.5
Mo	-	-	-	817 -896
Zn	58.1	115.2	47.2	-
Fe	249.6	2249.3	10599.4	-
Se	1.8	-	-	-

(- not given; [1]Zandvoort *et al.,* 2003; [2]Hullebusch *et al.,* 2005; [3]Uemura, 2010)

4.2.3 Influence of TEs on degradation of process intermediates: A more specific understanding of the influence of TEs is possible with VFA intermediates whose concentrations determine stability or otherwise of the AD. Investigations in this direction are limited. However, using propionate and acetate as substrates, Zitomer *et al.* (2008) investigated influences of Ni, Co and Fe supplementation to thermophilic and mesophilic digesters treating activated sludge or a mixture of primary- and activated sludge. The composition and concentrations of the elements that were supplemented to the batch reactors included 25 mg/L Ni, 25 mg/L Co and 25 mg/L Fe, and the results are summarized as follows:

- There was higher stimulation in the rate of propionate utilisation (86%) compared to rate of acetate utilization (33%); and
- Gains in CH_4 production were higher (4% - 51%) at thermophilic AD, compared to increase from 7% to 36% in mesophilic AD.

4.3 Factors influencing TEs availability

Substrate composition and source of the start-up inoculum are the main factors that affect digester TEs composition during AD. Whether what is present in the digestion mixture is utilisable by the microbial communities depends on other factors. These are reviewed next.

4.3.1 pH, anions, humics and inorganic solids: Table 4.5 shows solubility of different anions in aqueous solution and Figure 4.2 shows the effect of pH on the concentration of metal ion in aqueous solution. At certain pH values, anions may react with dissolved TEs to form precipitates. Anions such as CO_3^{2-}, SO_4^{2-}, Cl^-, PO_4^{3-}, OH^- and S^{2-} are importance due to their relative abundance in AD and the possibility of forming insoluble precipitates with TEs (Hullebusch *et al.,* 2005). When TEs form insoluble precipitate, the supplemented TEs become non-bioavailable and necessitate higher dosing concentration (Chen *et al.,* 2008).

TEs also form complex compounds with humics and solid inorganic minerals in the reactor sediments (Dong *et al.,* 2013; Alonso *et al.,* 2009; Lawson *et al.,* 1984). It is also reported that TEs are adsorbed to bacterial cell (Ferguson and Deisenhofer, 2004). These complexing capabilities of TEs complicate estimation of bioavailable proportions and suggest that there could be other uptake mechanisms aside direct utilization of the soluble ions.

Table 4.5 Solubility of the salts of different elements

Anions	Soluble salts	Insoluble salts
Acids	All	None
Cl^-	Most	$AgCl$, Hg_2Cl_2, $PbCl_2$
CO_3^{2-}	$(NH_4)_2CO_3$	Most
NO_3^-	All	None
O^{2-}	Ca, Sr, Ba	Most
OH^-	Ca, Sr, Ba	Most
PO_4^{3-}	$(NH_4)_3PO_4$	Most
S^{2-}	$(NH_4)_2S$	Most
NH_4^+	All	None
SO_4^{2-}	Most	$BaSO_4$, $SrSO_4$, $PbSO_4$

(NO_3^- nitrate; O^{2-} oxide; S^{2-} sulphide; Cl^- chloride; PO_4^{3-} phosphate, BCIT, 2006)

4.3.2 Antagonism and synergism: When two or more metallic ions interact in a mixture, the individual influences of one or all the interacting ions could weaken (antagonism) or strengthen (synergism) the response of interest, and influence reported optimum TEs concentration. TEs mixtures of Zn, Cu and Ni; Ni and Cu; and Ni, Mo and Co have been established as synergistic in AD (Lin, 1992; Babich and Stotzky, 1983). Ahring and Westermann (1985) reported mixtures of Ni and Cd; and Ni and Zn as antagonistic combinations for AD.

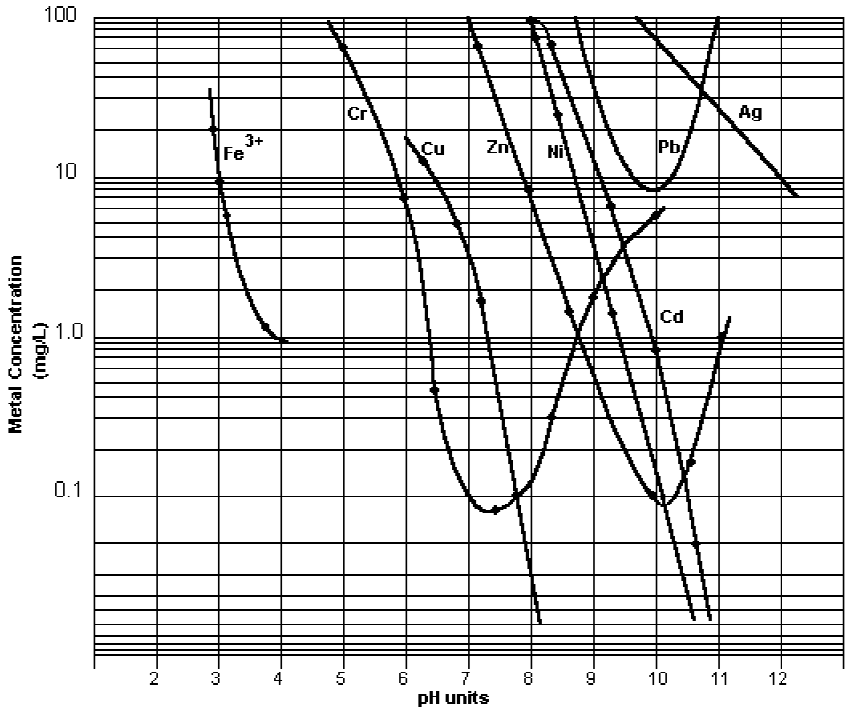


Figure 4.2 Relative concentrations of metal ions in aqueous solution at different pH (Hoffland Environmental, 2013)

CHAPTER 5 TRACE ELEMENTS AND METALLO-ENZYMES OF ANAEROBIC DIGESTION

5.0 Chapter overview

Generally, enzymes are protein molecules that catalyse biochemical reactions and are composed of units, which could be different or identical (Buchholz *et al.,* 2005). Some of the constituent units of an enzyme are the active sites for biocatalysis. Biocatalysis involves speeding up biochemical processes through reduction in the activation energy and enhancement of functional group transfers (Frey and Hegeman, 2007). According to Frey and Hegeman (2007), the structure of the active site of an enzyme is typically composed of reactive molecules such as:

- *Amino acid residues* of histidine, glutamate, glycine, aspartate, lysine, cysteine and arginine; *or*
- *Amino acids in complex with TEs*.

5.1 TEs and complexing relations with metallo-enzymes (MEs)

Enzymes that contain amino acids in complex with TEs or any other transition-type metals at the active site are referred to as metallo-enzymes (MEs) (Frey and Hegeman, 2007). One or more TEs may be required for the activity of MEs; and MEs generally depend on the redox state of the incorporated TEs to speed up biochemical reactions (Ragsdale and Pierce, 2008; Ragsdale and Wood, 1991). During methanization, MEs catalyse series of redox reactions that involve electron transfer through conduits of heme or Fe-S clusters in the MEs structure. These electron conduits also form different kinds of complexes with TEs (Ragsdale and Pierce, 2008; Menon and Ragsdale, 1999; Drennan *et al.,* 2004; Dobbek *et al.,* 2001). A number of TEs complexing patterns are generally identifiable in MEs of methanization, and the different phases of methanization also have some unique MEs. These are discussed next.

5.1.1 Se, W, Mo and Co in metallo-enzymes: Methanization MEs such as reductases and hydrogenases that reductively convert CO_2 to methyl group in series of redox reactions are Se, W or Mo-complexes (Da Silva *et al.,* 2011; Tan *et al.,* 2003). Those involved in non-reductive transfer of methyl group, e.g. Methyl-transferases and Carbon-monoxide dehydrogenase/Acetyl-CoA synthase (CODH/ACS) contain Co (Caspi *et al.,* 2008; Ragsdale, 2006).

5.1.2 Ni in metallo-enzymes: Ni forms weak non-covalent bonds with AD enzymes and substrates and participates in functional group transfer (Caspi *et al.,* 2008). In AD pathways, Ni is associated with the following enzymes and biochemical reactions (Drake *et al.,* 2008; Shima *et al.,* 2002; Ermler *et al.,* 1997):

- CODH/ACS: Cleavage of methyl group from methylated compounds;
- F_{430} moiety of methyl-CoM reductases: Reduction of methyl group; and
- Hydrogenases: Transfer of electrons to methyl group or its precursors.

5.1.3 Zn, Cu and Mg in metallo-enzymes: AD phases that are wide apart, such as hydrolysis and acetogenesis differ considerably in metabolites and enzymology. For instance, hydrolytic enzymes are tolerant to molecular O_2 and contain ions of Zn, Cu or Mg (Burgess and Pletschke, 2008; Kim *et al.,* 2001). These are not regular TEs of CO_2 fixation or acetate metabolism but might occur in enzymes that need to counter the formation of reactive radicals and peroxides associated with cellular O_2 reactions, e.g. CODH in the reduction of CO (Ragsdale, 2006).

5.2 TEs and MEs in the different phases of AD

Different MEs are associated with the different phases of AD. These MEs and their associated TEs are discussed next.

5.2.1 TEs and MEs of hydrolysis: Hydrolysis is a solubilisation reaction of the type shown in Equation 5.1. In AD, R is an organic compound and the transfer of the OH^- from H_2O in an aqueous solution could be influenced by pH, a catalyst, an enzyme or all three (Garret and Grisham, 2005).

Equation 5.1 $RX + H_2O \rightarrow ROH + HX$

Hydrolytic enzymes are called hydrolases and could hydrolyse AD substrates inside (intracellular) or outside (extracellular) the cell of the micro-organism. Hydrolases could also be both intra- and extracellular depending on the type of reaction that is catalysed (Frey and Hegeman, 2007). However, the important hydrolases of AD are extracellular enzymes and include lipases, glucosidases and proteases (Burgess and Pletschke, 2008; Frolund *et al.,* 1995). Extracellular enzymes in AD exist in any of the following forms:

- Released by micro-organisms and are adsorbed to extracellular polymeric substances such as carbohydrates, proteins, humic compounds, lipids, and deoxyribonucleic acids in the inoculum (Tchobanoglous *et al.,* 2003); and
- Originate from the inoculum via cell autolysis or are actively secreted by cells and dispersed freely in the inoculum or media (Higuchi *et al.,* 2005).

The active sites of hydrolases generally consist of charged amino acid residues, but might also contain one or two divalent metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+} (Heikinheimo *et al.,* 2001). The hydrolytic potential of the MEs varies with the number of coordinating divalent metal ions. According to Frey and Hegeman (2007), the hydrolytic effect of two divalent metal ions is twice as high as that of a single divalent metal ion. This explains the presence of two divalent metals ions participating in the hydrolytic mechanisms of MEs such as ureases, alkaline phosphatase and inorganic pyrophosphatases among others (Frey and Hegeman, 2007; Bortolato *et al.,* 1999).

5.2.2 TEs and MEs of acetogenesis: The predominant pathway for growth and metabolism in acetogens is the acetyl-CoA pathway (Ferry, 1999). Acetyl-CoA pathway involves metabolic processes that reduce CO_2 or CO using H_2 as electron donor to form acetate (Ragsdale, 2006; 2008). In the reverse direction, acetate is oxidized to its precursors. Some acetogens such as species of *Acetobacterium and Clostridium* can convert short chain fatty acids to only acetate in the acetyl-CoA pathway and are referred to as homoacetogens (Drake *et al.,* 2008; Diekert and Wohlfarth, 1994). Acetyl-CoA pathway is present in both acetogens and methanogens involved in the conversion of acetate to CH_4, and reduction of CO_2 to acetate using H_2 (Drake *et al.,* 2008).

The reduction of CO_2 to acetate using H_2 is called homoacetogenesis and offers the possibility to improve the energetics and kinetics of methanization through acetogenic removal of H_2 (Siriwongrungson *et al.,* 2007). Two distinct phases are identifiable during homoacetogenesis in the acetyl-CoA pathway:

- Systematic reduction of CO_2 to a methyl group ($-CH_3$); and
- Transfer of the $-CH_3$ to CODH for the synthesis of acetate.

The principal enzymes of the acetogenic and methanogenic acetyl-CoA pathway are MEs and include hydrogenases; FDH; MeTr; and CODH/ACS (Matthews, 2001; Drennan *et al.,* 2004; Ragsdale, 2006; 2008). The principal reactions of these MEs are discussed next.

a. Hydrogenases: Figure 5.1 shows the active site of hydrogenase (A) and its electron-conduit subunit (B). During CO_2 reduction, hydrogenases provide redox electrons by oxidation of H_2 as shown in Equation 5.2. Some hydrogenases contain metals, while others do not. The metals of importance in hydrogenases include Ni and Fe; other components include amino acid residues and sulphur. Ni-Fe complex is necessary for substrate binding; and Fe-S complex is required as conduit for electron transfer (Shima *et al.,* 2002).

Equation 5.2	H_2	↔	$2H^+ + 2e^-$

Figure 5.1 Ni-Fe catalytic centre in hydrogenases **(A)**; Fe-S cluster in hydrogenases **(B)** (Shima *et al.,* 2002; Thauer *et al.,* 2008)

b. Formate dehydrogenase (FDH): This MEs contains Se, W or Mo at the active site (Kletzin and Adams, 1996). The TEs found at the active site depends on the microbial species, the growth substrates or bioavailability of the TEs in the growth medium (Da Silva *et al.,* 2011). FDH catalyses thermodynamically unfavourable CO_2 reduction to formic acid (HCOOH) as shown in Equation 5.3. Redox electrons for the biocatalysis are obtained from reduced nicotinamide adenine dinucleotide phosphate (NADPH), H_2 or pyruvate (Reda *et al.,* 2008; Kletzin and Adams, 1996). When CO is the substrate instead of CO_2, FDH is associated with CODH oxidation of CO to CO_2 (Equations 5.4) and formic acid (Equations 5.5) (Seravalli and Ragsdale, 2000; Ragsdale, 2006).

Equation 5.3	$NADPH + CO_2 + H^+$	↔	$NADP^+ + HCOO^-$
Equation 5.4	$CO + H_2O$	→	$2e^- + 2H^+ + CO_2$
Equation 5.5	$2e^- + 2H^+ + CO_2$	→	$HCOOH$

c. Methyltransferase (MeTr): MeTr is associated with transfer of the group -CH_3, and is unique to micro-organisms that employ the acetyl-CoA pathway for acetate metabolism (Doukov *et al.,* 2008; Menon and Ragsdale, 1999). Its features are as follows:

- It has either of two types of active-site complexes: cobalamin or cobamide and both are activated by Co;
- The active site of MeTr is capable of accepting –CH_3 groups when the Co is in Co^+ state to form an enzyme bound CH_3-Co^{3+} complex (Equation 5.6);
- The -CH_3 group is transferred to coenzyme M (SH-CoM) to form CH_3-S-CoM, and the active Co^+ state is regenerated as shown in Equation 5.7 (Stock and Rother, 2009; Ragsdale and Speece 2008; Harms and Thauer, 1997; Ragsdale and wood, 1991); and
- Temperature range between 35°C and 40°C; and pH range between 7.5 and 7.7 are optimum for MeTr-dependent biocatalysis. The enzyme could be stable up to 45°C, and activity-decline sets in around 70°C (Caspi *et al.,* 2008).

Equation 5.6	$CH_3^+ + Co^+$	→	CH_3-Co^{3+}
Equation 5.7	CH_3-Co^{3+} + HS-CoM	→	CH_3-S-CoM + $Co^+ + H^+$

d. CODH/ACS complex: CODH/ACS complex is the main enzyme for the metabolism of acetate in both acetogens and methanogens. It comprises two sub-units: CODH and ACS (Drennan *et al.,* 2004). Figures 5.2a and b show the active units of CODH and ACS complex. CODH/ACS complex is involved in the metabolism of H_2, CO_2, CO and VFA. During CO_2 metabolism, one molecule of CO_2 is reduced to CO by CODH, and this becomes the carbonyl group of acetate (Equation 5.8a). Another molecule of CO_2 is reduced to formate ($HCOO^-$) (Equation 5.5), and further to a -CH_3 group (Equation 5.8b), and this forms the -CH_3 group of acetate (Svetlitchnyi *et al.,* 2001). The ACS unit condenses the CO and -CH_3 with CoA to form acetyl-CoA (Equation 5.9) (Lindahl, 2004).

Equation 5.8a	$CO_2 + H_2 + 2e^-$	→	$CO + H_2O$
Equation 5.8b	$HCOO^- + H_2$	→	$CH_3 + O_2^-$
Equation 5.9	$CH_3 + CO + CoA$	→	Acetyl-Co

Structurally, the active sites of the CODH/ACS complex in anaerobes contain Ni ions linked to the Fe-S electron conduit (Figure 5.2a and b) (Dobbek *et al.,* 2001; Svetlitchnyi *et al.,* 2001). CODH/ACS complex may additionally contain Mo and Cu to detoxify reactive oxygen species as shown in Equation 5.8b (Gnida *et al.,* 2003). The biochemical and kinetic properties of CODH/ACS complex depend on whether it is involved in acetogenesis or methanogenesis (Shin *et al.,* 2007; Lindahl, 2004; Tan *et al.,* 2003).

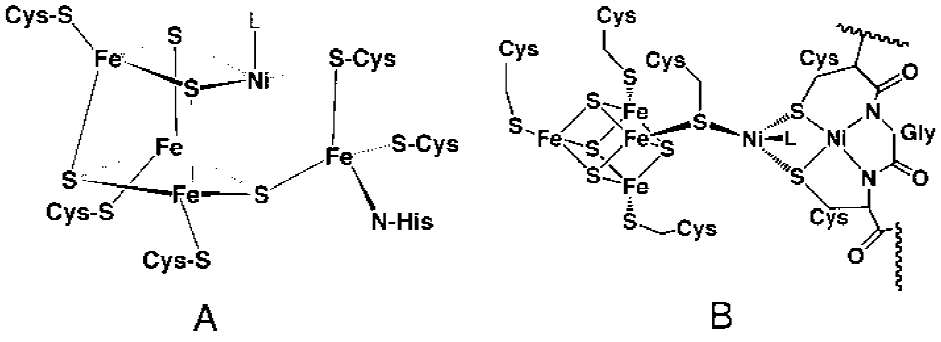


Figure 5.2 Active unit of **(A)** carbon-monoxide dehydrogenase **(B)** Acetyl CoA synthase (Drennan *et al.,* 2004)

Optimum temperature and pH for CODH are 60°C and 6.3 respectively for acetogenic CO_2 reduction; and about 70°C is optimum for ACS in aceticlastic methanogenesis (Shin *et al.,* 2007). This may explain the observation by Iranpour *et al.* (2005) that in extreme thermophilic AD (≥ 60°C), CO_2 and H_2 are converted to acetate instead of CH_4. It also explains the report by Uemura (2010) that CH_4 production in thermophilic AD was optimum compared to mesophilic AD which required TEs supplementation for optimum CH_4 production.

5.2.3 TEs and MEs of methanogenesis: Figure 5.3 shows the different routes by which CH_4 could be produced in AD. CH_4 production is possible through three different routes by different methanogens, and these include:

- Hydrogenotrophic methanogenesis: CH_4 formation from H_2 reduction of CO_2;
- Aceticlastic methanogenesis: The cleavage of acetate into CO_2 and CH_4; and
- Methylotrophic methanogenesis: Redox reactions using methylated substrates such as methanol, methylamines and dimethyl-sulphide to form CH_4.

Homoacetogenesis, aceticlastic methanogenesis and acetogenesis have acetate, CO_2 and H_2 in common as intermediate product of VFA degradation and substrate for

CH_4 or acetate production. Consequently, the processes also have similar enzymology; but the defining steps run in reverse directions (Bapteste *et al.,* 2005; Shima *et al.,* 2002). Some MEs that are common to both acetogenesis and methanogenesis such as MeTr and CODH/ACS have been discussed in sub-section 5.2.2. However, MEs associated with CH_4 formation from acetate or H_2 reduction of CO_2 are unique to methanogens (and to some extent, also involved in sulphate reduction). These unique MEs of methanogenesis and their TEs relations are discussed next.

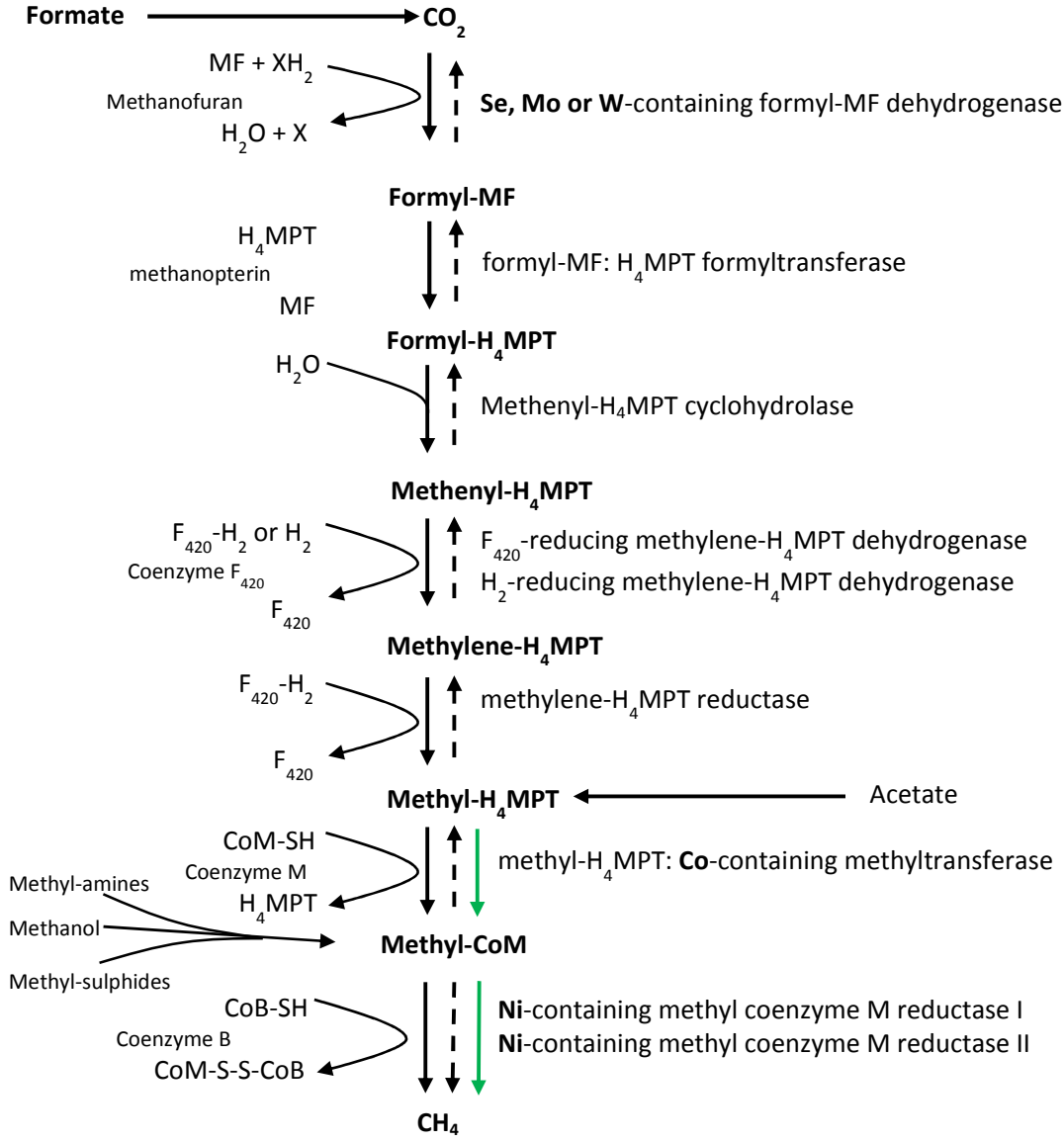


Figure 5.3 Pathways of Methanogenesis: **aceticlastic**- solid black arrows; **hydrogenotrophic-solid** green arrows; **methylotrophic-** broken black arrows (Adapted from Bapteste *et al.,* 2005)

5.2.3.1 Formyl-methanofuran dehydrogenase (FMD): FMD is the methanogenic analogue of FDH (see section 5.2.2). It is also called methanofuran

reductase (MR) (Ragsdale, 2006; Karrasch *et al.,* 1990). The reactions and structure of FMD are shown in Figures 5.4A and B and some important features of the ME include:

- The enzyme contains Mo or W at the active site (depending on which of the TEs is bio-available in the substrates), but it is more active with Mo than with W; and
- FMD is similar to Se-containing FDH in acetogens (Thauer, 1998; Karrasch *et al.,* 1990)

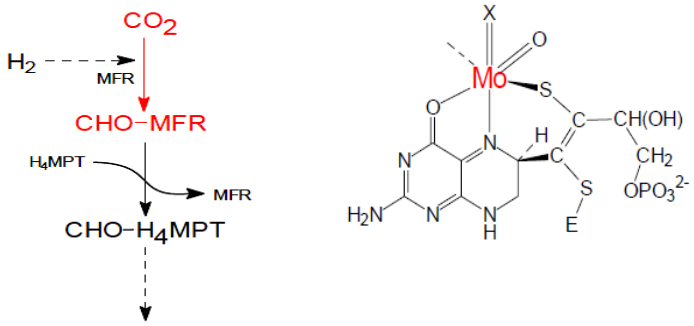


Figure 5.4 Biocatalysis of formyl methanofuran dehydrogenase (FMD) and central Mo in FMD (Ragsdale, 2006)

5.2.3.2 Methyl-CoM reductase (MCR): MCR is only found in methanogens and catalyses the final stage of CH_4 production from the reduction of $–CH_3$ as shown in Equation 5.10. This involves the reaction between methyl-coenzyme-M (CH_3-S-CoM) and coenzyme-B (SH-CoB) in the presence of MCR to form CH_4, SH-CoM and SH-CoB (Ferry, 1999).

Equation 5.10 CH_3-S-CoM + SH-CoB + MCR → CH_4 + CoM-S-S-CoB + MCR

According to Ferry (1999), two forms of MCR known as MCR-I and MCR-II exist, and their expression is influenced by H_2 concentration. Both forms have different biochemical properties (investigated under thermophilic conditions). MCR-I functions optimally in the pH range between 7.0 – 7.5, and has a maximum reaction rate of approximately 6 µmol min^{-1} mg^{-1}. The maximum reaction rate for MCR-II is approximately 21 µmol min^{-1} mg^{-1} and the optimum pH range is 7.5 – 8.0. Notwithstanding the form of existence, the general features of MCR include:

- MCR comprises a reactive co-factor known as F_{430}, which is dependent on its active Ni-complex for catalysis. Also, about 70% of the Ni found in some bacteria could be present in the F_{430} cofactor (Ermler *et al.,* 1997);

- The Ni-complex in MCR is similar to the Co-complex in MeTr;
- The active form of Ni in MCR is the Ni^{+} state, which forms CH_3-Ni^{3+} when it receives CH_3 group from MeTr ; and the active Ni^{+} state is regenerated when CH_3-Ni^{3+} is reduced to CH_4 (Caspi *et al.,* 2008).

Generally, substantial amount of the energy generated during methanization is trapped in CH_4 and is unavailable for biochemical work (Kleerebezem and Stams, 2000). MEs might have evolved in methanogens and homoacetogens to counteract the low energy exchange associated with AD in general and VFA degradation in particular (Ragsdale, 2006; Thauer, 1998). Low AD energetics implies that some of the AD reactions cannot go on spontaneously and must be catalyzed. The incorporation of TEs into the enzymes of micro-organisms that are involved in AD reactions with low energy exchange ensure that rate of these reactions meet thermodynamic requirements to make such reactions possible (Thauer *et al.,* 1977; Harms and Ermler *et al.,* 1997). The biocatalytic efficiency of the TEs can be evaluated by measuring parameters that are related to the activities of the MEs. These parameters are discussed next.

Table 5.1 Summary on the occurrence of trace elements in metallo-enzymes of anaerobic digestion

Trace elements	Associated metallo-enzymes
Ni	[3]CODH, MCR, hydrogenases and ureases
Co	[1, 2, 3]CODH and MeTr
Mo	[3]FDH and FMD
W	[1]FDH and FMD
Se	[3,4]nucleic acids, FDH, FMD and CODH/ACS
Fe	[3]FDH, FMD, hydrogenases and CODH/ACS
Zn	[1]FDH and hydrogenases [5]Kinases, phosphotransferases and hydrolytic enzymes
Cu	[4]Hydrogenases, superoxide dismutase and CODH
Mg	[4, 6,7]kinases, phosphotransferases and phosphatases
Mn	[4]Dehydrogenases and MeTr [6]Interchangeable with Mg in kinase and phosphatases

([1]Kayhanian and Rich, 1995; [2]Kida *et al.,* 2001; [3]Somitsch, 2007; [4]Schattauer *et al.,* 2011; [5]Burgess *et al.,* 1999; [6]Bortolato *et al.,* 1999; [7]Oleszkiewicz and Sharma, 1990) These enzymes have been described in the corresponding sections in Chapter 5.

5.3 Evaluating process efficiency during the stimulation of MEs

The activities MEs in the processes they mediate can be evaluated by measuring two parameters: maximum reaction rate or maximum velocity (MRR or V_{max}) of the conversion of the substrate being methanized, and inverse affinity (IA or K_m) (Illanes, 2008; Frey and Hegeman, 2007; Buchholz *et al.,* 2005). MRR, IA and substrate concentration (S) are related by Michaelis-Menten equation as shown in Equation 5.11, where V is rate of reaction at any time, [S] is the substrate concentration, a is MRR and b is IA. These parameters are discussed in 5.4.1 and 5.4.2.

Equation 5.11 $$V = \frac{a*[S]}{b+[S]}$$

5.3.1 Maximum reaction rate (MRR): MRR reflects the maximum rate of substrate utilization or product formation per time. Essentially, it is the maximum rate of the enzyme for a single enzyme assay, or the net process rate for a multi-enzyme process such AD. A relative increase in MRR could imply an increase in the concentration of the relevant enzyme(s) through synthesis of new enzymes or an optimization of the activities of existing enzyme(s). Thus for any substrate S, MRR can be compared for different operating conditions.

5.3.2 Inverse affinity: IA is the substrate concentration at which half the MRR is attainable. That is, it is the substrate concentration at which the reaction rate is at half the MRR. IA is influenced by both substrate binding or affinity, and substrate conversion efficiency. For a given process, a small IA indicates that the MRR will be reached more quickly compared to a relatively larger IA. Hence, relatively low IA is an indication of better affinity for the substrate being degraded (Bisswanger, 2004).

5.4 Research motive

AD has been reviewed in Chapters 4 and 5 with regard to TEs and their influences on MEs. The variations in TEs content in substrates of AD and the inoculum used as sources of nutrient, micro-organisms and TEs have been highlighted. TEs and their roles in the stimulation of methanization processes have also been appraised. Generally, there is lack of consensus on the concentrations of TEs that are optimum for methanization; but stimulatory influences are widely reported in TEs supplementation experiments. It is possible that the lack of agreement in optimum TEs concentration is related to the fact that apart from the concentration of the

elements that are supplemented, other factors influence TEs requirements during methanization.

Some of these factors might include composition of TEs, VFA concentration and methanization temperature and pH. Regarding TEs composition, section 4.3.2 discusses synergistic and antagonistic combinations of transition elements and TEs. The influence of VFA concentration can be deduced from the variations in TEs requirements for cellulosic substrates such as silages (4.2.1) and easily degradable substrates such as food waste (4.2.2). Temperature and pH are known to directly influence the activity of AD micro-organisms and enzymes (Shin *et al.,* 2007).

Thermophilic temperatures are optimum for some of the MEs of methanization (5.2.4 and 5.3.2); other MEs are optimum at mesophilic temperature (5.2.3). Hence, when methanization processes such as VFA degradation rate and CH_4 production are optimized at a certain temperature, TEs supplementation produce little or no further improvement. Furthermore, bioavailability of TEs determines whether or not the supplemented TEs are part of the nutrition of the micro-organisms in AD. Evaluating bioavailability of TEs during methanization will provide knowledge on what proportions of the supplemented TEs are available to the micro-organism. The influence of these individual factors and their interactions are generally neglected in reporting influences of TEs during methanization.

Additionally, there are no reports on the evaluation of TEs supplementation approaches to determine the most efficient TEs concentration and composition for different methanization goals. The mechanism of AD optimization by TEs supplementation has not been investigated. This leaves a large knowledge gap in determining the appropriateness or otherwise of TEs combinations. Most importantly, it is necessary to find the range of TEs concentration and composition (configuration) that is efficient across different concentrations of VFA during methanization. It is also necessary to ensure that the optimum TEs configuration does not jeopardize the use of digestate in agriculture. An optimum TEs configuration will eliminate the risk of imbalance in TEs composition. When the legal limits of TEs that are permitted in the European Directive 86/278/CEE for digestate to be used in soil enhancement is considered in the formulation of optimum TEs mixture, the risk of digestate unacceptability due to TEs overload is reduced.

Regarding influential elements, Ni, Co, Se and Mo have been selected for investigation because they occur in low concentrations in substrates compared to elements such as Fe, Zn, Mg, Cu and Mn, which are also stimulatory in AD. Also, Ni

Co, Se and Mo are important constituents of methanization MEs whose activities determine the efficiency of the different phases of AD. These have already been discussed in section 5.2. Therefore, the sufficiency or otherwise of Ni, Co, Se and Mo content in AD substrate for methanization are investigated. The methanization consequences of the interactions of the TEs with one another and with other factors such as temperature and VFA concentrations are also evaluated in this research.

The main objective of the experimental methods presented in Chapter 6 and the results in Chapter 7 is to fill the knowledge gap regarding Ni, Co, Se and Mo requirements in methanization in the areas that have been identified. The following investigations guide the realization of this objective:

1. TEs content and other characteristics of substrates of AD;
2. Supplementation of TEs during AD;
3. Evaluation of the efficiency of TEs supplementation approaches;
4. Determination of the mechanisms of AD optimization due to TEs supplementation;
5. TEs supplementation and use of digestate in agriculture; and
6. Formulation and validation of an Optimum TEs configuration.

CHAPTER 6 MATERIALS AND METHODS

6.0 Chapter overview

The main aspects of this investigation are listed in section 5.4. This chapter presents the materials and methods employed in the research. These include: reagents used for the research, experimental set-ups, and procedures applied for sample and data analyses. Multivariate experimentation, data analyses techniques and applied models are included as methods. Summary of the methods and materials for the different aspects of the investigations is presented Table 6.1. Detailed descriptions of the methods and materials are in the relevant sections.

Table 6.1 Overview of the methods and materials used in the optimization of methanization processes

Research methods and objectives	Substrates
1. TEs contents and other characteristics of AD substrates *Objectives:* Determine the TEs contents and other characteristics of selected AD substrates that can influence TEs bioavailability.	Shown in Table 6.2
2. Influences of TEs supplementation during AD *Objectives* **a.** Discuss the main and interaction effects of the TEs in hydrolysis-acidification rate (HAR), VFA degradation rate and CH_4 production, retention time and adaptation period; **b.** Highlight the TEs settings that influence methanization; **c.** Create supplementation scenarios from the optimum TEs settings identified for the mesophilic methanization and compare responses between scenarios; **d.** Identify the TEs configuration that can be supplemented across all levels of VFA; **e.** Highlight the underlying mechanisms of methanization enhancement due to TEs supplementation	Mixture of sodium salts of acetic, propionic and butyric acids as shown in Table 6.6
3. Sequential extraction of TEs in batch AD *Objectives* **a.** Evaluate bioavailability of the different species of TEs; **b.** Evaluate the impact of adsorbed TEs on the quality of digestate; and **c.** Recommend optimum ranges of Ni, Co, Se and Mo for methanization.	Digester content of the thermophilic investigation with TEs supplementation: Appendix 7.5a
4. Validation of 2a, b and c in a semi-continuous AD investigation *Objectives* **a.** Derive a VFA-independent optimum TEs configuration and develop its variants; **b.** Evaluate the efficiency of the derived TEs configuration and its variants in a semi-continuous mesophilic methanization.	Mixed fruit residue
5. Evaluate the change in microbial population due to TEs supplementation *Objective* Determine whether the mechanism of AD enhancement due TEs supplementation involves a change in microbial population or enhancement of the efficiency of resident microbial population.	Mixed fruit residue

6.1 Determination of the TEs contents and other characteristics of various AD substrates: Selected substrates of AD were analysed for Ni, Co, Se and Mo. Fe content in the substrates was also determined to show its relationship with the TEs. Other characteristics of the substrates that were analysed include C, H, O, N and S; and total carbohydrate. The substrates and the methods of analysis are discussed next.

6.1.1 Substrates analysed for TEs contents and other properties: The substrates include selected AD substrates shown in Table 6.2. The experimental substrates were selected to include relatively simple or mono-substrates and mixed or more complex substrates. Digestates were also included for reference to TEs contents after methanization. The dungs were considered as simple substrates because they are predominantly grass residues.

Table 6.2 Anaerobic digestion substrates analysed for trace elements and Fe contents

Substrate	Place of collection	*Sample form
Mono-substrates		
Lawn grass	Open field in Hamburg	Fresh
Spear grass	Open field in Hamburg	Fresh
Cow dung	Cattle farm in Hamburg	Slurry
Horse dung	Horse stable Hamburg	Dry
Mixed leaves	Fallen leaves in Hamburg	Mixture of fresh and dry
Complex-substrates		
Grease trap residue (GTR)	Company in Hamburg	Mix of fat and wastewater
Blackwater (BW)	Ecological settlement in Hamburg	Liquid from vacuum toilet
Restaurant biowaste (RW)	University in Hamburg	Slurry
Mixed fruit residue (MFR)	Company in Hamburg	Fresh residue
Silage (feedstock)	Biogas facility in Hamburg	Solid
Digestate		
Silage digestate	Biogas facility in Hamburg	Slurry
Sewage sludges	Sewage treatment facilities in Hamburg	Slurry

(*All samples were stored at temperature ≤ 4°C until required for use)

6.1.2 Methods for the determination of TEs contents and other properties of AD substrate: Table 6.3 shows the equipment, reagents and procedure used for substrate characterization, and TEs and Fe content analyses. Substrate characterization involved analyses for dry matter (DM) and organic dry matter (oDM); and quantification of C, H, O, N, S, simple sugars and total carbohydrate. Standard methods (SMs) were generally used for the analyses; hence, only the codes for the SMs are given in Table 6.3. Detailed procedures of the SMs are not presented, but

where applicable, the modifications to standard procedures are shown. In some cases, the analytical methods were developed or modified by the organizations that carried out the analyses; these are also indicated.

Table 6.3 Equipment and applied methods associated with substrate characterization and determination of trace elements and Fe contents of anaerobic digestion substrates

Substrate Parameter	Equipment	Applied Method
Dry matter (DM)	Oven: Heraeus B 5050E Germany	DIN EN 12880
Organic DM (oDM)	Muffle furnace: MR 170 E Germany	DIN EN 12879
Elemental composition C, H, O, N, S	Analyses was made at Thünen-Institute (TI) Lohbrügge, Hamburg	DIN EN 15104:2011-04 (E) Carried out at the Thünen-Institute (TI) Lohbrügge, Hamburg
Simple sugars and total carbohydrate	Borate complex ion-exchange chromatography: Biotronic, Frankfurt	Sinner and Puls (1978); Sinner *et al.* (1975). Carried out at the Thünen-Institute (TI) Lohbrügge, Hamburg (See Appendix 6.9)
TEs and Fe content N, Co, Se, Mo; and Fe	Substrate milling: Planetary ball mill pulverisette 5 (Fritsch) Germany Microwave digestion: Physica, Germany TEs and Fe detection by flame atomic absorption spectroscopy (FAAS): ContrAA 700 (Analytik Jena) Germany	DIN EN 13346:2001-04 (Scheme of steps in Appendix 6.1)

6.2 TEs supplementation experiments

Salts of Ni, Co, Se and Mo were supplemented to a mixture of sodium salts of VFA in batch experiments at 37°C and 55°C. The materials, including the reagents and the methods adopted for the investigations are discussed next.

6.2.1 Materials: These include reagents used for the preparation of basic nutrient medium, TEs solutions and VFA mixture; microbial seed culture (inoculum) and the set-up of the experiments. VFA mixture was used as substrate in the batch experiments. The individual compounds that constituted the reagents are contained in Appendix 6.2. Appendix 6.7b shows the average properties of the inocula used in the different investigations. Figure 6.1 and Figure 6.2 show the scheme of the experimental set-up and the actual set-up respectively, which were used in the investigations involving TEs supplementations in the batch AD. The features of the actual set-up are described in Table 6.4. The set-up is an adaptation of the German recommendation for the determination of biogas potential of a substrate (Verein

Deutscher Ingenieure, 2006). Except stated otherwise, this set-up was used for all the batch experiments involving TEs supplementations at 37°C and 55°C.

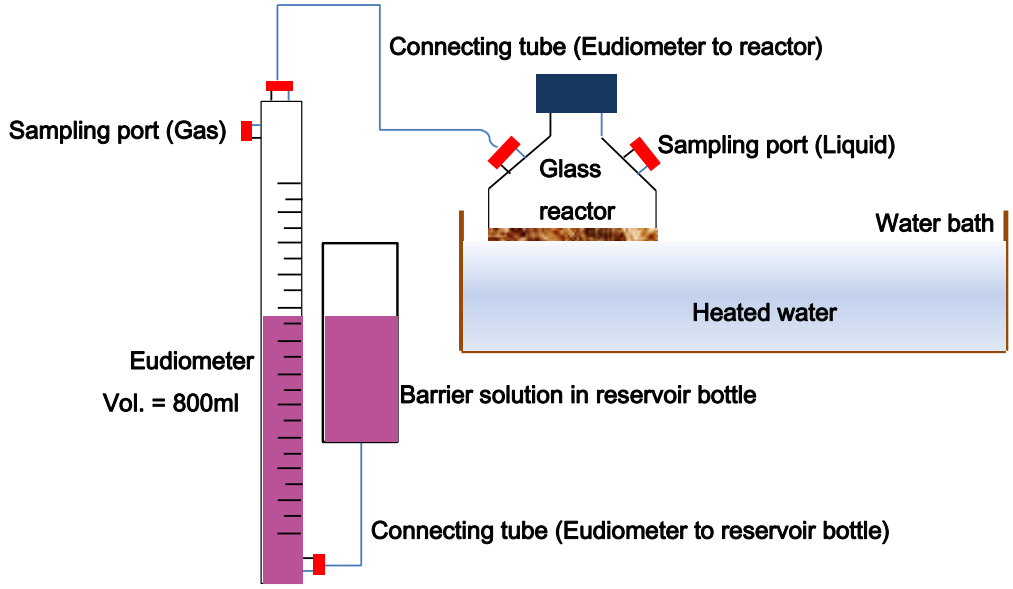


Figure 6.1 Basic scheme of the experimental test system used for the batch investigations

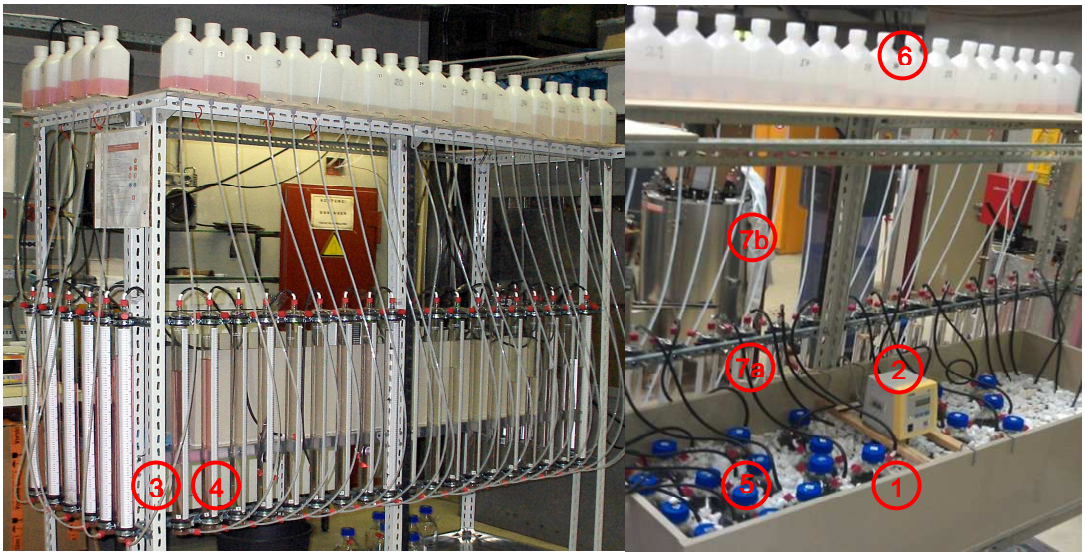


Figure 6.2 Photograph of the test system used for the batch investigations

Table 6.4 Main features of the set-up of the test system used for the batch investigation

Item Nr.	Components	Units	Composition/Function/Supplier
1.	Water bath	1	Polypropylene; contains water and the reactors.
2.	Thermostat	1	Lauda RE 120 thermostat from Lauda Dr. R. Wobser GmbH & CO. KG, Lauda-Königshofen, Germany; Keeps the water bath and reactors at experimental temperature.
3.	Metal frame	-	Supports the weight of the water bath and reservoir bottles; serves as attachment point for accessories.

Table 6.4 (cont'd) Main features of the set-up of the test system used for the batch investigations

Item Nr.	Components	Units	Composition/Function/Supplier
4.	**Eudiometers**	30	Calibrated 1-Litre glass tubes; manufactured by HWS Labortechnik, Mainz Germany; measures gas produced by liquid displacement
5.	**Glass reactors**	30	1-Litre Schott glass bottles with sampling port; purchased from HWS Labortechnik, Mainz Germany; provides containment and micro-environment for the investigations
6.	**Reservoir bottles**	30	1-Litre plastic bottles; purchased from VWR International, Germany; holds barrier solution displaced from the eudiometers
7.	**Tubing materials**	-	Nylon tubing from HWS Labortechnik, Mainz Germany for connecting the eudiometers to the reservoir bottles; Viton tubing from VWR International Germany, for connecting the reactors to the eudiometers
8.	**Septa**	-	Three-layered rubber septa purchased from VWR International, Germany; to make (5) airtight
9.	**Barrier solution**	-	Mixture of citric acid and NaCl prepared according to ISO/DIS 14853; contained in (4) and (6); prevents dissolution of CO_2

6.2.2 Method: Batch AD experiments were carried out at 37°C and 55°C with Ni, Co, Se and Mo supplementation to VFA mixture composed of sodium salts of VFA shown in Appendix 6.2. TEs supplementation with only Se and Mo were also carried out to compare partial TEs supplementation with full TEs supplementation involving Ni, Co, Se and Mo. Three main methods were involved in the supplementation experiments and these include:

I. Designing the supplementation matrices;
II. Implementing the designs; and
III. Data analyses.

Data analyses differ and depend on the aspect of the supplementation in focus. Details of the data analyses techniques and the applied statistical algorithms are discussed in relevant sections of this Chapter. Both the experimental design and data analyses were aided by JMP 10 (SAS Institute Inc., 2012). The main methods are discussed next.

6.2.2.1 Design of Experiment (DoE): The design of the experiments differs for partial supplementation involving Se and Mo; and full supplementation involving Ni, Co, Se and Mo. Important feature of both designs are the measured responses, the optimization goals associated with the responses, the factors that were varied, and the levels to which the factors were varied. Some of the responses were directly

measured while others were derived. Measured and derived responses, as well as the associated goals are shown in Table 6.5.

Table 6.5 Measured and derived responses, and the optimization goal of the individual responses in the trace elements supplementation experiments

Responses (dependent)	Response goal
Hydrolysis and acidification rate (HAR)[1]	Maximize
CH_4 production (SMP)[1]	Maximize
VFA degradation rate (DR)[1]	Maximize
Retention time (RT)[2]	Minimize
Adaptation period (AP)[2]	Minimize

([1]directly measured responses; [2] derived responses)

Table 6.6 Levels and concentration ranges of the factors used for designing the batch experiments for mesophilic and thermophilic operations

Factors	Levels		
	-1 (low)	0 (medium)	1 (high)
Ni (mg/L)	0.07	0.96	1.92
Co (mg/L)	0.03	1.85	3.70
Se (mg/L)	> 0.005	0.49	0.98
Mo (mg/L)	0.04	0.60	1.20
VFA mixture (mmol/L)	**≤ 25**	**≥ 100**	**≥ 200**
Acetate (mg/L)	≤ 600	≥ 2,250	≥ 4,500
Butyrate (mg/L)	≤ 250	≥ 1,000	≥ 2000
Propionate (mg/L)	≤ 1000	≥ 4,000	≥ 8,000
*Silage feedstock (mg/L)	4000	4000	4000

(*Silage feedstock was used for mesophilic investigation and not thermophilic)

Table 6.6 shows the range of the TEs and VFA (factors) used in the investigation. These ranges were chosen from literature review, especially from the report of DEFRA (2010). The investigated factors include VFA mixture, Ni, Co, Se and Mo and are also referred to as explanatory, independent and predictor variables; and there are 3 levels (concentration) of each factor. Each TE was supplemented in 3 levels to each of the 3 levels of VFA mixture. This supplementation arrangement formed low (-1), medium (0) and high (1) TEs supplementation categories defined by VFA levels. Supplementation matrixes for the above combination were generated using the DoE module of JMP 10 (SAS Institute Inc., 2012). Two DoE types were implemented and include:

- Full factorial for Se and Mo (partial) supplementation;
- Custom design for Ni, Co, Se and Mo (full) supplementation.

a. Full Factorial Design (FF): The Experimental design specification for the FF module in the Se and Mo supplementation is contained in Appendix 6.3. Table 6.7 shows the FF TEs supplementation matrix for Se, Mo and VFA. Se and Mo supplementation experiment was designed as FF using the FF DoE module of JMP 10.

Equation 6.1 $n^k = e$

Table 6.7 Full factorial coded matrix for Se, Mo and VFA in the mesophilic experiments

Treatment	Se	Mo	VFA	Group
*1	-1	-1	-1	
2	-1	0	-1	
3	-1	1	-1	
4	0	-1	-1	
5	0	0	-1	Low
6	0	1	-1	
7	1	-1	-1	
8	1	0	-1	
9	1	1	-1	
*10	-1	-1	0	
11	-1	0	0	
12	-1	1	0	
13	0	-1	0	
14	0	0	0	Medium
15	0	1	0	
16	1	-1	0	
17	1	0	0	
18	1	1	0	
*19	-1	-1	1	
20	-1	0	1	
21	-1	1	1	
22	0	-1	1	
23	0	0	1	High
24	0	1	1	
25	1	-1	1	
26	1	0	1	
27	1	1	1	

(* Control)

That is, all possible 3-level combinations of Se, Mo and VFA were made. 27 experiments where required according to the factorial in Equation 6.1 (SAS Institute Inc., 2012: DoE). In Equation 6.1, *n* is 3 (the number of levels of the factors), which are coded as high (1), medium (0), and low (-1); *k* is 3 (the number of factors that include Se, Mo and VFA); and *e* is the number of experiments required.

b. Custom Design (CD): Ni, Co, Se, Mo and VFA were the factors in the full TEs supplementation investigations. 3 levels of each of the 5 factors were involved in the design, and according to Equation 6.1, the required number of experiments would be 3^5 or 243 runs. To minimise the number of run without risking the predictive potential (power) of the investigation, the relevant fraction of the total number of required experiments was statistically selected using the CD module of the JMP 10 software. Details of the CD module as well as the experimental design specification for the full TEs supplementation are contained and discussed in Appendix 6.4. Choice of the representative number of runs is based on the following assumptions:

- The responses of interest are influenced by main effects (individual factors) and lower-order interactions (interactions involving 2 factors) of the factors investigated; and
- Higher-order interactions (interactions involving more than 2 factors) have negligible effects.

For example, the 243 runs will include main influences from the individual factors as well as combinations of any 2, 3, 4 and all 5 factors. In all these, the significant influences will be in runs involving the individual factors acting alone (main) and any two factors acting together (lower-order interaction). The 3, 4 and 5-factor interactions will influence the responses in ways resembling main effects or lower-order interactions effects (Collins *et al.,* 2009; Chakroborty *et al.,* 2009).

Equation 6.2 $CD\_implementable = Runs_{\_main,higher-order} * Runs_{\_lower-order}$

Equation 6.2 shows the relationship between the implementable CD design ($CD_{\_implementable}$); the runs that reflect the influences of the main effects and negligible higher-order interactions ($Run_{\_main,\ higher\text{-}order}$); and the runs that reflect the influences of the lower-order interactions ($Run_{\_lower\text{-}order}$). The $CD_{\_implementable}$ should predict the influence of the factors on the responses of interest as close as possible to what is obtainable implementing a full factorial. To compare this ability of the $CD_{\_implementable}$,

the relative prediction variance (RPV) is used. The FF (Equation 6.1) has a RPV value of 0; and the RPV of all the $CD\_{implementable}$ from Equation 6.2 can be compared. Details of the statistical algorithm for the RPV function are reported in JMP 10 software (SAS Institute Inc., 2012: DoE).

RPV analysis of the $CD\_{implementable}$ allows the designer to choose the number of runs that best suits experimental budget and objectives. The RPV analyses for the different $CD\_{implementable}$ are shown in Appendix 6.5. The coded design outcome for Ni, Co, Se, Mo and VFA interacting at 3 levels is shown in Table 6.8 for experiments at 37°C and Appendix 6.6 for experiments at 55°C. Similar to FF DoE, the matrix is divided into low, medium and high by VFA levels and each VFA level has a corresponding Control reactor.

Table 6.8 Custom design coded matrix for Ni, Co, Se, Mo and VFA used in the mesophilic investigations

Treatments	Ni	Co	Se	Mo	VFA	Groups
1	-1	-1	1	-1	-1	
2	-1	0	-1	0	-1	
3	-1	1	-1	0	-1	
4	-1	1	1	1	-1	Low
5	0	1	-1	1	-1	
6	1	-1	-1	-1	-1	
7	1	-1	1	1	-1	
8	1	1	1	-1	-1	
9	-1	0	0	-1	0	
10	0	-1	0	1	0	
11	0	0	1	0	0	Medium
12	1	1	0	0	0	
13	-1	-1	-1	0	1	
14	-1	-1	1	1	1	
15	-1	1	-1	1	1	
16	-1	1	1	-1	1	
17	0	0	0	0	1	High
18	0	1	-1	-1	1	
19	1	-1	1	-1	1	
20	1	0	-1	1	1	
21	1	1	1	1	1	
28	-1	-1	-1	-1	-1	Control-Low
29	-1	-1	-1	-1	0	Control-Medium
30	-1	-1	-1	-1	1	Control-High

6.2.2.2 Design Implementation: Implementation involves starting the runs, sampling and sample analysis.

Starting the experimental runs: The investigations include Control and treatment reactors operated at 37°C and 55°C following the respective matrices, and composed as follows:

Control reactors = Inoculum + nutrient media + VFA
Treatment reactors = Inoculum + nutrient media + TEs + VFA

a. Inoculum: This is the source of microbes and nutrients for the experiments. Mesophilic and thermophilic inocula were used. The mesophilic inoculum is the silage digestate described in Table 6.2 and Appendix 6.7b. The thermophilic inoculum was prepared by adapting the mesophilic inoculum according to the report of Ahn and Forster (2002); and Man-Chang *et al.* (2006). The inoculum was put in a 20 Litre plastic canister and placed in a waterbath. 2 units of 1 litre glass reactors were filled each with 500 ml of the inoculum and connected to the set-up as shown in Figure 6.2. 0.1 g acetate l^{-1} d^{-1} was fed to the reactors and gas production was measured. The temperature of the waterbath was increased gradually over time until the desired temperature was reached. The inoculum was considered adapted when the daily gas production remained constant. Table 6.9 shows the duration of each temperature increase.

Table 6.9 Adaptation procedure for the mesophilic inoculum used in the thermophilic experiments

Temperature transition (°C)	Number of days	Feeding
4	Storage	0.1g acetate (l^{-1} d^{-1})
20	3	
37	7	
45	3	
55	3	

(Method was adapted from Ahn and Forster, 2002; Man-Chang *et al.,* 2006)

b. Basic nutrient medium, TEs and VFA mixture: The basic nutrient medium was prepared as reported by Amani *et al.* (2011); the TEs solution as reported in DEFRA (2010); and VFA mixture according to the proportions shown in Table 6.6. The composition of the nutrient solution is shown in Table 6.10. Individual TE solutions were prepared according to Table 6.11. Both nutrient and TEs solution were acidified with 1M HCl and stored at 4°C until required. The reactors were grouped

into three blocks of low, medium and high VFA concentrations. TEs were added to each reactor following the matrix of Table 6.7 for Se, Mo and VFA at 37°C; Table 6.8 for Ni, Co, Se, Mo and VFA at 37°C; and Appendix 6.6 for Ni, Co, Se, Mo and VFA at 55°C. The actual concentrations of the TEs added in the medium and high levels according to the DoE are shown in Table 6.6. The low levels of the TEs are their respective average concentrations in the inocula used for the investigations.

For the evaluation of hydrolysis and acidification rate HAR, 4000 mg/L of silage feedstock (Appendix 6.7a) was added to each reactor during the implementation of the FF and CD matrices at 37°C. Details of the composition of the silage feedstock and the procedure for starting the batch experiments are presented in Appendix 6.7a and Appendix 6.7c respectively.

Table 6.10 Reagents and concentrations for the preparation of nutrient media

Reagent	Concentration (g/l)
KH_2PO_4	0.60
Na_2HPO_4	1.33
NH_4Cl	0.30
$CaCl_2.H_20$	0.11
$MgCl_2.6H_2O$	0.10
$NaHCO_3$	4.00
$Na_2S.9H_2O$	0.025
$FeCl_2$	0.45
H_3BO_4	0.04
$ZnCl_2$	0.03
$CuCl_2$	0.0063
$MnCl_2$	0.0295
Cysteine Hydrochloride	0.05

(Amani *et al.,* 2011)

Table 6.11 Reagents and concentrations used for preparing trace elements solution as well as the recommended dosing for trace elements supplementation

Reagents	Molar mass (g/L)	5.0mM Reagent (g/L)	TEs conc. (mg/ml)	*Recommended TEconcentration (mg/L)
$CoCl_2.6H_20$	237.93	1.190	0.2947	2.0
$NiCl_2.6H_20$	237.69	1.188	0.2935	1.0
$NH_4)_6Mo_7O_{24}.4H_2O$	1235.87	6.179	0.4797	-
$Na_2SeO_3.5H_2O$	263.02	1.315	0.3948	0.2

(*DEFRA, 2010)

b. Sample Collection and Analyses: Both liquid and gas samples were collected through the sampling port (Figure 6.1), which is a GL-14 port locked with a bored cap fitted with three-layer rubber septum. Liquid and gas samples were collected from the 1-L reactors using 20ml- and 50 ml syringes respectively, fitted with appropriate needles. Prior to sample collection, each reactor was manually shaken to homogeneity. The gas samples were analysed immediately after collection, and the liquid samples were either analysed shortly afterward or frozen at 18°C until required. Table 6.12 summarizes the equipment, standard methods and modification to the standards used to measure the responses in the batch experiments.

Table 6.12 Equipment, standard methods and modification to standard methods for sample analyses during the batch experiments

Parameter	Sample phase	Equipment/ Standard Method	Modifications to (standard) method
Primary Characteristics			
pH	Liquid	DIN 38404 – 5	-
Response Variables			
Biogas volume	Gas	VDI 2006: Eudiometer method	-
CH_4 content (%)	Gas	Spectra Mass Spectrometry	-
Total volatile fatty acids	Liquid	DIN 38414 – 19	15ml instead of 50ml
Individual VFA Acetic acid Propionic acid Normal butyric acid Iso-butyric acid Normal valeric acid Iso-valeric acid	Liquid	PerkinElmer Autosystem: column HP-FEAP 30m, 0.25mm ID, 0.25 FD (JAS); Carrier gas: H_2 5.0 1mL/min; Detector: FID; Injector temperature 280°C; FID temperature 300°C; Split ratio 15:1; Liner 4mm fine split completed and packed with glass wool; Oven temperature program: 80°C (1min), 6°C/min, 170°C, 20°C/min, 200°C (2mins)	-
TEs Speciation			
Sequential extraction	Liquid	Three-step protocol proposed by the Standards, Measurements and Testing programme (SM & T– formerly BCR) of the European Union as reported in Tokalioglu *et al.,* 2003.	Tokalioglu *et al.,* 2003 (Appendix 6.10).

6.2.2.3 **Estimating derived responses:** The measured and derived responses are shown in Table 6.5. The definitions and the equations of the derived responses are discussed next.

a. Hydrolysis and acidification rate (HAR): HAR was determined using Equation 6.3 by measuring VFA formation rate from the added silage feedstock (Appendix 6.7a) within 16 days of the implementation of the DoE in Tables 6.8. Since some of the VFA formed within the period of the hydrolysis-acidification (HA) experiments were converted to biogas, biogas produced per mmol/L VFA degraded ($Biogas_{\text{-per-VFA-deg.}}$) was calculated for each reactor as shown in Equation 6.3c and the total VFA converted to biogas ($TVFA_{\text{-HA-biogas}}$) was derived following Equation 6.3b for an observation periods ($t\text{-}_{HA}$) during the HA phase.

$TVFA_{\text{-HA-biogas}}$ (mmol/L) is Total VFA converted to biogas (Nml) in the $t\text{-}_{HA}$,
$TVFA_{\text{-HA-accum}}$ (mmol/L) is Total VFA accumulated in the $t\text{-}_{HA}$, in the HA phase,
$Total_{\text{-HA-biogas}}$ (Nml) is the total biogas produced in the $t\text{-}_{HA}$, in the HA phase,
$Total_{\text{-biogas-post-HA}}$ (Nml) is the total biogas produced in the post HA phase, and
$TVFA_{\text{deg-post-HA}}$ (mmol/L) is total VFA degraded in the post HA phase.

Equation 6.3a	HAR (mmol/L/d)	=	$(TVFA_{\text{-HA-accum}} + TVFA_{\text{-HA-biogas}}) / t\text{-}_{HA}$
Equation 6.3b	$TVFA_{\text{-HA-biogas}}$	=	$Total_{\text{-HA-biogas}} * (TVFA_{\text{deg-post-HA}} / Total_{\text{-biogas-post-HA}})$
Equation 6.3c	$Biogas_{\text{-per-VFA-deg}}$	=	$Total_{\text{-biogas-post-HA}} / TVFA_{\text{deg-post-HA}}$
Equation 6.3d	Rt (days)	=	$TVFA_{\text{-day16}} – TVFA_{\text{-measured-post-HA}} \leq 0.25\ (TVFA_{\text{-day16}})$
Equation 6.3e	Ap (days)	=	$CH_{4\text{-VOL-treatment}} \geq CH_{4\text{-VOL-control}}$

b. Retention time (Rt): The time required for the methanization of ≥ 75% of the post-hydrolysis VFA was estimated as retention time; that is, when the condition in Equation 6.3d was satisfied. $TVFA_{\text{-day16}}$ is total VFA on day 16, and $TVFA_{\text{-measured-post-HA}}$ is total VFA measured at any time in post HA phase.

c. Adaptation period: Adaptation time was derived from CH_4 yield as the time before one or more TEs treatments produced as much or more CH_4 than the Control treatment; that is, when condition in Equation 6.3e was satisfied in one or more treatments. $CH_{4\text{-VOL-treatment}}$ is volume (Nml) of CH_4 produced by a treatment reactor at any time during the experiment and $CH_{4\text{-VOL.control}}$ is volume (Nml) of CH_4 produced by the group Control reactor at any time during the experiment. Specific CH_4 production was calculated from Equation 6.3h, where VSS is the volatile suspended solids concentration of the reactor.

d. *Relative responses:* Measured and derived responses for the treatments are compared with the corresponding group Control reactor to obtain a ratio using Equation 6.3f. Treatments with relative response values > 1 are beneficial, 1 is equal to the Control and < 1 is inhibitory or non-beneficial for the measured response. The relative response value is converted to % gain (+) or % loss (-) using Equation 6.3g.

$y\_{rel.resp}$ is the relative response of treatment;
$y\_{treatment}$ is the measured response value in treatment;
$y\_{control}$ is the measured response value in Control; and
% y is the % gain or loss in response of interest.

Equation 6.3f	y_rel.resp	= $y\_{treatment}$ / $y\_{control}$
Equation 6.3g	%y (+ or -)	= (($y\_{treatment}$ / $y\_{control}$) – 1) * 100
Equation 6.3h	Specific CH_4 production	= Cumulative CH_4 content of biogas/VSS

The materials and method description continues with the specific methods relevant to specific research questions.

6.3 Evaluating important considerations for TEs supplementation

AD conditions are investigated for their influences on optimization of methanization processes using TEs. These include:

i. VFA concentrations (substrate concentration); and
ii. Process temperature.

The supplementation matrixes in Tables 6.7, 6.8 and Appendix 6.6 allow for dosing three levels of each TEs of interest to three levels of VFA mixture. Hence, main and interactions effects between VFA- (process condition I) and TEs concentration were deduced. By implementing Tables 6.7 and Appendix 6.6 at 37°C and 55°C respectively, the influence of temperature was determined (process condition II). The materials and methods are the same as discussed in section 6.2; and the temperature was regulated using a heating coil with thermostat from the Lauda Company (Table 6.4).

6.4 Determining the optimum range of TEs for AD

Two statistical functions of the JMP were used to determine the optimum range of the TEs for AD and these are response surface methodology and desirability function. These are discussed next.

6.4.1 Response surface methodology (RSM): The CD platform shown in Appendix 6.4 includes the response surface methodology (RSM) as a model for analysis of the data generated from the DoE (SAS Institute Inc., 2012: DoE). RSM is an algorithm for modelling the relationships between multiple factors and responses; and predicting responses based on the relationships between the factors (Douglas, 2001). The RSM function is shown in Equation 6.4. Y_i is any of the responses in Table 6.13, x_{1i} to x_{ki} are the factors (Ni, Co, Se, Mo and VFA) in the RSM function of response Y_i, β_0 is the intercept, β_i to β_k are the estimated coefficient of regression for the factors' main effects and interactions, and ε_i is the root mean square error (prediction error) for the response of interest.

Equation 6.4	$Y_i = \beta_0 + \beta_1 x_{1i} + \ldots + \beta_k x_{ki} + \varepsilon_i$
Equation 6.4a	$Y = \beta_o + \beta_1 VFA + \beta_2 Se + \beta_3 Mo + \beta_4 VFA\text{^}2 + \beta_5 VFA*Se + \beta_6 Se\text{^}2 + \beta_7 VFA*Mo + \beta_8 Se*Mo + \beta_9 Mo\text{^}2 + \varepsilon$
Equation 6.4b	$Y = \beta_o + \beta_1 VFA + \beta_2 Ni + \beta_3 Co + \beta_4 Se + \beta_5 Mo + \beta_6 VFA\text{^}2 + \beta_7 VFA*Ni + \beta_8 Ni\text{^}2 + \beta_9 VFA*Co + \beta_{10} Ni*Co + \beta_{11} Co\text{^}2 + \beta_{12} VFA*Se + \beta_{13} Ni*Se + \beta_{14} Co*Se + \beta_{15} Se\text{^}2 + \beta_{16} VFA*Mo + \beta_{17} Ni*Mo + \beta_{18} Co*Mo + \beta_{19} Se*Mo + \beta_{20} Mo\text{^}2 + \varepsilon$

Equation 6.4a and Equation 6.4b represents a typical RSM function for VFA, Se and Mo and VFA, Ni, Co, Se and Mo respectively. Depending on the supplementation matrix, either of the equations was fitted to the measured data for a response by backward stepwise regression as explained in JMP 10 (SAS Institute Inc., 2012: Fitting linear models); and the response of interest is predicted. The principle of fit is the least squares. Equations 6.4a and Equation 6.4b are implemented in the Custom design platform in the Design of Experiment Module of JMP 10 by clicking the RSM button. Details of the RSM are documented in JMP 10 (SAS Institute Inc., 2012: Fitting linear models).

a. RSM report: A typical RSM report comprises the 'Term', which refers to the individual factors' main effects and interactions. The values associated with the 'Terms' are the mean factor values used for the determination of the 'Estimates'. 'Estimates' are the coefficient of regression ($\beta_0 \ldots \beta_k$ in Equation 6.4). 'Estimate'

shows the orientation (positive or negative impact) and magnitude of influences of the factors and their interaction on the response of interest. ‘Std. Error’ is the standard error of the ‘Estimate’ for a ‘Term’. ‘t-Ratio’ is a standardized estimate that compares the mean effect of the factors (terms) to a hypothetical mean for the intent of significance test.

RSM distinguishes between factors with significant and non-significant influences on the responses of interest. The solid lines in the t-Ratio plot are the upper and lower boundaries of the hypothetical mean. Estimates with magnitude beyond these boundaries are influential, irrespective of the orientation (positive or negative). Prob.>|t| shows the ranking of factor(s) significance at a specified significance level. Significant factors are distinguished from non-influential terms by an asterisk (*). The significance level of 0.05 was used throughout the statistical analysis in this research.

b. RSM graph: In Chapter 7, results from the DoE analyzed by RSM are presented as rows and columns of curves (also referred to as traces). The rows contain curves that show the trends in response(s) and desirability as the individual factors change. The last row (bottom) shows the trends in desirability as the individual factors change. The columns show the concentrations of the factors that induce the trend in responses and the desirability. The trends are depicted as trace; and certain traces may have flat regions.

The flat regions correspond to the range of values of the factors that induce no change in response or desirability as a result of changes in factor concentration. They also show the tolerance limits of the associated factors or sensitivity of the desirability trace to small changes in the concentration of the associated factor. One factor is fixed (usually the VFA concentration), and the best setting for the other factors are determined. Solid vertical line across the columns of curves indicates the fixed value of the factor. The dotted horizontal lines show the corresponding values of the response(s) variables and desirability. The dotted vertical lines across the columns are the corresponding setting of the variable factors.

6.4.2 Response optimization by desirability function: While RSM predicts the behaviour of a response variable based on factors of influence, optimization of the response is implemented in the JMP 10 by the desirability function (D). D is a statistical algorithm that allows for the determination of the settings of the factors that produce the experimental goal of the response(s). D illustrates the proportion of the response(s) goals or experimental objectives that is achieved as a function of specific factor combination. To implement D, the responses were weighed according to their

importance to the process being evaluated (Table 6.13). D for an individual response that is to be maximized was computed as shown in Equation 6.5a (John, 2013; SAS Institute Inc., 2012: Multivariate analysis and modelling). D for multiple weighed responses is the geometric mean of the individual desirabilities of the individual responses as shown in Equation 6.5b. Equations 6.5a and 6.5b were implemented to get the maximum desirability for score for a response. Details on desirability function can be found in JMP 10 (SAS Institute Inc., 2012: Fitting Linear models).

Equation 6.5a $$D = \left(\frac{\hat{y} - A}{B - A}\right)^{w}, A \leq \hat{y} \leq B; D = 1, \hat{y} > B; D = 0, \hat{y} < A$$

Equation 6.5b $$D_{1\text{-}k} = [D^{e}_{1} \times D^{f}_{2} \ldots \times D^{z}_{k}]^{1/k}$$

A and B are respectively, the lowest and the highest values obtained for the response, and w is the weight of the response; e, f, z are the relative importance of the response desirabilities; and $\hat{y}$ is the response value predicted according to Equation 6.4a or Equation 6.4b. In the case of this research, the responses were ranked equally, so e, f, z are equal to 1. D ranges between 0, for a completely undesired response, and 1, for a perfect response. In this research, w is 1 for all the responses so that D varies linearly. The terms $D_1 \ldots D_k$ are the desirabilities of the individual responses 1 to k being evaluated at any given time.

Table 6.13 Optimization goals and importance weight of the methanization responses

Response	Goal	Importance weight
Hydrolysis and acidification rate (HAR)	Maximize	1
Methane production	Maximize	1
VFA degradation rate	Maximize	1
VFA retention time (Rt)	Minimize	1
Adaptation period (AP)	Minimize	1

Table 6.13 shows the desirability goals and importance weight of the evaluated responses. The responses could be equally weighed as in Table 6.13. In the case that some responses are more important than others for a given investigation, the weights are different and the most important response has the highest weight. By implementing the DoE in Tables 6.7, 6.8 and Appendix 6.6, and grouping the

experiments according to VFA levels, the TEs setting that produced the best D for the response(s) of interest in the low, medium and high VFA levels were derived.

a. Implementing desirability function: Desirability for a response is implemented as handles in the JMP 11 software and could be maximize, minimize or aimed at a target value as shown in Appendix 6.8. By pulling on the handles, a response goal can be changed. A response goal is set to minimize when the lowest possible value is most desirable. When higher values of the response are more beneficial, the D handle is set to maximize. When a specific value of the response is to be met, the D handle goal is set to the target value on the scale of the response. For any set response goal, the D varies within a range.

To derive the best D for the set response goal, the D is statistically maximized by implementing Equation 6.5a (not to be confused with maximizing the response goal using the D handle). When the D is maximized, the combination of factor settings that best satisfy the specified goal(s) of the response(s) are computed. For any response(s), maximizing the D produces the highest possible D and indicates the most optimum setting of the factors. The outcome of maximizing D depends on the relative weight (ranking) and goal of the individual responses.

b. Comparing desirability of factor settings: D ranges between 0 and 1, and the optimality of two or more factors settings can be evaluated by comparing their D values. D proportions close to 1 suggest that experimental conditions are in the optimum range, while scores near 0 indicate that the factor settings are not optimal. Values between 0 and 1 define varying degrees of factor optimality. The higher the proportion of D for a response or group of responses, the better the corresponding factor settings. In reporting the optimum settings of the factors in Chapter 7, D for response(s) was determined for different VFA levels, and the change in factors settings were evaluated.

VFA concentration of about 100 mmol/L is reported as optimum level during methanization, while ≥ 200 mmol/L is regarded as capable of inducing instability or failure during AD (Section 3.3.3 and Table 3.1). VFA concentration ≤ 25 mmol/L is lower than the recommended optimum and constitute the low VFA level, while VFA concentration of approximately 100 mmol/L and approximately 200 mmol/L are the medium and high concentrations respectively. Any of the VFA levels can be fixed and the TEs setting (also referred to as configuration) are varied to obtain the maximum D for the fixed VFA level.

6.5 Evaluating the efficiency of TEs supplementation approaches

The analysis of supplementation approaches aim to statistically derive a TEs configuration that is optimum for a wide range of VFA concentrations. TEs configurations and VFA concentrations were used to distinguish between the supplementation approaches. The influences of 4 TEs configurations were (independently) modelled as scenarios on 5 VFA concentrations ranging between 10 and 250 mmol/L. The influences of the different scenarios on the responses of interest, and the associated D proportion were compared. The scenarios include:

I. *TC-control:* This means TEs configuration in the Control reactors. It refers to TEs composition and concentration in the Control reactors. The TEs concentrations in this scenario correspond to the low values of the individual TEs (TEs concentrations in the inocula). In this approach, the aim was to find the means of the responses of interest and D across multi-levels of VFA concentration using the baseline TEs configuration.

II. *TC-compromise:* This means TEs Configuration in the compromise setting. This is the RSM derived TEs configurations that are suitable for methanization. The suitable TEs concentrations are not optimum and usually lie within the mid values of the experimental range of the TEs. In the RSM reports presented in Chapter 7, some values are in bracket after the main effects: these are the reference values upon which the estimates were based. These values were modelled as the TC-compromise. Suitable or reference factor setting is a compromise TEs configuration but not an optimized setting. Therefore, its influences on the response(s) of interest are subset of the optimum setting. Subsequently, suitable or the reference factor concentration will be referred to as TC-compromise.

III. *OTC-VFA_120:* This means Optimum TEs Configuration for VFA concentration of approximately 120 mmol/L. This supplementation approach is based on the optimum TEs concentration derived by fixing VFA at 120 mmol/L, which is within the range of optimum VFA concentration for methanization. The derived optimum TEs setting was modelled to determine its influences on the responses of interest across multi-levels of VFA concentrations. The aim was to evaluate the difference in responses between this TEs configuration and the configuration that produces the best responses and desirability. This is based on the assumption that TEs supplementation based on VFA level is most beneficial to methanization.

Iv *OTC-VFA_DL:* This means Optimum TEs Configurations for Different Levels of VFA. This involves derivation of the optimum TEs settings for each of the levels of

VFA concentration ranging between 10 and 250 mmol/L. The aim was to find the means of the responses of interest and D across the multi-levels of VFA concentration for the corresponding TEs settings. This scenario is expected to provide the upper limits (best values) in the responses and D across the multi-levels of VFA concentration.

6.6 Determining the mechanisms of AD optimization due to TEs supplementation

This aspect of investigation also depends on the data from the TEs supplementation experiments. MRR and IA are two important substrate (VFA mixture) utilization parameters (section 5.4). MRR and IA are parameters of the Michaelis-Menten equation (Equation 5.11) and were estimated by non-linear regression of VFA degradation rates measured at 37°C and 55°C, and multi-level VFA concentrations. MRR and IA were estimated and compared in the best TEs supplementation scenario and the TC- control scenario discussed in section 6.5.

6.7 TEs adsorption on digester solids and re-use of digestate in agriculture

The proportion of TEs dissolved in digestion mixture and available to micro-organisms for uptake, and the proportions of TEs that are bound to digestate and unavailable to micro-organisms can be quantified by sequential extraction (SE) (Dong *et al.,* 2013, Alonso *et al.,* 2009). The digester solids in the reactors supplemented with TEs were evaluated for suitability as agricultural digestate by comparing the TEs concentration in the digestate to legal limits of heavy metals in the European Directive 86/278/EEC for agricultural digestate and sludge.

6.7.1 Sequential extraction:

TEs exist in different chemical forms and are able to adsorb to the solids in the digester. Digester solids include humic materials from non-degradable fractions of the substrate, oxides of Fe and Mn, sulphides of Fe, carbonates and other insoluble particles. TEs-solids complex have different solubility properties. TEs complexes formed with carbonates and oxides of Fe and Mn are soluble. TEs complexes formed with sulphides of Fe and humics; and TEs bound in the crystal structure of mineral are insoluble. To determine the proportion of TEs adsorbed to different digester solids, sequential extraction was used.

SE is a method of determining the bioavailable and non-bioavailable proportions of metal in the solutions in which they occur. It involves the use of reagents of varying strength to leach the metals from digestate or sludge samples (Tessier *et al.,* 1979). The SE method used in this experiment is a 4-step protocol proposed by the

Standards, Measurements and Testing programme (SM & T–formerly BCR) of the European Union for the determination of the different fractions of metals in sludge and was reported in Tokalioglu *et al.* (2003). Details of the procedure are in Appendix 6.10. This method was used to determine the bioavailable and bound proportions of TEs in the digester solids after the batch experiments at 55°C. The samples were taken at the end of the experiment and the TEs concentrations were quantified in the 4 steps of SE as follows (Tokalioglu *et al.,* 2003):

SE-1: water and acid-soluble ions (exchangeable);
SE-2: ions adsorbed to oxides of Iron (reducible);
SE-3: ions adsorbed to humics and sulphides of Iron (oxidizable); and
SE 4: ions in crystal structure of primary and secondary minerals (residual).

SE-1 + SE-2 = total bioavailable TEs; and SE-3 + SE-4 = unavailable TEs.

6.7.2 Review of the legal limits for TEs in digestate and sludge meant for use in agriculture: The European Directive 86/278/EEC and the German adoption of the directive specify limits for heavy metal contents in digestate and sludge meant for use in agriculture. The optimum concentration of Ni, Co, Se and Mo derived from the batch experiments were compared with their corresponding limit in the European Directive 86/278/EEC where such limits exist.

6.8 Validation of the influence of the optimum TEs settings

Based on the influences of the main effects and the interactions the TEs on the measured responses in the batch AD, optimum TEs ranges for methanization at 37°C and 55°C were formulated. The strengths and weaknesses of the formulated optimum TEs configuration were highlighted by developing variants its variants. The variants reflected adjustment in concentrations of specific TEs to accommodate TEs interactions with [1]critical and stable VFA concentrations during methanization. The influences of the formulated optimum TEs configuration and its variants on VFA degradation rate and CH_4 production were confirmed in continuous AD investigations at 37°C. The materials and methods of the validation experiments differ from the batch experiments and are discussed next.

6.8.1 Materials: The materials include the reagents reported in Tables 6.11 and 6.12 for the preparation of basic nutrient and TEs solutions respectively; the MFR reported in Table 6.2; and the experimental set-up. Figure 6.3 shows the set-up for the

[1] The critical and stable concentrations of VFA for methanization are discussed in section 3.3.3 of Chapter 3

investigation. The functions of the features in Figure 6.3 are the same as described in Table 6.4.

6.8.2 Methods: The experimental method involves designing variants of the formulated optimum TEs mixture, running the experiments, inducing methanization changes that enable the determination of the differences in responses of interests and measurement of the responses of interest. The characterization parameters and methods of analyses for the MFR used as substrate are reported in Table 6.3.

6.8.2.1 Formulating the optimum TEs configuration and its variants: The optimum TEs configuration was formulated based on the results of the RSM of the individual responses and the optimization for multi-responses. The range of each TE that was determined as optimum for the methanization of VFA between 10 and 250 mmol/L was considered as the optimum range for the TE. Two variants of the formulated optimum TEs configuration were developed based on the main effects and interactions of the TEs for [2]VFA degradation rate.

6.8.2.2 Running the experiments: This involves supplementing the optimum TEs configuration and the two variants, starting the reactors, sampling and sample analyses. The procedure reported in Appendix 6.7c was modified to accommodate the change in reactor size from 1 L to 2 L, the substitution of VFA mixture with MFR and use of formulated optimum TEs mixture and its variants instead of DoE Matrices. The procedure for flushing with N to create anaerobic environment is the same as reported in Appendix 6.7c. The reactors were operated in duplicate, and comprised a Control reactor and 3 treatment reactors (formulated optimum TEs and 2 variants). That is,

Control reactors = Inoculum + nutrient media + MFR
Treatment reactors = Inoculum + nutrient media + TEs + MFR

a. *Sample collection:* Sample collection procedure was modified to enable collection of liquid sample with relatively high solid content due to change in substrate from VFA mixture to MFR (Figure 6.3b). A 50 ml catheter tip syringe shown in Figure 6.3c was used to draw samples from the continuously stirred 2 L reactors. Stirring was accomplished by magnetic rod inserted into each reactor and the magnetic stirrer attached to each reactor. Gas sampling and analysis were simultaneously done.

[2] VFA accumulation is the main cause of digester failure and influences TEs requirements; hence, TEs interactions with VFA were used as the basis for the design of the variants of the optimum configuration.

Figure 6.3 Experimental set-up for the semi-continuous validation investigation: (a) Gas sampling port on the eudiometer (b) Liquid sampling port on the 2 L reactor (c) 50 ml catheter tip syringe for liquid sample collection from (b)

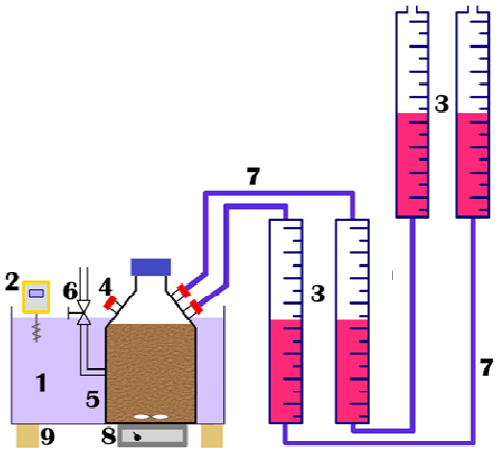


Figure 6.3 Basic scheme of the experimental test system used for the semi-continuous investigations (1) water bath (2) thermostat (3) eudiometers (4) supply port or nozzle (5) glass reactors (6) valve for feeding and sampling (7) tubes (8) magnetic stirrer and (9) water bath support

b. *Sample analyses:* The collected samples were analyzed as shown in Table 6.14. Liquid samples were analyzed for FOS/TAC, individual VFA concentrations and pH. FOS/TAC value between 0.15 and 0.45 indicate stability in methanization; and values above 0.6 suggest onset of instability in the methanization processes (Table 3.1). Individual VFA and pH were analyzed by the methods reported in Table 6.12. Biogas produced by each reactor was analyzed for CH_4 and H_2S content by

discharging the biogas in the eudiometers through a discharge valve (Figure 6.3a) that was connected to the equipment, GeoTech 5000 gas analyzer (Table 6.14). Biogas volume was measured by displacement method using calibrated eudiometers.

6.8.2.3 Inducing methanization changes and response measurement: In order to measure the methanization efficiency of the formulated optimum TEs configuration and the influence of the variations made to it, MFR loading rate was step-wisely increased until instability was induced in the methanization reactors. The responses of interest in the continuous AD experiment, equipment used for measurement and method of measurements are described in Table 6.14. Some responses such as individual VFA concentration and pH were measured as shown in Table 6.12. Changes in microbial communities were determined by denatured gel gradient electrophoresis (DGGE) using the method proposed by Muyzer *et al.* (1993). Samples for the DGGE were collected on the start of the experiments; when a significant change in CH_4 production was noticed and at the end of the experiment.

Table 6.14 Responses, sampling phases, equipment and analysis method for the semi-continuous experiment

Response	Sample phase	Equipment	Method
Volatile organic acids/Total anorganic carbon (FOSTAC)	Liquid	FOS/TAC 2000: Pronova Analysentechnik GmbH & Co. KG	Titration as instructed by the equipment manufacturer; see Voss *et al.* (2009)
Microbial Population	Liquid	Denatured Gel Gradient Electrophoresis (DGGE) Bacteria: PCR Primers: GM5 GC clamp/907RM; DGGE: gradient 20 - 80%, 55°C 200 V, 300 min. Archaea: PCR primers: Arc334f GC clamp/Arc915r; DGGE: gradient 30 - 80%, 60°C, 150 V, 180 min	Muyzer *et al.* (1993)
Biogas volume	Gas	Volume displacement in eudiometer	VDI, 2006
CH_4 and H_2S	Gas	GeoTech 5000 gas analyzer; Geotechnical Instrument (UK) Ltd	

CHAPTER 7 RESULTS AND DISCUSSIONS

7.0 Chapter overview

The characteristics of TEs and their respective roles in optimizing methanization were discussed In Chapter 4. The differences in the extent of optimization with TEs supplementation, and the lack of consensus in optimum concentration of TEs required for methanization were also highlighted in Chapter 4. The factors that could be responsible for the variations in the influences of TEs and that have created knowledge gaps in the subject of TEs supplementation were identified in Chapter 5. The TEs identified to be important were Ni, Co, Se and Mo, and this was due to their role in the metabolism of AD substrates, acetate and C-1 molecules. The enzymes that contain these TEs, and are responsible for the optimization of methanization were discussed in Chapter 5. Research motives were formulated in Section 5.5 to fill the identified knowledge gaps. The method of the investigations have been presented in Chapter 6; and in this Chapter, the results are presented and discussed. The following are the main sections of this report:

7.1 TEs contents and other characteristics of the substrates of AD;
7.2 Supplementation of TEs during AD;
7.3 Evaluation of the efficiency of TEs supplementation approaches;
7.4 Mechanisms of AD optimization due to TEs supplementation;
7.5 TEs supplementation and use of digestate in agriculture; and
7.6 Formulation and validation of the Optimum TEs configuration.

7.1 TEs contents and other characteristics of AD substrates

Task: *Determine the TEs contents of selected AD substrates and discuss substrate characteristics that can influence TEs bioavailability.*

7.1.1 TEs contents of selected AD substrates: The Selected substrates of AD that were analysed for TEs are presented in Table 7.1. Total concentrations of Ni, Co, Se and Mo were determined as shown in Table 6.3 and outlined in Appendix 6.1. The substrates were selected to include mono-substrates and complex substrates. The mono-substrates include grasses, dungs, and leaves. The complex substrates include liquid substrates such as grease trap residue (GTR) and blackwater (BW); and solid substrates such as restaurant biowaste (RW), mixed fruit residue (MFR) and silage feedstock (SF). The digestates from SF and digested sewage sludge (DS) were also included for reference to TEs contents after methanization. The dungs were considered as simple because they are predominantly grass residues. The

silage is a mixture of the constituents shown in Appendix 6.7a. DS 1 and 2, and GTR 1 and 2 are samples taken from different sources to indicate that TEs content may vary depending on the sources of the substrates. In Table 7.1, Fe is included, not as TEs, but for the purpose of evaluating the relationship between the contents of Fe and S, and bioaccumulation of TEs in liquid complex substrates. The TEs contents in the different substrate categories and digestates are discussed next.

Table 7.1 TEs and Fe contents of the selected AD substrates

Substrates	Ni	Mo	Co	Se	Fe
	mg/kg DM				
Lawn grass	0.18 ± 0.02	0.37 ± 0.04	0.13 ± 0.03	nd	282.09 ±8.05
Spear grass	0.13 ± 0.02	0.19 ± 0.03	0.27 ± 0.03	nd	147.50 ± 3.66
Cow dung	0.08 ± 0.01	0.27 ± 0.01	0.03 ± 0.01	nd	-
Horse dung	0.04 ± 0.01	0.15 ± 0.03	0.04 ± 0.02	nd	110.74 ± 4.61
Mixed leaves	0.14 ± 0.02	0.19 ± 0.03	0.54 ± 0.01	nd	631.75 ± 19.14
Grease trap residue-1	1.45 ± 0.03	0.27 ± 0.04	0.08 ± 0.01	nd	-
Grease trap residue-2	0.84 ± 0.04	0.12 ± 0.01	0.06 ± 0.01	nd	-
Blackwater	1.19 ± 0.13	0.28 ± 0.04	0.04 ± 0.03	nd	1378.0 ± 70.0
Restaurant biowaste	0.12 ± 0.08	0.10 ± 0.10	0.06 ± 0.08	nd	-
Mixed fruit residue	0.63 ± 0.08	0.60 ± 0.13	0.63 ± 0.18	nd	117.11 ± 21.31
Silage feedstock	3.62 ± 0.20	1.46 ± 0.13	1.17 ± 0.14	nd	513.03 ±12.71
Silage digestate	7.14 ± 0.91	4.95 ± 1.24	1.11 ± 0.18	nd	1804.30 ± 108.15
Digested sludge-1	3.10 ± 0.04	1.08 ± 0.05	16.71 ± 0.39	nd	4198.02 ± 11.03
Digested sludge-2	0.12 ± 0.03	0.54 ± 0.04	0.22 ± 0.04	nd	-

('nd' not detected; '-' not analysed)

a. TEs content of mono-substrates: Table 7.1 indicates that in all the substrates and digestates that were analysed, the Se content was below the FAAS detection limit (< 0.005 mg/L). Se content below detection limits were also reported by Clemens (2007) and Kayhanian and Rich (1995) for various substrates, and our results confirm these reports. Co content varied widely, and ranged between 0.03 mg/kg DM in cow dung and 0.54 mg/kg DM in mixed leaves. The dungs had the lowest content of Co, followed by the grass samples (0.13 - 0.27mg/kg DM). The highest content of Co (≈ 0.54 mg/kg DM) was analysed in the mixed leaves. Mo and Ni varied less in the mono-substrates analysed, compared to Co. Mo ranged between 0.15 mg/kg DM and 0.37 mg/kg DM. The upper limits of the range were in the lawn grass and cow dung. Ni ranged between 0.04 mg/kg DM and 0.18 mg/kg DM; and the lower limits of the range occurred in the dungs. Apparently, the dungs are low in TEs content compared to grass or leaves that may constitute the original feedstock. This could be due to assimilation of nutrients by the animals for growth and metabolism.

b. TEs content of complex-substrates: The mixed or complex substrates in Table 7.1 have Co contents that varied between 0.04 mg/kg DM and 1.17 mg/kg DM. The upper limits of the Co concentration were in MFR (0.63 mg/kg DM) and silage feedstock (1.17 mg/kg DM). GTR, BW and RW contain Co concentration of ≤ 0.08 mg/kg DM. The range of Mo was between 0.10 mg/kg DM and 1.46 mg/kg DM. Similar to Co content, the upper limits of the range of Mo were in MFR and silage feedstock. Ni content had the most variation compared to Mo and Co in the complex substrates that were analysed. Ni content varied between 0.12 mg/kg DM and 3.62 mg/kg DM; and the upper limits of the range occurred in GTR and silage feedstock. The maize silage had the highest content of Ni, Co and Mo among the complex-substrates. Banks and Zhang (2012) reported similar TEs concentrations in food waste (Table 4.4a) as are reported in Table 7.1.

c. TEs content of digestate: Se content in the digestates was below the FAAS detection limit as well. The contents of Co in the digestates ranged between 0.22 mg/kg DM and 16.71 mg/kg DM. DS-1 had relatively high Co content compared to DS-2 and silage digestate. This wide range is not unusual as Zandvoort *et al.* (2003) reported Co content of 1.7 mg/kg DM in sewage sludge; and Hullebusch *et al.* (2005) reported 13.7 mg/kg DM Co in paper mill sludge. Mo contents varied between 1.08 mg/kg DM and 4.95 mg/kg DM; and Ni contents varied between 3.10 mg/kg DM and 7.14 mg/kg DM. For both the Mo and Ni contents, the upper limits occurred in the silage digestate, which was the residue of the silage feedstock characterized in Appendix 6.7a. During the methanization of the silage feedstock, commercial TEs mixture was added to the digester and this explains the higher TEs content in the silage digestate compared to the silage feedstock.

Comparing complex-substrates, mono-substrates and digestates in terms of TEs content, Ni content was generally higher in the complex substrates compared to mono-substrates. GTR, BW and RW contained lower concentrations of Co than the mixed leaves and spear grass (mono-substrates). The Mo contents of the complex substrates were similar to Mo content in the mono-substrates, except for the MFR and silage feedstock. Similar to the mono-substrates, the complex substrates had Se contents below FAAS detection limit, and variation in TEs contents were wider in the Ni and Mo contents of the samples compared to the Co contents. The TEs content of complex substrates may depend on the source of the substrate. For example, the GTR obtained from two sources, showed significant variations in TEs content between the samples. TEs contents in the digestates were higher in concentrations and varied more than in mono-substrates and complex substrate.

7.1.2 Important considerations with regards to TEs contents of AD substrates

Oleszkiewicz and Sharma (1990) reported that during methanization, the total contents of TEs are significantly higher than their bioavailable proportions. Alonso *et al.* (2006) confirmed that 52% and 26% of the total Co and Ni respectively are bioavailable in sewage sludge. So, it is expected that bioavailable TEs in the substrates will be lower than the total values shown in Table 7.1. Pobeheim *et al.* (2010) reported that Ni and Co concentrations lower than 0.6 mg/kg DM and 0.02 mg/kg DM respectively are unsuitable for AD. Except for spear grass and mixed leaves of the mono-substrates; and GTR, MFR and silage feedstock of the complex substrates, the bioavailable proportion of the total TEs contents in the other substrates are likely to be unable to sustain methanization. Tessier *et al.* (1979) reported that the unavailable proportion of TEs are trapped in the crystal structures of mineral solids or absorbed to humics and FeS.

Consequently, Fe and S contents of the substrates, and the conditions that favour FeS formation during methanization might influence the bioavailable TEs proportions of the substrates shown in Table 7.1. Considering the Fe content, mixed leaves had the highest content of Fe (631.8 mg/kg DM) among the mono-substrates, which had Fe concentration between 110.7 mg/kg DM and 631.8 mg/kg DM. The Ni and Co contents of mixed leaves were in the upper limits of their respective ranges. Among the complex substrates with Fe concentration between 117.1 mg/kg DM and 1378 mg/kg DM, BW had the highest Fe content (1378 mg/kg DM). The Ni content of BW is in the upper limits of the range of Ni in the mixed-substrates. Among the digestates, DS-1 has the highest Fe content (4198 mg/kg DM) and significantly high Ni and Co contents. Since Fe contributes to TEs unavailability due to its reaction with S to form the insoluble FeS, it might be important to know how much S is contained in the substrates.

7.1.3 Other important compositions of the selected AD substrates: Other components whose concentrations and reactions may influence availability of the TEs of AD substrates are discussed next.

7.1.3.1 S, C and N contents of the selected substrates: Chandrappa and Das (2012) reported S, C and N as important constituents of AD substrates because their proportions influence the outcome of methanization. The S, C and N contents of the selected substrates were analysed as reported in Chapter 6, and presented in Table 7.2 in relation to the %DM of the substrate. During methanization, anaerobic bacteria oxidise S in the presence of Fe and precipitate FeS in the process (Suzuki *et al.,* 1990). FeS and TEs interact to form insoluble FeS-TEs complex (Alonso *et al.,* 2006; Dong *et al.,* 2013). The redox reaction leading to the formation of FeS is pH

dependent, and pH is regulated by the buffer capacity of a system. C and N content of substrates influence buffer capacity during methanization, because they influence the formation of NH_3/NH_4^+, HCO_3^- and VFA, which are the constituents of the buffer capacity of a medium (Mata-Alvarez, 2003). The content of S, C and N in the substrates and the implication of their interaction with the TEs during methanization are discussed next.

Table 7.2 DM, C and N content and C/N of the selected AD substrates

Sample	DM	S	Fe/S	C	N	C:N
	%FM	%DM		%DM		
Lawn grass	9.2 ± 0.19	0.26 ± 0.01	1085	43.5 ± 0.06	1.75 ± 0.04	25
Spear grass	9.26 ± 0.23	0.28 ± 0.01	527	45.52 ± 0.13	3.12 ± 0.04	15
Cow dung	9.90 ± 0.14	0.58 ± 0.01		43.56 ± 0.37	2.37 ± 0.01	18
Horse dung	11.83 ± 0.32	0.19 ± 0.01	583	45.80 ± 0.23	1.16 ± 0.01	39
Blackwater	0.66 ± 0.02	1.77 ± 0.05	779	33.83 ± 0.22	4.30 ± 0.06	8
Restaurant biowaste	28.83 ± 1.01	0.22 ± 0.01		45.97 ± 0.01	2.67 ± 0.04	17
Mixed fruit residue	10.79 ± 0.08	0.24 ± 0.01	488	45.52 ± 0.05	2.20 ± 0.03	21

(DM, dry matter; C, carbon; N, nitrogen)

a. Mono-substrates: These substrates have DM content between 9 and 10% of the fresh matter (FM), and the S contents range between 0.2% and 0.6% of the DM. Cow dung has a moderately higher S content (0.6%) compared to the other substrates. Compared to spear grass (527) and horse dung (582), Lawn grass has higher Fe/S ratio (1085) and presumably a higher possibility to form insoluble FeS-TEs complex. The N contents vary between 1.2% and 3.1%, and spear grass has a relatively high N content (3.1%) compared to the other substrates. The C contents of the mono-substrates range between 43.5% and 45.8%. C/N has implications that influence acidification, pH and buffer capacity during methanization; hence, optimum range(s) are usually recommended (Section 2.1.2).

Hill (1979) recommended optimum C/N range of 25; while Mata-Alvarez (2003) documented 30 - 35. With reference to the optimum ranges of C/N, spear grass (15) and cow dung (18) are low, and may be susceptible to NH_3 inhibition during methanization. Angelidaki and Ahring (1994) reported that NH_3 inhibition is associated with pH fluctuations. Fluctuations in pH induce variations in the extents of TEs and Fe dissolution and precipitation during methanization. Hoffland environmental (2013) documented that at pH 8, Ni is mostly in dissolved form, while Fe is mostly dissolved at pH of ≈ 3. The high C/N in horse dung (39) compared to the optimum range (25 – 35) suggest better buffering capacity during methanization with

horse dung compared to spear grass and cow dung. However, it might also induce reactor acidification and dynamics in bioavailability of TEs and Fe during methanization of horse dung.

b. Complex substrates: These substrates vary more in DM contents and range between 0.7% in BW and 28% in RW. Except BW, which has 1.8% of the DM as S, RW and MFR contain about 0.2% S. The Fe/S ratio is also higher in BW (779) compared to MFR (488). This suggests that the possibility of TEs precipitation during methanization of BW is higher than in MFR. MFR and RW have C content of about 46% compared to BW, which has 34% C. However, the N content in BW is relatively high (4.3%), resulting in a C/N ratio of 8 compared to MFR with N content of 2.2% and C/N ratio of 21, and RW with N content of 2.7% and C/N ratio of 17. pH fluctuations associated with NH_3 inhibition due to low C/N ratio is likely to influence TEs dynamics in BW more than in MFR and RW during methanization. Not all the C content of a substrate can be methanized. The ratio of acidity to alkalinity during methanization is principally controlled by the concentrations of acid intermediates and HCO_3^- (Sri Bala Kameswari *et al.,* 2012); and these are derived from the methanized C. The methanizable C contents of the substrates are discussed next.

7.1.3.2 Simple sugars and carbohydrate level: Sugar or carbohydrate level of a substrate is a good indicator of easily degradable C. The mono-substrates and one of the complex substrates in Table 7.2 with C content ≥ 40% and known Fe content were analysed for simple sugars by acid hydrolysis according to the method in Table 6.3 and the procedure in Appendix 6.9. Table 7.3 shows the percentage of xylose and glucose sugars. Xylose and glucose are in larger proportions compared to the other sugars in all the substrates analysed. Fructose is not among the hydrolysed sugars due to difficulties with its analysis using the procedure in Appendix 6.9. The hydrolysis residue contains the non-easily hydrolysable carbohydrate. The complete result containing all the analysed sugars and hydrolysis residue is shown in Appendix 7.1.

Table 7.3 indicates that about 30 - 50% of the > 40% C in the substrates is carbohydrate. The carbohydrate content of the mono-substrates lawn grass (42%), spear grass (43%) and horse dung (51%) are higher compared to cow dung (31%), and complex substrate MFR (37%). Simple sugars are easily degradable and constitute significant proportion of organic dry matter (oDM), which is the methanizable fraction of a substrate. Pobeheim *et al.* (2011) reported that TEs requirement during continuous methanization increased with increase in oDM loaded to a reactor. So, during methanization, substrates such as horse dung, spear grass and lawn grass may produce higher concentrations of acid intermediates; have

higher risk of acidification; or necessitate higher TEs requirement compared to MFR and cow dung.

Table 7.3 Sugar contents of some of the selected AD substrates

Sample	Xylose	Glucose	Total carbohydrates
		%DM	
Lawn grass	11.3	24.8	42.4
Spear grass	9.8	28.1	43.1
Cow dung	10.9	17.0	31.4
Horse dung	17.8	28.8	50.7
Mixed fruit residue	6.6	19.8	37.1

(all the analysed sugars and hydrolysis residue are shown in Appendix 7.1)

Whether the proportion of available TEs in an AD substrates is sufficient for methanization processes or not can only be determined by comparing TEs requirements for the methanization of the common VFA intermediates of these substrates. Consequently, the TEs requirements during the methanization of VFA are compared in Section 7.2.

Summary and conclusion: Table 7.1 shows that the TEs contents of AD substrates vary. It also shows that some TEs, e.g. Ni are higher in some substrates (GTR and BW) compared to others such as horse dung. It further shows that digestates are reservoirs of TEs. Other substrate characteristics such as Fe contents, and S, C and N also vary. The reported relationships between TEs dynamics and Fe, C, N and S during methanization suggest that differences in Fe, C, N and S contents of the substrate may influence proportion of the total TEs that are bioavailable during methanization.

7.2 Influences of TEs supplementation during AD

Task: *Discuss the main and interaction effects of the TEs in hydrolysis-acidification rate (HAR), VFA degradation rate and CH_4 production; as well as in retention time and adaptation period. Highlight the TEs settings that induce methanization gains or losses.*

The biochemical processes in the 4 steps of AD depend on the composition of the start-up inoculum, conditions of the experiment and substrates used as feedstock (Chapter 2). The source of the inocula for the experiments involving supplementation of TEs was described in Table 6.2. The TEs contents of the inocula did not vary significantly and have been reported in Table 7.1. In the subsequent sections, the influences of TEs supplementations on the responses in Table 6.5 are evaluated. For this purpose, each treatment of the DoE in Tables 6.7 and 6.8 is also referred to as reactor (R1 - R30 as applicable). Hence, reactor-number and treatments are used interchangeably. For ease of reference, reactor numbers 28, 29 and 30 have been reserved for the Control reactors of the low, medium and high VFA categories respectively.

7.2.1 Influence of TEs on hydrolysis-acidification rate (HAR): Hydrolysis and acidification (HA) phases of AD have been described in Sections 2.2.1 and 2.2.2. The experimental procedure for the batch experiments involving supplementation of the TEs is outlined in Appendix 6.7c. The concentrations of the TEs that were supplemented to different levels the VFA were shown in Table 6.6; and the DoE is shown in Table 6.8. Influences of Ni, Co, Se and Mo on hydrolysis-acidification rate (HAR) were investigated by measuring [3]VFA formation rate from the 4000 mg/L silage feedstock (Appendix 6.7a) added to the reactors during the implementation of the DoE in Tables 6.8. The experimental set-up has been described in Figure 6.1, 6.2 and Table 6.4.

The experimental reactors contain equal mass of silage feedstock so that any difference in HAR between treatments and Control reactor in a particular VFA level is a function of Ni, Co, Se and Mo configuration and VFA levels. The method for calculating the HAR from the silage digestate was discussed in Section 6.2.2.3. The peak HAR (maximum VFA formation rate) of each treatment reactor in a particular VFA level was compared with the peak HAR of the corresponding Control reactor to derive the relative HAR and the % gain or loss in HAR according to Equations 6.3f

[3]Average VFA degradation rates of the reactors show good correlation with average biogas production of the reactor during the HA experiment, hence, biogas produced was used to backwardly calculate the total VFA yielding.

and Equations 6.3g respectively. The peak HAR and the relative HAR for the different treatments following the DoE in Tables 6.8 are shown in Appendix 7.1b. Appendix 7.1b also shows the actual concentration of each TE supplemented to the treatments in all the mesophilic Ni, Co, Se and Mo supplementation.

7.2.1.1 Relative gains and losses in HAR: Figure 7.0 shows the relative losses and gains in HAR due to Ni, Co, Se and Mo supplementation at different levels of VFA.

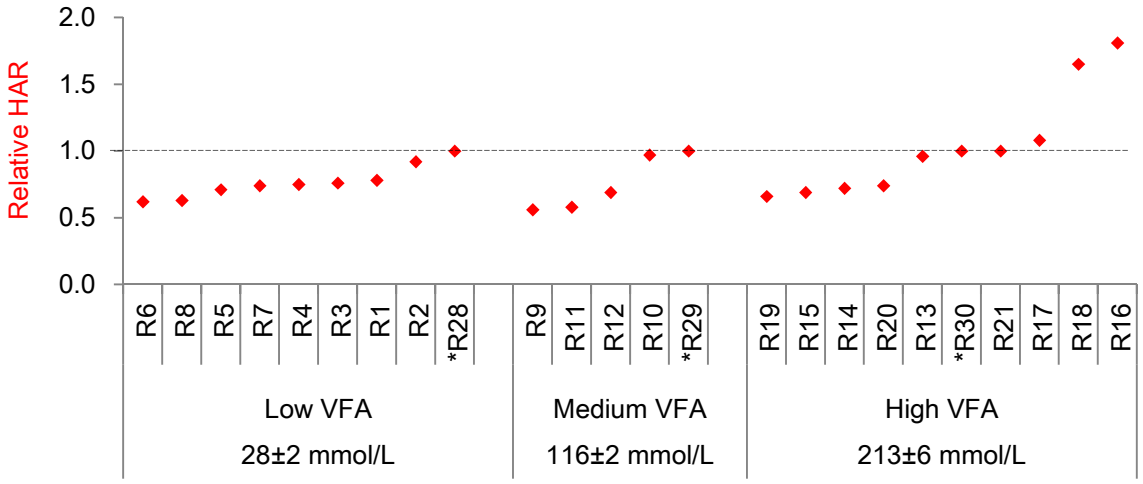


Figure 7.0 Influence of VFA concentrations and Ni, Co, Se and Mo supplementation on the relative hydrolysis and acidification rate (HAR) of silage feedstock at 37°C (*R28, R29 and R30 are the Control reactor in the low, medium and high VFA categories)

a. Low VFA level (28 ± 2 mmol/L): In the low VFA category, the treatments had relative HAR range between 0.62 – 0.92 compared to R28 (Control). This corresponds to between 8% and 38% loss in HAR compared to the low VFA Control reactor. The larger losses in HAR occurred in R6 and R8 (38% and 37% respectively) compared to the Control reactor (R28). R6 was supplemented with only Ni; whereas R8 was supplemented with Ni, Co and Se. R2 had the lowest loss in HAR (8%) and was supplemented with Co and Mo.

b. Medium VFA level (116 ± 2 mmol/L): In the medium VFA category, the treatments had relative HAR range between 0.56 – 0.97 compared to R29 (Control). This corresponds to between 3% and 44% loss in HAR compared to the medium VFA Control reactor. The larger losses in HAR occurred in R9 and R11 (44% and 42% respectively). R9 was supplemented with Co and Se; whereas, R11 was supplemented with all four TEs. The lowest loss in HAR (3%) was in R10, which was supplemented with Ni, Se and Mo.

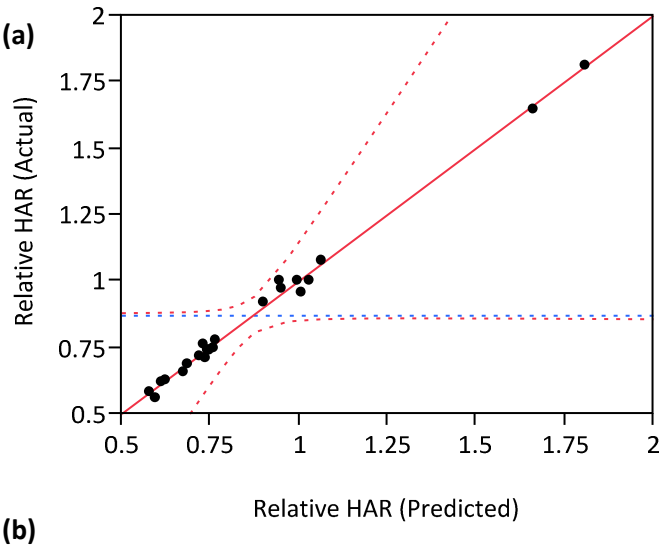
c. High VFA level (213 ± 6 mmol/L): The relative HAR values for the high VFA level were between 0.66 and 1.81. This corresponds to a wide range of influence spanning from a loss in HAR of 34% in R19 to a gain of 81% in R16. Relative HAR values lower than the HAR value of the Control reactor were observed in R13, R14, R15, R19 and R20, which showed losses in HAR of 4%, 28%, 31%, 34% and 26% respectively. Higher HAR values than the HAR value of the Control reactor were observed in R16 (81%), R17 (8%) and R18 (65%). R16 was supplemented with Co and Se, whereas R18 was supplemented with Ni and Co. R17 was supplemented with all four TEs. Co and Se supplementation improved HAR in R16 in the high VFA level but not in R9 in the medium VFA level.

The gains and losses in HAR in Figure 7.0 also suggest that as VFA level increases from low to high concentration, TEs supplementation become beneficial in HAR. The high gains in HAR in R16 and R18 (VFA ≥ 200 mmol/L) suggests that certain TEs interactions produce significant enhancements in HAR. The specific TEs settings whose interactions with VFA levels influenced the gains and the losses in HAR as shown in Figure 7.0 are discusses next.

7.2.1.2 Factors that induce losses and gains in relative HAR: To quantify the influence of the TEs and VFA on the gains and losses in HAR, the RSM was used. The RSM was described in Section 6.4.1. RSM was used to predict the relative HAR of the treatment reactors and Control reactors based on the factors Ni, Co, Se, Mo and VFA concentrations according to Equation 6.4b. The measured relative HAR (actual) due to TEs supplementation were plotted against the predicted relative HAR and the coefficients of regression ($\beta_0 \ldots \beta_k$ or estimates) were determined. Figure 7.1a shows a plot of the measured relative HAR (actual) vs. predicted relative HAR for each treatment and Figure 7.1b shows the estimates and significance of the influences of the factors.

The statistics for Figure 7.1a shows that the RSM fit has a coefficient of determination (R^2) of 0.99 with a fit error of 0.11, which indicates a good model fit between the predicted relative HAR and the actual relative HAR. The error in the fitted model is the average difference between the measured value and the value predicted by the fitted model (SAS Institute Inc., 2012). The R^2 adjusted or standardized R^2 value is 0.94 and is relevant for comparing the influences of the factors on different responses. The mean relative HAR is 0.87, corresponding to an average of 13% loss in HAR across all VFA levels due to TEs influences.

(a)



R^2	0.99
R^2 adjusted	0.94
Root mean square error (RMSE)	0.07
Mean relative HAR	0.87
No. of treatments	23

Line of fit (solid line), significance curve at α 0.05 (dotted curves), and line of response mean (dotted horizontal line)

(b)

Term	Estimate	Std Error	t Ratio	Prob>|t|
VFA (mmol/L)	0.0013837	0.000189	7.32	0.0182*
(Co(mg/L)-1.88)*(Mo(mg/L)-0.64)	-0.161015	0.022157	-7.27	0.0184*
(VFA (mmol/L)-123.827)*(Co(mg/L)-1.88)	0.0010036	0.000149	6.75	0.0212*
(VFA (mmol/L)-123.827)*(Mo(mg/L)-0.64)	-0.002789	0.000468	-5.96	0.0270*
(Co(mg/L)-1.88)*(Se(mg/L)-0.4687)	0.136371	0.025045	5.45	0.0321*
(Co(mg/L)-1.88)*(Co(mg/L)-1.88)	0.0646105	0.01415	4.57	0.0448*
(VFA (mmol/L)-123.827)*(VFA (mmol/L)-123.827)	0.0000324	7.115e-6	4.55	0.0450*

Figure 7.1 **(a)** Plot of actual vs. predicted relative HAR of silage feedstock at 37°C **(b)** Model terms, estimates of parameters and significance of terms in modelling of the relative HAR of silage feedstock at 37°C due to Ni, Co, Se and Mo supplementation and VFA concentration up to 213 mmol/L.

Figure 7.1b shows the mean Estimates of the influence of Ni, Co, Se, Mo and VFA on the relative HAR of silage feedstock in decreasing magnitude. The statistical features of Figure 7.1b are discusses in Section 6.4.1. The complete list of the main and interaction effects of the Terms, Estimates and significance of Terms shown in Figure 7.1b is contained in Appendix 7.2. Figure 7.1b shows that except for VFA, the main effects of the other factors are insignificant at α 0.05; conversely, some interactions between the factors are significant.

The most significant positive factor responsible for the gain in relative HAR is the VFA. In addition, VFA*VFA interaction has a positive influence on relative HAR. Similarly, Co*Co, VFA*Co and Co*Se interactions also have positive influences on relative HAR. Significant negative influence resulting in low relative HAR arises from the Co*Mo and VFA*Mo interactions. The Estimates of the factors in Figure 7.1b give a general indication of the importance of the factors and highlight the interactions of VFA, Co, Se and Mo as significantly influential on HAR. The optimum

ranges for the interactions of these TEs and VFA are not apparent from Figure 7.1b, and must be derived by response optimization.

7.2.1.3 Optimum TEs setting for HAR: The desirability function was used (Section 6.4.2) to determine the factor settings that maximize the gains in HAR. The function is shown in Equation 6.5a, the goal for HAR is shown in Table 6.5 and the importance level attached to HAR relative to other measured responses is shown in Table 6.13. Considering that VFA is the most important factor of influence, Figure 7.2a, b and c show the optimum TEs settings for HAR at different VFA levels.

a. Low VFA level: Figure 7.2a shows that the ranges of TEs concentrations that could induce gains in HAR at [4]low VFA level (10 mmol/L) were 0 – 1.0 mg/L Ni and 1.2 mg/L Mo in mixture. The optimum TEs configuration (TEs composition and concentration) is a mixture with 0.5 mg/L Ni and 1.2 mg/L Mo and this TEs configuration produced an average gain in HAR of 82% (relative HAR of 1.82) and a desirability of 0.87. Co and Se are not required for an optimum gain in HAR. The trend in relative HAR suggests an increase in relative HAR value as Mo concentration increases from 0 mg/L to the optimum concentration. Conversely, Co and Se concentration ≥ 0 mg/L induce significant loss in HAR. The none requirement of Co and Se for optimum HAR could be due to electrostatic inhibition (inhibition due to charge interaction between TEs).

b. Medium VFA level: Figure 7.2b shows the new hydrolytic roles of the TEs at VFA of 100 mmol/L. The range of TEs necessary for positive relative HAR is 0.2 mg/L – 1.5 mg/L Ni, and 0.9 mg/L – 1.4 mg/L Mo. An average gain of 13% (relative HAR of 1.13) was induced at the optimum TEs setting of 0.8 mg/L Ni and 1.3 mg/L Mo. The trend in the gain in HAR shows a linear increase in relative HAR as Mo concentration increases from 0 mg/L to 1.3 mg/L. Co and Se concentration > 0 mg/L induce loss in HAR. The lower value of desirability (0.43) at this VFA level compared to the low VFA level (0.87) suggests that interaction of TEs with reactor VFA of 100 mmol/L is less optimal for hydrolysis.

[4] The VFA concentrations of the inocula used for the investigations were generally in the range of 10 – 12 mmol/L and after hydrolysis of the added silage feedstock, an average of 28 ± 2 mmol/L VFA was measured in the low VFA level. Hence, (10 mmol/L) the pre-hydrolysis VFA concentration was chosen as low VFA level for this analysis.

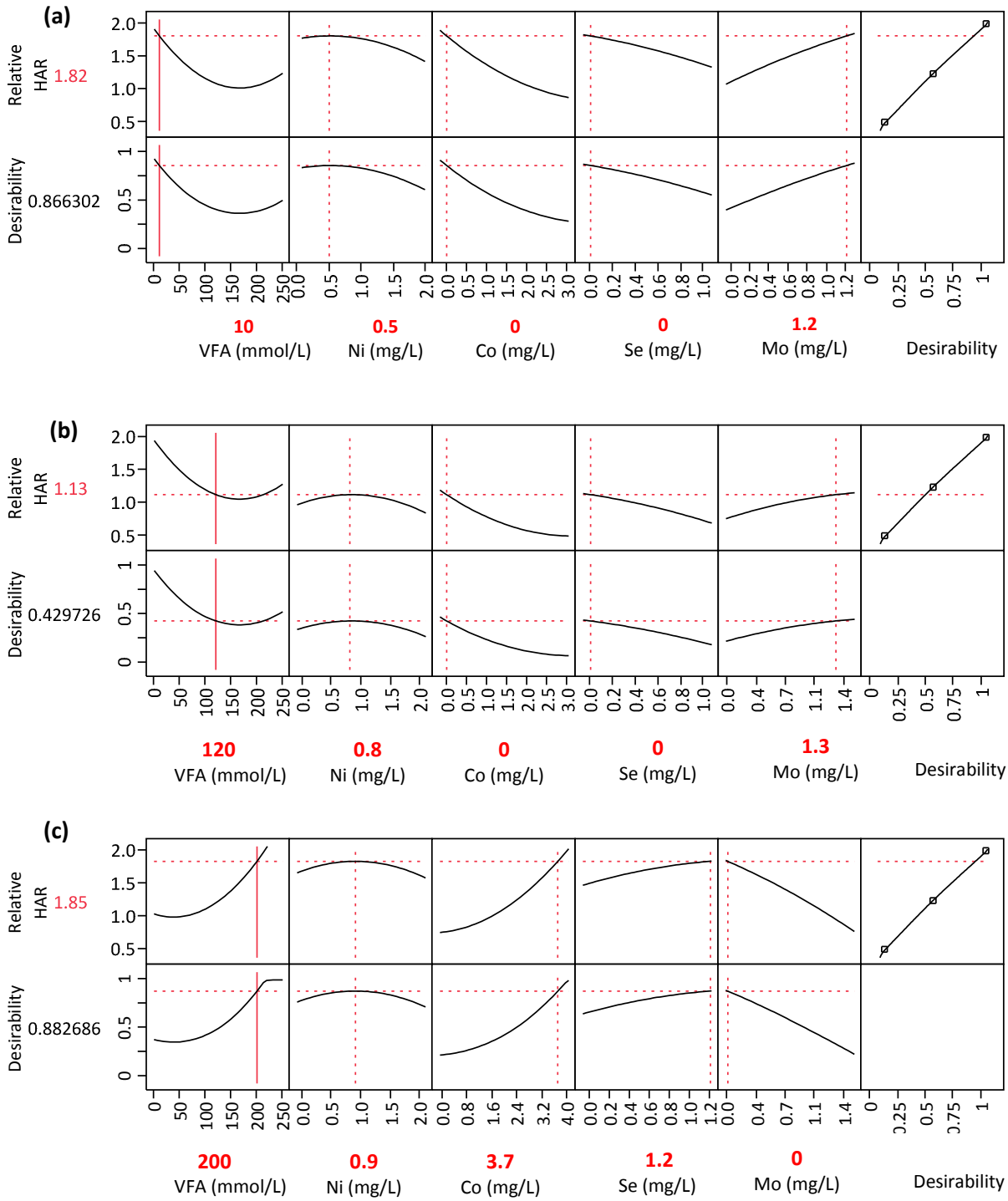


Figure 7.2 Prediction profile and optimum factor settings for the relative HAR of silage digestate at 37°C due to Ni, Co, Se and Mo supplementation and different VFA concentrations **(a)** 10 mmol/L VFA **(b)** 120 mmol/L VFA **(c)** 200 mmol/L VFA

c. High VFA level: Figure 7.2c shows the TEs configuration responsible for the gains and losses in HAR at VFA of 200 mmol/L. Ni, Co and Se interaction induced an average gain of 85% (relative HAR 1.85) in HAR. The range of the TEs necessary for the gain in HAR at this VFA level include 0.4 mg/L – 1.5 mg/L Ni; about 3.7 mg/L Co;

and 0.8 mg/L – 1.2 mg/L Se. The optimum TEs configuration is 0.9 mg/L Ni, 3.7 mg/L Co and 1.2 mg/L Se. The desirability of the optimum TEs configuration is 0.88. The trends in the relative HAR in Figure 7.2c show that Mo > 0 mg/L and Ni ≥ 1.5 mg/L induce losses in HAR, while gain in HAR increases as Co concentration increases from 0 mg/L to 3.7 mg/L, and as Se concentration increases from 0 mg/L to 1.2 mg/L.

Since hydrolysis is initiated by nucleophilic attack of an OH^- group on a substrate, it could be presumed that the interaction of Ni and Mo ions aids the OH^-nucleophilic attack on substrates at low VFA level. The efficiency of this configuration at improving HAR decreased as VFA concentration increased from 10 mmol/L to 120 mmol/L. Figure 7.1b shows that VFA*Mo has a negative influence on HAR. Therefore, as VFA increased from 10 mmol/L to 120 mmol/L, the effect of VFA*Mo interaction resulted in decline in the relative HAR value. Ni and Mo are the most important TEs for VFA 10 mmol/L – 120 mmol/L. Apparently, Mo interaction with Ni, Co and Se is even much less favourable for HAR in AD when the VFA level is 200 mmol/L. At 200 mmol/L VFA, Co and Se interactions with VFA are beneficial and could optimize HAR by 85%.

Figure 7.1b shows that Co*Se and VFA*Co can stimulate HAR; and this is evident in the increase in relative HAR with Co and Se becoming necessary as VFA increased from 120 mmol/L to 200 mmol/L. HA requirement for Ni changed only slightly (0.5 mg/L – 0.9 mg/L) compared to Co, Se and Mo for VFA between 10 and 200 mmol/L. Pair wise activities of the TEs are in agreement with the documentation by Frey and Hegeman (2007) that the hydrolytic effect of two divalent metals coordinated to a single water molecule is twice as high as single divalent metal-water coordination. Higuchi *et al.* (2005) also documented that two divalent metals ions participating in the hydrolytic mechanisms of amylase and ureases account for enhanced hydrolysis relative to single metal ion coordination in the enzymes.

7.2.1.4 Implications of the optimum TEs configuration on HA of AD substrates

Table 7.1 showed the TEs content of selected AD substrates. The need for Ni and non-requirement for Mo at VFA concentrations of 200 mmol/L in Figure 7.2c has implications for AD with Ni- and Mo-rich sludges as start-up inoculum. Table 7.1 showed that sewage sludges could have Ni and Mo content up to 7.14 mg/kg DM and 4.95 mg/kg DM respectively. It also showed that substrates such as silage feedstock could contain up to 3.62 mg/kg DM Ni and 1.46 mg/kg DM Mo. The relatively high Mo content of the sludge and silage feedstock could promote HAR in the beginning of AD when VFA level is low; however, as VFA accumulate, further HA could be impaired due to negative influence of VFA*Mo interaction. Inhibition of HAR

as VFA accumulates may be further compounded by the occurrence of Co and Se at lower concentration compared to Ni and Mo in AD substrates (Table 7.1).

It could also be presumed that depending on the concentrations of Ni, Co and Se, different substrates will exhibit varying HAR due to differences in Mo concentration. Variations in Ni, Co, Se and Mo contents and VFA production potential of sludges from different sources are documented (Christ *et al.,* 2000; Rajagopal and Beline, 2011); but no explanations are given for such variations. The trends in Co and Se (Figure 7.2c) suggest that higher gains in HAR will be possible as Co and Se concentrations increase either by supplementation or co-digestion during AD. Figures 7.0, 7.2a, b and c suggest that changes in TEs configuration and reactor VFA levels are important causes of variations in HAR. Considering that the substrates in Table 7.1 have levels of Se below detection and that elevated Co concentration (up to 3.7 mg/L in Figure 7.2c) are beneficial for HAR, Se and Co supplementation to AD might be necessary to enhance substrate HAR.

7.2.2 Influence of TEs on substrate conversion at 37°C: In substrate conversion, the influence of TEs on VFA degradation rate, cumulative CH_4 production, microbial adaptation period and VFA retention time are discussed in terms of the relative gains and losses during AD in treatment reactors over the Control reactors. VFA degradation rate and CH_4 production were the measured responses while the derived responses include VFA retention time and microbial adaptation period. CH_4 production was measured from the biogas volume (Nml) and CH_4 content (%) of biogas. The analytical standards and the procedure for the experiments are discussed in Chapter 6.

7.2.2.1 Influence of TEs on microbial adaptation period in AD at 37°C: Some time is required for microbial adaptation to TEs concentrations and incorporation of the TEs into the enzymes of the micro-organisms (Frausto da Silva and Williams, 1991). This is the microbial adaptation period and was derived from data on CH_4 production when the condition in Equation 6.3e shown in Section 6.2.2 was satisfied in one or more reactors. For the purpose of calculating the microbial adaptation period, the cumulative CH_4 production included CH_4 produced between day 1 and day 29. The relative CH_4 production for the different reactors were calculated from post-HA CH_4 production (day 16 – day 29) as shown in Appendix 7.3a for Ni, Co, Se and Mo supplementation and Appendix 7.4a for Se and Mo supplementation. Different time period were applied to the cumulative CH_4 production and relative CH_4 production for the following reasons:

1. To highlight the possible lag in CH_4 production due to the influence of TEs supplementation, which could be perceived as loss in CH_4 production if the

experiments were carried out in a shorter time than the microbial adaptation period; and

2. To specifically highlight the gains in CH_4 production due to TEs supplementation after the microbial adaptation period.

The use of CH_4 production data within the entire time span of the experiment, without differentiating into phases, masks the actual influence of the TEs on optimizing CH_4 production. Consequently, the weakness of TEs supplementation is clearly highlighted in long adaptation periods; whereas the strengths of TEs supplementation are evident in the relative gains. The influences of TEs on microbial adaptation period at the 3 levels of VFA and the relative CH_4 production are discussed.

a. Ni, Co, Se and Mo supplementation to low VFA level: Figures 7.3a shows cumulative CH_4 production up to day 21 in the low VFA level when the condition in Equation 6.3e was satisfied in one or more treatments; and the relative CH_4 production at the end of the experiment (day 29). On day 21, R28 had a cumulative CH_4 production of 541 Nml. R1, R5 and R8 were adapted and had cumulative CH_4 production of 541, 547 and 545 Nml respectively within the same period. R2, R3, R4, R6 and R7 had cumulative CH_4 production lower than 541 Nml on day 21. In spite of weak CH_4 production in R6 and R7 prior to adaptation period, at the end of the batch experiment, R6 and R7 had a relative CH_4 production of 1.46 and 1.40 respectively. R1, R5 and R8 that adapted on day 21 had higher relative CH_4 production of 1.54, 1.58 and 1.71 respectively. R4 never adapted to the influence of TEs; whereas R2 and R3 showed low relative CH_4 production of 1.09 and 1.10 respectively.

The remarkable features of the treatments with ≥ 1.5 relative CH_4 production (R1, R5 and R8) are that R1 was supplemented with only [5]Se, R5 was supplemented with Ni, Co and Mo but not Se and R8 was supplemented with Ni, Co and Se but not Mo. The remarkable features of the non-adapted treatments in the low VFA level include:

- Lack of Ni supplementation (R2, R3 and R4);
- Lack of Ni and one or more TEs supplementation (R2 and R3);
- Co-supplementation of Mo and Co without Ni addition (R2 and R3); and
- Co-supplementation of Se and Mo without Ni addition (R4 in contrast to R7).

[5] Ni, Co, Mo content are respective concentrations in the experimental inoculum

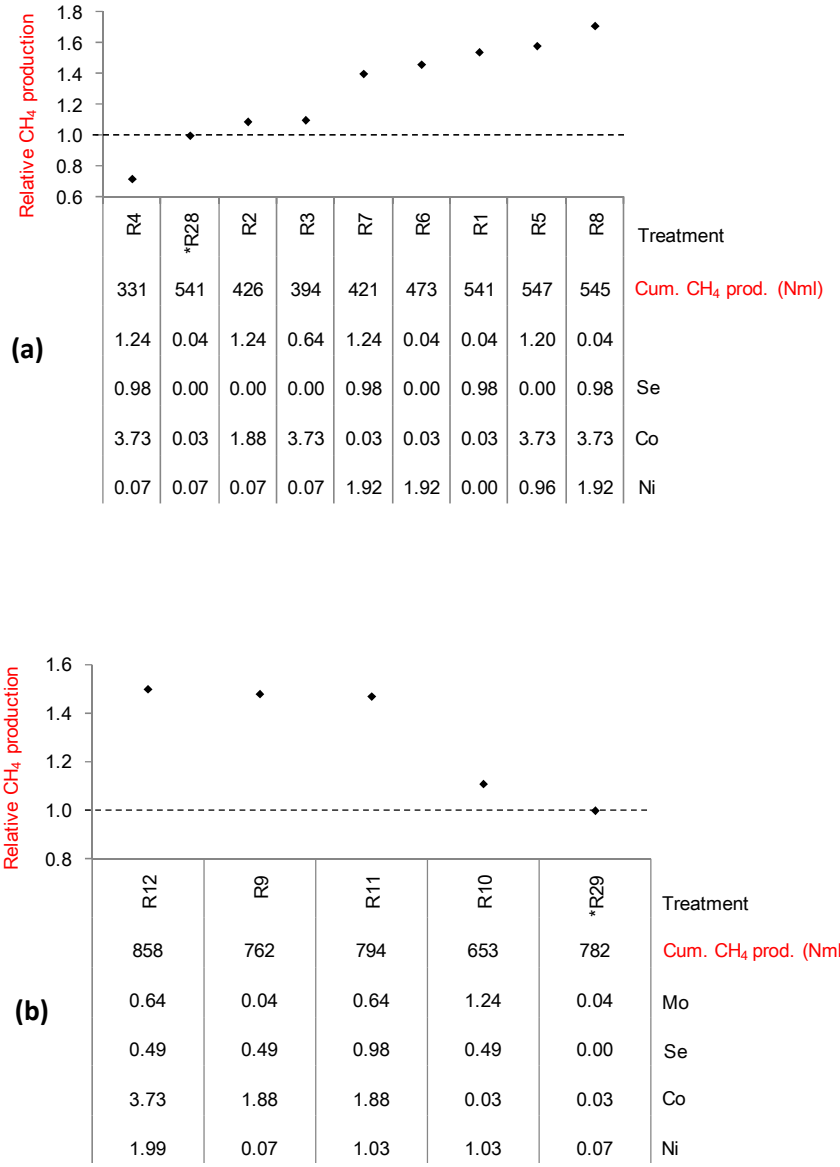


Figure 7.3 Cumulative CH_4 production on day 21 and relative CH_4 production at the end of the experiment at 37°C due to Ni, Co, Se and Mo supplementation to different levels of VFA **(a)** Low VFA level (28 ± 2 mmol/L) **(b)** Medium VFA level (116 ± 2 mmol/L)

b. Ni, Co, Se and Mo supplementation to medium VFA level: Figures 7.3b shows cumulative CH_4 production (Nml) on day 21 in the medium VFA level when the condition in Equation 6.3e was satisfied in one or more treatments; and the relative CH_4 production at the end of the batch experiment on day 33. By day 21, R29 had a cumulative CH_4 production of 782 Nml. R11 and R12 had cumulative CH_4 production of 794 Nml and 858 Nml respectively on day 21; whereas R9 and R10 had 762 Nml and 653 Nml respectively on same day. In spite of weak cumulative CH_4 production prior to the microbial adaptation period, at the end of the batch experiment, R9 had a relative CH_4 production of 1.48; whereas R10 had 1.11. R11 and R12 had relative CH_4 production of 1.47 and 1.50 respectively at the end of the experiment.

The remarkable features of the treatments with ≥ 1.4 relative CH_4 production (R9, R11 and R12) is that they all contain Co; in addition, R9 contains Se. R11 and R12 have all the elements supplemented and Mo was supplemented at medium level (0.64 mg/L). R9 has neither Ni nor Mo but was well adapted like R11 and R12. The remarkable feature of the treatments with less CH_4 production on day 21 (R9 and R10) compared to the Control reactor (R29) is the absence of Co and supplementation of Mo at high level (1.2 mg/L) in R10; and the absence of Ni and Mo in R9.

c. Ni, Co, Se and Mo supplementation to high VFA level: The treatments in the high VFA level adapted to the TEs supplementation on different days so that a direct comparison of the microbial adaptation periods is meaningful. Figures 7.4a shows adaptation periods and relative CH_4 production at the end of the experiment (day 41) in the high VFA level. The earliest microbial adaptation to the influences of TEs occurred on day 27 in R18, while R17, R20 and R21 adapted to influences of TEs on days 32, 35 and 36 respectively. At the end of the experiment, R17 and R18 had relative CH_4 production of 1.21, while R20 and R21 had 1.04 and 1.14 respectively. The duration of the experiment was 41 days and the other treatments in the group (R13, R14, R15 and R16, and R19) had relative CH_4 production < 1.0 (Appendix 7.3b).

The remarkable features of the treatments with relative CH_4 production ≥ 1.2 (R18 and R17) are that R17 had medium level TEs content and R18 is a partial supplementation involving only Ni and Co. Treatments R20 and R21 contain 1.24 mg/L Mo (high level). Apparently, Ni and Co supplementation (R18) and medium level supplementation of all the elements (R17) are significantly beneficial for both adaptation to TEs influence and gains in CH_4 production when the VFA concentration is high (≥ 200 mmol/L). The common feature of the non-adapted treatments includes absence of Ni in R13, R14, R15 R16 and R19; and in addition, absence of Co in R13, R14 and R19 (See Appendix 7.1b for Ni, Co, Se and Mo supplementation matrix at 37°C; Appendix 7.3a for CH_4 production; and Appendix 7.3b for relative CH_4 production). R19 was supplemented with Ni and Se; and R14 suggests that the partial supplementation with Se and Mo will result in longer adaptation periods to the influence of TEs. This is discussed next.

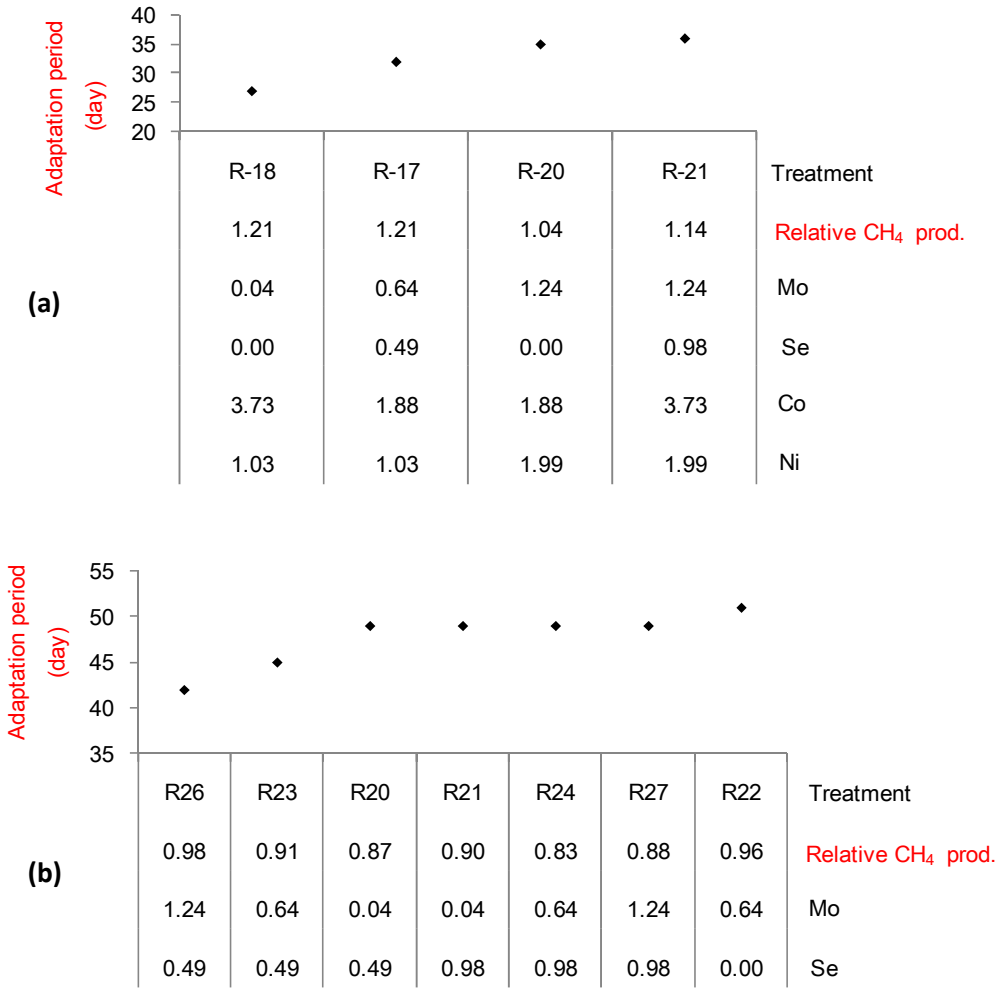


Figure 7.4 Adaptation time and relative CH_4 production at 37°C in the high VFA level due to **(a)** Ni, Co, Se and Mo supplementation (VFA = 213 ± 6 mmol/L) **(b)** Se and Mo (VFA = 217 ± 9 mmol/L)

d. Se and Mo supplementation to high VFA level: [6]Figure 7.4b shows the microbial adaptation period to TEs influences after Se and Mo supplementation. The earliest adaptation time to the influence of TEs was on day 42 and occurred in R26, while 51 days was required in R22 for the same purpose. R20, R21, R24 and R27 adapted on day 49. At the end of the experiment (day 52), R20 – R24, R26 and R27 had relative CH_4 production not significantly different than 1.0 (See Appendix 7.4a for Se and Mo supplementation matrix at 37°C; cumulative CH_4 production, and Appendix 7.4b for relative CH_4 production). The duration of the experiment was 52 days and R25 had relative CH_4 production < 1.0.

The remarkable features of the treatments with the shortest adaptation period after Se and Mo supplementation include that R26 was a high level Mo (1.24 mg/L)

[6] The adaptation trend in low and medium VFA categories is similar in the partial supplementation involving only Se and Mo. Hence, only the full supplementation is shown.

supplementation; and R23 was supplemented with 0.64 mg/L Mo, and 0.49 mg/L Se in mixture. The remarkable features of the treatments with the longest adaptation period after Se and Mo supplementation are that R22 was supplemented with 0.64 mg/L Mo (compared to 0.49 mg/L Se in R20). R25 did not adapted to the influence of the TEs in terms of CH_4 production and was supplemented with high level Mo (1.24 mg/L). This contrasts with R21 that had high level Se (0.98 mg/L) and adapted to the treatment after 49 days (See Appendix 7.4b for relative CH_4 production at the end of the experiments).

Generally, Figure 7.4b illustrates a methanization process with longer adaptation need (42 – 51 days) due to partial supplementation compared to Figure 7.4a with a full supplementation (27 – 35 days). Supplementation of Ni, Co, Se and Mo to the low and medium VFA categories produced similar adaptation behaviour and suggests that inhibitory influences of Co and Mo; and Se and Mo in TEs mixtures are TEs concentrations dependent. High levels of Se and Mo (0.98 mg/L and 1.2 mg/L respectively) in co-supplementation tend to prolong the microbial adaptation period to the influences of TEs in methanization. Relative CH_4 production in treatments with either Se or Mo were higher and suggest that either Se or Mo is required (not both) in TEs mixtures for rapid microbial adaptation to TEs supplementation during methanization.

Furthermore, at low levels of VFA, Se is preferred in mixture with Ni and Co compared to Mo in mixture with Ni and Co. However, in the medium VFA level, Co and Se are more important for microbial adaptation to TEs influences compared to the other treatments. In the high level, Mo concentration is an important determinant of adaptation period: the higher the concentration of Mo, the longer the adaptation period. Notwithstanding the supposed involvement of Se and Mo in methanogenesis, relative CH_4 production were generally weak in Se and Mo supplementations compared to Ni, Co, Se and Mo supplementation to high VFA level.

7.2.2.2 Influence of TEs on CH_4 production at 37°C: In Section 7.2.2.1, CH_4 production in the treatment reactors were used (in relation to Equation 6.3e) to derive microbial adaptation period to the influences of TEs during methanization. Therefore, the relative CH_4 production and cumulative CH_4 production were discussed for partial and full supplementations and need no repetition. However, Figure 7.5 shows the summary of the relative CH_4 production in the different levels of VFA due to partial and full TEs supplementations. Ni, Co, Se and Mo (full) supplementation has a group average of 1.30 relative CH_4 production in the low VFA level, and 1.39 relative CH_4 production in the medium VFA level.

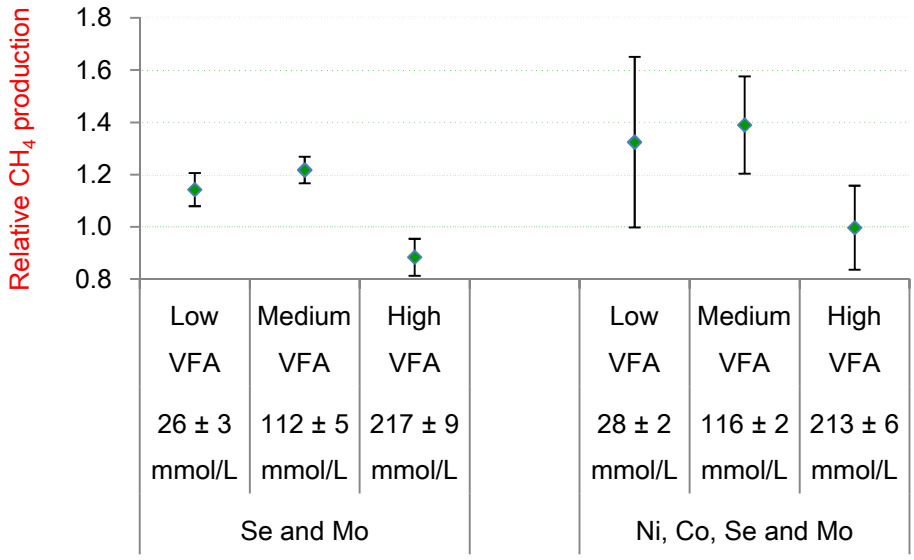
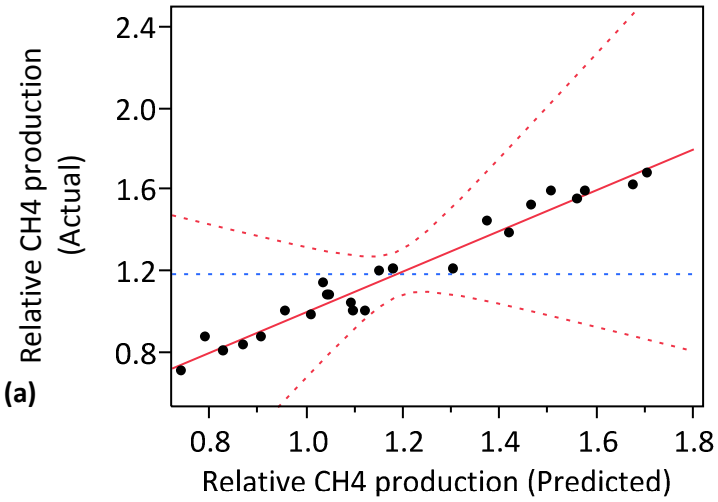


Figure 7.5 Summary of the relative CH_4 production at 37°C due to partial supplementation with Se and Mo and full supplementation with Ni, Co, Se and Mo for different levels of VFA

In the low and medium VFA levels of the Se and Mo supplementation (partial), the group averages for relative CH_4 production are 1.14 and 1.22. In the high VFA category (VFA ≥ 200 mmol/L), the group average relative CH_4 productions is about 1.0 for full supplementation, and 0.88 for partial supplementation. Generally, the variations in the relative CH_4 production for the levels of VFA are wider in Ni, Co, Se and Mo supplementation compared to Se and Mo. It is noteworthy that relative CH_4 production are higher in the medium VFA group (116 mmol/L) compared to the low and high VFA groups for Se and Mo as well as Ni, Co, Se and Mo supplementations. The influences of the TEs are quantified and optimum TEs concentrations are derived in the subsequent sections.

7.2.2.3 Identification of the factors of influence during CH_4 production: The variations in relative CH_4 production in Figure 7.5 indicate that some of the TEs configurations caused a reduction in relative CH_4 production. To identify and quantify the specific influences of the TEs on the gains and losses in CH_4 production, RSM was used to predict the relative CH_4 production according to Equation 6.4b in Section 6.4.1 and estimate the influences of the factors. The data for the fit are in Appendix 7.3b for full supplementation with Ni, Co, Se and Mo; and in Appendix 7.4b for partial TEs supplementation with Se and Mo.



(a)

R^2	0.96
R^2 Adjusted	0.67
Root Mean Square Error	0.17
Relative CH_4 production	1.19
Observations)	24

(b)

Term	Estimate	Std Error	t Ratio	Prob>|t|
VFA (mmol/L)	-0.001941	0.000428	-4.53	0.0201*

Figure 7.6 **(a)** Plot of actual vs. predicted relative CH_4 production at 37°C **(b)** Model terms, estimates of parameters and significance of terms in modelling of the relative CH_4 production at 37°C due to Ni, Co, Se and Mo supplementation to VFA levels between 28- and 213 mmol/L.

a. Partial supplementation with Se and Mo: The plot of the actual vs. predicted CH_4 production and the statistics of the plot; model estimates of the factors; and the prediction profile for low and high VFA levels are contained in Appendix 7.4c, d, e and f respectively for the partial supplementation. The prediction was done with Equation 6.4a in Section 6.4.1; and the optimum settings were derived using Equation 6.5a in Section 6.4.2. Most importantly, the optimum setting for Se and Mo for CH_4 production confirm methanization preference of Se over Mo at VFA level of 240 mmol/L; and Mo over Se at 25 mmol/L VFA.

b. Full supplementation with Ni, Co, Se and Mo: Figure 7.6a shows the plot of actual relative CH_4 production vs. predicted CH_4 production for full supplementation following the RSM (Section 6.4.1) and using Equation 6.4b for prediction of the relative CH_4 production. The statistics for Figure 7.6a shows that the prediction has a coefficient of determination (R^2) of 0.96 with an error of 0.17, which indicates a good model fit between the predicted relative CH_4 and the actual relative CH_4 production. The R^2 adjusted or standardized R^2 value is 0.67. It is considerably lower than R^2 and suggests that many factors in the model have lower relevance. R^2 adjusted is pertinent for comparing the influence of the factors on different responses. The higher the number of significant factors in the model, the larger is the R^2 adjusted. The mean relative CH_4 is 1.19, corresponding to an average of 19% gain in CH_4 production due to TEs influences across all VFA levels.

Figure 7.6b shows the important terms and Estimates for the influence of Ni, Co, Se, Mo and VFA on the relative CH_4 production in decreasing magnitude. Explanation of the features of Figure 7.6b is offered in Section 6.4.1. VFA concentration is the most influential factor that influences CH_4 production. The net influence of VFA concentration is negative and significant: so that an increase in VFA concentration results in low relative CH_4 production. Consequently, the main effects and interactions of the TEs with different VFA levels are evaluated and optimum settings are derived.

7.2.2.4 Optimum TEs setting for relative CH_4 production: Considering that VFA is the most important factor of influence in CH_4 production, Figures 7.7a, b and c show the prediction profiles and the optimum TEs settings in the low VFA level, medium VFA level and high VFA level respectively, using the method described in Section 6.4.2 and Equations 6.5b. The importance level for CH_4 production is shown in Table 6.13 and optimization goal is to maximize the relative CH_4 production in all levels of VFA, and this is shown in Table 6.5.

a. Low VFA level: Figure 7.7a shows the prediction profiler for the relative CH_4 production and the influence of the TEs on VFA concentration of 23 mmol/L. The optimum TEs configuration (TEs composition and concentration) is a mixture containing about 2.2 mg/L Ni, 3.7 mg/L Co and 1.6 mg/L Mo. This configuration produced an average gain in relative CH_4 production of 108% (relative CH_4 production of 2.08) and a desirability of 0.98.

b. Medium VFA level: Figure 7.7b shows the prediction profiler for the relative CH_4 production and the influence of the TEs on VFA concentration of 125 mmol/L. The optimum TEs configuration is a mixture containing 2.6 mg/L Ni, 3.8 mg/L Co and 1.6 mg/L Mo. This configuration produced an average gain in relative CH_4 production of 81% (relative CH_4 production of 1.81) and a desirability of 0.81. The optimum requirements for TEs in the low and medium VFA levels are similar, and suggest that for VFA concentration between 23 and 125 mmol/L, low concentrations of Se (< 0.1 mg/L) are more beneficial for CH_4 production but Se concentration up to 0.3 mg/L can be tolerated (Figure 7.7a and b). Banks and Zhang (2012) reported similar effect for Se and observed that Se concentrations above 0.4 mg/L reduced CH_4 yield in food waste methanization.

In addition, gains in CH_4 production achievable with this configuration of TEs decreases as the VFA concentration increases. This is evident in the decline in desirability as VFA concentration increases from 23 to 125 mmol/L. In the low and medium VFA levels, increase in Ni and Co concentrations generally resulted in proportional increase in relative CH_4 production and desirability. The requirement of

relatively high concentrations of Ni and Co for this range of VFA concentration during CH_4 production suggests that both TEs play complementary roles in enhancing CH_4 production from intermediates of VFA degradation. The prediction profiles also suggest that changes in concentrations of Mo and Se have opposite effects, and indicate that increase in concentration of one, will necessitate a reduction in the concentration of the other. Obviously, Mo is preferred in mixture at this VFA range to Se. Lo *et al.* (2010) reported similar concentration ranges for Ni and Co as optimum for stable methanization, and also indicated that Mo is required for methanization but its influences are not substantial (Section 4.2.2).

c. High VFA level: The high VFA level (≥ 200 mmol/L) is particularly important because it is the VFA range reported to be associated with digester failure during methanization processes (See Table 3.1 in Section 3.3.3). Figure 7.7c shows the prediction profile for the relative CH_4 production and the influence of the TEs on 240 mmol/L VFA. The optimum TEs configuration is a mixture containing 1.2 mg/L Co and 1.0 mg/L Se. This configuration produced an average gain of 35% in CH_4 production (relative CH_4 production of 1.35) and a desirability of 0.51. Figure 7.7c indicates that at high VFA concentration (240 mmol/L), the requirements for TEs change so that Co and Se induce high relative CH_4 production.

The prediction profiles for CH_4 production and desirability suggest that Se concentration between 0.70 mg/L and 1.2 mg/L; and Co concentration between 0.6 mg/L and 1.5 mg/L will induce gains in CH_4 production. Ni up to 0.4 mg/L could be tolerated within the process at this VFA level (240 mmol/L). Conversely, Mo > 0 mg/L induced decline in CH_4 production. It can be inferred from the gains due to the TEs configuration in Figure 7.7c that supplementation with Co and Se is capable of reviving a digester that is experiencing methanization failure as a result of accumulated VFA. Co and Se are associated with the MeTr and FDH respectively and these are the main enzymes for the metabolism of H_2 and -CH_3 (Ragsdale and Pierce, 2008).

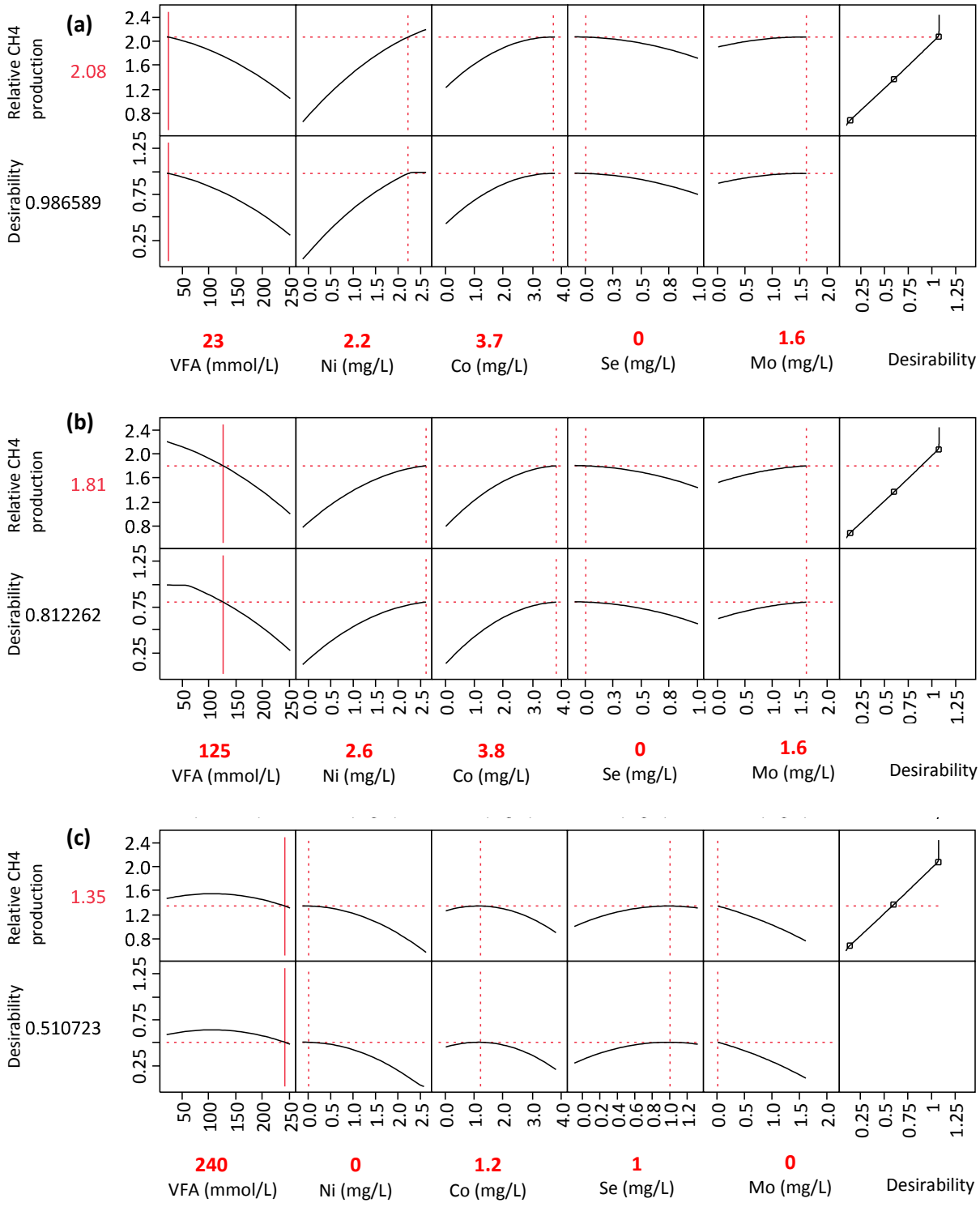


Figure 7.7 Prediction profile and optimum factor settings for relative CH_4 production at 37°C due to Ni, Co, Se and Mo supplementation to different levels of VFA **(a)** 23 mmol/L **(b)** 125 mmol/L **(c)** 240 mmol/L

7.2.2.5 Influence of TEs on VFA retention time and VFA degradation rate at 37°C: VFA concentration was measured by distillation and titration according to the method described in DIN 38414 – 19. Degradation rate was measured as the change

in VFA concentration (Δ*VFA* concentration) per change in time (Δ*time*) as shown in Equation 7.1. VFA retention time in the batch operations was calculated as shown in Equation 6.3d in Section 6.2.2.3, as the time required to degrade 75% of the post-HA VFA. The relative responses are the ratios of the responses in the treatment reactors for a particular VFA level, compared to the Control reactors of the same VFA level.

Equation 7.1 Δ*VFA* concentration / Δ*time*

Appendix 7.1b contains the actual TEs supplemented to the reactors; the supplementation procedure, including starting the experiments are described in Appendix 6.7c. Appendix 7.3a contains the average VFA degradation rates and the VFA retention times for the treatments and Control reactors. The relative VFA degradation rates and relative VFA retention time are contained in Appendix 7.3b. Figure 7.8a, b, and c show differences in relative VFA degradation rate and relative VFA retention times due to TEs supplementation in low, medium and high VFA levels respectively.

a. Low VFA level: Figure 7.8a shows the retention time, relative VFA retention time and the relative VFA degradation rate in the low VFA level after Ni, Co, Se and Mo supplementation at 37°C. The range of the retention time was between 7 and 15 days. R2 and R3 had retention time of 15 and 14 days respectively, which were higher than the retention time of 12.5 days in R28 (Control). R2 and R3 also had relative VFA retention times of 0.8 and 0.88 respectively, which indicate 20% and 12% longer VFA digestion time respectively, compared to the Control.

Lower retention times than in Control were observed in R1, R5, R6 and R8, with corresponding relative VFA retention times of 1.28, 1.44, 1.12 and 1.44 respectively. In Figure 7.8a the relative VFA degradation rate ranges between 0.90 and 1.92, with a mean of 1.54 ± 0.35. Except in R4 that had relative VFA degradation rate of 0.9, all the treatments had ≥ 1.35 relative VFA degradation rate. Relative VFA degradation rate ≥ the group average of 1.54, or ≥ 54% gain in VFA degradation, was observed in R2, R3, R5 and R8; whereas R1 and R7 had relative VFA degradation rate of 1.42 and 1.41 respectively. The lowest relative VFA degradation rate of 1.35 occurred in R6.

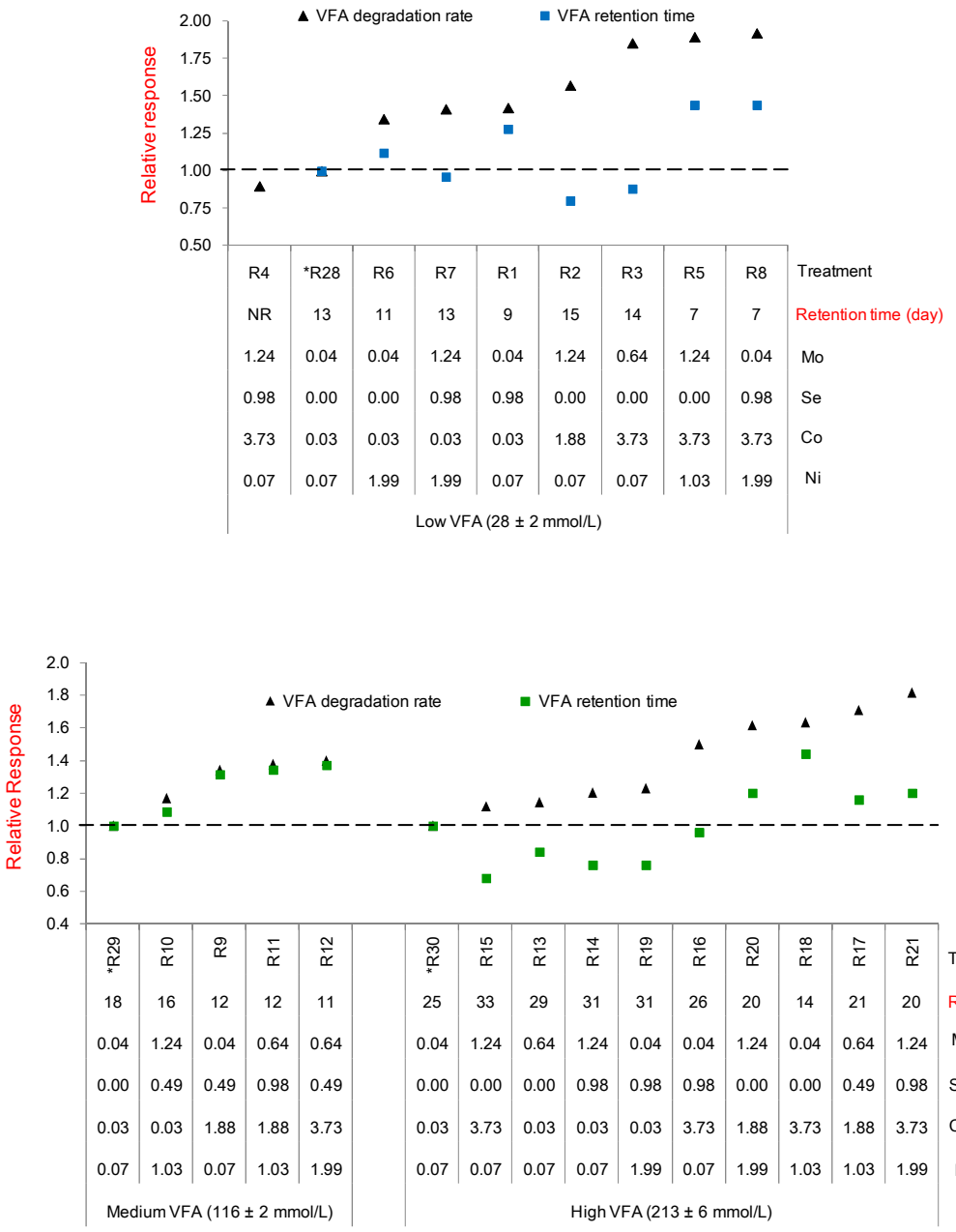


Figure 7.8 Retention time, relative retention time and relative VFA degradation rate at 37°C due to Ni, Co, Se and Mo supplementation to different levels of VFA **(a)** Low VFA level **(b)** Medium and High VFA levels (NR < 75% VFA concentration was degraded at the end of the experiment; Control= *R28)

b. Medium VFA level: Figure 7.8b shows the relative VFA degradation rate, relative VFA retention time and the retention time in the medium and high VFA levels after Ni, Co, Se and Mo supplementation. The range of the VFA retention time in the medium VFA level is between 11 and 18 days. All the treatments in this VFA level have lower VFA retention times compared to the Control (R29), which have 18 days VFA retention time. Except R10 with 16 days VFA retention time, R9 and R11 had 12 days VFA retention, while R12 had 11 days. The group average for the relative VFA

retention time was 1.28 ± 0.13 or 28% reduction in VFA retention time over the Control reactor as a result of TEs supplementation. The relative VFA retention time in R9, R11 and R12 were higher than the group average, and were 1.31, 1.34 and 1.37 respectively. R10 was lower than the group average and had a relative VFA retention time of 1.09. Relative VFA degradation rate in the medium VFA level followed similar trend as relative VFA retention time, and the group average was 1.32 ± 0.10 or 32% increases in VFA degradation rate compared to Control reactor. The corresponding relative VFA degradation rate in R9, R10, R11 and R12 were 1.17, 1.34, 1.38 and 1.40 respectively.

c. High VFA level: Figure 7.8b also shows the retention time, relative VFA retention time and the relative VFA degradation rate in the medium and high VFA levels after Ni, Co, Se and Mo supplementation. The range of the retention time is between 14 and 33 days, and indicates that not all the treatments had lower retention time due to TEs supplementation compared to R30, which had 25 days retention time. R13, R14, R15 and R19 had VFA retention times higher than the 25 days retention time in the Control reactor; whereas R17, R18, R20 and R21 had VFA retention times lower than that of the Control reactor. The group average for the relative VFA retention time as a result of TEs supplementation was 1.00 ± 0.26. The relative VFA retention times in R17, R18, R20 and R21 (with shorter retention time than the Control) were 1.16, 1.44, 1.20 and 1.20. R13, 14, 15, 16 and 19 had relative VFA retention times < 1.0; and had the common TEs supplementation feature of the lack of Ni in the mixture.

The relative VFA degradation rate in the high VFA level followed dissimilar trend as relative VFA retention time, and the group average was 1.44 ± 0.27 or 44% increases in VFA degradation rate compared to Control. Contrary to the observation in the relative VFA retention time, all the other treatments had relative degradation rate > 1.0 at the end of the experiments. However, R13, R14, R15 and R19 had lower relative VFA degradation rate (between 1.12 and 1.23) than the group average; while the relative VFA degradation rate in R16, R17, R18, R20 and R21 (between 1.50 and 1.82) were higher than the group average. Two obvious trends can be observed in the high VFA level of Figure 7.8b:

1. long retention time resulting in relative VFA retention time < 1.0:
   - Ni deficiency (R13, 14, 15, 16);
   - Partial supplementation with Ni and Se (R19), Co and Mo (R15), Co and Se (R16), Se and Mo (R14), and Mo (R13).
2. Short retention time resulting in relative VFA retention time > 1.0:
   - Partial supplementation with Ni and Co (R18); and Ni, Co and Mo (R20)
   - Full supplementation with Ni-Co-Se-Mo (R17, R21)

Co is required for propionate degradation in the methyl-malonyl-CoA pathway (De Bok *et al.,* 2005). This might account for poor degradation rate and long retention time in Co-deficient treatments such as R10, R13, R14 and R19. In addition, two or more TEs were lacking in R13, 14, 15, 16 and R19, which resulted in long retention time and poor degradation rate. R15 further indicates that partial mixture of Co and Mo resulted in lower relative VFA degradation rate and induced longer retention time than in the Control reactor. R19 shows that long retention time and VFA accumulations could occur in Co and Mo deficient reactors. Compared to the positive influence of partial mixture of Ni and Co in R18, partial mixture of Se and Mo in R14 is less beneficial in enhancing gains in retention time and degradation rate.

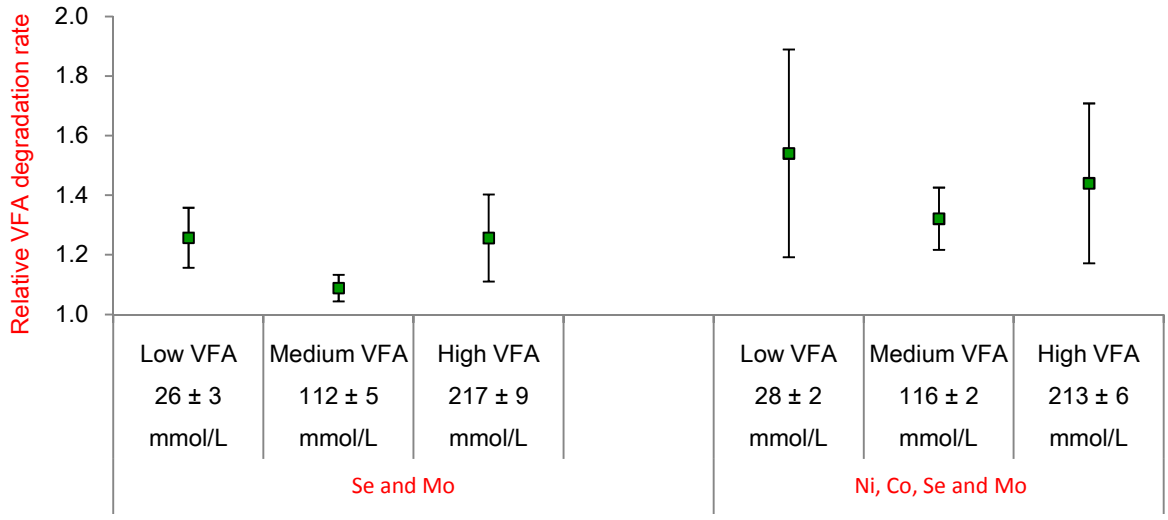


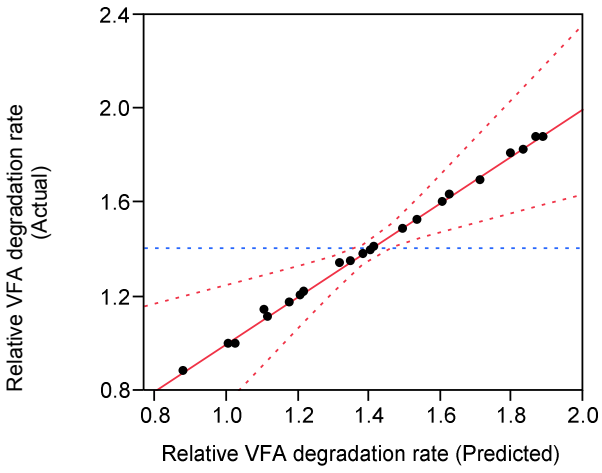
Figure 7.9 Relative VFA degradation rates at 37°C due to partial supplementation with Se and Mo and full supplementation with Ni, Co, Se and Mo to different levels of VFA

7.2.2.6 Partial Se and Mo vs. full Ni, Co, Se and Mo supplementation and relative VFA degradation rate: Figure 7.9 summarizes the relative degradation rate in the low, medium and high VFA levels after partial and full TEs supplementation at 37°C. The average relative VFA degradation rates were 1.54, 1.32 and 1.44 in the low, medium and high VFA levels respectively for the Ni, Co, Se and Mo treatments. For Se and Mo supplementation, the average relative VFA degradation rates were about 1.26, 1.09 and 1.26 in the low, medium and high VFA levels respectively. The relative VFA degradation rates were considerably lower in partial Se and Mo supplementations compared with Ni, Co, Se and Mo supplementation. In both the partial and the full TEs supplementations, the medium VFA level had lower relative VFA degradation rate compared to the low and high VFA levels. This may be related

to the fact that the VFA concentration at this level is optimum for stable methanization as shown in Table 3.1 in Section 3.3.

7.2.2.7 Identification and quantification of the factors of influence during VFA degradation rate: The Variations in the influence of TEs supplementations on VFA degradation rate due to partial and full TEs supplementation suggest that the individual TEs configurations in the investigations have different extents of influence. To estimate the magnitude of influence that individual factors or combination of factors exert on VFA degradation rate, the RSM was implemented as discussed in Section 6.4.1, and the relative VFA degradation rate was predicted using Equation 6.4b. The actual relative VFA degradation rates are shown in Appendix 7.3b. Figure 7.10a shows a plot of the actual relative VFA degradation rate vs. predicted relative VFA degradation rate; and Figure 7.10b shows the estimates and significance of the factors.

(a)



R^2	0.99
R^2 Adjusted	0.98
Root Mean Square Error	0.04
Mean of Response	1.41
Observations	23

Line of fit (solid line), significance curve at α 0.05 (dotted curves), and line of response mean (dotted horizontal line)

(b)

Term	Estimate	Std Error	t Ratio	Prob>|t|
(VFA (mmol/L)-127.888)*(VFA (mmol/L)-127.888)	0.0000451	3.526e-6	12.79	0.0061*
Ni (mg/L)	0.1749563	0.013744	12.73	0.0061*
(VFA (mmol/L)-127.888)*(Se(mg/L)-0.4687)	0.0027372	0.000239	11.43	0.0076*
Co(mg/L)	0.0705259	0.00622	11.34	0.0077*
(Ni (mg/L)-0.86304)*(Mo(mg/L)-0.64)	0.2367762	0.024884	9.52	0.0109*
(Ni (mg/L)-0.86304)*(Ni (mg/L)-0.86304)	-0.223257	0.030293	-7.37	0.0179*
(Co(mg/L)-1.88)*(Mo(mg/L)-0.64)	-0.088795	0.012672	-7.01	0.0198*
Mo(mg/L)	-0.126642	0.018658	-6.79	0.0210*
(VFA (mmol/L)-127.888)*(Mo(mg/L)-0.64)	0.0018035	0.000277	6.52	0.0227*
VFA (mmol/L)	-0.00064	0.000114	-5.60	0.0305*
(Ni (mg/L)-0.86304)*(Se(mg/L)-0.4687)	0.1403069	0.027198	5.16	0.0356*
(Co(mg/L)-1.88)*(Co(mg/L)-1.88)	-0.037988	0.008043	-4.72	0.0420*

Figure 7.10 **(a)** Plot of actual vs. predicted relative VFA degradation rate at 37°C **(b)** Model terms, estimates of parameters and significance of terms in modelling of the relative VFA degradation rate at 37°C due to Ni, Co, Se and Mo supplementation to VFA levels between 28 and 213 mmol/L.

The statistics for Figure 7.10a shows that the prediction has a coefficient of determination (R^2) of 0.99 with an error of 0.17. The adjusted R^2 or standardized R^2 value is 0.98. It is comparable to the R^2 and suggests that many factors in the model have significant influence on relative VFA degradation rate (compared to similar plot for relative CH_4 production in Figure 7.6a). The higher the number of significant factors in the model, the larger the adjusted R^2. The mean relative VFA degradation rate is 1.41, and corresponds to an average of 41% gain in VFA degradation rate compared to the Control reactors.

Figure 7.10b shows the mean Estimates of the effects of Ni, Co, Se, Mo and VFA on the relative VFA degradation rate in decreasing magnitude. See Section 6.4.1 for explanation of the features of Figure 7.10b. Ni, Co, Mo and VFA are significant individual factors responsible for the gains and losses in relative VFA degradation. The individual factors responsible for the gains include Ni and Co, while those responsible for the losses are Mo and VFA. This agrees with the report of Gustavsson *et al.* (2010) that Ni and Co are the most positively influential TEs during mesophilic methanization of silages.

The order of relevance for the individual TEs in enhancing VFA degradation rate is Ni > Co > Se. Se has an insignificant influence; nevertheless, it is significantly and positively influential in interactions with VFA (VFA*Se) and Ni (Ni*Se). The most significantly positive interaction is VFA*VFA (extreme concentration of VFA). This implies that TEs supplementations would produce high relative VFA degradation rate in low and high concentrations of VFA. Other positively influential factors include VFA*Se, VFA*Mo, Ni*Mo and Ni*Se. Significant negative influences on relative VFA degradation rate arise from Mo, and from the Ni*Ni, Co*Mo and Co*Co interactions. The Estimates of the factors in Figure 7.10b highlight the main and interaction effects of the factors on VFA degradation rate but not the optimum settings. The optimum settings are discussed next.

7.2.2.8 Optimum TEs setting for VFA degradation rate at 37°C: To determine the optimum factors setting that maximized the relative VFA degradation rates, the RSM described in Section 6.4.2 was used. Desirability function was used for the optimization and the function is shown in Equations 6.5a. The optimization goal for VFA degradation rate is shown in Table 6.5 and the importance level attached to VFA degradation rate relative to other measured responses is shown in Table 6.13. Figure 7.11a, b and c show the prediction profiles and optimum TEs settings for relative VFA degradation rate at low, medium and high VFA levels.

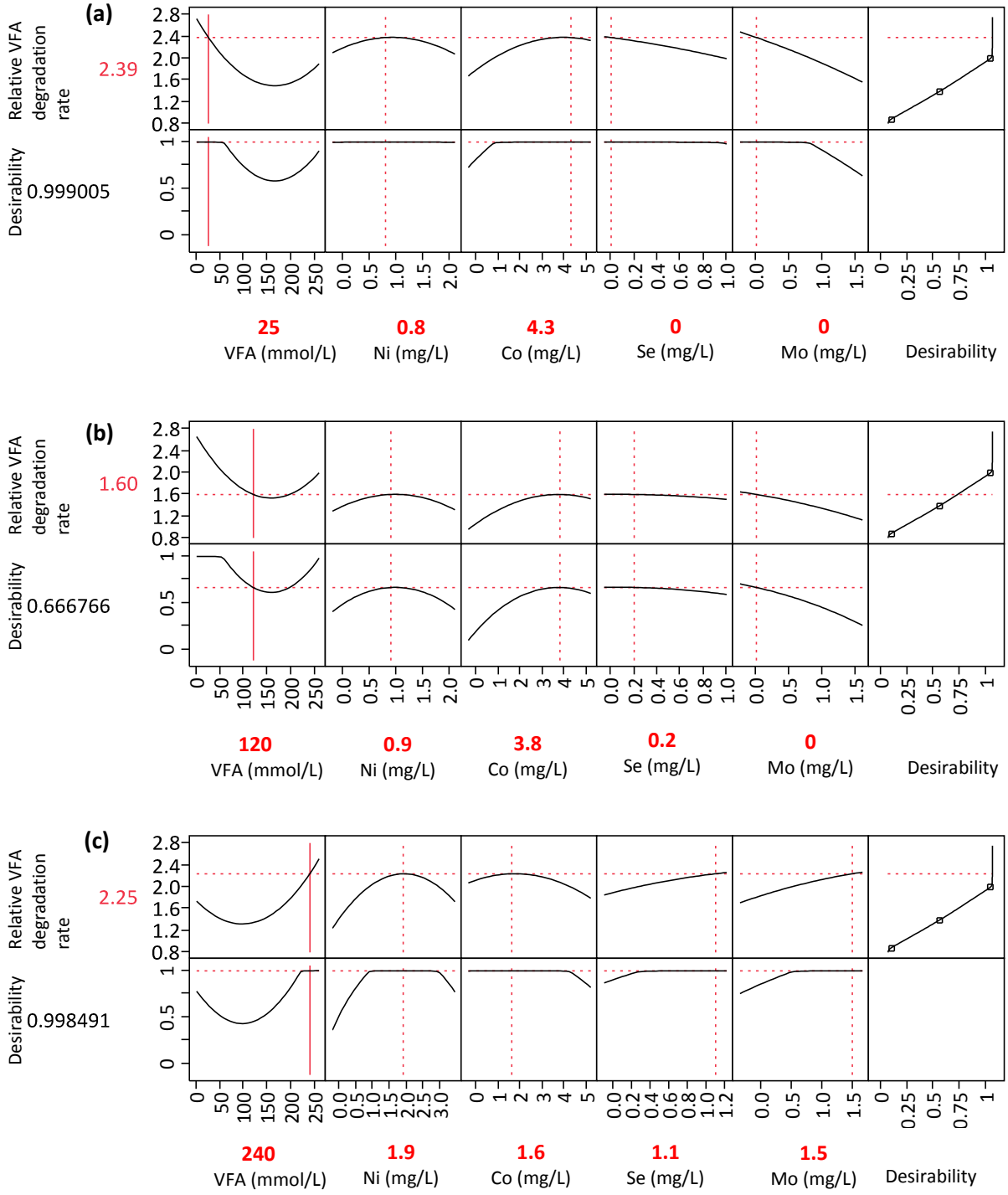


Figure 7.11 Prediction profile and optimum factor settings for relative VFA degradation rate at 37°C due to Ni, Co, Se and Mo supplementation to different levels of VFA **(a)** 25 mmol/L VFA **(b)** 120 mmol/L VFA **(c)** 240 mmol/L VFA

a. Low VFA level: Figure 7.11a shows the prediction profile for the relative gain in VFA degradation rate and the influence of the factor settings at VFA concentration of 23 mmol/L. Excluding Se and Mo from TEs supplementation mixtures will not induce

negative influences on the relative VFA degradation rate. About 0.5 mg/L – 1.2 mg/L Ni and 3.5 mg/L - 4.2 mg/L Co would enhance relative VFA degradation rate. The optimum TEs setting is 0.8 mg/L Ni and 4.3 mg/L Co, and results in a relative VFA degradation rate of 2.39 (139% gain in VFA degradation rate). The associated desirability is 0.99.

The prediction profiles show that a relative gain of up to 1.6 is possible without the supplementation of any TEs, since the enhancements due to Ni and Co are predicted to be necessary for relative VFA degradation rate above 1.6 (when Co and Ni concentrations are 0 mg/L respectively). For example, Ni is required for relative VFA degradation rate above 2.0 whereas Co is required for relative VFA degradation rates above 1.6 for this VFA concentration. Co concentration of about 0.8 mg/L in mixture will induce relative VFA degradation rate up to 1.90, and only higher with the inclusion of Ni in the mixture. The requirement of Ni and Co is consistent with the findings of Tan *et al.* (2003) who reported that during CH_4 formation, the metabolism of -CH_3 by the enzyme CODH is slow when non-catalysed by Ni and Co.

b. Medium VFA level: As the VFA concentration increases from 23 mmol/L, the relative VFA degradation rate and desirability of the TEs setting declines (Figure 7.11a), and new factor settings are required. Figure 7.11b shows the prediction profile for the relative gain in VFA degradation rate and the influence of the factor settings at VFA concentration of 120 mmol/L. The ranges of important TEs include 0.5 mg/L – 1.5 mg/L Ni and 3 mg/L – 4.5 mg/L Co. The optimum TEs setting include 0.9 mg/L Ni, 3.8 mg/L Co and 0.2 mg/L Se. The desirability of the optimum TEs setting is 0.67; and the corresponding relative VFA degradation rate is 1.6. Though Se concentration of 0.2 is optimum, up to 0.5 mg/L is tolerable. Mo > 0 mg/L is undesirable. The prediction profiles also suggest that about 1.0 mg/L Co with no Ni or Se in mixture could result in relative VFA degradation gain of up to 1.20; however, further increases up to 1.6 will require Ni and Se in the TEs mixture. The significance of Mo in this investigation is in agreement with Mo requirement during methanization of maize silage as reported by Demirel and Scherer (2011) (Table 4.2 in Section 4.2.1).

c. High VFA level: Figure 7.11c shows the prediction profile for the relative gain in VFA degradation rate and the influence of the factor settings at VFA concentration of 240 mmol/L. The beneficial range of the important TEs include 0.8 mg/L – 3.0 mg/L Ni, 0.0 mg/L – 4.0 mg/L Co, 0.2 mg/L – 1.2 mg/L Se, and 0.5 mg/L – 1.6 mg/L Mo. The optimum TEs configuration is 1.9 mg/L Ni, 1.6 mg/L Co, 1.1 mg/L Se and 1.5 mg/L Mo. This configuration produced a desirability of 0.99, and a relative VFA degradation rate of 2.25 (about 125% increases in VFA degradation rate). The

prediction profiles for Se and Mo suggest that the high VFA category shows tolerance to higher Se and Mo concentrations than are optimum. Figure 7.10a shows Mo as having negative influence on relative VFA degradation rate; conversely, VFA*Mo has a significant positive influence. VFA*Se also has a significant positive influence despite Se being negatively influential (though insignificant). It can be inferred that the inhibitory tendencies of Mo and Se are attenuated by increase in VFA concentration. Furthermore, about 0.3 mg/L – 0.7 mg/L Ni could induce relative VFA degradation rate of about 1.5 – 1.8. However, higher gains require Co, Se and Mo in mixture.

7.2.2.9 Optimum TEs configuration for multi-responses at 37°C: So far, influences of TEs on individual responses have been evaluated in Section 7.2.2.1 – 7.2.2.8; and the optimum TEs configurations for individual methanization processes have been derived. However, AD processes are overlapping and interdependent and more than one response needs to be optimized per time. Optimizing more than an individual response at a time is called multi-response optimization and is implemented as described in Section 6.4.2 using Equation 6.5b. Table 7.4 shows the output of the multi-response optimization for relative VFA degradation rate, relative VFA retention time and relative CH_4 production at different levels of VFA.

Table 7.4 Optimum and suitable Ni, Co, Se and Mo configuration for relative CH_4 production, relative retention time and relative degradation at 37°C in VFA levels between 10 and 250 mmol/L

Supplementation	VFA	Ni	Co	Se	Mo	Relative VFA degradation rate	Relative VFA retention time	Relative CH_4 production	Desirability
	mmol/L	mg/L							
Suitable	124	0.77	1.78	0.45	0.58	1.41	1.30	1.56	0.58
Optimum	10	1.88	4.21	0.32	1.61	1.96	1.93	1.93	0.91
	50	1.92	4.01	0.19	1.61	1.78	1.74	1.94	0.82
	100	1.94	3.74	0.10	1.61	1.62	1.59	1.83	0.75
	150	2.15	3.75	0.00	1.61	1.60	1.46	1.68	0.70
	200	2.04	3.62	0.03	1.61	1.67	1.44	1.40	0.64
	250	0.80	2.20	0.53	0.00	1.85	1.31	1.22	0.59

The suitable configuration contains the mean values of the factors used in the prediction of the individual responses (also see Section 6.5). The different levels of VFA have specific TEs configurations that are optimum for the combination of responses shown in Table 7.4. For VFA between 10 and 200 mmol/L, Ni concentrations between 1.9 mg/L and 2.2 mg/L are beneficial; between 4.2 mg/L – 3.6 mg/L Co are beneficial; and between 0 mg/L and 0.3 mg/L Se are beneficial. Mo concentration of 1.6 mg/L is optimum for VFA between 10 and 200 mmol/L. The

optimum TEs configuration at VFA concentration of 250 mmol/L is 0.8 mg/L Ni, 2.2 mg/L Co, 0.5 mg/L Se and 0 mg/L Mo.

An obvious trend in the optimum requirement for Se and Mo is that as VFA concentration increases from 10 to 200 mmol/L, the optimum concentration of Se also decreases. However, at 250 mmol/L, 0.53 mg/L Se is optimum; and for Mo, the concentration is steady at 1.6 mg/L. It is also apparent that increase in VFA concentration from 10 to 200 mmol/L requires either Se or Mo in mixture and not both; and this is most expressed at VFA concentrations of 150 mmol/L and 250 mmol/L. Conversely, both Ni and Co are required in the optimum TEs settings for VFA concentration between 10 mmol/L and 200 mmol/L, and are synergistic with Mo in the mixture of TEs. This was earlier confirmed by Lin (1992); and Babich and Stotzky (1983) and was discussed in Section 4.3.2.

The corresponding relative VFA degradation rate, relative VFA retention time, relative CH_4 production and desirability for the beneficial range of the TEs for the different VFA levels are also shown in Table 7.4. Being optimum factor settings, all the responses have relative values > 1.0. For multi-response optimization, it is easier to compare the desirability values of the setting that result in the observed responses. So the desirability value for VFA concentrations 10 and 50 mmol/L are 0.91 and 0.82, which are higher than desirability value for VFA concentration of 100, 150, 200 and 250 mmol/L (0.75, 0.70, 0.64 and 0.62 respectively). The declining desirability as VFA concentration increases is reflected in the decrease in the relative values of the responses as the VFA increases from 10 mmol/L to 250 mmol/L (except for relative VFA degradation rate at 200 mmol/L).

7.2.3 Influence of TEs on VFA degradation rate and CH_4 formation at 55°C: The experimental procedure outlined in Appendix 6.7c also applies to thermophilic batch investigations. The experimental set-up has been described in Figure 6.1, 6.2 and Table 6.4 and the operating temperature of the water bath was 55.5 ± 0.5°C. The methods for the measurements of VFA and CH_4 production have been described in the Table 6.12. The concentrations of the TEs that were supplemented to different levels of the VFA; the measured values for VFA degradation rate and CH_4 production are shown in Appendix 7.5a. Relative VFA degradation rate and relative CH_4 production, and the corresponding % gains and losses were calculated from Equations 6.3f and 6.3g respectively. The relative values of the responses are shown in Appendix 7.5b. Figure 7.12 shows the summary of relative VFA degradation rate and relative CH_4 production at 55°C in different VFA levels supplemented with Ni, Co, Se and Mo.

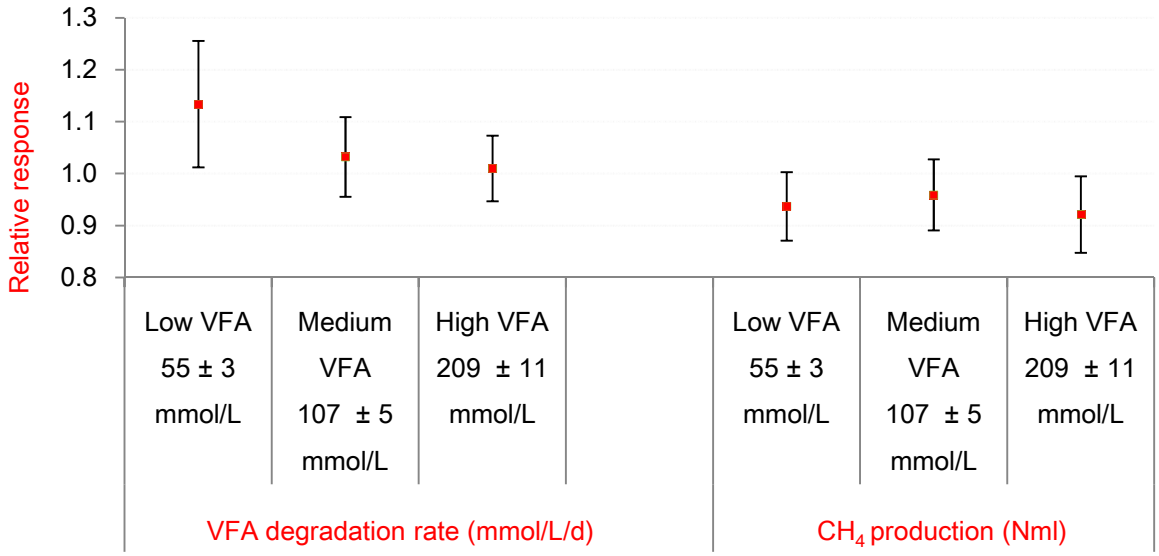
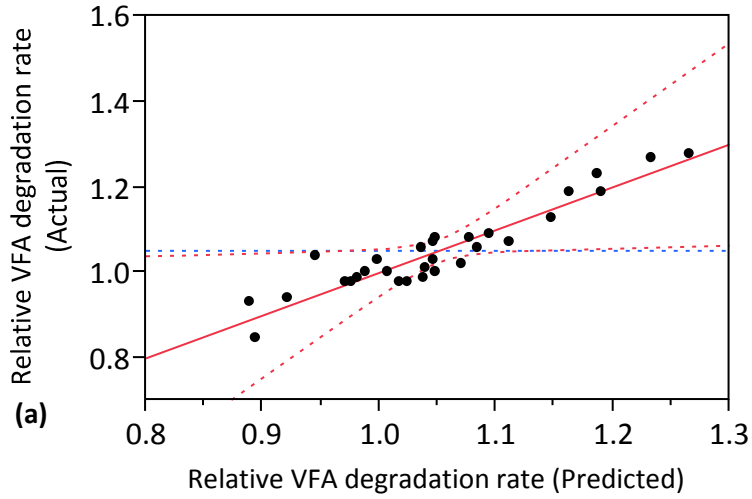


Figure 7.12 Relative VFA degradation rate and CH_4 production at 55°C due to Ni, Co, Se and Mo supplementation to different levels of VFA

The average relative VFA degradation rates in the low, medium and high VFA levels are 1.13, 1.03 and 1.01 respectively. The relative CH_4 productions in the low, medium and high VFA levels are 0.94, 0.96 and 0.92 respectively. The relative VFA degradation rate decreases as VFA concentration increases; and the average relative CH_4 production across all VFA levels is < 1. The most obvious effects are weaker gains in degradation rate and CH_4 production compared to Ni, Co, Se and Mo supplementation at 37°C (Figures 7.5 and 7.11 respectively).

7.2.3.1 Identification and quantification of the factors of influence for VFA degradation rate at 55°C: To quantify the influences of the TEs on VFA degradation rate at 55°C, RSM was used according to Equation 6.4b in Section 6.4.1 for the prediction of the relative VFA degradation rate based on the actual data in Appendix 7.5b. Figure 7.13a shows a plot of the actual relative VFA degradation rate vs. predicted relative VFA degradation rate; and Figure 7.13b shows the model estimates and significance of the factors (Ni, Co, Se, Mo and VFA).



(a)

R^2	0.88
R^2 Adjusted	0.61
Root Mean Square Error	0.062
Mean of Response	1.05
Observations	30

Line of fit (solid line), significance curve at α 0.05 (dotted curves), and line of response mean (dotted horizontal line)

(b)

Term	Estimate	Std Error	t Ratio	Prob>|t|
VFA(mmol/L)	-0.000632	0.000186	-3.39	0.0080*
(Ni(mg/L)-0.871)*(Ni(mg/L)-0.871)	-0.111137	0.0392	-2.84	0.0196*
(Ni(mg/L)-0.871)*(Co(mg/L)-1.79933)	0.0271935	0.009709	2.80	0.0207*
(Co(mg/L)-1.79933)*(Co(mg/L)-1.79933)	0.0249353	0.009034	2.76	0.0221*
Ni(mg/L)	0.0443228	0.017438	2.54	0.0316*
(Co(mg/L)-1.79933)*(Se(mg/L)-0.45733)	-0.046495	0.018489	-2.51	0.0331*

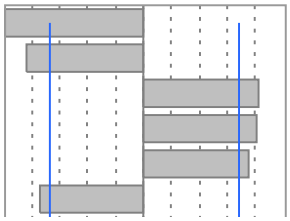


Figure 7.13 **(a)** Plot of actual vs. predicted relative VFA degradation rate at 55° **(b)** Model terms, estimates of parameters and significance of terms in modelling of the relative VFA degradation rate at 55°C due to Ni, Co, Se and Mo supplementation to VFA levels between 55 and 209 mmol/L.

The statistics for Figure 7.13a shows that the prediction has a coefficient of determination (R^2) of 0.88 with a fit error of 0.062. The adjusted R^2 or standardized R^2 value is 0.61. The mean relative VFA degradation rate across all VFA levels due TEs influences is 1.05. Figure 7.13b shows the mean Estimates of the influences of Ni, Co, Se, Mo and VFA on the relative VFA degradation rate in decreasing magnitude (see Section 6.4.1 for explanation of the features of RSM reports and graphs).

The significant main factors include VFA and Ni concentrations. The significant interaction factors include Ni*Co and Co*Se. Positive influences were induced by Ni, Ni*Co and Co*Co. VFA has the most significant influence and this is negative. Other negative TEs influences include extreme Ni*Ni, and interactions between Co*Se. The Estimates of the factors in Figure 7.13b are derived from the mid-concentrations of the factor ranges (also referred to as compromise setting in this report). They give a general indication of the importance and influences of the factors; but do not indicate optimum settings for VFA degradation rate.

7.2.3.2 **Optimum TEs settings for VFA degradation rate at 55°C:** To determine the factor settings that maximize the relative VFA degradation rate, the desirability function was used (Section 6.4.2). The function is shown in Equation 6.5a; the goal for VFA degradation rate is shown in Table 6.5; and the importance level attached to VFA degradation rate relative to other measured responses is shown in Table 6.13. Figure 7.14a, b and c show the optimum TEs settings for VFA degradation rate at low, medium and high VFA levels.

a. Low VFA level: Figure 7.14a shows the prediction profiles for the relative VFA degradation rate and the influences of the factors at low VFA level. The ranges of the beneficial TEs are between 0.8 mg/L and 1.6 mg/L Ni; at least 1.0 mg/L Se; and between 0.4 mg/L and 1.3 mg/L Mo. The optimum TEs configuration is 1.2 mg/L Ni, 1.4 mg/L Se and 1.0 mg/L Mo. The resultant relative VFA degradation rate is 1.32, which corresponds to 32% gain in VFA degradation rate over a non-TEs supplemented AD. The desirability is 0.99.

The prediction profile for Ni suggests that changes in Ni concentration outside the beneficial range will induce a sharp decline in relative VFA degradation rate. Co concentration > 0 mg/L will also induce a significant decline in relative VFA degradation rate, whereas Se concentration ≥ 0.5 mg/L will enhance relative VFA degradation rate. Conversely, changes in Mo concentration within the beneficial range are weakly influential.

b. Medium VFA level: Figure 7.14b shows the prediction profiles for the relative VFA degradation rate and the influences of the factors at medium VFA level. The ranges of beneficial TEs are 0.8 mg/L - 1.6 mg/L Ni; at least 0.5 mg/L Se; and 0.4 mg/L – 1.3 mg/L Mo. The optimum TEs configuration is 1.1 mg/L Ni and 1.6 mg/L Se and 0.8 mg/L Mo. This setting resulted in a relative VFA degradation rate of 1.28; and a desirability of 0.94. The prediction profiles for Ni in Figure 7.14b suggest that changes in Ni concentration outside the beneficial range will induce a sharp decline in relative VFA degradation rate. Co concentration > 0 mg/L will also induce a significant decline in VFA degradation rate, whereas an increase in Se concentration from 0.5 mg/L results in proportional increase in relative VFA degradation rate. Conversely, changes in Mo concentration within the beneficial range are weakly influential.

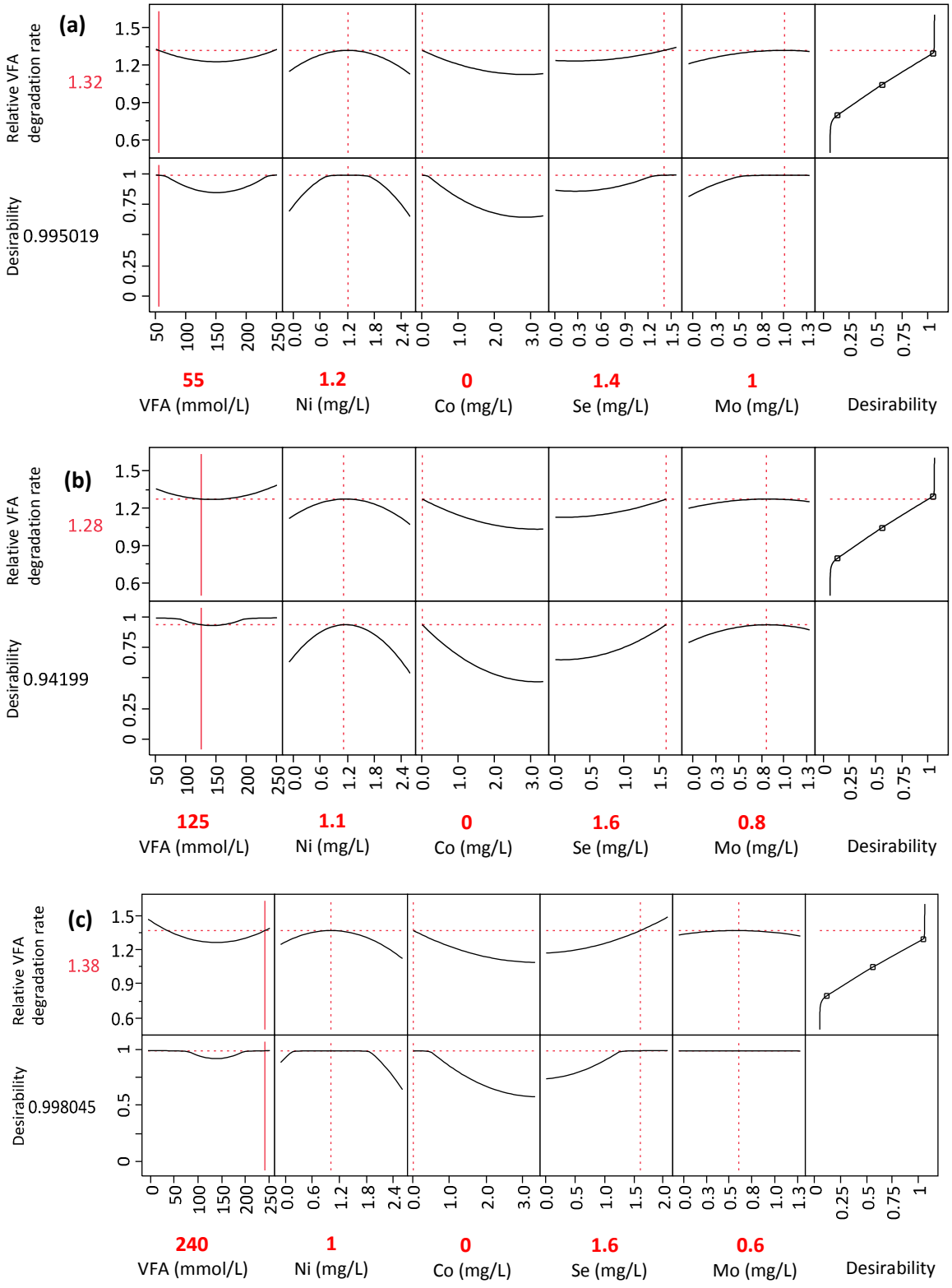


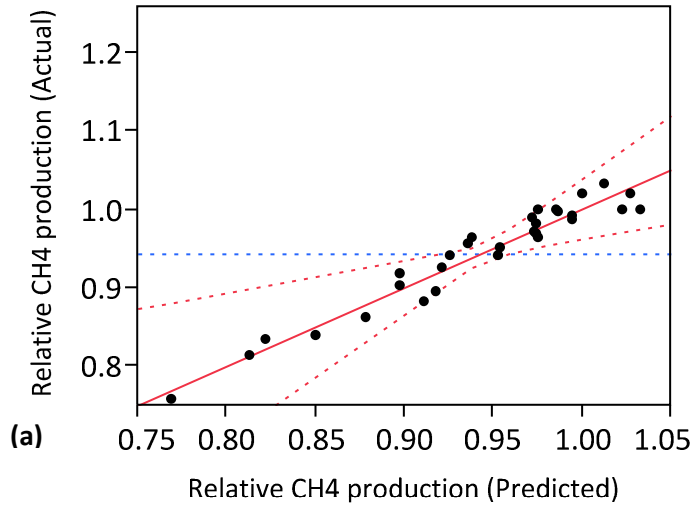
Figure 7.14 Prediction profile and optimum factor settings for relative VFA degradation rate at 55°C due to Ni, Co, Se and Mo supplementation to different levels of VFA **(a)** 55 mmol/L **(b)** 125 mmol/L **(c)** 240 mmol/L

c. High VFA level: Figure 7.14c shows the prediction profiles for the relative VFA degradation rate and the influences of the TEs at high VFA level. The range of the beneficial TEs are 0.2 mg/L – 2.0 mg/L Ni; at least 1.0 mg/L Se; and 0.2 mg/L – 1.2 mg/L Mo. The optimum TEs configuration is 1.0 mg/L Ni, 1.6 mg/L Se, and 0.6 mg/L Mo. This factor setting resulted in relative VFA degradation rate of 1.38; and a desirability of 0.99. The prediction profile for Ni suggests that an increase in Ni concentration beyond 1.6 mg/L induces a sharp decline in relative VFA degradation rate.

Increase in Co concentration from 0 mg/L induces proportional decrease in relative VFA degradation rate; however, the desirability profile suggests that up to 0.5 mg/L Co is tolerable. In contrast to Co, increase in Se concentration results in proportional increase in relative VFA degradation rate. Conversely, changes in Mo concentration within and beyond the beneficial range are weakly influential. The TEs requirements for optimum VFA degradation rate are similar for VFA concentration between 55 mmol/L and 240 mmol/L. However, Mo concentration decreased as VFA concentration increased from 55 mmol/L – 240 mmol/L. This implies that 1.0 mg/L – 1.2 mg/L Ni, 0 mg/L Co, 1.6 mg/L Se, and 0.6 mg/L – 1.0 mg/L Mo would be optimum for methanization at 55°C.

7.2.3.3 Identification and quantification of the factors of influence for relative CH_4 formation at 55°C: To estimate the magnitude of influence that individual factors or combination of factors exert on relative CH_4 production at 55°C, the RSM was implemented as discussed in Section 6.4.1. The relative CH_4 production was predicted using Equation 6.4b. The actual relative CH_4 production is shown in Appendix 7.5b.

Figure 7.15a shows a plot of the actual vs. predicted relative CH_4 production for Ni, Co Se and Mo supplementation at 55°C resulting from the TEs supplementation matrix and relative CH_4 production data contained in Appendix 7.5b. The statistics for Figure 7.15a shows that the model prediction of the relative CH_4 production has a coefficient of determination (R^2) of 0.94 with a fit error of 0.03. The adjusted R^2 or standardized R^2 value is 0.81. The mean relative CH_4 production is 0.94, corresponding to an average of 6% loss in CH_4 production across all VFA levels due TEs influences, compared to a situation of no TEs supplementation.



(a)

R^2	0.94
R^2 Adjusted	0.81
Root Mean Square Error	0.03
Mean of Response	0.94
Observations	30

Line of fit (solid line), significance curve at α 0.05 (dotted curves), and line of response mean (dotted horizontal line)

(b)

Term	Estimate	Std Error	t Ratio	Prob>|t|
(Ni(mg/L)-0.922)*(Ni(mg/L)-0.922)	-0.099155	0.016972	-5.84	0.0002*
(Co(mg/L)-1.81833)*(Mo(mg/L)-0.6)	0.0306952	0.006814	4.50	0.0015*
Ni(mg/L)	0.0305367	0.007634	4.00	0.0031*
(VFA(mmol/L)-130.203)*(Se(mg/L)-0.45733)	0.0006648	0.000188	3.53	0.0064*
(VFA(mmol/L)-130.203)*(Mo(mg/L)-0.6)	0.0004487	0.000156	2.87	0.0185*
Se(mg/L)	-0.03844	0.013969	-2.75	0.0224*
Co(mg/L)	-0.010314	0.003756	-2.75	0.0226*
(VFA(mmol/L)-130.203)*(Co(mg/L)-1.81833)	-0.000134	0.000052	-2.58	0.0299*
(Se(mg/L)-0.45733)*(Mo(mg/L)-0.6)	-0.062769	0.026118	-2.40	0.0397*

Figure 7.15 **(a)** Plot of actual vs. predicted relative CH_4 production at 55°C **(b)** Model terms, estimates of parameters and significance of terms in modelling of the relative CH_4 production at 55°C due to Ni, Co, Se and Mo supplementation to VFA levels between 55 and 209 mmol/L.

Figure 7.15b shows the significant terms and mean Estimates of the influence of Ni, Co, Se, Mo and VFA on the relative CH_4 production in decreasing magnitude (see Section 6.4.1 for explanation of the features of RSM report and graphs). The significant main factors include Ni, Co and Se concentrations. The significant factor interactions include extreme concentrations of Ni, Co and Mo (Ni*Ni, Co*Co and Mo*Mo respectively); interactions of VFA with and Se, Mo and Co (VFA*Se, VFA*Mo VFA*Co); and interaction of Se with Mo (Se*Mo). Positive influences were induced by Ni, Co*Mo, VFA*Se, and VFA* Mo. Extreme Ni concentration has the most significant negative influence on relative CH_4 production. Other negative TEs influences originate from the factors Co and Se, and from the interactions VFA*Co, and Se*Mo.

It is noteworthy that in spite of the enhancing influences of VFA*Se, and VFA*Mo, the interaction of Se*Mo causes significant reduction in CH_4 production. This suggests that simultaneous increase in the concentrations of Se and Mo will cause reduction in CH_4 production. Consequently, it is expected that optimum TEs configurations for CH_4 production in thermophilic methanization will incorporate either Se or Mo, but not both. It can be inferred from Figure 7.15b that the positive influences of TEs on CH_4 production processes are counterbalanced by the negative influences due to TEs toxicity. The Estimates of the factors in Figure 7.15b give a general indication of the importance and influences of the factors; but these do not indicate optimum TEs and VFA interactions for gains in CH_4 production. Therefore, the optimum factor settings are derived next.

7.2.3.4 Optimum TEs setting for relative CH_4 production at 55°C: To determine the factors that maximize CH_4 production, the desirability function was used (Section 6.4.2). The function is shown in Equation 6.5a, the goal for CH_4 production is shown in Table 6.5 and the importance level attached to CH_4 production relative to other measured responses is shown in Table 6.13. Figure 7.16a, b and c show the optimum TEs settings for CH_4 production at low, medium and high VFA levels.

a. Low VFA level: Figure 7.16a shows the prediction profiles for the relative CH_4 production and the influences of the TEs for the low VFA level. The range of beneficial TE is 0.5 mg/L – 1.5 mg/L Ni. The optimum TEs configuration is 0.9 mg/L Ni and resulted in relative CH_4 production of 1.11; and a desirability of 0.99. The desirability profile of VFA suggests that this configuration of TE can maintain desirability of 0.99 up to VFA concentration of 175 mmol/L. The prediction profile for relative CH_4 production suggests that a change in Ni concentration outside the beneficial range results in steep decline in relative CH_4 production. Concentrations of Co, Se and Mo > 0 mg/L induce decline in relative CH_4 production. Comparatively, the decline in relative CH_4 production due to increase in Se is more significant than for Co and Mo. However, the desirability profiles for Co, se and Mo suggest that though 0 mg/L is optimum for these TEs, concentrations up to 1.3 mg/L Co, 0.5 mg/L Se and 0.6 mg/L Mo will not result in significant decline in desirability.

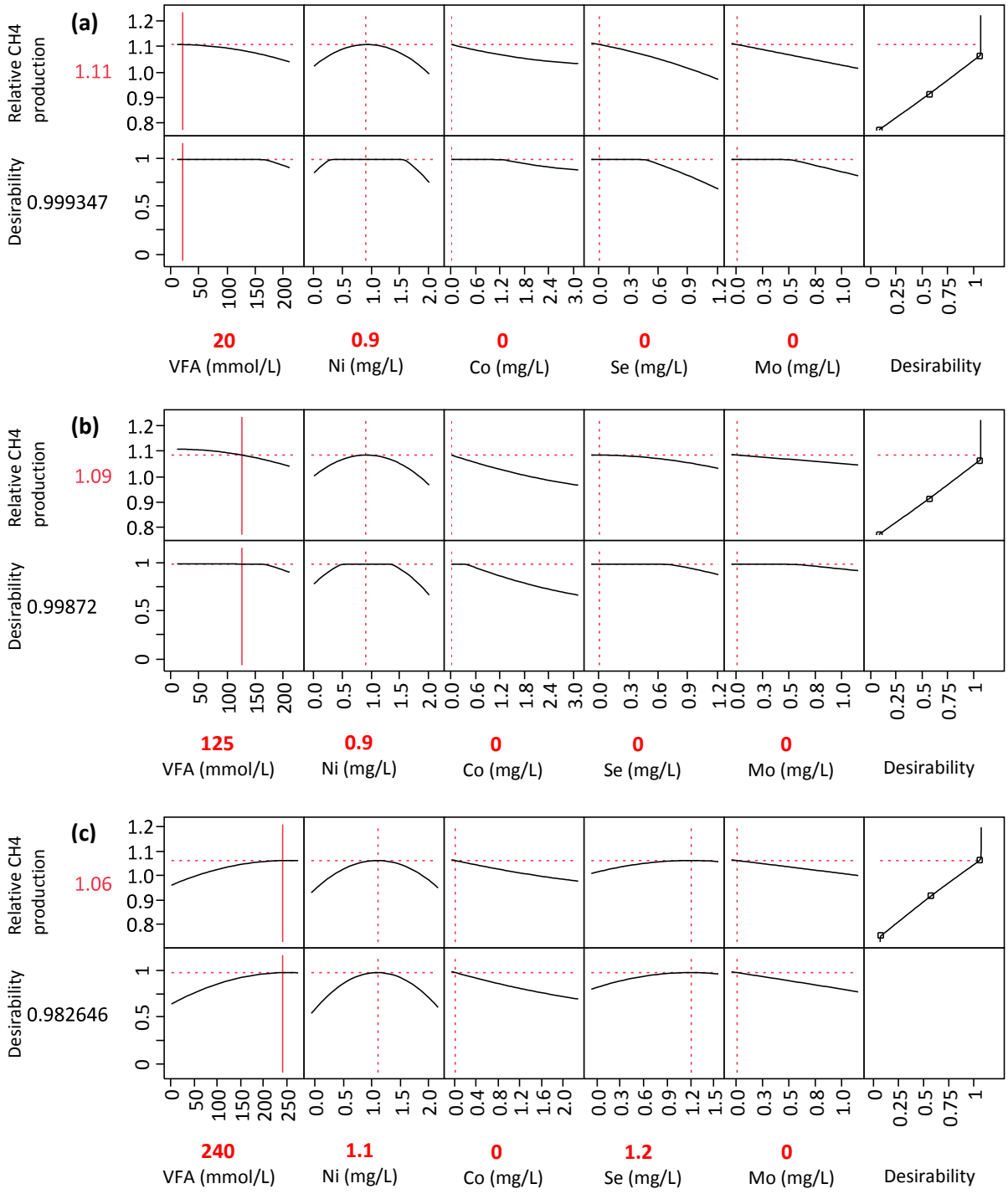


Figure 7.16 Prediction profile and optimum factor setting for relative CH_4 production at 55°C due to Ni, Co, Se and Mo supplementation to different levels of VFA **(a)** 20 mmol/L **(b)** 125 mmol/L **(c)** 240 mmol/L

b. Medium VFA level: Figure 7.16b shows the prediction profile for the relative CH_4 production and the influences of the TEs for the medium VFA level. The range of the beneficial TE and the optimum factor setting is similar to low VFA level at 55°C in (a); and the desirability is also 0.99 but the relative CH_4 production due to the

optimum setting is 1.09 or 9% increase in CH_4 production compared to a system without TEs supplementation. The desirability profiles for Co, Se and Mo suggest that the concentrations of Co, Se and Mo up to 0.4 mg/L, 0.7 mg/L and 0.8 mg/L respectively will not induce sharp decline in desirability. It is noteworthy that Figure 7.15b indicates that Se*Mo has a significant negative influence on CH_4 production. Therefore, the tolerance limits of desirability for Se and Mo concentration imply that an increase in either Se or Mo is tolerated and not both. Since Co is rarely 0 mg/L in AD substrates (Table 7.1), Mo or Se is tolerated, and the influences of Co*Mo and VFA*Mo in Figure 7.15b are positive and significant, then Mo addition to the mixture is preferred to Se.

c. High VFA level: Figure 7.16c shows the prediction profile for the relative CH_4 production and the influence of the TEs for the high VFA level. The ranges of beneficial TEs is about 0.5 mg/L – 1.5 mg/L Ni and 0.6 mg/L – 1.5 mg/L Se. The optimum TEs configuration is 1.1 mg/L Ni and 1.2 mg/L Se, and this TEs configuration resulted in relative CH_4 production of 1.06; and a desirability of 0.98. The desirability profiles suggest that this configuration of TEs is optimum for VFA concentrations ≥ 200 mmol/L. The prediction profile for relative CH_4 production suggests that a change in Ni concentration outside the beneficial range results in significant decline in relative CH_4 production. Concentrations of Co and Mo ≥ 0 mg/L induce decline in optimum relative CH_4 production.

The factor settings in Figure 7.16a, b and c suggest that concentrations of Ni between 0.9 mg/L – 1.1 mg/L are optimum for CH_4 production in a thermophilic operation for digester VFA level up to 240 mmol/L. It also indicates that the absence of Co and Mo in TEs mixture during thermophilic methanization will not result in decline in CH_4 production. Se requirement increased from 0 mg/L in (Figure 7.16a and b) to 1.2 mg/L (Figure 7.16c) as VFA concentration increased from 20 mmol/L – 240 mmol/L. It is noteworthy that in Figure 7.15b, VFA*Se has a significant positive influence on relative CH_4 production. This explains the increase in optimum Se concentration from 0 mg/L at VFA concentrations between 20 and 125 mmol/L to 1.2 mg/L at VFA concentration of 240 mmol/L.

It is obvious that gains in thermophilic methanization due to TEs supplementation were minimal compared to mesophilic methanization discussed in Section 7.2.2.4. It is possible that the low gains were because the MEs of the MPB function optimally at 55°C. Caspi *et al.* (2008) reported that Cobalt-dependent MeTr, which is responsible for transfer of $-CH_3$ to CODH for acetate formation or to MCR for CH_4 production is optimum at 45°C and becomes unstable at 70°C. Similarly, Shin *et al.* (2007) reported that Ni-dependent acetogenic CODH that is responsible for VFA

degradation to acetate, is optimum at 60°C and methanogenic ACS is optimum at 70°C. Consequently, methanization processes in thermophilic operations might be temperature-optimized. It could also be that the interactions between the TEs (Figure 7.15b) are harmful to the MPB; hence they perform sub-optimally.

7.2.3.5 Optimum TEs configuration for multi-responses at 55°C: So far, the influences of TEs on relative VFA degradation rate and relative CH_4 production have been independently evaluated and optimum TEs settings for the individual responses have also been derived and discussed. However, AD processes are overlapping and interdependent; and multi-responses need to be optimized. Consequently, the influence of TEs on relative VFA degradation rate and relative CH_4 production are co-evaluated to derive the optimum factor setting for both responses. The procedure for multi-response optimization was described in Section 6.4.2. The output of the multi-response optimization for different levels of VFA is shown in Table 7.5 for relative VFA degradation rate and relative CH_4 production. The suitable configuration contains the mean values of the factors used in the prediction of the individual responses (Also see Section 6.5).

Table 7.5 Optimum and suitable Ni, Co, Se and Mo configuration for relative VFA degradation rate and relative CH_4 production at 55°C for VFA levels between 10 and 250 mmol/L

Supplementation	VFA	Ni	Co	Se	Mo	Relative VFA degradation rate	Relative CH_4 production	Desirability
	mmol/L	mg/L						
Suitable	130	0.87	1.80	0.46	0.58	1.02	1.01	0.61
Optimum	10	1.14	3.73	0.00	1.24	1.44	1.08	1.00
	50	1.25	3.73	0.00	1.16	1.32	1.07	1.00
	100	1.23	3.73	0.00	1.06	1.21	1.05	0.89
	150	1.08	0.03	1.24	0.17	1.17	1.03	0.81
	200	1.05	0.03	1.24	0.20	1.21	1.04	0.88
	250	1.03	0.03	1.24	0.21	1.30	1.05	0.99

The different levels of VFA have specific TEs configurations that are optimum for the combination of responses as shown in Table 7.5. Nonetheless, optimum Ni concentration is between 1.03 mg/L and 1.25 mg/L. Optimum Co concentration ranges between 0.03 mg/L and 3.73 mg/L; optimum Se concentration is between 0 mg/L and 1.24 mg/L; and optimum Mo ranges between 0.21 mg/L and 1.44 mg/L. Ni has a narrow optimum range; conversely, Co, Se and Mo require significantly different optimum concentrations for different levels of VFA. For VFA concentration between 10 mmol/L and 100 mmol/L, about 3.73 mg/L Co and 0 mg/L Se are required; and between 150 mmol/L and 250 mmol/L VFA, 0.03 mg/L Co and 1.24 mg/L Se are required.

It is noteworthy that in Figure 7.15b, VFA*Co and Se*Mo have significant negative influences on CH_4 production; whereas VFA*Se has a significant positive influence on CH_4 production. This suggests that optimum TEs mixture for enhanced relative CH_4 production will have lower Co concentrations as VFA increases, and increased Se concentration as VFA increases. However, considering that Se*Mo has significant negative influence on CH_4 production in Figure 7.15b, an increase in Se as VFA increases will require a decrease in Mo concentration. Furthermore, in Figure 7.13b, Co*Se has a significant negative influence on VFA degradation rate. This implies that for improved relative VFA degradation rate, an increase in Co concentration will require a decrease in Se concentration.

Therefore, the optimum TEs configuration for enhancement of both CH_4 production and VFA degradation accommodated the relevance of the interactions VFA*Co and VFA*Se; Se*Mo; and Co*Se. This resulted that, as VFA increased from 0 - 250 mmol/L, Se concentration increased from 0 - 1.24 mg/L; and correspondingly, Co concentration decreased from 3.73 - 0.03 mg/L and Mo concentration decreased from 1.24 – 0.21 mg/L. The desirability of the multi-response optimization varies between 0.81 and 1.0. For VFA between 100 and 200 mmol/L, lower desirabilities were obtained (0.81 – 0.89) compared to 1.0 in 10 and 50 mmol/L, and 0.99 at VFA concentration of 250 mmol/L. It seems like the efficiency of the Co*Se interaction at enhancing CH_4 production and VFA degradation decreased as VFA increased from 10 mmol/L – 100 mmol/L; and the efficacy of VFA*Se interaction increased as VFA concentrations increased from 150 – 250 mmol/L.

Summary and conclusion: The gains from supplementation of TEs during mesophilic methanization are strong enough to conclude that it is beneficial to augment substrate TEs content by supplementation of Ni, Co, Se and Mo. Supplementation gains in HAR at high VFA level suggest that reduction in substrate hydrolysis due to accumulation of VFA could be overcome by Co and Ni, or Co and Se supplements. Degradation rate, retention time and CH_4 yield are significantly enhanced by different combinations of TEs. Thermophilic methanization showed weaker gains in CH_4 production and VFA degradation rate as a result of TEs supplementation compared to mesophilic methanization. Optimum TEs settings for methanization at different VFA levels were established for single (independent) and multiple (overlapping) methanization processes at 37°C and 55°C.

7.3 Evaluation of TEs supplementation approaches

Task: *Create supplementation scenarios from the optimum TEs settings identified for the mesophilic methanization; compare relative VFA degradation rate, relative CH_4 production and relative retention time between scenarios; and identify the TEs configuration that can be supplemented across all levels of VFA and that offers optimization in methanization processes that are comparable to TEs supplementation based on different VFA concentration.*

In the analyses of the influences of TEs on the various processes of methanization for mesophilic and thermophilic operation, compromise configurations were shown in the tables of factor settings (Table 7.4 and Table 7.5). The compromise TEs configurations have lower gains in the responses of interest than the optimum TEs configurations. In Tables 6.6, the TEs configuration of the Control reactors was given (Level: low), and in Table 7.4, the compromise and VFA-dependent optimum TEs configurations were shown. In Figures 7.7, 7.11, 7.14 and 7.16 the optimum TEs configurations for CH_4 productions and VFA degradation rates were shown for fixed VFA levels. Control TEs configuration (TC-control), Compromise TEs configuration (TC-compromise), VFA-dependent optimum TEs configuration (OTC-VFA_DL) and the optimum TEs configuration based on the VFA concentration of 120 mmol/L (OTC-VFA_120) have been described in Section 6.5 as scenarios of TEs supplementation.

Appendix 7.6 shows the scenarios, the TEs configurations of the scenarios, and the VFA levels. It also shows the predicted relative responses and the corresponding desirability. Equation 6.4b (Section 6.4.1) was used for the prediction of the relative responses based on actual data in Appendix 7.3b. The coefficients of regression (Estimates) are shown in Appendix 7.3c, d and e for relative CH_4, relative VFA degradation rate and relative VFA retention time respectively. Maximum desirability for the multi-responses was derived using Equation 6.5b as discussed in Section 6.4.2. The statistical analyses were done with JMP 10 software (SAS institute Inc.).

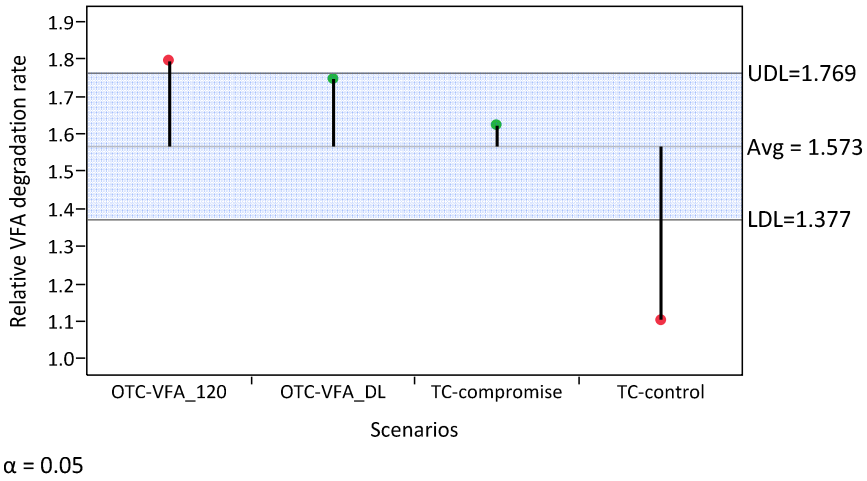
In this Section, the predicted relative VFA degradation rate, relative VFA retention time and relative CH_4 production of the scenarios are compared statistically for methanization at 37°C using the Dunnett's method for Analysis of Mean (ANOM as reported in Nelson *et al.* (2005) and implemented in JMP 10 (SAS Institute Inc.). The statistical details for the calculation of upper and lower decision limits (UDL and LDL) are reported in Nelson *et al.* (2005). Following the procedure of Nelson *et al.* (2005), TEs configuration(s) whose mean(s) for a particular response lie outside the UDL or LDL are significantly different from the other TEs configurations.

7.3.1 Comparing relative VFA degradation rates across the TEs supplementation scenarios: Figure 7.17a shows a comparison of the relative VFA degradation rate across the four scenarios (See Appendix 7.6 for data of the plot). The average relative VFA degradation rate of the scenarios is 1.58, and the LDL and UDL are 1.38 and 1.77 respectively. The average relative VFA degradation rate for the TC-control scenario is 1.11. The TC-compromise dosing has a relative value of 1.63. OTC-VFA_120 scenario has an average relative VFA degradation rate of 1.80; while the OTC-VFA_DL scenario has an average relative VFA degradation rate of 1.75. OTC-VFA_120 scenario has a significantly higher average relative VFA degradation rate compared to the other TEs supplementation scenarios ($\alpha = 0.05$). Conversely, the TC-control scenario has a significantly lower average relative VFA degradation rate compared to the other scenarios ($\alpha = 0.05$).

7.3.2 Comparing relative VFA retention time across the TEs supplementation scenarios: The predicted relative VFA retention times are shown in Appendix 7.6. Figure 7.17b shows a comparison of the relative VFA retention time across the four scenarios. The average relative VFA retention time of the scenarios is 1.31, and the LDL and UDL are 1.20 and 1.42 respectively. The average relative VFA retention time for the TC-control is 1.04. The TC-compromise scenario has a relative VFA retention time of 1.29. OTC-VFA_120 scenario has an average relative VFA retention time of 1.42; while in the OTC-VFA_DL scenario, it is 1.51. OTC-VFA_DL scenario has a significantly higher average relative VFA retention time compared to the other TEs supplementation scenarios ($\alpha = 0.05$). Conversely, the TC-control has a significantly lower average relative VFA retention time compared to the other scenarios ($\alpha = 0.05$).

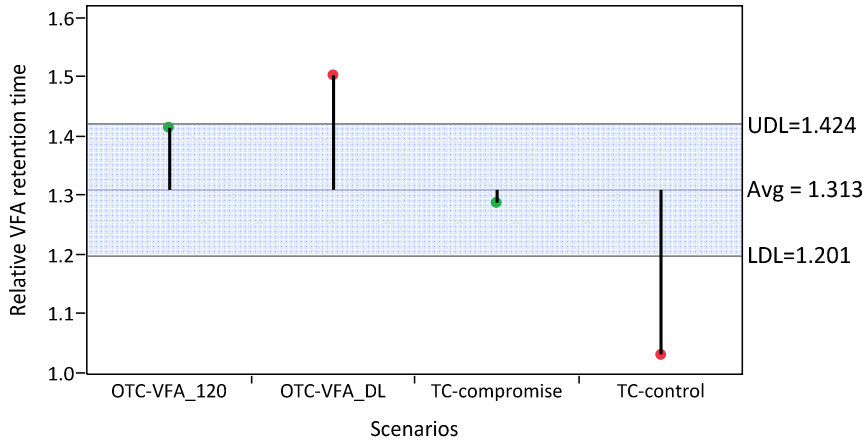
7.3.3 Comparing relative CH_4 production across the TEs supplementation scenarios: The predicted relative CH_4 productions are shown in Appendix 7.6. Figure 7.17c shows a comparison of the relative CH_4 production across all the scenarios. The average relative CH_4 production of the four scenarios is 1.43, and the LDL and UDL are 1.20 and 1.66 respectively. The average relative CH_4 production for the TC-control scenario is 1.04 (4% increase in CH_4 production compared to a reference situation of 0 mg/L Ni, 0 mg/L Co, 0 mg/L Se, 0 mg/L, and 0 mg/L Mo).

(a)



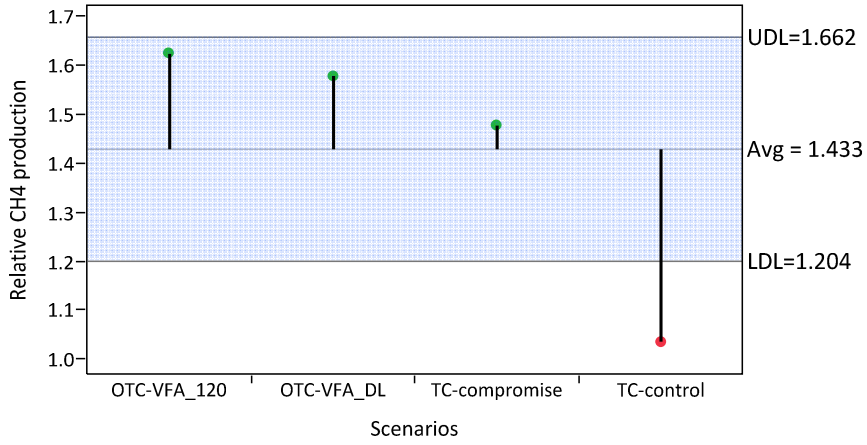
α = 0.05

(b)



α = 0.05

(c)



α = 0.05

Figure 7.17 Comparison of the predicted methanization responses in the four scenarios **(a)** Relative VFA degradation rate **(b)** Relative VFA retention time **(c)** Relative CH_4 production (Avg. = Average; significantly different scenarios are marked in red)

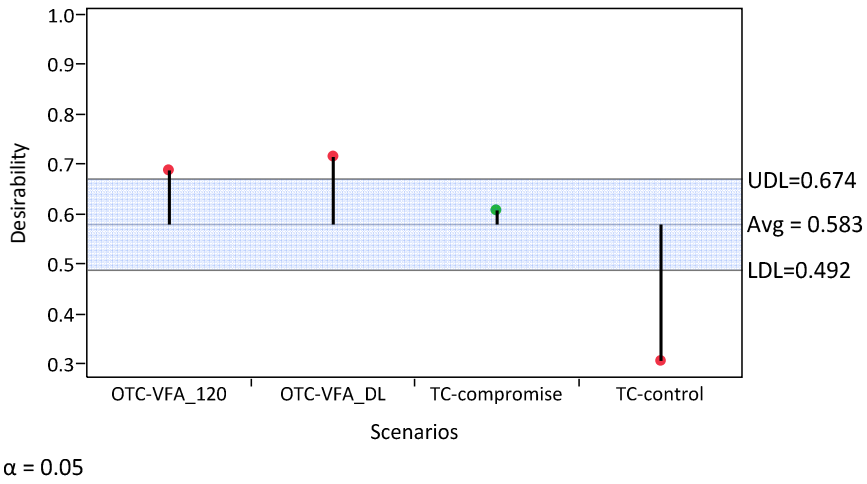


Figure 7.17d Comparison of the desirability for the different supplementation approaches during methanization at 37°C (Avg. = Average; significantly different scenarios are marked in red)

The TC-compromise scenario has a relative CH_4 production of 1.48 (48% increase in CH_4 production). OTC-VFA_120 scenario has an average relative CH_4 production of 1.63 (63% increase in CH_4 production); while the OTC-VFA_DL has 1.58 (58% increase in CH_4 production). Differences in relative CH_4 production exist between the TC-compromise, OTC-VFA_120 and OTC-VFA_DL scenarios; however these are insignificant ($\alpha = 0.05$). The TC-control scenario has a significantly lower average relative CH_4 production compared to the other scenarios ($\alpha = 0.05$). The differences in CH_4 production can only be significantly different when degradation rates are distinctly different or supplementation has an influence on the use of CO_2 for CH_4 formation. The moderately high relative CH_4 production in the OTC-VFA_120 compared to the other scenarios draws from high relative VFA degradation rate shown in Figure 7.17a. Differences in the relative responses in all the scenarios of interest in Figures 7.17a, b and c determine the desirability. This is discussed.

7.3.4 Comparing desirability across the TEs supplementation scenarios: Figure 7.17d shows a comparison of the desirability (proportion of the experimental goal achievable due to the implementation of a scenario) when relative VFA degradation rate, relative VFA retention time and relative CH_4 production were simultaneously optimized. The experimental goal for each scenario was to maximize the relative responses. The desirabilities of the scenarios are shown in Appendix 7.6. TC-control scenario has an average desirability of 0.31 compared to 0.61 in the TC-compromise scenario. The two scenarios with optimum TEs configuration, OTC-VFA_120 and OTC-VFA_DL have average desirability values of 0.69 and 0.72 respectively. The desirability values indicate that an average of 31%, 61%, 69% and 72% of the maximum VFA degradation rate, VFA retention time and CH_4 production is

achievable by implementing TC-control, TC-compromise, OTC-VFA_120 and OTC-VFA_DL respectively.

The low desirability in the TC-control scenario compared to TC-compromise, OTC-VFA_120 and OTC-VFA_DL is consistent with reported low yields in AD operating under conditions that are not optimum (Appels *et al.,* 2008; Chen *et al.,* 2008). The desirability values of the two scenarios with optimum TEs configuration, OTC-VFA_120 and OTC-VFA_DL are similar (0.69 and 0.72 respectively), but differ significantly from the desirability of TC-compromise (0.60). The similarity in desirability between OTC-VFA_120 and OTC-VFA_DL suggests that OTC-VFA_120 could meet TEs methanization requirements for a wide range of VFA concentration, but with weaknesses in certain VFA concentrations.

In Appendix 7.6, the desirability values in the OTC-VFA_120 scenarios for the VFA concentrations of 10 mmol/L and 250 mmol/L are 0.83 and 0.54 respectively; whereas in the OTC-VFA_DL the desirability values for the corresponding VFA levels are 0.89 and 0.59 respectively. The differences in the desirability values are statistically insignificant and could be due to differences in adaption period and VFA retention time (Sections 7.2.2.1 and 7.2.2.5). Nevertheless, the observed enhancements in methanization agree with the reports of Demirel and Scherer (2011) and Pobeheim *et al.* (2010) on improvements in the stability of methanization processes due to TEs supplementation (See Table 4.2 in Section 4.2.1).

Summary and conclusion: The supplementation scenarios OTC-VFA_120 (optimum TEs configuration based on the VFA concentration of 120 mmol/L) and OTC-VFA_DL (VFA-dependent optimum TEs configuration) are comparable in terms of optimization potentials (average desirability value of 0.69 and 0.72 respectively), but less comparable to the TC-compromise scenario (desirability value of 0.60). Therefore, the optimum ranges of Ni, Co, Se and Mo concentrations that are applicable to a wide range of VFA levels will be within the TEs concentrations of the OTC-VFA_120 and OTC-VFA_DL scenarios.

7.4 Mechanisms of gains in responses due to TEs supplementation

Task: *Highlight the underlying mechanisms of enhancement in methanization due to supplementation of TEs by comparing VFA degradation kinetics in the TC-control and OTC-VFA_120 scenarios.*

Figure 7.17a, b, c and d indicate that OTC-VFA_120 scenario is comparable to OTC-VFA_DL scenario and is better than TC-compromise and TC-control scenarios: this has already been discussed in Section 7.3.4. Hence, OTC-VFA_120 scenario has been chosen to represent the TEs supplemented systems, while the TC-control represents methanization without supplementation. In this Section, TC-control and OTC-VFA_120 scenarios are compared to determine the mechanisms of TEs improvement of methanization processes by estimating the kinetic parameters in Michaelis-Menten model. Michaelis-Menten model was discussed in Section 5.3 and the model was shown in Equation 5.11. Michaelis-Menten kinetic parameters (MRR and IA) are related to substrate degradation rate and affinity, and have been described in Sections 5.3.1 and 5.3.2 respectively.

The TEs configurations for TC-control and OTC-VFA_120 scenarios are shown in: Appendix 7.7a for Ni, Co, Se and Mo supplementation at 55°C; Appendix 7.7b for Ni, Co, Se and Mo supplementation at 37°C; Appendix 7.7c for Se and Mo supplementation at 37°C. Equation 6.4a and b for RSM were fitted to measured data for [7]CH_4 production and VFA degradation rates shown in Appendix 7.3a for Ni, co, Se and Mo supplementation at 37°C; Appendix 7.4a for Se and Mo supplementation at 37°C; and Appendix 7.5a for Ni, Co, Se and Mo supplementation at 55°C.

VFA degradation rates were predicted based on the TEs configurations of TC-control and OTC-VFA_120 scenarios for Ni, Co, Se and Mo supplementation at 55°C (Appendix 7.7a); Ni, Co, Se and Mo supplementation at 37°C (Appendix 7.7b); and Se and Mo supplementation at 37°C (Appendix 7.7c). Michaelis-Menten model was fitted to the predicted VFA degradation rate for each scenario and MRR and IA were estimated. MRR and IA for each scenario were compared using Dunnett's test for the analysis of mean as documented by Nelson *et al.* (2005) and implemented in JMP 10 (SAS Institute Inc.).

7.4.1 Michaelis-Menten kinetics for Ni, Co, Se and Mo supplementation at 55°C:

Figure 7.18a shows a fit of the Michaelis-Menten model to the predicted VFA degradation rates for VFA concentrations between 50 mmol/L and 250 mmol/L; and Table 7.6 shows the model estimates for the MRR and IA for methanization at 55°C. MRR and IA are compared and shown in Figure 7.18b and c.

[7] CH_4 production and VFA degradation were regressed jointly since both are interdependent methanization responses.

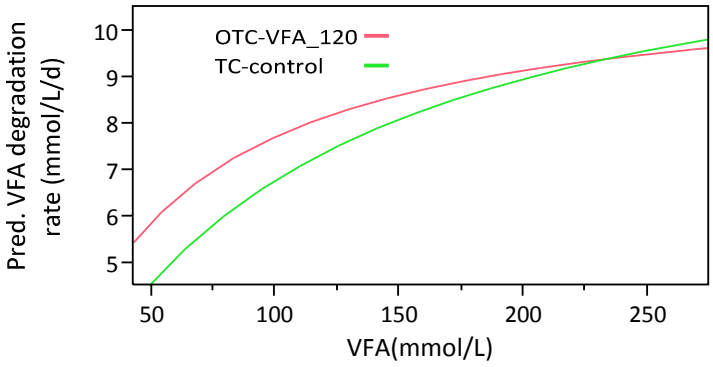


Figure 7.18a Michaelis-Menten model plot of VFA concentrations vs. thermophilic VFA degradation rates due to Ni, Co, Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios

Table 7.6 Estimates of the thermophilic kinetic parameters due to Ni, Co, Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios (CI = Confidence Interval)

Process	Supplementation approach	Parameter	Estimate	Lower 95% CI	Upper 95% CI	Relative parameter
55°C: Ni, Co, Se and Mo	TC-control	MRR (mmol/L/d)	13.2	11.5	14.9	1.00
		IA (mmol/L)	94.6	63.4	125.7	1.00
	OTC-VFA_120	MRR (mmol/L/d)	11.2	10.3	12.1	0.85
		IA (mmol/L)	45.3	31.5	59.1	2.09

7.4.1.1 MRR at 55°C: Figures 7.18b shows a comparison of the MRR between TC-control and OTC-VFA_120 scenarios using the Dunnett's test for analysis of means. The average MRR for both scenarios is 11.7 mmol/L/d: TC-control has an average MRR of 13.3 mmol/L/d, whereas OTC-VFA_120 has an average MRR of 11.2 mmol/L/d. Table 7.6 indicates that the MRR in OTC-VFA_120 scenario declined by 15% (relative MRR 0.85) compared to TC-control; however, this is insignificant (Figure 7.18b), since the MRR in both scenarios are within the limits of the UDL and LDL. The lower and upper 95% CI of the MRR in the OTC-VFA_120 scenario (10.3 and 12.1 mmol/L/d) in Table 7.6 indicate that it is a subset of a wider MRR inherent in the TC-control scenario (11.5 and 14.9 mmol/L/d).

7.4.1.2 IA at 55°C: Figures 7.18c shows a comparison of the IA between TC-control and OTC-VFA_120 scenarios using the Dunnett's test for analysis of means. The average IA for both scenarios is 54.9 mmol/L: TC-control has an average IA of 94.5 mmol/L, whereas OTC-VFA_120 has an average IA of 45.3 mmol/L. Table 7.6 indicates that the IA in OTC-VFA_120 scenario increased by 109% (relative IA of 2.09) compared to TC-control, despite the reduction in MRR. The IA in both scenarios is significantly different from each other at α 0.05. The lower and upper 95% CI of the IA in the OTC-VFA_120 scenario (31.5 and 59.1 mmol/L) in Table 7.6 indicate that it is not a subset of a wider IA inherent in the TC-control scenario (63.4

and 125.7 mmol/L). This suggests that the IA of the microbial population in the OTC-VFA_120 scenario was enhanced and this resulted in better affinity for the VFA substrate compared to the TC-control scenario.

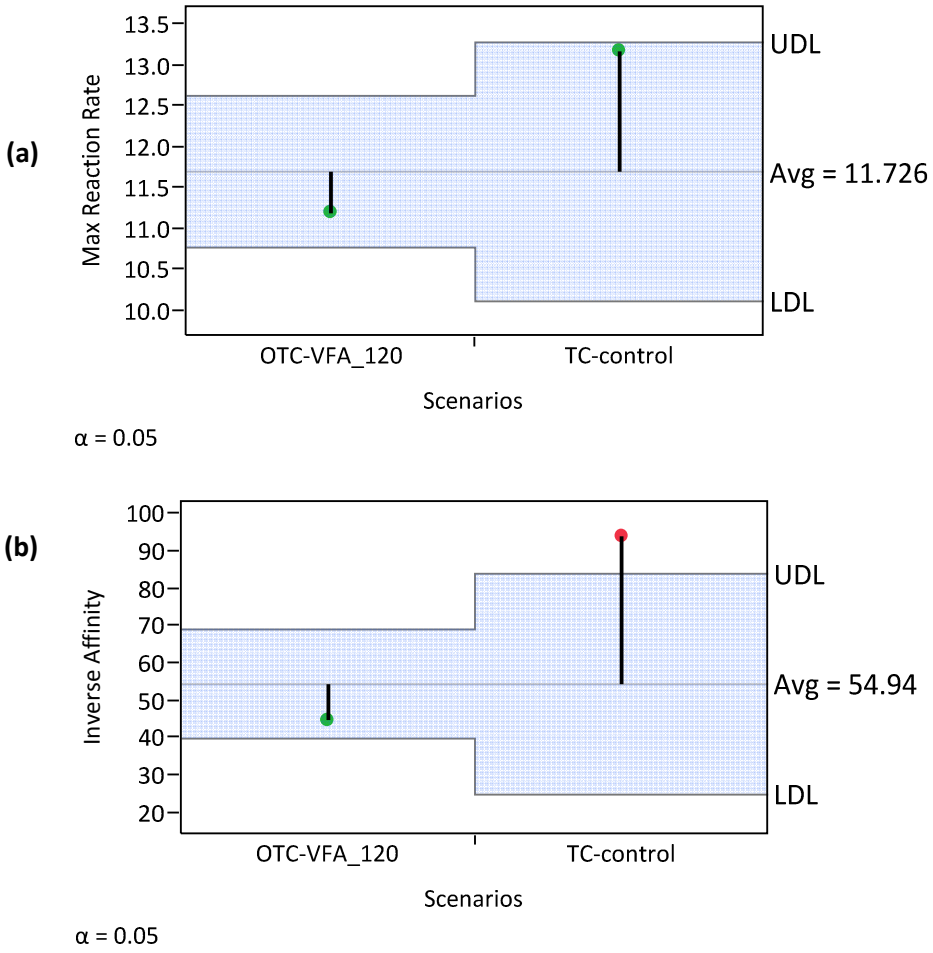


Figure 7.18 Comparison of thermophilic kinetic parameters due to Ni, Co, Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios by ANOM using the Dunnett's test **(b)** maximum reaction rate (mmol/L/d) **(c)** inverse affinity (mmol/L)

7.4.2 Michaelis-Menten kinetics for Ni, Co, Se and Mo supplementation at 37°C:

Figure 7.19a shows a fit of the Michaelis-Menten model to the predicted VFA degradation rates for VFA concentrations between 50 and 250 mmol/L; and Table 7.7 shows the model estimates for the MRR and IA for methanization at 37°C. Figure 7.19b and c show comparison of the MRR and IA in TC-control and OTC-VFA_120 scenarios for VFA degradation rate at 37°C.

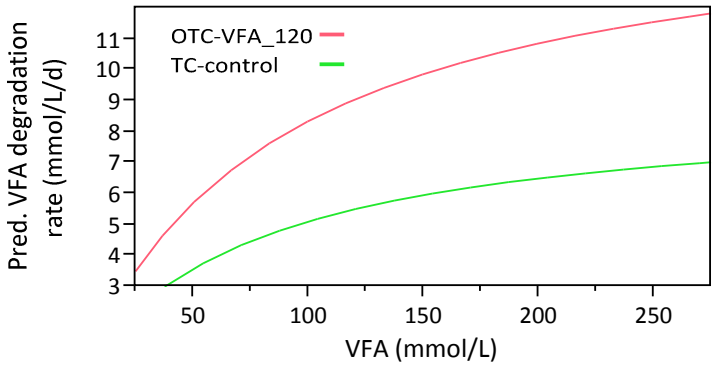


Figure 7.19a Michaelis-Menten model plot of VFA concentrations vs. mesophilic VFA degradation rates due to Ni, Co, Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios

Table 7.7 Estimates of the mesophilic kinetic parameters due to Ni, Co, Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios (CI = confidence interval)

Process	Supplementation approach	Parameter	Estimate	Lower 95% CI	Upper 95% CI	Relative parameter
37°C: Ni, Co, Se, Mo	TC-control	MRR (mmol/L/d)	8.9	8.0	9.8	1.00
		IA (mmol/L)	75.1	52.3	98.0	1.00
	OTC-VFA_120	MRR (mmol/L/d)	15.5	14.5	16.6	1.74
		IA (mmol/L)	86.4	70.7	102.2	0.87

7.4.2.1 MRR at 37°C: Figures 7.19b shows a comparison of the MRR between TC-control and OTC-VFA_120 scenarios using the Dunnett's test for analysis of means. The average MRR for both scenarios is 11.8 mmol/L/d: TC-control has an average MRR of 8.9 mmol/L/d, whereas OTC-VFA_120 has an average MRR of 15.5 mmol/L/d. Table 7.7 indicates that the average MRR in OTC-VFA_120 scenario increased by 74% (relative MRR of 1.74) compared to the TC-control.

The MRR in both scenarios is significantly different from each other at α 0.05. The lower and upper 95% CI of the MRR in the OTC-VFA_120 scenario (14.5 and 16.6 mmol/L/d) in Table 7.7 indicate that it is not a subset of a wider MRR inherent in the TC-control scenario (8 and 9.8 mmol/L/d). This suggests that the MRR of the microbial population in the OTC-VFA_120 scenario was enhanced and the improvement resulted in better VFA degradation rate compared to the TC-control scenario.

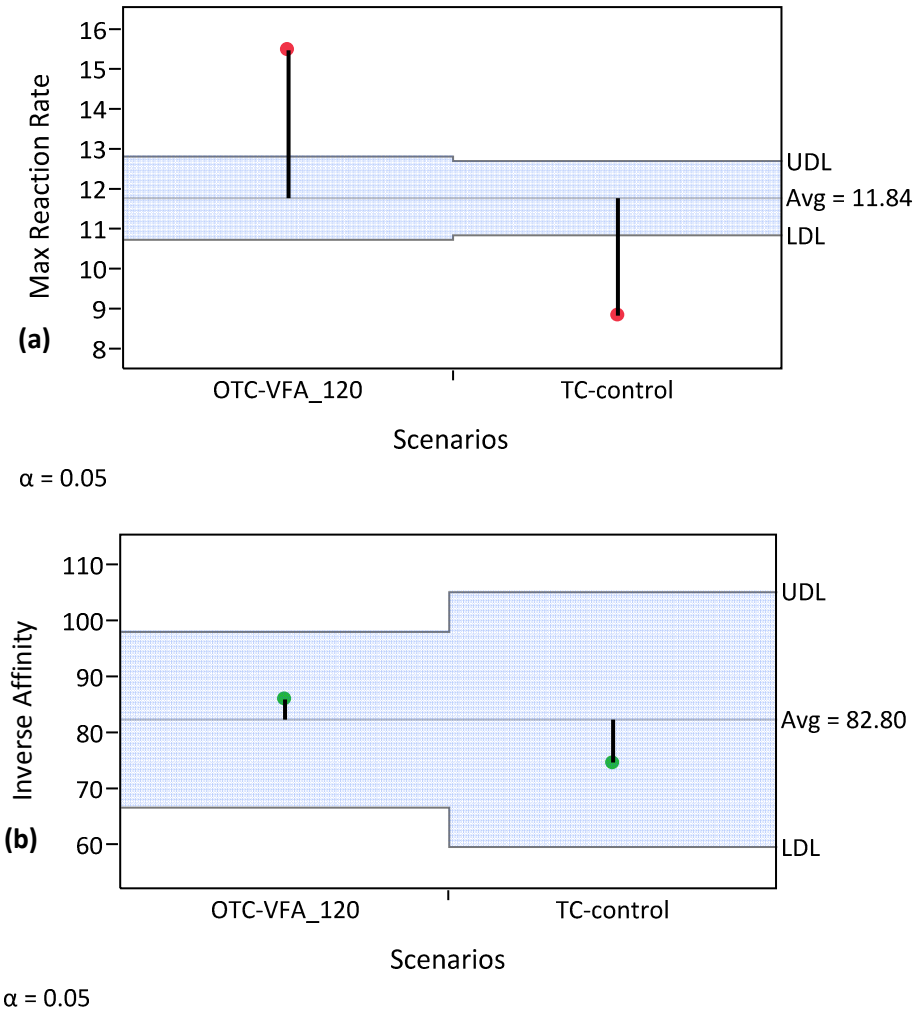


Figure 7.19 Comparison of mesophilic kinetic parameters due to Ni, Co, Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios by ANOM using Dunnett's test **(b)** maximum reaction rate (mmol/L/d) **(c)** inverse affinity (mmol/L)

7.4.2.2 IA at 37°C: Figures 7.19c shows a comparison of the IA between TC-control and OTC-VFA_120 scenarios using the Dunnett's test for analysis of means. The average IA for both scenarios is 82.8 mmol/L: TC-control has an average IA of 75.1 mmol/L, whereas OTC-VFA_120 has an average IA of 86.4 mmol/L. Table 7.7 shows that the IA in OTC-VFA_120 scenario declined by 13% (relative IA 0.87) despite an increase in MRR, when compared to the TC-control scenario. The lower 95% CI (70.7 mmol/L) of the IA in the OTC-VFA_120 scenario in Table 7.7 indicates that the IA of this scenario is a subset of a wider IA inherent in the TC-control scenario (52.3 mmol/L to 98.0 mmol/L). Figure 7.19c confirms that the IA of the TC-control (75.1 mmol/L) is not significantly lower than the IA of the OTC-VFA_120 scenario (86.4 mmol/L/d) at α 0.05. This suggests that the IA of the microbial population in the OTC-VFA_120 scenario was not significantly impaired by TEs supplementation, but some degree of loss in VFA affinity was induced.

Besides, both acetate and butyrate are contained in the experimental VFA mixture (Table 6.6 in Section 6.2.2). So, moderate inhibition of propionate degradation due to acetate and butyrate degradation might have resulted in the loss in affinity for the dominant VFA specie (propionate). Amani *et al.* (2011) also confirmed that in a mixture of VFA, acetate and butyrate inhibit propionate degradation during mesophilic methanization. In addition, according to Ingram-Smith *et al.* (2005), the similarity in the binding pockets of acetate and propionate at the active sites of their respective kinases are similar, and the binding of one could retard the binding of the other.

Ingram-Smith *et al.* (2005) also reported that Ni and other divalent metals activate and modify kinases. Presumably, activation and modification of the VFA binding pockets in the kinases might be responsible for the > 70% increase in MRR during mesophilic methanization in the OTC-VFA_120 scenario. It is expected that the modification would also allow for degradation of acetate, which is an intermediate bye product in both butyrate and propionate oxidation. This could result in minimal reduction in affinity for the dominant VFA specie.

7.4.3 Michaelis-Menten kinetics for Se and Mo supplementation at 37°C: Figure 7.20a shows a fit of the Michaelis-Menten model to the predicted VFA degradation rates for VFA concentrations between 50 and 250 mmol/L (Appendix 7.7c); and Table 7.8 shows the model estimates for the MRR and IA for methanization at 37°C for Se and Mo supplementation. Figure 7.20b and c show comparison of the MRR and IA in TC-control and OTC-VFA_120 scenarios for VFA degradation rate at 37°C for partial supplementation with Se and Mo.

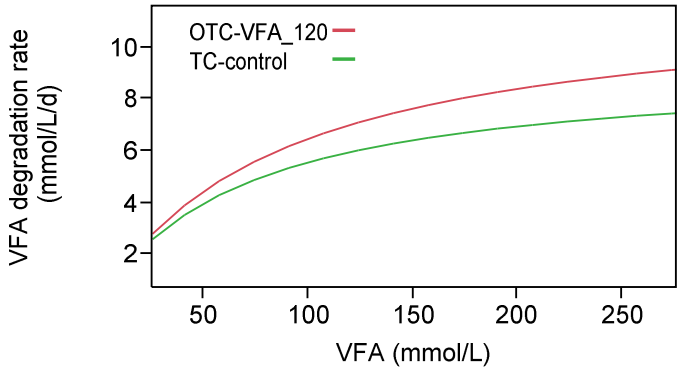


Figure 7.20a Michaelis-Menten model plot of VFA degradation rates vs. VFA concentrations for Se and Mo supplementation at 37°C for TC-control and OTC-VFA_120 scenarios

Table 7.8 Estimates of the mesophilic kinetic parameters due to Se and Mo supplementation in TC-control and OTC-VFA_120 scenarios

Process	Scenarios	Parameter	Estimate	Lower 95%	Upper 95%	Relative parameter
37°C: Se and Mo	TC-control	MRR (mmol/L/d)	9.3	8.0	10.5	1.00
		IA (mmol/L)	66.8	39.1	94.6	1.00
	OTC-VFA_120	MRR (mmol/L/d)	12.0	10.5	13.4	1.29
		IA (mmol/L)	84.8	55.8	113.9	0.79

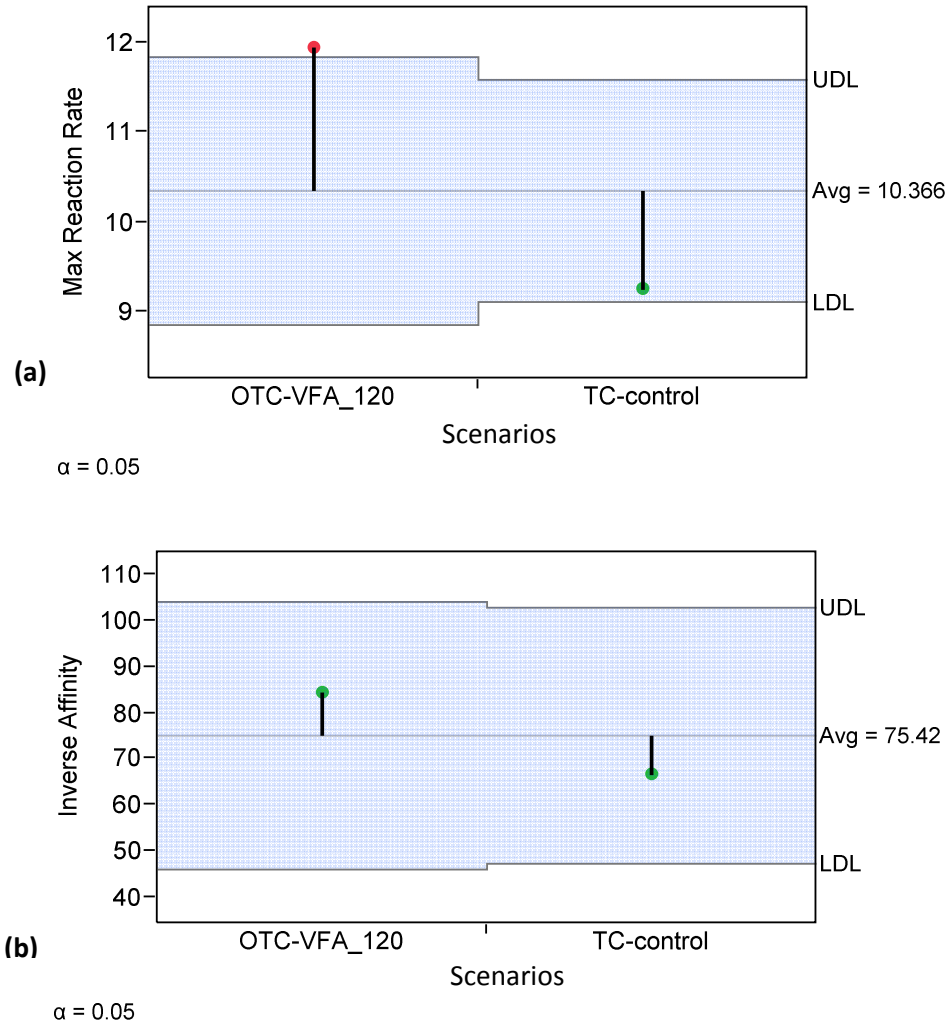


Figure 7.20 Comparison of the mesophilic kinetic parameters due to Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios by ANOM using Dunnett's test **(b)** maximum reaction rate (mmol/L/d) **(c)** inverse affinity (mmol/L)

a. MRR at 37°C: Figure 7.20b shows a comparison of the MRR between TC-control and OTC-VFA_120 scenarios using the Dunnett's test for analysis of means. The average MRR for both scenarios is 10.4 mmol/L/d: TC-control has an average MRR of 9.3 mmol/L/d, whereas OTC-VFA_120 has an average MRR of 12 mmol/L/d

(Table 7.8). Table 7.8 also indicates that the average MRR in OTC-VFA_120 scenario increased by 29% (relative MRR of 1.29) compared to the TC-control scenario. The average MRR in both scenarios is significantly different from each other at α 0.05 (Figure 7.20b). The lower and upper 95% CI of the MRR in the OTC-VFA_120 scenario (10.5 mmol/L/d and 13.4 mmol/L/d) in Table 7.8 indicate that it is not a subset of a narrower MRR inherent in the TC-control scenario (8.0 and 10.5 mmol/L/d). This suggests that the average MRR of the microbial population in the OTC-VFA_120 scenario was enhanced and the improvement resulted in better VFA degradation rate compared to the TC-control scenario.

b. IA at 37°C: Figures 7.20c shows a comparison of the IA between TC-control and OTC-VFA_120 scenarios using the Dunnett's test for analysis of means. The average IA for both scenarios is 75.4 mmol/L: TC-control has an average IA of 66.8 mmol/L, whereas OTC-VFA_120 has an average IA of 84.8 mmol/L. Table 7.8 shows that the IA in OTC-VFA_120 scenario declined by 21% (relative IA 0.79) despite an increase in MRR, when compared to the TC-control scenario. Table 7.8 indicates that the average IA of OTC-VFA_120 (84.8 mmol/L) is within the lower and upper 95% CI of the IA in the TC-control scenario (39.1 and 94.6 mmol/L). Figure 7.20c confirms that the IA of the TC-control (66.8 mmol/L) is not significantly lower (α 0.05) compared to the IA of the OTC-VFA_120 scenario (84.8 mmol/L). This suggests that the IA of the microbial population in the OTC-VFA_120 scenario was not significantly impaired by TEs supplementation, but some degree of loss in VFA affinity was induced.

7.4.4 AD Implications of the kinetic parameters due to TEs supplementation: The implications of the mechanisms of enhancing mesophilic and thermophilic methanization are discussed.

a. Implications of the kinetic parameters for VFA degradation rate at 55°C: Figure 7.21 shows the plot of the predicted VFA degradation rates for scenarios TC-control and OTC-VFA_120 for VFA concentrations between 50 mmol/L and 250 mmol/L. As the VFA increases in concentrations from 50 mmol/L to 250 mmol/L, the difference in rates of VFA degradation decline. At 250 mmol/L, there is no difference in VFA degradation rate between OTC-VFA_120 and TC-control.

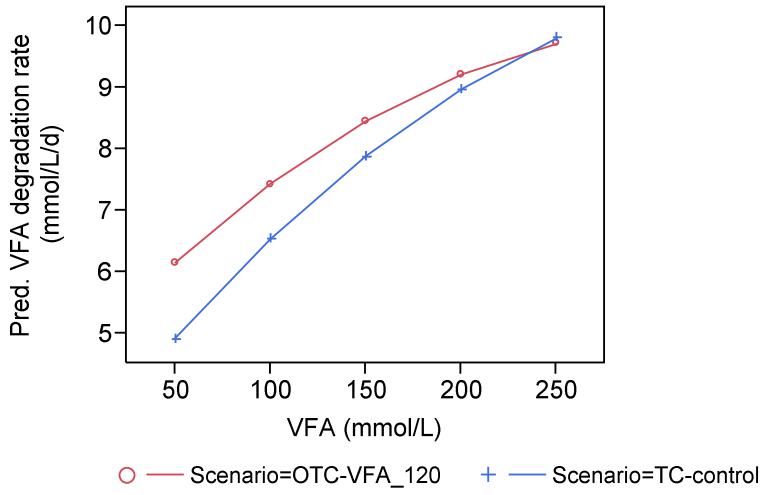


Figure 7.21 Predicted thermophilic VFA degradation rates due to Ni, Co, Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios for VFA concentrations between 50 mmol/L and 250 mmol/L

The TEs supplementation in OTC-VFA_120 scenario enhanced VFA degradation rate until about 150 mmol/L. Apparently, the increase in degradation rate was due to enhanced IA as shown in Figure 7.18c (IA is related to enzymatic affinity for a substrate this was discussed in Section 5.3.2 of Chapter 5). This suggests that substrate affinity is a major challenge in thermophilic methanization at VFA concentration ≤ 150 mmol/L. Figures 7.18b indicates that there is no difference in MRR between TC-control and OTC-VFA_120 due to TEs supplementation at 55°C. This is evident in the similarity in VFA degradation rates in VFA concentrations of 200 and 250 mmol/L (when there is sufficient VFA in the reactor).

Higher VFA degradation rates in the VFA concentrations of 50 mmol/L – 150 mmol/L in OTC-VFA_120 compared to TC-control suggest that TC-control has lower substrate affinity at such VFA concentrations compared to OTC-VFA_120. Nevertheless, at VFA concentrations of 200 and 250 mmol/L, the loss in VFA affinity is non-existent. Consequently, it is possible to overcome VFA affinity challenge in thermophilic methanization by increased substrate loading. In any case, as the VFA concentration decreases over time, VFA degradation rate will decline due to loss in VFA affinity. Therefore, TEs supplementation in methanization at 55°C is necessary to increase the VFA degradation rate at lower substrate concentrations.

b. Implications of the kinetic parameters due to TEs supplementation at 37°C

Figure 7.22a and b show the plots of the predicted VFA degradation rate for scenarios TC-control and OTC-VFA_120 for VFA concentrations between 50 and 250 mmol/L due to TEs supplementation (a) Ni, Co, Se and Mo; and (b) Se and Mo.

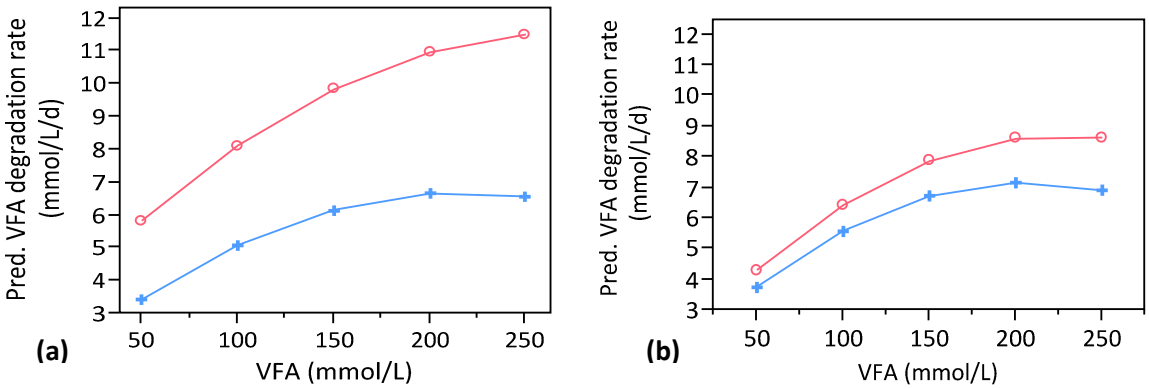


Figure 7.22 Predicted mesophilic VFA degradation rates in OTC-VFA_120 (o) and TC-control (+) scenarios due to **(a)** Ni, Co, Se and Mo supplementation **(b)** Se and Mo supplementation

In Figure 7.22a (Ni, Co, Se and Mo supplementation) scenario OTC-VFA_120 has VFA degradation rates of 5.8 mmol/L/d, 9.8 mmol/L/d and 11.5 mmol/L/d at VFA concentrations of 50, 150 and 250 mmol/L respectively; whereas TC-control has 3.4 mmol/L/d, 6.2 mmol/L/d and 6.6 mmol/L/d respectively for the corresponding VFA concentrations. In Figure 7.22b (Se and Mo supplementation) scenario OTC-VFA_120 has VFA degradation rates of 4.3 mmol/L/d, 7.9 mmol/L/d and 8.6 mmol/L/d at VFA concentrations of 50, 150 and 250 mmol/L respectively; whereas TC-control has 3.8 mmol/L/d, 6.7 mmol/L/d and 6.9 mmol/L/d respectively for corresponding VFA concentrations. The trends in difference between OTC-VFA_120 and TC-control scenarios are similar in both Figure 7.22a and b; but the magnitudes of the differences are different.

OTC-VFA_120 in (a) has higher VFA degradation rates compared to OTC-VFA_120 in (b), and the difference in both is reflected in the MRR of OTC-VFA_120 in the different supplementations as shown in Table 7.7 and Table 7.8. This implies that partial supplementation with Se and Mo has relatively lower enhancement potential in VFA degradation rate for methanization at 37°C compared to a full supplementation with Ni, Co, Se and Mo. This further suggests that Ni and Co were deficient during methanization with Se and [8]Mo supplementation; and this deficiency could have resulted in lower degradation rates when compared to OTC-VFA_120 scenario. The roles of Ni and Co in methanization have been discussed in Section 7.2.

7.4.5 Thermophilic vs. mesophilic methanization with Ni, Co, Se and Mo supplementation: Figure 7.23 compares the predicted VFA degradation rates of the OTC-VFA_120 and TC-control scenarios due to Ni, Co, Se and Mo supplementation at 55°C and 37°C. Scenario OTC-VFA_120 has VFA degradation rates of 5.8

[8] Ni and Co were present in Se and Mo supplementation to the levels contained in the TC-control scenario.

mmol/L/d, 9.8 mmol/L/d and 11.5 mmol/L/d at VFA concentrations of 50, 150 and 250 mmol/L respectively for methanization at 37°C.

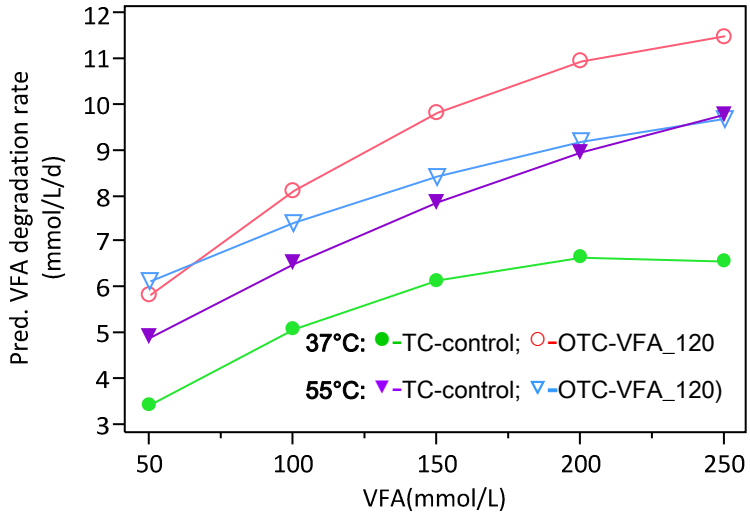


Figure 7.23 Comparison of the predicted mesophilic and thermophilic VFA degradation rates due to Ni, Co, Se and Mo supplementation in OTC-VFA_120 and TC-control scenarios

At 55°C, the predicted VFA degradation rates for OTC-VFA_120 scenario are 6.1 mmol/L/d, 8.4 mmol/L/d and 9.7 mmol/L/d respectively for the corresponding VFA concentrations. The TC-control has VFA degradation rates of 3.4 mmol/L/d, 6.2 mmol/L/d and 6.6 mmol/L/d at VFA concentrations of 50, 150 and 250 mmol/L respectively during methanization at 37°C; whereas at 55°C the rates were 4.9 mmol/L/d, 7.9 mmol/L/d and 9.8 mmol/L/d respectively for the corresponding VFA concentrations. It is evident in Figure 7.23 that the VFA degradation rates in TC-control at 55°C are higher than the VFA degradation rates in TC-control at 37°C.

The VFA degradation rates of TC-control and OTC-VFA_120 are similar at VFA concentrations between 150 and 250 mmol/L for methanization at 55°C. However, at 37°C, the OTC-VFA_120 scenario has higher VFA degradation rates compared to TC-control at 37°C, TC-control at 55°C and OTC-VFA_120 at 55°C. The observation in Figure 7.23 leads to the conclusion that without TEs supplementation, high VFA degradation rates can be achieved at thermophilic methanization temperature. However, the enhancement in VFA degradation rate due to Ni, Co, Se and Mo supplementation in mesophilic methanization is higher than the enhancement due to methanization at thermophilic temperature, with and without TEs supplementation. Furthermore, Ni, Co, Se and Mo supplementations have less positive influence on VFA degradation rate in thermophilic methanization than in mesophilic methanization. This may be related to TEs uptake at different temperatures: this could be

investigated for methanization at 37°C and 55°C. It is noteworthy that the enhancement in VFA degradation rate at 37°C require adaptation period (see Section 7.2.2.1).

Summary and conclusion: The kinetics of VFA degradation enabled the estimation of parameters related to maximum substrate conversion rate and substrate affinity. Supplementation increased maximum reaction rate but did not enhance substrate affinity in mesophilic methanization. The implication of maximizing reaction rate due to TEs supplementation holds huge potential for mesophilic AD, especially for digesters experiencing acidification as a result of propionic acid accumulation. Thermophilic methanization is enhanced through increase in VFA affinity but not maximum reaction rate of VFA degradation.

7.5 Speciation of TEs and use of TE-enriched digestate in agriculture

Task: *Evaluate bioavailability of the different species of TEs; highlight the influence of TEs adsorption to digester solids on bioavailability of Ni, Co, Se and Mo during methanization; evaluate the impact of adsorbed TEs on the quality of digestate used in agriculture; and recommend optimum ranges of Ni, Co, Se and Mo for methanization.*

7.5.1 Speciation of TEs in AD: TEs speciation refers to the different chemical forms in which TEs exist. In AD, the different chemical forms of TEs are adsorbed to different forms of digester solids. The fraction or proportion of TEs that is adsorbed to the different solids in a digester can be quantified through a procedure referred to as sequential extraction (SE). TEs speciation has been discussed in Section 6.7.1 in Chapter 6. The SE method used in this experiment is a 4-step protocol proposed by the Standards, Measurements and Testing programme of the European Union for the determination of the different species of metals in sludge and was reported in Tokalioglu *et al.* (2003).

The procedure of the SE used in this experiment is shown in Appendix 6.10 and was carried out to determine the proportions of Ni, Co, Se and Mo that were bio-available during methanization at 55°C using VFA as substrates (Table 6.6) and silage digestate as inoculum (Appendix 6.7b). The concentrations of Ni, Co, Se and Mo supplemented to the reactors are shown in Appendix 7.5a. The samples for the SE were taken at the end of the experiment from the reactors with medium and high level supplementation of the TEs. The proportion of Ni, Co, Se and Mo ions were quantified in the following stages of the SE and are presented in Figure 7.24:

SE-1: water and acid-soluble ions (exchangeable);
SE-2: ions adsorbed to oxides of Iron (reducible);
SE-3: ions adsorbed to humics and sulphides of Iron (oxidizable); and
SE 4: ions in crystal structure of primary and secondary inorganic minerals (residual).

SE-1 + SE-2 = total bioavailable TEs; and SE-3 + SE-4 = total unavailable TEs.

a. Average proportions of TEs in the different stages of SE during methanization at 55°C: Figure 7.24 shows the average proportion of Ni, Co, Se and Mo in the different stages of the SE. The average proportions of Ni and Mo in the exchangeable stage (SE-1) are 19% and 20% respectively. 28% and 49% of Co and Se respectively, exist in the reactor as exchangeable ions. The proportion of Ni and Co that are bound to oxides of Fe (SE-2) are 26% and 38% respectively. About 10% Mo is bound to oxide of Fe (SE-2); whereas only about 1% of the supplemented Se is

bound to the oxide of Fe (SE-2). Ni has about 15% of its ions bound to FeS (SE-3), while 27% and 37% of Co and Se respectively are adsorbed to FeS (SE-3) in the reactors. About 67% of Mo is adsorbed to FeS. About 4% Mo, 7% Co and 13% Se are adsorbed to crystal structures of mineral particles (SE-4); whereas 39% of Ni is locked up in the crystals structures of inorganic minerals in the reactors.

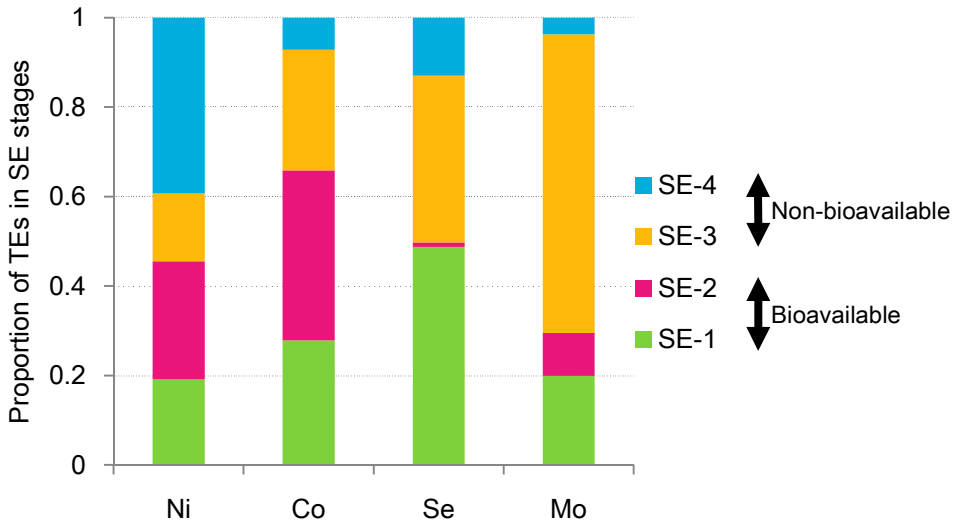


Figure 7.24 Average proportions of Ni, Co, Se and Mo determined by SE after AD at 55°C using VFA as substrate and silage digestate as inoculum

b. Proportions of bioavailable and non-bioavailable TEs during methanization at 55°C: The proportions of Ni, Co, Se or Mo species in SE-1 and SE-2 constitute the total bio-available TE, while the proportions of the species of each of the TEs in SE-3 and SE-4 constitute the proportion of the non-bioavailable TE. Mo is the most non-bioavailable TEs with about 71% of its ions adsorbed to FeS and mineral crystals (SE-3 and SE-4). Next is Ni, with about 54% non-bioavailability. Se and Co are about 40% and 34% non-bioavailable. Co ions are the most bioavailable in the digester (66%), followed by Se (60%). Ni has a total bioavailability of about 45%. Co is more bioavailable than Ni, while Mo is least bioavailable.

Relatively low bio-availability of Mo might be responsible for the report by Banks and Zhang (2012) that Mo supplementation has no influence on methanization. Frausto da Silva and Williams (1991) documented that large proportions of Ni are immobilized in silicate lattices. The authors assumed that such immobilizations are responsible for the rare occurrence and non-availability of Ni in biological environment. The results obtained from this work on the differences in bioavailability of the TEs are in agreement with the findings of Alonso *et al.* (2006) who reported 21% exchangeable Ni and 45% exchangeable Co for sewage sludge, and 56% Mo adsorbed to FeS.

Similar fractions of TEs were reported to be adsorbed to digester solids by Gustavsson (2012) for maize silage digestate and Dabrowska (2012) for sewage sludge digestate.

7.5.2 Digestate use for soil amendment: The European Directive 86/278/CEE specified limits for the content of heavy metals such as lead, cadmium, copper and chromium in digestates and sludges for countries of the European Union (EU). Countries of the EU also set specific concentrations for these heavy metals based on the specifications in this directive. In order to determine whether the optimum ranges of TEs in Table 7.4 and Table 7.5 will impair legal acceptability of the resultant digestates, the concentrations of the optimum TEs were compared with legal limits specified in the European Directive 86/278/CEE.

a. Reviewing the legal limits for TEs in digestates and sludges: A legal limit of 300 - 400 mg/kg DM is specified for Ni concentration in sludges and digestates meant for soil amendment in the EU member states by the European Directive 86/278/CEE. The limit varies in different EU countries since each member country adopts a limit not more than the general limit specified in the directive. There are no legal limits for Mo, Co and Se concentrations in digestates and sludges used for soil enhancement in agriculture in this directive. In Germany, the limit for Ni in sludge meant for soil amendment is 200 mg/kg DM. Similarly, according to the European Directive 86/278/CEE, 3 kg/ha/year of Ni is allowed to be introduced to soil.

b. The optimal experimental Ni concentrations and legal limit of Ni in digestate
During the TEs supplementation in the batch experiments analysed in Sections 7.2, 7.3 and 7.4, the maximum concentration of supplemented Ni was 1.99 mg/L. The maximum total Ni concentration that was required for enhancement of VFA degradation rate and CH_4 production in thermophilic AD was 1.25 mg/L (Table 7.5) equivalent to 54.6 mg Ni/kg [9]DM; and in mesophilic methanization, it was 2.15 mg/L (Table 7.4) equivalent to 94 mg Ni/kg DM. These are less than the permissible limit for Ni in sludge meant for soil amendment in Germany, and much less than is allowed in the European Directive 86/278/CEE.

Since no such limits exist for concentrations of Co, Se and Mo in digestates and sludges, nothing can be said of their supplemented concentrations. Therefore, it can be assumed that based on the current European directive, the optimum Ni configurations for AD, which are shown in Table 7.4 and 7.5 will not impair legal and agricultural acceptability of the resultant digestates.

[9] The conversion of the TEs from mg/L to mg/kg DM is based on a digester TS content of 9.4 ± 1.4%. See Appendix 6.7b.

7.5.3 Setting TEs supplementation ranges based on limits allowed in digestate: The influences of TEs on degradation of VFA and CH_4 production have been clarified in Section 7.2. The influences of VFA and temperature on the requirements of TEs during methanization were also discussed in Section 7.2. Different supplementation approaches have been examined and the proportion of the process goals that is achievable by each approach has been compared in Section 7.3. The mechanisms by which methanization processes are enhanced due to TEs supplementation have been evaluated in Section 7.4. Speciation and bioavailability of TEs have also been discussed in Section 7.5.1 and 7.5.2. Based on the overall observations, supplementation ranges for Ni, Co, Se and Mo can be recommended for AD.

Table 7.9 Proposed ranges for Ni, Co, Se and Mo concentrations in mesophilic and thermophilic methanization

Temperature	Units	Limits	Ni	Co	Se	Mo
37°C	Total (mg/L)	Lower	0.80	2.20	0.00	0.00
		Upper	2.15	4.21	0.53	1.61
	Total (mg/kg DM)	Lower	34.97	96.17	0.00	0.00
		Upper	93.99	184.04	23.14	70.38
55°C	Total (mg/L)	Lower	1.03	0.03	0.00	0.17
		Upper	1.25	3.73	1.24	1.24
	Total (mg/kg DM)	Lower	45.03	1.31	0.00	7.43
		Upper	54.64	163.06	54.21	54.21

Table 7.9 gives the ranges of Ni, Co, Se and Mo in mesophilic and thermophilic methanization that will optimize AD processes. These ranges are derived from the TEs concentrations predicted to be optimum in methanization involving 10 mmol/L - 250 mmol/L VFA as discussed in the various Sections of this document and shown in Table 7.4 and Table 7.5 for mesophilic and thermophilic methanization respectively. Since the interactions between these elements are important for AD with different concentrations of VFA, the choice of specific concentrations for each TE should be in relation to other TEs. Consequently, the full RSM model terms, estimates and significance for CH_4 production, VFA degradation rate and VFA retention time at 37°C due to Ni, Co, Se and Mo supplementation are provided in Appendix 7.3c, d and e. For Ni, Co, Se and Mo supplementation at 55°C, the full RSM model terms, estimates and significance for CH_4 production and VFA degradation rate are provided in Appendix 7.5c and d; and for Se and Mo supplementations at 37°C, these are found in Appendix 7.4c and d.

Summary and conclusion: This Section showed that different TEs have different proportions that are bioavailable during methanization. Se and Co are most bioavailable, while Ni and Mo are least bioavailable. The proportions of TEs that are non-bioavailable are adsorbed on the digestate that could be utilized in agriculture. Optimum TEs concentration for methanization presented in this report will not result in higher Ni content in digestate than the legal limit stipulated in the European Directive 86/278/CEE. Finally, optimum ranges for Ni, Co, Se and Mo have been recommended for mesophilic and thermophilic methanization.

7.6 Validation of the optimum TEs formulations for mesophilic methanization

Task: *Derive VFA-independent optimum TEs configuration from the VFA-dependent optimum mesophilic TEs configurations shown in Table 7.4; develop variants of the VFA-independent optimum TEs configuration; evaluate the efficiency of the VFA-independent optimum TEs configuration and its variants in stabilizing mesophilic methanization in a semi-continuous substrate loading operation; and validate the mechanism of enhancement in methanization processes.*

This section presents the result of supplementing a derived VFA-independent optimum TEs configuration and its variants in a semi-continuous AD operation using mixed fruit residue (MFR) as an example of a complex AD substrate (Section 7.1.1). The VFA-independent optimum TEs configuration was derived from the TEs configurations shown in Table 7.4 as optimum for different concentrations of VFA in a batch system as analysed in Sections 7.2. The VFA-independent optimum configuration was further modified to reflect the weakness that is possible in the use of one configuration of TEs for the optimization of AD. The VFA-independent optimum TEs configuration that was derived from the TEs configurations in Table 7.4, and the modifications to the individual concentrations of the TEs were used in a semi-continuous AD in order to validate their efficiency in VFA degradation, process stability and CH_4 production. Changes in bacterial population were monitored during the semi-continuous AD operation as a way of validating mechanisms of TEs enhancement of methanization processes.

7.6.1 Formulating the VFA-independent Optimum TEs configuration: The VFA-dependent TEs configurations for multi-response optimization during methanization of VFA in batch AD operation were shown in Table 7.4 and discussed in Section 7.2.2.9. Based on these concentrations, a single optimum TEs configuration that is independent of VFA concentration was derived and its variants were developed to highlight inherent weaknesses in the use of single TEs configuration for the optimization of AD that involves changing VFA concentration or varying organic loading rate (OLR). The derived VFA-independent Optimum TEs configurations simply referred to as ‘Optimum’ in the subsequent texts and the variants of the ‘Optimum’ are shown in Table 7.10. The Control is the TEs configuration in the inoculum used for the investigation. The considerations for the derivation of the concentrations of TEs in the Optimum and its variants are discussed next.

Table 7.10 TEs configuration of the 'Optimum' and its variants used for dosing in the semi-continuous validation investigation

Supplementation arrangements (Reactors)	mg/L			
	Ni	Co	Se	Mo
Control (R-1)	0.09	0.04	0.00	0.04
Optimum (R-2)	1.46	2.70	0.40	0.50
Optimum –Co (R-3)	1.46	0.50	0.40	0.50
Optimum +Se (R-4)	1.46	2.70	1.50	0.50

a. Optimum Ni concentration: From Table 7.4, the concentration range of Ni that is beneficial for VFA concentration of 10 mmol/L – 200 mmol/L is 1.88 – 2.15 mg/L, whereas 0.8 mg/L Ni is optimum for 250 mmol/L VFA. Apparently, Ni requirement decreases as VFA concentration increases. Hence, any Ni concentration between 1.88 and 2.15 mg/L will enhance VFA degradation when the VFA concentration is between 10 mmol/L and 200 mmol/L, but ≤ 0.8 mg/L Ni is required for 250 mmol/L VFA. Therefore, any Ni concentration that is ≥ 0.80 mg/L but ≤ 2.15 mg/L is a good compromise for VFA concentration between 10 mmol/L and 250 mmol/L. Based on this, 1.46 mg/L was preferred for Ni concentration (Table 7.10).

b. Optimum Co concentration: From Table 7.4, the concentration range of Co that is beneficial for VFA concentration of 10 mmol/L – 200 mmol/L is 3.62 mg/L – 4.21 mg/L, whereas 2.20 mg/L Co is optimum for 250 mmol/L VFA. Apparently, Co requirement decreases as VFA concentration increases. Hence, Co concentrations between 3.62 mg/L and 4.21 mg/L will enhance VFA degradation when the VFA concentration is between 10 mmol/L and 200 mmol/L; however, < 3.62 mg/L Co is required at VFA concentration of 250 mmol/L. Therefore, any Co concentration ≥ 2.20 mg/L but ≤ 4.21 mg/L is a good compromise for VFA concentration between 10 mmol/L and 250 mmol/L. Based on this, 2.70 mg/L was preferred for Co concentration (Table 7.10).

c. Optimum Se concentration: From Table 7.4, the concentration range of Se that is beneficial for VFA concentration of 10 mmol/L – 200 mmol/L is 0 mg/L – 0.32 mg/L, whereas 0.53 mg/L Se is optimum for 250 mmol/L VFA. Apparently, Se requirement decreases as VFA concentration increases up to 200 mmol/L. However, Se requirement increases at VFA concentration of 250 mmol/L. Hence, Se concentration ≤ 0.32 mg/L will be a good compromise for VFA concentration between 10 mmol/L and 200 mmol/L. However, at VFA concentration of 250 mmol/L, > 0.32 mg/L is more beneficial. Since high organic loading rate will be favoured in this research, Se requirement for VFA concentration of 250 mmol/L is given priority. Therefore, Se concentration ≥ 0.32 mg/L but ≤ 0.53 mg/L is a good compromise for VFA

concentration between 10 mmol/L and 250 mmol/L. Based on this, 0.4 mg/L was preferred for Se concentration (Table 7.10).

d. Optimum Mo concentration: From Table 7.4, the concentration range of Mo that is beneficial for VFA concentration of 10 mmol/L – 200 mmol/L is 1.61 mg/L, whereas for 250 mmol/L VFA, 0 mg/L Mo is optimum. Apparently, Mo requirement is constant for VFA concentration between 10 mmol/L and 200 mmol/L, but decreases to 0 mg/L as VFA concentration increases from 200 mmol/L to 250 mmol/L. Hence, Mo concentration < 1.61 mg/L will be a good compromise for VFA concentration between 10 mmol/L and 250 mmol/L. To find the preferred concentration of Mo that will not produce negative interaction with the concentrations decided for Ni, Co and Se, Figure 7.25 is considered (See Section 7.6.2 for an understanding of the reason for the choice of this RSM).

Using the values for the determination of the Estimates as reference (values in bracket), VFA*Mo and Ni*Mo interactions have positive Estimate values, suggesting that as VFA concentration increases or decreases from 127 mmol/L, corresponding increase or decrease in Mo concentration from 0.64 mg/L is required. Also, as Ni concentration increases or decreases from 0.86 mg/L, corresponding increase or decrease in Mo from 0.64 mg/L is required. However, the Estimates for Se*Mo and Co*Mo interaction are negative. Se concentration > 0.47 mg/L requires that Mo concentration is < 0.64 mg/L to avoid negative influence of Se*Mo interaction on VFA degradation rate, and vice versa. Since 0.4 mg/L Se was decided upon (< 0.47 mg/L), Mo > 0.64 mg/L could be supplemented without negative Se*Mo influences. However, Co concentration > 1.88 mg/L was decided upon; hence, to avoid negative Co*Mo interactions, Mo < 0.64 mg/L is required. Since Se*Mo is not significant in the model, but Co*Mo is, priority was given to Co*Mo interaction over Se*Mo interaction. Based on these considerations, 0.5 mg/L was decided upon for Mo concentration (Table 7.10).

7.6.2 Modifying the Optimum TEs configuration for various VFA concerns: In formulating the TEs concentrations for the Optimum treatment and its variants, it was assumed that since FOS/TAC is the ratio of VFA concentration to alkalinity, enhancing VFA degradation rate will prevent VFA accumulation and keep FOS/TAC within the optimum range (see Section 3.3.3). Hence, the RSM for relative VFA degradation rate, which was discussed in Section 7.2.2.7, was used for the TEs formulation. Figure 7.25 shows the full model terms, Estimates of parameters and significance of terms in modelling of relative VFA degradation rate at 37°C as a result of Ni, Co, Se and Mo supplementation to VFA concentrations between 28 and 213 mmol/L.

Term	Estimate	Std Error	t Ratio	Prob>|t|
(VFA (mmol/L)-127.888)*(VFA (mmol/L)-127.888)	0.0000451	3.526e-6	12.79	0.0061*
Ni (mg/L)	0.1749563	0.013744	12.73	0.0061*
(VFA (mmol/L)-127.888)*(Se(mg/L)-0.4687)	0.0027372	0.000239	11.43	0.0076*
Co(mg/L)	0.0705259	0.00622	11.34	0.0077*
(Ni (mg/L)-0.86304)*(Mo(mg/L)-0.64)	0.2367762	0.024884	9.52	0.0109*
(Ni (mg/L)-0.86304)*(Ni (mg/L)-0.86304)	-0.223257	0.030293	-7.37	0.0179*
(Co(mg/L)-1.88)*(Mo(mg/L)-0.64)	-0.088795	0.012672	-7.01	0.0198*
Mo(mg/L)	-0.126642	0.018658	-6.79	0.0210*
(VFA (mmol/L)-127.888)*(Mo(mg/L)-0.64)	0.0018035	0.000277	6.52	0.0227*
VFA (mmol/L)	-0.00064	0.000114	-5.60	0.0305*
(Ni (mg/L)-0.86304)*(Se(mg/L)-0.4687)	0.1403069	0.027198	5.16	0.0356*
(Co(mg/L)-1.88)*(Co(mg/L)-1.88)	-0.037988	0.008043	-4.72	0.0420*
(Ni (mg/L)-0.86304)*(Co(mg/L)-1.88)	0.0352149	0.008667	4.06	0.0556
(Co(mg/L)-1.88)*(Se(mg/L)-0.4687)	-0.04363	0.014607	-2.99	0.0962
(Se(mg/L)-0.4687)*(Mo(mg/L)-0.64)	-0.097343	0.042507	-2.29	0.1492
Se(mg/L)	-0.049307	0.022585	-2.18	0.1607
(VFA (mmol/L)-127.888)*(Co(mg/L)-1.88)	-0.000119	8.58e-5	-1.39	0.2984
(Mo(mg/L)-0.64)*(Mo(mg/L)-0.64)	-0.056982	0.070525	-0.81	0.5039
(Se(mg/L)-0.4687)*(Se(mg/L)-0.4687)	-0.105383	0.133352	-0.79	0.5122

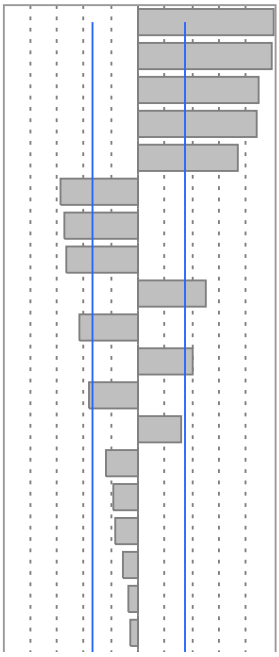


Figure 7.25 Model terms, Estimates of parameters and significance of terms in modelling of the relative VFA degradation rate at 37°C due to Ni, Co, Se and Mo supplementation to VFA concentrations between 28 and 213 mmol/L

With emphasis on the orientation (positive or negative values of Estimate) of VFA*TE interactions, VFA*Se, VFA*Mo and VFA*Ni are positive. This suggests that for optimum VFA degradation rate, Se, Mo and Ni concentrations are required to increase as VFA concentration increases. However VFA*Co is negative and indicates that for optimum VFA degradation, an increase in VFA from 128 mmol/L requires corresponding decrease in Co from 1.88 mg/L. Therefore, two variants of the TEs configuration of the Optimum were derived to reflect an increase in the concentration of Se as VFA concentration increases (Optimum +Se); and to reflect a decrease in the concentration of Co as VFA concentration increases (Optimum –Co). Consequently, the Se concentration was increased from 0.4 mg/L in the TEs configuration of the Optimum to 1.5 mg/L in the Optimum +Se variant; and Co concentration was reduced from 2.7 mg/L in the TEs configuration of the Optimum to 0.5 mg/L in the Optimum –Co variant (Table 7.10).

7.6.3 Optimization efficiencies of the Optimum TEs configuration and its variants: In the text, Optimum –Co and Optimum +Se are also referred to as '–Co' and '+Se' variants of the TEs configuration of the Optimum: the – and + signs indicate less Co and more Se respectively compared to the Co and Se concentrations of the Optimum. Mixed fruit residue (MFR) was used as the complex substrate in this semi-continuous AD operation in contrast to the VFA mixture used in the batch experiments described in Sections 7.2 – 7.5. The TEs content, C-N-S- and sugar

contents of the MFR have been discussed in Section 7.1 and shown in Tables 7.1, 7.2 and 7.3 respectively. The experimental inoculum used in the semi-continuous experiments was obtained from the same AD plant in Hamburg where inocula used for the batch investigations were collected (Appendix 6.7b). The experimental procedure has been discussed in Section 6.8.2 of Chapter 6; and outlined in Appendix 6.7c. The experimental set-up was shown in Figure 6.3 in Chapter 6. The methods of analyses for the parameters discussed in this section are shown in Table 6.14 of Chapter 6.

7.6.3.1 Influence of the TEs configurations on FOS/TAC: The stability of the methanization process was influenced by frequently varying the organic loading rate (OLR) of the MFR that was fed to the reactors. Figure 7.26a shows OLR that were loaded to the reactor and the total organic material fed to the Control, Optimum, Optimum –Co and Optimum +Se reactors in any given time. Figure 7.26a also shows the plot of FOS/TAC vs. OLR of the reactors. The methanization time is divided into four intervals based on the OLR: 2g oDM/L/d, 4g oDM/L/d, 8g oDM/L/d, and 6g oDM/L/d. FOS/TAC values between 0.15 – 0.45 are regarded as indicative of stability in the methanization process; and FOS/TAC > 0.6 indicate the onset of instability (Voss *et al.,* 2009).

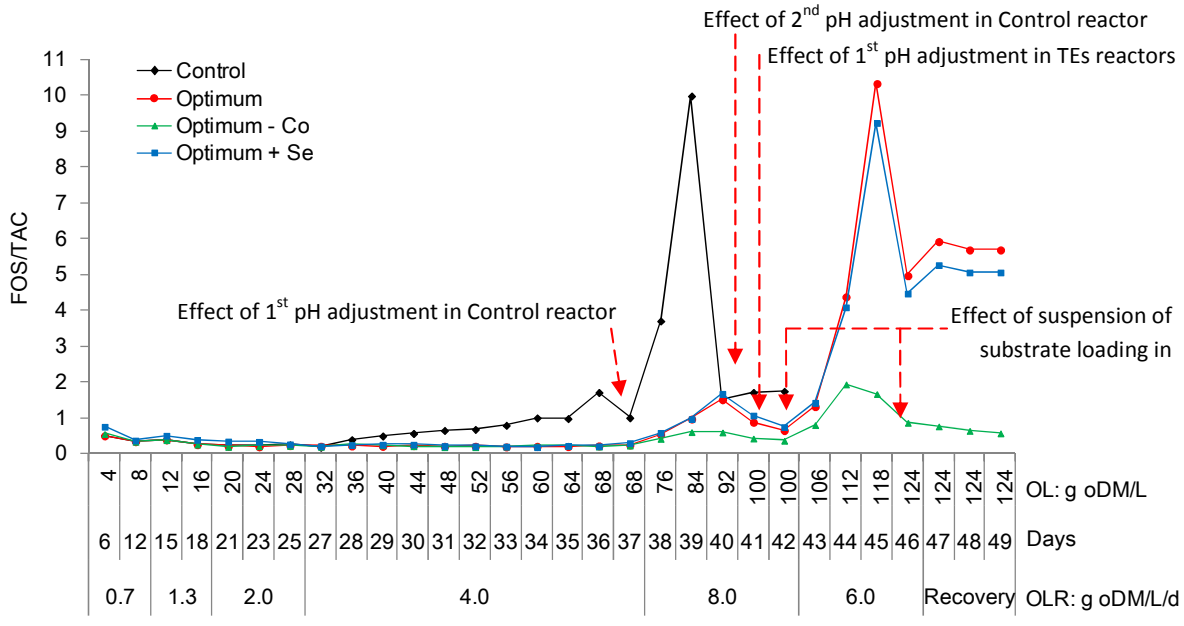


Figure 7.26a Overview of FOS/TAC of the reactors due to Ni, Co, Se and Mo supplementation and different OLR in the mesophilic semi-continuous experiment with mixed fruit residue.

Figure 7.26b shows the relationship between FOS/TAC, pH, TAC (alkalinity measured as the equivalent of $CaCO_3$ in the reactor), and FOS. In Figure 7.26a and

b, there is a general increase in FOS/TAC value as the OLR increases. Furthermore, there is a general decrease in alkalinity (TAC) as the acid content (FOS) of the reactors increase. Apparently, decrease in pH generally accompanies increases in FOS/TAC and FOS.

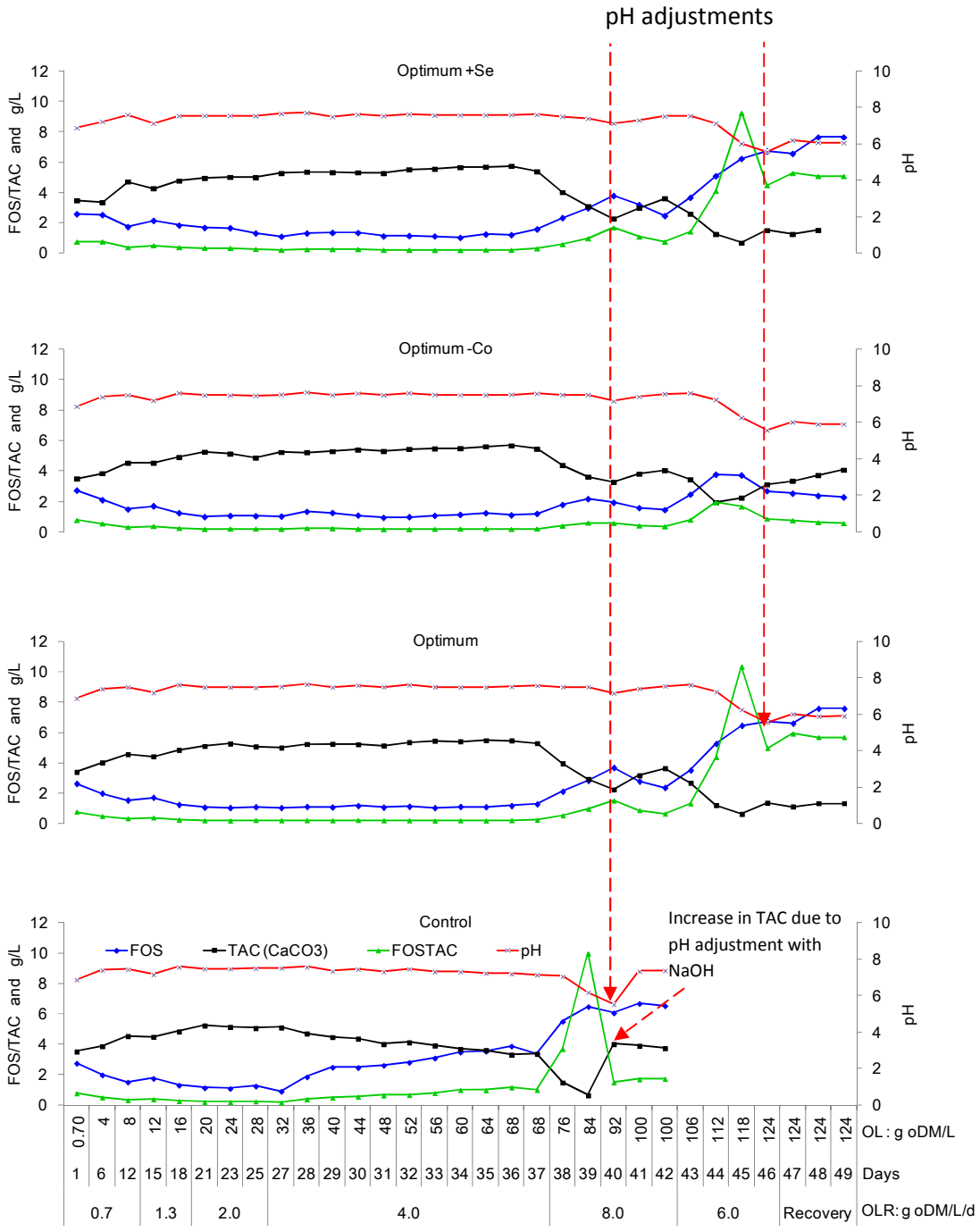


Figure 7.26b Overview of FOS/TAC, FOS, TAC and pH of the reactors due to Ni, Co, Se and Mo supplementation and different OLR in the mesophilic semi-continuous experiment with mixed fruit residue. (TEs configurations of the Control, Optimum, Optimum +Se and Optimum –Co reactors are shown in Table 7.10)

a. Influence of the TEs configurations on FOS/TAC during OLR of 2g oDM/L/d

Figure 7.27 shows the FOS/TAC values of the Control, Optimum, Optimum –Co and Optimum +Se reactors from start-up of the experiment to the end of the OLR of 2g oDM/L/d. The OLR was maintained at 0.7g oDM/L/d for 12 days; 1.3g oDM/L/d for 6 days and 2.0g oDM/L/d for 7 days in this phase of the substrate loading. Control, Optimum and Optimum –Co reactors had FOS/TAC values of 0.51, 0.50 and 0.55 respectively in the beginning; whereas the Optimum +Se reactor had FOS/TAC value of 0.76. The FOS/TAC values in all four reactors dropped to between 0.33 and 0.37 at the end of the OLR of 0.7g oDM/L/d.

When OLR was increased from 0.7 g oDM/L/d to 1.3g oDM/L/d for 6 days, the FOS/TAC value in the Optimum +Se increased from 0.37 to 0.51 but returned to 0.39 at the end of the 1.3g oDM/L/d OLR on day 18. Conversely, within the same period, the FOS/TAC values in the Control, Optimum, and Optimum –Co reactors dropped further to between 0.26 and 0.27 from about 0.3, which was measured at the end of 0.7 g oDM/L/d OLR. Increasing OLR from 1.3g oDM/L/d to 2g oDM/L/d for 7 day did not increase FOS/TAC above 0.3 in any of the reactors, including the Optimum +Se reactor. The Control, Optimum and Optimum –Co reactors also had FOS/TAC values within the regions of instability ($0.45 < FOS/TAC \leq 0.60$) but this was only within the first 4 days. Subsequently, the methanization stabilized and FOS/TAC values returned to < 0.45; and there was no significant difference in FOS/TAC between these three reactors for the OLR between 0.7g oDM/L/d and 2g oDM/L/d.

Baring other influences, it could be assumed that the 1.5 mg/L Se in the Optimum +Se slowed substrate degradation and resulted in instability at the beginning of the investigation, even at an OLR as low as 0.7g oDM/L/d. Furthermore, the inhibitory influence of Se at 1.5 mg/L required about 18 -27 days before stability in the methanization process became equal to the other treatments, including the Control. However, Optimum +Se treatment was stable after 18 days ($FOS/TAC \leq 0.45$) but relatively less stable compared to the other treatments (including the Control) until full adaptation on day 27.

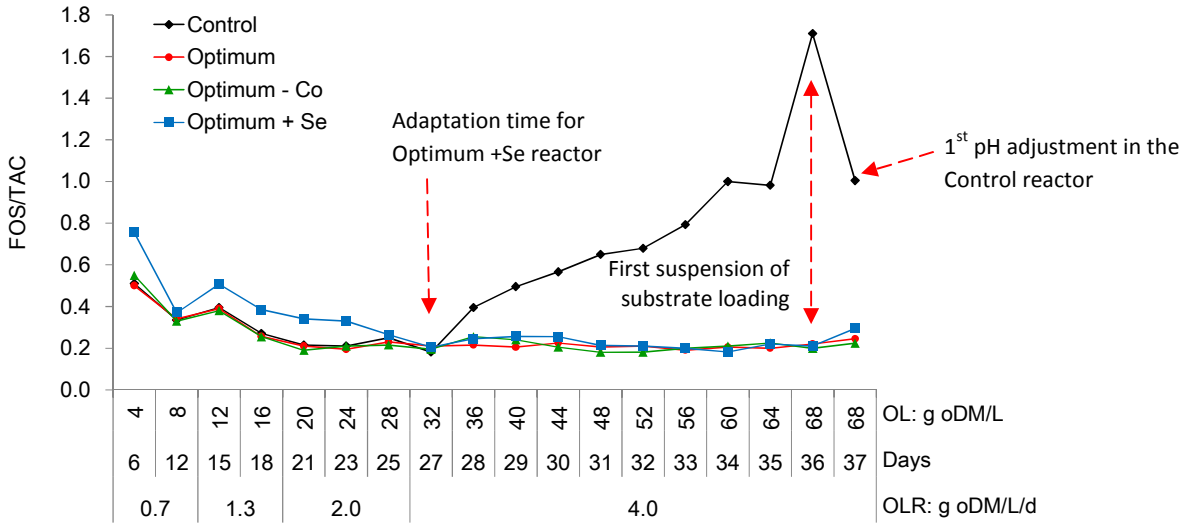


Figure 7.27 [10]FOS/TAC of the reactors due to Ni, Co, Se and Mo supplementation and OLR of 2g - 4g oDM/L/d in the mesophilic semi-continuous experiment with mixed fruit residue (TEs configurations of the Control, Optimum, Optimum +Se and Optimum –Co reactors are shown in Table 7.10)

b. Influence of the TEs configurations on FOS/TAC during OLR of 4g oDM/L/d: The Control, Optimum and Optimum –Co reactor showed no significant differences in process stability (FOS/TAC was < 0.3) until the OLR was changed from 2g oDM/L/d to 4g oDM/L/d on day 25 after a total OL of 28g oDM/L. Figure 7.27 shows that at the OLR of 4g oDM/L/d for 12 days, there was a steady increase in FOS/TAC in the Control reactor. The FOS/TAC in the Control reactor was 0.18 in the start of the OLR of 4g oDM/L/d, but rose steadily to 1.71 at the end of this OLR when the total OL was 68g oDM/L. This necessitated the first suspension of substrate loading and pH adjustment to 7.0 in the Control reactor, and the FOS/TAC decreased to 0.98. On the contrary, FOS/TAC was < 0.30 at any time of measurement in the Optimum, Optimum –Co and Optimum +Se reactors until the end of the OLR of 4g oDM/L/d. Obviously, the Optimum +Se reactor maintained relatively same level of stability in methanization as the Optimum and Optimum –Co reactors once adaptation to the influence of the Se concentration was achieved.

c. Influence of the TEs configurations on FOS/TAC during OLR of 8g oDM/L/d: The Optimum, Optimum –Co and Optimum +Se reactors showed no significant differences in process stability (FOS/TAC was < 0.3) until the loading rate was changed from 4g oDM/L/d to 8g oDM/L/d on day 37 after a total of 68g oDM/L had been loaded to the reactors. Figure 7.28 shows that at the OLR of 8g oDM/L/d for 5

[10] FOS/TAC between 0.15 – 0.45 are regarded as indicative of stability in the methanization process; FOS/TAC > 0.6 indicate onset of instability (Voss *et al.,* 2009). Days 0-25: the reactors were lightly loaded to enable adaptation to the mixed fruit residue. 0.7, 1.3 and 2 g oDM/L/d were loaded as shown (low feeding); days 26 - 37: 4g oDM/L/d was loaded steadily to each reactor as shown (steady feeding).

days, there was a significant instability in all the reactors. A peak FOS/TAC of 9.99 was recorded in the Control reactor within this OLR and this was accompanied by a pH of 6.2 and TAC of 0.65 mg/L $CaCO_3$ equivalent. The high FOS/TAC value was due to the low pH that resulted in loss of alkalinity (see Figure 7.29a, b and c for FOS/TAC and pH plots of the Optimum –Co, Optimum +Se, Optimum and Control reactors respectively.

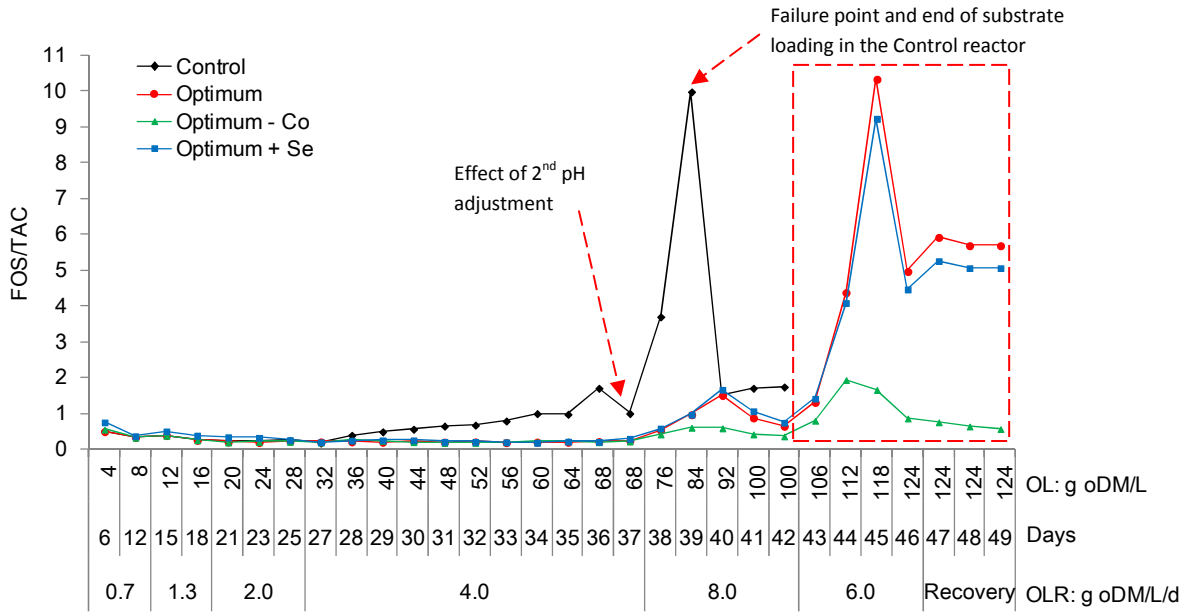


Figure 7.28 FOS/TAC of the reactors due to Ni, Co, Se and Mo supplementation and OLR of 8g oDM/L/d in the mesophilic semi-continuous experiment with mixed fruit residue. Day 38 - 42: the feeding rate was doubled to induce shock in loading rate. (TEs configurations of the Control, Optimum, Optimum +Se and Optimum –Co reactors are shown in Table 7.10)

The pH of the Control reactor was adjusted to 7.0 with 1M NaOH, and this caused a rise in alkalinity of the reactor (Figure 7.26b). The pH adjustment caused the FOS/TAC of the Control reactor to decrease from 9.99 (prior to pH adjustment) to 1.51. Substrate loading was stopped in the Control reactor at a total organic loading of 84g oDM/L and recovery was monitored. Notwithstanding the indefinite suspension of further substrate loading to the Control reactor, the FOS/TAC in this reactor varied between 1.51 and 1.93 until the end of the investigation on day 49. The shock loading in the Control reactor could have acidified the reactor and resulted in permanent or protracted biochemical impairments to the bacteria and archaea population.

Similarly, Figure 7.28 shows that differences in FOS/TAC were also high in the Optimum, Optimum –Co and Optimum +Se reactors during the OLR of 8g oDM/L/d compared to OLR of 4g oDM/L/d. The Optimum –Co had a peak FOS/TAC of 0.61 within this period, whereas the Optimum and Optimum +Se reactors had peak FOS/TAC values of 1.51 and 1.67 respectively. At a total organic load of 92g oDM/L, the Optimum and the Optimum +Se had passed the critical FOS/TAC of 0.6 (1.51 in Optimum and 1.67 in Optimum +Se) but the Optimum –Co was at the critical limit (0.61). Apparently, the Optimum -Co treatment resulted in better stabilization of the methanization process when the OLR was 8g oDM/L/d compared to Optimum and Optimum +Se treatment.

Due to the instability in the Optimum and the Optimum +Se reactors, all the three functional reactors were operated one day without substrate loading (after OL of 100g oDM/L) in order to degrade accumulated organic load. The one-day break in organic loading induced recovery and returned the FOS/TAC values to 0.35 (Optimum -Co), 0.65 (Optimum) and 0.75 (Optimum +Se). Presumably, the shock loading acidified the reactors, especially Optimum and the Optimum +Se (Figure 7.30: FOS > 3.8g), but did not impair the functionality of the micro-organisms. Optimum –Co reactor showed a better recovery to shock organic loading compared to the Optimum and the Optimum +Se reactor.

d. Influence of the TEs configurations on FOS/TAC during OLR of 6g oDM/L/d and recovery phase: Feeding resumed with a relatively low OLR of 6g oDM/L/d in the Optimum, Optimum -Co and Optimum +Se reactors after substrate loading was stopped in the three supplemented reactors for one day due to instability in the methanization process. The FOS/TAC values associated with this OLR are in the highlighted region in Figure 7.28. This region is represented in Figure 7.29a, b and c. Figure 7.29b and c show that peak FOS/TAC of 9.2 and 10.3 were recorded in the Optimum +Se and the Optimum reactors respectively after organic loading resumed. Figure 7.29a shows that a peak FOS/TAC value of 1.66 was recorded in the Optimum –Co within the same period.

The sharp rise in FOS/TAC was accompanied by noticeable decrease in pH and loss of alkalinity similar to the Control treatment at failure point. The corresponding pH in the reactors at peak FOS/TAC were 6.26 (Optimum), 6.02 (Optimum +Se) and 7.0 (Optimum –Co). Consequently, substrate loading was stopped and pH was adjusted to 7.0 with 1M NaOH in the Optimum +Se and Optimum reactors. Notwithstanding the pH adjustment after organic loading was stopped, FOS/TAC in the Optimum +Se and Optimum reactors stayed at 5.07 (Optimum +Se) and 5.69 (Optimum) at the end

of the investigation. On the contrary, the FOS/TAC value dropped from 1.66 to 0.57 in the Optimum –Co reactor on day 49.

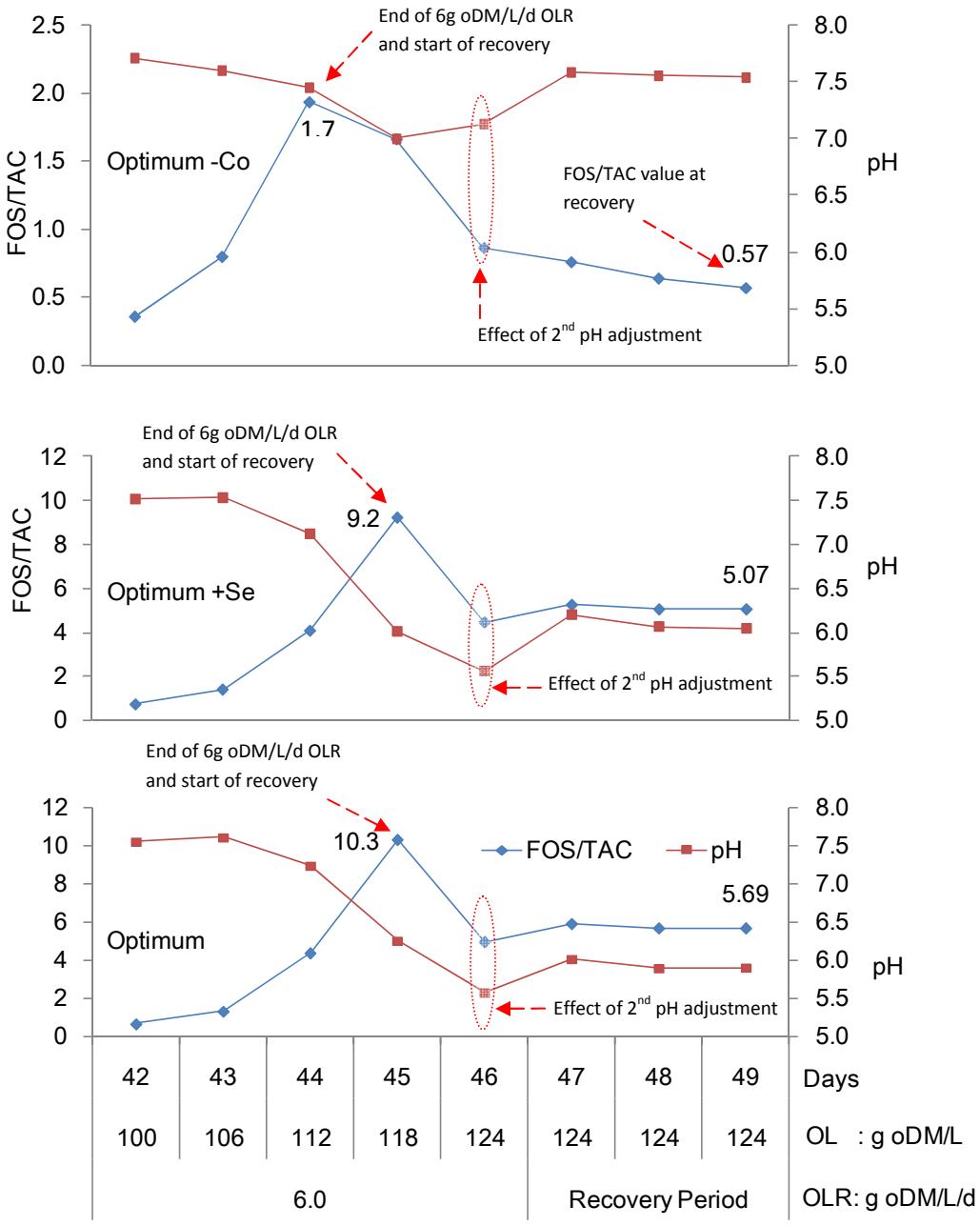


Figure 7.29 FOS/TAC of the reactors due to Ni, Co, Se and Mo supplementation and OLR of 6g oDM/L/d in the mesophilic semi-continuous experiment with mixed fruit residue. (TEs configurations of the Optimum, Optimum +Se and Optimum –Co reactors are shown in Table 7.10)

Note: Days 43 - 46: OLR was reduced to 6g oDM/L/d and recovery was monitored after loading was stopped.

7.6.3.2 Influence of the TEs configurations on VFA accumulation: The VFA was measured as shown in Table 6.14 in Chapter 6. VFA concentration is a component of the FOS/TAC measurement and is responsible for the acidity of the reactors. Reactor acidification due to VFA accumulation is better evaluated in relation to the ratio of VFA to alkalinity. Hence, there is no specific VFA concentration that is acceptable as critical during AD. However, VFA concentrations could be compared with FOS/TAC values to ascertain the cause of the changes in FOS/TAC values.

Figure 7.30 shows the concentration of VFA (FOS) in the reactors within the time of the investigation. The trend in VFA accumulation is similar to trend in FOS/TAC values in all the reactors. In the Control reactor, the high VFA concentrations during OLR of 4g oDM/L/d were between 3.1 g/L and 3.9 g/L. During the same OLR, the peak VFA concentrations in the Optimum, Optimum +Se and Optimum -Co reactors were < 1.5 g/L. The peak VFA concentrations during OLR of 8 g/L oDM/L/d were as follows: Optimum (5.3 g/L), Optimum +Se (5.1 g/L), Optimum –Co (3.8 g/L) and Control (6.5 g/L).

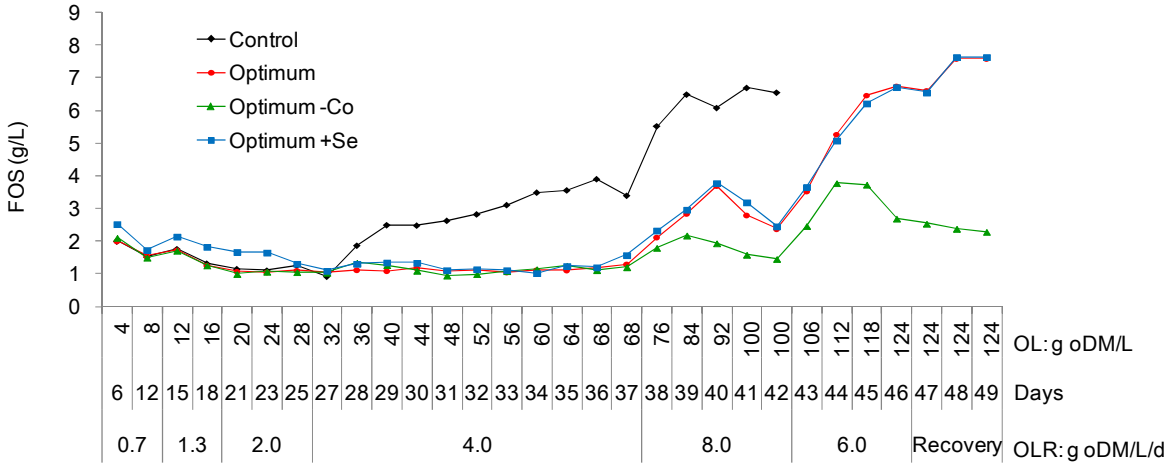


Figure 7.30 FOS concentrations (VFA) of the reactors due to Ni, Co, Se and Mo supplementation and different OLR in the mesophilic semi-continuous experiment with mixed fruit residue. (TEs configurations of the Control, Optimum, Optimum +Se and Optimum –Co reactors are shown in Table 7.10)

Further VFA accumulation occurred due to OLR of 6g oDM/L/d in Optimum up to 7.5 g/L, and up to 7.6 g/L in the Optimum +Se; whereas in the Optimum –Co, VFA concentration dropped to 2.9 g/L. Apparently, notwithstanding the adjustment in pH of the Optimum, Optimum –Co and the Optimum +Se reactors (Section 7.6.3.1d), Optimum and Optimum +Se were already acidic due to VFA concentration > 7 g/L and pH < 6.3. Without a doubt, the biochemical machinery of methanization was

impaired in the Optimum and Optimum +Se reactors but not in the Optimum –Co reactor.

7.6.3.3 Influence of the TEs configurations on the individual VFA concentrations: The individual VFA that were measured during the investigations include acetic acid, propionic acid, n-butyric acid, iso-butyric acid, n-valeric acid and iso-valeric acid. The concentrations of these acids are shown in Appendix 7.8a, b, c and d for Optimum, Control, Optimum +Se and Optimum –Co reactors respectively. The VFA concentrations were mainly due to accumulations of acetic acid, propionic acid and butyric acid. In the Control reactor, acetic acid concentration was between 4.2 g/L and 5.2 g/L during the OLR of 8g oDM/L/d (days 38 and 42). During the same OLR, acetic acid concentrations were 0.5 g/L and 2.0 g/L in the Optimum; 0.5 g/L and 2.5 g/L in Optimum +Se; and 0.3 g/L and 1.2 g/L in the Optimum -Co reactors. Peak propionic acid and butyric acid concentrations during the OLR of 8g oDM/L/d (days 38 and 42) were as follows: Control (0.1 g/L and 1.2 g/L respectively); Optimum (0.2 g/L and 0.4 g/L respectively), Optimum +Se (0.4 g/L and 0.9 g/L respectively), Optimum –Co (0.6 g/L and 0.2 g/L respectively).

During the OLR of 6g oDM/L/d, the individual VFA had accumulated from the OLR of 8g oDM/L/d so that in the Optimum reactor, the peak individual VFA concentrations were 6.3 g/L for acetic acid; 0.2 g/L for propionic acid and 0.7 g/L for butyric acid. In the Optimum –Co reactor these were 3.1 g/L for acetic acid; 0.7 g/L for propionic acid and 0.6 g/L for butyric acid; whereas in Optimum +Se reactor, these were 6.1 g/L for acetic acid; 0.3 g/L for propionic acid and 0.9 g/L for butyric acid. The Control reactor was not operational in this phase. Optimum –Co reactor seems to have a lower propionic acid oxidation capability compared to the other reactors.

The Optimum –Co reactor had the lowest acetic acid and butyric acid accumulation compared to the Optimum and Optimum +Se reactors. However, it had the highest accumulation of propionic acid. This is highlighted in Figure 7.31 where the propionic acid concentrations between day 40 and 49 are shown. Within this period, the Optimum had propionic acid peak of 0.3 g/L and Optimum +Se had propionic acid peak of 0.4 g/L; correspondingly, Optimum –Co had propionic acid peak of 0.7 g/L. Considering that Co is important for propionic acid oxidation (See Section 7.2.2), lower propionic acid oxidation rates in the Optimum –Co reactor compared to Optimum and the Optimum +Se reactors were anticipated. The Optimum reactor showed better propionic acid oxidation compared to the Optimum –Co and the Optimum +Se reactors (See further discussion on Co and propionate oxidation in Section 7.2.2.5c).

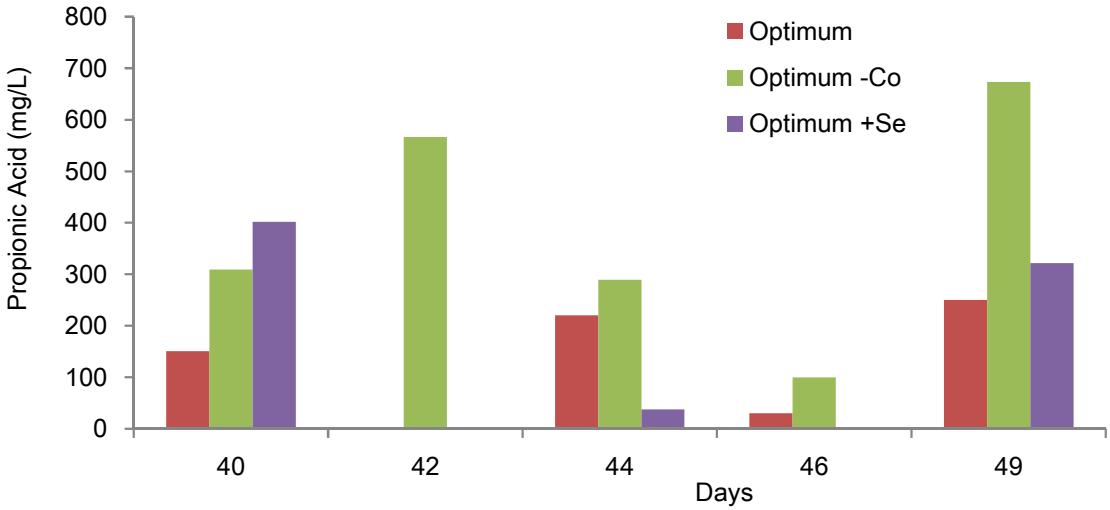


Figure 7.31 Propionic acid concentrations during mesophilic methanization of mixed fruit residue in the Optimum, Optimum –Co and Optimum +Se reactors between days 40 and 49

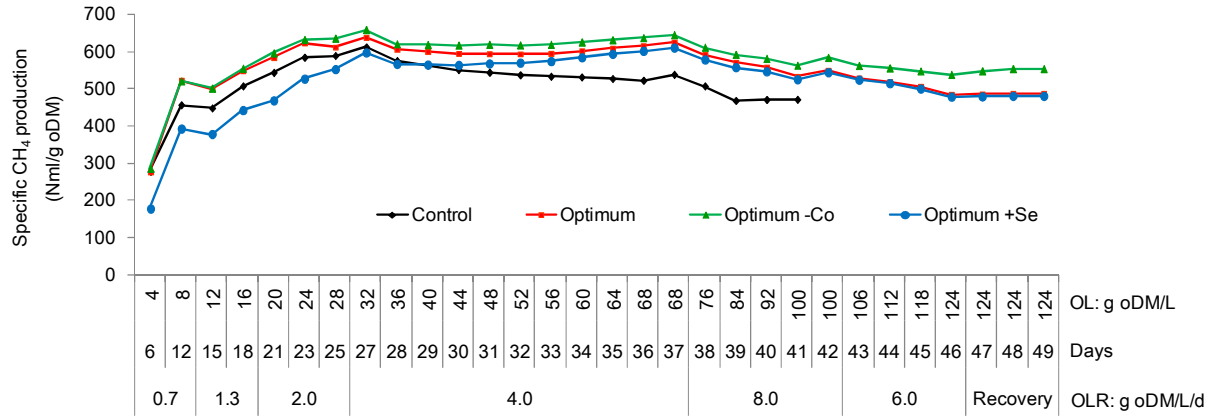


Figure 7.32 Overview of specific CH_4 production of the reactors due to Ni, Co, Se and Mo supplementation and different OLR in the mesophilic semi-continuous experiment with mixed fruit residue. (TEs configurations of the Control, Optimum, Optimum +Se and Optimum –Co reactors are shown in Table 7.10)

7.6.3.4 Influence of the TEs configurations on CH_4 production: Figure 7.32 shows an overview of the trend in specific CH_4 production during the changes in OLR. It is obvious that as the OLR increased from 0.7g to 2g oDM/L/d, the specific CH_4 production in Control, Optimum, and Optimum –Co and Optimum +Se reactors also increased. However, continuous increase in OLR up to 8g oDM/L/d resulted in decline in specific CH_4 production. Apparently, specific CH_4 production was steady at OLR of 4g oDM/L/d in the TEs supplemented reactors, but the Control reactor was already showing decline in production at this OLR. The overview of the cumulative CH_4 and biogas production in relation to FOS/TAC is shown in Appendix 7.9. Both cumulative biogas and CH_4 followed similar trend and showed a decline in volume as

FOS/TAC value increased above stability region. Details of the influences of the TEs supplementations on specific CH_4 production are discussed next.

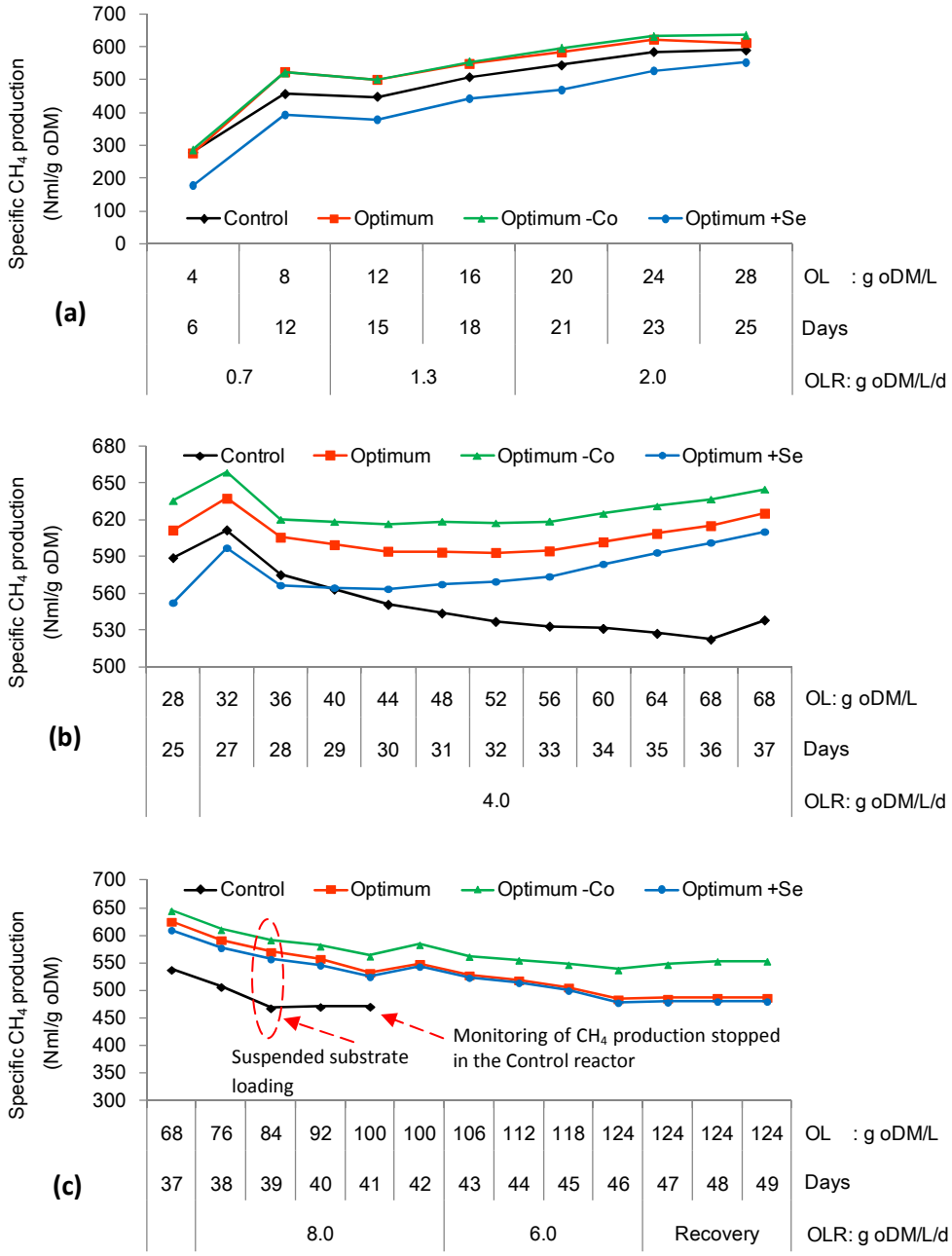


Figure 7.33 Specific CH_4 production of the reactors due to Ni, Co, Se and Mo supplementation and different OLR: **(a)** OLR: 2g oDM/L/d **(b)** OLR: 4g oDM/L/d **(c)** OLR: 8g and 6g oDM/L/d

Figure 7.33a shows the specific CH_4 production during the OLR of 2g oDM/L/d. Specific CH_4 production shows the biochemical efficiency of the MPB to convert organic carbon source to CH_4. It is an indication of the substrate methanization efficiency of the MPB. Apart from the Optimum +Se reactor that showed relative weakness in specific CH_4 production due to instability arising from the need to adapt

to the influences of Se (See Section 7.6.3), the other reactors showed only small differences in specific CH_4 production. At the end of the OLR of 1.3g oDM/L/d and 2g oDM/L/d, the specific CH_4 produced by the reactors were as follows: Control (507 and 589 Nml/g oDM respectively); Optimum (548 and 612 Nml/g oDM respectively); Optimum -Co (555 and 635 Nml/g oDM respectively); and Optimum +Se (442 and 552 Nml/g oDM respectively). Generally, the specific CH_4 production increased with increase in OLR from 0.7 g to 2 g oDM/L/d. At the end of the 2g oDM/L/d, the Optimum and Optimum –Co produced 4% and 8% more CH_4 respectively, compared to the Control reactor; whereas the Optimum +Se produced 6% less specific CH_4 production compared to the Control reactor.

Figure 7.33b shows the specific CH_4 production during the OLR of 4g oDM/L/d. At the end of this period, the specific CH_4 produced by the reactors were as follows: Control (538 Nml/g oDM); Optimum (625 Nml/g oDM); Optimum -Co (645 Nml/g oDM); and Optimum +Se (610 Nml/g oDM). At the end of the 4g oDM/L/d, the Optimum, Optimum –Co and Optimum +Se had 16%, 20% and 13% more CH_4 per organic matter methanized compared to the Control reactor. Furthermore, the TEs supplemented reactor showed increase in CH_4 production as the OLR increased from 2g oDM/L/d to 4g oDM/L/d, whereas the Control showed a decline in CH_4 production with the increase in OLR.

Figure 7.33c shows the specific CH_4 production between the OLR of 8g oDM/L/d and the end of the investigation when the OLR was reduced to 6g oDM/L/d due to VFA accumulation in the TEs supplemented reactors (See Section 7.6.3.2). The specific CH_4 production of the Control reactor was 538 Nml/g oDM at the end of the 4g oDM/L/d (day 37), but decreased to 507 Nml/g oDM after the first OLR of 8g oDM/L/d (day 38). The specific CH_4 production of the Control reactor further decreased to 468 Nml/g oDM on day 39 and organic loading was suspended indefinitely due to the instability (FOS/TAC > 9 discussed in Section 7.6.3). At this time (day 39), the TEs supplemented reactors had specific CH_4 production of 571 Nml/g oDM (Optimum); 591 Nml/g oDM (Optimum –Co) and 557 (Optimum –Se) respectively compared to 468 Nml/g oDM in the Control. This corresponds to 22% (Optimum), 26% (Optimum –Co) and 19% (Optimum +Se) more CH_4 per day than the Control reactor. In spite of the suspension of feeding in the Control reactor, the specific CH_4 production of this reactor did not improve (471 Nml/g oDM on day 41); hence, gas measurement was stopped.

With further organic loading, decrease in specific CH_4 production also occurred in the TEs supplemented reactors. At the end of the OLR of 8g oDM/L/d (day 42), the specific CH_4 produced by the TEs supplemented reactors were as follows: Optimum

(549 Nml/g oDM); Optimum -Co (584 Nml/g oDM); and Optimum +Se (544 Nml/g oDM). The instability in methanization associated with this phase (Section 7.6.3.1c) induced general decline in specific CH_4 production in the TEs supplemented reactors. Consequently, the OLR was reduced to 6g oDM/L/d on day 43; organic loading was stopped finally on day 46 when FOS/TAC value stayed above the critical value of 0.6 in spite of pH adjustment. Parameter measurement was stopped on day 49 in all the TEs supplemented reactors. The specific CH_4 produced by the TEs supplemented reactors at the end of the experiment on day 49 were: Optimum (487 Nml/g oDM); Optimum -Co (554 Nml/g oDM); and Optimum +Se (481 Nml/g oDM). The Optimum –Co reactor had 14% and 15% more specific CH_4 production than the Optimum and the Optimum +Se reactors respectively.

It is obvious that the Control reactor had lower specific CH_4 production compared to Optimum, Optimum –Co and Optimum +Se reactors even within the time of stable methanization (2g oDM/L/d). The Optimum –Co and Optimum +Se reactors also showed differences in specific CH_4 production: Optimum +Se reactor had lower specific CH_4 production all through the experiment when compared to Optimum and Optimum –Co reactors. The Optimum –Co had the highest specific CH_4 production among the reactors: this is related to the stability in the methanization process during the investigation. There were also some differences in CH_4 and H_2S contents of the reactors and these are discussed next.

7.6.3.5 Influence of the TEs configurations on CH_4 and H_2S content: Figure 7.34, b, c and d show the CH_4 and H_2S contents of the reactors during the entire investigation. The average CH_4 content of the reactors during the OLR of 2g, 4g and 8g oDM/L/d were as follows: Control (53%, 46% and 37% respectively); Optimum (54%, 53% and 54% respectively); Optimum -Co (54%, 54% and 58% respectively); and Optimum +Se (50%, 54% and 55% respectively). The average CH_4 content in the TEs supplemented reactors during the 6g oDM/L/d and recovery period was as follows: Optimum (47%); Optimum -Co (62%); and Optimum +Se (45%). All the TEs supplemented reactors had similar CH_4 content with the Control at OLR of 2g oDM/L/d except Optimum +Se which had 3% less CH_4 compared to the Control. However, the average CH_4 content in the Control reactor declined with increase in the ORL, such that during the OLR of 4g and 8g oDM/L/d, Optimum, Optimum –Co and Optimum +Se reactors had significantly higher CH_4 content compared to the Control reactor.

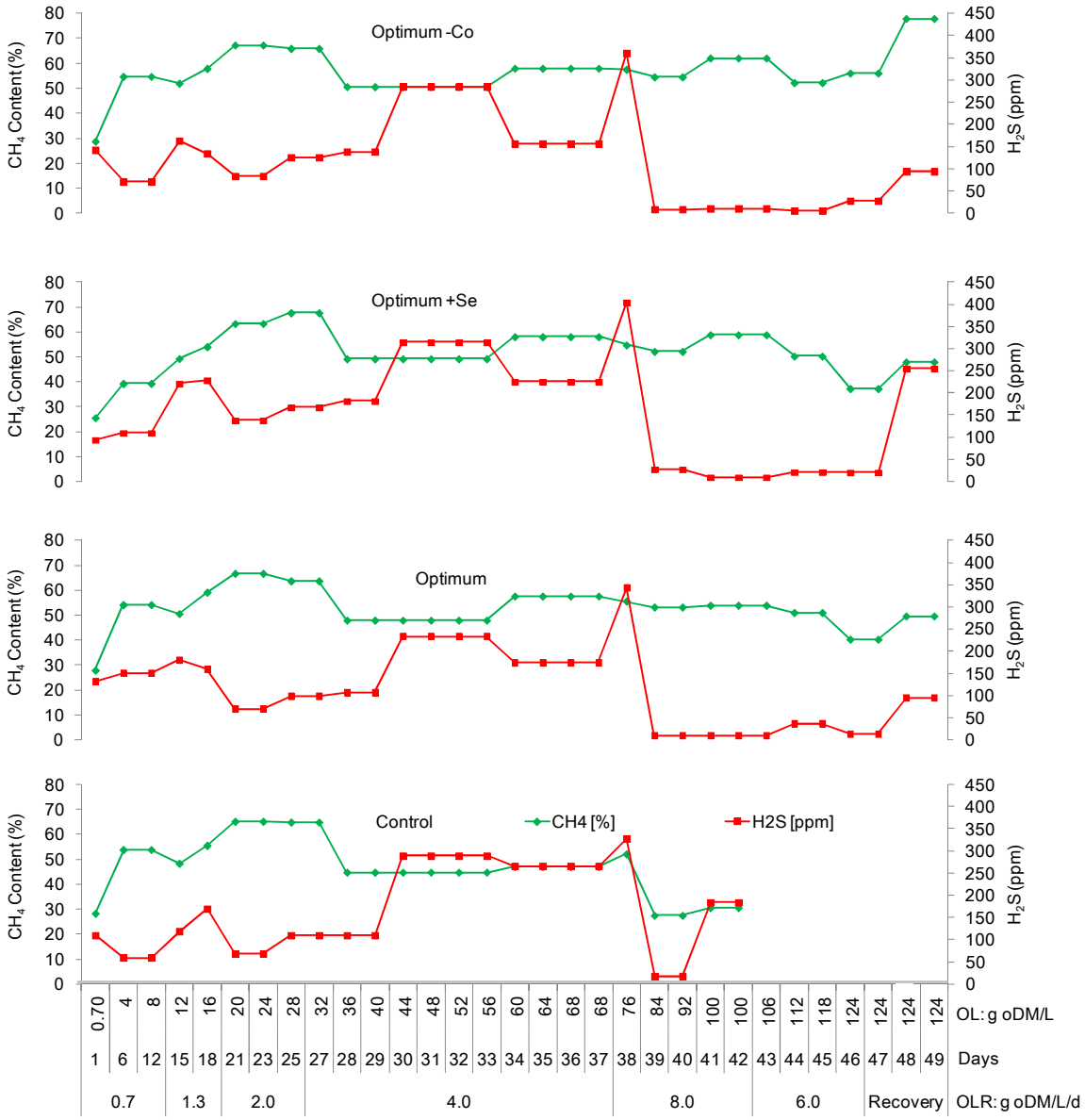


Figure 7.34 CH_4 and H_2S content of the reactors due to Ni, Co, Se and Mo supplementation and different OLR in the mesophilic semi-continuous experiment with mixed fruit residue (TEs configurations of the Control, Optimum, Optimum +Se and Optimum –Co reactors are shown in Table 7.10)

Apparently, during the OLR of 4g and 8g oDM/L/d, there was no significant difference in CH_4 content between the TEs supplemented reactor. However, after the instability in the TEs supplemented reactors due to the OLR of 8g oDM/L/d, there was a decline in CH_4 content of the Optimum and the Optimum +Se reactors. Consequently, the CH_4 contents in the Optimum –Co reactor during the 6g oDM/L/d was significantly higher compared to the Optimum and the Optimum +Se reactors.

H_2S content in biogas may vary between 10 – 3000 ppm depending on the S content of the substrate and the activities of the SRB (Mata-Alvarez, 2003). The S content of

the MFR used in this experiment is shown in Table 7.2. Generally, it seems that the population of SRB, which are responsible for H_2S production, increased with increase in OLR up to 4g oDM/L/d; peaked on the first OLR of 8g oDM/L/d; declined afterwards as methanization became unstable in the reactors; and increased during 6g oDM/L/d and the recovery period. The average H_2S content (ppm) of the reactors during the OLR of 2g, 4g and 8g oDM/L/d were as follows: Control (106, 233 and 177 ppm respectively); Optimum (132, 172 and 121 ppm respectively); Optimum -Co (120, 184 and 127 ppm respectively); and Optimum +Se (160, 237 and 148 ppm respectively). The average H_2S content in the TEs supplemented reactors at the 6g oDM/L/d and recovery period was as follows: Optimum (48 ppm); Optimum -Co (43 ppm); and Optimum +Se (100 ppm).

The average H_2S concentration was high in Control, Optimum, Optimum –Co and Optimum +Se reactors during the OLR of 4g oDM/L/d compared to 2g and 8g oDM/L/d; and the Control reactor had the highest concentration of H_2S during this period followed by the Optimum +Se reactor. The lowest H_2S concentration during the OLR of 4g oDM/L/d occurred in the Optimum reactor. During the OLR of 6g oDM/L/d, the lowest H_2S concentration occurred in the Optimum –Co reactor while the highest occurred in the Optimum +Se reactor. The Control reactor was not operated with the OLR of 6g oDM/L/d. The relatively high H_2S content of the Control reactor suggests that TEs supplementation in the Optimum reactor and it variants enhanced the activities of the MPB over the SRB in H_2 utilization. Enhanced the activities of the MPB over the SRB in H_2 utilization is most obvious in the Optimum –Co reactor. When CH_4 and H_2S contents during the OLR of 8g and 6g oDM/L/d are jointly evaluated for the supplemented reactors, it appears that the failure in the Optimum and Optimum +Se reactor is related to inhibition in the activities of both MPB and the SRB.

7.6.4 VFA, Co and Se interactions and the Optimum TEs configuration: The behaviour of the Optimum, Optimum –Co and Optimum +Se reactors could be explained in relation to the VFA*Co, VFA*Se and Co*Se interactions. Based on the significance of the RSM Estimates (Figure 7.25), the inhibition due to Co*Se interaction is expected to be negative but insignificant at an OLR that supported stable methanization in the TEs supplemented reactors. The OLR of 0.7g - 4g oDM/L/d showed stable FOS/TAC in the reactors and could be regarded as normal or stable OLR for the reactors. Negative but insignificant Co*Se interaction could be responsible for the instability and lower CH_4 production during the ORL of 0.7g – 2.0g oDM/L/d in the Optimum +Se reactor, which was readily overcome by adaptation between days 18 and 27 (See Section 7.6.3.1a).

In addition, VFA*Se is positive and significant in Figure 7.25. This suggests that as VFA concentration increased in the reactors, Se concentration should increase for an improved VFA degradation. This may account for the similarity in FOS/TAC values (Figure 7.27) and CH_4 production (Figure 7.32b) between the Optimum, Optimum -Co and the Optimum +Se after the later reactor had adapted. Considering that in the Optimum +Se reactor, the Se concentration was 1.5 mg/L, it is possible that the relatively high Se concentration in this reactor contributed to its lower stability and CH_4 production compared to the Optimum and the Optimum –Co reactors. All the same, the post adaptation performance of Optimum +Se suggests that it has similar influence on methanization as the Optimum. The influence and significance of VFA*TE and TE*TE interactions during methanization suggest the possibility of equivalent TEs configuration: i.e., TEs mixtures with different concentrations and compositions of TEs that have similar methanization effects.

Finally, VFA*Co is negative in Figure 7.25. This required that in the Optimum –Co reactor, an increase in VFA or OLR from 0.7g oDM/L/d to 8g oDM/L/d should be accompanied by a decrease in Co concentration. This was accommodated in the formulation of Optimum -Co. Apparently, the VFA*Co interaction was responsible for the superior stability and CH_4 production in the Optimum –Co reactor compared to the Optimum and the Optimum +Se reactors with similar TEs configuration.

Essentially, the results of the validation experiments demonstrate the bio-catalytic potential of the TEs configuration of the Optimum reactor to reduce long adaptation time common to TEs supplementation in mesophilic methanization (Section 7.2.2.1), and maintain substantial gains in methanization. The results also portray that the TEs configuration of the Optimum reactor is a compromise setting for all methanization phases and could be weak in certain processes. It further highlights the need for adjustment in TEs configuration in special circumstances. Regardless, the gain in CH_4 production and relative stability of the supplemented reactors confirm the potentials of TEs supplementations in optimizing mesophilic methanization processes.

7.6.5 Influence of the TEs configurations on bacteria and archaea population: The DGGE results illustrating the influence of the TEs configuration on the microbiology of the methanization process are shown in Figure 7.35a for bacteria and in Figure 7.35b for archaea population. The DGGE was done according to the procedure of Muyzer *et al.* (2003). Three samples were taken from Control, Optimum, Optimum –Co and Optimum +Se reactors for the DGGE on days 2, 30 and 39. The sample on day 2 was taken to show the population structure in the beginning of the investigation. The sample on day 30 was taken to show the population structure when methanization

was stable in the Control, Optimum, Optimum –Co and Optimum +Se reactors (FOS/TAC < 0.6). The sample on day 39 was taken to show the population structure when methanization was unstable in the four experimental reactors (FOS/TAC > 0.6).

In Figure 7.35a and b, the numbers represent lanes, which reflect patterns of bands corresponding to the different sequences of DNA from the bacteria or archaea populations present in a reactor on the day of sample collection. A band on a lane represents the DNA finger print a specific group of bacteria or archaea. The coloured boxes are used to identify the bands on the lanes. The letters are the initials of the colours of the boxes: e.g., *'r'* for the red, *'b'* for blue and so on. Hence, the different DNA sequences represented by the bands will be described by the corresponding letters of the boxes. The presence or absence of a particular band or DNA sequence from the baseline sequences suggests changes in population (Muyzer *et al.,* 1993).

7.6.5.1 **Influence of the TEs configurations on bacteria population:** Figure 7.35a shows the DGGE results of the DNA sequence for bacteria population in the experimental reactors. It shows the reactors, the days of sampling and the lanes from a particular sampling day. Lanes 1, 4, 7 and 10 were from the samples collected on day 2 from each of the reactors as shown in Figure 7.35a. Lanes 2, 5, 8 and 11 were samples collected on day 30 from all the four reactors; whereas lanes 3, 6, 9 and 12 were collected from the corresponding reactor on day 39 of the experiment. Lanes 1, 4, 7 and 10 were the baseline bacterial population at start-up (day 2) in the Control, Optimum, Optimum –C and Optimum +Se reactors respectively and are similar in number and distribution of bands. In the Control reactor, bands *r*, *g* and *b* were present in the samples collected on the 3 sampling days. Band *y* was present in the sample collected on day 30, while bands *y* and *v* were present in the samples collected on days 30 and 39. Apparently, the number of bacteria species increased in the Control reactor as the OLR increased from 2g to 8g oDM/L/d within the experimental period.

In the Optimum reactor, bands *r* and *b* were present at all the sampling times but band *g* appeared only after day 30 of the experiment and was detected on day 39. Bands *y* and *v* were present on days 30 and 39 in the Optimum reactor but not on day 2. Similar to the Control, there was an increase in bacterial species in the Optimum reactor as OLR increased from 2g to 8g oDM/L/d in the course of the experiment. In the Optimum –Co reactor, band *r* was present on all three sampling days, whereas bands *b* and *g* were present on day 2 and day 39 respectively. Bands *y* and *v* were present on both days 30 and 39 but not on day 2. It can also be observed that bacteria population increased in Optimum –Co as the OLR increased within the time of the experiment. Optimum +Se reactor had the bacterial population

corresponding to bands *r*, *g* and *b* from day 2 until day 39; and bands *y* and *v* were both present on days 30 and 39 but not on day 2. Apparently, just like in the Control, Optimum, and Optimum –Co, the bacterial species in the Optimum +Se reactor increased with increase in OLR within the period of the experiment.

Comparatively, the Control, Optimum and Optimum +Se reactors had more diverse bacteria population than Optimum –Co reactors. With emphasis on days 30 and 39 (the period of 4g and 8g oDM/L/d), the Control, Optimum and the Optimum +Se reactor shared similar population of bacteria species. The Optimum –Co reactor had the least diversity in bacterial species. All species of bacteria represented by the bands had appeared by day 30 in the Optimum +Se reactor; whereas in the Control reactor, all the species appeared on day 39 in spite of the relatively high FOS/TAC value associated with this day (See Section 7.6.3). Apparently, bands *y* and *v* appeared in the course of the experiment since they were absent on day 2 in all the reactors. Furthermore, it seems that TEs supplementation enabled the bacteria populations of band *v* to flourish earlier in the treatment reactors compared to the Control reactor. Regardless, the composition of the bacterial species is the same in all four reactors but there were differences in temporal abundance of the species in the reactors.

Exempting other influences, it can be alleged that TEs supplementation led to decline in a few population in the Optimum –Co reactors (lanes 8 and 9), compared to the Control (lanes 2 and 3), Optimum (lanes 5 and 6) and Optimum +Se (lanes 11 and 12). Appart from this difference, it appears that the same kinds of species occurred in all the reactors but at different times. This suggests that the optimization in methanization processes of the TEs supplemented reactors compared to the Control reactor is the outcome of the effects of the TEs on the bacteria population. Apparently, the TEs strengthened the methanization potentials of the resident bacterial species in the treated reactors. This enhancement was lacking in the Control reactor due to lower TEs concentration compared to the TEs supplemented reactors (Table 7.10).

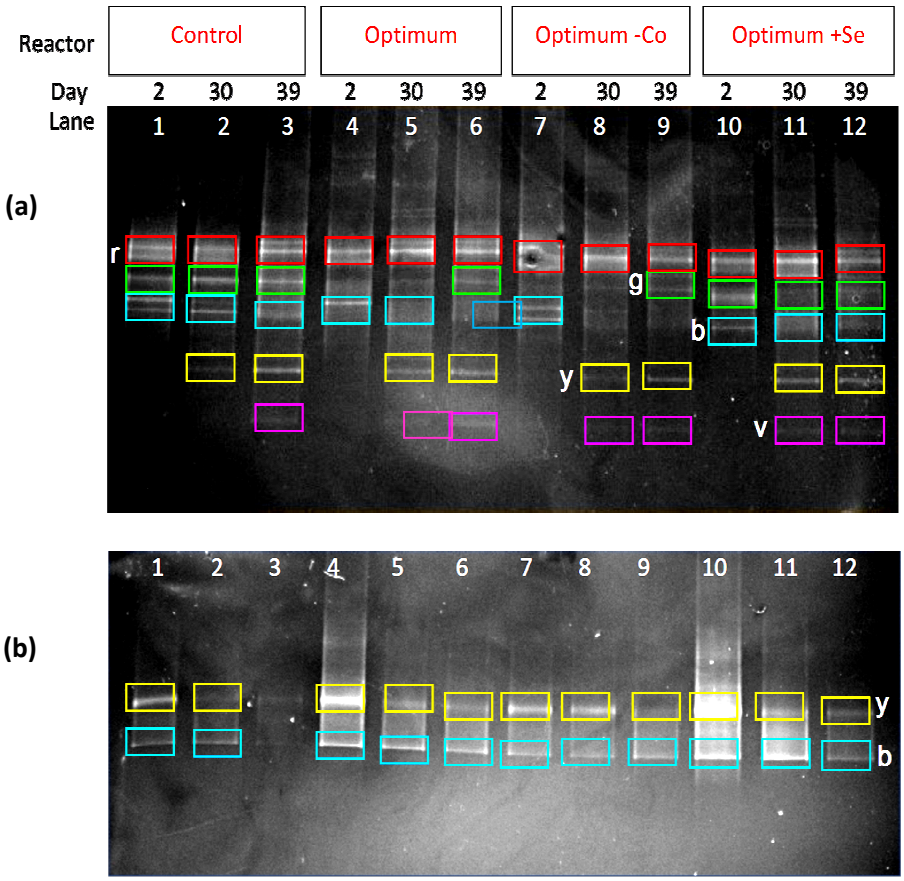


Figure 7.35 DGGE of different microbial population in the reactors due to Ni, Co, Se and Mo supplementation in the mesophilic semi-continuous experiment with mixed fruit residue **(a)** Bacteria population **(b)** Archaea population (TEs configurations of the Control, Optimum, Optimum +Se and Optimum –Co reactors are shown in Table 7.10)

Note: (a) PCR Primers: GM5 GC clamp/907RM; DGGE: gradient 20 - 80%, 55°C 200 V, and 300 min (b) PCR primers: Arc334f GC clamp/Arc915r; DGGE: gradient 30 - 80%, 60°C, 150 V, 180 min

7.6.5.2 Influence of the TEs configurations on archae population: Figure 7.35b shows the DGGE results of the archaea population in the experimental reactors. Bands *y* and *b* are common to all the lanes except lane 3, which was the sample from the Control reactor on day 39. Lanes 6, 9 and 12, which were samples on day 39 have the same bands as days 2 and 30 but with less intensity of bands. It is apparent that as the OLR increased from 2g to 8g oDM/L/d there was decline in the population of the methanogens in the Control reactor as is evident in lack of bands on lane 3.

Apart from this change, it seems that the CH_4 forming population were conserved in Optimum, Optimum –Co and Optimum +Se reactors. This suggests that the instability during the methanization did not induce significant change in the population of MPB in the reactors supplemented with TEs. This may also imply that the mechanism of

the enhancement or the reduction in CH_4 production was by activation or deactivation of bio-catalysis in the same group of archaea population, and not through the emergence of new archaea population in the TEs supplemented reactors. Conversely, in the Control reactor, the disappearance of the bands on lane 3 that were present in the TEs supplemented reactors during the period of instability (day 39) suggests losses in diversity of important microbial population. This may account for the larger decline in CH_4 production and CH_4 content in the Control reactor during the period of instability compared to the reactors with TEs supplementation (Section 7.6.3).

Summary and conclusion: The results of the validation experiments confirm the bio-catalytic potential of the TEs configurations proposed for the optimization of the methanization processes of VFA degradation rate, CH_4 production and process stability. The results also confirm the need for modifications in TEs concentrations to accommodate specific changes in VFA concentration during methanization. The bacterial species appeared at different times in the reactors during the semi-continuous methanization of mixed fruit residue but the total number of species was the same in all the four reactors. The TEs supplemented reactor with less diverse population (Optimum –Co) was associated with better stability during the investigation. The archae population were stable within the investigation time in the TEs supplemented reactor, but not in the Control reactor. Loss or gain in CH_4 production in the TEs supplemented reactors was due to changes in OLR and the associated pH changes, and not as a result of change in microbial population. However, the disappearance of certain archaea population in the Control reactor suggests that the instability in the methanization process in that reactor led to loss of important CH_4 producing population.

CHAPTER 8 CONCLUSION AND RECOMMENDATIONS

The results presented in this report suggest that TEs content of AD substrates cannot support optimum methanization. General balance in TEs composition is very important for optimizing methanization by TEs supplementation. Imbalances in TEs composition will have such effects as inhibition to hydrolysis, decline in CH_4 production, reduction in maximum substrate conversion potential, and loss in substrate affinity. Toxicity to the CH_4 forming micro-organisms due to lack of attenuation effects from the limiting TEs constituents is also very likely. Hence, it is strongly recommended that Ni, Co, Se and Mo are supplemented to methanization reactors in both mesophilic and thermophilic operations.

TEs supplementation based on VFA concentration in a reactor is the best supplementation approach. Nevertheless, VFA-dependent TEs supplementation is difficult because some TEs are required in low concentrations in high VFA level, and others are required in relatively high concentrations in low VFA level. Hence, reducing the concentration of already supplemented TEs is impractical. However, TEs supplementation based on the normal operating VFA level of a reactor will provide comparable benefits as TEs supplementation based on observed VFA levels, but with weaknesses in the improvement of methanization at certain VFA concentrations.

In formulating TEs mixture, it is strongly recommended that the background TEs content of the reactor is known. Also, the interactions effect of the TEs; and VFA*TEs interactions should be considered when deciding the TEs and the TEs concentrations to be supplemented. This is important because the optimum concentration of any TE in a mixture TEs will vary depending on the relative abundance of the constituent elements; and this is always reflected in the TE*TE and VFA*TE interactions. As a result of TE*TE and VFA*TE interactions, different TEs configurations could produce similar results (as seen in Optimum and Optimum +Se TEs configuration). Hence, the concentration of any TE in a mixture of TEs should be in relation to other TEs. A guide to TEs formulation is the RSM model terms and Estimates provided in the Appendix for all the responses investigated in the batch experiments.

Microbial populations participating in mesophilic methanization have low reaction rate and this is considerably enhanced by TEs supplementation during mesophilic methanization. Conversely, temperature increases the rate of reaction in thermophilic methanization. However, the microbial affinity for VFA is less in a non-TEs supplemented thermophilic methanization compared to mesophilic methanization.

Hence, microbial affinity for VFA is considerably increased in thermophilic methanization by TEs supplementation. Microbial adaptation to the influences of TEs is necessary for optimum performance of the bacteria and archaea population during methanization.

Consequently, it is hereby recommended that to maximize the biochemical impacts of TEs supplementation on methanization processes, it is necessary to incorporate an adaptation chamber to AD facilities. An added chamber, analogous to the leachate percolation chamber used in certain dry fermentation facilities will be appropriate. TEs could be supplemented to this leachate or inoculum chamber and the adaptation is monitored for specific range of organic loading rate. The adapted inoculum could be intermittently mixed with in-coming substrate in a continuous system, to provide the micro-organisms necessary for enhanced methanization. This also provides replacement for the microbial population that is lost during the discharge of digester content in continuous feeding operations. In dry batch fermentation, such adapted culture could be periodically recirculated by infiltration through the bulk of the digester content.

It has been documented in this report that optimum TEs configuration varies with VFA concentrations. Consequently, it is also recommended that for a stable methanization, it is better to supplement TEs to an organic loading rate that has been established as optimum for a particular substrate and digester. For example, when an OLR of 4g oDM/L/d for a substrate is optimum without TEs supplementation, increase in degradation rate of 50% due to TEs supplementation could allow for an OLR of 6g oDM/L/d. However, it is a better practice to load the digester at an OLR of 4g oDM/L every 12 - 16 hours than to load 6g oDM/L every 24 hours (i.e. 6g oDM/L/d). Maintaining an OLR of 4g oDM/L in every 12 - 16 hours keeps the VFA level within the concentration necessary to maximize the substrate affinity of the micro-organisms. It also keeps the VFA level of the digester from varying outside the range of concentration that is optimum for the VFA*TE interactions. A significant change in the VFA level generally induces new TEs interactions and might necessitate re-adaptation of the micro-organisms.

CHAPTER 9 SUMMARY

In **Chapters 1 – 3**, it was noted that AD offers sustainable bio-conversion technique for the methanization of organic residues from municipal-, agricultural- and industrial sectors. Variations in substrate composition and operating conditions induce decline in efficiency during methanization. In **Chapters 4 - 5**, it was noted that to maintain high process output in spite of variations during AD, the bio-catalytic activity of the microbial population must be kept optimum. Ni, Co, Se and Mo were identified as important for bio-catalysis of enzymes associated with methanization. The low concentrations and imbalance in composition of Ni, Co, Se and Mo in most substrates of AD cannot support optimum bio-catalysis during methanization, and should be supplemented. When these TEs are limiting, methanization could be inefficient even when other factors are optimum. Limited knowledge exist on how AD can be optimized using TEs. Consequently, experiments were designed to optimize methanization by the supplementation of Ni, Co, Se and Mo during mesophilic and thermophilic operations. The experimental procedures were discussed in **Chapter 6**.

In **Sections 7.1 of Chapter 7**, the Ni, Co, Se and Mo contents of AD substrates were discussed. Complex substrates such as grease trap residue and blackwater where shown to have elevated TEs contents compared to the mono-substrates. Mono-substrates such as grass and dungs are generally low in Ni, Co, Se and Mo compared to non-TEs elements such Fe, and require TEs supplementation for enhanced methanization. Co was particularly low in concentrations (0.03 -1.2 mg/kg DM) in the mono-substrates discussed in **Section 7.1 of Chapter 7**, compared to the Co concentration in the digestates (1.1 – 16.7 mg/kg DM). Se concentration was below the detection limit of the FAAS (0.005 mg/L). Ni concentration was lower in mono-substrates (0.04 – 0.2 mg/kg DM) compared to complex substrates (0.1 – 3.6 mg/kg DM) and digestates (0.1 – 7.1 mg/kg DM); and Mo also showed similar distribution as Ni.

In **Sections 7.2 – 7.4 of Chapter 7**, influences of Ni, Co, Se and Mo during batch methanization were investigated and response surface methodology (RSM) was used to analyse the results. RSM enabled in-depth understanding of the roles of the main effects and interactions of the TEs. In **Section 7.2**, important TEs were identified for different methanization parameters at mesophilic and thermophilic temperatures. Furthermore, the influences of Ni, Co, Se and Mo supplementation to different VFA concentration during mesophilic and thermophilic methanization in batch operation were evaluated. In mesophilic methanization, influences of Ni, Co, Se and Mo supplementation on hydrolysis and acidification rate, microbial adaptation time, CH_4

production and VFA degradation rate were evaluated. Similarly, the influences of Ni, Co, Se and Mo supplementation on CH_4 production and VFA degradation rate where evaluated in thermophilic methanization.

In the mesophilic methanization, hydrolysis of maize silage was influenced by VFA concentration and the interactions of Co, Se and Mo in the reactor. Co*Se increased hydrolysis and acidification rate of maize silage in the different VFA levels, whereas Co*Mo reduced hydrolysis and acidification rate of the maize silage. The concentrations of the TEs that increased hydrolysis and acidification rate during the mesophilic batch operations were Ni (0.5 – 0.9 mg/L); Co (0 mg/L for VFA between 20 and 120 mmol/L, and 3.7 mg/L for VFA > 200 mmol/L); Se (0 mg/L for VFA between 20 and 120 mmol/L, and 1.2 mg/L for VFA > 200 mmol/L); and Mo (0 mg/L for VFA between 20 and 120 mmol/L, and 1.3 mg/L for VFA > 200 mmol/L).

Adaptation time allows for the microbial uptake and incorporation of TEs into the active sites of the enzymes involved in methanization and to adapt to the toxic influences of the excess and unwanted TEs. Microbial adaptation time was shortened by increase in Ni concentration and elongated by an increase in the concentration of Mo. Enhancing VFA degradation rate and CH_4 formation during mesophilic supplementation required adaptation to the influences of TEs. After microbial adaptation, CH_4 production was influenced by the concentration of the VFA in the reactors, and Ni, Co and Mo were important for CH_4 production in VFA concentrations between 20 and 120 mmol/L, whereas Co and Se were important in VFA concentration ≥ 200 mmol/L. VFA degradation rate was enhanced by Ni and Co. Similarly, Ni*Mo and VFA*Se interactions increased VFA degradation rate, whereas Mo and Co*Se interaction decreased VFA degradation rate. The specific influences of the important TEs and the interactions of the TEs were dependent on the VFA concentrations in the batch reactors.

In mesophilic methanization, full supplementation with Ni, Co, Se and Mo was compared with partial supplementation with Se and Mo; and in all the measured responses, partial supplementation with Se and Mo showed significantly lower efficiency at optimizing methanization processes relative to full supplementation with Ni, Co, Se and Mo. The concentrations of the TEs for multi-response optimization involving CH_4 production and VFA degradation rate during the mesophilic batch operations were Ni (0.8 – 2.2 mg/L); Co (3.6 – 4.2 mg/L for VFA < 200 mmol/L, and 2.2 mg/L for VFA > 200 mmol/L); Se (0 – 0.3 mg/L for VFA < 200 mmol/L, and 0.53 mg/L for VFA > 200 mmol/L); and Mo (1.6 mg/L for VFA < 200 mmol/L, and 0 mg/L for VFA > 200 mmol/L).

In the thermophilic methanization, adaptation period was not required. Ni (1 -1.2 mg/L); Se (1.4 – 1.6 mg/L); and Mo (0.6 – 1 mg/L) increased VFA degradation rate in VFA concentrations between 50 and 250 mmol/L. Co was not required to increase VFA degradation rate during thermophilic methanization. CH_4 production in thermophilic methanization was also increased by Ni, and concentrations between 0.1 and 1.1 mg/L had positive influences. Co and Mo decreased CH_4 production and were not required. Se also decreased CH_4 production in VFA concentrations between 20 and 120 mmol/L, but up to 1.2 mg/L Se was required at VFA concentration > 200 mmol/L. The concentrations of the TEs for multi-response optimization involving VFA degradation rate and CH_4 production in thermophilic methanization were Ni (1 – 1.3 mg/L); Co (3.7 mg/L for VFA < 200 mmol/L, and 0.3 mg/L for VFA > 200 mmol/L); Se (0 mg/L for VFA < 150 mmol/L, and 1.2 mg/L for VFA > 150 mmol/L); and Mo (1.1 – 1.2 mg/L for VFA < 150 mmol/L, and 0.2 mg/L for VFA > 150 mmol/L).

The influences of TEs supplementation in mesophilic methanization resulted in between 60% and 130% increase in VFA degradation rate; and between 35% and 108% increase in CH_4 production. In thermophilic methanization, TE supplementations resulted in between 28% and 38% increase in VFA degradation rate; and between 6% and 11% increase in CH_4 production. Judging by the differences in gains in methanization parameters, it seems that thermophilic methanization is optimized due to operation at a higher temperature than mesophilic methanization. Thermophilic methanization also require lower concentration of TEs supplementation compared to mesophilic methanization, especially when operating in the range of 100 – 150 mmol/L VFA concentrations.

RSM was also used for the derivation of TEs configurations that are suitable but not optimum for mesophilic methanization. It also enabled the derivation of the optimum TEs configurations for mesophilic methanization as a function of VFA concentration in the reactors. In Section 7.3, the different derivations were used in simulation of supplementation approaches, which were compared by analysis of means (ANOM) using the Dunnett's method. Four TEs supplementation approaches including the suitable or compromise scenario (TC-compromise), optimum TEs configuration for 100 – 120 mmol/L VFA (OTC-VFA_120), optimum TEs configuration based on different VFA levels (OTC-VFA_DL) and no TEs supplementation (TC-control) were compared as possible TEs supplementation scenarios.

Gains in mesophilic methanization efficiency were compared for CH_4 production, VFA degradation rate and VFA retention time between the scenarios. Desirability is the proportion of an experimental objective that is achieved as a result of implementing a particular TEs configuration. It served as the basis for simultaneously evaluating the

influences of the different TEs configuration of the scenarios on CH_4 production, VFA degradation rate and VFA retention time. Desirability has a maximum value of 1: TC-control scenario had a desirability of 0.31 compared to 0.60 in the TC-compromise scenario, whereas OTC-VFA_120 and OTC-VFA_DL had 0.69 and 0.72 desirability values respectively.

The desirability values indicated that an average of 31%, 60%, 69% and 72% of the maximum VFA degradation rate, VFA retention time and CH_4 production is achievable by implementing TC-control, TC-compromise, OTC-VFA_120 and OTC-VFA_DL respectively. TEs supplementation based on VFA levels in a reactor (OTC-VFA_DL) is labour-intensive but is the most efficient TEs supplementation approach for the reduction of substrate methanization time. The OTC-VFA_120 TEs supplementation scenario is comparable in methanization enhancement to OTC-VFA_DL. However, lower methanization efficiency occurs with OTC-VFA_120 TEs supplementation approach under certain VFA concentrations. Regardless the risk of lower methanization efficiency compared to OTC-VFA_DL, OTC-VFA_120 minimised the adverse effects of electrostatic interactions due to TEs supplementation and was significantly more desirable than the TC-compromise and the TC-control. Consequently, it was selected for further investigations on the mechanisms of TEs improvement during methanization.

The mechanisms of the enhancements in methanization due to TEs supplementation were investigated for mesophilic and thermophilic operations by comparing the OTC-VFA_120 to TC-control TEs configurations in mesophilic and thermophilic operations. The influence of TEs supplementation on the Michaelis-Menten kinetic parameters of maximum reaction rate (MRR) and inverse affinity (IA) for VFA degradation rates were evaluated in **Section 7.4**. The mechanisms by which TEs enhance methanization processes are temperature dependent. Mesophilic methanization was enhanced by an increase in MRR, but showed an insignificant loss in IA due to supplementation of OTC-VFA_120 TEs configuration. This indicates that minor loss in substrate affinity is possible in mesophilic methanization with the supplementation of the OTC-VFA_120 TEs configuration, and highlights the bio-catalytic weakness in the use of single TEs configuration during methanization involving varying VFA concentrations.

Conversely, in thermophilic methanization, VFA. OTC-VFA_120 TEs configuration did not enhance MRR but reduced the IA by up to 109% compared to the TC-control. This thermophilic enhancement mechanism suggests that TEs supplementation significantly improves microbial affinity for VFA in thermophilic AD but does not improve the maximum VFA utilization rate. Looking at the AD implications of the

kinetic influences of mesophilic and thermophilic OTC-VFA_120 TEs configuration, it was obvious that the mesophilic OTC-VFA_120 induced higher VFA degradation rates compared to the thermophilic OTC-VFA_120 and TC-control TEs configurations. Conversely, thermophilic OTC-VFA_120 and TC-control had comparable VFA degradation rates, and thermophilic TC-control had higher VFA degradation rate than mesophilic TC-control.

The bioavailability of TEs in the batch experiments was evaluated since not all the TEs that are supplemented to a reactor can be utilized by the micro-organisms for the optimization of methanization processes. In **Section 7.5 of Chapter 7**, it was shown that some proportions of the supplemented TEs are bioavailable and are taken up by the micro-organisms for growth and metabolism, whereas some proportions are adsorbed to digester solids. Digester solids include sulphides and oxides of Fe, and insoluble minerals in the reactors. Co and Se were the most bioavailable TEs, whereas Ni and Mo where the least bioavailable because they were more adsorbed to digester solids. Regardless the TEs adsorption to digester solids, the quality of the digestate obtained after AD by implementing the recommended TEs concentrations in this report is not compromised by TEs content. Optimum supplementation range for Ni that is recommended in this report is below the legal limits of 300 – 400 mg/kg DM set by the European Directive 86/278/EEC and the German adoption of the directive for total Ni concentration in methanization sludge intended for soil amendment. There are no such limits for Co, Se and Mo in digestate.

The results of semi-continuous experiments, which were aimed at validating the TEs configurations derived in the batch operations, were presented in **Section 7.6 of Chapter 7**. VFA-independent optimum TEs configuration was derived from the range of VFA-dependent TEs concentrations that were considered optimum for mesophilic methanization. This VFA-independent optimum TEs configuration was modified into variant with less Co concentration and more Se concentrations. These variants of the optimum TEs configuration were named Optimum –Co and Optimum +Se respectively. The VFA-independent optimum TEs configuration or simply referred to as 'Optimum', Optimum –Co, Optimum +Se variants and the Control TEs configuration were tested in a semi-continuous investigation using mixed fruit residue (MFR) as substrate. The organic loading rate (OLR) was varied and FOS/TAC, CH_4 production and VFA accumulation were monitored.

The Optimum, Optimum –Co, Optimum +Se variants produced > 20% more CH_4 per day and accumulated less VFA than the Control reactor. The Optimum, Optimum –Co and Optimum +Se reactors also had higher organic loading rates, lower FOS/TAC values and were more stable during the methanization period compared to the

Control reactor that failed due to VFA accumulation even at relatively low organic loading rate. Acetic acid, propionic acid and butyric acid were the most abundant VFA species; and Optimum –Co had the lowest acetic acid accumulation but the highest propionic acid accumulation. A reduction in Co concentration in one of the variants of the VFA-independent optimum TEs configuration (Optimum –Co reactor) enabled quick recovery from toxic influences of acid accumulation when the OLR was increased from 4g oDM/L/d to 8g oDM/L/d. This capability was absent in the Control reactor and relatively low in the Optimum and Optimum +Se reactors.

DGGE was used to monitor the change in population of the micro-organism during the semi-continuous experiments and the results corroborate the mechanisms of methanization enhancements in our batch investigations. There were no emergence of new populations of bacteria and archaea during the process. However, there was disappearance of certain bacterial population due to TEs supplementation and changes in OLR, and this was most evident in the Optimum –Co reactor. The microbial population structure was generally conserved in the archaea population in the TEs supplemented reactors but loss in population of archaea was observed in the Control reactor.

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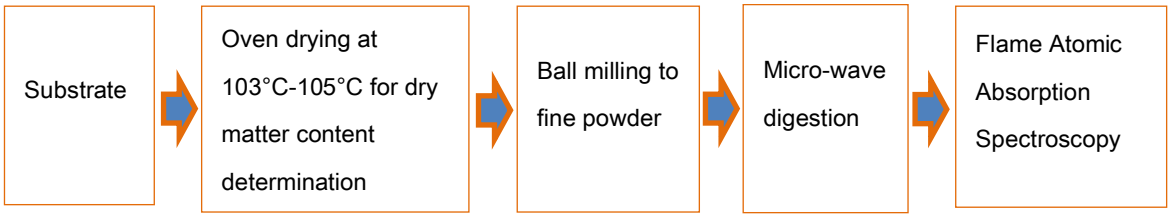
APPENDICES

Appendix: Chapter 6

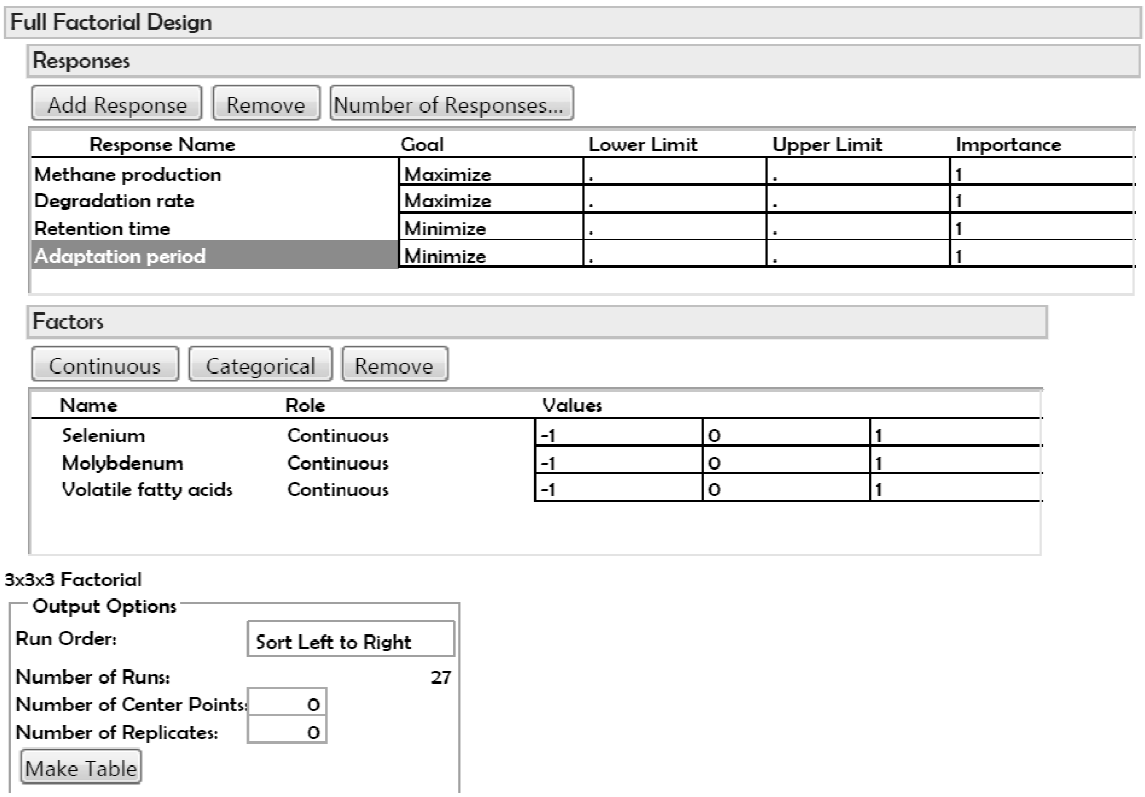
Appendix 6.1 Scheme of procedure for trace elements and Fe determination in degradable organic substrates



Appendix 6.2 Reagents for nutrient media, trace elements solution and VFA mixture used in the mesophilic and thermophilic experiments

Material	Source	Comment
Basic nutrient solution KH_2PO_4 Na_2HPO_4 NH_4Cl $CaCl_2.H_20$ $MgCl_2.6H_2O$ $NaHCO_3$ $Na_2S.9H_2O$ $FeCl_3$ H_3BO_4 $ZnCl_2$ $CuCl_2$ $MnCl_2$ Cysteine Hydrochloride	VWR International GmbH	Analytic grade
TEs solution $CoCl_2.6H_2O$ $NiCl_2.6H_2O$ $Na_2SeO_3.5H_2O$ $(NH_4)_6Mo_7O_{24}.4H_2O$	VWR International GmbH	Analytic grade
Silage digestate (Inoculum)	Company, Hamburg	Mesophilic (37°C)
Substrates (VFA mixture) $C_4H_7NaO_2$ $C_3H_5NaO_2$ $C_2H_3NaO_2$	VWR International GmbH	Analytic grade

Appendix 6.3 Full factorial (FF) platform of JMP 10 showing factor and response specifications used for the generation of the DoE for the investigations with Se, Mo and VFA as factors


Full Factorial Design

Responses

Add Response | Remove | Number of Responses...

Response Name	Goal	Lower Limit	Upper Limit	Importance
Methane production	Maximize	.	.	1
Degradation rate	Maximize	.	.	1
Retention time	Minimize	.	.	1
Adaptation period	Minimize	.	.	1

Factors

Continuous | Categorical | Remove

Name	Role	Values		
Selenium	Continuous	-1	0	1
Molybdenum	Continuous	-1	0	1
Volatile fatty acids	Continuous	-1	0	1

3x3x3 Factorial

Output Options

Run Order: Sort Left to Right

Number of Runs: 27

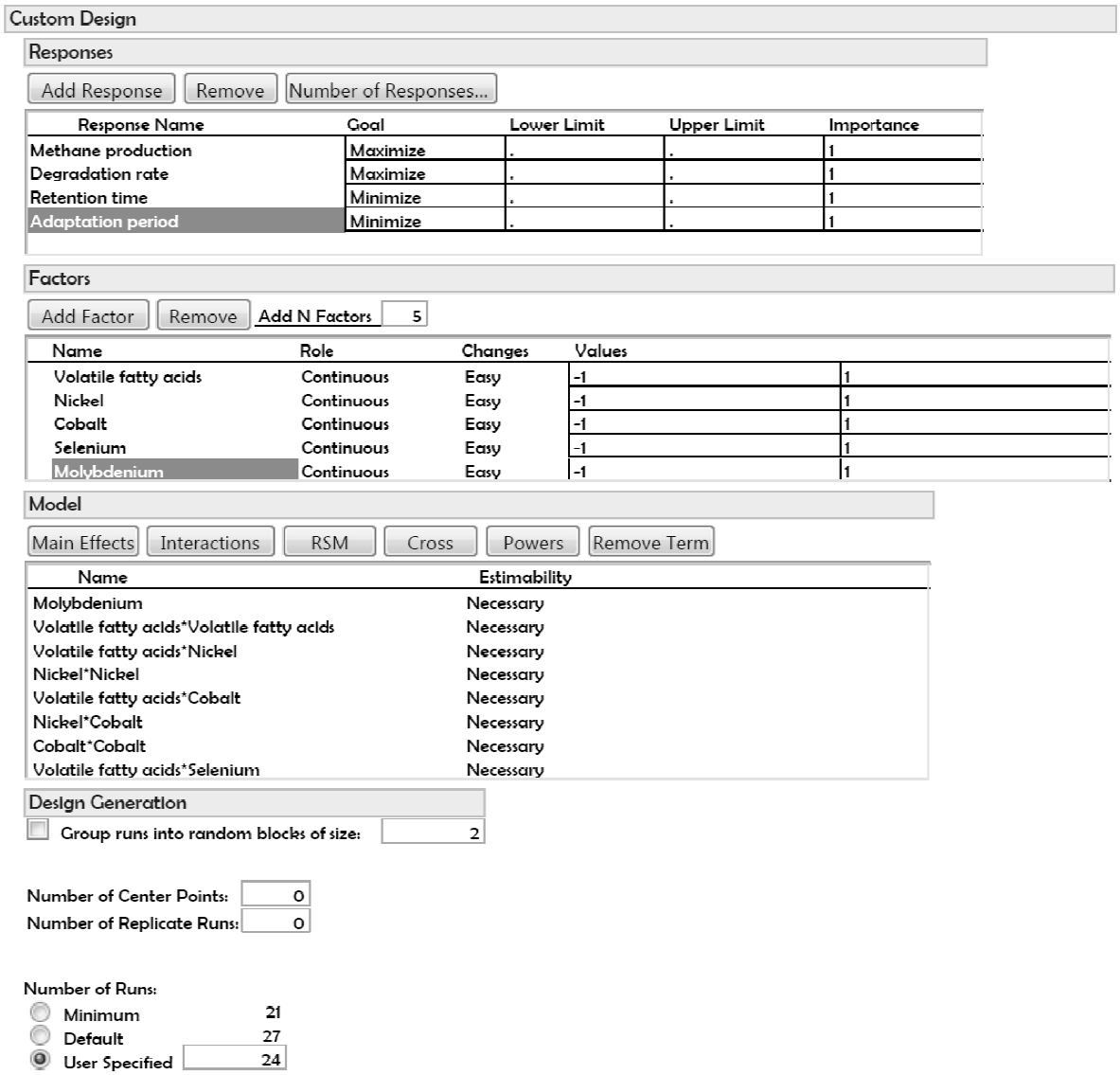
Number of Center Points: 0

Number of Replicates: 0

Make Table

The full factorial platform allows for definition of goals for the responses of interest. Maximum methane production and VFA degradation rate were most desirable; lower substrate retention time was targeted and a short period for process adaptation to TEs supplementation was preferred. All response variables were considered of equal relevance to methanization efficiency. -1, 0 and 1 are design codes referring to low-, medium- and high factor concentrations respectively.

Appendix 6.4 Custom design (CD) platform of JMP 10 showing factor and response specifications used for the generation of the design of experiments (DoE) for the investigations with Ni, Co, Se, Mo and VFA as factors



Appendix 6.4 shows features of the CD module of JMP 10. Details are contained in JMP10 (SAS Institute Inc. 2012: Design of Experiments). The main features of CD include:

- Possibility to create designs that optimize the measures of goodness of fit in responses of interest;
- Minimization of the average variance of prediction
- Flexibility in choice of affordable number of experimental runs; and
- Accuracy of prediction increases with increase in number of experimental runs;
- Ranking of responses based on the optimization need of the designer; and
- Application of statistical models to data analysis.

The models enable the determination of the following effects:
- Main effects (influence of individual factors);
- Factor interaction effects (joint influences of two or more factors);
- Quadratic effects or response surface modelling, RSM (influence of a wide range of factor settings, including extreme positive and negative values that define limits of factor effects);
- Cross effects (interaction between groups of factors); and

- Experimental power (the ability of the experimental design to detect small changes in responses of interest).

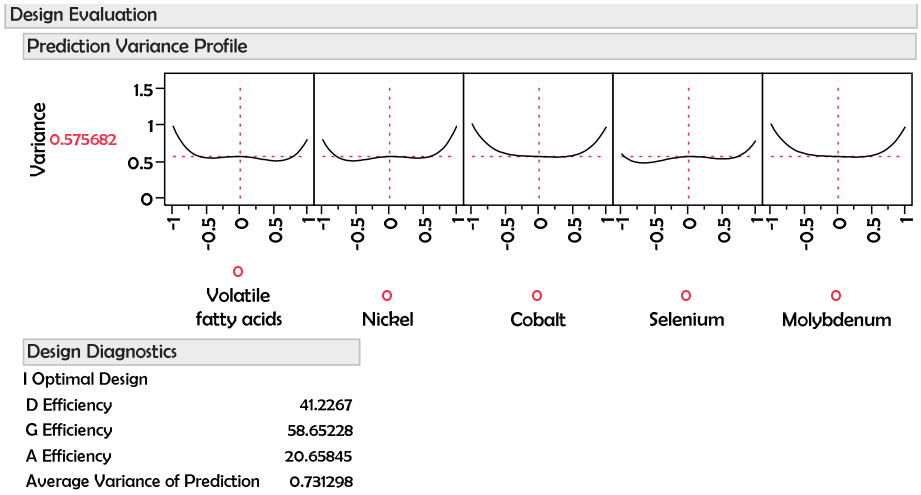
The platform in Appendix 6.4 also shows the minimum number of experimental runs necessary for the investigation. It also allows the designer to add runs to this minimum (user defined). The default or recommended number of runs is also shown. In principle, any number between the minimum and the number of runs in the FF design is implementable. Based on PV, the efficiency of any chosen number of experimental runs or design can be visualized.

The PV for any factor setting is the product of the error variance (*e*), and a quantity that depends on the design (*d*), and the factor setting (*s*). Prior to data collection, *e* is unknown, so PV is also unknown. However, the ratio of PV to *e* is not a function of *e*, but of *d* and *s*. This ratio is called the relative prediction variance (RPV) and can be calculated before acquiring the data. In principle, the FF design has RPV of 0, since all possible combinations of the factors are explored.

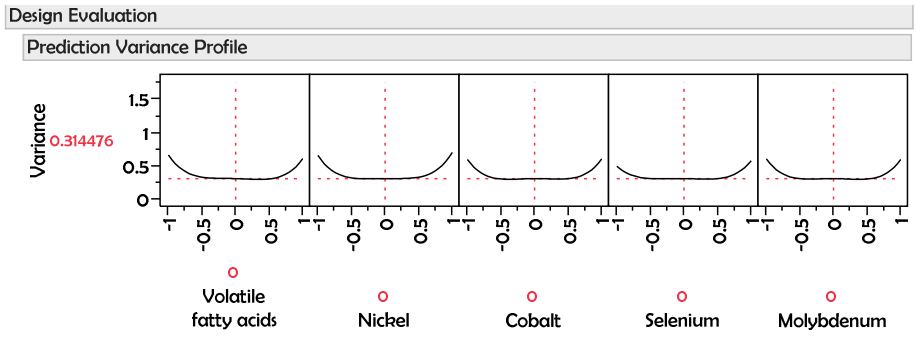
PV $= e * ds$

The larger the number of runs, the smaller the RPV value; hence, the more efficient is the design. The RPV approaches zero as the number of runs increases. Any number of runs between least- (FF design) and highest RPV values (minimum $CD_{\_implementable}$) can be used for the investigation (SAS Institute Inc. 2012: DoE). The RPV can be displayed as profiles of the entire response surface. These profiles are called the Prediction Variance Profile (PVP). PVP plots the RPV as a function of each factor at fixed values of the other factors. Using a built-in statistical algorithm, the average variance of prediction across the factors can be determined and used for design comparison. Appendix 6.5a, b and c shows the profiles of RPV for 21-, 24-, and 27 experimental runs corresponding to minimum-, user specified-, and default number of experimental runs in the design.

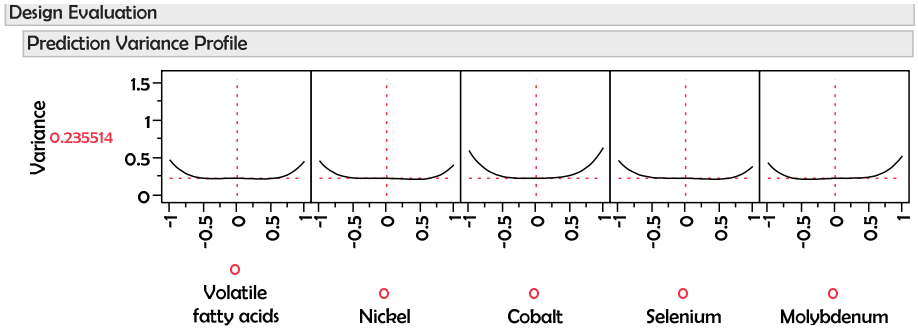
Appendix 6.5 Profiles of the relative prediction variance for the design of experiments (DoE) of the Ni, Co, Se, Mo and VFA investigations generated in the custom design platform of the JMP 10 for **(a)** 21 experimental runs (minimum) **(b)** 24 experimental runs (user specified) **(c)** 27 experimental runs corresponding to (default)



Design Diagnostics	
I Optimal Design	
D Efficiency	41.2267
G Efficiency	58.65228
A Efficiency	20.65845
Average Variance of Prediction	0.731298



Design Diagnostics	
I Optimal Design	
D Efficiency	44.72552
G Efficiency	74.73185
A Efficiency	26.22006
Average Variance of Prediction	0.490391



Design Diagnostics	
I Optimal Design	
D Efficiency	45.51546
G Efficiency	88.57706
A Efficiency	28.77463
Average Variance of Prediction	0.395985

The values in red on the variance axis (Y-axis) are relative factor variance at mid-concentrations of the factors. The Average Variance of Prediction shown in the Design Diagnostics is the average of the maximum variance across the factors.

Appendix 6.6 Design of experiments (DoE) from the custom design (CD) platform for Ni, Co, Se, Mo and VFA in the thermophilic investigation

Treatment	Ni	Co	Se	Mo	VFA	Group
R1	-1	-1	-1	1	-1	
R2	-1	-1	1	-1	-1	
R3	-1	0	-1	0	-1	
R4	-1	1	-1	1	-1	
R5	0	1	-1	-1	-1	Low
R6	0	1	1	0	-1	
R7*	-1	-1	-1	-1	-1	
R8	1	-1	1	1	-1	
R9	1	0	1	-1	-1	
R10	1	1	-1	1	0	
R11	-1	0	1	1	0	
R12	-1	1	-1	-1	0	
R13	0	-1	-1	0	0	
R14	0	0	-1	0	0	Medium
R15	0	0	-1	1	0	
R16	0	0	1	0	0	
R17	1	1	-1	0	0	
R18	-1	-1	-1	0	0	
R19	-1	-1	1	1	1	
R20	-1	0	-1	0	1	
R21	-1	1	-1	1	1	
R22	-1	1	1	-1	1	
R23	0	0	-1	-1	1	
R24	0	0	-1	0	1	
R25	1	-1	-1	1	1	High
R26	1	-1	1	-1	1	
R27	1	1	-1	-1	1	
R28	1	1	1	1	1	
R29*	-1	-1	-1	-1	1	
R30*	-1	-1	-1	-1	1	

Appendix 6.7a Feed constituents of the silage feedstock and digestate in Table 7.1

Constituents	%
Maize silage	75
Lawn grass	15
Wine residue	5
Cow manure (milking cow)	2.5
Others	2.5

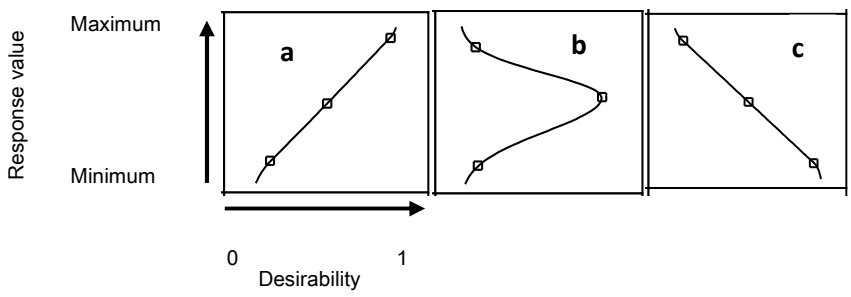
Appendix 6.7b Average properties of the inoculum used for the supplementation experiments

Inocula characteristics	
pH	7.7 ± 0.1
DM (%)	9.34 ± 1.4
C (%DM)	38.53 ± 0.04
H (%DM)	4.98 ± 0.12
N (%DM)	2.77 ± 0.02
S (%DM)	0.41 ± 0.01
O (%DM)	53.51 ± 0.05
Organic Dry Matter (o DM) (%)	74.5 ± 0.60
Substrate o DM (%)	9.69 ± 0.43
Ash (%)	2.63 ± 0.06
Water content (%)	89.70 ± 0.04

Appendix 6.7c Procedure for implementing the experimental runs in the batch and semi-continuous trace elements supplementation investigations

a. Weigh into a 2000 ml flask containing small amount of distilled water, *n+1* times the quantities of the individual VFA salts required for the block being prepared, (*n* is the number of treatments in the VFA block being prepared, +1 extra reactor that serves for any necessary baseline analysis for a block of treatments, example: total VFA);
b. Stir with a magnetic stirrer until salts dissolve;
c. Add distilled water up to 2000 ml mark;
d. Weigh in ≈7.4 g VSS (≈ 200 ml depending on DM) equivalent of the inoculum into a 1000 ml ± 20 measuring cylinder;
e. Add (*2000/n+1*) ml of VFA solution in step c into cylinder;
f. Add 250 ml nutrient solution and stir gently to homogeneity using a glass rod;
g. Add distilled water up to 750 ml mark;
h. Note all quantities added up to this point;
i. Turn the mixture in step *g* into a reactor;
j. Repeat the procedure for the other treatments in the group by adding the exact quantities of all inputs as noted in step *h* directly to the reactors. This should be 750ml of content in all the reactors;
k. Add calculated volume of each TEs required for implementing the matrix, from the prepared nutrient solution (see Table 6.6);
l. The total reactor volume should be 800ml; the volume of Ni, Co, Se and Mo added to each reactor vary but should be less than 50ml for the sum of all TEs addition for any treatment;
m. Add 50 ml - ∑TEs (ml) distilled water to each reactor;
n. Adjust the pH of all reactors to between 6.8 and 7.2;
o. Store the content of the extra reactor from each of the VFA blocks at ≤0°C or 4°C depending on whether microbial analysis is required or not;
p. Flush each experimental reactor with nitrogen gas for one minute, connect to the eudiometer and take first reading.
q. In the semi-continuous investigations, 2 litre glass reactors were used instead of the 1 litre glass reactors. However, the procedures are the same, except that in the semi-continuous operation, the TEs were also added periodically to compensate for TEs removed when reactor content was removed to during organic loading. Also in the continuous operation, reactor content was mixed by a magnetic stirrer instead of manual stirring.

Appendix 6.8 Shapes of desirability function for response variable **(a)** maximize; **(b)** target value and **(c)** minimize (SAS Inc. NC, 2012: Multivariate analysis and modelling).



Appendix 6.9 Procedure for the determination of the carbohydrate composition by hydrolysis and high performance liquid chromatography (HPLC)

Hydrolysis:

The following two-stage acid hydrolysis was optimized for woody monomeric sugar recovery (Puls, 1993).

The hydrolysis was performed in triplicate using 200 mg of the sample calculated as dry material for each hydrolysis experiment. 2 ml of cold H_2SO_4 (72 %) was added to the sample and incubated 1 hour at 30 °C in a water bath. The reaction mixture was stirred with a glass rod to insure an even impregnation with the acid and a uniform pre-hydrolysis. The pre-hydrolysis was stopped by addition of 6 ml water. The mixture was transferred with additional 50 ml of water into a volumetric flask (100 ml). The volumetric flask was placed into an autoclave and the post-hydrolysis was performed at 120 °C, 1,2 bar for 30 minutes (The heating up and cooling down time is not included into the hydrolysis time. After post-hydrolysis the flasks were cooled down, filled up to the mark and the hydrolysis residue was filtered on a G-4 crucible. The filtrate was frozen to the time of HPLC analysis. The residue was washed extensively with water, dried at 105 °C over night and determined gravimetrically.

HPLC:

The different monosaccharides were separated on a 6.6 mm bore column of 115mm length (Omnifit) filled with the strong anion exchange resin (MCI Gel CA08F (Mitsubishi) at 60°C). The mobile phase (0.7 ml min−1) was made of A: 0.3M potassium borate buffer pH 9.2 and B: 0.9M potassium borate buffer pH 9.2. After sample injection the separation was started with 90%A and 10%B. A linear gradient was run within 35 min to 10%A and 90%B. Data acquisition was stopped after 47 min. Sugar quantification was achieved by after-column derivatization with Cu-bichinconinate (0.35 ml min−1) at 105°C in a 30m crocheted Teflon coil of 0.3mm inner diameter and detection at 560 nm. Data were processed with the Dionex Chromeleon 6.80 software.

Appendix 6.10 Procedure for the sequential extraction of Ni, Co, Se and Mo in the silage digestate used for thermophilic methanization with VFA mixture as substrates.

The BCR three stage extraction procedure for trace- and other metals (BCR -community bureau of reference): (Modified from Tokalioglu *et al.,* 2003)

Sample collection and pre-treatment

Samples were collected and dried at 105 ◦C in an oven acceding to the procedure for the determination of total solids (DM); then the dried samples were crushed to a fine powder using a ball mill. They were stored at 4°C until required for analysis. Precautions were taken to avoid contamination during sampling, drying, grinding, sieving and Storage.

Exchangeable metals: 40 ml of 0.11 mol L^{-1} acetic acid was added to 1.00 g of sample (dried at 105°C) in a 50-ml polypropylene tube. The mixture was shaken for 16 hrs at 22 ± 3°C (overnight) at 400 rpm. The extract was separated from the solid phase by centrifugation at 3800 rpm for 20 min. The supernatant liquid was decanted into a 100-ml beaker and then covered with a watch-glass. The residue was washed by adding 20 ml of double-distilled water, shaking for 15 min, and then centrifuging at 3800 rpm for 20 min. The second supernatant liquid was discarded without any loss of residue.

Metals bound to iron and manganese oxides: Metals bound to iron and manganese oxides were extracted by adding 40 ml of 0.1 mol L^{-1} hydroxyl-ammonium chloride (adjusted to pH 2 with 2 mol L^{-1} nitric acid) onto the residue from the first step. After shaking the mixture for 16 hrs at 22 ± 3°C, it was centrifuged for 15 min, and then decanted into a beaker. Using 20 ml of distilled water, the residue was washed, centrifuged, and the supernatant discarded.

Metals bound to organic matter and sulphides: 10 ml of 8.8 mol L^{-1} hydrogen peroxide was carefully added in small aliquots to the residue in the centrifuge tube. The tube ingredients were digested at room temperature for 1hr with occasional manual shaking. The procedure was continued for 1 hour at 85°C and the volume reduced to a few ml by further heating in a water bath. A second aliquot of 10 ml of hydrogen peroxide was added to the residue and the digestion procedure was repeated. The solution was heated to near dryness, and 50 ml of 1.0 mol L^{-1} ammonium acetate solution (adjusted to pH 2 with nitric acid) was added to the moist residue. The sample solution was shaken and centrifuged, and the extract was separated as described above.

Residual: The analysis of the residue was performed using aqua regia for metals insoluble in the previous steps. For this purpose, first 6 ml of double-distilled water and then aqua regia solution in a sequence of 15 ML (HNO3) and 10 ML (HCl) were added to the remaining residue. After adding each aqua regia solution, the residue was evaporated to near dryness on a water bath. The extract was filtered through filter paper by adding 1 mol L^{-1} HNO3 solution in small amounts on the last residue in the centrifuge tube. The tube walls were carefully rinsed with the same acid solution and then the dregs were collected in a beaker.

Extracts analysis: The extracts acquired after each extraction stage applied sequentially were evaporated to near dryness. Each extract was completed to 5 ml with 1 mol L^{-1} HNO_3; the extract of ammonium acetate was made up to 6 ml. The determinations of Ni, Co, Se and Mo in the extracts were performed by FAAS.

Appendix: Chapter 7

Appendix 7.1a Sugar contents of selected anaerobic digestion substrates

Sugar/Sample	Lawn grass	Spear grass	Cow dung	Horse dung	Mixed fruit residue
4-O-Me-Glc-Acid [%]	0.00	0.00	0.00	0.00	0.00
Cellobiose [%]	0.20	0.21	0.25	0.00	0.02
Rhamnose [%]	0.45	0.25	0.30	0.26	1.02
Arabinose [%]	3.16	2.56	1.70	2.20	4.09
Galactose [%]	1.84	2.02	0.97	1.00	3.30
Mannose [%]	0.60	0.17	0.27	0.61	2.32
Xylose [%]	11.30	9.83	10.94	17.80	6.60
Glucose [%]	24.83	28.05	16.98	28.79	19.79
Sum carbohydrates [%]	42.38	43.09	31.40	50.65	37.14
Hydrolysis residue [%]	24.80	21.10	35.47	32.40	10.00

Appendix 7.1b Design of experiments (DoE) for mesophilic Ni, Co, Se and Mo supplementation to different VFA levels; peak HAR and relative HAR (SD- standard deviation; * Control reactor)

R. Nr.	VFA (mmol/L)		Ni	Co	Se	Mo	Peak HAR (mmol/L/d)		Relative HAR	
	Avg.	SD	mg/L				Avg.	SD	Avg.	SD
R1	28	2	0.07	0.03	0.98	0.04	3	0.14	0.78	0.04
R2			0.07	1.88	0.00	1.24	3.57	0.31	0.92	0.09
R3			0.07	3.73	0.00	0.64	2.91	0.32	0.76	0.09
R4			0.07	3.73	0.98	1.24	2.89	0.26	0.75	0.08
R5			1.03	3.73	0.00	1.24	2.72	0.33	0.71	0.10
R6			1.99	0.03	0.00	0.04	2.39	0.21	0.62	0.06
R7			1.99	0.03	0.98	1.24	2.86	0.25	0.74	0.07
R8			1.99	3.73	0.98	0.04	2.43	0.14	0.63	0.04
R9	116	2	0.07	1.88	0.49	0.04	3.41	0.20	0.56	0.04
R10			1.03	0.03	0.49	1.24	5.91	0.40	0.97	0.08
R11			1.03	1.88	0.98	0.64	3.56	0.14	0.58	0.03
R12			1.99	3.73	0.49	0.64	4.21	0.21	0.69	0.04
R13	213	6	0.07	0.03	0.00	0.64	5.28	0.36	0.96	0.08
R14			0.07	0.03	0.98	1.24	3.97	0.26	0.72	0.06
R15			0.07	3.73	0.00	1.24	3.79	0.19	0.69	0.04
R16			0.07	3.73	0.98	0.04	9.96	0.28	1.81	0.06
R17			1.03	1.88	0.49	0.64	5.96	0.24	1.08	0.05
R18			1.03	3.73	0.00	0.04	9.06	0.46	1.65	0.10
R19			1.99	0.03	0.98	0.04	3.61	0.23	0.66	0.05
R20			1.99	1.88	0.00	1.24	4.09	0.24	0.74	0.05
R21			1.99	3.73	0.98	1.24	5.51	0.28	1.00	0.06
R28*	28	2	0.07	0.03	0.00	0.04	3.86	0.22	1.00	-
R29*	116	2	0.07	0.03	0.00	0.04	6.12	0.30	1.00	-
R30*	213	6	0.07	0.03	0.00	0.04	5.5	0.13	1.00	-

Appendix 7.2 Full model terms, estimates of parameters and significance of terms in modelling of HAR of silage feedstock at 37°C due to Ni, Co, Se and Mo supplementation to VFA levels between 28 and 213 mmol/L

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	0.574627	0.064594	8.90	0.0124*
VFA (mmol/L)	0.0013837	0.000189	7.32	0.0182*
Ni (mg/L)	-0.023253	0.024224	-0.96	0.4384
Co(mg/L)	0.015289	0.010857	1.41	0.2944
Se(mg/L)	-0.057564	0.041517	-1.39	0.2999
Mo(mg/L)	-0.088682	0.032801	-2.70	0.1139
(VFA (mmol/L) 123.827)*(VFA (mmol/L) 123.827)	0.0000324	7.115e 6	4.55	0.0450*
(VFA (mmol/L)-123.827)*(Ni (mg/L)-0.86304)	0.0010115	0.000282	3.59	0.0696
(Ni (mg/L)-0.86304)*(Ni (mg/L)-0.86304)	-0.17872	0.052237	-3.42	0.0758
(VFA (mmol/L)-123.827)*(Co(mg/L)-1.88)	0.0010036	0.000149	6.75	0.0212*
(Ni (mg/L)-0.86304)*(Co(mg/L)-1.88)	0.0249158	0.015264	1.63	0.2442
(Co(mg/L)-1.88)*(Co(mg/L)-1.88)	0.0646105	0.01415	4.57	0.0448*
(VFA (mmol/L)-123.827)*(Se(mg/L)-0.4687)	0.0007053	0.00041	1.72	0.2273
(Ni (mg/L)-0.86304)*(Se(mg/L)-0.4687)	-0.090108	0.04601	-1.96	0.1893
(Co(mg/L)-1.88)*(Se(mg/L)-0.4687)	0.136371	0.025045	5.45	0.0321*
(Se(mg/L)-0.4687)*(Se(mg/L)-0.4687)	-0.131485	0.249335	-0.53	0.6506
(VFA (mmol/L)-123.827)*(Mo(mg/L)-0.64)	-0.002789	0.000468	-5.96	0.0270*
(Ni (mg/L)-0.86304)*(Mo(mg/L)-0.64)	0.032706	0.043702	0.75	0.5323
(Co(mg/L)-1.88)*(Mo(mg/L)-0.64)	-0.161015	0.022157	-7.27	0.0184*
(Se(mg/L)-0.4687)*(Mo(mg/L)-0.64)	-0.113034	0.074514	-1.52	0.2686
(Mo(mg/L)-0.64)*(Mo(mg/L)-0.64)	-0.098076	0.125589	-0.78	0.5166

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Appendix 7.3a [11]Cumulative CH_4 production, CH_4 content, VFA degradation rate and VFA retention time at 37°C due to Ni, Co, Se and Mo supplementation to different VFA levels (SD-standard deviation; NR – never recovered; * Control reactor)

R. Nr.	VFA (mmol/L)		Cumulative CH_4 production (Nml)		CH_4 (%)		VFA degradation rate (mmol/L/d)		Retention time (Days)	
	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
R1	28	2	666.04	18.84	59.0	1.7	2.40	0.07	9	0
R2			473.07	28.10	60.0	3.6	2.60	0.15	15	1
R3			475.36	30.92	59.0	3.8	3.10	0.20	14	1
R4			311.78	15.87	58.0	3.0	1.50	0.08	NR	-
R5			681.69	46.27	56.0	4.0	3.20	0.22	7	1
R6			632.21	25.03	57.0	2.3	2.30	0.09	11	0.5
R7			606.63	30.88	57.0	3.0	2.40	0.12	13	1
R8			737.84	20.87	57.0	1.6	3.20	0.09	7	0
R9	116	2	2181.87	129.60	60.0	3.6	7.80	0.46	12	1
R10			1643.04	111.53	60.0	4.0	6.80	0.46	16	1
R11			2175.42	86.14	60.0	2.4	8.00	0.32	12	0.5
R12			2221.58	113.10	59.0	3.0	8.10	0.41	11	0.5
R13	213	6	2082.06	141.33	61.0	4.1	7.20	0.49	29	2
R14			1978.97	128.74	61.0	4.0	7.60	0.49	31	2
R15			2067.94	105.28	61.0	3.1	7.00	0.36	33	2
R16			2318.24	65.57	60.0	1.7	9.40	0.27	26	1
R17			2876.60	113.91	61.0	2.4	10.70	0.42	21	1
R18			2874.12	146.33	60.0	3.1	10.30	0.52	14	1
R19			1914.85	124.57	60.0	3.9	7.70	0.50	31	2
R20			2476.23	147.08	60.0	3.6	10.10	0.60	20	1
R21			2693.83	137.15	61.0	3.1	11.40	0.58	20	1
R28*	28	2	432.44	28.13	57.0	3.7	1.70	0.11	12.5	2
R29*	116	2	1477.82	87.78	58.0	3.5	5.80	0.34	17.5	1
R30*	213	6	2370.44	67.05	59.0	1.7	6.30	0.18	25	1

[11] Cumulative CH_4 production and VFA degradation rate were measure in the post-hydrolysis and acidification phase.

Appendix 7.3b Relative CH_4 production, relative VFA degradation rate and relative VFA retention time at 37°C due to Ni, Co, Se and Mo supplementation to different levels of VFA (SD-standard deviation; * Control reactor)

R. Nr.	VFA (mmol/L)		Relative CH_4 production		Relative VFA degradation rate		Relative retention time	
	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
R1	28	2	1.54	0.04	1.41	0.04	1.28	0.04
R2			1.09	0.06	1.53	0.09	0.80	0.05
R3			1.10	0.07	1.82	0.12	0.88	0.06
R4			0.72	0.04	0.88	0.04	2.00	0.10
R5			1.58	0.11	1.88	0.13	1.44	0.10
R6			1.46	0.06	1.35	0.05	1.12	0.04
R7			1.40	0.07	1.41	0.07	0.96	0.05
R8			1.71	0.05	1.88	0.05	1.44	0.04
R9	116	2	1.48	0.09	1.34	0.08	1.31	0.08
R10			1.11	0.08	1.17	0.08	1.09	0.07
R11			1.47	0.06	1.38	0.05	1.34	0.05
R12			1.50	0.08	1.40	0.07	1.37	0.07
R13	213	6	0.88	0.06	1.14	0.08	0.84	0.06
R14			0.83	0.05	1.21	0.08	0.76	0.05
R15			0.87	0.04	1.11	0.06	0.68	0.03
R16			0.98	0.03	1.49	0.04	0.96	0.03
R17			1.21	0.05	1.70	0.07	1.16	0.05
R18			1.21	0.06	1.63	0.08	1.44	0.07
R19			0.81	0.05	1.22	0.08	0.76	0.05
R20			1.04	0.06	1.60	0.10	1.20	0.07
R21			1.14	0.06	1.81	0.09	1.20	0.06
R28*	28	2	1.00		1.00		1.00	-
R29*	116	2	1.00		1.00		1.00	-
R30*	213	6	1.00		1.00		1.00	-

Appendix 7.3c Full model terms, estimates of parameters and significance of terms in modelling relative CH_4 production at 37°C due to Ni, Co, Se and Mo supplementation to VFA levels between 28 and 213 mmol/L.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.6275294	0.135257	12.03	0.0012*
VFA (mmol/L)	-0.001941	0.000428	-4.53	0.0201*
Ni (mg/L)	0.1746408	0.059262	2.95	0.0602
Co(mg/L)	0.0508382	0.026148	1.94	0.1471
Se(mg/L)	0.1014606	0.096827	1.05	0.3717
Mo(mg/L)	-0.163068	0.078286	-2.08	0.1286
(VFA (mmol/L)-123.548)*(VFA (mmol/L)-123.548)	-1.117e-5	1.327e-5	-0.84	0.4615
(VFA (mmol/L)-123.548)*(Ni (mg/L)-0.83)	-0.001905	0.000637	-2.99	0.0582
(Ni (mg/L)-0.83)*(Ni (mg/L)-0.83)	-0.106603	0.118313	-0.90	0.4340
(VFA (mmol/L)-123.548)*(Co(mg/L)-1.80292)	0.0001547	0.000337	0.46	0.6772
(Ni (mg/L)-0.83)*(Co(mg/L)-1.80292)	0.0710213	0.034587	2.05	0.1323
(Co(mg/L)-1.80292)*(Co(mg/L)-1.80292)	-0.062184	0.031902	-1.95	0.1464
(VFA (mmol/L)-123.548)*(Se(mg/L)-0.44917)	0.0002217	0.000928	0.24	0.8266
(Ni (mg/L)-0.83)*(Se(mg/L)-0.44917)	-0.052923	0.10364	-0.51	0.6448
(Co(mg/L)-1.80292)*(Se(mg/L)-0.44917)	-0.117463	0.056567	-2.08	0.1294
(Se(mg/L)-0.44917)*(Se(mg/L)-0.44917)	-0.289201	0.527113	-0.55	0.6214
(VFA (mmol/L)-123.548)*(Mo(mg/L)-0.615)	0.0001487	0.00106	0.14	0.8974
(Ni (mg/L)-0.83)*(Mo(mg/L)-0.615)	0.1235328	0.09887	1.25	0.3001
(Co(mg/L)-1.80292)*(Mo(mg/L)-0.615)	0.0475853	0.050144	0.95	0.4126
(Se(mg/L)-0.44917)*(Mo(mg/L)-0.615)	-0.10797	0.168269	-0.64	0.5668
(Mo(mg/L)-0.615)*(Mo(mg/L)-0.615)	-0.072226	0.281314	-0.26	0.8140

Appendix 7.3d Full model terms, estimates of parameters and significance of terms in modelling relative VFA degradation rate at 37°C due to Ni, Co, Se and Mo supplementation to VFA levels between 28 and 213 mmol/L.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.3050273	0.037153	35.13	0.0008*
VFA (mmol/L)	-0.00064	0.000114	-5.60	0.0305*
Ni (mg/L)	0.1749563	0.013744	12.73	0.0061*
Co(mg/L)	0.0705259	0.00622	11.34	0.0077*
Se(mg/L)	-0.049307	0.022585	-2.18	0.1607
Mo(mg/L)	-0.126642	0.018658	-6.79	0.0210*
(VFA (mmol/L)-127.888)*(VFA (mmol/L)-127.888)	0.0000451	3.526e-6	12.79	0.0061*
(VFA (mmol/L)-127.888)*(Ni (mg/L)-0.86304)	0.0001326	0.000168	0.79	0.5124
(Ni (mg/L)-0.86304)*(Ni (mg/L)-0.86304)	-0.223257	0.030293	-7.37	0.0179*
(VFA (mmol/L)-127.888)*(Co(mg/L)-1.88)	-0.000119	8.58e-5	-1.39	0.2984
(Ni (mg/L)-0.86304)*(Co(mg/L)-1.88)	0.0352149	0.008667	4.06	0.0556
(Co(mg/L)-1.88)*(Co(mg/L)-1.88)	-0.037988	0.008043	-4.72	0.0420*
(VFA (mmol/L)-127.888)*(Se(mg/L)-0.4687)	0.0027372	0.000239	11.43	0.0076*
(Ni (mg/L)-0.86304)*(Se(mg/L)-0.4687)	0.1403069	0.027198	5.16	0.0356*
(Co(mg/L)-1.88)*(Se(mg/L)-0.4687)	-0.04363	0.014607	-2.99	0.0962
(Se(mg/L)-0.4687)*(Se(mg/L)-0.4687)	-0.105383	0.133352	-0.79	0.5122
(VFA (mmol/L)-127.888)*(Mo(mg/L)-0.64)	0.0018035	0.000277	6.52	0.0227*
(Ni (mg/L)-0.86304)*(Mo(mg/L)-0.64)	0.2367762	0.024884	9.52	0.0109*
(Co(mg/L)-1.88)*(Mo(mg/L)-0.64)	-0.088795	0.012672	-7.01	0.0198*
(Se(mg/L)-0.4687)*(Mo(mg/L)-0.64)	-0.097343	0.042507	-2.29	0.1492
(Mo(mg/L)-0.64)*(Mo(mg/L)-0.64)	-0.056982	0.070525	-0.81	0.5039

Appendix 7.3e Model terms, estimates of parameters and significance of terms in modelling relative VFA retention time at 37°C due to Ni, Co, Se and Mo supplementation to VFA levels between 28 and 213 mmol/L.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.1620518	0.092958	12.50	0.0011*
VFA (mmol/L)	-0.001237	0.000294	-4.20	0.0246*
Ni (mg/L)	0.1594698	0.040729	3.92	0.0296*
Co(mg/L)	0.0812071	0.017971	4.52	0.0203*
Se(mg/L)	0.176331	0.066546	2.65	0.0770
Mo(mg/L)	-0.107604	0.053804	-2.00	0.1393
(VFA (mmol/L)-123.548)*(VFA (mmol/L)-123.548)	-1.265e-6	9.117e-6	-0.14	0.8985
(VFA (mmol/L)-123.548)*(Ni (mg/L)-0.83)	0.0006706	0.000438	1.53	0.2233
(Ni (mg/L)-0.83)*(Ni (mg/L)-0.83)	-0.217929	0.081313	-2.68	0.0750
(VFA (mmol/L)-123.548)*(Co(mg/L)-1.80292)	-0.000416	0.000231	-1.80	0.1701
(Ni (mg/L)-0.83)*(Co(mg/L)-1.80292)	0.0282217	0.02377	1.19	0.3206
(Co(mg/L)-1.80292)*(Co(mg/L)-1.80292)	-0.011332	0.021925	-0.52	0.6409
(VFA (mmol/L)-123.548)*(Se(mg/L)-0.44917)	-0.002972	0.000638	-4.66	0.0187*
(Ni (mg/L)-0.83)*(Se(mg/L)-0.44917)	-0.306265	0.071229	-4.30	0.0231*
(Co(mg/L)-1.80292)*(Se(mg/L)-0.44917)	0.0699737	0.038877	1.80	0.1697
(Se(mg/L)-0.44917)*(Se(mg/L)-0.44917)	-0.276025	0.362269	-0.76	0.5015
(VFA (mmol/L)-123.548)*(Mo(mg/L)-0.615)	-0.000715	0.000729	-0.98	0.3989
(Ni (mg/L)-0.83)*(Mo(mg/L)-0.615)	0.0127558	0.067951	0.19	0.8631
(Co(mg/L)-1.80292)*(Mo(mg/L)-0.615)	0.0677004	0.034462	1.96	0.1442
(Se(mg/L)-0.44917)*(Mo(mg/L)-0.615)	0.3268606	0.115646	2.83	0.0664
(Mo(mg/L)-0.615)*(Mo(mg/L)-0.615)	0.265683	0.193338	1.37	0.2631

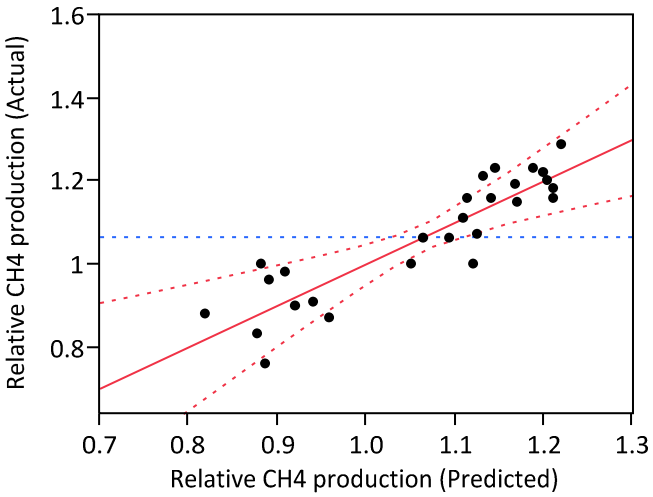
Appendix 7.4a Design of experiments (DoE) for mesophilic Se and Mo supplementation to different VFA levels; Cumulative CH_4 production; CH_4 content and VFA degradation rate (SD-standard deviation; * Control reactor)

R. Nr.	VFA (mmol/L)		Se	Mo	Cumulative CH_4 production (Nml)		CH_4 (%)		VFA degradation rate (mmol/L/d)	
	Avg.	SD	mg/L		Avg.	SD	Avg.	SD	Avg.	SD
R28*	26	3	0	0.04	432.44	12.23	57	3.7	1.33	0.09
R2			0.49	0.04	511.68	30.39	61	3.6	1.66	0.1
R3			0.98	0.04	462.95	30.12	60	3.9	1.63	0.11
R4			0	0.64	513.88	26.16	60	3.1	1.49	0.08
R5			0.49	0.64	451.73	30.66	59	4	1.62	0.11
R6			0.98	0.64	454.18	17.98	58	2.3	1.6	0.06
R7			0	1.24	521.86	26.57	58	3	1.64	0.08
R8			0.49	1.24	544.05	15.39	59	1.7	1.83	0.05
R9			0.98	1.24	448.35	26.63	58	3.5	1.91	0.11
R29*	112	5	0	0.04	1404.31	39.72	58	3.5	5.69	0.34
R11			0.49	0.04	1821.11	72.11	61	2.4	6.52	0.26
R12			0.98	0.04	1878.26	95.63	61	3.1	6.41	0.33
R13			0	0.64	1782.94	121.03	61	4.1	5.96	0.4
R14			0.49	0.64	1779.18	115.74	61	4	6	0.39
R15			0.98	0.64	1750.97	89.15	61	3.1	6.24	0.32
R16			0	1.24	1781.36	50.38	60	1.7	5.78	0.16
R17			0.49	1.24	1931.96	76.50	63	2.5	6.28	0.25
R18			0.98	1.24	1924.00	97.95	60	3.1	6.36	0.32
R30*	217	9	0	0.04	2294.04	64.89	59	1.7	6.28	0.18
R20			0.49	0.04	2187.67	129.94	61	3.6	7.98	0.47
R21			0.98	0.04	2233.72	113.72	61	3.1	7.24	0.37
R22			0	0.64	2323.26	118.28	61	3.1	6.86	0.35
R23			0.49	0.64	2291.47	64.81	61	1.7	8.64	0.24
R24			0.98	0.64	2095.71	124.48	61	3.6	7.24	0.43
R25			0	1.24	1869.32	121.61	61	4	7.13	0.46
R26			0.49	1.24	2471.99	125.85	61	3.1	8.67	0.44
R27			0.98	1.24	2222.10	62.85	62	1.8	9.38	0.27

Appendix 7.4b Relative CH_4 production and relative VFA degradation rate at 37°C due to Se and Mo supplementation to different VFA levels (SD- standard deviation; * Control reactor)

R. Nr.	VFA (mmol/L)		Relative CH_4 production		Relative VFA degradation rate	
	Avg.	SD	Avg.	SD	Avg.	SD
R28*	26	3	1.00	0.03	1	-
R2			1.18	0.07	1.25	0.07
R3			1.07	0.07	1.23	0.08
R4			1.19	0.06	1.12	0.06
R5			1.04	0.07	1.22	0.08
R6			1.05	0.04	1.2	0.05
R7			1.21	0.06	1.23	0.06
R8			1.26	0.04	1.38	0.04
R9			1.04	0.06	1.44	0.09
R29*	112	5	1.00	0.03	1	-
R11			1.30	0.05	1.15	0.05
R12			1.34	0.07	1.13	0.06
R13			1.27	0.09	1.05	0.07
R14			1.27	0.08	1.05	0.07
R15			1.25	0.06	1.1	0.06
R16			1.27	0.04	1.02	0.03
R17			1.38	0.05	1.1	0.04
R18			1.37	0.07	1.12	0.06
R30*	217	9	1.00	0.03	1	-
R20			0.95	0.06	1.27	0.08
R21			0.97	0.05	1.15	0.06
R22			1.01	0.05	1.09	0.06
R23			1.00	0.03	1.38	0.04
R24			0.91	0.05	1.15	0.07
R25			0.81	0.05	1.14	0.07
R26			1.08	0.05	1.38	0.07
R27			0.97	0.03	1.49	0.04

Appendix 7.4c Actual vs. predicted plot of relative CH_4 production at 37°C due to Se and Mo supplementation to VFA levels between 26 and 217 mmol/L.



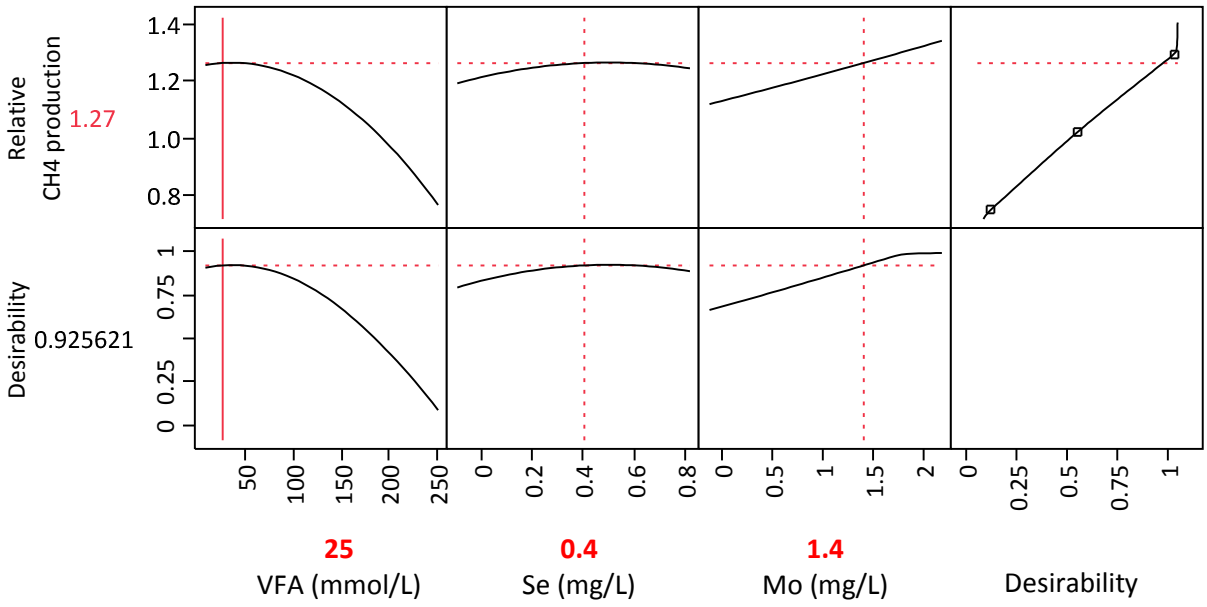
R^2	0.81
R^2 Adj.	0.71
Root Mean Square Error	0.08
Mean of Response	1.07
Observations	27

Line of fit (solid line), significance curve at α 0.05 (dotted curves), and line for mean of response (dotted horizontal line)

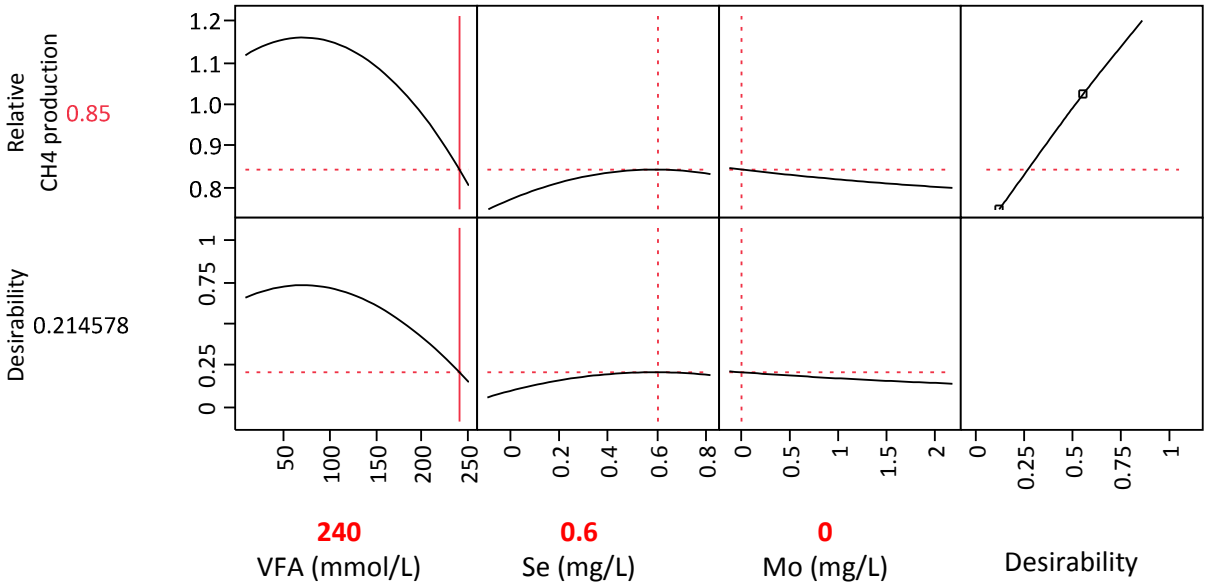
Appendix 7.4d Model terms, estimates of parameters and significance of terms in modelling of relative CH_4 production at 37°C due to Se and Mo supplementation to VFA levels between 26 and 217 mmol/L

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.3158612	0.053387	24.65	<.0001*
VFA (mmol/L)	-0.001079	0.000201	-5.36	<.0001*
Se (mg/L)	-0.002268	0.038721	-0.06	0.9540
Mo (mg/L)	0.0425926	0.031622	1.35	0.1957
(VFA (mmol/L)-118.333)*(VFA (mmol/L)-118.333)	-1.88e-5	3.645e-6	-5.16	<.0001*
(VFA (mmol/L)-118.333)*(Se (mg/L)-0.49)	0.0001074	0.000496	0.22	0.8311
(Se (mg/L)-0.49)*(Se (mg/L)-0.49)	-0.185108	0.136872	-1.35	0.1940
(VFA (mmol/L)-118.333)*(Mo (mg/L)-0.64)	-0.000662	0.000405	-1.63	0.1205
(Se (mg/L)-0.49)*(Mo (mg/L)-0.64)	-0.02551	0.07904	-0.32	0.7508
(Mo (mg/L)-0.64)*(Mo (mg/L)-0.64)	0.0154321	0.091286	0.17	0.8678

Appendix 7.4e Prediction profile and optimum factor settings for relative CH_4 production at 37°C due to Se and Mo supplementation to VFA level of 25 mmol/L



Appendix 7.4f Prediction profile and optimum factor settings for relative CH_4 production at 37°C due to Se and Mo supplementation to VFA level of 240 mmol/L



Appendix 7.5a Design of experiments (DoE) for thermophilic Ni, Co, Se and Mo supplementation to different VFA levels (red); CH_4 content; Cumulative CH_4 production; and VFA degradation rate

R. Nr.	VFA (mmol/L)		Ni	Co	Se	Mo	CH_4 (%)		Cumulative CH_4 production (Nml)		VFA degradation rate (mmol/L/d)	
	Avg.	SD	mg/L				Avg.	SD	Avg.	SD	Avg.	SD
R1	55	3	0.09	0.03	0.00	1.24	61.7	1.7	1178.15	33.32	5.9	0.2
R2			0.09	0.03	0.98	0.04	61.4	3.6	1159.65	68.88	5.2	0.3
R3			0.09	1.88	0.00	0.64	60.2	3.9	1160.93	75.52	4.9	0.3
R4			0.09	3.73	0.49	1.24	59.8	3.0	1102.92	56.15	4.7	0.2
R5			1.05	3.73	0.00	0.04	60.5	4.1	1271.79	86.33	6.1	0.4
R6			1.05	3.73	0.98	0.64	60.3	2.4	1228.70	48.65	5.7	0.2
R7*			0.09	0.03	0.00	0.04	60.1		1233.92	62.82	4.8	0.2
R8			2.01	0.03	0.98	1.24	61.4	1.7	1004.53	28.41	5.7	0.2
R9			2.01	1.88	0.98	0.04	61.4	3.6	1089.09	64.69	4.7	0.3
R10			2.01	3.73	0.00	1.24	60.8	4.1	1215.33	82.50	6.1	0.4
R11	107	5	0.09	1.88	0.98	1.24	59.8	2.4	2062.26	81.66	6.2	0.3
R12			0.09	3.73	0.49	0.04	59.5	3.0	2232.79	113.67	6.2	0.3
R13			1.05	0.03	0.49	0.64	59.8	4.1	2467.74	167.52	7.5	0.5
R14			1.05	1.88	0.00	0.64	60.0	3.9	2517.95	163.80	7.0	0.5
R15			1.05	1.88	0.49	1.24	60.1	3.1	2522.59	128.43	6.7	0.34
R16			1.05	1.88	0.98	0.64	59.9	1.7	2427.12	68.65	7.1	0.2
R17			2.01	3.73	0.49	0.64	60.3	2.4	2380.59	94.27	7.2	0.29
R18	209	11	0.09	0.03	0.00	0.64	59.4	3.0	4721.45	240.38	9.4	0.5
R19			0.09	0.03	0.98	1.24	58.4	3.8	4220.98	274.59	9.4	0.6
R20			0.09	1.88	0.49	0.64	59.3	3.5	4497.06	267.11	9.1	0.5
R21			0.09	3.73	0.00	1.24	58.6	3.0	4537.71	231.02	8.6	0.4
R22			0.09	3.73	0.98	0.04	58.7	3.0	4113.09	209.40	7.5	0.4
R23			1.05	1.88	0.49	0.04	59.5	1.7	4749.15	134.33	9.0	0.3
R24			1.05	1.88	0.49	0.64	59.1	3.5	4859.80	288.66	8.6	0.5
R25			2.01	0.03	0.00	1.24	53.9	3.5	4661.25	303.23	8.6	0.6
R26			2.01	0.03	0.98	0.04	59.2	3.0	4842.63	246.55	9.3	0.5
R27			2.01	3.73	0.00	0.04	58.9	1.7	3707.34	104.86	9.0	0.3
R28			2.01	3.73	0.98	1.24	60.0	3.9	4755.20	309.34	8.6	0.6
R29*	107	5	0.09	0.03	0.00	0.04	61.8		2473.29	146.91	6.6	0.4
R30*	209	11	0.09	0.03	0.00	0.04	63.3		4899.50	138.58	8.7	0.3

Appendix 7.5b Relative VFA degradation rate and relative CH_4 production at 55°C due to Ni, Co, Se and Mo supplementation to different VFA levels (SD- standard deviation; * Control reactor)

R. Nr.	VFA (mmol/L)		Relative VFA degradation rate		Relative CH_4 production	
	Avg.	SD	Avg.	SD	Avg.	SD
R1	55	3	1.23	0.03	0.95	0.03
R2			1.08	0.06	0.94	0.06
R3			1.02	0.07	0.94	0.06
R4			0.98	0.05	0.89	0.05
R5			1.27	0.09	1.03	0.07
R6			1.19	0.05	1.00	0.04
R7*			1.00	-	1.00	-
R8			1.19	0.03	0.81	0.02
R9			0.98	0.06	0.88	0.05
R10			1.28	0.09	0.98	0.07
R11	107	5	0.93	0.04	0.83	0.03
R12			0.94	0.05	0.90	0.05
R13			1.13	0.08	1.00	0.07
R14			1.06	0.07	1.02	0.07
R15			1.01	0.05	1.02	0.05
R16			1.07	0.03	0.98	0.03
R17			1.09	0.04	0.96	0.04
R18	209	11	1.08	0.05	0.96	0.05
R19			1.07	0.07	0.86	0.06
R20			1.04	0.06	0.92	0.05
R21			0.99	0.05	0.93	0.05
R22			0.85	0.04	0.84	0.04
R23			1.03	0.03	0.97	0.03
R24			0.99	0.06	0.99	0.06
R25			0.98	0.06	0.95	0.06
R26			1.06	0.05	0.99	0.05
R27			1.03	0.03	0.76	0.02
R28			0.98	0.06	0.97	0.06
R29*	107	5	1.00	-	1.00	-
R30*	209	11	1.00	-	1.00	-

Appendix 7.5c Model terms, estimates of parameters and significance of terms in modelling of relative CH_4 production at 55°C due to Ni, Co, Se and Mo supplementation to VFA levels between 55 and 209 mmol/L.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.0284268	0.021252	48.39	<.0001*
VFA(mmol/L)	-7.464e-5	8.872e-5	-0.84	0.4219
Ni(mg/L)	0.0305367	0.007634	4.00	0.0031*
Co(mg/L)	-0.010314	0.003756	-2.75	0.0226*
Se(mg/L)	-0.03844	0.013969	-2.75	0.0224*
Mo(mg/L)	-0.004495	0.011206	-0.40	0.6977
(VFA(mmol/L)-130.203)*(VFA(mmol/L)-130.203	-1.616e-6	2.759e-6	-0.59	0.5726
(VFA(mmol/L)-130.203)*(Ni(mg/L)-0.922)	-2.176e-5	9.757e-5	-0.22	0.8285
(Ni(mg/L)-0.922)*(Ni(mg/L)-0.922)	-0.099155	0.016972	-5.84	0.0002*
(VFA(mmol/L)-130.203)*(Co(mg/L)-1.81833)	-0.000134	0.000052	-2.58	0.0299*
(Ni(mg/L)-0.922)*(Co(mg/L)-1.81833)	0.0032603	0.004376	0.74	0.4753
(Co(mg/L)-1.81833)*(Co(mg/L)-1.81833)	0.0048212	0.004237	1.14	0.2846
(VFA(mmol/L)-130.203)*(Se(mg/L)-0.45733)	0.0006648	0.000188	3.53	0.0064*
(Ni(mg/L)-0.922)*(Se(mg/L)-0.45733)	0.0315443	0.016096	1.96	0.0817
(Co(mg/L)-1.81833)*(Se(mg/L)-0.45733)	0.0161003	0.00875	1.84	0.0989
(Se(mg/L)-0.45733)*(Se(mg/L)-0.45733)	-0.033023	0.060377	-0.55	0.5977
(VFA(mmol/L)-130.203)*(Mo(mg/L)-0.6)	0.0004487	0.000156	2.87	0.0185*
(Ni(mg/L)-0.922)*(Mo(mg/L)-0.6)	0.0207972	0.01314	1.58	0.1479
(Co(mg/L)-1.81833)*(Mo(mg/L)-0.6)	0.0306952	0.006814	4.50	0.0015*
(Se(mg/L)-0.45733)*(Mo(mg/L)-0.6)	-0.062769	0.026118	-2.40	0.0397*

Appendix 7.5d Model terms, estimates of parameters and significance of terms in modelling of relative VFA degradation rate at 55°C due Ni, Co, Se and Mo supplementation to VFA levels between 55 and 209 mmol/L.

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	1.0869427	0.04459	24.38	<.0001*
VFA(mmol/L)	-0.000632	0.000186	-3.39	0.0079*
Ni(mg/L)	0.0395365	0.016018	2.47	0.0357*
Co(mg/L)	-0.014266	0.007881	-1.81	0.1037
Se(mg/L)	-0.036497	0.029309	-1.25	0.2445
Mo(mg/L)	0.0435181	0.023511	1.85	0.0972
(VFA(mmol/L)-130.203)*(VFA(mmol/L)-130.203	1.0042e-5	5.79e-6	1.73	0.1169
(VFA(mmol/L)-130.203)*(Ni(mg/L)-0.922)	-0.000232	0.000205	-1.13	0.2862
(Ni(mg/L)-0.922)*(Ni(mg/L)-0.922)	-0.09912	0.03561	-2.78	0.0213*
(VFA(mmol/L)-130.203)*(Co(mg/L)-1.81833)	-0.000112	0.000109	-1.02	0.3326
(Ni(mg/L)-0.922)*(Co(mg/L)-1.81833)	0.0258628	0.009182	2.82	0.0202*
(Co(mg/L)-1.81833)*(Co(mg/L)-1.81833)	0.0243761	0.00889	2.74	0.0228*
(VFA(mmol/L)-130.203)*(Se(mg/L)-0.45733)	0.0002654	0.000395	0.67	0.5182
(Ni(mg/L)-0.922)*(Se(mg/L)-0.45733)	0.0425882	0.033771	1.26	0.2390
(Co(mg/L)-1.81833)*(Se(mg/L)-0.45733)	-0.046195	0.018359	-2.52	0.0330*
(Se(mg/L)-0.45733)*(Se(mg/L)-0.45733)	0.0645242	0.12668	0.51	0.6228
(VFA(mmol/L)-130.203)*(Mo(mg/L)-0.6)	-0.000398	0.000328	-1.21	0.2554
(Ni(mg/L)-0.922)*(Mo(mg/L)-0.6)	0.00954	0.027569	0.35	0.7373
(Co(mg/L)-1.81833)*(Mo(mg/L)-0.6)	-0.016871	0.014297	-1.18	0.2682
(Se(mg/L)-0.45733)*(Mo(mg/L)-0.6)	-0.031779	0.0548	-0.58	0.5762

Appendix 7.6 Influences of different mesophilic Ni, Co, Se and Mo supplementation approaches on relative VFA degradation rate, relative VFA retention time, relative CH_4 production and desirability

Scenarios	VFA	Ni	Co	Se	Mo	Relative VFA degradation rate	Relative VFA retention time	Relative CH_4 production	Desirability
	mmol/L	mg/L							
[12]TC-control	10	0.07	0.03	0.00	0.04	1.44	0.94	1.09	0.39
	50	0.07	0.03	0.00	0.04	1.15	0.98	1.13	0.35
	100	0.07	0.03	0.00	0.04	0.94	1.02	1.14	0.29
	150	0.07	0.03	0.00	0.04	0.89	1.06	1.09	0.26
	200	0.07	0.03	0.00	0.04	0.99	1.09	0.98	0.28
	250	0.07	0.03	0.00	0.04	1.25	1.12	0.81	0.29
TC-compromise	10	0.77	1.78	0.45	0.58	1.89	1.43	1.64	0.76
	50	0.77	1.78	0.45	0.58	1.63	1.39	1.65	0.68
	100	0.77	1.78	0.45	0.58	1.44	1.33	1.60	0.61
	150	0.77	1.78	0.45	0.58	1.42	1.27	1.51	0.56
	200	0.77	1.78	0.45	0.58	1.55	1.20	1.35	0.54
	250	0.77	1.78	0.45	0.58	1.84	1.13	1.14	0.52
OTC-VFA_120	10	1.94	3.74	0.10	1.61	2.20	1.50	1.99	0.83
	50	1.94	3.74	0.10	1.61	1.89	1.48	1.93	0.79
	100	1.94	3.74	0.10	1.61	1.65	1.45	1.80	0.72
	150	1.94	3.74	0.10	1.61	1.56	1.41	1.61	0.66
	200	1.94	3.74	0.10	1.61	1.63	1.36	1.37	0.61
	250	1.94	3.74	0.10	1.61	1.87	1.31	1.07	0.54
OTC-VFA_DL	10	1.88	4.21	0.32	1.61	1.96	1.81	1.82	0.89
	50	1.92	4.01	0.19	1.61	1.91	1.58	1.72	0.82
	100	1.94	3.74	0.10	1.61	1.62	1.54	1.70	0.73
	150	2.15	3.75	0.00	1.61	1.58	1.38	1.63	0.66
	200	2.04	3.62	0.03	1.61	1.60	1.42	1.41	0.62
	250	0.80	2.20	0.53	0.00	1.85	1.31	1.22	0.59

[12] In the RSM model, the, responses are predicted per unit concentration of the predictor variable (VFA, Ni, Co, Se and Mo) and the relative values are in comparison with a situation of 0 mg/L Ni, Co, Se and Mo in the reactor (See SAS Institute Inc., 2013: JMP 11 Fitting linear models page 262 for more details).

Appendix 7.7a Predicted VFA degradation rate at 55°C due to Ni, Co, Se and Mo supplementation that was used for the determination of the kinetic parameters MRR and IA with the Michaelis–Menten mode

Scenarios	VFA (mmol/L)	Ni (mg/L)	Co(mg/L)	Se(mg/L)	Mo(mg/L)	Pred. VFA degradation rate (mmol/L/d)
TC-control	50	0.09	0.03	0	0.04	4.90
TC-control	100	0.09	0.03	0	0.04	6.52
TC-control	150	0.09	0.03	0	0.04	7.87
TC-control	200	0.09	0.03	0	0.04	8.96
TC-control	250	0.09	0.03	0	0.04	9.79
OTC-VFA_120	50	1.23	3.73	0.00	1.06	6.14
OTC-VFA_120	100	1.23	3.73	0.00	1.06	7.42
OTC-VFA_120	150	1.23	3.73	0.00	1.06	8.44
OTC-VFA_120	200	1.23	3.73	0.00	1.06	9.20
OTC-VFA_120	250	1.23	3.73	0.00	1.06	9.70

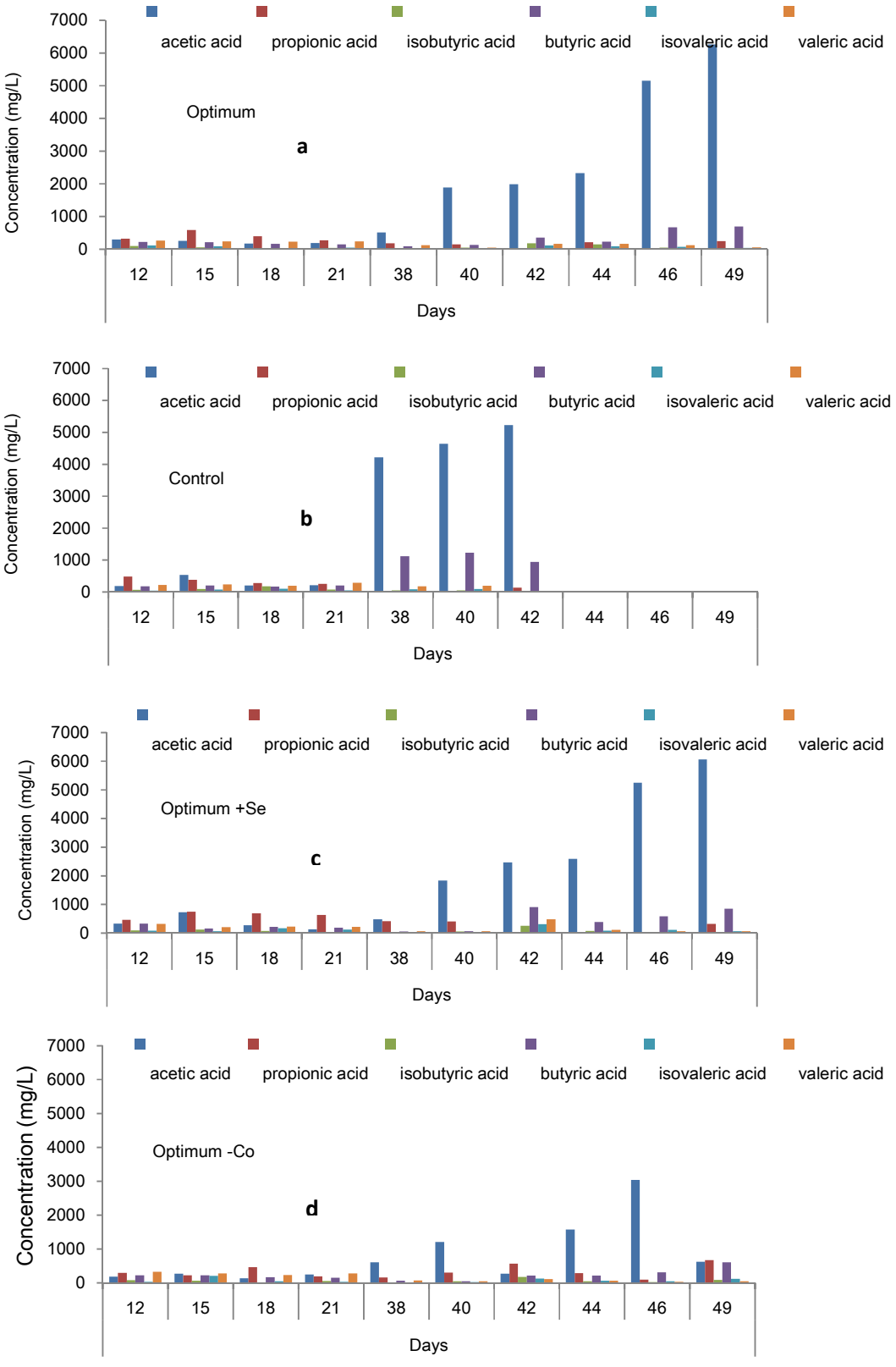
Appendix 7.7b Predicted VFA degradation rate at 37°C due to Ni, Co, Se and Mo supplementation that was used for the determination of the kinetic parameters MRR and IA with the Michaelis–Menten model

Scenarios	VFA (mmol/L)	Ni (mg/L)	Co(mg/L)	Se(mg/L)	Mo(mg/L)	Pred. VFA degradation rate (mmol/L/d)
TC-control	50	0.07	0.03	0.00	0.04	3.43
TC-control	100	0.07	0.03	0.00	0.04	5.08
TC-control	150	0.07	0.03	0.00	0.04	6.16
TC-control	200	0.07	0.03	0.00	0.04	6.66
TC-control	250	0.07	0.03	0.00	0.04	6.57
OTC-VFA_120	50	1.94	3.74	0.10	1.61	5.83
OTC-VFA_120	100	1.94	3.74	0.10	1.61	8.12
OTC-VFA_120	150	1.94	3.74	0.10	1.61	9.83
OTC-VFA_120	200	1.94	3.74	0.10	1.61	10.96
OTC-VFA_120	250	1.94	3.74	0.10	1.61	11.51

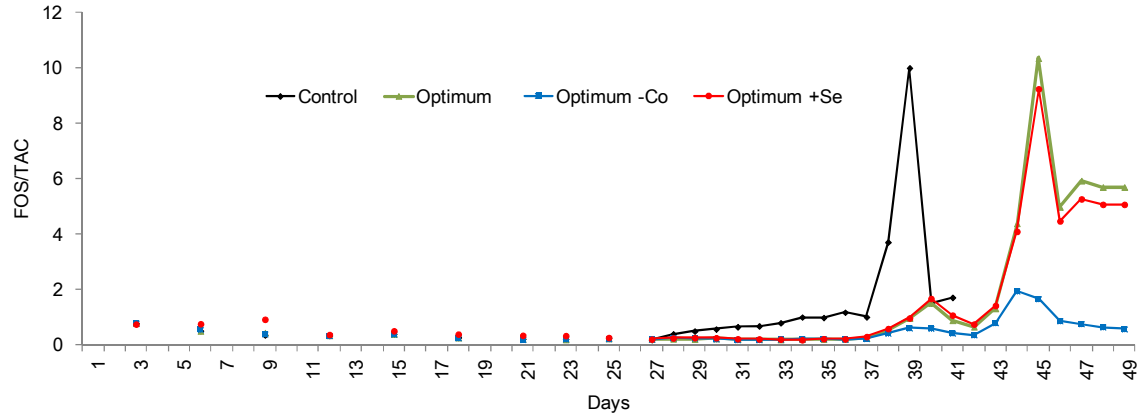
Appendix 7.7c Predicted VFA degradation rate at 37°C due to Se and Mo supplementation that was used for the determination of the kinetic parameters MRR and IA with the Michaelis–Menten model

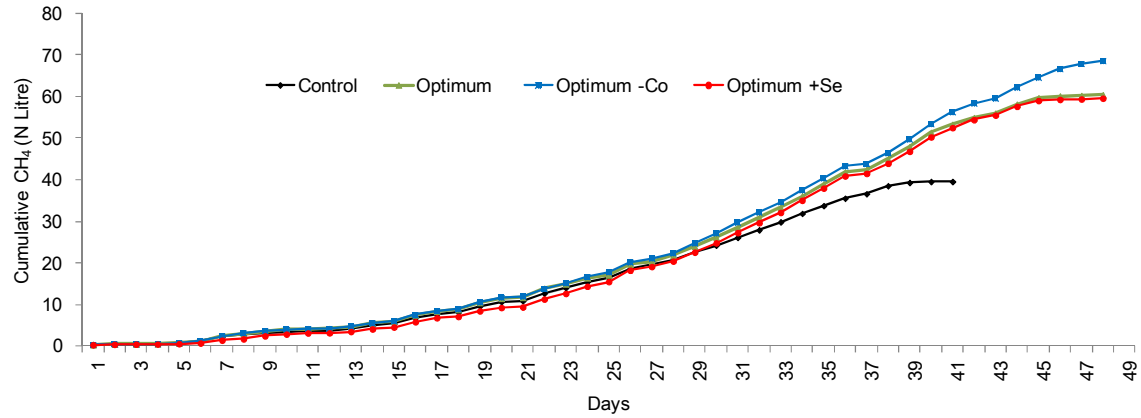
Scenarios	VFA (mmol/L)	Se(mg/L)	Mo(mg/L)	Pred. VFA degradation rate (mmol/L/d)
TC-control	50	0	0.04	3.76
TC-control	100	0	0.04	5.59
TC-control	150	0	0.04	6.73
TC-control	200	0	0.04	7.17
TC-control	250	0	0.04	6.91
OTC-VFA_120	50	0.7	1.2	4.31
OTC-VFA_120	100	0.7	1.2	6.43
OTC-VFA_120	150	0.7	1.2	7.86
OTC-VFA_120	200	0.7	1.2	8.60
OTC-VFA_120	250	0.7	1.2	8.64

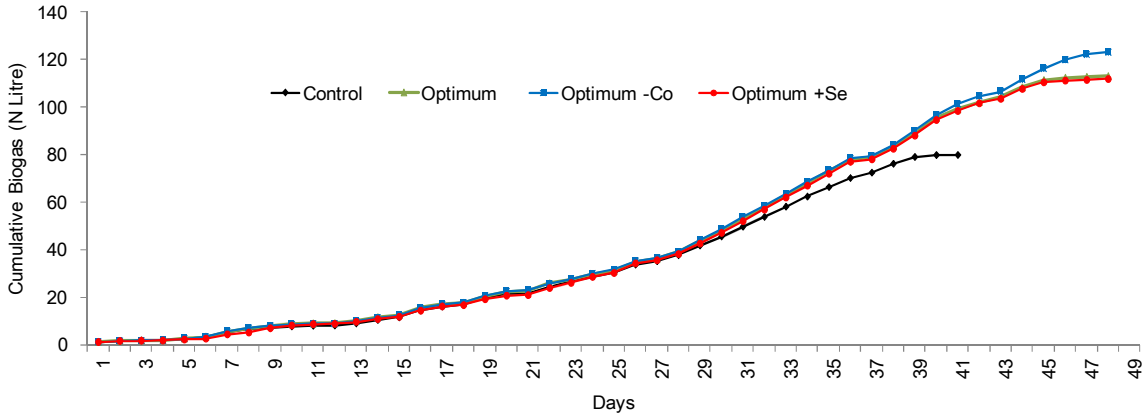
Appendix 7.8 Individual VFA concentrations in the experimental reactors due to Ni, Co, Se and Mo supplementation (as shown in Table 7.10) in the mesophilic semi-continuous experiment with mixed fruit residue: **a)** Optimum reactor; **b)** Control reactor; **c)** Optimum +Se reactor; **d)** Optimum –Co reactor



Appendix 7.9 Overview of cumulative biogas, cumulative CH_4 and FOS/TAC due to Ni, Co, Se and Mo supplementation (as shown in Table 7.10) and different OLR in the mesophilic semi-continuous experiment with mixed fruit residue for the Control, Optimum, Optimum –Co and Optimum +Se reactors







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