UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING AND THE ENVIRONMENT

REQUIREMENT AND DISTRIBUTION OF TRACE ELEMENTS IN MESOPHILIC ANAEROBIC DIGESTION

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

FACULTY OF ENGINEERING AND THE ENVIRONMENT

Civil and Environmental Engineering

Thesis for the degree of Doctor of Philosophy

REQUIREMENT AND DISTRIBUTION OF TRACE ELEMENTS IN MESOPHILIC ANAEROBIC DIGESTION

Nanthanat Sriprasert

For AD process control and optimisation, significant attention must be paid to long-term process stability. TE are essential and play a crucial role in the metabolism of anaerobic microorganisms. The TE requirement has been recognised widely, but the challenge about how to optimise TE dosing, which is a multifaceted question involving metal chemistry, physical interactions of metal and solids, microbiology and technology optimisation, still remained. The availability of TE for microbial activities is very important to obtain efficient and stable biogas processes. Therefore, the factors regulating TE bioavailability need to be further understood in order to determine proper TE dosage to be supplied in anaerobic systems. Moreover, an appropriate quantification of the required TE dosing and suitable ranges during long-term operation has been given little attention. Therefore, this research aimed to quantify the required TE dosing ranges under designated operational conditions in mesophilic AD using model substrate. A special attention was given to identify the critical TE concentrations and the dynamic changes of their distribution in different fractionations over the course of TE washing process. TE availability was examined via digester performance and TE fractionation using sequential extraction. Because of the fluctuation of TE concentrations in real-world organic waste, model substrate comprised of whole milk powder, whole egg powder and rice flour on a 20:20:60 ratio (VS basis) was employed. The low nutrient contents indicated the suitability of substrate used for this research. TKN concentration in feed was unlikely to induce inhibitory effect and therefore this would not become an interfering issue when investigating the TE effect on AD.

The requirement of TE for long-term AD were determined in eight 5-L CSTRs at OLR of 3.0 kg VS m^{-3} d^{-1}, HRT of 33.3 days and VS of 10% in the feed. The digesters were subject to 8 different TE dosing strategies in sufficient amount. Among them, digester with no TE addition and with full set of 11 TEs were acted as control. Co, Ni and Fe were selected as the main TE to investigate due to the long recognition of their role on stable AD, especially when TAN is not high. The results confirmed that single element dosing of Co or Ni was unable to prevent VFA accumulation when baseline TE concentrations were Co 0.01, Ni 0.03, Fe 2.88, Se 0.04 and Mo 0.06 mg kg^{-1} FM. The digester with both Co and Ni supplementation could not maintain long-term stable performance either, but its VFA accumulation appeared later than the control digester or digesters with single TE dosing. Fe showed an antagonistic effect when supplemented with either Co or Ni and reduced their availability, whilst at the same time it proved to be an essential TE. When supplemented with a mix of Co, Ni and Fe digesters operated well for 400 days but showed VFA accumulation after 12 HRTs. Se was also found to be essential for long-term stable operation of this substrate. Digesters supplemented with Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM was confirmed to be sufficient for stable performance. Other TE provided by model substrate was sufficient and there was no evidence that any of them was required in concentration greater than their baseline level. The system operated for 18 HRTs and clearly showed that the TE requirement was uncoupled from the hydraulic characteristics of the digester. This suggested that the chemical species and bioavailability of the TEs is a critical element in their function and is, to a certain degree, independent of washout as the results showed that nutrients are not lost from the system simply as a hydraulic function.

The critical concentrations of Co, Ni, Fe and Se to maintain stable digestion at moderate OLR when the rest TE existed in sufficient quantity (Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM) were quantified. Four digesters were operated at OLR of 3.0 kg VS m^{-3} d^{-1}, HRTs of 33.3 day, supplied with sufficient TE dosing to establish digestion baseline. The general approach of this study was to supplement 3 from 4 TE in sufficient amount in order to identify the minimum requirement for selected TE. The critical TE concentration will induce VFA accumulation, then, step-wise increased of selected TE dosing strength when it was re-introduced to recover process stability. Results indicated that the impact of Fe deficiency appeared earlier while Ni, Co and Se seemed to affect the process at later stages under the higher OLR. Co concentration became critical at its baseline level of 0.01 mg kg^{-1} FM at OLR 3.5 kg VS m^{-3} d^{-1}. Ni was critical at 0.03 mg kg^{-1} FM, equal to the baseline concentration at OLR 3.0 kg VS m^{-3} d^{-1}. Ni at the strength of 0.6-0.8 mg kg^{-1} FM is recommended to maintain stability at OLR 3.5 kg VS m^{-3} d^{-1}. Fe was critical at 5.0 and 6.2 mg kg^{-1} FM at OLR of 3.0 and 3.5 kg VS m^{-3} d^{-1}, respectively. Se was critical at its baseline level of 0.06 mg kg^{-1} FM at OLR 4.0 kg VS m^{-3} d^{-1}. This critical concentration, however, was not sustainable and not sufficient if instability initiated for instance by OLR increase. A sufficient safety factor should be applied if the critical concentrations are to be used for developing the TE supplementation strategies. Recovery and continuing stable operation, however, required much higher TE strength compared with their critical concentrations at high level of VFA. After long-term washed out, TE deficiency appeared earlier in higher OLR digester.

In parallel with the work as abovementioned, the fractionations of Co, Ni, Fe and Se in AD under different operational conditions were assessed, in order to identify the dynamic changes of their distribution over a range of main fractions (i.e. in liquid, organically bound, precipitated with sulphide, and in microbial biomass) when their total concentrations were gradually decreased in digesters over time. Results illustrated that microbial biomass maintained relatively stable amount of Co and Ni for metabolic activities although all the extracellular TE fractions were washed-out gradually. Sulphide fraction competed with intracellular fraction for Fe. This caused the accumulation of VFA when the total Fe concentration was still high. The fact that TE availability to certain extent decoupled from simple hydraulic washing out effect was explained by the high affinity of microbial biomass for TE in this experiment.

Apart from its readily biodegradability, another important feature of the model substrate was that OLR was able to decouple from HRT by adjusting the organic matter to water ratio. This allowed the investigation of the effect of OLR on the requirement of Co, Ni and Fe supplementation for stable AD without the interference of HRT (constant at 33.3 days). This trial was started at OLR 1.0 and step-wise increased to 3.0 Kg VS m^{-3} d^{-1}. The initial TE dosing was set at the strength of Co 0.03, Ni 0.03 and Fe 0.3 mg kg^{-1} FM and it was increased later in response to digester performance and loading increase. Se dosing strength was kept constant at 0.1 mg kg^{-1} FM. This ensured that Se was sufficient up to an OLR of 3.0 kg VS m^{-3} d^{-1}. Performance of digesters at different OLR levels was therefore directly related to the dosing strengths of Co Ni and Fe. Results indicated that the critical TE levels (defined as the concentration when VFA start to appear) appeared to be much higher than those obtained from washing-out experiment as aforementioned. When OLR increases, the concentration of microbial biomass increases accordingly if HRT is fixed, and microbes may have to take more TE from their environment. But when TE concentration in their environment is low, there are two problems: 1) the concentration gradient or energy required to find and carry TE to the inside of the cells; 2) the availability/speciation of TE in their environment. Therefore, the total TE concentration used to control this set of experiment should be much higher than that of the effective TE which should belong to microbe-incorporated TE. The minimum Co, Ni and Fe requirement relevant to OLR when Se was supplied in sufficient quantity are quantified as follow: OLR 1.0 kg VS m^{-3} d^{-1}, a mix of Co 0.03, Ni 0.06 and Fe 0.6 mg kg^{-1} FM; OLR 1.5 kg VS m^{-3} d^{-1}, a mix of Co 0.06, Ni 0.06 and Fe 0.6 mg kg^{-1} FM; OLR 2 kg VS m^{-3} d^{-1}, a mix of Co 0.09, Ni 0.09 and Fe 0.9 mg kg^{-1} FM and OLR 2.5 kg VS m^{-3} d^{-1}, a mix of Co 0.12, Ni 0.12 and Fe 1.2 mg kg^{-1} FM. Not only the total strength, but the optimal ratio of a mix of Co, Ni and Fe in supplementation mixture played a significant role on digester

stability. The ratio of Co+Ni and Fe in supplementation mixture in range of 1:5-1:6.67 was found optimal to recover process stability and ensure stable performance. Higher ratio (1:2.5, 1:3.33, 1:3.75 and 1:4) gave rise to disturbance and function decline probably due to less amount of available Fe.

The research provided new insight on optimising essential TE supplementation to mesophilic anaerobic digestion, considering the availability of TE for microbial activities, which is very important to obtain efficient and long-term stable biogas processes.

Keyword: anaerobic digestion, trace element, cobalt, nickel, iron, selenium, bioavailability, organic loading rate, VFA accumulation

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Definitions and Abbreviations

AAS	Atomic absorption spectroscopy
ACS	Acetyl-CoA Synthase
AD	Anaerobic Digestion
ATP	Adenosine Triphosphate
BMP	Biochemical Methane Potential
CFeSP	Fe/S cluster-containing protein
C/N	Carbon to Nitrogen
CoA	Coenzyme A
CoM	Coenzyme M
COD	Chemical Oxygen Demand
CODH	Carbon Monoxide Dehydrogenase
COD: N	Chemical Oxygen Demand to Nitrogen
CSTR	Continuously Stirred Tank Reactor
DBP	Daily Biogas Production
DI	Deionised
DW	Dry weight
EDTA	Ethylenediaminetetraacetic Acid
EPS	Extracellular Polymeric Substances
FAN	Free (unionised) Ammonia Nitrogen (NH_3-N)
FDH	Formate Dehydrogenase
FID	Flame Ionisation Detectors
FM	Fresh Matter
FW	Food Waste
GC	Gas Chromatography
H_4MPT	Tetrahydromethanopterin
HAc	Acetic Acid
HDR	Heterodisulphide Reductase
HEM	Hexane Extractable Material
HPr	Propionic Acid
HRT	Hydraulic Retention Time
IA	Intermediate Alkalinity
IA/PA	Intermediate Alkalinity to Partial Alkalinity
IC_{50}	50% Inhibitory Concentration
LCFA	Long Chain Fatty Acid

ICP-MS	Inductively Coupled Plasma Mass Spectrometry
MF	Methanofuran
MSW	Municipal Solid Waste
MV	Methyl Viologen
ODM	Organic Dry Matter
OHPA	Obligate Hydrogen-Producing Acetogens
OLR	Organic Loading Rate
PA	Partial Alkalinity
POB	Propionate Oxidising Bacteria
$R\text{-}NH_2$	Amino Nitrogen
RT	Retention Time
SBP	Specific Biogas Production
SE	Sequential Extraction
SCOD	Soluble Chemical Oxygen Demand
SMP	Specific Methane Production
SoMP	Soluble Microbial Products
SRB	Sulphate Reducing Bacteria
SRT	Solids Retention Time
SS-DFW	Source Segregated Domestic Food Waste
STP	Standard Temperature and Pressure
TA	Total Alkalinity
TAN	Total Ammoniacal Nitrogen
TCOD	Total Chemical Oxygen Demand
TE	Trace Element
TKN	Total Kjeldahl Nitrogen
TS	Total Solids
UASB	Upflow Anaerobic Sludge Blanket
VBP	Volumetric Biogas Production
VFA	Volatile Fatty Acid
VMP	Volumetric Methane Production
VS	Volatile Solids, also known as Organic Dry Matter (ODM)
VSD	Volatile Solids Destruction
VSS	Volatile Suspended Solids

Chapter 1: **Introduction**

1.1 Background

For anaerobic digestion (AD) process control and optimisation, significant attention must be paid to long term process stability. One of the principal issues associated with the digestion stability is nutritional requirements of microbial biomass in this process (Takashima, Speece and Parkin, 1990; Wheatley, 1990; Scherer *et al.*, 2009; Demirel and Scherer, 2011; Takashima, Shimada and Speece, 2011; Banks *et al.*, 2012; Qiang, Lang and Li, 2012; Qiang *et al.*, 2013). It is well known that those microorganisms require a range of nutrients, including micronutrients or trace elements (TEs). TEs are essential and play a crucial role in the metabolism of anaerobic microorganisms due to their functions in their key enzymes, co-factors and electron carriers. For example, it is estimated that about 30% of all enzymes in methanogens have a metal ion as a functioning participant (White and Stuckey, 2000).

Although it has been known for decades that TE are necessary for microorganisms involved in AD and thus biogas production (Takashima, Speece and Parkin, 1990; Kayhanian and Rich, 1995; Feng *et al.*, 2010; Gustavsson, Svensson and Karlsson, 2011; Schattauer *et al.*, 2011; Takashima, Shimada and Speece, 2011; Banks *et al.*, 2012), it was used to be assumed that organic waste feedstocks contained adequate supplies of these essential nutrients. Without appropriate TE supplementation, their deficiencies resulted in unstable AD performance, low biogas productivity, high volatile fatty acid (VFA) concentration, incomplete stabilisation of the organic substrates and probably digester failure (Takashima, Speece and Parkin, 1990; Kayhanian and Rich, 1995; Hinken *et al.*, 2008).

The requirement for essential TEs has now been recognised widely, but challenging research issues remain with regard to how to optimise TE supplementation. This is a multifaceted question involving metal chemistry, physical interactions of metal and solids, microbiology and technology optimisation. It is important to minimise TE dosing with respect to both the elements and the concentration used, due to concerns about their release into the environment including agricultural land along with the land application of digestate, as well as to digester operating cost.

Among the issues to be considered, the availability of trace elements for microbial activities is very important in order to achieve efficient and stable biogas production processes (Zandvoort *et al.*, 2006; Aquino and Stuckey, 2007; Romera, González Otazua and Romero Rossi, 2007; Fermoso *et al.*, 2008; Gustavsson *et al.*, 2013b; Shakeri Yekta *et al.*, 2014a; Ortner *et al.*, 2015). In general, the availability of essential TEs to anaerobic microorganisms varies in different anaerobic digestion processes due to different operating regimes, environmental conditions

applied, and the quantity of TEs introduced from inoculum and substrate. Most research work on this topic has been focused on metal speciation in anaerobic granular sludge (Osuna *et al.*, 2004; Hullebusch *et al.*, 2005; Virkutyte *et al.*, 2005; Zandvoort *et al.*, 2006; Jansen, Gonzalez-Gil and van Leeuwen, 2007; Romera, González Otazua and Romero Rossi, 2007; van der Veen, Fermoso and Lens, 2007; Bartacek *et al.*, 2008) and anaerobic sludge (Carliell-Marquet and Wheatley, 2002; Alonso *et al.*, 2006; Aquino and Stuckey, 2007; Lenz *et al.*, 2008; Dąbrowska, 2012; Dong *et al.*, 2013; Gustavsson *et al.*, 2013b; Shakeri Yekta *et al.*, 2014a; Yekta *et al.*, 2017). There is still lack of investigations on metal fractionation of digestate from anaerobic digesters treating high solids substrate.

Therefore, the factors regulating TE bioavailability need to be understood in order to determine proper dosage for anaerobic reactors fed with high solids feedstock. Several sets of experiments were carried out in this study to quantify the required trace elements dosing ranges under long term designated operational conditions in mesophilic anaerobic digestion using low TE content in feedstock. A special attention was given to identify the critical total TE concentrations and the dynamic changes of their distribution in different digestate fractionations over the course of TE washing-out process. TE availability was examined via digester performance and TE fractionation, which were determined by sequential extraction, which has been proved to be an effective way of studying metal fractionations in digestate (Aquino and Stuckey, 2007; Gustavsson *et al.*, 2013a; Shakeri Yekta *et al.*, 2014a; Yekta *et al.*, 2017).

Because of the fluctuation of TE concentrations in real-world organic waste (He, 2016), model substrate was formulated in this study to allow better quantification of the required TE dosing ranges and their fractionations in digestate under designed operational conditions. To the best of our knowledge, the present study is the first effort to compare the dynamic changes of TE distribution over a range of main fractions (i.e. soluble, organically bound, sulphide, and microbial biomass) using sequential extraction method when their total concentration were gradually decreased in digesters over time due to washing-out effect.

1.2 Research aim and objectives

1.2.1 **Research Aim**

This research aimed to quantify the required essential TE dosing ranges under designated operational conditions using model substrate with low TE content, and special attention was given to identify the dynamic changes of their distribution in different digestate fractions in anaerobic digesters over the course of TE washing-out process.

1.2.2 **Research Objectives**

The following objectives were identified as necessary to achieve the above aim.

1. To operate an 100-L breeder digester using model substrate to generate an acclimated inoculum for model substrate with low TE concentrations;

2. To quantify the critical concentrations of Co, Ni, Fe and Se to maintain stable digestion at moderate organic loading rate (OLR);

3. To identify the fractionation of Co, Ni, Fe and Se in anaerobic digestion under different TE dosing schemes and OLR, and to investigate the dynamic changes of TE distribution over different fractions when their total concentrations were gradually decreased in digesters over time;

4. To determine the effect of OLR on the requirement for Co, Ni and Fe supplementation at constant hydraulic retention time (HRT).

Chapter 1

Chapter 2: **Literature review**

In this chapter the anaerobic digestion fundamentals are presented and reviewed in detail, including the nutritional requirements of the process. An effort has also been made to critically review the state of the art of the research on TE bioavailability in anaerobic digestion system and how sequential extraction and metals fractionation approach can be employed for this research.

2.1 Anaerobic Digestion Process Fundamentals

Anaerobic digestion is the biological conversion by a complex microbial ecosystem of organic substrates in the absence of an oxygen source to biogas and digestate. During the process, biodegraded organic material is converted mainly to methane, carbon dioxide, and microbial biomass. Nitrogen released from converted organics is in the form of ammonia.

Anaerobic processes for organic waste treatment have advantages over aerobic ones in that there are no power requirements for air supply, production of sludges requiring treatment and disposal is much lower, and the methane generated can be used for energy production.

In order to achieve efficient digestion process, a range of operational parameters and environmental conditions need to be properly designed and controlled, including: retention time, mixing, operating temperature, characteristics of feedstock, pH, nutrients and the presence of toxic materials.

Among the operational parameters, operating temperature is without doubt an important one: it not only influences microbial growth, but also affects physicochemical parameters of digestate such as equilibrium position, viscosity, surface tension and mass transfer properties (Angelidaki, Ellegaard and Ahring, 2003). To avoid the detrimental effect on microbial population in digestion process, particularly on methanogens, temperature is usually maintained stably without fluctuation and shock (Appels *et al.*, 2008; Ward *et al.*, 2008).

Two temperature ranges are normally adopted in AD: mesophilic (30 - 45 ºC) and thermophilic (50-60 ºC) (Angelidaki, Ellegaard and Ahring, 2003; Speece, 2008; Weiland, 2010). Operating AD under thermophilic condition may increase biochemical reaction rates, enhance pathogen destruction and improve digestate dewaterability (Mackie and Bryant, 1995; Mata-Alvarez, 2002; Appels *et al.*, 2008; Khanal, 2009). However, thermophilic digestion also suffers from higher parasitic heating requirement, higher odor formation resulting from a higher volatile fatty acid (VFA) concentration, increased sensitivity to thermal shock, slow start-up, susceptibility to load variation and substrate changes, and more sensitive to toxicity such as ammonia inhibition (Angelidaki and Ahring, 1994). It therefore has been suggested that the benefits of thermophilic

digestion do not outweigh the disadvantages (de Lemos Chernicharo, 2007). This is especially true for certain types of substrate such as manure and food waste with high nitrogen content; this is because mesophilic operation alleviates ammonia toxicity and therefore offers more stable and efficient digestion (Angelidaki and Ahring, 1994; Hansen, Angelidaki and Ahring, 1998; Banks, Chesshire and Stringfellow, 2008).

Theory of anaerobic process: the microbial food chain and conversion process

The process can be summarised as follows: (1) multiple microbial steps, mediated by different organisms; (2) different steps that can be rate limiting under specific conditions; (3) interaction with the physicochemical system, particularly weak acid and base inhibition of microbial processes; and (4) highly nonlinear behaviour, particularly with respect to pH regulation and inhibition.

It is conceptually correct and convenient to characterise complex organics using their biochemical composition such as carbohydrates, proteins, and lipids. The organic fraction of any AD feedstock can be described by these components, which preserves full information of feedstock including its calorific value, and nitrogen content (Nopens *et al.*, 2009). To achieve stable anaerobic digestion, several groups of microorganisms forming a syntrophic relationship play a very important role. In the process, complex organic material in substrate is broken down and converted to biogas in four biologically-mediated stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Veeken *et al.* (2000).

1. Hydrolysis is an enzyme-mediated extracellular step which solubilises particulate organic material that cannot be directly utilised by anaerobic organisms. These enzymes such as amylase and cellulase are excreted by hydrolytic microorganisms.

2. Acidogenesis is the breakdown of soluble substrates including the products of hydrolysis (e.g. soluble amino acids and sugars) without external electron acceptors. The products are largely organic acids, alcohols, hydrogen, and carbon dioxide. Ammonia and hydrogen sulphide are also produced by this step.

3. Acetogenesis is the further degradation of acidogenesis products to acetic acid, hydrogen and carbon dioxide. This process has a syntrophic relationship with the next step, methanogenesis.

4. Methanogenesis is the production of methane via three pathways: (1) fermentation of acetic acid; (2) reduction of carbon dioxide or formic acid by hydrogen; and (3) dismutation of methanol or methylamines.

In a well-balanced anaerobic digestion process, all products of a previous metabolic stage are converted into the next one without significant build-up of intermediary products. The overall result is a nearly complete conversion of the anaerobically biodegradable organic materials into end products like methane, carbon dioxide, hydrogen sulphide, ammonia, as well as microbial biomass. A few steps, however, can potentially become rate-limiting and negatively affect the digestion process. A detailed analysis on each step is therefore necessary to achieve stable and effective digestion process.

2.1.1 **Hydrolysis**

Hydrolysis is a term that is used to refer to degradation and solubilisation of complex particulate materials which include carbohydrates (cellulose, hemicellulose and starch), proteins, and fats. The products of the hydrolysis process are organic monomers, mainly simple sugars, amino acids and fatty acids, which can then be used as substrates by either anaerobic fermentative organisms (Demirel and Scherer, 2008).

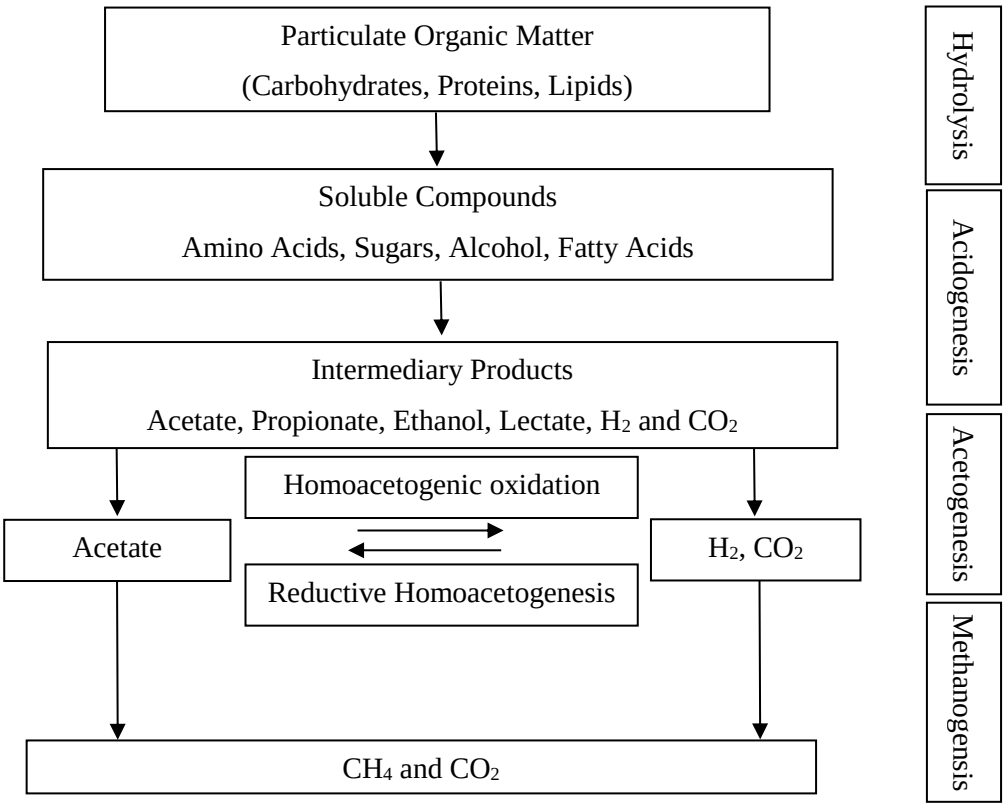


Figure 2-1 Key steps of anaerobic digestion for the conversion from biomass to methane modified from Demirel and Scherer (2008)

The rate of hydrolysis is affected by a range of environmental factors and operational parameters such as pH, temperature, particle size, biochemical composition of the substrate, hydraulic

retention time (HRT), solids retention time (SRT), composition of microbial population, and concentration of hydrolysis products (Miron *et al.*, 2000).

A number of studies have stated that the hydrolysis can be the rate limiting step in the anaerobic digestion of certain types of solid substrate materials such as biosolids from wastewater treatment works and lignocellulosic materials (Parkin and Owen, 1986; Henze and Mladenovski, 1991; Vavilin, Rytov and Lokshina, 1996; Veeken *et al.*, 2000; Veeken and Hamelers, 2000). On the other hand some substrates are easily hydrolysed; food waste is an example for this (Banks, Chesshire and Stringfellow, 2008; Banks *et al.*, 2012).

Various models have been proposed to model hydrolysis. First-order kinetics with respect to the remaining biodegradable particulate substrate is the simplest and most widely applied approach in describing the hydrolysis rate (Veeken *et al.*, 2000). According to Eastman and Ferguson (1981) a first-order hydrolysis function is purely an empirical expression that reflects the cumulative effect of many processes. The Monod equation is also used to describe degradation of particulate matter (Ghosh and Klass, 1977). Hobson (1983) proposed a model with two Monod equations that allows the distinction to be made between non-degradable, rapidly degradable, and slowly degradable fractions.

Subsequent studies have confirmed the conclusions of previous work but using different models for the more accurate simulation. Vavilin, Rytov and Lokshina (1996) reviewed four types of kinetic models describing hydrolysis (first-order, Monod, Contois, and two-phase), taking into account colonisation of the particles by bacteria. It was concluded that all types of hydrolysis kinetics could fit a variety of experimental data comparatively well. Several modifications of the simple first-order kinetics were proposed by Mata-Alvarez (1987) and Llabrés-Luengo and Mata-Alvarez (1988), to take into account high concentrations of VFA accompanied by low pH which may inhibit hydrolysis. They tested the Monod, first-order, and Hashimoto models but these all showed a significant lack of fit to the experimental data. Llabrés-Luengo and Mata-Alvarez (1988) thus proposed a kinetic model where the hydrolysis rate was proportional to the substrate volatile solids (VS), whilst the hydrolytic biomass concentration was inversely proportional to the VFA concentration. The proposed model, together with fitted parameters, adequately represented the hydrolysis process under all conditions tested, and it was concluded that hydrolysis kinetics could be described by their VFA inhibition model.

2.1.2 **Acidogenesis**

Acidogenesis or fermentation refers to the conversion of soluble monomers, such as amino acids and sugars, to a variety of fatty acids, alcohols, hydrogen, and carbon dioxide without the

requirement for an additional electron acceptor or donor (Mara, 2003). Among them, the principal acidogenesis products are acetic acid (CH_3COOH), hydrogen (H_2) and carbon dioxide (CO_2). These products will skip the acetogenesis step, and will be directly utilised by methanogens in the terminal stage.

Acidogenesis process is carried out through various fermentative pathways by facultative and obligate acidogenic bacteria, which include a large variety of fermentative genera, such as *Clostridium, Barteroides, Rumminococcus, Butyribacterium, Propionibacterium, Eubacterium, and Lactobacillus* (Mara and Horan, 2003). The facultative members of this group also help protect the oxygen-sensitive methanogens by consuming traces of oxygen that may enter the digester with the feed (Mara and Horan, 2003).

Acidogenesis is considered to be the fastest step in the anaerobic digestion process (Mata Álvarez, 2003) and the microorganisms involved in acidogenesis are least susceptible to inhibition: as often observed in acidified digesters, although methane production ceases, the fatty acids present in the digestate can continue to increase. However, according to Zhang *et al.* (2005), acidogenesis works at its best at a pH neutral condition. The results of a batch experiment showed pH adjustment to 7 almost doubled acidogenesis rates of kitchen wastes, compared with pH at 5, 9 and 11. There were a variety of factors affecting the improvement of hydrolysis and acidogenesis such as substrate characteristics, seed inoculum, dilution rate, pH and temperature.

2.1.3 **Acetogenesis**

Acetogenesis is carried out by acetogenic bacteria and produces acetic acid, with carbon dioxide and hydrogen as co-products in some cases. Acetogens can be classified, on the basis of their metabolism, into two distinct groups, i.e. the obligate hydrogen-producing acetogens (OHPA) and homoacetogens (Mara and Horan, 2003).

The main stream of acetogenesis in AD system is to degrade VFA with a carbon-chain length of 3 or more, alcohols and long chain fatty acids (LCFA) to acetic acid, carbon dioxide and hydrogen by OHPA (de Lemos Chernicharo, 2007). OHPA only function in an environment when a low concentration of metabolic products such as hydrogen is maintained due to thermodynamic reasons. For example, the standard Gibbs free energy change of butyric acid oxidisation to acetic acid, hydrogen and carbon dioxide is +482 kJ mol^{-1} which indicates the reaction is not feasible at standard conditions. However, when H_2 partial pressure is as low as 10^{-5} atm, the Gibbs free energy change will become -8.9 kJ mol^{-1} which makes the reaction thermodynamically feasible. The Gibbs free energy can be even lower if acetic acid concentration keeps low in the digester.

The other acetogenesis is named as homoacetogenesis, which utilises hydrogen and carbon dioxide to generate acetic acid. The homoacetogens responsible for this are obligate anaerobes that use the unique reductive Wood-Ljungdahl pathway. Acetate-oxidising bacteria can also accomplish the reverse reaction of homoacetogenesis and this step is vital in digesters with the acetoclastic methanogenic population inhibited or suppressed, e.g. at high total ammoniacal nitrogen (TAN) concentration (Demirel and Scherer, 2008; Schnurer and Nordberg, 2008). Several key metalloproteins/metalloenzymes have been identified in the reverse Wood-Ljungdahl pathway, such as formate dehydrogenase (FDH), corrinoid- and Fe/S cluster-containing protein (CFeSP), Carbon monoxide dehydrogenase (CODH), and Acetyl-CoA synthase (ACS) (Ragsdale and Pierce, 2008; Zhu and Tan, 2009).

2.1.4 **Methanogenesis**

Methanogenesis is the final stage of AD, which is strictly performed by methanogenic archaea, namely methanogens. All methanogenic archaea investigated to date rely on methanogenesis as their catabolic pathway for energy production and growth (Thauer, 1998). The types of substrates utilised by methanogens are quite limited reflecting the narrow ecological niche this group occupies: most methanogens are only able to grow with H_2 and CO_2/formate, some can utilise methylated compounds, and some can grow with acetate.

Three methanogenesis pathways have so far been discovered (Galagan *et al.*, 2002): 1) acetoclastic pathway with acetate as sole reactant: acetate is cleaved to a methyl group and an enzyme-bound CO, with the CO subsequently being oxidised to provide electrons for the reduction of the methyl group to methane; 2) hydrogenotrophic pathway with CO_2/formate and H_2 as reactants: CO_2/formate is reduced to methane using electrons provided by H_2; 3) methylotrophic pathway with C-1 compounds such as methanol or methylamines as reactants: one molecule of C-1 compound is oxidised to CO_2 to provide electrons for reducing three additional molecules to methane in the case of methanol. These pathways share a common final step, i.e. the reduction of methyl-CoM to methane (Galagan *et al.*, 2002). Acetoclastic and hydrogenotrophic pathways are the two dominant pathways involved in the digestion of natural organic materials and therefore are described in more detail below.

Acetoclastic methanogenesis

In this pathway, acetate is cleaved to a methyl group and a carboxyl group. The methyl group is converted directly to methane, whereas the carboxyl group is oxidised to CO_2.

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \text{Equation 2-1}$$

Acetoclastic methanogens include two genera of the methanogenic archaea: *Methanosaeta* and *Methanosarcina* (Ferry, 2010). It is often stated that this group of methanogens is the dominant methanogen group in sewage sludge digestion and contributes to 70% of methane production (Gujer and Zehnder, 1983). Therefore, digester operation and optimisation are often based on maintaining acetoclastic methanogen populations (Karakashev *et al.*, 2006).

The acetoclastic pathway employs a set of highly specialised enzymes and coenzymes and proceeds via several biochemical reactions in a stepwise manner, as described below (Ferry, 1992;1999; Ferry, 2010;2011).

Firstly, as shown in Equation 2-2 acetate binds with coenzyme A (CoA) to form acetyl-CoA ($CH_3COSCoA$), which has been identified as the activated form of acetate. The enzymes involved in this step are adenosine monophosphate-forming acetyl-CoA synthetase or by the combined actions of acetate kinase and phosphotransacetylase.

$$CH_3COOH + CoA \longrightarrow CH_3COSCoA + H_2O \qquad \text{Equation 2-2}$$

The methyl group of acetyl-CoA is then transferred to H_4SPT to generate methyltetrahydromethanopterin (CH_3-H_4SPT), accompanying with the oxidisation of carbonyl group to CO_2 and the release of free CoA (Equation 2-3). Carbon monoxide dehydrogenase/acetyl-CoA synthase complex (CODH/ACS) is the enzyme responsible for this reaction.

$$CH_3COSCoA + H_4SPT \rightarrow CH_3\text{-}H_4SPT + CO_2 + CoA + 2[H] \qquad \text{Equation 2-3}$$

Subsequently, the methyl group of CH_3-H_4SPT is transferred to coenzyme M (CoM), by substitution of the hydrogen atom in the thiol group of coenzyme M (Equation 2-4). This reaction is catalysed by coenzyme M methyltransferase.

$$CH_3\text{-}H_4SPT + H\text{-}S\text{-}CoM \rightarrow CH_3\text{-}S\text{-}CoM + H_4SPT \qquad \text{Equation 2-4}$$

The final step (Equation 2-5), common for all methanogenic pathways, is the reduction of methyl group by combining coenzyme B with coenzyme M via the catalysis of Methyl-CoM reductase, yielding a heterodisulphide (CoM-S-S-CoB) and CH_4.

$$CH_3\text{-}S\text{-}CoM + H\text{-}S\text{-}CoB \rightarrow CoM\text{-}S\text{-}S\text{-}CoB + CH_4 \qquad \text{Equation 2-5}$$

Hydrogenotrophic methanogenesis

In this pathway, hydrogenotrophic methanogens form methane by chemical reduction of carbon dioxide (CO_2) with hydrogen (H_2) as the electron donor (Equation 2-6), and this group is therefore essential for ensuring the syntrophy with acetogens.

$4\ H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ Equation 2-6

Biochemical reactions in this pathway involve several enzymes and cofactors (DiMarco, Bobik and Wolfe, 1990; Ferry, 1999; Shima *et al.*, 2002; Deppenmeier and Muller, 2008; Thauer *et al.*, 2008; Ferry, 2010). Firstly, CO_2 attaches to methanofuran (MF) which is then reduced to formyl-MF (Equation 2-7) by the catalysis of formyl-MF dehydrogenase (Fmd).

$CO_2 + MF + H_2 \longrightarrow$ formyl-MF $+H_2O$ Equation 2-7

The formyl group of formyl-MF is then transferred to tetrahydromethanopterin (H_4MPT) catalysed by formyl-MF: H_4MPT formyltransferase (Equation 2-8).

Formyl-MF + H_4 MPT $\longrightarrow$ 5-formyl-H_4 MPT + MF Equation 2-8

This is followed by conversion of 5-formyl-H_4MPT to 5, 10-methenyl-H_4MPT^+ (Equation 2-9), then 5, 10-methylene-H_4MPT (Equation 2-10) and 5-methyl-H_4MPT (Equation 2-11). In the conversion from 5, 10-methenyl-H_4MPT^+ to 5-methylene-H_4MPT, F_{420} acts as a coenzyme for hydride transformation in these two reactions. Subsequently, the methyl group is transferred to CoM (Equation 2-12) which is catalysed by methyl-H_4MPT: coenzyme M methyltransferase. Finally, methyl-CoM is reduced to methane by methyl-CoM reducetase (Equation 2-13).

5-formyl-H_4MPT + H+$\longrightarrow$ 5, 10-methenyl-H_4 $MPT^+ + H_2O$ Equation 2-9

5, 10-methenyl-$H_4MPT^+ + F_{420}H_2 \rightarrow$ 5, 10-methylene-H_4MPT + F_{420} + H^+ Equation 2-10

5, 10-methylene-H_4MPT + $F_{420}H_2 \rightarrow$ 5-methyl-H_4MPT + F_{420} Equation 2-11

5-methyl-H_4MPT+HS-CoM $\rightarrow$ CH_3-S-CoM+H_4MPT Equation 2-12

CH_3-S-CoM + HS-CoB $\rightarrow$ CH_4 + CoM-S-S-CoB Equation 2-13

2.2 Nutritional requirements

Nutrients can be taken up by microorganisms from their environment during both catabolism and anabolism (White and Stuckey, 2000). For a stable and effective digestion process, the nutrients required must be present in the digester in the correct forms and concentrations (Kayhanian and Rich, 1995). It has often been assumed that most substrates contain sufficient quantities of the nutrients required for microbial growth to maintain stable process operation, however, some solid substrates or certain industrial wastewater cannot provide all required nutrients. The deficiency of nutrients can limit the functioning of anaerobic process significantly.

The required nutrients in AD can be categorised into macro-nutrients and micro-nutrients depending on the amount needed to maintain microbial growth and a well working anaerobic

process. It has now been ascertained that, depending on the specific substrate constituency, element supplementation may be required for microbial activities. The macro nutrients (i.e. C, O, H, N, S, P, K, Mg, Ca, Na and Cl) build up the bulk of the biomass, while the micro nutrients (i.e. Fe, Zn, Mn, Mo, Se, Co, Cu, Ni, W and V) mostly are present in cofactors and enzymes (Takashima, Speece and Parkin, 1990; Zandvoort *et al.*, 2006). The following review addresses all of the specific nutrients requirements reported for microorganism in AD.

2.2.1 **Macronutrients**

In AD process, carbon, nitrogen, phosphorus and sulphur are included in macro-nutrients as these elements are required in substantial quantities. According to Kayhanian and Rich (1995), the function of macronutrients in anaerobic digestion is as below.

Carbon (C) is the basic building block of microbial cell material and is the primary source of energy for heterotrophic organisms. Because organic substrates are carbon-rich, carbon will generally not be a limiting nutrient. Instead, the ratios of carbon to nitrogen (C/N) and carbon to phosphorus may better define the nutritional requirements.

Nitrogen (N) is the primary nutrient required for microbial synthesis. Nitrogen occurs in the cell material in the reduced-form as amino nitrogen ($R\text{-}NH_2$). Amino-nitrogen is essential for the synthesis of proteins and nucleic acid.

Phosphorus (P) and sulphur (S) requirements for bacterial synthesis are generally much less than that of carbon and nitrogen. Phosphorus aids in the synthesis of nucleic acids and also used for ATP, and sulphur is required for protein synthesis.

In addition to the above mentioned major macronutrients, other elements such as potassium, calcium and magnesium, although required to a lesser extent, also have important roles in anaerobic microorganisms.

Although the requirements of macronutrients for cell growth in anaerobic processes are much lower than the requirements for aerobic processes because of the lower cell yield from the anaerobic degradation of equal quantities of substrate, deficiency or imbalance in macro-nutrients may cause inadequate microbiological activity in AD process. Recommended C:N:P:S proportion for the growth and survival of microorganisms were proposed in the ratio of 600:15:5:3 (Fricke *et al.*, 2007) or 600:15:5:1 (Weiland, 2010) on a mass balance. The carbon requirement is higher than what is calculated from the empirical formula of cell biomass ($C_5H_7O_2NP_{0.06}S_{0.1}$) (Speece (1996); this is because carbon is also consumed for energy production purpose.

Special attention needs to be given to the C/N ratio of substrates. In practice, the C/N ratio has proven to be critical in the operation of the anaerobic digesters process. If the C/N of the substrate is too high, the digester will develop several problems such as deficiency in nitrogen to build-up of the bacterial mass, loss of buffering capacity (Yenigün and Demirel, 2013), or triggering the production of EPS which reduces the carbon recovery as biogas (Miqueleto *et al.*, 2010). If the C/N ratio is too low, the degradation of the substrate leads to increase in ammonia formation and result in inhibition to the anaerobic microbes due to ammonia toxicity (Kim *et al.*, 2006). Compared with the optimal ratio for microbial growth, a lower C/N ratio of 25~30 is preferred due to a certain level of buffering capacity by ammonia nitrogen is required in actual digestion process (Kim *et al.*, 2006). For food waste, the C/N ratio, based on the biodegradable organic carbon, is below 20 (Han and Shin, 2002; Zhang *et al.*, 2007; Zhang, Lee and Jahng, 2011; Banks *et al.*, 2012; Zhang *et al.*, 2013), whereas with mixed paper, the ratio can be over 100. The optimum C/N ratio therefore can be achieve by co-digestion by mixing substrate with a higher C/N ratio (e.g. paper, wood and cardboard, etc.) with those with a lower C/N ratio. For anaerobic treatment of wastewater, COD is usually used instead of carbon content, and COD to nitrogen ratios of 40~50 and 140 were recommended for high rate and low rate systems, respectively (Villain *et al.*, 2010; Dai *et al.*, 2013). It was also suggested a COD to P ratio in the range of 80:1 to 200:1 (Mara and Horan, 2003), and a S requirement of 0.001 - 1.0 mg kg^{-1} FM (Speece, 1996).

2.2.2 **Micronutrients**

Micro-nutrients or TEs are required only in smaller amounts, but functionally can be very important to anaerobic microorganism in AD. As discussed in section 2.1, under anaerobic conditions, microorganisms utilise a set of unique enzyme systems. TEs are often present in these enzyme systems as part of a cofactor or electron carriers, and therefore they are of vital importance for the enzyme system. On the non-enzymatic level, TEs are also involved in microbial anaerobic respiration processes, bound to cell wall or extracellular electron acceptors (Zandvoort *et al.*, 2006). A range of TE, such as Co, Ni, Fe, Se, Mo and W were reported to be essential for AD operation (Schönheit, Moll and Thauer, 1979; Speece, 1983; Speece, Parkin and Gallagher, 1983; Takashima, Speece and Parkin, 1990; Lenz, Janzen and Lens, 2008; Fermoso *et al.*, 2009; Worm *et al.*, 2009; Takashima, Shimada and Speece, 2011; Banks *et al.*, 2012).

It has been generally accepted that sufficient quantities of required TEs exist in system for microbial growth and metabolism. However, previous studies from many researches (Takashima, Speece and Parkin, 1990; Singh, Kumar and Ojha, 1999; Scherer *et al.*, 2009; Uemura, 2010; Demirel and Scherer, 2011; Takashima, Shimada and Speece, 2011; Zhang, Lee and Jahng, 2011; Banks *et al.*, 2012; Qiang, Lang and Li, 2012; Zhang and Jahng, 2012; Zhang, Ouyang and Lia,

2012; Zhang, Banks and Heaven, 2012b; Qiang *et al.*, 2013; Zhang *et al.*, 2015; Zhang, Zhang and Li, 2015) have reported that TE supplementation with proper quantity may also be advantageous for improving specific biogas production and were found to significantly influence and increase process performance stability. Although required only in trace amounts, in the case of deficiency, they may make the system under the condition of nutritional deficiency (Callander and Barford, 1983a;b) resulting in significant limitation of microbial activity that can result in the failure of AD processes. On the other hand, all the TEs are potential toxicants to AD process which is another important issue and has been studied mainly using feedstock from certain industrial processes, usually contaminated by heavy metals. In several studies, the problem of TE overdosing was also raised when the substrate already contained relatively high background levels of TE. Once over the tolerable concentrations, they became inhibitory to anaerobic microorganisms including methanogens (Hobson and Shaw, 1976; Ahring and Westermann, 1985; Mori *et al.*, 2000; Sai Ram *et al.*, 2000). In this case TE imposed negative effect on AD process (Hinken *et al.*, 2008; Facchin *et al.*, 2013), resulting in significant loss of methane yield. This has been proved on Se (Lenz, Janzen and Lens, 2008), Ni (Bartacek *et al.*, 2010) and Co (Bartacek *et al.*, 2008). Although heavy metal toxicity in anaerobic reactors has been studied for decades, reported values of toxic concentrations vary considerably between different authors. Examples for TE toxicity and their inhibitory effect on microorganisms are listed as followed:

Co: Bartacek *et al.* (2008) investigated the influence of Co speciation on the toxicity of Co to methylotrophic methanogenesis in anaerobic granular sludge. The Co speciation was studied with three different media that contained varying concentrations of complexing ligands [carbonates, phosphates and ethylenediaminetetraacetic acid (EDTA)]. Three fractions (nominal added, dissolved and free) of Co were determined in the liquid media and were correlated with data from batch toxicity experiments. The results indicated that the average concentration of Co that was required for 50% inhibition of methanogenic activity (IC_{50}) for free Co^{2+} in the three sets of measurements was 0.77 mg L^{-1} with a standard deviation of 22 % and a similarity of 72 % between the data obtained in the three different media for the range of Co concentrations investigated. The standard deviation of the IC_{50} for the other two fractions was much higher, i.e. 85 and 144% for the added Co and dissolved Co, respectively, and the similarity was almost 0% for both fractions. Complexation (and precipitation) with EDTA, phosphates and carbonates was shown to decrease the toxicity of Co on methylotrophic methanogenesis. The free Co concentration is proposed to be the key parameter to correlate with Co toxicity.

Cu: Karri, Sierra-Alvarez and Field (2006) evaluated the inhibitory effect of Cu to the activities of acetoclastic and hydrogenotrophic methanogensin sludge obtained from a full-scale sulphate reducing bioreactor. The IC_{50} of Cu^{2+} to acetoclastic and hydrogenotrophic methanogens was 20.7 and 8.9 mg L^{-1}, respectively.

Se: Inhibitory effects of selenite and selenate towards hydrogenotrophic and acetoclastic methanogenesis were evaluated in anaerobic toxicity assays (Lenz, Janzen and Lens, 2008). The results indicated that the 50 % inhibitory concentration (IC_{50}) of both selenium oxyanions was below 4.8 mg L^{-1} in hydrogenotrophic assays, whereas acetoclastic methanogens were less inhibited: IC_{50} = 6.6 and 43 mg L^{-1} for selenite and selenate, respectively. Selenite completely inhibits both hydrogenotrophic and acetoclastic methanogenesis at concentrations ≥ 79 mg L^{-1} selenite, while marginal methanogenic activities occur at equimolar concentrations of selenate. Consequently, methane recovery can be seriously hampered or even impossible during anaerobic treatment of highly selenium contaminated waste streams.

The toxicity-resistance of sludge biogranules to heavy metals was studies and found that the methanogenic biogranules' activity reduces by 50 % for each gram of biomass that comes into contact with 1.6 mg Cd, 1.0 mg Cr, 0.9 mg Cu, 2.3 mg Ni, 40 mg Pb or 1.6 mg Zn. (Lin and Chen, 1997).

Early work evaluating the toxicity of heavy metals during AD of sewage sludge indicated severe inhibition at concentrations ranging from 70 to 400 mg L^{-1} for Cu, 200 to 600 mg L^{-1} for Zn and 10 to 2000 mg L^{-1} for Ni (Lawrence and McCarty, 1965; Hayes and Theis, 1978; Ahring and Westermann, 1985). In experiments conducted under more defined conditions, toxic concentrations of heavy metals are generally lower. The activity of an acetate-degrading methanogenic enrichment culture was inhibited by 50% at 7.7, 12.5, 16 and 67.2 mg L^{-1} of Cd, Cu, Zn and Rb, respectively (Lin, 1992). A mixed methanogenic culture was inhibited by 50 % by 10, 40 and 60 mg L^{-1} of Co, Zn and Ni, respectively (Zayed and Winter, 2000). Cu and Zn caused 50% inhibition of a sulphate reducing mixed culture at initial dissolved concentrations of 10.5 or 16.5 mg L^{-1} of Co and Zn, respectively (Utgikar *et al.*, 2001).

In summary, all microorganisms have a range of elements requirements for optimum and robust growth. The supplementation of nutrients into the AD is necessary when the macro-nutrients are not in balance or the TE contents of substrate are not sufficient for the metabolic processes of AD under the operational conditions. In a comprehensive review on mineral requirements for methane production, Takashima, Speece and Parkin (1990) noted that mineral supplementation in systems treating industrial wastewaters has been limited to nitrogen and phosphorous and also suggested that quantity of nutrients to promote microbial growth for AD depend on specific characteristic of waste being treated. The specific TE requirement, however, in terms of function and optimum dosage, is strongly dependent upon the speciation and the bioavailability of the element to the microorganisms as well as the specific methanogens in the process (Zandvoort *et al.*, 2006; Romera, González Otazua and Romero Rossi, 2007). Oleszkiewicz and Sharma (1990) indicated that TE supplementation requirement depends not only on their role in biochemical pathways, but

also on concentration, type of TE and speciation. Similar conclusions were given by Schattauer *et al.* (2011).

2.3 Trace elements requirement for anaerobic digestion

A trace element can be defined as "any chemical element that occurs in very small amounts in organisms, but is essential for many physiological and biochemical processes" (Zandvoort *et al.*, 2006). TEs play an important role in the growth and metabolism of anaerobic microorganisms (Takashima, Speece and Parkin, 1990) and can accumulate in anaerobic system through physical, chemical, or biological processes (Osuna *et al.*, 2004). The roles of TE in anaerobic processes are significant in terms of process stability and continuous biogas production. TEs, both metals and non-metals, function as structural elements and are essential constituents of cofactors and enzymes, as well as electron carriers, for (anaerobic) microorganisms. Iron is almost ubiquitously required for life, but other metals and Se are associated more closely with specific microbial physiologies (Zerkle, House and Brantley, 2005b). The requirement for TEs during AD can be explained according to the metabolic processes of anaerobic bacteria and methanogens. More information has been found for methanogens than for bacteria in this case probably for the following two major reasons (Ferry, 1993; Deppenmeier, 2002; Deppenmeier and Muller, 2008; Thauer *et al.*, 2008). Firstly, biological methanogenesis is the terminal step in the mineralisation of organic materials under many anoxic environments, and therefore becomes an important part of the maintenance of the carbon cycle on earth. Secondly, from an evolutionary point of view, methanogens are close to the origin of life on earth, and live close to the thermodynamic limit.

The supplementation of TEs to AD is necessary when the TE contents of the substrates are not sufficient for the metabolic processes of AD. The specific requirements for TE supplementation, however, can be affected by their species, bioavailability, digester operation mode and dosing strategy; and inhibition effects will arise if they are overdosed (Zandvoort *et al.*, 2006; Lenz, Janzen and Lens, 2008; Uemura, 2010). It appears that although the optimisation of TE supplementation still has to be based on quantitative experimental trials, a fundamental knowledge of TE functions in the process should provide a more theoretical basis to solve any TE deficiency problem. The literature reviewed in this section is organised as follows: the requirements of TE for the well-studied methanogenesis are introduced first, followed by the TE requirement in other anaerobes in AD, including syntrophic acetate oxidising and propionate degradation pathways. Finally the studies on the TE effect on overall AD process are reviewed.

2.3.1 Trace elements requirement in Methanogens

As discussed previously, the requirement for TEs is very specific and dependent largely on the phylogenetic groups and metabolic pathways that are adopted. In a review of the enzymes involved in acetoclastic and hydrogenotrophic methanogenesis (section 2.1.4), it is understood that the specific TEs requirement is usually because of their irreplaceable function in enzyme systems associated with each of these pathways. Recently, TEs including selenium, Co, Ni, Fe, Mo and/or W have been identified as a constituent of enzymes or are a required cofactor involved in methanogenesis.

Methanogenesis is the final and a most critical step of the AD process for biogas production, and was commonly regarded as the step most likely to be subject to TE deficiency in the AD process (Takashima, Speece and Parkin, 1990; White and Stuckey, 2000). This was thought to be because of their requirement in elaborate enzyme system associated with the biochemical reactions of methanogenesis. As methanogens carry out the terminal steps in the AD process, whenever their consumption of acetate, hydrogen, or formate is slower than the production of these intermediate products due to TE deficiency, further VFA accumulation will appear due to product-induced feedback inhibition and can adversely affect the overall process.

All methanogenic archaea investigated to date rely strictly on methanogenesis for energy production and growth, and methanogenesis is regarded as one of the most metal-rich enzymatic pathways in biology (Zerkle, House and Brantley, 2005a; Glass and Orphan, 2012). As mentioned in section 2.1.4, the number of substrates utilised for methanogenesis is quite limited reflecting the narrow ecological niche methanogens occupy: most methanogens are only able to grow with H_2 and CO_2/formate, some can utilise methylated compounds, and some can grow with acetate. Although the specific metal requirements differ to a certain extent depending on the pathway involved, they follow the same trend, : Fe is used most abundantly, followed by Ni and Co, and then smaller amounts of Se, Mo (and/or W) and Zn (Zerkle, House and Brantley, 2005a; Glass and Orphan, 2012).

According to several studies and reviews (Mossop and Davidson, 2003; Zhang *et al.*, 2003; Fermoso *et al.*, 2009) all methanogens appear to require Co, Ni and Fe for growth; however, most of these studies are qualitative regarding the TEs requirements.

Scherer, Lippert and Wolff (1983) has quantitatively determined methanogen elemental composition in 10 methanogen species cultured using defined media for hydrogenotrophic, methylotrophic and acetoclastic pathways. The 10 species were representative of all three orders of the methanogens and were cultivated under defined conditions. Special emphasis was given to *Methanosarcina barkeri*, represented by 5 strains and cultivated on various substrates. Micro and

macro nutrients were measured by inductively coupled plasma mass spectrometry (ICP-MS) and an elemental analyser. The results showed that the metal content varied considerably between the different species of methanogens even when they were from the same genus and converting the same substrate. The ranges of TE contents in these 10 methanogens were: Mg (900 - 5,300 ppm), Fe (700 - 2,800 ppm), Ni (65 - 180 ppm), Co (10 - 120 ppm), Mo (10 - 70 ppm), Zn (50 - 630 ppm), Cu (<10 - 160 ppm), Mn (<5 - 25 ppm) on TS basis. All species investigated contained high zinc contents, whereas copper seemed to be present only in some species.

Additional TE requirements, i.e. Se and W have been reported for some methanogens (Fermoso *et al.*, 2009). Se is present in a variety of selenoproteins, e.g. in hydrogenase and formate dehydrogenases (*Methanococcus jannaschii*) (Jones, Dilworth and Stadtman, 1979) and formylmethanofuran dehydrogenase (*Methanopyrus kandleri*) (Vorholt, Vaupel and Thauer, 1997). According to Ching *et al.* (1984), Se containing tRNAs account for 13 to 20% of the total tRNA in *Methanococcus vannielii*.

The essential biological functions of W have been discovered relatively late, compared to its chemically analogous element Mo (Kletzin and Adams, 1996). A stimulatory effect of W on the growth of methanogens was first reported in the late 1970s using *Methanococcus vannielii* (Jones and Stadtman, 1977*)*. Its growth was dramatically enhanced by the addition of W, but not by Mo, to the growth medium when formate was used as the carbon and energy source, although the effect of W was not observed when the organism was grown on H_2 and CO_2. This suggested the involvement of a W-containing formate dehydrogenase (FDH). The stimulatory effect of W on cell growth to some methanogen species is very concentration dependent. Winter *et al.* (1984) reported an obligate autotrophic methanogen, *Methanobacterium wolfei* whose growth rate had a linear correlation with W content up to a concentration of 1.4 mg W L^{-1} in the medium.

As reviewed previously in section 2.1.4, methanogenesis shares several biochemical reactions in common due to the overlapping routes, and therefore various TEs involved in enzymatic systems responsible for these reactions are ubiquitously required in all methanogenic pathways. On the other hand, certain reactions are unique to each methanogenic pathway, therefore special elements are required. All methanogenic pathways converge to the enzymatic reduction of methyl coenzyme M to methane. This reduction is catalysed by the Methyl-coenzyme M reductase complex, which includes a Ni containing cofactor F_{430} (Friedmann, Klein and Thauer, 1990).

Another metallo-enzyme that is present in all methanogenic pathways is the Co/corrinoid containing methyl-H_4MPT: Coenzyme M methyltransferase complex (Thauer, 1998). The first step of methanogenesis from methanol is also catalysed by a specific cobalt dependent methyltransferase, methanol:coenzyme M. In addition to cobalt, this enzyme also contains Zn (Sauer and Thauer, 2000).

Heterodisulphide reductase (HDR) is found in a number of methanogenic archaea, particularly in *Methanosarcina* species, which are also involved in all methanogenic pathways. It is an iron-sulphur protein that catalyses the reversible reduction of the heterodisulphide (CoM-S-S-CoB) of the methanogenic thiol-coenzymes, coenzyme M (CoM-SH) and coenzyme B (CoB-SH) (Hedderich, Hamann and Bennati, 2005).

From enzyme purification studies carried out on *Methanosarcina thermophila* (Murakami et al., 2001), *Methanothermobacter marburgensis* (Duin *et al.*, 2002), *Methanosarcina barkeri* (Heiden *et al.*, 1994) *and Methanothermobacter thermautotrophicus* (Setzke *et al.*, 1994), it is understand that Fe is found in all HDR purified from the 4 methanogens, and Ni was found in *Methanosarcina barkeri* (Heiden *et al.*, 1993) *and Methanothermobacter thermautotrophicus* (Setzke *et al.*, 1994).

Formate dehydrogenase (FDH) is a member of the dimethyl sulphoxide (DMSO) reductase family. It catalyses the reversible two-electron oxidation of formate or reduction of CO_2 which is the first step of the hydrogenotrophic pathway. In some anaerobic microorganisms, FDH was reported to have a Mo or W *bis*-(pyranopterin guanidine dinucleotide) cofactor and Fe-S clusters (Hille, 2002; Romao, 2009) and shows great variability in quaternary structure, physiological redox partner, and cellular location (da Silva *et al.*, 2011). Jones, Dilworth and Stadtman (1979) also reported two types of FDH in *Methanococcus vannielii*: a Mo-W-Se-Fe-S protein and a Mo-Fe-S protein with neither Se nor W.

Hydrogenases play key roles in the metabolism of methanogenic archaea in the hydrogenotrophic pathway. These comprise of a group of Fe-S flavoprotein (FAD) which contain Ni or in some cases also contain selenocysteine (Thauer, Hedderich and Fischer, 1993). Two distinct hydrogenases have been detected in all methanogens capable of chemolithoautotrophic growth (Baron and Ferry, 1989; Michel *et al.*, 1995). One (F_{420}-hydrogenase) reduces the physiological low potential two-electron acceptor coenzyme F_{420} and also, to a lesser extent, the artificial electron acceptor methyl viologen (MV), the riboflavin analogue of F_{420} and flavins. The other (MV-hydrogenase) reduces MV but not F_{420}. All the hydrogenases purified from methanogens so far form large aggregates with molecular weights of 720,000 to 1,300,000 and contain Ni and Fe-S centres. Selenocysteine, in particular, was discovered in hydrogenase purified from *Methanococcus vannielii* (Yamazaki, 1982) and *Methanosarcina barkeri* (Michel *et al.*, 1995).

Formylmethanofuran dehydrogenase is a Mo-containing enzyme that catalyses the terminal step in the oxidation of methanol to CO_2 and the first step in CO_2 reduction to CH_4 in autotrophic CO_2 fixation (Bertram and Thauer, 1994). A study carried out by Hochheimer *et al.* (1995) on pure culture shows that *Methanobacterium thermoautotrophicum* contains two formylmethanofuran dehydrogenase iso-enzymes, a W form and a Mo containing form. The Mo enzyme is synthesised

only when Mo is available in the growth medium. The W enzyme is synthesised when either W or Mo is present. If the growth medium contains Mo, the W enzyme will contain Mo instead of W (Hochheimer *et al.*, 1995).

The key enzyme complex in the acetoclastic pathway is carbon monoxide dehydrogenase (CODH). CODH cleaves the C-C and C-S bonds in the acetyl moiety of acetyl-CoA, oxidises the carbonyl group to CO_2 and transfers the methyl group to Coenzyme M. The CODH complex is composed of two enzyme components: a Ni/Fe-S component and a corrinoid/Fe-S component (Ferry, 1992). This enzyme complex is also involved in the formation of acetate by acetogens from H_2/CO_2 and methanol (Zandvoort *et al.*, 2006).

The essential TEs which have been identified in the acetoclastic methanogenesis pathway and in the hydrogenotrophic methanogenesis pathway are summarised in Table 2-1 and Table 2-2, respectively (Ferry, 1992;1999; Ferry, 2010;2011).

Table 2-1 Major metalloenzymes identified in acetoclastic methanogenesis.

Metalloproteins/metalloenzymes	Trace elements
CO dehydrogenase/acetyl-CoA decarbonylase	Co, Ni, Fe
Methyl-H_4SPT:HS-CoM methyltransferase	Co, Ni, Fe, Zn
Heterodisulfide reductase	Fe
Methyl-CoM reductase	Ni

Table 2-2 Major metalloenzymes identified in hydrogenotrophic methanogenesis pathway

Metalloproteins/metalloenzymes	Trace elements
Formate dehydrogenase	Se, Mo/W, Fe
Hydrogenase	Se, Ni, Fe
Formyl-methanofuran dehydrogenase	Se, Mo/W, Fe
Heterodisulfide reductase	Fe
Methyl-CoM reductase	Ni

2.3.2 **Trace elements requirement in other anaerobes**

Apart from being essential to methanogens, several TE species are required by syntrophic acetate oxidising bacteria adopting the reverse Wood-Ljungdahl pathway (oxidative acetyl-CoA pathway) which has been intensively studied both due to the important role it takes in energy production and autotrophic carbon assimilation which also contributes to global carbon cycle (Ragsdale and Pierce, 2008); and due to current trends in industrial biotechnology research related to CO_2 or syngas utilisation for chemical production (Thauer, 2015; Bengelsdorf *et al.*, 2016).

The other anaerobic acetogenesis routes, as well as acidogenesis and hydrolysis involved in AD, however, have not been widely studied with respect to their TE requirement apart from propionate degradation pathways (syntrophic propionate oxidising route).

2.3.2.1 **Syntrophic acetate oxidising pathway**

In syntrophic acetate oxidising pathway, four key metalloproteins/metalloenzymes have been identified in the reverse Wood-Ljungdahl pathway: formate dehydrogenase (FDH), corrinoid- and Fe/S cluster-containing protein (CFeSP), carbon monoxide dehydrogenase (CODH), and acetyl-CoA synthase (ACS). The TEs identified to be used in these protein/enzymes are listed in Table 2-3 (Zhu and Tan, 2009).

Table 2-3 Major metalloenzymes identified in syntrophic acetate oxidising pathway

Metalloproteins/metalloenzymes	Trace elements
Formate dehydrogenase	Se, Mo/W, Fe
Corrinoid- and Fe/S cluster-containing protein	Co, Fe
Carbon monoxide dehydrogenase	Ni, Fe
Acetyl-CoA synthase	Ni, Fe

FDH of *Clostridium pasteurianum* was tested for this pathway and the results showed that this enzyme from *C. pasteurianum* is a Mo Fe-S protein containing 1 mol of Mo and 24 mol of non-heme Fe and acid labile S in 1 mol of enzyme (Scherer and Thauer, 1978). A W-containing FDH in *Desulfovibrio gigas* was reported as well, in its active side W was bound to Mo atoms and selenoproteins. This W enzyme follows the same catalytic mechanism as Mo-containing FDH (Raaijmakers *et al.*, 2002; Ragsdale and Pierce, 2008; Stock and Rother, 2009).

CFeSP was reported to connect the methyl and carbonyl branch in this pathway as a methyl-acceptor, then donate it to acetyl-CoA synthase (Ragsdale and Pierce, 2008). Svetlitchnaia *et al.* (2006) isolated the crystal structure of CFeSP from *Carboxydothermus hydrogenoformans*, illustrating its component as 1 mol Co containing corrinoid cofactor and a single Fe_4S_4 cluster. In addition, CFeSP was assumed to be homologous to another Co-containing enzyme methyltransferase in methanogenesis as they have similar functions (Jablonski *et al.*, 1993).

Two types of CODH were involved in AD: 1) monofunctional nickel CODH, containing 10 Fe and 1 Ni per monomer, physiologically functions in the direction of CO oxidation; 2) bifunctional CODH/ACS, containing 14 Fe and 3 Ni per monomeric unit (Ragsdale and Pierce, 2008). In the Wood-Ljungdahl pathway, association of ACS with CODH forms a bifunctional CODH/ACS, to catalyse CO_2 reduction coupled with acetyl-CoA synthesis (Ragsdale, 2007). In nature, acetyl-

CoA synthase appears to associate tightly with CODH to form a heterotetrameric complex CODH/ACS. The structure of ACS itself could be described as a binuclear Ni-Ni centre bridged to a [4Fe-4S] (Nicolet *et al.*, 2000; Ragsdale, 2007).

The TEs identified as used in these protein/enzymes are Fe, Ni, Co, Se, Mo and W. There are also some studies showing the involvement of Cu and Zn in the Wood-Ljungdahl pathway, although other investigations demonstrated that the presence of Cu and Zn inactivated the enzymes (Doukov *et al.*, 2002; Seravalli *et al.*, 2003; Zhu and Tan, 2009)

2.3.2.2 **Propionate degradation pathways**

The syntrophic propionate oxidising pathway is adopted by syntrophic propionate oxidising bacteria (POB) present in the AD process. In a number of studies, *Syntrophobacter* spp., *Pelotomaculum* spp. and *Smithella* spp. have been recognised as the main stream of syntrophic propionate oxidising bacteria (McMahon *et al.*, 2004; Worm *et al.*, 2009; Muller *et al.*, 2010).

Two formate dehydrogenases were isolated from the syntrophic propionate oxidising bacterium *Syntrophobacter fumaroxidans* (de Bok *et al.*, 2003). Both enzymes were produced in axenic fumarate-grown cells as well as in cells which were grown syntrophically on propionate with *Methanospirillum hungatei* as the H_2 and formate scavenger. The purified enzymes exhibited extremely high formate oxidation and CO_2 reduction rates, and low K_m values for formate (0.01~0.04 mM). Both enzymes contained W and Se, while Mo was not detected. This matches with the genome analysis of *Syntrophobacter fumaroxidans* which indicated that *S. fumaroxidans* could code for a cytoplasmic [NiFe]-hydrogenase, two cytoplasmic [NiFeSe]- hydrogenases, a [NiFe]-hydrogenase maturation protein, and two cytoplasmic formate dehydrogenases (Muller *et al.*, 2010).

Several TE supplementation/deficiency experiments were carried out to distinguish the real TE requirement for syntrophic propionate oxidisation. Plugge *et al.* (2009) studied the effect of W and Mo on the growth of *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei* co-culture and also in their individual pure strain cultures. The study concluded that the effect of W and Mo on the activity of formate dehydrogenase was considerable in both the organisms, whereas hydrogenase activity remained relatively constant. Depletion of W and/or Mo, however, did not affect the growth of the pure culture of *S. fumaroxidans* on propionate plus fumarate significantly, although the specific activity of hydrogenases and especially formate dehydrogenase were influenced by the absence of Mo and W. The result suggests a more prominent role for H_2 as electron carrier in the syntrophic conversion of propionate, when the essential TMs, W and Mo for the functioning of formate dehydrogenase are depleted.

Worm *et al.* (2009) observed a propionate degrader shift from *Syntrophobacter* spp. to *Pelotomaculum* spp. and *Smithella* spp. in an Upflow Anaerobic Sludge Blanket (UASB) digester fed with synthetic propionate medium without Mo, W and Se. The authors argued that one of the reasons for the species shift was because *Pelotomaculum* spp. and *S. propionica* may need Mo for formate dehydrogenase activity whereas *Syntrophobacter* spp. needed W for formate dehydrogenase activity and Mo can even have an antagonistic effect as was described for *S. fumaroxidans*. The medium, however, was deficient in both Mo and W which could not support the authors' explanation, especially since *Syntrophobacter* spp. was the dominant propionate oxidising species at the beginning of the experiment.

Boonyakitsombut *et al.* (2002) tested the effect of TE on propionate degradation in mesophilic conditions and showed the addition of Fe, Co and Ni (10, 1 and 1 mg L^{-1}, respectively) enhanced the propionate utilisation rate. The addition of Mo individually, however, reduced the propionate degradation rate.

According to Osuna *et al.* (2003b), the addition of trace elements to UASB reactors significantly stimulated the conversion of propionate in a mixture of VFA (acetate, propionate, butyrate, in a ratio 3:1:1). The effect of individual TE to propionate degradation could not be distinguished in this study, however, because the trace elements listed below were added simultaneously (concentrations are shown in brackets in the units of mg L^{-1}): $FeCl_2{\cdot}4H_2O$ (2000), H_3BO_3 (50), $ZnCl_2$ (50), $MnCl_2{\cdot}4H_2O$ (500), $CuCl_2{\cdot}2H_2O$ (38), $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (50), $CoCl_2{\cdot}6H_2O$ (2000), $NiCl_2{\cdot}6H_2O$ (142), and $Na_2SeO_3{\cdot}5H_2O$ (164). At the end of the experiment, the COD removal efficiencies were 99 and 77% for the trace elements supplemented and deprived reactors, respectively.

2.3.3 Effect of trace element supplementation on AD performance

Although TEs are essential for anaerobic treatment processes, the supply of these to bioreactors has received less attention than their toxicity. There are, however, many studies reporting stimulatory effects of TEs on biogas process performance, including increased specific methane production, less VFA accumulation, and better substrate utilisation and process stability.These allow for application of higher organic loading rates (OLRs) of the process and thus, increasing the capacity of anaerobic digesters for volumetric methane production and substrate utilisation rate (Murray and Vandenberg (1981); Takashima, Speece and Parkin (1990); Feng *et al.* (2010); Fermoso *et al.* (2010); Demirel and Scherer (2011).

Stimulatory and stabilising effects of TEs on AD process depend on type and dose of their supplementation (Takashima, Speece and Parkin, 1990; Gustavsson, Svensson and Karlsson,

2011; Lindorfer, Ramhold and Frauz, 2012; Vintiloiu *et al.*, 2012). Among TEs, Fe, Co and Ni are the most studied and often show stimulatory effects on biogas process, while Se, W and Mo so far have been given less attention (Worm *et al.*, 2009; Feng *et al.*, 2010; Worm *et al.*, 2011). For example, increased CH_4 production was achieved by addition of Co to a UASB reactor, compared to a control without Co supply (d'Abzac *et al.*, 2013). Similar results were obtained by Gustavsson *et al.* (2013a), who showed that addition of Co to a laboratory-scale reactor digesting grass clover silage made it possible to increase the OLR, improve gas production and stabilise pH and increasing the treatment capacity of biogas reactors. Murray and Vandenberg (1981) reported an increase in CH_4 production due to Ni, Co and Mo addition to a reactor digesting food processing waste. Moreover, Feng *et al.* (2010) demonstrated that addition of Se/W in combination with low levels of Co increased biogas production.

The quantitative requirements of metals to achieve improved AD process performance vary to a great extent (Demirel and Scherer, 2011). This is likely due to the variation in process conditions (e.g. pH, OLR, HRT, substrate composition, etc.) and the complex chemical and biological processes controlling trace metals bioavailability (Zandvoort *et al.*, 2006).

Florencio (1994) found that the specific methanogenic activity of anaerobic sludge for methanol degradation was greatly stimulated by adding trace elements, especially cobalt. The maximum activity was found at a cobalt concentration of 0.1 mg L^{-1}. Gonzalez-Gil, Kleerebezem and Lettinga (1999) also confirmed that microbial biomass in UASB and expanded granular sludge bed reactors can be nickel and cobalt limited during the degradation of methanol.

According to Qiang, Lang and Li (2012), the highest production of methane was observed when the substrate was treated by a combination of cobalt and nickel, or iron, cobalt, and nickel in batch experiment. The results from both batch and continuous experiments also showed that iron, cobalt, and nickel have a significant effect on methane fermentation. It was revealed that the addition of Co, Ni at concentrations of 1, 1 mg L^{-1} and Fe, Co, Ni at 10, 1, 1 mg L^{-1} to the mainly carbohydrate-based food waste resulted in improvements in the methane production by factors of 7.8- and 7.5-folds, respectively, compare to control in which the TEs were not added in batch experiment. Moreover, digesters achieved stable performance and sudden decrease in VFA concentration after the addition of certain amounts of Fe, Co, and Ni in continuous experiment.

Demirel and Scherer (2011) reviewed the micronutrient requirements of farm-based digestion systems fed with energy crops and manure, concluding that a lack of nutrients can lead to process instability and disruption of the energy production in agricultural biogas plants, and the addition of single trace elements or their combinations showed significant effects on anaerobic digestion. The effect of trace elements supplementation is most important when energy crops are used as sole substrates for the production of biogas.

Interestingly, Williams, Shih and Spears (1986) observed that the stimulating effects of metals can occur even when high total concentrations of these metals are present in the reactor system: this implies that the metals can be present in a non-bioavailable form. In their study nickel at a dosing strength of 0.587 mg L^{-1} stimulated the biogas production of a chicken manure digester, while nickel was present in the digester at a concentration of 14.851 mg L^{-1} before nickel was added externally.

Since the addition of TE may increase the operating costs of the process, considerable attention has been given to the effect of TE dosing strength on the performance of anaerobic bioreactor systems in order to achieve a more economic and effective control of the process. The minimum amount of TE required for optimal performance of the anaerobic system, however, is still not well defined (Osuna *et al.*, 2003b). If insufficient TE is present then process compensation must be made by either lowering the loading rate or accepting a lower treatment efficiency. In other words, the metabolic capacity of the digester is determined by the availability of TE which ultimately determine the enzyme activity which is available for the catabolic reactions required to deal with the organic load applied (Banks and Heaven, 2013). Thus, in the case of a reactor having proper operating regime and macro-nutrients, the microbial cell density and methane production were highly dependent on the trace elements concentration in the reactor (Zhang *et al.*, 2003).

2.4 Trace Elements Uptake and their Bioavailability in anaerobic system

Bioavailability is the degree to which elements are available to interact with biological part of the system (Marcato *et al.*, 2009), and it depends on their chemical speciation and fractionation in the system. Generally in anaerobic digestion system, it is assumed that dissolved TE as free metal ions and as certain soluble organic metal complexes (Jansen, 2004) are readily available and can be taken up directly by microorganisms. Models used to predict TE bioavailability usually consider the free ions as the major bioavailable species (Worms *et al.*, 2006).

TEs enters digesters in different forms depending on if they are introduced with substrates or by direct supplementation. In order to have a biological function in microorganisms, TEs firstly need to be taken up by microoganisms. The uptake is assumed to proceed mainly via the transport of free ions across the cell membrane via two steps (Zandvoort *et al.*, 2006): a passive adsorption step onto the biomass surface, followed by an energy-dependent transport into the cell. Once internalised, TE is usually readily available to conduct its biological functions (van Hullebusch *et al.*, 2016). The former step for transport of TE across cell membrane is usually unspecific and driven by the concentration gradient between bulk solution and the cytoplasmatic membrane of microorganisms; whereas the latter is highly substrate specific, slower and inducible. The second

step often consumes energy, and is only used by a cell in times of need, starvation, or a special metabolic situation (Aquino and Stuckey, 2007). The first step is complicated and discussed in more detail in the following paragraph; a transporter in the cell membrane is usually required for the second step, and its quantity determines the maximum transfer rate.

Before the essential elements actually reach the microbial biomass present in a biofilm, granular sludge or suspended floc, they are subjected to complex (bio)chemical processes including: 1) precipitation and complexation with sulphides (S^{2-}) (Jong and Parry, 2003; La *et al.*, 2003), carbonate (CO_3^{2-}) and phosphate (PO_4^{3-}) (van Hullebusch, Zandvoort and Lens, 2003) to form inorganic and organic complexes (Callander and Barford, 1983a;b; Jansen, 2004; van der Veen, Fermoso and Lens, 2007); 2) binding with organic chelators of microbial origin, the so-called soluble microbial products (SoMP) or extracellular polymeric substance (EPS) (Kuo and Parkin, 1996; Barber and Stuckey, 2000; Gonzalez-Gil *et al.*, 2003; Patidar and Tare, 2006; Aquino and Stuckey, 2007); and 3) These processes can reduce the free metal concentration or other readily bioavailable form in solution to extremely low values. Then the mobility and availability of TEs becomes an important aspect.

Gustavsson *et al.* (2013a) reported that the bioavailability of TEs for microbial uptake and growth depends on their speciation which is controlled by both the environmental and operational conditions. Many parameters such as shifts in pH-value, temperature, OLR and redox-potential may lead to precipitation and/or chelation of TEs thus reducing bioavailability (Speece, 1996; Zandvoort *et al.*, 2005). The bioavailability of TEs for metabolic pathways of the anaerobic bacteria is in most cases not related to the total amount measured in the medium since only a fraction is present in solution (Pobeheim *et al.*, 2010). As a result, TE requirements will vary among different biogas production processes due to different environmental conditions and substrate composition.

The bioavailability of TEs needs to be analysed on a case-by-case basis due to a wide variety of substrate type and digester operational practices, and different opinions exist even for a single chemical process. Sulphur-containing organic matter and sulphate in substrate are converted to sulphide during AD (J.W.H *et al.*, 1994), and several studies reported the importance of sulphide as a regulator for metal bioavailability due to high affinity of sulphide for binding metal ions (Callander and Barford, 1983b;a; Rinzema and Lettinga, 1988; Morse and Luther Iii, 1999; Gonzalez-Gil *et al.*, 2003; Jansen *et al.*, 2005; Patidar and Tare, 2006; Aquino and Stuckey, 2007; Jansen, Gonzalez-Gil and van Leeuwen, 2007).

Morse and Arakaki (1993) indicated that the high Fe concentrations in digesters promoted co-precipitation and adsorption of Co and Ni on FeS. Hence, in digesters where Fe is often supplied in relatively large quantities compared to Co and Ni, adsorption/co-precipitation of Co and Ni on FeS may play an important role on their bioavailability for microbial uptake and process stability

(Shakeri Yekta *et al.*, 2012; Shakeri Yekta *et al.*, 2014a; Shakeri Yekta *et al.*, 2014b; Yekta *et al.*, 2017). The formation of metal-sulphides is often considered to be the most important aspect with regard to metal speciation and limitation (Schmidt *et al.*, 2014).

As metal sulphides have a very low solubility product, it would be expected that these metals are non-bioavailable to the methanogenic consortia when soluble sulphide is available (van der Veen, Fermoso and Lens, 2007). Jansen (2004) and Jansen, Gonzalez-Gil and van Leeuwen (2007), however, proposed that in most cases the dissolution rates of Co and Ni sulphides are not limiting to methanogenic activity in anaerobic wastewater treatment and suggested that these complexes may act as metals sources of Ni and Co for the methanogens. It should be noted that this was based on batch tests with methanogenic enrichments and freshly precipitated metal sulphides. However, the presence of different organic and inorganic chelators, and the variety of particle size and the age of the metal sulphide precipitates in real-world digester operation, was considered to lower dissolution rates of metal sulphides and therefore reduce bioavailability of metals (Gonzalez-Gil *et al.*, 2003).

The function of TEs in the AD process therefore means only those available to microbial biomass represent useful TE. The total TE concentration in digester is not a direct indication of this and cannot give precise information about the bioavailability and mobility of the TEs which can be up taken by microoganisms. With limited knowledge on bioavailability, however, the quantification of the optimum TEs range to maintain an optimised cell functions has to rely on absolute/total TE concentrations, especially for digestion systems treating complex solid organic waste streams. Although the total concentration may not present the readily available TE concentration in AD, the findings based on total TE remain reliable (Choong *et al.*, 2016). In addition, the continuous supplementation of TEs as soluble elemental solution makes sure that free metals are available for biomass to uptake at any time (Thanh *et al.*, 2016). As the physicochemical conditions of a specific digestion system do not vary much, the determination of total TE required for supplementation should provide sufficient information on other digesters of the same kind. The digester response to total TE addition applied also helps to clarify the mechanisms of TE to each microbial group involved in AD process, which is the first step to gain more insight and knowledge, i.e. the availability of TE, of this research topic.

Although the complex pool of inorganic and organic matter in anaerobic digesters makes it difficult to predict TE bioavailability, the research on natural and engineered systems has proved that metal speciation and fractionation is a useful tool for the investigation of their bioavailability (Stover, Sommers and Silviera, 1976; Tessier, Campbell and Bisson, 1979; Álvarez *et al.*, 2002; Filgueiras, Lavilla and Bendicho, 2002; Camel, 2003; Mossop and Davidson, 2003; Wright, Parker and Amrhein, 2003; Hullebusch *et al.*, 2005).

2.5 Metals fractionation and sequential extraction

Fractionation analysis refers to separation procedures to classify analytes according to their physical (e.g., size, solubility) or chemical (e.g., bonding and reactivity) properties (van Hullebusch *et al.*, 2016). Different from speciation analysis, it has insufficient separation power to differentiate between individual chemical species. Metals fractionation is achieved by sequential extraction (SE), a sequence of selective chemical extraction techniques with increasingly aggressive reagents which is to convert the elements bound in solid phase into soluble forms with the extractant used at each step (Howells, 1995).

It is now widely recognised that the trace element fractionation, their mobility and bioavailability in anaerobic system depend not only on their total concentration but also on the association form in the solid/liquid phase to which they are bound (Filgueiras, Lavilla and Bendicho, 2002). The need to understand metal fractionation in order to predict the fate, the potential mobility and bioavailability of trace metals during anaerobic digestion ecosystems led to the development of experimental sequential extraction (SE) schemes. Although a thorough determination of the entire speciation profiles of TE in AD ecosystem is often not attainable, TE fractionation by applying the SE techniques can provide valuable information on hierarchy of TE pools useful to assess bioavailability (Ortner *et al.*, 2014b; Ortner *et al.*, 2015). The fractions possess a decreasing solubility/reactivity from the first to the last step, although the fractions are operationally-defined and not phase/species-specific (Quevauviller *et al.*, 1997; Harmsen, 2007).

These methodological schemes have been criticised due to the following issues: time consuming, lack of uniformity in the procedure, lack of selectivity of extracting reagents, lack of quality control, and the significant influence of operational parameter (e.g. temperature, pH, extracting time, reagent concentration, stirring system, the ratio of solid mass to volume of extractants) selection on result (Förstner, 1993; Gleyzes, Tellier and Astruc, 2002; Hsu, Liu and Tzou, 2015). However, SE provides valuable information about the biological and physicochemical availability, as well as the mobilisation and transport, of trace metals.

In the last decades, many metal fractionation method have been applied to study anoxic sediments and their interstitial waters; however, information on other anaerobic ecosystems, such as in anaerobic digesters, is still lacking. Filgueiras, Lavilla and Bendicho (2002) provided a very comprehensive review on different sequential extraction schemes used to fractionate metals in different environmental solid samples, e.g. soil, sediment, sewage sludge, and fly ash. The main differences between them are the extractant reagents, extraction conditions, and number of fractions.

One of the most widely applied schemes for SE is Tessier scheme (Tessier, Campbell and Bisson, 1979), which was developed for determining the fractionation of TE (Cd, Co, Cu, Ni, Pb, Zn, Fe,

and Mn) in particulate materials into five fractions: exchangeable, bound to carbonates, bound to Fe-Mn oxides, bound to organic matter/sulphide, and residual. The contribution of the sulphide fraction was not singled out, as the samples tested in the original study were oxic fluvial bottom sediments. The Tessier procedure was implemented by Angelidis and Gibbs (1989) on anaerobically treated sludge and it was revealed that the organic matter and/or sulphide fraction was the most important carrier of metals in these matrixes. Therefore, it is worth investigating the individual contribution of organic matter and sulphide fractions, rather than allowing them to be simultaneously extracted (van Hullebusch *et al.*, 2016).

Another popular scheme was developed by Stover, Sommers and Silviera (1976) for determining the forms and amounts of Pb, Zn, Cu, and Ni in anaerobically digested wastewater sludges obtained from 12 municipal treatment plants. The Stover SE scheme provides information on six different fractions: soluble/exchangeable, adsorbed, organic matter bound, carbonate, sulphide fractions and residual. This scheme was then modified by Lake, Kirk and Lester (1985) and used in many studies on metal extraction for anaerobic sludges (Rudd *et al.*, 1988; Carliell and Wheatley, 1997; Carliell-Marquet and Wheatley, 2002; Hullebusch *et al.*, 2005; Aquino and Stuckey, 2007; Hu *et al.*, 2008a). An advantage of this scheme is that it allows discriminating metal distribution between the organic matter and sulphide fraction (van Hullebusch *et al.*, 2016).

Many other SE procedures were developed later, including the BCR SE scheme proposed by the Commission of the European Communities Bureau of Reference in the attempt to harmonise the methodology used in different schemes (Ure *et al.*, 1993) and certify SE through inter-laboratory trials (Rauret *et al.*, 2000). The accelerated BCR SE scheme was then developed (Pérez-Cid, Lavilla and Bendicho, 1999), which significantly reduced the extraction time: from 2.5 days for the original BCR scheme to half a day. The BCR scheme extracts metals into four fractions, i.e. exchangeable plus water and acid soluble, Fe and Mn oxides, organic matter/sulphides fraction and residual. The main difference between the BCR and Tessier schemes is the first fraction; the metal content extracted from the acid soluble fraction in the BCR scheme should be equivalent to the sum of metal contents extracted from the acid soluble and exchangeable fractions in the Tessier scheme (Thanh *et al.*, 2016). A later modified BCR scheme (Sahuquillo *et al.*, 1999) is able to extract the Fe-based components of the reducible fraction (Mossop and Davidson, 2003). The original and modified BCR have been used to fractionate metals in anaerobic sludge (Rauret *et al.*, 2000; Alonso *et al.*, 2006; Fuentes *et al.*, 2008; Dąbrowska, 2012) and in sediment (Quevauviller *et al.*, 1994; Quevauviller *et al.*, 1997), and the results showed that Fe and Mn oxides were not commonly found in anaerobic sludge (van der Veen, Fermoso and Lens, 2007). The sequential extraction procedure used in Aquino and Stuckey (2007) also provided an effective tool to distinguishing the metal participation in different fractions of anaerobic digestion system.

2.6 Research gaps

Although a great amount of studies have been conducted on TE effect on anaerobic digestion process, some issues still have not been fully investigated. For example, little knowledge was available on the relation between metal speciation/fractionation and their bioavailability in anaerobic bioreactors; therefore rendering extremely difficult the determination of the optimum trace metal concentration range to maintain optimised microbial cell functions. This was especially true for continuously stirred digesters treating solid feedstocks. The information was also lacking on how the operating factors affects TE supplementation requirement. This study was therefore conducted to investigate the TE requirement and the dynamic changes of their distribution in different digestate fractions in anaerobic digesters under different conditions. Special focus was paid to the TE distribution over the course of TE washing-out process to identify which fractions had the highest affinity to TE when this resource was limiting, which helped to examine the TE availability to microbial consortium in AD.

Chapter 3: **Materials and Methods**

3.1 Digester operation and monitoring

3.1.1 **Semi-continuous digestion experiments**

Digesters used in the study were of a continuously stirred tank reactor (CSTR) design. The reactors had two sizes: 5-litre and 100-litre capacity with 4-litre and 75-litre liquid working volume respectively. Semi-continuous operation was achieved by removing digestate through a small outlet port at the bottom of reactor before adding essential trace elements and feeding substrate via the feed port on the top of reactor. Deionised water was added to the substrate to lower the VS to target value. During this process a small amount of atmospheric air was introduced to the headspace of reactor but in insufficient quantities to affect digester performance: any air detected in the gas composition is corrected for, as this is not normally produced as a result of the digestion process. Feeding was done on a daily basis throughout the experimental period, and the digestate was disposed of from the reactors at least once a week to maintain the liquid working volume.

5-litre CSTR digester

The 5-litre digesters were constructed of PVC tube with gas-tight top and bottom plates. The top plate was fitted with a gas outlet, a feed port sealed with a rubber bung, and a draught tube liquid seal through which an asymmetric bar stirrer was agitated continuously using an 80 rpm motor mixer mounted directly on the top plate to mix the reactor content. Figure 3-1 shows the schematic and photo of this type of digester. Mesophilic condition was maintained by controlling temperature at 36 ± 1°C using thermostatic bath to circulate water through a heating coil surrounding the digesters. Biogas production was measured using tipping bucket gas counters with continuous data logging (Walker *et al.*, 2009). The gas counter calibration was carried out weekly by collecting biogas produced over a feeding cycle using Tedler bags after the gas went through the tipping bucket gas counters, and measuring the volume of gas collected using a weight-type water displacement gasometer (Walker *et al.*, 2009). All gas volumes reported were corrected to standard temperature and pressure (STP) of 0°C and 101.325 kPa. The biogas composition was also determined using the gas collected in the Tedler bags.

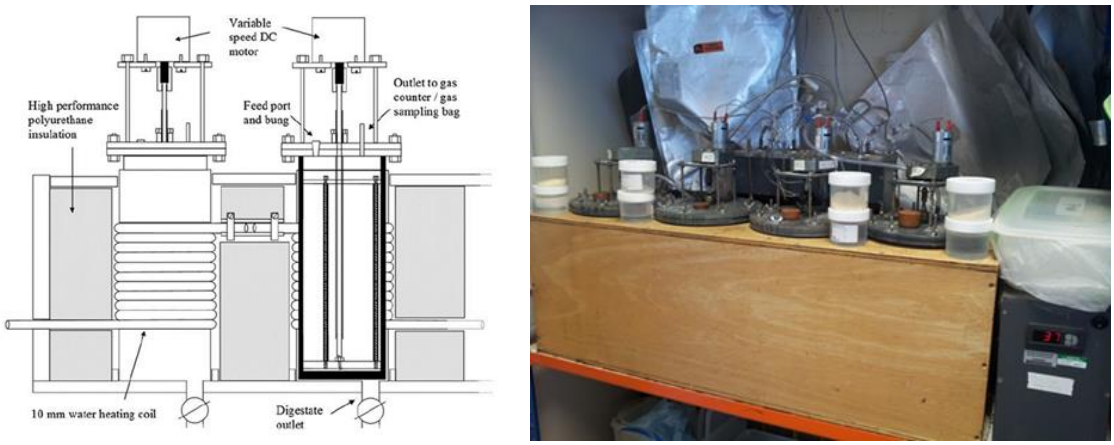


Figure 3-1 Typical schematic of the 5-litre digesters used with cross-section showing details of heating (left) and stirring systems (Banks, Zotova and Heaven, 2010; Jiang, Heaven and Banks, 2012) and photo of anaerobic digester of 5-litre digesters

100-litre CSTR digester

This type of digesters had a total volume of 100-L and a liquid working volume of 75-L, and was constructed from 40 cm inner diameter PVC pipe sealed at its top and bottom with plates incorporating feed and drainage ports. Digester temperature was controlled at 36 ± 1 °C by recirculating water from a thermostatic bath through an internal heating coil. The digesters were sealed from the outside atmosphere by a draught tube through which an offset bar stirrer was inserted to allow low speed mixing at 26 rpm by means of geared motors (Parvalux, UK). Biogas production was measured using tipping bucket gas counters with continuous data logging (Walker *et al.*, 2009). The configuration of the system is shown in Figure 3-2.

Figure 3-2 Typical schematic (left) 1 feedstock inlet; 2 motor to proper a stirrer; 3 heater; 4 heating coil; and 5 digestate outlet and photo (right) of the 100-litre digesters

3.1.2 **Digester monitoring**

The above mentioned digesters were used for the study as discussed in Chapter 5-8. Feeding amount, digestate removed and TE supplemented for each digester were thoroughly recorded for each experiment. Digestate samples were used for analysing pH, total ammoniacal nitrogen (TAN), alkalinity, solids, VFA and trace elements concentrations. Biogas samples were collected for gas composition analysis and gas counter calibration, as described in section 3.1.1. As shown in Table 3-1, The performance of the reactors was evaluated at least once a week and more frequently in some cases, especially when digester were under unstable condition, using the analysis listed above. Daily biogas production (DBP), specific biogas production (SBP), specific methane production (SMP), volumetric biogas production (VBP) and volumetric methane production (VMP) were also calculated daily to evaluate the digestion efficiency (Table 3-2).

Table 3-1 Digester monitoring plan

Parameter	Frequency
Total Ammoniacal Nitrogen (TAN)	Once a week
Alkalinity	
Total Alkalinity (TA)	Once a week
Partial Alkalinity (PA)	Once a week
Intermediate Alkalinity (IA)	Once a week
Solid	Once a week
Total Solids (TS)	Once a week
Volatile Solids (VS)	Once a week
VFA	Twice a week
pH	Three times a week
Temperature	Three times a week
Biogas production	Twice a week
Biogas composition	Twice a week

Table 3-2 Calculated parameters for the evaluation of digester performance

Parameter	Frequency
Daily Biogas production (DBP)	Daily
Specific Biogas Production (SBP)	Daily
Specific Methane Production (SMP)	Daily
Volumetric Biogas Production (VBP)	Daily
Volumetric Methane Production (VMP)	Daily
Volatile Solid Destruction (VSD)	Once a week
Free Ammonia Nitrogen (FAN)	Once a week
IA/PA	Once a week

3.2 Digester substrate

The model substrate used in this research was an assembly of organic materials with low TE content: whole milk powder (Nido instant full cream milk powder, Nestle, UK), whole egg powder (whole egg powder, MyProtein) and rice flour (free from gluten, Doves Farm). These components were bought in big batch to ensure the substrate for each long-term experiment was from same batch of materials. The model substrate was made by mixing them at a ratio of 20 : 20 : 60 on a VS basis to give a biochemical composition of 63.6 % of carbohydrate (as simple sugars and starch), 20.7 % of protein and 15.7 % of lipids on an organic dry weight basis as shown in Table 3-3. All of the components were lack of lignocellulosic materials. The substrate was kept in dry condition after the components were weighed and mixed to ensure homogenisation, as shown in Figure 3-3. The quantity of the working substrate and the deionised water added to each digester was calculatd according to the designed organic loading rate (OLR) and hydraulic retention time (HRT). For example, Table 3-4 below shows the digester daily feeding regime for an experiment using 5-L digesters at an OLR of 3.0 kg VS m^{-3} d^{-1} and HRT of 33.3 days. In that case, deionised water was added daily and the VS level of the whole feeding blend (model substrate and water) was 10%.

Table 3-3 Biochemical composition of model substrate and its components on an organic dry weight basis

Materials	Typical Composition (% organic dry weight)		
	carbohydrate	protein	lipids
whole milk powder	42.12	26.84	31.05
whole egg powder	7.50	52.54	39.95
rice flour	89.41	8.11	2.47
Model substrate	63.6	20.7	15.7

Note: *Carbohydrate from sugar and starch

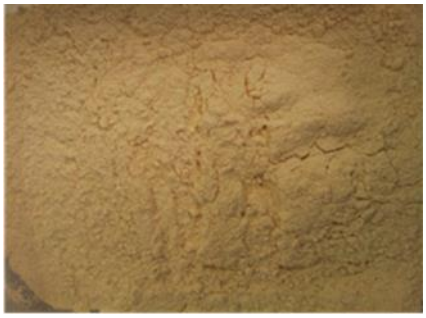

(a)

(b)

Figure 3-3 Components of the model substrate (a) top-left is egg powder, bottom-left is milk powder and right is rice flour and their mixture (b)

Table 3-4 Digester daily feeding regime: an example on a 5-L digester at an OLR of 3.0 kg VS m^{-3} d^{-1} and HRT of 33.3 days

Materials	Daily input (g VS d^{-1})	Daily input (g fresh matter (FM) d^{-1})
Model substrate, includeing	12.0	13.52
whole milk powder	2.4	2.62
whole egg powder	2.4	2.66
rice flour	7.2	8.24
Deionised water	0	106.48

Izumi *et al.* (2010) suggested that one of the most important factors in anaerobic digestion is particle size of substrate. Smaller particles increase the specific surface area available to the microorganisms, resulting in increased food availability and accelerated hydrolysis rate (Mshandete *et al.*, 2006). The model substrate used in this study was in powder form which was therefore subject to rapid digestion (Palmowski and Muller, 2000; Mshandete *et al.*, 2006).

Estimation of biochemical methane potential and sulphur content of the model substrate

The Biochemical Methane Potential (BMP) test and sulphur (S) content of the model substrate did not experimentally carry out in this study. Then, the predicted BMP and S values are estimated from available data of source segregated organic material and also calculated from the biochemical composition of model substrate presented in Table 3-3. This is because the proportion of three main compositions in model substrate is similar to food waste which generally composed of several parts such as starch, proteins, lipids and cellulose etc (Zhang, Lee and Jahng, 2011; Zhang, Banks and Heaven, 2012a;b; Wei *et al.*, 2014; Zhang *et al.*, 2015). The biochemical composition of substrate used in this study is 63.6 % carbohydrate, 20.7 % protein and 15.7 % lipid similar to source segregated domestic food waste (SS-DFW) which reported in terms of 58.6 % carbohydrate (including lignin, cellulose and hemicellulose), 26.6 % protein and 14.8 % lipid (Zhang, Banks and Heaven, 2012a;b) and 66.3 % carbohydrate, 13.9 % protein and 19.6 % lipid (Zhang, Lee and Jahng, 2011).

The theoretical BMP values could be estimated from the biochemical composition of the wastes (Angelidaki and Ellegaard, 2003), using the Buswell equation (Symons and Buswell, 1933) based on an empirical formula derived from the elemental composition and using a carbon mass balance based on the measured VS destruction, biogas composition and elemental carbon measurements (Zhang, Banks and Heaven, 2012a). Zhang, Banks and Heaven (2012a) studied the BMP tests results of source segregated domestic food waste and found that the measured BMP was 10 % lower than theoretical BMP calculated from the biochemical composition of the wastes (0.494 STP m^3 CH_4 kg^{-1} VS_{added}) due to some fibre and lipid materials still remained and could account for this discrepancy in digestate residue at the end of the test. The measured BMP values were much lower than those predicted using the Buswell equation (0.547 STP m^3 CH_4 kg^{-1} VS_{added}), reflecting its relatively high lignin content which is known not to break down under anaerobic conditions. Whilst, the latest estimation method gave a predicted value (0.467 STP m^3 CH_4 kg^{-1} VS_{added}) within 5 % of the experimentally determined value (0.456 m^3 CH_4 kg^{-1} VS_{added}), which supports the accuracy of the analyses for volatile solids, biogas composition and elemental carbon measurements.

Measured BMP values were also reported at 0.471 (Yirong, 2014), 0.480 (Zhang, Lee and Jahng, 2011) and 0.435 m^3 CH_4 kg^{-1} VS_{added} (Zhang *et al.*, 2007) within ± 10% of those found by Zhang, Banks and Heaven (2012a) for similar source segregated domestic food waste, reflecting their consistent of the biochemical composition and elemental composition as can be seen in Table 3-5.

The slightly higher BMP values implied that the higher protein and lipid in substrate leading to a higher percentage of C and H. However, these values are similar to the results which BMP were in the range from 0.401 to 0.489 m^3 CH_4 kg^{-1} VS_{added} for separately collected and source

segregated organic material (Cecchi *et al.*, 2003). Therefore, the estimated BMP value for the model substrate used in this study should also be in this range (0.401 to 0.489 $m^3 CH_4 kg^{-1} VS_{added}$). This would provide a measure of comparison of the efficiency of the digesters with the theoretical value expected, however, specific methane production (SMP) in this research under stable digestion were around 0.45 ± 0.03 $m^3 CH_4 kg^{-1} VS_{added}$

As a result, the elemental composition of model substrate could be predicted by using data from source segregated domestic food waste in Table 3-5. Therefore, sulphur content in the model substrate should be in the range around 0.1 - 0.81 % or 1.0 - 8.1 g kg^{-1} (TS basis).

Table 3-5 The characteristic of food waste

References	Zhang, Banks and Heaven (2012a) and Zhang, Banks and Heaven (2012b)	Wei *et al.* (2014) and Zhang *et al.* (2015)	Qiang *et al.* (2013) and Qiang, Lang and Li (2012)	Han and Shin (2004)	Zhang, Lee and Jahng (2011)	Zhang *et al.* (2007)	Yirong, Heaven and Banks (2015)	Zhang, Zhang and Li (2015)	Zhang *et al.* (2013)	Nagao *et al.* (2012)
Biochemical composition (VS basis), g kg^{-1}										
Carbohydrates	453 ± 17[a]	-	-	-	111.7 ± 6.2	-	-	-	-	-
Lipids	151 ± 18[b]	283 ± 13 (crude fat)	-	-	23.3 ± 0.45	-	-	-	-	-
Crude proteins	253 ± 3	-	-	-	32.9 ± 1.4	-	-	-	-	-
Hemi-cellulose	38.1 ± 3.7	10.1 ± 0.9	-	-	-	-	-	-	-	-
Cellulose	50.4 ± 1.6	4.3 ± 0.3	-	-	-	-	-	-	-	-
Lignin	16.5 ± 0.2	2.2 ± 0.2	-	-	-	-	-	-	-	-
Elemental composition (TS basis), %										
N	3.44 ± 0.04	2.50 ± 0.11	1.90 ± 0.09	3.5	3.54	3.16 ± 0.22	3.00 ± 0.01	2.7 ± 0.2	2.2 ± 0.3	3.2
C	47.6 ± 0.5	48.24 ± 0.17	47.4 ± 0.01	51.4	46.67	46.78 ± 1.15	52.3 ± 1.1	50.3 ± 0.4	46.5 ± 1.15	45
H	7.04 ± 0.63	6.90 ± 0.16	6.65 ± 0.28	6.1	6.39	-	6.89 ± 0.16	7.1 ± 0.2	-	-
S	0.15 ± 0.01	0.44 ± 0.02	0.41 ± 0.06	0.1	0.33	0.81 ± 0.03	-	-	-	-
O	-	34.13 ± 0.31	43.7 ± 0.28	38.9	36.39	-	-	29.1 ± 0.3	-	-

Note: [a] in equivalent glucose and [b] n-Hexane extractable material (HEM)

3.3 Digester inoculums

There were 3 sources of inoculum used in this study for long-term semi-continuous digestion experiments and short-term batch trials. The inoculums were analysed for pH, alkalinity, ammonia, TS and VS and the results were given in Table 3-6.

3.3.1 Digestate from the Millbrook wastewater treatment plant

One 100-L CSTR digester and eight 5-L CSTR digesters (N1-N8) as discussed in Chapter 4 and Chapter 5 were inoculated with digestate obtained from a full scale digester treating municipal wastewater biosolids at Millbrook wastewater treatment plant, Southampton, UK. This inoculum was sieved before inoculation to eliminate particles larger than 1 mm.

3.3.2 Digestate from the 100-L laboratory digester

The inoculum for the four 5-L CSTR digesters as discussed in Chapter 8 (R1-R4) was the low TE digestate taken from the 100-L digester, which was started with the inoculum described in section 3.3.1 and had been running for more than 600 days using model substrate. The detailed information on the operation and performance of this digester is given in section 4.3 of Chapter 4.

3.3.3 Digestate from 5-L laboratory digesters

Four 5-L digesters discussed in Chapter 6 and 7 (A1-A4) were started using the digestate of digesters N5, N4, N7 and N8 (details presented in Chapter 5)

Table 3-6 Inoculums properties

Sources of inoculum	Millbrook	100-litre digester	Digester N5	Digester N4	Digester N7	Digester N8
Digester inoculated	100-L digester, N1-N8	R1-R4	A1	A2	A3	A4
pH	7.39 ± 0.03	7.60 ± 0.04	7.51 ± 0.02	7.51 ± 0.03	7.52 ± 0.01	7.52 ± 0.03
TKN (g kg^{-1} FM)	3.79 ± 0.29	-	-	-	-	-
TAN (g N kg^{-1} FM)	1.29 ± 0.03	2.79 ± 0.05	2.50 ± 0.01	2.49 ± 0.06	2.62 ± 0.04	2.55 ± 0.07
Solid						
Total Solid, TS (% FM)	4.07 ± 0.59	1.29 ± 0.06	1.62 ± 0.02	1.65 ± 0.01	1.57 ± 0.02	1.57 ± 0.01
Volatile Solid, VS (% FM)	2.44 ± 0.20	0.99 ± 0.06	1.32 ± 0.02	1.34 ± 0.01	1.29±0.02	1.28 ± 0.02
Volatile Solid, VS (% TS)	63.07 ± 3.99	76.94 ± 0.99	81.81 ± 0.42	80.97 ± 0.54	82.42±0.21	81.49 ± 0.91
Alkalinity (g kg^{-1} FM)						
Total Alkalinity, TA	6.39 ± 0.29	10.36 ± 0.07	9.64 ± 0.01	9.34 ± 0.11	9.25±0.31	9.40 ± 0.09
Partial Alkalinity, PA	3.78 ± 0.17	7.78 ± 0.06	7.47 ± 0.07	7.17 ± 0.04	6.94±0.33	7.19 ± 0.06
Intermediate Alkalinity, IA	2.61 ± 0.15	2.59 ± 0.05	2.17 ± 0.08	2.17 ± 0.15	2.30±0.02	2.20 ± 0.15
IA/PA	0.69 ± 0.06	0.33 ± 0.01	0.29 ± 0.01	0.30 ± 0.02	0.33±0.02	0.31 ± 0.02
TE concentrations (mg kg^{-1} FM)						
Co	0.13	0.01	1.0	1.0	1.0	1.0
Ni	0.76	0.03	1.0	1.0	1.0	1.0
Fe	1,123	2.87	10.0	10.0	10.0	10.0
Se	0.07	0.00	0.1	0.1	0.1	0.1
Other elements	-	-	0.1 (Mo)	0.1 (Al, B, Cu, Mn, Mo, W, Zn)	-	0.1 (Al, B, Cu, Mn, Mo, W, Zn)

3.4 Digester supplements

11 trace elements were tested in this research but not all of them were added to every digester. Instead, a range of TE mixes was investigated in this research work. As shown in Table 3-7, the dosing strength of the elements can be grouped into three levels: 1) high strength of 10 mg kg^{-1} FM, which includes Fe only, 2) medium strength of 1.0 mg kg^{-1} FM, which includes Co and Ni, and 3) low strength of 0.1 mg kg^{-1} FM, which includes the rest of the elements tested (Al, B, Cu, Mn, Mo, Se, W and Zn). These dosing strengths were chosen based on the fractional factorial design results of previous studies (Banks *et al.*, 2012; Jiang *et al.*, 2017), apart from those for Co, Ni, Fe and Se when they were tested for their critical levels required for stable operation at different conditions, as described in Chapter 6 and 8. In the first case, the TE washing out experiment was conducted in order to investigate the optimal supplementation strengths of Co, Ni, Fe and Se at a range of OLRs. In the latter case, the dosing strengths of Co, Ni and Fe were started from very low levels of Co 0.03, Ni 0.03 and Fe 0.3 mg kg^{-1} FM by keeping Se dosing strength constant at 0.1 mg kg^{-1} FM during the course of this experiment in order to determine the effect of OLR on the requirement of Co, Ni and Fe at constant HRT.

3.4.1 Stock solutions for Co, Ni, Fe, Se and Mo individually

Each of the compounds listed in Table 3-7 was weighed individually using a small glass beaker, and then dissolved in the beaker by adding around 100 mL of deionised water. The resulting solution was transferred to a 1-litre volumetric flask, and the volume was made up by the deionised water, including that used to rinse the beaker. For iron solution, its pH was adjusted to 3 using 1 M HCl to prevent the precipitation. Each solution was stored a plastic storage bottle and kept in fridge when not in use.

Table 3-7 Concentration of cation and oxyanion elements in single element stock solution

Trace element	Compound used	Supplemented concentration in the working condition (after diluted by 10,000 times), mg kg^{-1} FM	Compound concentration in stock solution, g L^{-1}
Cobalt (Co)	$CoCl_2.6H_2O$	1.0	40.38
Nickel (Ni)	$NiCl_2.6H_2O$	1.0	40.50
Iron (Fe)	$FeCl_2.4H_2O$	10.0	355.97
Selenium (Se)	Na_2SeO_3	0.1	2.19
Molybdenum (Mo)	$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	0.1	1.84

3.4.2 **Stock solution for Al, B, Cu, Mn and Zn as a mixture**

Each of the compounds shown in Table 3-8 was weighed individually using a small glass beaker, and then the compound was dissolved in the beaker by adding around 100 mL of deionised water. Put all the solutions into one 1-litre volumetric flask. The beakers were rinsed with deionised water and the rinsing water was also added to the flask. After the volume was made up to 1-litre, the solution was transferred to a plastic storage bottle and kept in fridge when not in use.

Table 3-8 Concentration of cation in other mixed elements stock solution

Trace element as cation	Compound used	Suppemented concentration in the working condition (after diluted by 10,000 times), mg kg^{-1} FM	Compound concentration in stock solution, g L^{-1}
Aluminum (Al)	$AlCl_3.6H_2O$	0.1	8.95
Boron (B)	H_3BO_3	0.1	5.72
Copper (Cu)	$CuCl_2.2H_2O$	0.1	2.68
Manganese (Mn)	$MnCl_2.4H_2O$	0.1	3.60
Zinc (Zn)	$ZnCl_2$	0.1	2.08

Note: boron is arranged in the cation stock solution as its pKa is 5.2.

3.4.3 **Stock solution for oxyanions**

Each of the compounds shown in Table 3-9 was weighed individually using a small glass beaker, and then the compound was dissolved in the beaker by adding around 100 mL of deionised water. Put all the solutions into one 1-litre volumetric flask. The beakers were rinsed with deionised water and the rinsing water was also added to the flask. After the volume was made up to 1-litre, the solution was transferred to a plastic storage bottle and kept in fridge when not in use.

Table 3-9 Concentration of oxyanion elements in stock solution

Trace element as oxyanion	Compound used	Supplemented concentration in the working condition (after diluted by 10,000 times), mg kg^{-1} FM	Compound concentration in stock solution, g L^{-1}
Molybdenum (Mo)	$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$	0.1	1.84
Selenium (Se)	Na_2SeO_3	0.1	2.19
Tungsten (W)	$Na_2WO_4\cdot 2H_2O$	0.1	1.79

3.5 General Analytical Methods

3.5.1 Chemical Reagents and Glassware Used

All glassware used was washed with detergent and rinsed with tap water followed by deionised water before reuse. The deionised water was produced by a reverse osmosis system (Barnstead RO system, Thermo Scientific, UK).

All glassware and apparatus used in the analysis of trace concentrations of metals was washed with detergent, rinsed with tap water and deionised water, then soaked in an acid bath of 20 % nitric acid for at least 12 - 24 hours. Water used in the acid bath, subsequent rinsing and all wet chemistry analyses (alkalinity, ammonia and Kjeldahl nitrogen) was deionised water.

Both nitric and hydrochloric acids used in analysis were trace analysis grade. All reagents, standards and diluted samples were prepared using ultra-pure deionised water (Barnstead Nanopure ultrapure water purification system, Thermo Scientific, UK). Chemicals used were from Fisher Scientific (Loughborough, UK) except where otherwise specified.

3.5.2 Total Solids (TS) and Volatile Solids (VS)

Inoculum, digestate and model substrate samples were analysed according to the following procedure: after thorough homogenisation of the sample, approximately 50 g of sample was transferred into a weighed crucible by pouring (inoculum and digestate samples) or spatula (substrate samples: rice flour, whole milk powder, whole egg powder and model substrate). Samples were weighed using a balance with accuracy of ± 0.001 g (Sartorius LC6215 balance, Sartorius AG, Gottingen Germany) and placed in an oven (Vulcan laboratory oven, LTE Scientific Ltd., 68 Oldham UK) for drying overnight at 105°C ± 2°C. After drying the samples were transferred to a desiccator to cool for at least 40 minutes. Samples were then weighed again with the same balance, before being transferred to a muffle furnace (Carbolite Furnace 201, Carbolite UK, Hope Valley UK) and heated to 550°C ± 10°C for two hours. After this ashing step, samples were again cooled in a desiccator for at least one hour, before weighing a third time.

After all analyses, crucibles were washed with detergent, rinsed with tap water and again rinsed with deionised water, and stored in an oven until required for the next analysis. Crucibles were transferred from the oven to a desiccator for cooling to room temperature before each analysis. TS and VS were calculated according to Equation 3-1 to 3-3:

$$\%TS = \frac{W_3 - W_1}{W_2 - W_1} \times 100\% \qquad \text{Equation 3-1}$$

$$\%VS\ (\text{based on FM}) = \frac{W_3 - W_4}{W_2 - W_1} \times 100\% \qquad \text{Equation 3-2}$$

$$\%VS\ (\text{based on TS}) = \frac{W_3 - W_4}{W_3 - W_1} \times 100\% \qquad \text{Equation 3-3}$$

Where:

TS is the total solids;
VS is the volatile solids;
W_1 is the weight of the empty crucible, g;
W_2 is the weight of the crucible containing fresh sample, g;
W_3 is the weight of the crucible and sample after drying at 105°C for more than 10 hours, g;
W_4 is the weight of the crucible and sample after heating at 550°C for 2 hours, g.

3.5.3 **pH**

pH of digestate was measured using a Jenway 3310 pH meter (Bibby Scientific Ltd, Essex UK). This was done immediately after sample collection to avoid pH changes due to temperature changes and loss of dissolved CO_2. The meter has a sensitivity of 0.01 pH units and accuracy to 0.01 ± 0.005 units. Calibration of the probe was carried out before pH measurement using buffer solutions of pH 7.0 and 9.2. Buffer solutions were prepared weekly by dissolving pH buffer tablets (Fisher Scientific) in 100 mL of deionised water. Cross contamination was avoided by through rinsing of the probe between each measurement with deionised water, and storage of the probe in a pH 7 buffer solution between uses.

3.5.4 **Alkalinity**

A 2 - 5 grams of homogenous digestate sample was added to 40 mL of deionised water and titrated with 0.25 N H_2SO_4, with constant mixing using a magnetic stirrer. Titration was done using a Schott Titroline Easy automatic digital titration burette system (Schott, Mainz, Germany). The sample was titrated to endpoints 5.75 and 4.3, allowing calculation of partial alkalinity (PA), intermediate alkalinity (IA) and total alkalinity (TA) (Ripley, Boyle and Converse, 1986). Among them, PA is a measure of bicarbonate buffering, while IA indicates the buffering capacity attributable primarily to the salts of VFA. The ratio of IA to PA (Ripley Ratio) refers the VFA concentrations compared to the buffering capacity of bicarbonate (Ripley, Boyle and Converse, 1986).

Before alkalinity titration, the pH probe was calibrated using the buffer solutions noted above for pH measurement. Cross-contamination was avoided by thorough rinsing of the probe between each measurement, and storage of the probe in a pH 7 buffer solution between uses.

The measurement is based on the Standard Method 2320B for Alkalinity (APHA, 2005). Alkalinity in mg $CaCO_3$ kg^{-1} FM was calculated based on the Equation 3-4 to 3-6:

$$TA = \frac{(V_{4.3}+V_{5.7})\times 0.25\times 50{,}000\times a}{m} \quad \text{Equation 3-4}$$

$$PA = \frac{V_{5.7}\times 0.25\times 50{,}000\times a}{m} \quad \text{Equation 3-5}$$

$$IA = \frac{V_{4.3}\times 0.25\times 50{,}000\times a}{m} \quad \text{Equation 3-6}$$

Where:

TA is total alkalinity, mg $CaCO_3$ kg^{-1} FM;
PA is partial alkalinity or bicarbonate alkalinity, mg $CaCO_3$ kg^{-1} FM;
IA is intermediate alkalinity or volatile fatty acid alkalinity, mg $CaCO_3$ kg^{-1} FM;
$V_{4.3}$ is the volume of acid (H_2SO_4) required to reach the pH value of 4.3, mL;
$V_{5.7}$ is the volume of acid (H_2SO_4) required to reach the pH value of 5.75, mL;
0.25 is normality of H_2SO_4 used to titrate the sample;
a is correction factor for normality of H_2SO_4 solution;
50,000 is the conversion factor of 50,000 mg $CaCO_3$ to 1 equivalent alkalinity;
m is the weight of the sample, g.

3.5.5 **Total Ammonia Nitrogen (TAN)**

TAN was measured in accordance with the Standard Method 4500-NH_3 B and C (APHA, 2005) immediately after the digestate samples were collected to avoid any ammonia losses. Basically, 3 - 5 g of a sample, quantified by mass for an accuracy of 0.1 g, was added into a glass distillation tube with 40 mL DI water. Both blank (40 mL DI water) and standard samples (10 mL of 1,000 mg L^{-1} NH_4Cl plus 40 mL DI water) were used for calibration. Several drops of 10 M sodium hydroxide were added to the sample to raise the pH above 9.5 to make sure transformation of ammonium ion to free ammonia. The distillation was performed with a Foss Tecator Kjeltec system 1002 distillation (Foss Tecator AB, Hoganas, Sweden). Erlenmeyer flask previously filled with 25 mL indicating boric acid solution, containing 20 g L^{-1} of H_3BO_3 and 10 ml L^{-1} of mixed methyl red and methylene blue indicator, was used to collect the distillate. The collecting solution was then titrated manually with H_2SO_4 (0.25 N) using a digital automatic titration system (Schott Titroline, Gerhardt UK Ltd) until a lavender colour was achieved, and the volume of H_2SO_4 consumed was recorded. The calculation of TAN is as shown in Equation 3-7:

$$TAN\ (g\ N\ kg^{-1}FM) = \frac{(V_{sample}-V_{blank})\times 14.0\times N\times a}{m} \quad \text{Equation 3-7}$$

Where:

TAN is concentration of total ammoniacal nitrogen, g N kg^{-1} FM;
V_{sample} is volume of 0.25 N H_2SO_4 used to titrate the sample, in mL;
V_{blank} is volume of 0.25 N H_2SO_4 used to titrate the blank, in mL;
N = normality of standard H_2SO_4 solution, in N;
a is standardisation factor of H_2SO_4 solution;
m is mass of the sample, g

To obtain the free ammonia nitrogen (FAN) concentration, the equation from Hansen, Angelidaki and Ahring (1998) was used to calculate the FAN as shown in Equation 3-8.

$$\frac{\text{FAN}}{\text{TAN}} = (1 + \frac{10^{-\text{pH}}}{10^{-(0.09018+\frac{2729.92}{\text{T}})}} \quad \text{Equation 3-8}$$

Where:

FAN is concentration of free ammonia nitrogen, mg N kg^{-1} FM ;
TAN is concentration of total ammoniacal nitrogen, mg N kg^{-1} FM;
pH is pH of the samples;
T is absolute temperature of the samples, K.

3.5.6 **Volatile Fatty Acids (VFA) by gas chromatography**

The method is based on SCA (1979): Determination of Volatile Fatty Acids in Sewage sludge. Basically, digestate samples were prepared for VFA analysis by centrifugation (Eppendorf 5417 C/R, Eppendorf, Hamburg Germany) at 17,900 g (13,000 rpm) for 30 minutes and 0.9 mL of the supernatant was transferred by pipette (Finnpipette, Thermo Fisher Scientific, UK) to vials with 0.1 mL formic acid to give a concentration of 10% formic acid to acidify samples (Zhang, Banks and Heaven, 2012a; Jiang *et al.*, 2017). Where dilution was necessary, milli-Q water was used and formic acid was added to a concentration of 10% of the total volume for analysis. The purpose of sample acidification for VFA determination is to achieve a good chromatographic separation of peaks: after acidification, peaks are symmetrical without tailing on the chromatograms, and thus are easier to quantify accurately (van den Bogaard, Hazen and Van Boven (1986). A concentration of at least 5% formic acid also helps to eliminate the disturbing ghosting effect to an insignificant level (Cottyn and Boucque, 1968). If the samples after acidification were still turbid they were centrifuged again at 13,000 rpm for 20 minutes to obtain a clearer liquid.

VFA concentrations were quantified in a Shimazdu-2010 gas chromatography (GC) (Shimadzu, Milton Keynes, UK) using a flame ionisation detector. This instrument was equipped with a capillary column type SGE BP 21 with helium as the carrier gas at a flow of 190.8 mL min^{-1}, with

a split ratio of 100 giving a flow rate of 1.86 mL min^{-1} in the column and a 3.0 mL min^{-1} purge. The GC oven temperature was programmed to increase from 60 to 210°C in 15 minutes, with a final hold time of 3 minutes. The temperatures of injector and detector were 200 and 250°C, respectively. Three standard solutions containing 50, 250 and 500 mg L^{-1} of acetic, propionic, iso-butyric, n-butyric, isovaleric, valeric, hexanoic and heptanoic acids were used for VFA calibration.

Besides concentrations of individual VFA species, total VFA concentration was also used in the study to express the extent of total VFA produced during anaerobic digestion. The total VFA concentration was calculated as a summation of the eight individual VFAs (acetic, propionic, iso-butyric, n-butyric, isovaleric, valeric, hexanoic and heptanoic) quantified by gas chromatograph, as shown in Equation 3-9. This is in accordance with a number of previous studies and reviews on anaerobic digestion (Oh, Zhang and Jahng, 2008; Zhang, Lee and Jahng, 2011; Banks *et al.*, 2012; Jiang, Heaven and Banks, 2012; Qiang, Lang and Li, 2012; Zhang and Jahng, 2012; Zhang, Banks and Heaven, 2012b;a; Qiang *et al.*, 2013; Wei *et al.*, 2014; Zhang *et al.*, 2015; He, 2016; Jiang *et al.*, 2017).

$$C\ (T)\ (mg\ L^{-1}) = C(acetic) + C(\text{propionic}) + \text{C}(\text{iso} - \text{butyric}) + C(\text{n} - \text{butyric}) + C(\text{isovaleric}) + C(\text{valeric}) + C(\text{hexanoic}) + \text{C}(\text{heptanoic}) \quad \text{Equation 3-9}$$

Where:

C(T) is the total VFAs concentration, mg L^{-1};
C(acetic) is acetic acid concentration, mg L^{-1};
C(propionic) is propionic acid concentration, mg L^{-1};
C(iso-butyric) is iso-butyric acid concentration, mg L^{-1};
C(n-butyric) is n-butyric acid concentration, mg L^{-1};
C(isovaleric) is isovaleric acid concentration, mg L^{-1};
C(valeric) is valeric acid concentration, mg L^{-1};
C(hexanoic) is hexanoic acid concentration, mg L^{-1};
C(heptanoic) is heptanoic acid concentration, mg L^{-1}.

The total VFAs concentration can also be expressed in other ways, for instance in acetic acid equivalent (Murali, Fernandez and Ahring (2017). This acetic acid equivalent approach was not employed in the study because this research did not aim to calculate the chemical oxygen demand balance.

3.5.7 **Total Kjeldahl Nitrogen (TKN)**

All components of model substrate (whole milk powder, whole egg powder and rice flour) were analysed for TKN individually in solid form while inoculum and digestate samples were analysed in liquid form. TKN analysis was carried out in duplicate in parallel with blanks and standard as follows: a mass of 1 - 3 g was weighed to an accuracy of 1 mg into a glass digestion tube, and two Kjeltab catalyst tablets (FOSS Analytical) added to facilitate acid digestion by lowering the activation energy of the reaction. 15 ml of low nitrogen concentrated sulphuric acid was added carefully to each digestion tube and the tubes were gently agitated to ensure contact of the entire sample with the acid. One tube containing the Kjeltab tablets and acid with no sample was used as a blank. Another tube containing 0.3 - 0.5 g of glutamic acid, Kjeltab tablets and acid was treated as a positive control. All tubes were then subjected to acid digestion on a heating block with exhaust system (Foss Tecator 1007 Digestion System 6, Foss Analytical, Hoganas Sweden) at 420°C ± 5°C until the content of each tube converted to a clear blue-green solution, the duration of which was at least two hours. The tubes were cooled to around 50°C, and then 40 mL of deionised water was gently poured to each tube to prevent later crystallisation on further cooling. The content of each tube was steam distilled using a Foss Tecator Kjeltec System 1002 distillation unit (Foss Tecator AB, Hoganas Sweden), after the addition of 10 M sodium hydroxideto raise the pH above 9.5. The following process and calculation formula for the concentration of nitrogen is the same as stated in 3.5.5.

3.5.8 **Gas Composition by gas chromatography**

Biogas composition (CH_4 and CO_2) was measured periodically using a Varian Star CP-3400 CX gas chromatograph (Varian, Oxford, UK) with a gas sampling loop and thermal conductivity detector. The GC was fitted with a Hayesep C column and a molecular sieve 13 × (80-100 mesh) operating at a temperature of 50^{o}C. Argon was used as the carrier gas at a flow of 50 mL min^{-1} and the run time was 1.5 min per sample The GC was calibrated using a standard gas containing 35% CO_2 and 65% CH_4 (v/v) (BOC, UK). During analysis, 5 mL sample was directly taken from the Tedler bag for semi-continuous experiments and was injected into the gas sampling loop of this instrument.

3.5.9 **Gas volume**

Volume of biogas in Tedlar bags was quantified by a water displacement gasometer (Walker *et al.*, 2009). In this device the biogas flowed from Tedlar bag to a water column under vacuum and the water displaced was introduced into a tank on a balance which allowed weight determination of discharged water. The procedure included the recording of initial height of the water column

before the gas collected with Tedlar bag was introduced into the column from its headspace, and the weight of water displaced after the Tedlar bag was empty. The ambient temperature (T) and pressure (P) were recorded at the same time. The volume of biogas was calculated using equation below, and reported as the volume under standard temperature and pressure (STP) of 0 °C, 101.325 kPa. Weight Gasometer Governing Equation (Walker *et al.*, 2009) was shown in Equation 3-9.

$$V_{stp} = \frac{T_{stp}A}{T_{atm}P_{stp}}\left[\left((P_{atm} - P_{H_2O(T_{atm})}\right) - \rho_{H_2O}g(H - h_1 - \frac{m_{H_2O}}{A\rho_{H_2O}}))(h_1 + \frac{m_{H_2O}}{A\rho_{H_2O}}) - (P_{atm} - P_{H_2O(T_{atm})} - \rho_{H_2O}g(H - h_1))h_1)\right] \quad \text{Equation 3-10}$$

Where:

V_{stp} is biogas volume at standard temperature and pressure, m^3;
P_{stp} is standard pressure, 101325 Pa;
P_{atm} is ambient pressure, Pa;
T_{stp} is standard temperature, 273.15 K;
T_{atm} is ambient temperature, K;
$P_{H2O(Tatm)}$ is saturated water vapour pressure at temperature T_{atm}, Pa;
H is total height of gasometer, m;
h_1 is distance from the top of gasometer to liquid surface in gasometer, m;
A is cross-sectional area of water column in gasometer, m^2;
m_{H_2O} is mass of water displaced, kg;
ρ_{H_2O} is density of water, kg m^{-3};
g is gravitational acceleration, m s^{-2}.

3.5.10 **Trace elements extraction and analysis**

3.5.10.1 **Pretreatment method for the determination of total TE concentration**

Hydrochloric-nitric acid digestion was used to extract trace elements from substrate and digested samples, in accordance with EPA method 3010A (acid digestion of sediments, sludges, and soils; Analysts and Great Britain 1987). This sample pre-treatment was carried out using a heating block (Gerhardt Kjeldatherm) in parallel with blanks and standard. For each digestion tube, a known quantity (~30 g) of fresh sample/ DI water / standard TE solution was introduced into it first, and then 15 mL of 35 ~ 36 % w/v HCl and 5 mL 70 % w/v HNO_3 were added into the tube sequentially and mixed gently. Tubes were placed into heating block, connected to the condenser system, and then digested at room temperature for 48 hours prior to heating. The key step of acid digestion

involved gradually increasing the temperature first to 100 °C and then to the final temperature of ~ 200 °C for about 2 hours. After cooling, each acid digestate was filtered using Whatman No.1 filter paper into a 50 mL volumetric flask. The digestion tube and remaining residues on filter paper was rinsed with warm 12.5 % HNO_3 which was also filtered into the same 50 mL flask. The volume was made up to 50 mL with 12.5 % HNO_3 when the content cooled down. The filtrate was then transferred into a polyethylene terephthalate (PET) bottle and stored at 4 ºC until TE determination.

3.5.10.2 **Sequential extraction (SE) method**

The applied method delivers four different fractions which correspond to different levels of bioavailability in attempt to evaluate the various forms in which elements might exist. The four fractions consist of metals in liquid (water-soluble), organically bound metals, metals precipitated with sulphide and intracellular metals. This method was carried out following the procedure which was developed from Aquino and Stuckey (2007) who modified the original fractionation scheme from Stover, Sommers and Silviera (1976) and Lake, Kirk and Lester (1984), as outlined in Figure 3-4. The scheme used in this study was an adapted TE extraction from centrifuged wet samples. This version is the more appropriate approach to represent the bioavailable fraction (Ortner *et al.*, 2015) and better reflect the native TE fractionation of the sample (van Hullebusch *et al.*, 2016).

The four operationally fractionations of TE were separated using the procedure below. It should be noted that the SE method used in this study was applied for metals (Co, Ni and Fe) fractionations regarding the selective extraction reagents used in each step. Se which mainly presented as a highly soluble anion form (selenite Se (IV), SeO_3^{2-}), therefore, the interpretation regarding Se in other fractions apart from in liquid fraction can be biased by unselective extraction of the targeted species.

Water-soluble fraction: The samples freshly taken from anaerobic digesters were centrifuged at 18,533 g (11000 rpm) at 4°C for 30 min. The supernatant was then collected for the determination of water-soluble fraction of TE, and the pellet left was used for the following extraction steps. This fraction contains the water-soluble species made up of free ions and ions complexed with soluble organic matter or other substances. It constitutes the relative mobile and potentially bioavailable metal and metalloid species (Filgueiras, Lavilla and Bendicho, 2002). Several studies showed that metals availability for microorganisms was closely related to their presence in the liquid phase (Jansen, Gonzalez-Gil and van Leeuwen, 2007; Fermoso *et al.*, 2009).

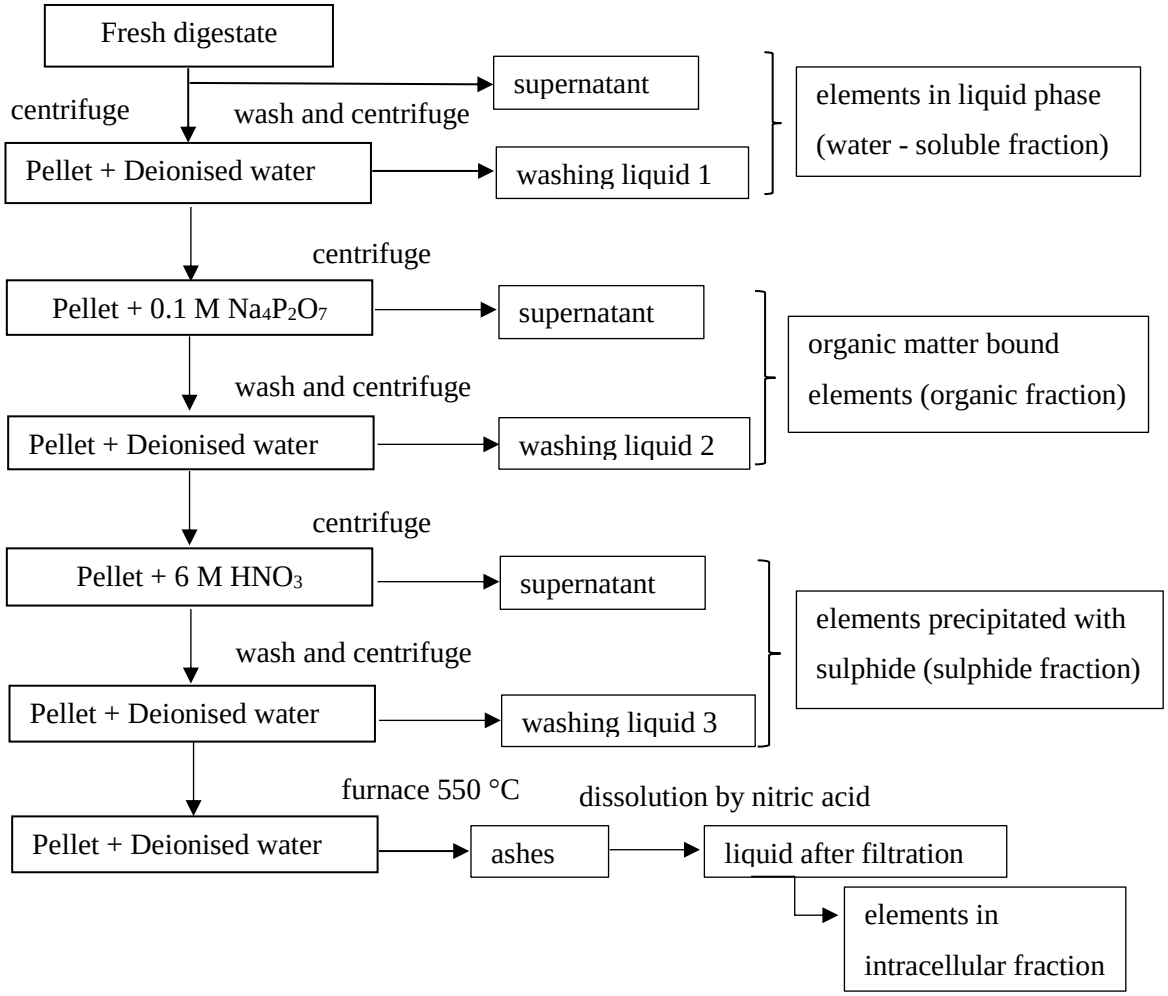


Figure 3-4 Sequential chemical procedure used to fractionate the elements in digestate samples during their washed-out phase

Organically bound fraction: The reagent employed for leaching elements bound with organic matter in this study was sodium pyrophosphate buffer at pH 9.8 (0.1 M $Na_4P_2O_7$), which is selective for the easily soluble organic fraction (i.e. elements associated with humic and fulvic acids). It is a well-known chelating reagent to extract organic substances precipitated by metallic cations by solubilizing organic matter through complexation and dispersion. Metals present in organic matter bound fraction would be adsorbed with negative charged groups on microbial biomass or particle surfaces such as phenolic and carboxylic groups which is the majority functional groups. This fraction is an important fraction especially in sediment and sewage sludge which can even dominate trace metals distribution (Filgueiras, Lavilla and Bendicho, 2002). Oleszkiewicz and Sharma (1990) demonstrated that metals bound with organics produced by biomass could be taken up and used by microorganisms.

Sulphide fraction: Based on pH stability properties 6 M HNO_3 was used to dissolve all elements precipitated with sulphide fraction with enhance selectivity. The first three fractions have been defined as elements presented in extracellular fraction.

Intercellular fraction: after the above extraction steps, the remaining TE represented the elements in intracellular fraction. This fraction was extracted by ashing the sample residue from the first three fractions at 550°C for 2 hours in order to destroy cells and hence free elements. Carbon, hydrogen and oxygen (organic fraction) of the cells were converted to CO_2 and H_2O, and other elements (e.g. P, Na, K, and TE) were left as ash. After that, the ash was mixed with concentrated nitric acid to release the interested elements.

For each analysis fresh digestate around 100 g FM was used. All extraction steps were done by using incubator shaker at a speed of 200 rpm at 20°C for 16 h (Hullebusch *et al.*, 2005; Hu *et al.*, 2008b) followed by centrifugation step in order to collect the liquid samples sequentially extracted. All centrifugation steps were done with a Sorvall Legend XTR device at the rotation speed of 18,533 g (11000 rpm) at 4°C for 30 mins using polypropylene centrifuge tubes. The centrifugation rate was to ensure complete separation of the solid and liquid phases which reported at 10,000 rpm (Gustavsson *et al.*, 2013b). After each extraction, the sample residue pellet was washed twice with milliQ water: re-suspended the pellet in a certain amount to milliQ water, incubated the mixture in an orbital shaker at a speed of 200 rpm at 20°C for 30 min before centrifugation to separate supernatant and pellet. The element contents in the liquid from the washing procedure after each SE step was included in those individual fractions for all elements. All liquid solutions from the extraction procedure were stored at 4°C until TE determination.

3.5.10.3 **Co, Ni, Fe and Se analysis**

Digested samples as described in section 3.5.10.1 were prepared for determination of total measured TE concentrations. All liquid solution samples from SE method were acidified with high purity nitric acid (below pH 2). These can be stored for a certain time without additional treatment. Quantification of Co, Ni, Fe and Se concentrations were analysed by AAS (PerkinElmer Aanalyst200 Atomic Absorption). For digestate samples, some samples have been sent out for elemental analysis using High Resolution ICP-MS (Thermo Element 2XR at Ocean and Earth Science, National Oceanography Centre Southampton, UK) to validate results from in-house AAS. Substrate samples and original inoculums (section 3.3.1) were analysed using ICP-MS at a UKAS accredited commercial laboratory (ALS Environmental Ltd, Coventry, UK).

All glassware and containers used in this experiment were acid washed in advance, and the reagents used were trace grade. Glassware and plastic staff were acid washed in 20% HNO_3 for at least 48 hours before rising with milliQ water at least twice. The obtained results of the analytical determinations was provided in mg kg^{-1} FM. Spiked samples were prepared to identify possible matrix interferences and machine detection limit in different matrixes were analysed for quality

control of the elements analysis. The machine detection limits of the elements were shown in Table 3-10.

Table 3-10 The detection limit of ICP-MS and AAS

Elements	ICP-MS		AAS	
	Reporting Limit (mg L^{-1})	Detection Limit (mg element kg^{-1} VS)	Reporting Limit (mg L^{-1})	Detection Limit (mg element kg^{-1} VS)
Co	0.0006	0.04 ± 0.00	0.019	0.06 ± 0.00
Ni	0.002	0.12 ± 0.00	0.105	0.32 ± 0.00
Fe	0.19	11.27 ± 0.27	0.078	0.24 ± 0.00
Se	0.0016	0.09 ± 0.00	-	-
Mo	0.002	0.12 ± 0.00	-	-
Al	0.032	1.90 ± 0.05	-	-
B	0.12	7.12 ± 0.17	-	-
Cu	0.001	0.06 ± 0.00	-	-
Mn	0.004	0.24 ± 0.01	-	-
Zn	0.003	0.18 ± 0.00	-	-

3.5.10.4 **Data analysis and recovery rate analysis**

It was important to develop an accurate fractionation scheme capable of recovering elements from each fractionate form. To evaluate the extraction efficiency, the percentage of recovery rate for the measured total element concentrations in fresh digestate using hydrochloric-nitric acid digestion related to the total amount of elements extracted from single stage during sequential extraction procedure was determined. A check on the results of applied sequential extraction procedure was performed by comparing the sum of the four fractions: C(liquid) + C(organic matter) + C(sulphide) + C(intracellular) with the total concentrations of elements from four digestion procedure as shown in Equation 3-11.

$$\text{Recovery rate } (\%) = \frac{\text{C(liquid)+C(organic matter)+C(sulphide)+C(intracellular)}}{\text{C(T)}} \quad \text{Equation 3-11}$$

Where:

C(T) is the measured total concentration of metals determined without fractionation, mg kg^{-1} FM; C(liquid), C(organic matter), C(sulphide) and C(intracellular) are the concentrations of elements extracted in each fraction, mg kg^{-1} FM.

The recovery rates of Co, Ni, Fe (and Se) were within ± 20% for each element indicating the element determination method used was accurate underlining the validity of the measured values (Gustavsson *et al.*, 2013b). The obtained data on TE fractionation in different chemical extraction approaches might be used to provide information regarding the potential bioavailability and mobility of those TE.

3.6 Data Analysis using mass balance approach

3.6.1 Percentage of VS destruction

VSD, the ratio of VS removed to VS added can be calculated by Equation 3-10, indicates the efficiency of volatile solids degradation in anaerobic digestion. It was calculated on a weekly basis by mass balance in this study. The VS removed every day equalled to the wet weight of digestate removed per day multiplying VS of digestate, and the VS added every day was calculated by multiplying wet weight of model substrate added per day with VS of model substrate. The wet weight of digestate removed was equal to that of model substrate added per day minus the weight of biogas produced per day. The weight of daily biogas produced was estimated from the weekly average volume and gas composition in terms of total weight of CH_4 and CO_2 produced. Water vapour and other gases were ignored in this calculation, and therefore the results obtained might be slightly lower than the real values.

$$\mathrm{VSD} = \frac{(\mathrm{VS_{sub}} \times \mathrm{W_{sub}}) - (\mathrm{VS_{digestate}} \times \mathrm{W_{digestate}})}{(\mathrm{VS_{sub}} \times \mathrm{W_{sub}})} \times 100 \qquad \text{Equation 3-12}$$

The digestate removal rate ($W_{digestate}$, kg d^{-1}) can be calculated using Equation 3-11.

$$\mathrm{W_{digestate}} = \mathrm{W_{sub}} - \frac{(16 \times \mathrm{C_{methane}} \times \mathrm{V_{biogas}}) + 44 \times (100\% - \mathrm{C_{methane}}) \times \mathrm{V_{biogas}})}{22.4} \qquad \text{Equation 3-13}$$

Where:

VS_{sub} is the VS content of substrate, %;
$VS_{digestate}$ is the VS content of digestate, %;
W_{sub} is the weight of substrate including water added into digester every day, kg;
$W_{digestate}$ is the weight of digestate removed from digester every day, kg;
$C_{methane}$ is the methane composition in biogas, %;
V_{biogas} is the volume of daily biogas production, STP m^3.

3.6.2 **Trace elements concentration during washing out**

Concentration of trace element during their washing out was modelled based on the following approximations: 1) the flow pattern of the digester followed CSTR model; 2) the density of digestate was 1 kg L^{-1}; and 3) the hydraulic retention time equalled to the working volume of digester divided by the volume of model substrate including water added each day, which was calculated from the weight input. According to mass balance, the amount of trace elements in digester on day n+1 was equal to the amount of trace element in digester in previous day (day n) minus that discharged with digestate on the day, then plus that introduced from daily substrate waste input on day n+1.

$$C_{n+1} = \frac{C_n \times (TW_{digestate} - W_{sub}) + (C_{sub} \times W_{sub})}{TW_{digestate}} \quad \text{Equation 3-14}$$

Where:

C_n is TE concentration on day n, mg kg^{-1};
n is the operational day which started from day 0;
C_{sub} is TE concentration of substrate, mg kg^{-1};
W_{sub} is the weight of substrate including water added into digester every, kg;
$TW_{digestate}$ is the total weight of digestate in digester, kg.

Assuming the density of digestate was 1 kg L^{-1}, its default value was 4 kg for 4 L liquid working volume digesters, kg

3.7 Development of preparation techniques for VFA determination

Keeping digestate immediately after samples have been removed from digesters in freezing condition is the proper practice to preserve samples for VFA determination. This is because frozen method could assist solid/liquid to be easily separated by centrifugation.

Therefore, the experiments were designed to examine a screening procedure for assessing and describes effect of samples status (frozen or defrosted, cold or ambient temperature) when samples are put into centrifuge and to determine effect of the order of samples preparation steps (centrifugation first, dilution second vs. dilution first, centrifugation second) on VFA recovery rate. These VFA concentration tests used VFA spikes by mixing of substrate spike included acid form of acetic acid; CH_3COOH and propionic acid; CH_3CH_2COOH and salts of both acid (sodium acetate; $C_2H_3NaO_2$ and sodium propionate; $C_3H_5NaO_2$). The tests used digestate from a stable performance 100-L CSTR (detail of digester operation can be seen in section 4.3 Chapter 4).

From Figure 3-5, there was no significant different in VFA recovery rate from sample preparation steps (centrifugation first, dilution second vs. dilution first, centrifugation second) and a spike of VFA addition (acid or salt of acid).

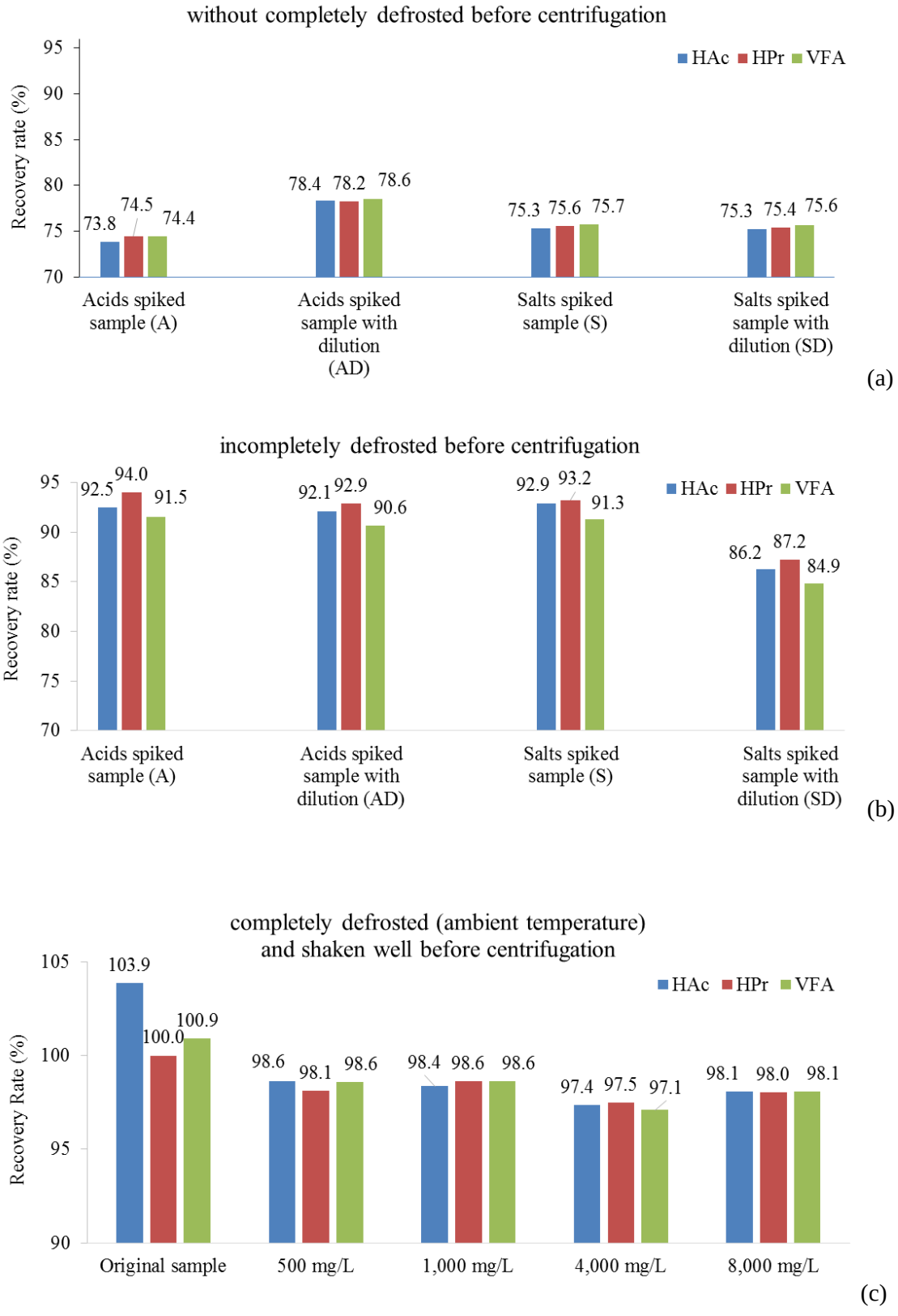


Figure 3-5 VFA recovery rate in different samples status under the different preparation steps

VFA recovery rate was determined 100% for original frozen sample (without VFA spiked) (Figure 3-5 (c). For spiked samples, VFA recovery rate was only 74 - 79 % for samples without completely defrosted (frozen sample) (Figure 3-5 (a), 85 - 93 % by incompletely defrosting (cold sample) (Figure 3-5 (b) and 97 - 98 % for overnight defrosting and homogenised samples have been put for centrifugation (Figure 3-5 (c). By using the latter procedure, more than 98% recovery rate could be achieved (by salts spiked) for VFA in range of 500 - 8,000 mg L^{-1} (Figure 3-5 (c))

Preserving digestate by freezing is an efficient procedure for VFA determination. According to the demonstrated results, it is suggested that, in order to achieve high VFA recovery rate, especially for defrosting process, it would be better to allow samples completely defrosted reaching ambient temperature when samples are put into centrifuge. However, some of the effects might be sample specific and may not cause too much trouble to others analysis, but it is always good to be aware of the issues.

Chapter 4: **Digester substrate and anaerobic digestion of model substrate in 100-L CSTR digester without TE supplementation**

4.1 Introduction

The aim of this part is to describe the characteristics of the model substrate as a digester feed. Due to the fluctuation of TE concentrations in real - world organic waste, model substrate was employed in this study to allow better quantification of the required TE dosing ranges under specific operational conditions. Key substrate characteristics for anaerobic digestion, TS, VS, TKN and several essential TE were determined as baseline TE concentrations.

In order to generate an acclimated inoculum for model substrate with low TE concentrations to conduct 5-L scale CSTR laboratory experiments (Chapter 8, R1 - R4) and also to confirm that VFA accumulation was a problem in anaerobic digestion without TE supplementation at OLR of 1.0 - 3.0 kg VS m^{-3} d^{-1}, a long-term CSTR digestion experiment was carried out.

4.2 Substrate characterisation

The analytical testing of model substrate characteristics was carried out for individual characteristics before they were used as digester substrate, including TS, VS, macronutrients (Ca, Mg, K, Na, P, TKN) and micronutrients (Co, Cu, Fe, Mn, Mo, Ni, Se, Zn). Among them, the nutrient analysis was carried out using ICP-MS (at a UKAS accredited commercial laboratory: ALS Environmental Ltd, Coventry, UK) after in - house digestion. The values presented in Table 4-1 were average and range of the above duplicate measurements. The low nutrient contents reflected the high VS to TS ratio (97.24%) of the model substrate.

Table 4-1 The solids and nutrient analysis of model substrate and its components

	whole milk powder	whole egg powder	rice flour	model substrate
TS (% of FM)	96.95 ± 0.04	96.77 ± 0.04	88.45 ± 0.03	10.31 ± 0.07
VS (% of FM)	91.44 ± 0.00	90.38 ± 0.12	87.37 ± 0.11	10.02 ± 0.07
VS (% TS)	94.32 ± 0.04	93.40 ± 0.16	98.78 ± 0.15	97.24 ± 0.07
Micronutrients (mg element kg^{-1} VS basis)				
Cobalt (Co)	1.1 ± 0.0	1.1 ± 0.0	0.6 ± 0.0	0.09 ± 0.00
Copper (Cu)	1.4 ± 0.0	17.1 ± 0.0	23.8 ± 0.0	2.04 ± 0.03
Iron (Fe)	122.3 ± 0.0	789.4 ± 0.1	125.3 ± 0.1	28.8 ± 2.72
Manganese (Mn)	43.3 ± 0.0	39.8 ± 0.0	243.8 ± 0.0	18.6 ± 1.04
Molybdenum (Mo)	2.7 ± 0.0	7.4 ± 0.0	5.5 ± 0.0	0.60 ± 0.02
Nickel (Ni)	0.3 ± 0.0	4.7 ± 0.0	2.2 ± 0.0	0.26 ± 0.10
Selenium (Se)	3.1 ± 0.0	8.4 ± 0.0	2.6 ± 0.0	0.43 ± 0.03
Zinc (Zn)	249.3 ± 0.0	397.3 ± 0.0	130.4 ± 0.0	23.2 ± 0.39
Chromium (Cr)	4.6 ± 0.0	13.6 ± 0.0	2.6 ± 0.0	0.58 ± 0.17
Macronutrients (g element kg^{-1} VS basis)				
Calcium (Ca)	82.4 ± 1.2	21.6 ± 1.3	0.8 ± 1.2	2.33 ± 0.00
Magnesium (Mg)	7.2 ± 0.1	4.5 ± 0.3	6.7 ± 0.1	0.72 ± 0.00
Potassium (K)	77.1 ± 1.0	36.6 ± 2.3	10.3 ± 0.6	3.21 ± 0.03
Sodium (Na)	22.3 ± 0.2	49.1 ± 2.8	0.1 ± 0.0	1.58 ± 0.06
Phosphorous (P)	25.5 ± 25.1	34.2 ± 36.6	18.3 ± 17.5	2.57 ± 1.36
Total Kjeldahl Nitrogen (TKN)	9.8 ± 1.8	19.4 ± 2.5	13.3 ± 1.7	42.5 ± 0.20

Note: The machine detection limits of each element were shown in Table 3-10.

The nutrient concentrations in digester feed at different OLR are shown in

Table **4-2**. Due to the fixed HRT of 33.3 days, the nutrient concentrations increased proportionally with the increased loading rate. The concentrations of some essential trace element, however, were still lower than what are necessary for efficient AD process. For example, Co, Ni and Fe concentrations at OLR of 3.0 kg VS m^{-3} d^{-1} were 0.01, 0.03 and 2.87 mg kg^{-1} FM, respectively, which were lower than the previous recommended concentrations for similar digestion processes (Uemura, 2010; Pobeheim *et al.*, 2011; Banks *et al.*, 2012; Facchin *et al.*, 2013; Evranos and Demirel, 2014; Zhang *et al.*, 2015; Moestedt *et al.*, 2016). The TKN concentrations in feed was less than 4.25 g kg^{-1} FM which was unlikely to induce inhibitory effect (Rajagopal, Massé and

Singh, 2013) and therefore this would not become an interfering issue when investigating the TE effect on AD.

Table 4-2 Concentration of macro and micro nutrients in digester fed at different OLR

Trace elements	OLR (kg VS m^{-3} d^{-1})							
	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
Micronutrients (mg element kg^{-1} FM)								
Cobalt (Co)	0.003	0.005	0.01	0.01	0.01	0.01	0.01	0.01
Copper (Cu)	0.07	0.10	0.14	0.17	0.20	0.24	0.27	0.31
Iron (Fe)	0.96	1.44	1.92	2.40	2.87	3.35	3.83	4.31
Manganese (Mn)	0.62	0.93	1.24	1.55	1.86	2.17	2.48	2.79
Molybdenum (Mo)	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Nickel (Ni)	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04
Selenium (Se)	0.01	0.02	0.03	0.04	0.04	0.05	0.06	0.06
Zinc (Zn)	0.77	1.16	1.55	1.93	2.32	2.71	3.09	3.48
Chromium (Cr)	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
Macronutrients (mg element kg^{-1} FM)								
Calcium (Ca)	77.8	116.7	155.6	194.5	233.4	272.3	311.2	350.1
Magnesium (Mg)	24.0	36.0	48.0	60.0	72.1	84.1	96.1	108.1
Potassium (K)	106.9	160.3	213.8	267.2	320.7	374.1	427.5	480.9
Sodium (Na)	52.6	78.9	105.2	131.5	157.8	184.0	210.3	236.6
Phosphorous (P)	85.7	128.6	171.4	214.3	257.2	300.0	342.9	385.7
Total Kjeldahl Nitrogen (TKN)	1,417	2,125	2,833	3,541	4,250	4,958	5,666	6,374
Solid properties (% FM)								
Total Solid, TS	3.44	5.15	6.87	8.59	10.31	12.03	13.75	15.46
Volatile Solid, VS	3.33	5.00	6.67	8.33	10.02	11.67	13.33	15.00

4.3 100-L CSTR digester for anaerobic digestion of model substrate without TE supplementation

4.3.1 **Objective**

To carry out semi-continuous digestion of model substrate in 100-L semi-continuously stirred tank reactor (CSTR) to observe whether VFA accumulation was a problem in AD without TE supplementation even running at low organic loading. For the purpose of TE supplementation trials (Chapter 8), an inoculum with low TE concentration was desired in order to exclude the effect of background TE from inoculum.

4.3.2 **Methodology for the pilot-scale CSTR digester of model substrate AD**

The pilot-scale CSTR digester with a 100-L (75-L liquid working volume capacity) was employed in a semi-continuous mode: sewage sludge digestate was used as an original inoculum as described in section 3.3 Chapter 3. The inoculum characteristic are shown in Table 3-6. There was no TEs supplementation aimed to dilute out metals introduced from original inoculum. The VFA profile and loading history in detail was shown in Figure 4-1. Digestate were removed once a week and samples were analysed for pH, alkalinity, TAN, TS/VS and VFA. On day 637 under stable operation, digestate was used as an inoculum for lab-scale CSTR digesters (Chapter 8, R1 - R4). The VS of feed was fixed to 10% for this experiment, therefore HRT changed with changed OLR.

4.3.3 **Results of 100 L anaerobic digester trial**

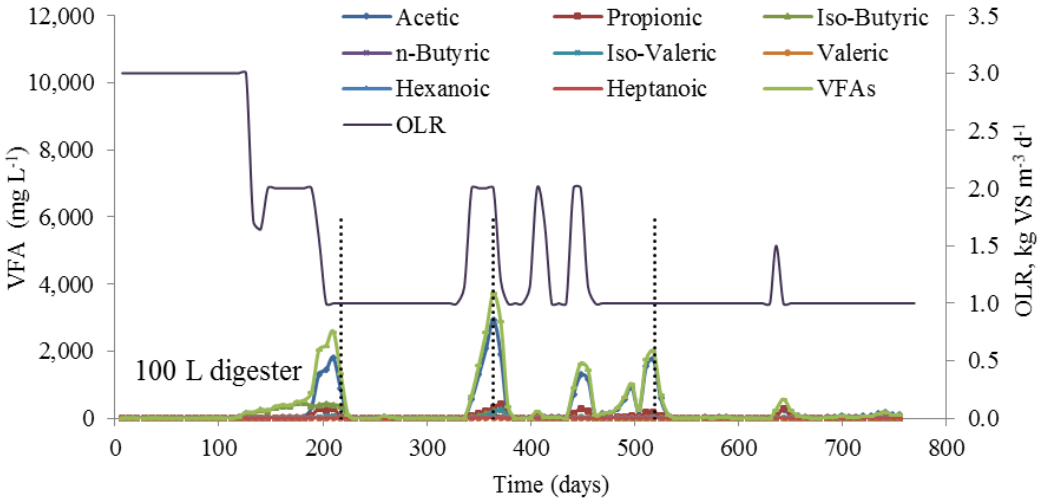


Figure 4-1 OLR history and VFA profiles of 100-L CSTR digester (vertical dashed lines indicated when Co and Ni supplementation were applied to the digester)

As shown in Figure 4-1, OLR was initially set at a moderate level of 3.0 kg VS m^{-3} d^{-1}. The following OLR adjustments were based on VFA concentration in digester. When the feed lacks a trace element, the concentration of that element in the sludge drops and decreases the biological activity. VFA was accumulated after digester was operated at OLR 3.0 kg VS m^{-3} d^{-1} for about 3 HRT (119 days). OLR decrease to 2.0 kg VS m^{-3} d^{-1} was introduced when VFA showed increase. OLR was then further decreased from 2.0 to 1.0 kg VS m^{-3} d^{-1} stepwise from day to day. In an attempt to maintain process stability, an one-off dosing at a strength of Co 0.01 and Ni 0.03 mg kg^{-1} digestate FM, respectively, was also applied to the digester on day 218 when digester was suffering a rapid fall in pH and a decline in daily biogas production (Figure 4-3 (a) and (f)) with VFA accumulated to around 2,500 mg L^{-1}. As shown in Figure 4-2, Co and Ni concentrations in

the digester were only 0.01 and 0.03 mg kg^{-1} FM, respectively, equal to the baseline concentration from the feed. This TE dosing helped VFA consumption and stable operation for 1 HRT at OLR 1.0 kg VS m^{-3} d^{-1}. However, when OLR was raised to 2.0 kg VS m^{-3} d^{-1} on day 336, sharp increase in VFA was observed to a concentration of 4,000 mg L^{-1} within 32 days. Acetic acid was still the main component of this VFA peak with other VFA species presented in small amount. Co and Ni supplementation and OLR decrease were used again to control VFA accumulation for this and following attempts to increase OLR. OLR finally was kept at 1.0 kg VS m^{-3} d^{-1} which could maintain VFA under 100 mg L^{-1}. After digestate was taken to inoculate four 5-L CSTR digesters (Chapter 8, R1-R4) on day 637, therefore, around 33% of liquid working volume was reduced (from 75 L to around 50 - 55 L), while feeding rate had been remained, which means loading rate was increased from 1.0 to 1.5 kg VS m^{-3} d^{-1}. As a result, VFA showed some accumulation to a concentration of above 600 mg L^{-1}, however, after that digester could be recovered to the normal operation by further feeding at the previous load (1.0 kg VS m^{-3} d^{-1}) until the end of experiment. From day 637 onwards, digestate samples have been observed only for VFA concentrations and this digester was terminated on day 755.

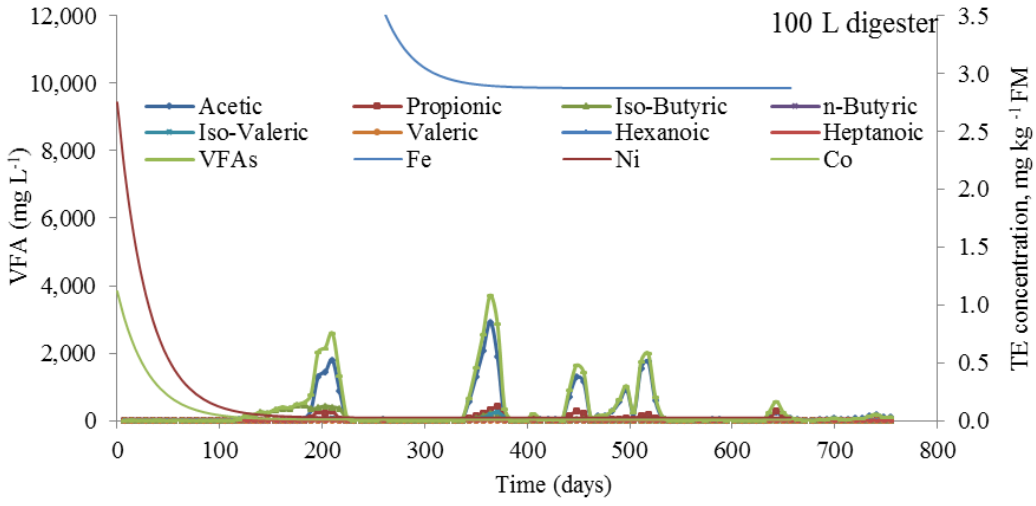


Figure 4-2 VFA and simulated TEs concentration profile in digester N4

As shown in Figure 4-3 (a), pH dropped to around 7.20 - 7.35 to reflect the first VFA accumulation on day 206 and 365 when TAN was around 2,600 and 2,900 mg L^{-1}, respectively. pH, however, maintained stable with value of 7.7 ± 0.1 and insensitive to later VFA accumulation due to the buffering capacity of elevated TAN concentration of around 3,000 mg L^{-1}. IA/PA ratio (Figure 4-3 (c)) increased up to 0.59, 0.89 and 0.47 around day 206, 367 and 507, respectively corresponding to VFA accumulation at that time. However, IA/PA ratio could be decreased and maintain constant around 0.3-0.4 when digester has been operated at OLR 1.0 kg VS m^{-3} d^{-1}. The detailed for alkalinity profiles was shown in Figure 4-3 (b).

As shown in Figure 4-3 (d) TS-VS profile, the average values of solid content were TS 1.16 % and VS 0.85 % on FM basis, with a VS/TS ratio of 73.1 %. VS destruction was around 93.2 %. TAN concentration was 1,300 mg kg^{-1} FM at the beginning of the experiment, and increased gradually caused by TKN concentration supply from model substrate. TAN and FA concentration (Figure 4-3 (e)) kept relatively constant around 3,100 and below 200 mg kg^{-1} FM, respectively at the final stage of this trial. Considering a feed TKN of 4.25 g kg^{-1} FM as shown in Table 4-1, and final TAN 3.1 g kg^{-1} as presented in Table 4-3, and nitrogen content of microbial biomass is 12.4 % (using an empirical microorganism formula of $C_5H_7O_2N$), then the microbial biomass concentration is 9.3 %, very close to final VS concentration. As such all the substrate has been converted either to biogas or to microbial biomass. Average values for performance and monitoring parameters during day 539 to day 643 for digester running at OLR 1.0 kg VS m^{-3} d^{-1} were shown in Table 4-3. The digester showed good performance and has been operated at OLR 1.0 kg VS m^{-3} d^{-1} until the end of trial. The digester performance profiles were shown in Figure 4-3.

Table 4-3 Digester efficiency and monitoring parameter (average from day 539 - 643)

Parameters	Unit	Average ± Standard deviation
OLR	kg VS $m^{-3}d^{-1}$	1.0 ± 0.0
SBP	m^3 biogas kg^{-1} VS_{added} d^{-1}	0.86 ± 0.12
VBP	STP m^3 m^{-3} digestate d^{-1}	0.87 ± 0.11
TAN	mg N kg^{-1} FM	3,100 ± 50
FAN	mg N kg^{-1} FM	171 ± 54
TS	% FM	1.16 ± 0.02
VS	% FM	0.85 ± 0.03
VS	% TS	73.1 ± 1.2
VSD	%	93.2 ± 1.7
pH	-	7.71 ± 0.06
TA	g $CaCO_3$ kg^{-1} FM	11.5 ± 0.53
PA	g $CaCO_3$ kg^{-1} FM	8.6 ± 0.42
IA	g $CaCO_3$ kg^{-1} FM	2.8 ± 0.25
IA/PA		0.30 ± 0.03
Total VFA	mg L^{-1}	19.5 ± 10.9

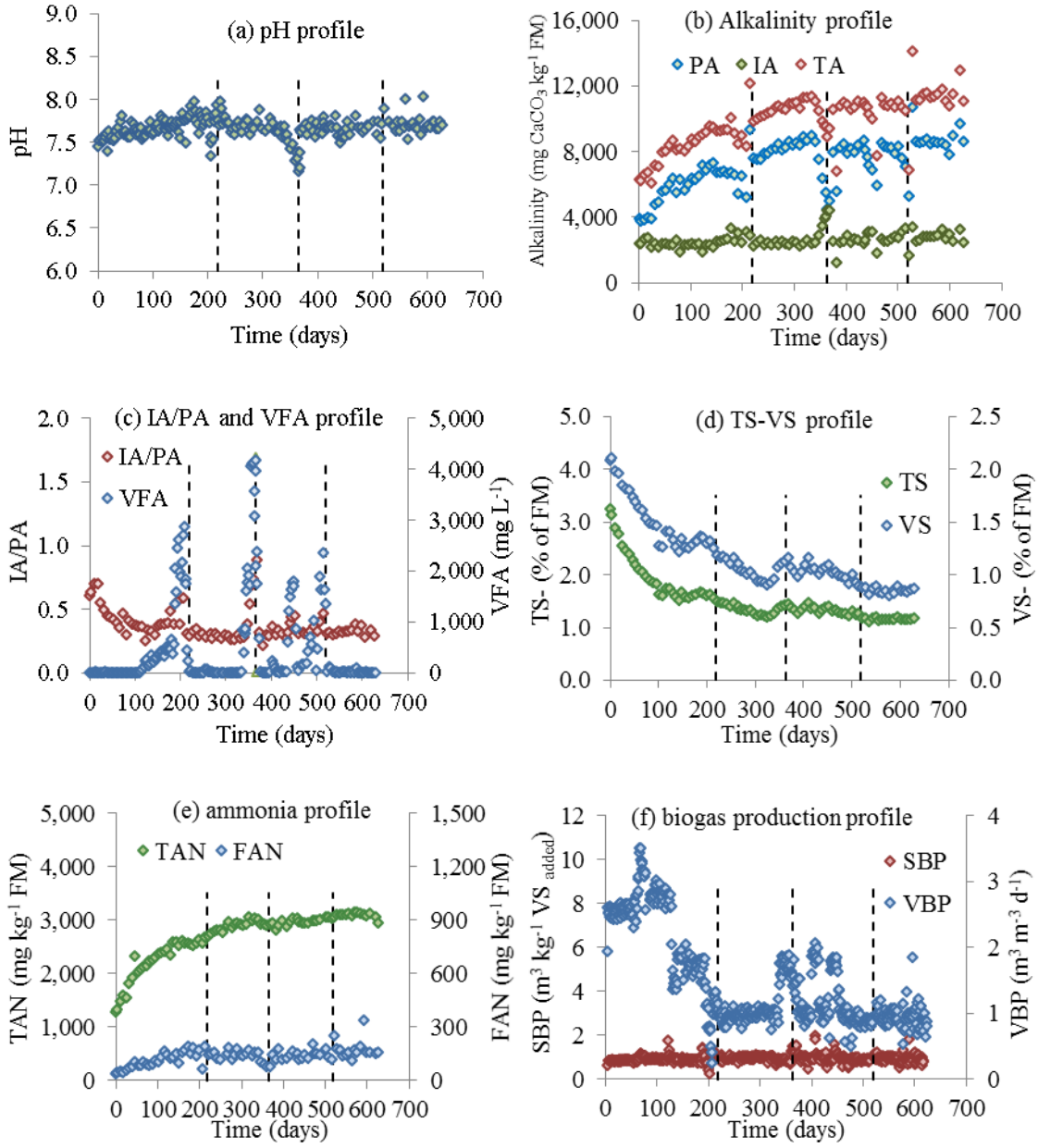


Figure 4-3 100-L CSTR digester performance profiles (a) pH, (b) alkalinity, (c) IA/PA ratio and VFA, (d) TS-VS, (e) Ammonia and (f) biogas production

4.4 Concluding remarks

1. The low nutrient contents of the model substrate indicated the suitability of substrate used for this research. TKN concentration in feed was unlikely to induce inhibitory effect and therefore this would not become an interfering issue when investigating the TE effect on AD. Another reason for the use of the model substrate is that it is readily biodegradable.

2. The results obtained from CSTR experiment indicated that without TE supplementation, digestion could not overcome the instability issue caused by VFA accumulation. Digester could not perform stably over OLR of 1.0 kg VS m^{-3} d^{-1}.

3. This trial successfully provided the acclimated inoculum with low TE content to conduct 5-L scale CSTR laboratory experiments (Chapter 8, R1 - R4).

Chapter 5: **Trace element requirement for long-term mesophilic digestion at OLR 3.0 kg VS m^{-3} d^{-1}**

5.1 Introduction

The experiment was designed to distinguish which trace elements play important role on process stability to anaerobic digestion using model substrate and to investigate the potential TE interaction on digestion performance under designated operational conditions. Eight 5-L CSTR digesters were employed in this study each running in a semi-continuous mode at a moderate OLR of 3.0 kg VS m^{-3} d^{-1} and HRT of 33.3 days for over 600 days. With VS of 10% in the feed, the TAN concentration in well-performed digesters was around 2,500 mg kg^{-1} FM, well below the inhibitory level which is around > 3,000 - 4,000 mg kg^{-1} FM (Rajagopal, Massé and Singh, 2013). The general approach of this study was to supply individual TE or their combination to different digesters in sufficient amount in order to identify the TE supplementation required and to restore stability to the digester from the point where VFA accumulation by addition of the other essential trace elements which can be used in reducing VFA levels. Co, Ni and Fe were selected as the main trace elements to investigate in this study due to the long recognition of their role on stable anaerobic digestion, especially when TAN is not high (Demirel and Scherer, 2011; Nges and Björnsson, 2012; Meyer and Edwards, 2014; Lee *et al.*, 2017). A wide range of digestion parameters were measured during this study, but VFA was monitored intensively as it is the most important and prompt one to indicate the stability of digesters and therefore the effect of trace elements deficiency.

To achieve the aim, the following objectives were set:

1) To operate eight digesters (N1-N8) identically at the beginning of the experiment to ensure they have comparable performance;

2) To apply different TE supplementations strategies (individual and different combinations of TEs) to different digesters and to monitor the digester performance with different strategies;

3) To supply additional trace element(s) to digesters that suffered from VFA accumulation even with a TE dosing action to distinguish the essential TE to supplement and/or TE interaction;

4) To run this experiment for a sufficient duration to ensure that the identification of the whole range of TE that was necessary to add for long-term stable digestion and that the potential antagonistic TE interaction was distinguished under the designated conditions.

5.2 Experimental Method

At the beginning of the experiment, eight 5-L digesters (N1-N8) were seeded with biosolids digestate taken from Millbrook wastewater treatment plant (section 3.3.1 Chapter 3). These digesters were then operated in a semi-continuous mode at a moderate OLR of 3.0 kg VS m^{-3} d^{-1} and a HRT of 33.3 days. Model substrate with a VS level of 10 % was fed to digesters once per day. No TE was added to these digesters from the beginning of the experiment to day 42. From day 43 onwards, the digesters were subject to 8 different TE supplementation strategies, as shown in Table 5-1, and the dosing strength of each element was shown in Table 3-7 - Table 3-9. Among them, digester N1 (no TE addition) and N8 (full set of 11 elements) were acted as negative and positive control, respectively. The supplementation of Co, Ni, and Fe and their interaction were tested in N2 - N7.

Table 5-1 Initial TE supplementation strategies

Digester	TE supplemented
N1	-
N2	Ni
N3	Co
N4	Co and Ni
N5	Co and Fe
N6	Ni and Fe
N7	Co, Ni and Fe
N8	Co, Ni, Fe and Al, B, Cu, Mn, Mo, Se, W, Zn

Apart from N8, the operating arrangements for N1 - N7 were modified later in this experiment to respond to the VFA accumulation, including additional element (s) supplementation, TE dosing strength adjustment, and OLR reduction. The summary of the operational changes over the entire course of the experiment is given in Table 5-2, and the justification of these changes is given in section 5.3, along with the results and discussion.

Table 5-2 Summary of the TE supplementation arrangement throughout the experiment

<table>
<tr><td></td><td colspan="16">Days</td></tr>
<tr><td></td><td>43</td><td>128</td><td>164</td><td>175</td><td>181</td><td>183</td><td>196</td><td>204</td><td>240</td><td>262</td><td>272</td><td>286</td><td>310</td><td>339</td><td>412</td><td>423</td><td>630</td></tr>
<tr><td>N1</td><td>No TE,
OLR 3.0</td><td colspan="3">No TE
OLR 1.5</td><td colspan="2">No TE,
OLR 1.0</td><td colspan="2">Ni (1)
OLR 1 .0</td><td colspan="8">Feeding ceased</td></tr>
<tr><td>N2</td><td colspan="8">Ni (1)</td><td colspan="3">Ni (1) Co (0.02)</td><td colspan="3">Ni (0.5) Co (0.5)</td><td colspan="3">Feeding ceased</td></tr>
<tr><td>N3</td><td colspan="6">Co (1)</td><td colspan="4">Co (1) Ni (1)</td><td>Co (1)
Ni (1)
Fe (10)</td><td colspan="4">Full set of 11 elements</td></tr>
<tr><td>N4</td><td colspan="10">Co (1) Ni (1)</td><td colspan="7">Full set of 11 elements</td></tr>
<tr><td>N5</td><td colspan="4">Co (1) Fe (10)</td><td colspan="6">Co (1) Fe (10) Ni (1)</td><td colspan="3">Co (1) Fe (10) Ni (1)
Se (0.1) Mo (0.1)</td></tr>
<tr><td>N6</td><td colspan="2">Ni (1) Fe (10)</td><td>Ni (1)</td><td colspan="13">Feeding ceased</td></tr>
<tr><td>N7</td><td colspan="15">Co (1) Ni (1) Fe (10)</td><td>Co (1) Ni (1)
Fe (10)
Se (0.1)</td></tr>
<tr><td>N8</td><td colspan="16">Full set of 11 elements</td></tr>
</table>

Note: 1. No TE was added to N1 - N8 from the beginning of the experiment to day 42; 2. The numbers shown in brackets are the TE dosing strength in mg kg^{-1} FM; 3. The OLR of N2 - N8 was maintained at 3.0 kg VS m^{-3} d^{-1} unless feeding completely ceased, as shown in the table; the OLR of N1 is shown in the table directly, in units of kg VS m^{-3} d^{-1}

5.3 **Results and discussion**

According to SMP and VFA data shown in Figure 5-1, all eight digesters performed in the same manner during the first 43 days, which provided an identical start point for the operational changes on day 44. The performance of each digester and their contribution to the aim of this study was then examined separately as follows due to the complex nature of this set of experiment. The TE and VFA concentrations in each digester were the main parameters used in the analysis. A comparison was then carried out between digesters to extract key findings. Under stable condition, the SBP was around 0.77 ± 0.02 m^3 biogas kg^{-1} VS_{added} (SMP 0.45 ± 0.02 m^3 CH_4 kg^{-1} VS_{added}), methane content was around 58.3 ± 1.2 %, Average VFA was below 100 mg L^{-1}; pH was around 7.5 ± 0.1.

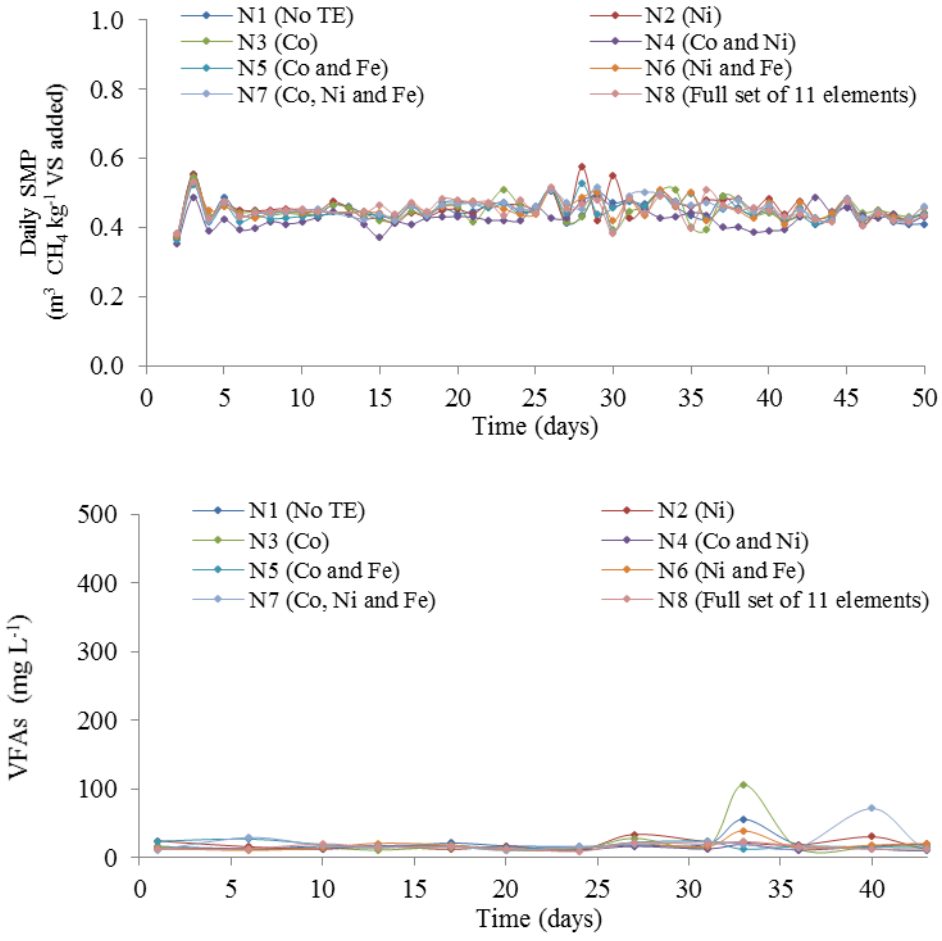


Figure 5-1 (a) SMP and (b) Total VFA profiles in digester N1-N8 for the first 43 days of experiment

5.3.1 Control digesters

5.3.1.1 Without TE supplementation (N1)

There was no significant difference noted in the performance of digester N1 during the first 3 HRTs of operation (day 0 - 100). The average specific methane production (SMP) was 0.45 ± 0.03 m^3 CH_4 kg^{-1} VS_{added} (corresponding to about 0.76 ± 0.05 m^3 biogas kg^{-1} VS_{added}), the VS-reduction was 85 ± 2 % and VFA-concentration was below 100 mg L^{-1}.

Same as the 100-L digester described in section 4.3, VFA started to accumulate after the initial three retention times when the TE introduced by inoculum was simply washed out and their concentration in N1 approached to the equilibrium concentration determined by the TE content of the feed (Figure 5-2). The total VFA increased from below 100 mg L^{-1} on day 112 to above 3,800 mg L^{-1} (2,500 mg HAc L^{-1} and 560 mg HPr L^{-1}) on day 127 indicating a VFA accumulation rate of 253 mg L^{-1} day^{-1} and 8.43 % of substrate VS was wasted. As seen from Figure 5-2, the simulated concentrations of Fe 101, Ni 0.09, Co 0.03 and Se 0.05 mg kg^{-1} FM when VFA started accumulating, which is greater than those found from baseline concentration (Fe 2.89, Ni 0.03, Co 0.01 and Se 0.04 mg kg^{-1} FM), although it is not clear if this VFA increase was caused by single TE deficiency or the combined effect from different TE. OLR was then reduced from 3.0 to 1.5 kg VS m^{-3} d^{-1} from day 128 to avoid the digestion failure. The VFA accumulation trend was stopped for 19 days after this loading decrease, however VFA started to climb up again from day 147 to above 5,100 mg L^{-1} with accompanied pH decrease to 7.21 and methane content to 47.3 % (on day 182). A further loading reduction from 1.5 to 1.0 kg VS m^{-3} d^{-1} was therefore applied from day 183, as shown in Figure 5-3. Without clear effect from this second loading drop, the Ni dosing at a strength of 1.0 mg kg^{-1} FM was given to N1 by an injection at the beginning of this action from day 204 to day 261, as a further attempt to control its VFA level. The reason to supplement Ni to N1 was because N3 had shown single Co dosing was not sufficient for stable digestion but digester with only Ni supplementation (N2) still perform well at that point (section 5.3.2). After temporary VFA fluctuation to respond to the remedial actions, the total VFA concentration rose to over 12,000 mg L^{-1} from day 223 and acetic and propionic acid concentration kept increasing with continuously decreased the biogas yield (Figure 5-12). To maintain pH a phase of intermittent feeding was adopted, which could not stop performance deterioration neither: the pH dropped to around 6.0 and the methane content of biogas was less than 40%. Feeding was then stopped at day 262 but the digester mixing and temperature control were maintained. It took the digester around 200 days after feeding stop to recover from its VFA stress and restore its pH level to above 7.0. From this and the previous results (section 4.3 Chapter 4) it appeared that operation at OLR over 1.0 kg VS m^{-3} d^{-1} would probably not be achievable without TEs supplementation or even with Ni supplementation after VFA had been accumulated.

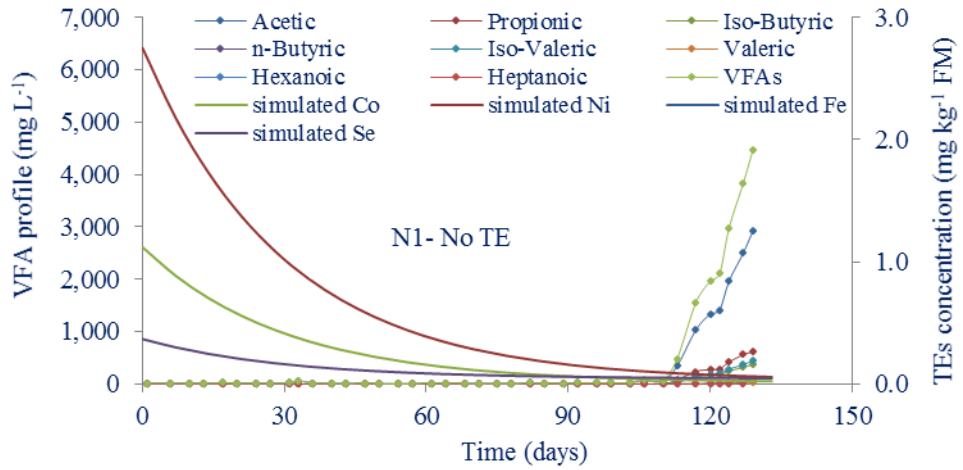


Figure 5-2 Simulated Co, Ni, Fe and Se concentrations and VFA profiles in the control digester (N1) for TE washed-out from inoculum phase

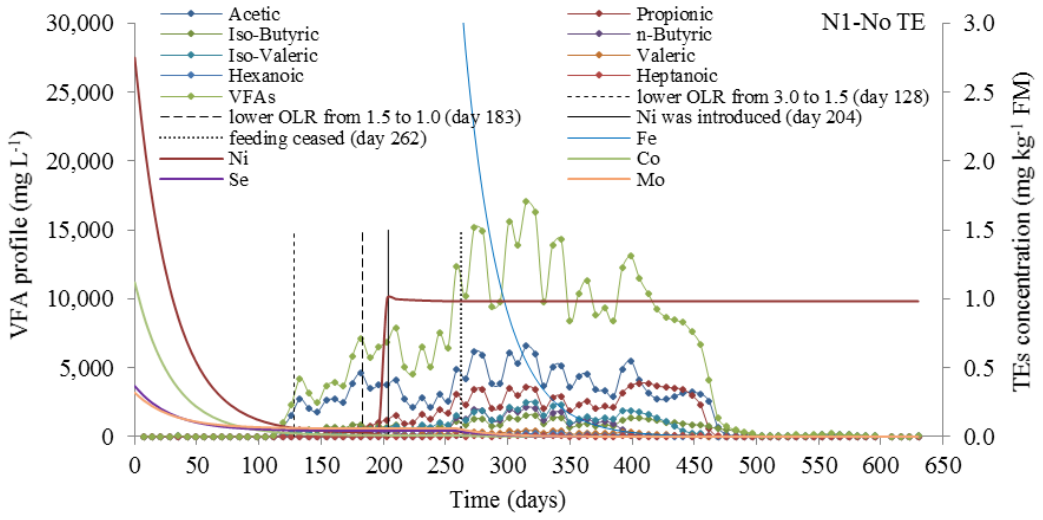


Figure 5-3 VFA and simulated TEs concentrations profiles in digester N1 running at an OLR of 3.0 - 1.0 kg VS m^{-3} d^{-1}

5.3.1.2 **Full set of 11 TE supplementation**

Digester N8 continued to perform normally with no significant difference noted in the performance and digestion stability would be achieved when operated with sufficient TE supplementation at OLR 3.0 kg VS m^{-3} d^{-1} over the 630 days (18 HRTs). These findings were also supported by the behaviour of digester N3 and N4 which did result in process recovery from un-balanced process performance by dosing with full set of 11 elements by the end of trials which will be discussed later. The parameter values for the stable operating period were: specific CH_4-production 0.45 ± 0.03 m^3 CH_4 kg^{-1} VS added (corresponding to about 0.77 ± 0.03 m^3 biogas kg^{-1} VS_{added}); volumetric CH_4-production 1.30 ± 0.05 STP m^3 m^{-3} d^{-1} (corresponding to about 2.30 ± 0.05 STP m^3m^{-3} digestate d^{-1}); VSD 87.7 ± 1.5 %; TAN 2,505 ± 69 mg kg^{-1} FM; the biogas

methane content 58.5 % as shown in Figure 5-12 - Figure 5-14. The concentration of each VFA monitored never exceed 300 mg L^{-1} as presented in Figure 5-4.

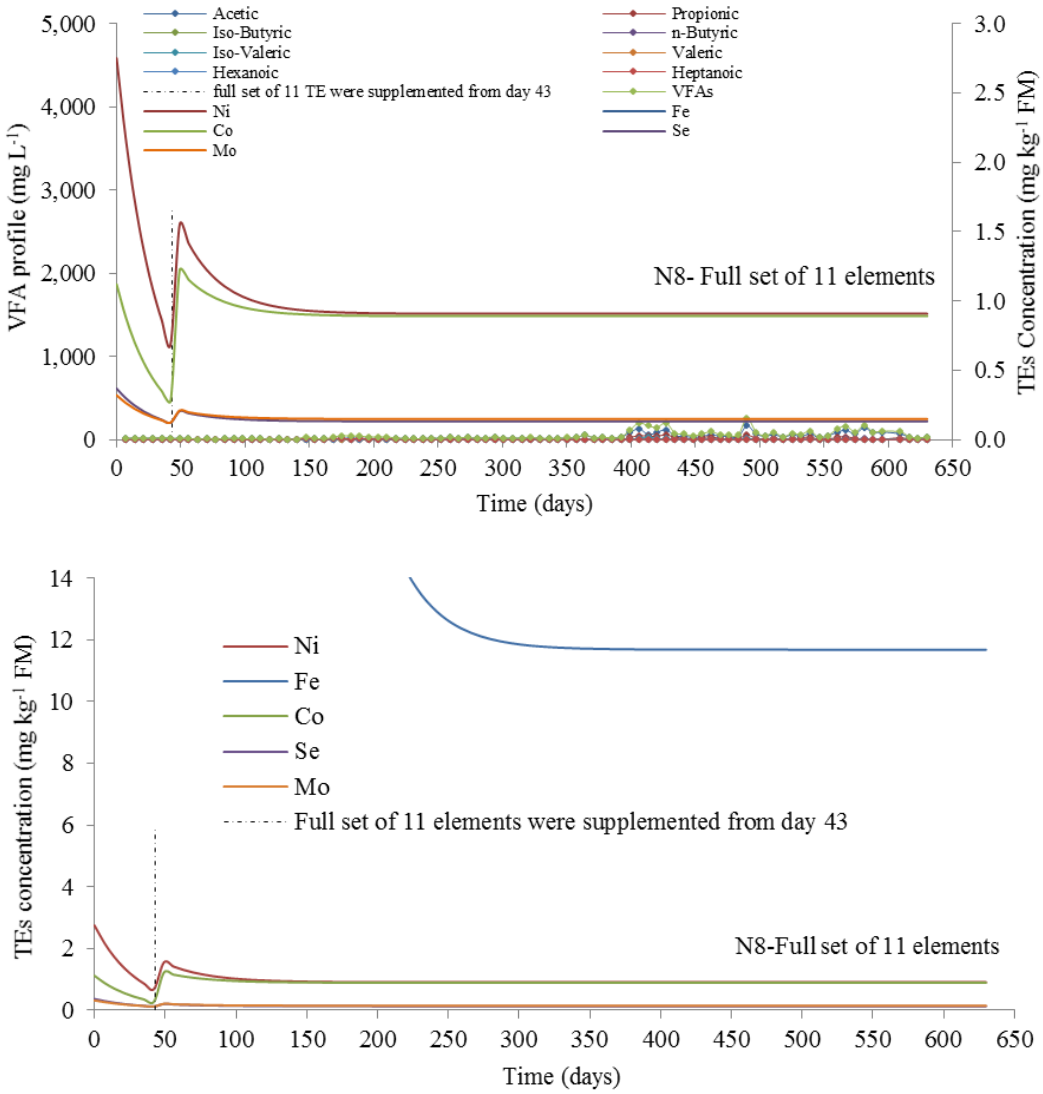


Figure 5-4 VFA and simulated TE concentrations profiles in digester N8

5.3.2 **Single element supplementation digesters**

Single element supplementation, Ni-supplemented (N2) and Co-supplemented (N3) digesters were started dosed with single Ni and single Co from day 43 of the experiment. This was also unable to satisfy the TE requirement. From Figure 5-5, after which time (223 days), digester N2 was suffering from VFA accumulation to around 3,000 mg L^{-1} reflected a rapid fall in pH from 7.60 to 7.37 (Figure 5-14) and a decline in daily biogas production and methane content (Figure 5-12). The Co concentration when VFA started accumulating were 0.01 mg kg^{-1} FM, equal to the baseline concentration from the feed as shown in Figure 5-5. Therefore, Co at strength of 0.02 mg kg^{-1} FM was introduced to digester N2 on day 240 of the operation. The reason to supplement Co

to N2 was because digester with Co and Ni dosing (N4) still perform well at that point. These was in attempt to achieve stability by reducing VFA level.

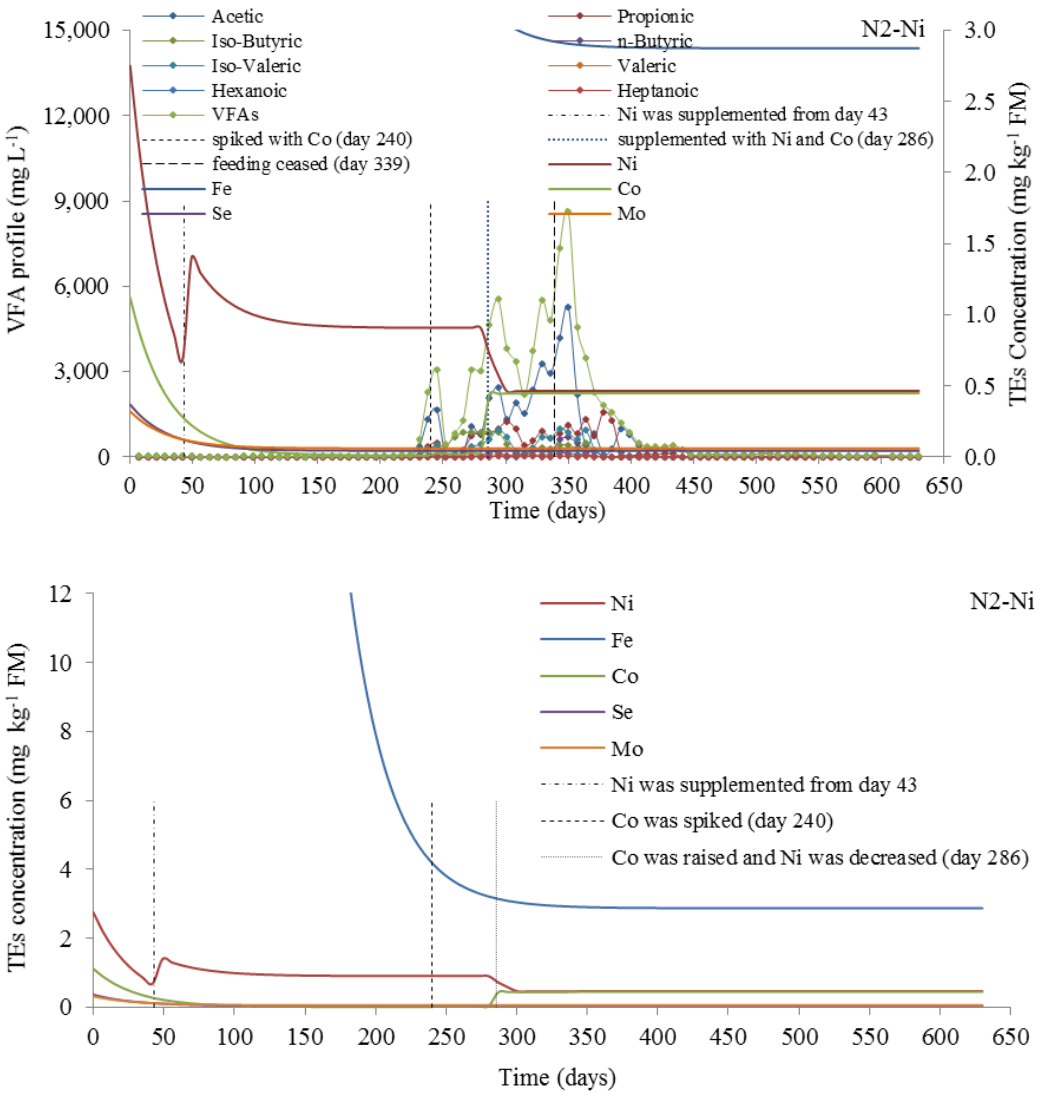


Figure 5-5 VFA and simulated TE concentrations profiles in digester N2

These results from N2 match those observed from digester N3. Digester performance remained stable for around 4 HRTs. As can be seen in Figure 5-6, the total VFA started to increase from below 100 mg L^{-1} on day 150 to above 5,700 mg L^{-1} (3,200 mg HAc L^{-1} and 1,000 mg HPr L^{-1}) on day 196 with a gradually dropped in pH from 7.60 to 7.14 (Figure 5-14), a decreasing in daily gas production and methane content to 47.7 % (Figure 5-12). As seen from Figure 5-6, the Ni concentration when VFA started accumulating were 0.03 mg kg^{-1} FM, equal to the baseline concentration from the feed. As a result, the Ni dosing at a strength of 1.0 mg kg^{-1} FM was given to N3 by an injection at the beginning of this action from day 196 of operation followed by weekly supplementation, as an attempt to control its VFA level. The reason to supplement Ni to N3 was

because digester with only Ni supplementation (N2) also digester with Co and Ni dosing (N4) still perform well at that point

From this stage of operational process, however, it was demonstrated that after a period of un-stable process performance, an immediate and significant effect can be attributed to the Co and Ni addition to N2 and N3, respectively. The processes responded to Co and Ni supplementation by suddenly decrease of acetic acid concentration from above 3,000 mg L^{-1} (Figure 5-5) and 5,700 mg L^{-1} (Figure 5-6) to less than 300 mg L^{-1} and this was followed later by a decline in propionic acid concentration within 7 days and 28 days for N2 and N3, respectively. Results regarding biogas production during recovery period indicated an increase in process stability. In these cases, the stable digestion was achieved and pH increased to around normal range at 7.6 (Figure 5-14), the higher biogas yield, better methane content (Figure 5-12) and lower VFA concentration can be attributed to the Co (0.02 mg kg^{-1} FM) adding to N2 and Ni (1.0 mg kg^{-1} FM) adding to N3. These two digesters were no longer rate-limited by methanogenesis, leading to digester efficiency and stable process performance.

From these results, it appeared that digesters supplemented with Ni only or Co only cannot allow stable performance in the absence of other TEs. Therefore both Co and Ni seem to be needed to maintain stable process conditions. It seems that for Ni-supplemented digester (N2) has no longer effect of the digester performance compared to Co-supplemented digester (N3) while the digester initially supplemented with both Co and Ni (N4) had stable operation for more than 280 days (8 HRTs) without any VFA accumulation (Figure 5-7). The similar results was proposed from Gustavsson *et al.* (2013a) who found that the earlier and the more rapid VFA accumulation occurred in Ni-deficiency digester (Co alone) than Co-deficiency digester (Ni alone).

The improvement of process performance in N2 and N3 was short–lived in this condition after receiving Co and Ni dosing. VFA, however, continuously increased for N2 from day 252 to 4,700 mg L^{-1} on day 286 (Figure 5-5) with reducing in pH to 7.30 (Figure 5-14) and methane content to 51.7 % (Figure 5-12) while the VFA accumulation trend was stopped for 1 retention times (day 223 to day 254) for N3 as shown Figure 5-6, however, VFA started to climb up again from day 262 to 3,600 mg L^{-1} on day 286 with accompanied pH decrease to 7.28 (Figure 5-14) and methane content to 51.2% (Figure 5-12).

There was no reduction and the accumulation of VFA became more severe from day 286 in digesters N2 and N3. It was initially hypothesised that this unbalanced operation might be due to these digesters lack of other essential TEs. They may require other TE apart from Co and Ni dosing. To test this, different actions had been taken.

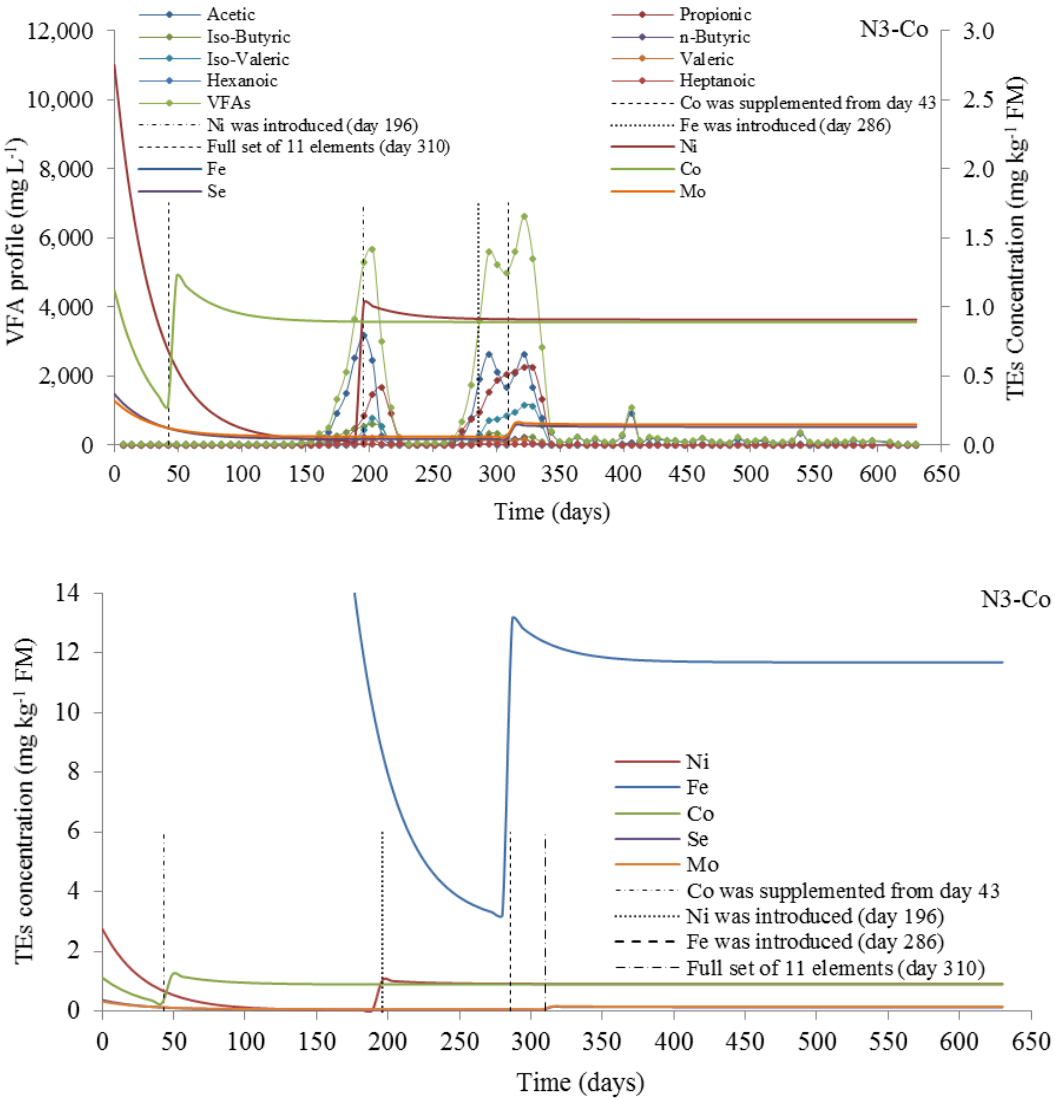


Figure 5-6 VFA and simulated TE concentrations profiles in digester N3

Digester N2 supplemented with Ni 1.0 and Co 0.02 mg kg^{-1} FM cannot maintain long term stable process performance due to Co deficiency. At that point, Co concentration was less than 0.03 mg kg^{-1} FM when VFA started to accumulate in control digester (N1) Figure 5-2. The Co dosing was introduced to N2 to a strength of 0.5 mg kg^{-1} FM while Ni was washed out from day 286 and has been re-supplemented when Ni concentration reached 0.5 mg kg^{-1} FM on day 307. The reason to supplement both Co and Ni at a strength of 0.5 mg kg^{-1} FM to N2 was because at that point (286 days; 9 HRTs), digester with Co and Ni supplementation (N4) received both Co and Ni at the strength of 1.0 mg kg^{-1} FM observed a sharply increase in VFA to 2,500 mg L^{-1}. The test was designed to determine the effect of overdosing of Co and Ni may cause the unstable process performance to N4. These would probably not be achievable by supplementation with both Co and Ni at the strength of 0.5 mg kg^{-1} FM. VFA accumulation trend was stopped for 21 days (day 294 to day 315) as shown Figure 5-5, however, VFA started to climb up again from day 315 to 5,500 mg L^{-1} on day 329 with accompanied pH and methane content decrease (Figure 5-14 and

Figure 5-12). To maintain pH a phase of intermittent feeding was necessary adopted, which could not stop performance deterioration neither: the pH dropped to around 7.08 and the methane content of biogas was 49.5 %. Feeding was then stopped at day 339 but the digester mixing and temperature control were maintained. VFA concentration persistently increased to around 8,600 mg L^{-1}. It took the digester around 67 days after feeding stop to recover from its VFA stress and restore its pH level to normal range at 7.80. From this and the previous results, it can be concluded that digester N2 seems to be required other elements apart from Co and Ni to maintain stable process conditions rather than it received overdosing of them at the strength of 1.0 mg kg^{-1} FM.

Fe was introduced to digester N3 on day 286 (Figure 5-6) at a strength of 10 mg kg^{-1} FM. The reason to supplement Fe to N3 because at that point, digester with Co, Ni and Fe dosing (N7) showed very stable process performance. However, Fe-supplementation to N3 did not result in a process recovery when VFA had been accumulated at 3,900 mg L^{-1}. No reduction in VFA was observed. The VFA concentration continued to increase to above 5,600 mg L^{-1} with accompanied pH decreased to 7.28 (Figure 5-14) and methane content to 51.2 % (Figure 5-12). This might be because under un-balanced stability, digester may require more than those the combination of Co, Ni and Fe to recover stability to normal operation. From this results indicated that TE addition, to a large extent, cannot initiate the VFA consumption process in digester with high VFA concentrations. It appeared that after a digester has been subjected to accumulation of VFA for a period of time, the onset of VFA degradation depends on other factors in addition to those of TE concentrations. Once the VFA degradation process has started the supplementation of a specific TE or multicomponent TE matrix can, however, accelerate the VFA consumption rate. Then, the timing of TE addition need to be taken into consideration to seek a balance between effect of VFA production and VFA consumption. As such strategy for stable digestion should focus on the prevention of initial VFA accumulation in the digester by TE supplementation, rather than the recovery of a severely VFA-laden digester. Full set of 11elements was applied to this digester on day 310 (> 9 HRTs) in attempt to reduce VFA level, however, these would probably be achievable stable process performance with the total VFA concentration could be dropped and fluctuating around less than 300 mg L^{-1} as can be seen in Figure 5-6.

5.3.3 **Binary elements supplementation digesters**

5.3.3.1 **Co and Ni supplementation**

As shown in Figure 5-7, digester with Co and Ni supplementation (N4) also showed unstable performance although with some delay compared with N1 (no TE) and single TE dosing digesters (N2 and N3). It appeared that N4 was suffering from VFA accumulation to around 2,500 mg L^{-1} on day 289 (8 HRTs) reflected as a rapid fall in pH from 7.60 to 7.40 (Figure 5-14) and a decline

in daily biogas production and methane content (Figure 5-12). The hypothesis for this unstable operation was that it required other elements.

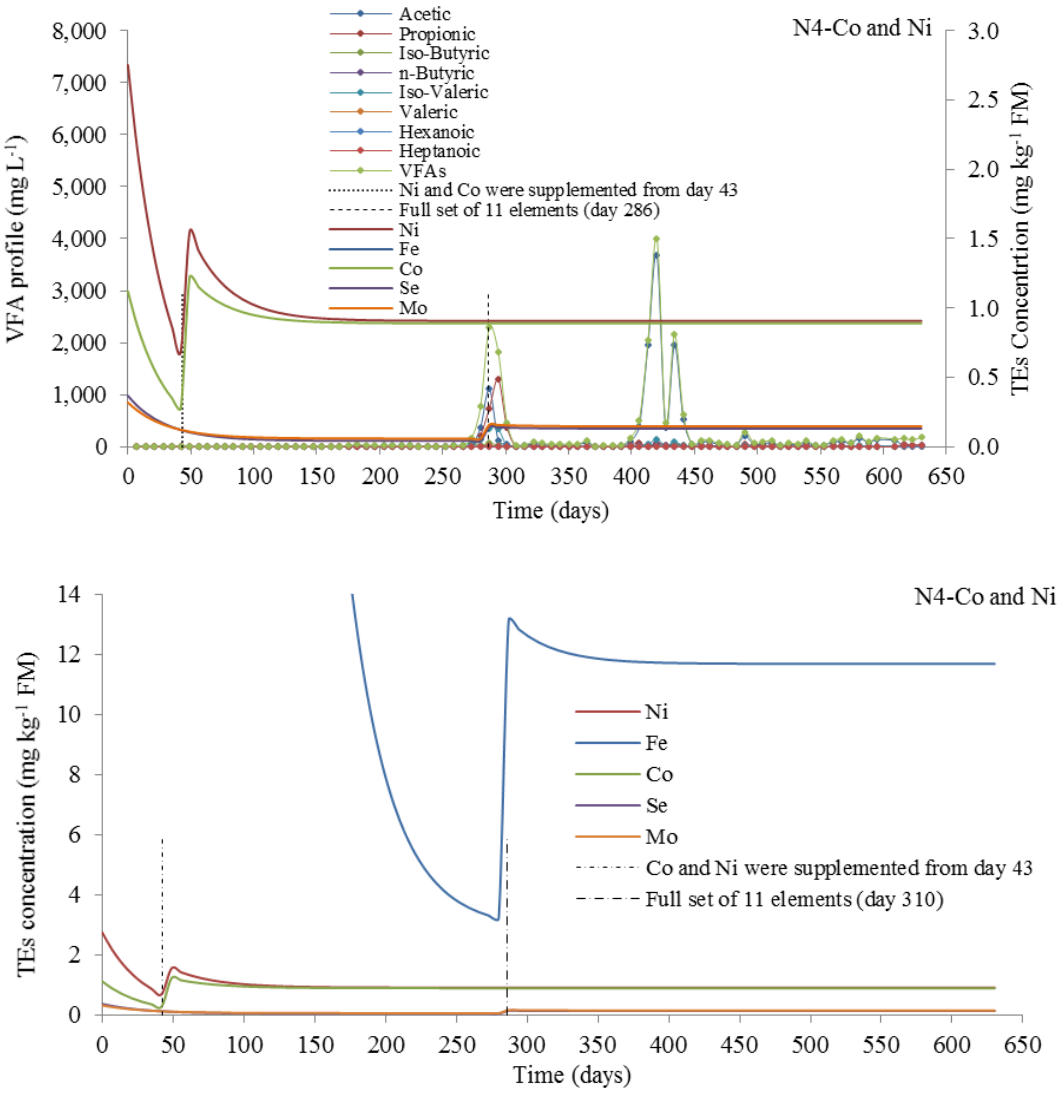


Figure 5-7 VFA and simulated TE concentrations profiles in digester N4

A set of 11 elements was given to N4 from day 286 in attempt to reduce VFA level. The reason to supplement 11 elements to N4 was because at that point digester N8 still performed well. The process immediately responded to a set of 11 elements supplementation by suddenly decrease of VFA from above 2,500 mg L^{-1} (Figure 5-7) to less than 300 mg L^{-1} in 22 days. Results regarding biogas production during recovery period indicated an increase in process stability. In this case, the stable digestion was achieved and pH increased to normal range which is around 7.5 - 7.6 (Wheatley, 1990), the higher biogas yield and the better methane content can be attributed to the set of 11 elements supplementation.

The acetic acid concentration (>90 % of VFA) of N8, N3 and N4 suddenly increased from less than 100 to 400 , 1,000 and 1,000 mg L^{-1} from day 392 (12 HRT) as can be seen in Figure 5-4,

Figure 5-6 and Figure 5-7, respectively. It was initially hypothesised that this unbalanced operation might be due to temperature change effect because at that point these set of digesters received full set of 11 elements. To test this, digestate temperature profile had been investigated as shown in Figure 5-8 and found that VFA accumulation (from day 392) when actual temperature of the digestate higher than average range at 36°C which means methanogen being temperature sensitive and have been affected. However, process performance recovered after average temperature change back to mesophilic condition without other any actions have been taken.

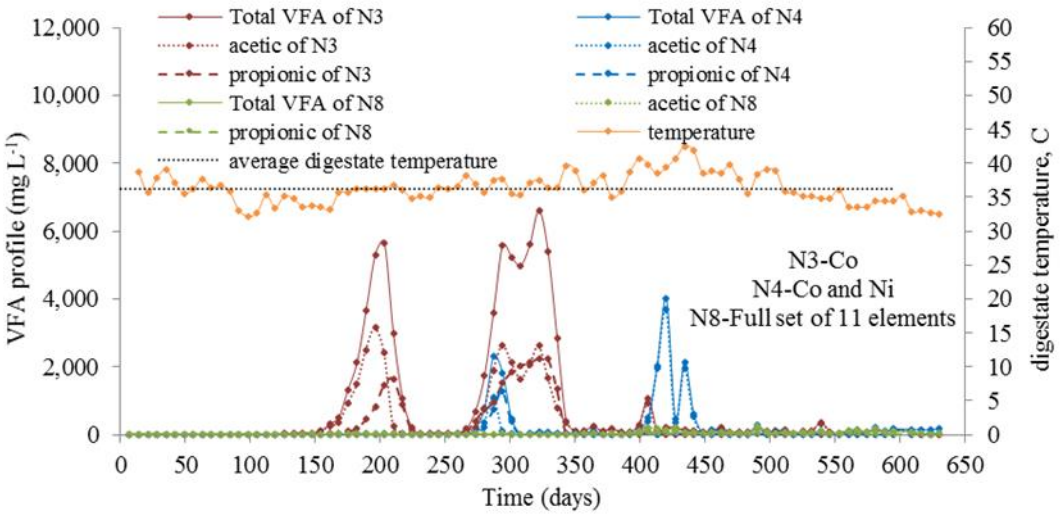


Figure 5-8 VFA concentration and digestate temperature profiles in digesters N3, N4 and N8

The findings of the current results from N2, N3 and N4 do support the fact that digesters require at least combination of Co, Ni and Fe or a set of full 11 elements supplementation to maintain long term stable process conditions without VFA accumulation and did the result in a process recovery from unstable performance.

5.3.3.2 **Fe either with Co or Ni**

Digester supplemented with Co and Fe (N5)

N5 performed normally for 4 HRTs. After that time, VFA started to increase from day 140 to 5,300 mg L^{-1} (Figure 5-9) resulted in pH gradual drop from 7.6 to 7.4 (Figure 5-14), and a decreasing in daily gas production and methane content (Figure 5-12). The hypothesis for this imbalance was that N5 required other elements other than Co and Fe to sustain a stable process. The Ni concentration when VFA started accumulating in N5 were 0.05 mg kg^{-1} FM, a bit higher than the baseline concentration from the feed (0.03 mg kg^{-1} FM) as shown in Figure 5-9 but less than when VFA started to accumulate in N1 (0.09 mg kg^{-1} FM). Then, the Ni dosing at a strength of 1.0 mg kg^{-1} FM was given to N5 from day 181, as an attempt to control its VFA level. The reason to supplement Ni to N5 was because digester with Co, Ni and Fe dosing (N7) still operated

well at that point (section 5.3.4). As presented in Figure 5-9, process performance showed immediately response to Ni dosing. The very rapid reduction on accumulated VFA from 5,500 to less than 100 mg L^{-1} within 22 days was observed following this action. Digestion could be recovered from un-balanced operation. It was confirmed by improvement in the IA/PA ratio (Figure 5-14), pH increased to around 7.60 (Figure 5-14), gas production and methane content were recovered (Figure 5-12). These indicated an increase in process stability can be attributed to the Ni addition. N5 performed optimally with Co, Fe and Ni supplementation for around 200 days (day 200 to day 399) which supported the very stable operation without any major digestion instability for more than 12 HRTs (399 days) of N7 (Figure 5-11), with receiving a combination of Co, Ni and Fe supplementation.

N5, with Co, Ni and Fe supplementation, suddenly suffered from VFA accumulation from day 399 to above 4,700 mg L^{-1} on day 412 (Figure 5-9), in which propionic acid accounted for around 600 mg L^{-1} accompanied with a sharply decreased in pH from above 7.50 to less than 7.30 (Figure 5-14), reduction in biogas production and methane content reduced to 54 % (Figure 5-12). This indicated that Co, Ni and Fe as well as other elements existed in N5 worked on prevention of VFA accumulating, however, without sufficient of some key elements, digestion had been disturbance. This unbalanced operation might be due to N5 lack of other essential TEs which is required above baseline concentration. The Se and Mo concentration when VFA started accumulating (day 399) were 0.04 and 0.06 mg kg^{-1} FM, respectively, equal to baseline concentration from the feed as shown in Figure 5-9. It was obvious that if no actions was taken digestion would fail. Therefore, the Se and Mo dosing at a strength of 0.1 mg kg^{-1} FM were introduced to N5 from day 412, as an attempt to reduce VFA level. The reason to supplement Se and Mo to N5 was because Se is related to key enzymes involved in hydrogenotrophic methanogesis and propionic degradation. Mo was also reported to stimulate methane production. This has been primarily because of their role in the formate dehydrogenase enzyme system as mentioned in Chapter 2. The depletion of Se and Mo may decrease microbial activity. This hypothesis can be supported by aforementioned researches which indicated that propionate consumption and methanogenic activity decreased when Mo, W and Se in influent of biological process were deficient (Worm *et al.*, 2009). Plugge *et al.* (2009) proposed that the reduction of Mo and W can decrease formate dehydrogenase activity.

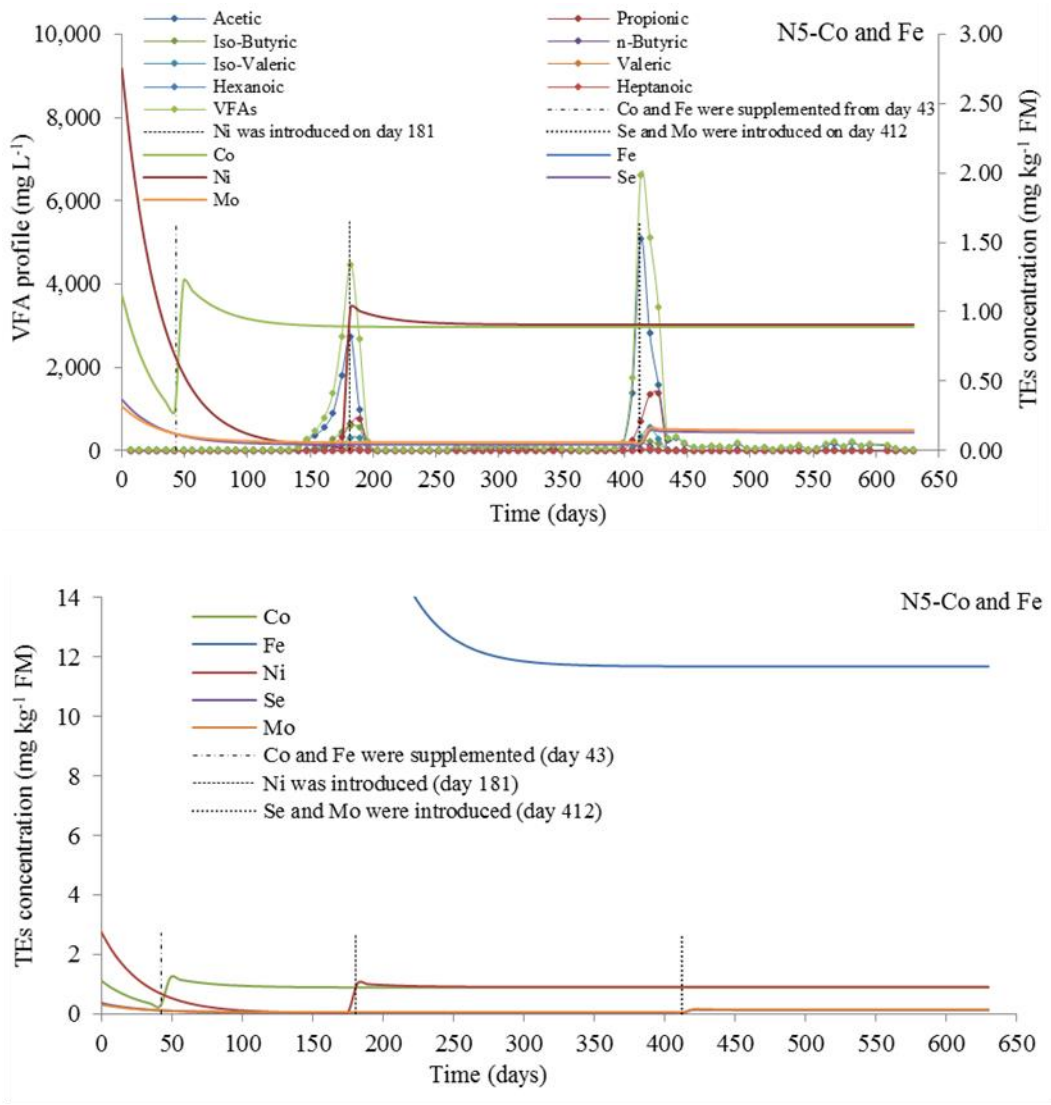


Figure 5-9 VFA and simulated TE concentrations profiles in digester N5

An immediate and significant effect can be attributed to Se and Mo addition to N5. The process responded by suddenly decreasing of total VFA from 6,600 to less than 300 mg L^{-1} (Figure 5-9) and this was followed later by a decline in propionic acid concentration within 21 days which resulted in process recovery from un-balanced process performance. Results regarding biogas production during recovery period indicated an increase in process stability. In this case, it appeared that the stable digestion was achieved and pH increased to around normal range at 7.60 (Figure 5-14). The higher biogas yield, better methane content (Figure 5-12) and lower VFA concentration can be attributed to the strength of Co 1.0, Ni 1.0, Fe 10, Se 0.1 and Mo 0.1 mg kg^{-1} FM dosing.

Digester supplemented with Ni and Fe (N6)

N6 suffered from VFA accumulation from day 154 (4 HRTs) of operation to a concentration of 3,300 mg L^{-1} on day 164 along with a sharp decrease in pH (Figure 5-14), a reduction in gas production and methane content (Figure 5-12). It was initially hypothesised that this unbalanced

operation might be due to N6 lack of Ni by some reasons. This is because digester with only Ni supplementation: N2 still performed well at that point (section 5.3.2). To test this, a double dose of Ni at the strength of 1.0 mg kg^{-1} FM was applied and therefore the existing Fe in the digester would have been gradually diluted out from day 164, as an attempt to recover the digestion balance by controlling its VFA level. This action aimed to test the antagonistic effect of Fe when supplemented with Ni.

As can be seen in Figure 5-10, no reduction in VFA was observed and the accumulating of VFA became more severe to the concentration of 9,200 mg L^{-1} which acetic acid was the predominant specie on day 174. There was no apparent effect has been observed; all these attempts failed. Performance deterioration could not be stopped: the pH dropped to around 6.90 and the methane content was 37.8 % (Figure 5-12).

Stable digestion was not be achievable. This might be because under un-balanced conditions, the digester may require more than Ni and Fe to recover stability to normal operation. The results indicated that TE addition, to a large extent, cannot initiate the VFA consumption process in digester with high VFA concentrations. It appears that after a digester has been subjected to accumulation of VFA for a period of time, the onset of VFA degradation depends on other factors in addition to those of TE concentrations. Once the VFA degradation process has started the supplementation of a specific TE or multicomponent TE matrix can, however, accelerate the VFA consumption rate. Then, the timing of TE addition need to be taken into consideration to seek a balance between effect of VFA production and VFA consumption. Strategy for stable digestion should focus on the prevention of initial VFA accumulation in the digester by TE supplementation, rather than the recovery of a severely VFA-laden digester. Feeding was then stopped at day 175 but the digester mixing and temperature control were maintained. It took the digester around 154 days after feeding stop to recover from its VFA stress which fluctuated around 7,000 - 11,000 mg L^{-1} and restore its pH level to normal range around 7.80 (Figure 5-14).

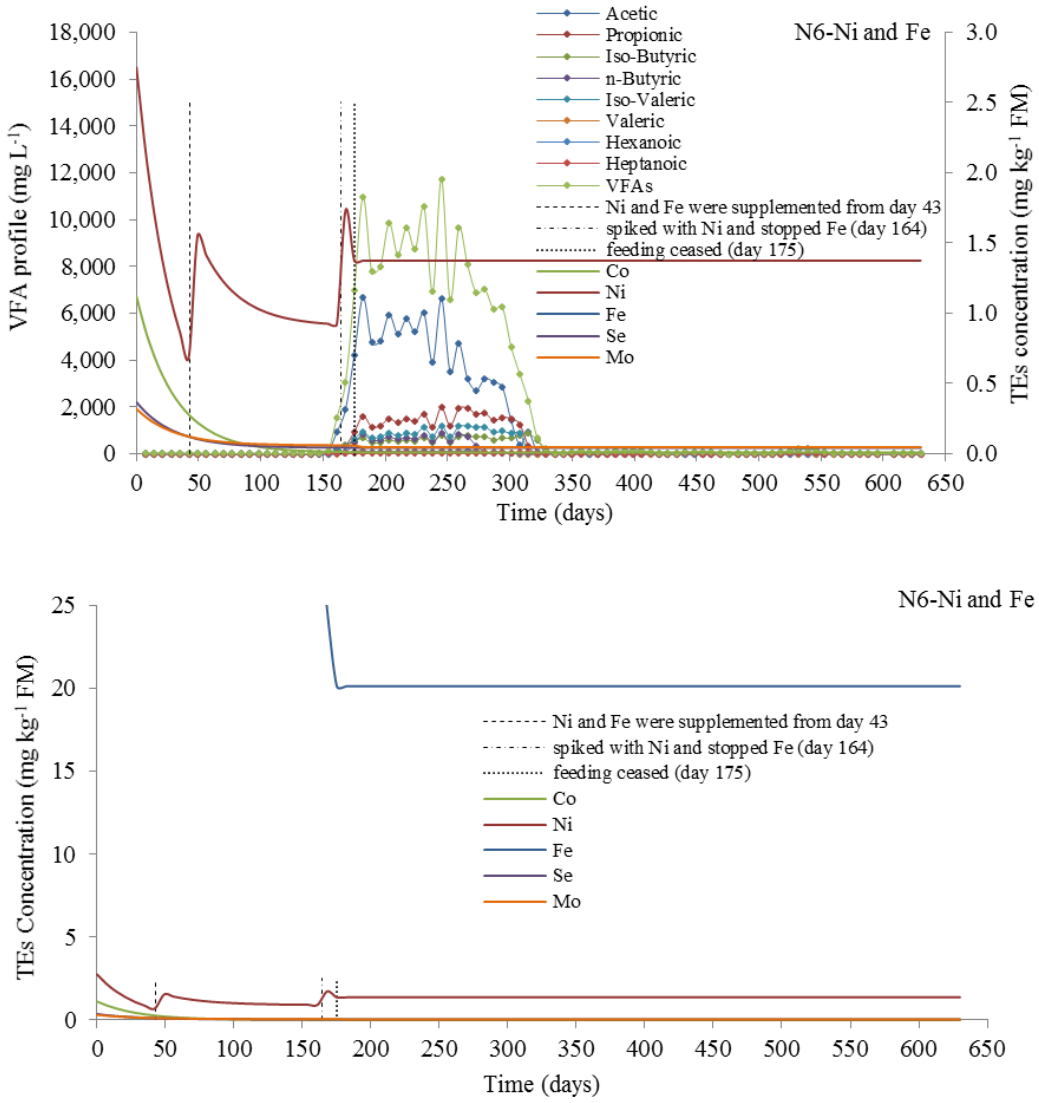


Figure 5-10 VFA and simulated TE concentrations profiles in digester N6

An antagonistic TE interaction

As discussed above, digesters dosing with Co and Fe (N5) and with Ni and Fe (N6) suffered from VFA accumulation from day 140 and day 154, respectively. These were risking process failure compared to digesters receiving only Co (N3) and only Ni (N2) which still operated normally at that point. It appears that Fe showed antagonistic effect when initially supplemented with either Co (N5) or Ni (N6) which suggested that at this condition some other component has become limiting.

As mentioned in Chapter 2, sulphur - containing organic matter and sulfate in substrate are converted to sulphide during AD (J.W.H *et al.*, 1994). Several studies reported the importance of sulphide as a regulator for metal bioavailability due to high affinity of sulphide for binding metal ions (Callander and Barford, 1983b;a; Rinzema and Lettinga, 1988; Morse and Luther Iii, 1999; Gonzalez-Gil *et al.*, 2003; Jansen *et al.*, 2005; Patidar and Tare, 2006; Aquino and Stuckey, 2007;

Jansen, Gonzalez-Gil and van Leeuwen, 2007). The precipitation of Co-and Ni-sulphides has been suggested to affect Co and Ni bioavailability (Gonzalez-Gil *et al.*, 2003; Jansen, Gonzalez-Gil and van Leeuwen, 2007) due to their very low solubility products (Callander and Barford, 1983a;b; Rinzema and Lettinga, 1988; Shen, Kosaric and Blaszczyk, 1993; Rickard and Luther, 2006). It would be expected that these metals are non-bioavailable to the methanogenic consortia (van der Veen, Fermoso and Lens, 2007). Gustavsson *et al.* (2013a) reported that the main S species of the sludge solid phase was FeS which is around 63 % of total S. Co and Ni can be adsorbed and/or co-precipitated on FeS (Morse and Arakaki, 1993; Gustavsson *et al.*, 2013a; Shakeri Yekta *et al.*, 2014a) which play an important role on their bioavailability for microbial uptake and process stability (Gustavsson *et al.*, 2013b; Shakeri Yekta *et al.*, 2014a).

It is possible that the unstable operation on N5 and N6 on day 140 and 154 might be due the available Co and Ni presented in N5 and N6, respectively being below supplemented level of 1.0 mg kg^{-1} FM before VFA-accumulation commenced which would likely give rise to process disturbance and function decline. This may lead to Co-deficiency in N5 and Ni-deficiency in N6. As a result, N5 and N6 showed faster decreasing in process stability before N3 (Co alone) and N2 (Ni alone) which received single element dosing.

5.3.4 **Co, Ni and Fe supplementation digester**

The digester with Co, Ni and Fe supplementation (N7) could be maintained stably without encountering any major operational difficulties for almost 400 days. VFA sharply increased to 4,700 mg L^{-1} (1,800 mg L^{-1} of acetic acid and 2,400 mg L^{-1} of propionic) on day 399 (Figure 5-11) and methane content dropped to 51.9 % (Figure 5-12). These supported the un-stable operation of N5 on day 399. It seemed that some TEs were deprived after 12 HRTs. Same as discussed in section 5.3.3.2 for N5, this unbalanced operation might be due to N7 lack of other essential TEs particularly Se and Mo which is required above baseline concentration. The Se and Mo concentration when VFA started accumulating (day 399) were 0.04 and 0.06 mg kg^{-1} FM, respectively, equal to baseline concentration from the feed as shown in Figure 5-11. In this case, the Se supplementation at a strength of 0.1 mg kg^{-1} FM was given to N7 from day 423, in an attempt to reduce VFA level. As mentioned above, the reason to supplement Se because Se is related to key enzymes (FDH: formate dehydrogenase) involved in hydrogenotrophic methanogesis and propionic degradation.

This would likely bring process performance back to normal process operation. An immediate and significant effect can be attributed to Se addition to N7. The process responded by sudden decrease of total VFA from 5,600 to less than 300 mg L^{-1} (Figure 5-11) within 1 retention time which resulted in process recovery from unbalanced process performance. Results regarding biogas production

during recovery period indicated an increase in process stability. In this case, it appeared that the stable digestion was achieved and pH increased to around normal range at 7.60 (Figure 5-14), the higher biogas yield, better methane content (Figure 5-12) and lower VFA concentration can be attributed to the strength of Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM dosing.

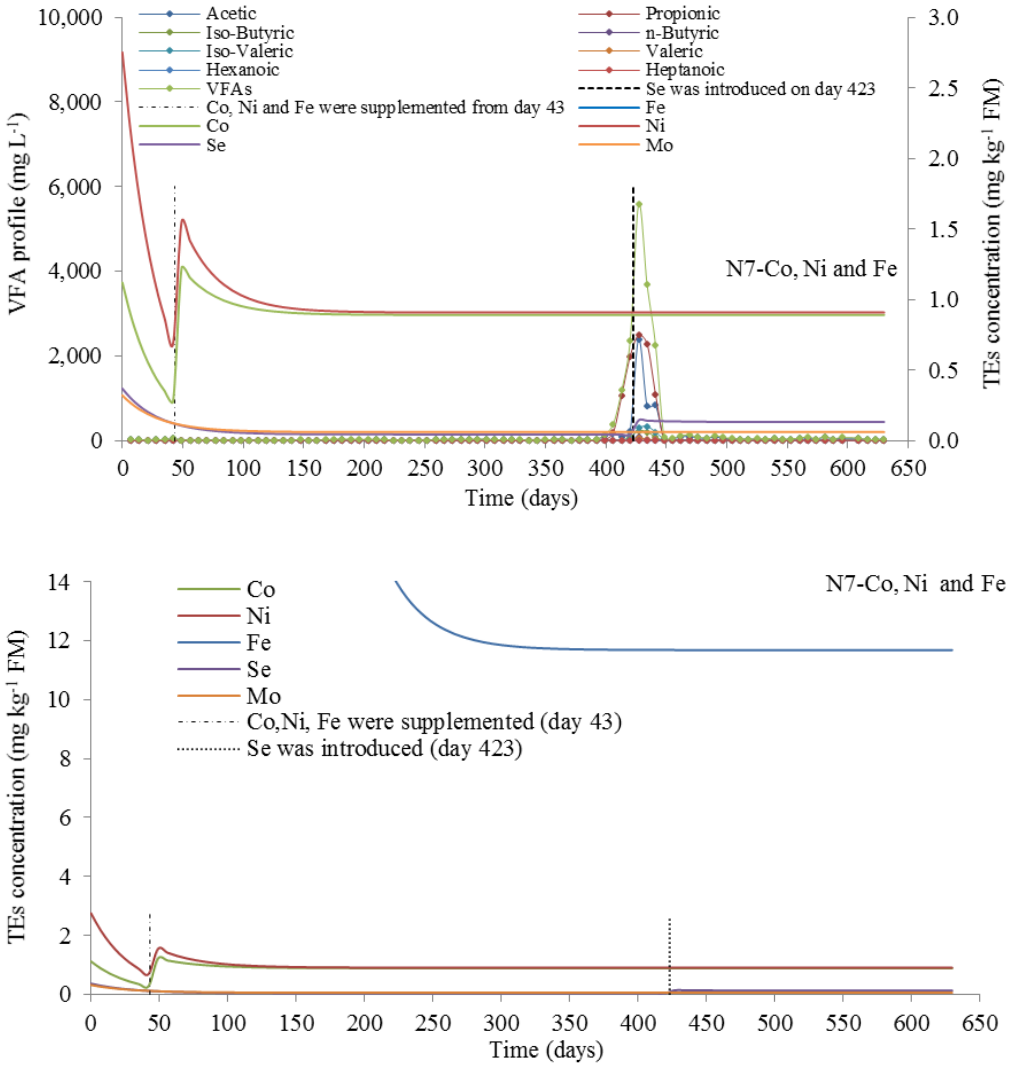


Figure 5-11 VFA and simulated TE concentrations profiles in digester N7

Further discussion

The most important relevant finding from the results obtained from N5 and N7 was that Se seems to be the limiting TE and the recommended concentration need to be supplemented for OLR 3.0 kg VS m^{-3} d^{-1} should be 0.1 mg kg^{-1} FM (0.14 mg kg^{-1} FM including substrate providing). This was in good agreement with the suggested concentration in Banks *et al.* (2012) who demonstrated critically Se concentration of 0.16 mg kg^{-1} FM at moderate loading of 3.0 kg VS m^{-3} d^{-1}. It appeared that the combination of Co, Ni, Fe and Se was thus required for long-term mesophilic

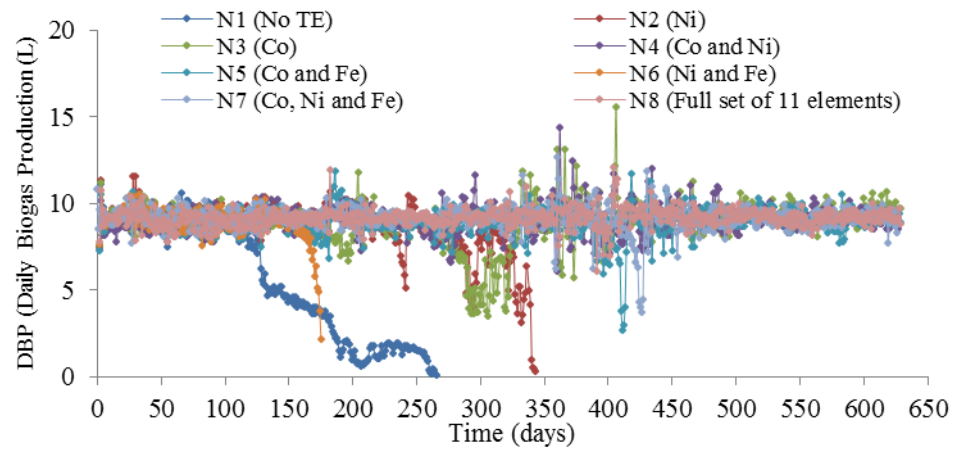
digestion (18 HRTs) under designed condition, at the strength of Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM which was greater than those found to be naturally presented in the model substrate.

It is, however, stressed that this was under the imposed loading rate of 3.0 kg VS m^{-3} d^{-1} and the demands under different loading conditions could vary depending both on metabolic pathways and overall metabolic activity. Results indicated that the other TE provided by model substrate was sufficient and there was no evidence that any of them was required in concentration greater than their baseline concentration. The system was operated for more than 18 HRTs and clearly showed that the requirement for TE was uncoupled from the hydraulic characteristics of the digester, suggesting that the chemical species and bioavailability of the TEs is a critical element in their function and is, to a certain degree, independent of washout as the results showed that nutrients are not lost from the system simply as a hydraulic function.

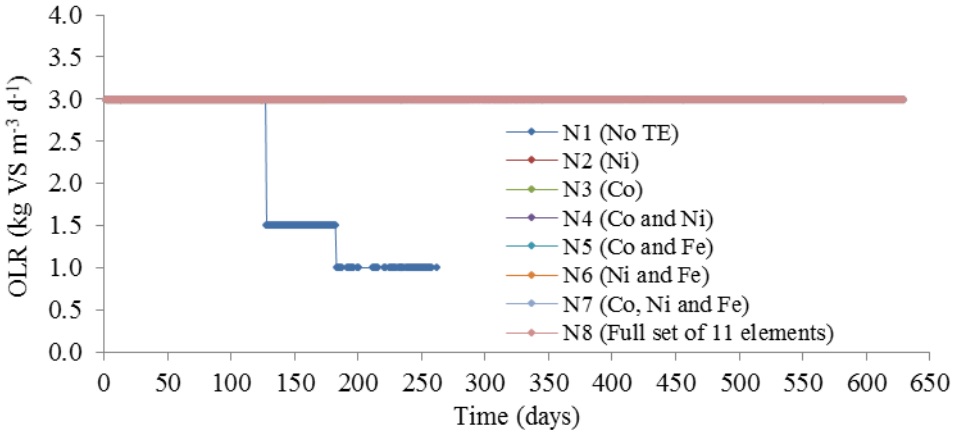
It was likely that a deficiency of TE resulted in the interruption of the syntrophic metabolic pathways leading to methane production. It is noteworthy that Se supplementation stimulated both acetic and propionic acid degradation, especially when supplied with sufficient strength which could ensure its complete and timely degradation. The rest of other VFA species existed at very low levels throughout the experiment. The results demonstrated that a balanced relation between the concentrations of these elements was of greater importance than the presence of individual elements for a digester to be able to operate for a long term at moderate OLR and maintain low VFA concentrations.

5.3.5 **General parameters and biogas performance indicator**

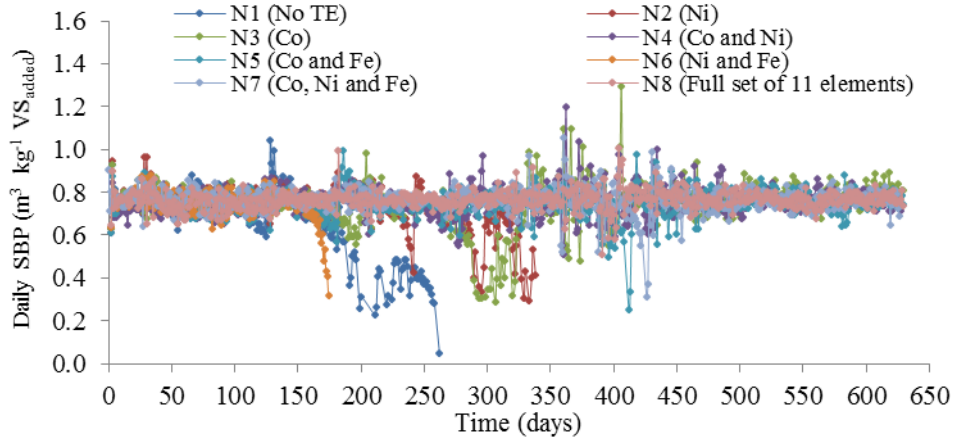
The performance profiles of semi-continuous anaerobic digestion of model substrate supplied with TE compared with control (without TE and with full set of 11 TE) can be seen in Figure 5-1-Figure 5-14. Using this substrate, for stable biogas process performance, the specific CH_4-production (SMP) was 0.44 ± 0.03 m^3 CH_4 kg^{-1} VS_{added} (corresponding to about 0.76 ± 0.03 m^3 biogas kg^{-1} VS_{added}), the volumetric CH_4 production was 1.30 ± 0.05 STP m^3 m^{-3} d^{-1} (2.28 ± 0.07 STP m^3 m^{-3} digestate d^{-1}) and the biogas methane content was 58.4 ± 0.9 % (Figure 5-12).



(a)

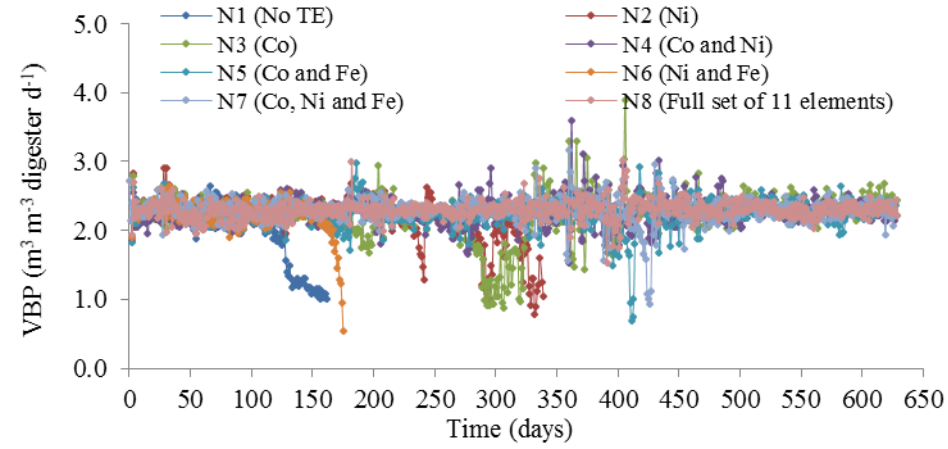


(b)

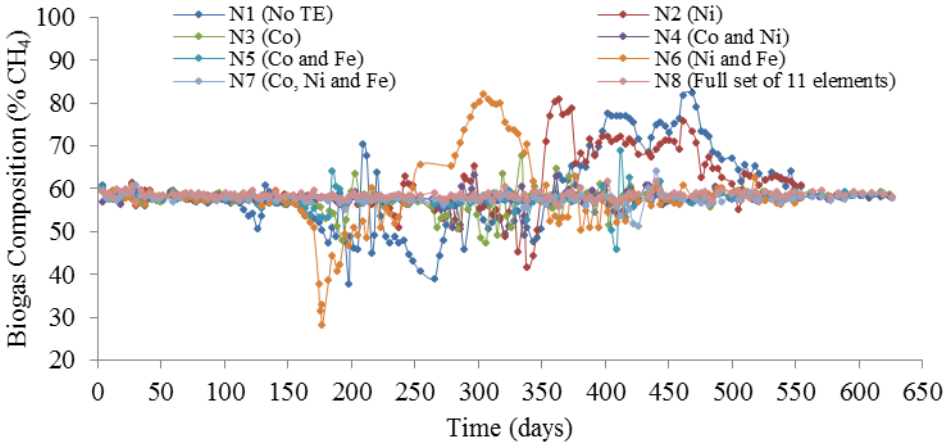


(c)

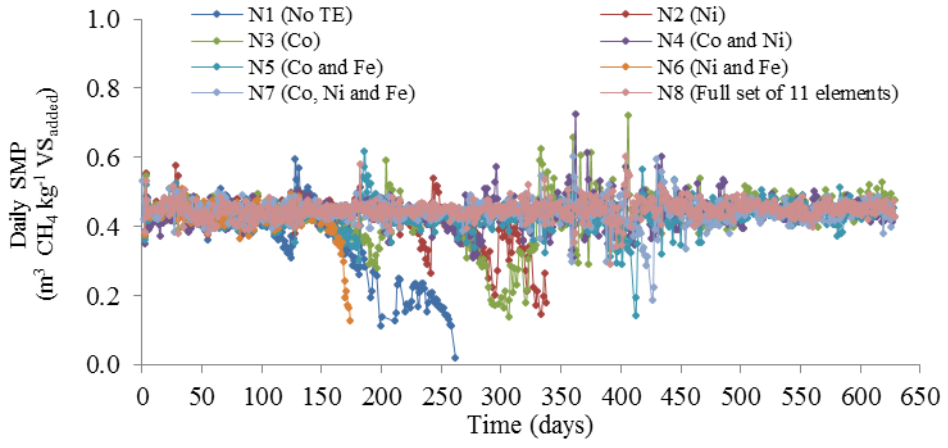
Figure 5-12 (a) DBP, (b) OLR, (c) SBP, (d) VBP, (e) % CH_4, (f) SMP and (g) VMP



(d)

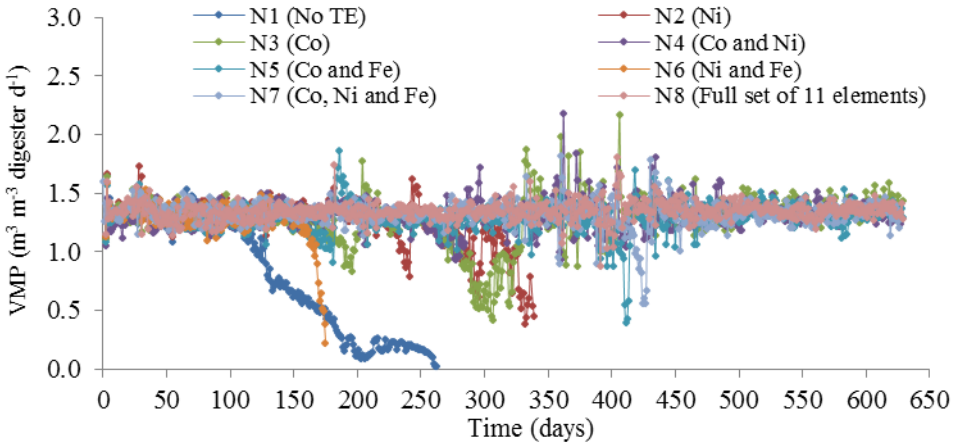


(e)



(f)

Figure 5-12 (a) DBP, (b) OLR, (c) SBP, (d) VBP, (e) % CH_4, (f) SMP and (g) VMP (continued)

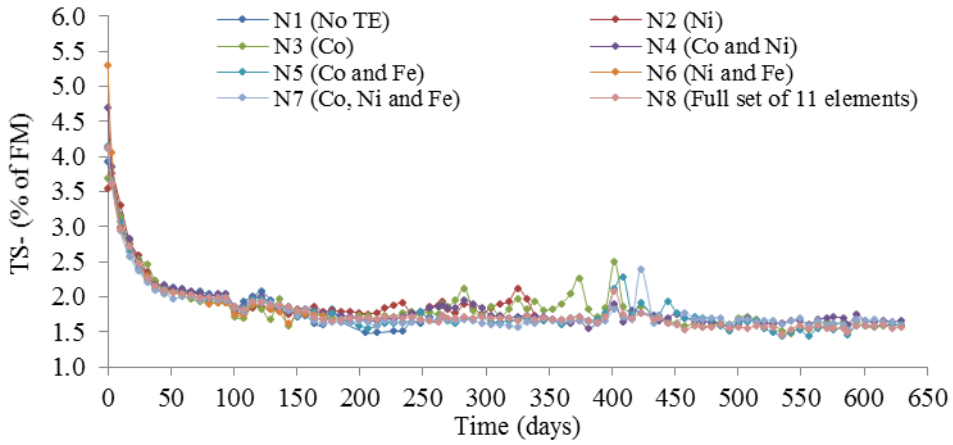


(g)

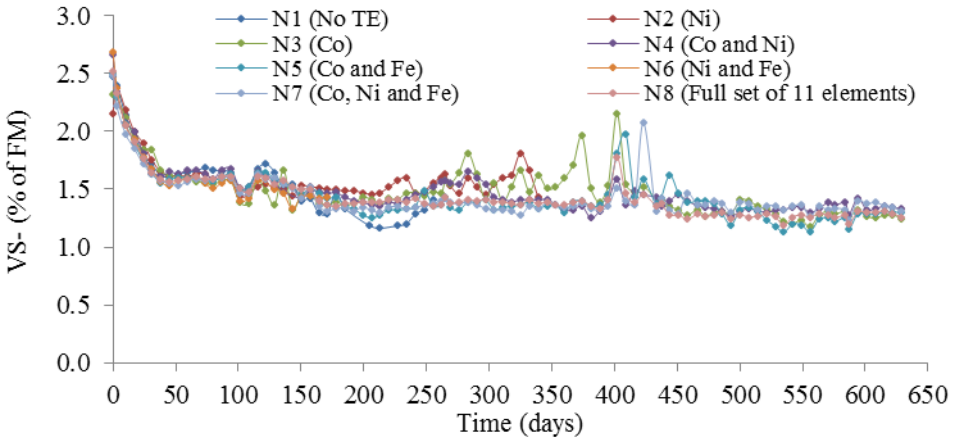
Figure 5-12 (a) DBP, (b) OLR, (c) SBP, (d) VBP, (e) %CH_4, (f) SMP, (g) VMP (continued)

The other general parameters were shown in Figure 5-13 and Figure 5-14. When digesters showed unstable performance reflected in VFA accumulation, various attempts were taken in order to restore their stability from TE deficient issue. IA/PA ratio for the stable digestion was around 0.4 ± 0.1 and the change in IA/PA ratio can be seen in Figure 5-14, indicating both the accumulation of VFA and the development of digestion instability (Ripley, Boyle and Converse, 1986). The pH in the well performed digesters remained around 7.5 ± 0.1 (Figure 5-14). To maintain pH a phase of intermittent feeding was adopted in certain digesters.

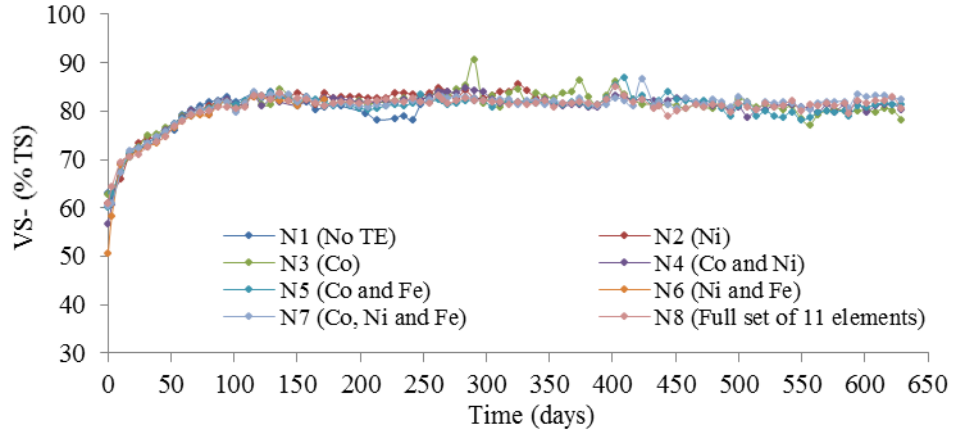
From Figure 5-14, TAN for instable digesters were higher than the well performed digesters (2,505 ± 69 mg N kg^{-1}). This is because when digester could not operate normally, microbial biomass concentration was lower than under stable condition. This resulted in the reducing in nitrogen content of microbial biomass. Considering the same OLR reflected the constant organic nitrogen from substrate, TAN is higher in unstable digesters as such. VFA accumulation in digesters under unstable digestion which mean TAN released to liquid digestate indicated that substrate has already degraded completely, however, when operated with sufficient TE supplementation the concentration of each VFA monitored never exceeded 100 mg L^{-1} (Figure 5-1 - Figure 5-11).



(a)

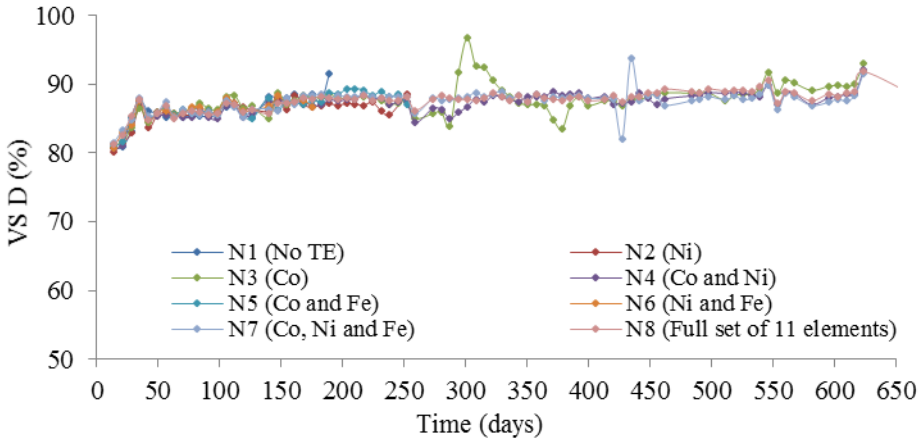


(b)



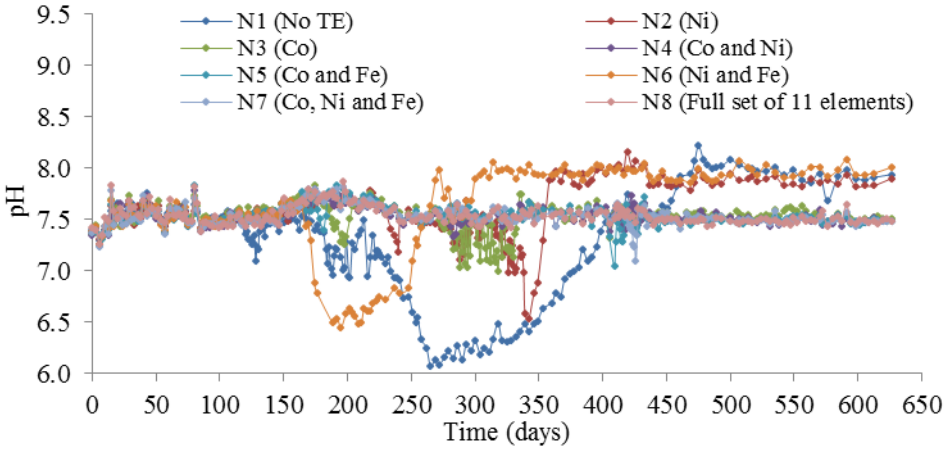
(c)

Figure 5-13 (a) TS% of FM, (b) VS% of FM, (c) VS as a% of TS and (d) %VSD

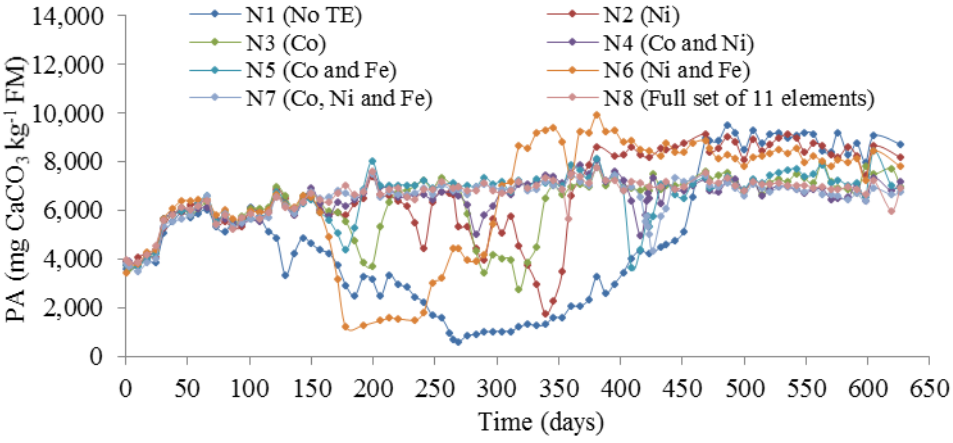


(d)

Figure 5-13 (a) TS% of FM, (b) VS% of FM, (c) VS as a% of TS and (d) %VSD (continued)

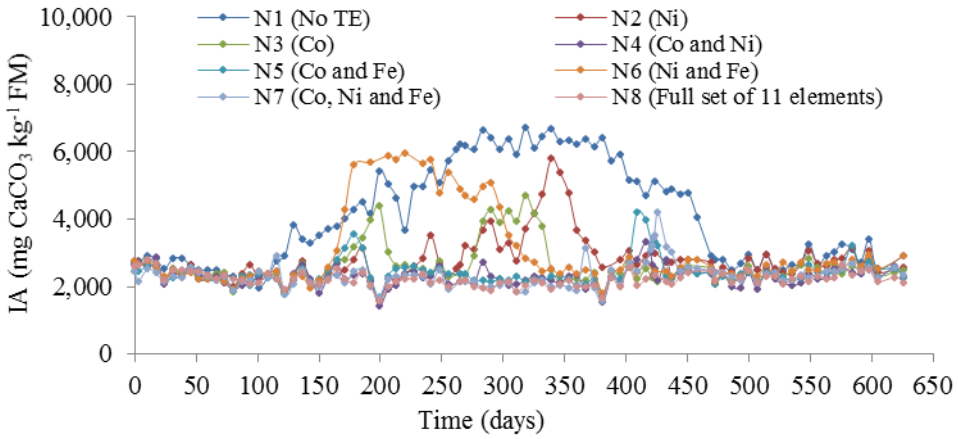


(a)



(b)

Figure 5-14 (a) pH, (b) PA, (c) IA, (d) TA, (e) IA/PA ratio, (f) TAN and (g) FAN



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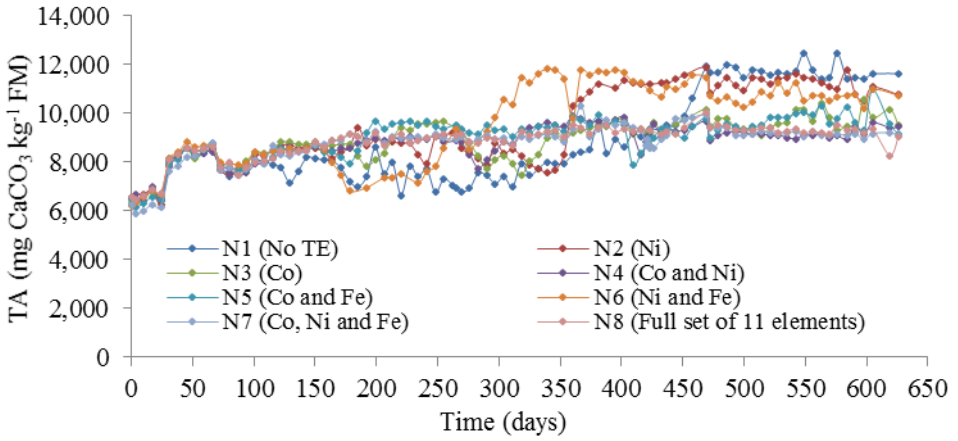

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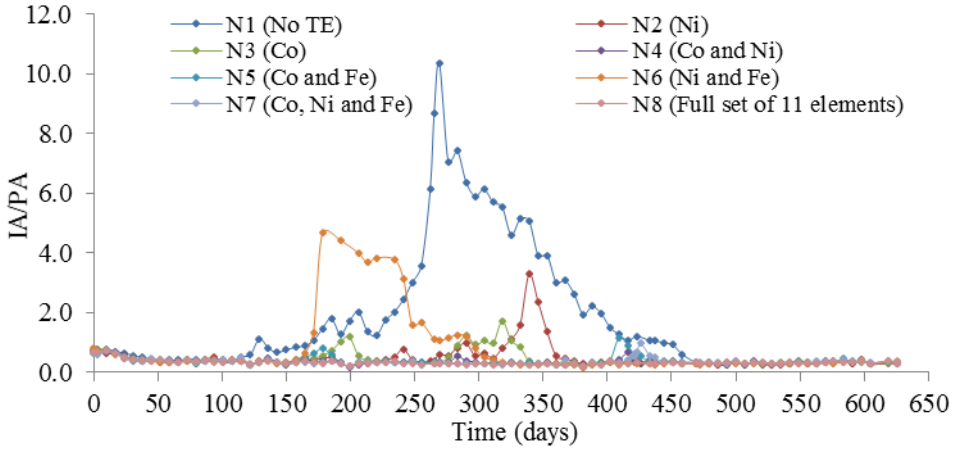
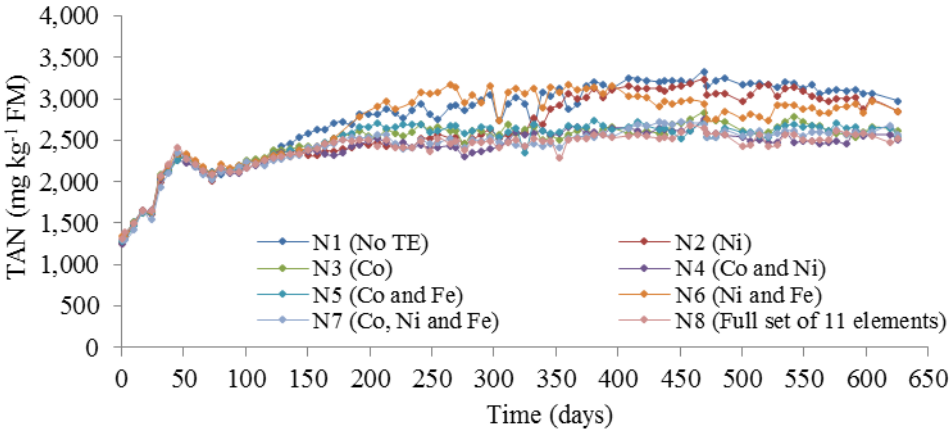
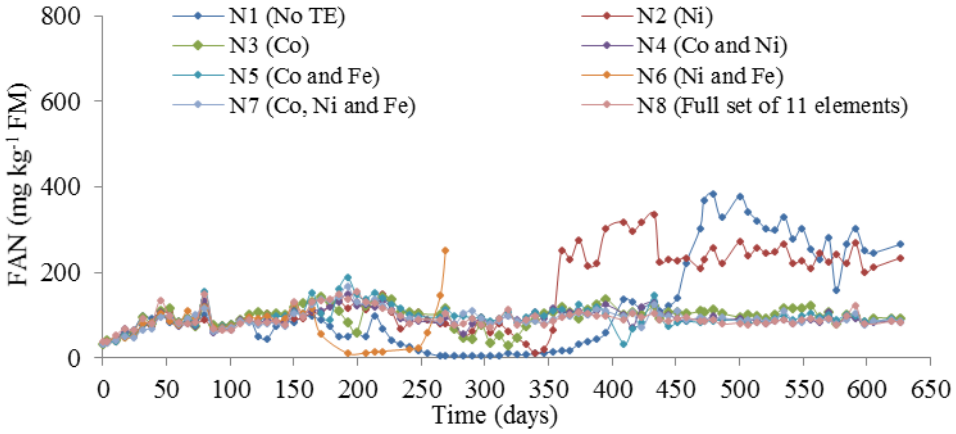


(e)

Figure 5-14 (a) pH, (b) PA, (c) IA, (d) TA, (e) IA/PA ratio, (f) TAN and (g) FAN (continued)



(f)



(g)

Figure 5-14 (a) pH, (b) PA, (c) IA, (d) TA, (e) IA/PA ratio, (f) TAN and (g) FAN (continued)

5.4 Concluding remarks

1. Single element supplementation of Co or Ni was unable to prevent VFA accumulation when baseline TE concentrations were Co 0.01, Ni 0.03, Fe 2.88, Se 0.04 and Mo 0.06 mg kg^{-1} FM.

2. The digester with both Co and Ni supplementation could not maintain long-term stable performance either, but its VFA accumulation appeared later than the control digester (without TE) or digesters with single TE dosing (i.e. Ni alone and Co alone).

3. Fe showed antagonistic effect when supplemented with either Co or Ni and reduced their availability, whilst at the same time it proved to be an essential TE. When supplemented with a mix of Co, Ni and Fe digesters operated well for 400 days but showed VFA accumulation after 12 HRTs.

4. Se was also found to be essential for long-term stable operation of this model substrate.

5. Digester supplemented with Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM was confirmed to be sufficient for stable and optimal digester performance at OLR of 3.0 kg VS m^{-3} d^{-1}.

6. Other TE provided by model substrate was sufficient and there was no evidence that any of them was required in concentration greater than their baseline concentration over 18 HRTs (630 days).

7. The system was operated for more than 18 HRTs and clearly showed that the requirement for TE was uncoupled from the hydraulic characteristics of the digester, suggesting that the chemical species and bioavailability of the TEs is a critical element in their function and is, to a certain degree, independent of washout as the results showed that nutrients are not lost from the system simply as a hydraulic function.

Chapter 6: **Effect of Co, Ni, Fe and Se on stabilisation of mesophilic digestion at moderate OLR of 3.0 - 4.5 kg VS m^{-3} d^{-1}: their critical concentrations determination by washing out experiment**

6.1 Introduction

This work continued the work described in Chapter 5 on the required essential TEs dosing ranges under designated operational conditions using a model substrate for long-term sustainable operation at OLR 3.0 kg VS m^{-3} d^{-1}. The obtained results showed that there was a requirement to supplement Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM which was greater than those found to be naturally present in the model substrate.

According to the previous results Co, Ni, Fe and Se were highlighted as the essential TE for model substrate digestion at the OLR used and they were proven to be required to be supplemented and stable digesters had been achieved by the end of run. Although the significance of beneficial effect of TE on digestion was identified, that study left some research questions for further clarification. If they or one of them was lower than the minimum requirement, this will induce VFA production significantly. In an attempt to quantify the critical concentrations of Co, Ni, Fe and Se for stable digestion operating at moderate OLR of 3.0 - 4.5 kg VS m^{-3} d^{-1} when the rest TE existed in sufficient quantity, therefore, this set of experiment was conducted.

Four of the eight digesters previously used for the study described in Chapter 5 were directly used to start this experiment, as described in section 3.3.3. These four digesters (A1 - A4) were operated at OLR of 3.0 kg VS m^{-3} d^{-1}, HRTs of 33.3 day, supplementation with the strength Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM, for the first 175 days to establish digestion. During TE washing-out stage, in order to determine the critical total TE concentrations, from day 176 - 450, these digesters were then operated without any further selected TE dosage. The OLR and the concentration of TE supplements that remained were the same as previously used. From day 451 onwards, step wise increasing OLR from 3.0 to 4.5 kg VS m^{-3} d^{-1} had been adopt to determine if there is a higher dose requirement or whether other TE are needed.

The general approach of this study was to supplement three from four elements in sufficient amount in order to identify the minimum requirement for selected TE under designed operation. The assumption was that, the critical total TE concentration would induce VFA accumulation,

then, step-wise increase of selected TE supplementation strength when it was re-introduced would to recover process stability.

The simulation of total TE concentrations profiles calculated used mass balance approach (section 3.6.2) was compared with measured concentrations in the same sampling day for the whole experiment. A wide range of digestion parameters were measured during this study, but VFA was monitored intensively as it is the most important and prompt one to indicate the stability of digesters and therefore the effect of TE deficiency; SMP was used to evaluate the substrate conversion efficiency and VMP was employed to indicate the digestion productivity.

To achieve this, four objectives were set:

1) To dilute individual element to the point where VFA accumulation starts. This has been taken as the critical concentration for the element (Co, Ni, Fe or Se) at the loading of 3.0 kg VS m^{-3} d^{-1} used;

2) To relate the TE concentration to the digesters performance at the point of VFA accumulation (TE deficient condition) in comparing with stable performance (TE sufficient condition);

3) To restore stability to the digester by addition of the selected TE which can be used in reducing VFA levels;

4) To increase the organic loading from 3.0 to 3.5, 4.0 and 4.5 kg VS $m^{-3}d^{-1}$ in order to determine if there is a higher dose requirement or whether other TE are needed.

6.2 Experimental method

Previously TE supplementation for A1-A4 were shown in Table 6-1. The detailed operational history of each inoculum digesters were given in Chapter 5.

Table 6-1 Previously TE supplements for A1 - A4

Digesters'name used in Chapter 6 and 7	Digesters's name previously used in Chapter 5 (inoculum)	Previously TE supplements
A1	N5	Co, Ni, Fe, Se and Mo
A2	N4	Co, Ni, Fe, Al, B, Cu, Mn, Mo, Se, W, Zn
A3	N7	Co, Ni, Fe and Se
A4	N8	Co, Ni, Fe, Al, B, Cu, Mn, Mo, Se, W,

Note: TE concentration of 1.0, 1.0, 10 mg kg^{-1} FM for Co, Ni and Fe and 0.1 mg kg^{-1} FM for other elements

Methodology for process operation

6.2.1 Establishing a digestion baseline (day 0 - 175)

Briefly, lab-scale of CSTR digesters were conducted by choosing 4 of previous 8 existing digesters (from digesters N1 - N8, Chapter 5) and all digesters (A1 - A4) were then operated in a semi-continuous mode and still run according to the previous loading rate of 3.0 kg VS $m^{-3}d^{-1}$. All other elements were removed from these 4 digesters by ceasing their supplementation with the exception of Co, Ni, Fe and Se. The digesters were initially performed with sufficient TE supplementation at the strength of Co 1.0; Ni 1.0; Fe 10; Se 0.1 mg kg^{-1} FM to establish a performance and stability baseline.

6.2.2 Critical total TE concentrations determination by TE washing out experiment (day 176 onwards)

The critical total TE concentration always determined by the depletion method, in which the reactor has been run with one element washed out while still being supplemented with other essential elements (Fermoso, Bartacek and Lens, 2010; Schmidt *et al.*, 2014). Two approaches were adopted for this to identify their critical concentrations for maintaining stable digestion: TE were washed out at the beginning of the experiment and then step-wise increase of TE supplementation strength when it was re-introduced to clarify the optimum TE concentrations and determine effect of TE on VFA degradation and methane production during their washed-out and re-introduction phases.

Digesters were run at previous loading rate of 3.0 kg VS $m^{-3}d^{-1}$ without any further Co (as named A1, Co washed-out digester), Ni (as named A2, Ni washed-out digester), Fe (as named A3, Fe washed-out digester), and Se (as named A4, Se washed-out digester) dosage from day 176 onwards to the point where VFA accumulation started which was taken as the critical concentration for the element. The concentration of TE supplements that remained were the same as previously used apart from the selected one which their strengths were changing over time.

Stepwise increase of OLR from 3.0 to 3.5, 4.0 and 4.5 kg VS m^{-3} d^{-1} was applied to digesters from day 451 onwards because there was no apparent VFA accumulation. A1 - A4 were then continuously monitored to investigate if VFA accumulation resulted due to OLR increase and selected TE washing out. After TEs have been washed out, TE deficiencies can limit the functioning of anaerobic process and result in unstable performance at the critical total TE concentrations. To restore stability to the digesters, re-addition of the selected TE in step-wise manner need to be applied which could be used in reducing VFA levels. This was in an attempt

to determine the optimal dosing concentration by testing the spontaneous effect of its dosing on VFA degradation.

The measured total TE concentrations in digestate were obtained by taking representative samples for hydrochloric-nitric acid digestion followed by elemental analysis by AAS and ICP-MS (detail as described in section 3.5.10).

Digesters parameters for stably operated digesters have been carried out routinely. The digester performance were evaluated by daily measurements of biogas and CH_4 production, routine analysis of VSD, pH, alkalinity, TAN and VFA concentrations. Detail about digesters construction, substrate used, inoculum properties and TE solutions were described in section 3.1, 3.2, 3.3 and 3.4 (Chapter 3). Table 6-2 showed detail of experimental design.

Table 6-2 Experimental design and operation scheme of the digestion trial for A1 - A4

Digester	Day	Organic Loading Rate kg VS m^{-3} d^{-1}	Selected TE concentration mg kg^{-1} FM	TE addition and OLR increase
Co washed-out; A1	1 - 175	3.0	Co 1.06	Baseline establishing (section 6.2.1)
	176 - 450	3.0	Co 1.06 - 0.01	Co washing out experiment to determine the critical total Co concentration (section 6.2.2).
	451 - 492	3.5	Co 0.01	OLR increased to 3.5 kg VS m^{-3} d^{-1} from day 451, as VFA concentration were low with baseline TE concentration (0.011 mg kg^{-1} FM), to address the critical total Co concentration (section 6.2.2).
	493 - 572	4.0	Co 0.01 - 0.25	OLR increased to 4.0 kg VS m^{-3} d^{-1} from day 493 as VFA concentration were low with baseline TE concentration (0.012 mg kg^{-1} FM), to address the critical total Co concentration. This resulted in VFA accumulation, then, Co was re-introduced from day 532 in step-wise manner to the recommended concentration to test if this addition could lower VFA concentration. This was to investigate if there is a higher Co dosage requirement or whether other TE were needed when OLR has been increased (section 6.2.2).
Ni washed-out; A2	1 - 175	30	Ni 1.07	Baseline establishing

Digester	Day	Organic Loading Rate kg VS m^{-3} d^{-1}	Selected TE concentration mg kg^{-1} FM	TE addition and OLR increase
	176 - 450	3.0	Ni 1.07 - 0.03	Ni washing out experiment to determine the critical total Ni concentration.
	451 - 572	3.5	Ni 0.03 - 0.84	OLR increased to 3.5 kg VS m^{-3} d^{-1} from day 451 as VFA concentration were low with baseline TE concentration (0.03 mg kg^{-1} FM), to address the critical total Ni concentration. This resulted in VFA accumulation, then, Ni was re-added from day 479 in step-wise manner to the recommended concentration to test if this addition could lower VFA concentration. This was to investigate if there is a higher Ni dosage requirement or whether other TE were needed when OLR has been increased.
Fe washed-out; A3	1 - 175	3.0	Fe 12.88	Baseline establishing
	176 - 238	3.0	Fe 12.88 - 4.07	Fe washing out experiment to determine the critical total Fe concentration.
Fe washed-out; A3	239 - 308	3.0	Fe 4.07 - 7.04	Fe was re-introduced in step-wise manner to test if this addition could lower VFA concentration. Critical total Fe concentration could be determined.
	309 - 350	3.0	Fe 6.16 - 3.89	Fe was washing out again to confirm the critical total Fe concentration.

Digester	Day	Organic Loading Rate kg VS m^{-3} d^{-1}	Selected TE concentration mg kg^{-1} FM	TE addition and OLR increase
	351 - 450	3.0	Fe 3.89 - 5.84	Fe was added in step-wise manner to the point where VFA could be reduced. Critical total Fe concentration and recommended Fe dosing range can be addressed.
	451 - 492	3.5	Fe 5.95 - 6.24	OLR increased to 3.5 kg VS m^{-3} d^{-1} from day 451, as VFA concentration were low. Fe was added and maintained at around 6.2 mg kg^{-1} FM with success to keep stable process performance.
	493 - 572	4.0	Fe 6.24 - 20.27	OLR increased to 4.0 kg VS m^{-3} d^{-1} from day 493 resulted in VFA accumulation, then, Fe was added from day 519 in step-wise manner to above 20 mg kg^{-1} FM to test if this addition could lower VFA concentration. This was to investigate if there is a higher Fe dosage requirement or whether other TE were needed when OLR has been increased.
Se washed-out ; A4	1 - 175	3.0	Se 0.14	Baseline establishing
	176 - 450	3.0	Se 0.14 - 0.04	Se diluting out experiment to determine the critical total Se concentration.
	451 - 492	3.5	Se 0.04 - 0.05	OLR increased to 3.5 kg VS m^{-3} d^{-1} from day 451, as VFA concentration were low at baseline TE concentration of 0.05 mg kg^{-1} FM. This was to

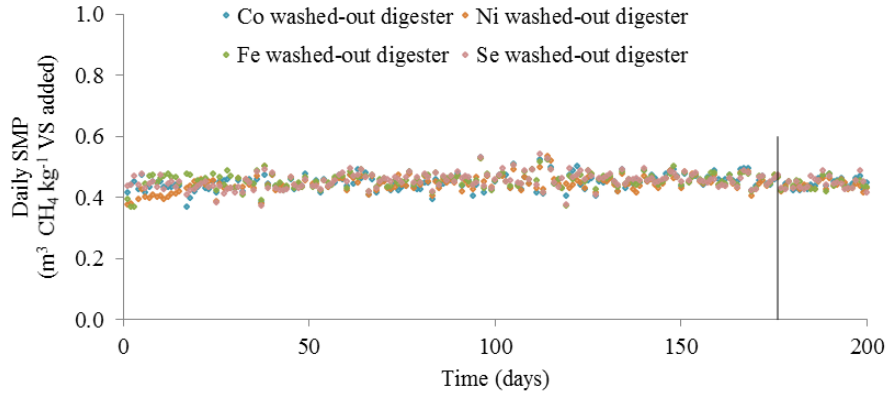
Digester	Day	Organic Loading Rate kg VS m^{-3} d^{-1}	Selected TE concentration mg kg^{-1} FM	TE addition and OLR increase
				investigate if there is a higher Se dosage requirement or whether other TE were needed when OLR has been increased.
	493 - 526	4.0	Se 0.05	OLR again increased to 4.0 kg VS m^{-3} d^{-1} from day 493, as VFA concentration were low at baseline TE concentration of 0.05 mg kg^{-1} FM. This was to investigate if there is a higher Se dosage requirement or whether other TE were needed when OLR has been increased.
	527 - 572	4.5	Se 0.06 - 0.27	OLR increased to 4.5 kg VS m^{-3} d^{-1} from day 527 resulted in VFA accumulation, then, Se was added from day 543 in step-wise manner to the recommended concentration (0.2 - 0.3 mg kg^{-1} FM) to test if this addition could lower VFA concentration. This was to investigate if there is a higher Se dosage requirement or whether other TE were needed when OLR has been increased.

Note: The concentration of TE supplements that remained were the same as previously used, Ni 1.0, Co 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM

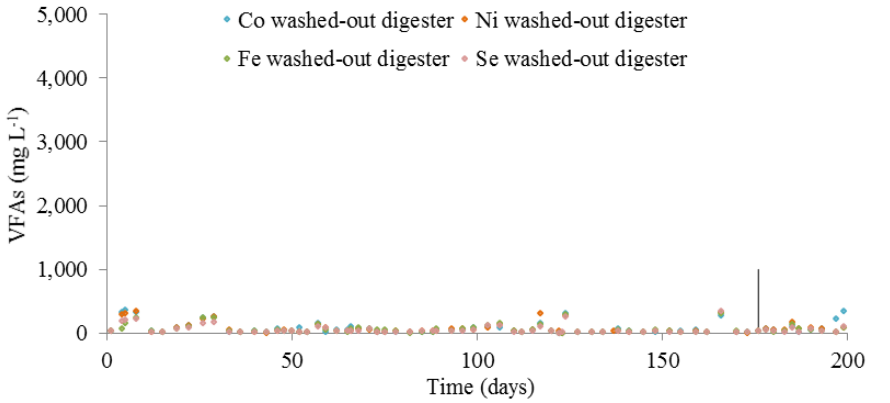
6.3 Results and discussion

6.3.1 Baseline performance and stability assessment (day 0 - 175)

There was no noticeable instability associated with the starting period. According to SMP and VFA data shown in Figure 6-1, TE washed-out digesters (A1 - A4) performed in the same manner during the first 175 days, which provided an identical start point for the operational changes on day 176. All digesters with TE supplementation at the strength of Co 1.0; Ni 1.0; Fe 10; Se 0.1 mg kg^{-1} FM were stable. From this and the previous results (Chapter 6), it indicated that these dosing strength were sufficient for stable and optimal digester performance. All the TE in feedstock were consumed, and VS of digestate was contributed by microbial biomass solely which means TE in feedstock is available to microbes in digesters, no TE washing out in un-degraded feedstock. The performance of each digester and their contribution to the aim of this study was then examined separately as follows. The TE and VFA concentrations in each digester were the main parameters used in the analysis.



(a)



(b)

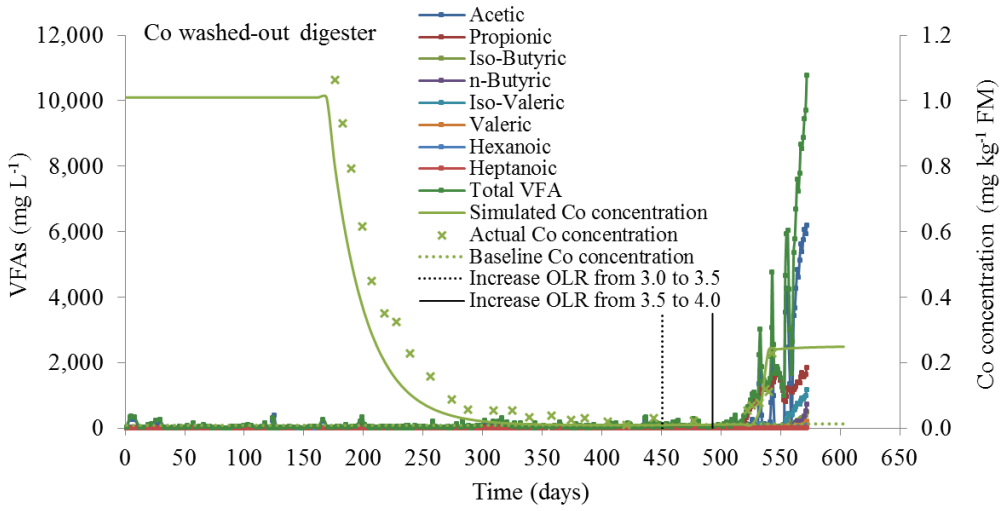
Figure 6-1 SMP and VFA profiles during baseline scenario. The vertical solid line denoted the initial TE washing out point (day 176)

During baseline scenario, biogas production and methane yield was stable with the specific CH_4 production (SMP) was 0.45 ± 0.03 m^3 CH_4 kg^{-1} VS_{added} (0.77 ± 0.04 m^3 biogas kg^{-1} VS_{added}); volumetric CH_4 production (VMP) was 1.36 ± 0.04 STP m^3 m^{-3} d^{-1} (2.32 ± 0.04 STP m^3 m^{-3} digestate d^{-1}), and the biogas CH_4 content was 58.6 ± 0.9 % (Figure 6-3). pH remained practically constant at 7.6 ± 0.1 close to optimum range for mesophilic methanagenic activity (Wheatley, 1990), digester TAN concentration was 2,642 ± 113 mg N kg^{-1}, well below the inhibitory level (Rajagopal, Massé and Singh, 2013), the IA/PA was less than 0.31 ± 0.02 in the range of optimal operation (Ripley, Boyle and Converse, 1986) (Figure 6-4). VSD was 88.7 ± 1.8 % (Figure 6-5) and no VFA accumulation was detected. All stability parameters remained in a safe range with the concentration of each volatile fatty acids (VFA) monitored never exceeded 100 mg L^{-1} which was 71 ± 12 (Figure 6-2). The average performance and monitoring parameters for optimal digestion during day 0 - 175 were shown in Table 6-3.

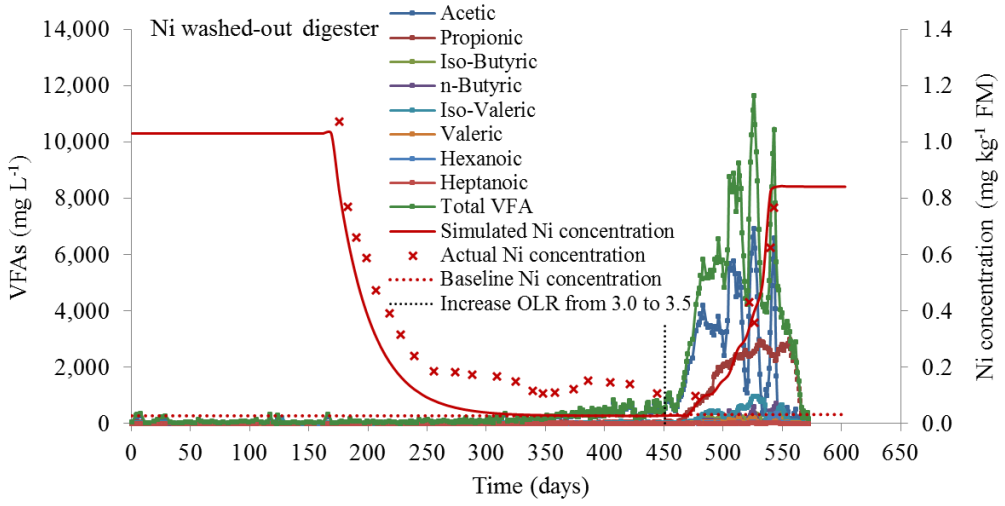
6.3.2 **Critical total TE concentrations**

It needs pointing out that critical dosage from this experimental stage was different from optimal concentration of TE required for stable process control. In this washing out stage, critical TE concentration was regarded as the minimal dosage needed to maintain stable process however the process is less tolerant to circumstance changes. Optimal concentration of TE was also determined, at which dosage stable process was achieved.

Figure 6-2 showed the concentration of VFA, measured and simulated total Co, Ni, Fe and Se concentration profiles and OLR changes throughout the trial for Co, Ni, Fe and Se washed-out digesters respectively. The washed-out curves for the digesters in which selected TE supplementation ceased on day 176. Their omission resulted in a continuous leaching of the elements. This was modelled assuming the digester to be a CSTR with HRT calculated from the working volume and the wet weight of model substrate added each day (assuming a density of 1 kg L^{-1}). The total VFA concentrations were shown on the same graph of TE analysis over the time, and the critical concentrations of the elements are taken to be at the intersection of the TE washout and VFA concentration curves. The complex experimental data obtained has been interpreted and calculated using mass balance approach (section 3.6.2). As shown in Figure 6-2 (a)-(d), VFA and the total TE concentration depletion curves, both simulated and measured values of TE concentrations have a good agreement. It could be confirmed that simulated values were similar to the actual elementals concentrations measured experimentally, which proved reliability of the simulated TEs concentration of elements from equation model used compare with results from the laboratory TE analysis. Consequently, the equation of TE concentrations calculation during TE washing out experiment could be used for same type of experiments to simulate the TE profiles.



(a)



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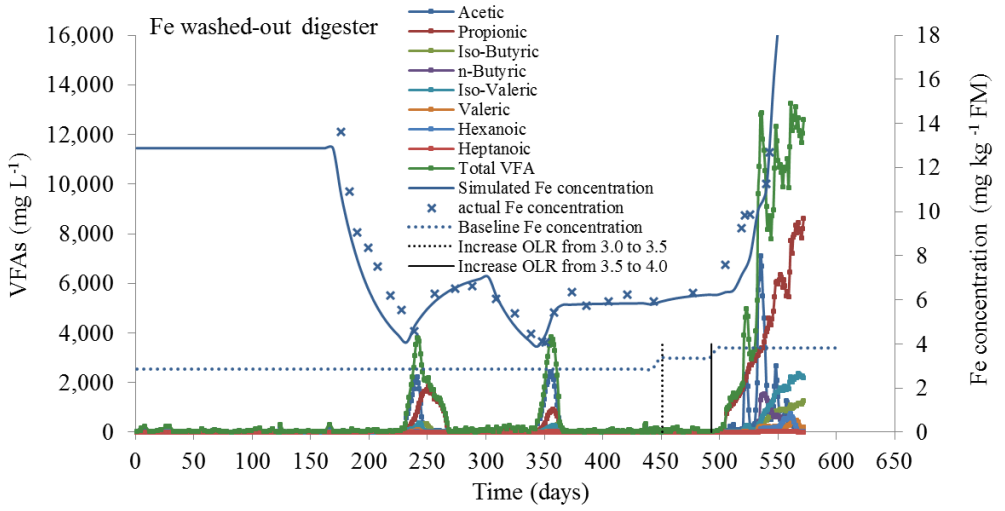
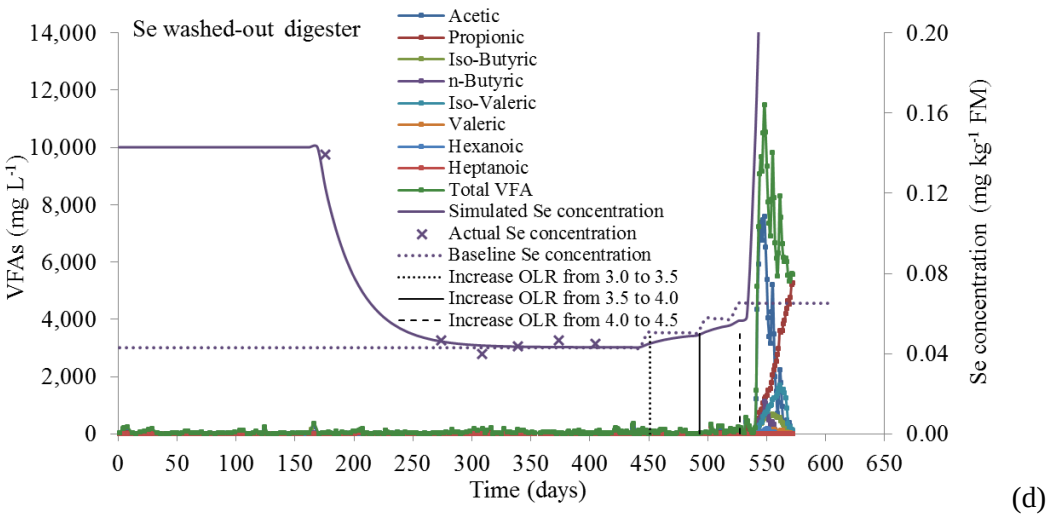


(c)

Figure 6-2 (a) - (d) VFA and TE concentration (simulated and measured values) profiles and OLR changes throughout the trial for Co, Ni, Fe and Se washed - out digesters, respectively running at OLR 3.0 - 4.5 kg VS m^{-3} d^{-1}



(d)

Figure 6-2 (a) - (d) VFA and TE concentration (simulated and measured values) profiles and OLR changes throughout the trial for Co, Ni, Fe and Se washed - out digesters, respectively running at OLR 3.0 - 4.5 kg VS m^{-3} d^{-1} (continued)

6.3.2.1 **Co washed-out digester (A1)**

6.3.2.1.1 **Critical Co concentration**

There was no noticeable instability (Figure 6-3 - Figure 6-5) associated in the form of accumulation of VFA in Co washed-out digester running at OLR 3.0 kg VS m^{-3} d^{-1} (day 176 - 450) and 3.5 kg VS m^{-3} d^{-1} (day 451 - 492) during its washed out stage. The VFA profiles were maintained at relatively low level as shown in Figure 6-2 (a). This result suggested that supplementation at the strength of Ni 1, Fe 10 and Se 0.1 mg kg^{-1} FM and Co 0.01 mg kg^{-1} FM, equal to the baseline concentration from the feed as shown in Figure 6-2 (a) was sufficient to maintain process stability.

Increase in OLR from 3.5 to 4.0 kg VS m^{-3} d^{-1} was further applied from day 493 after Co had been washed-out for 317 days. VFA accumulated corresponding to increase in OLR, unstable operation was observed in this digester within 40 days. There was a sharp increase in VFA concentration up to 2,300 mg L^{-1} on day 532 as seen in Figure 6-2 (a), reflecting a Co deficiency issue. From this result, critical Co concentration at OLR 3.5 - 4.0 kg VS m^{-3} d^{-1} were determined to be 0.01 mg kg^{-1} FM. To be more specific, at OLR 3.5 kg VS m^{-3} d^{-1}, supplementation at the strength of Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM, Co seemed to be critical when less than 0.01 mg kg^{-1} FM, equal to the baseline concentration from the feed as shown in Figure 6-2 (a) and after loading increased to 4.0 kg VS m^{-3} d^{-1}, VFA accumulated immediately.

Critical Co concentration was previously tested (Banks *et al.*, 2012). In that experiment 0.22 mg Co kg^{-1} FM was demonstrated to be critical at a moderate loading 3.0 kg VS m^{-3} d^{-1}, which is much higher than this study. The differences might be because Co and Se were washed-out

together in that study, whereas only Co was examined in this study. He (2016) also indicated that at OLR 4.0 kg VS m^{-3} d^{-1}, with 0.2 mg Se kg^{-1} FM supplementation, Co seemed to be critical when was less than 0.06 mg kg^{-1} FM.

6.3.2.1.2 **Effect of Co on VFA degradation and methane production during its washed out and re-dosed period**

Stability indicator - VFA

VFA accumulation occurred in the Co washed-out digester after OLR increase from 3.5 to 4 kg VS m^{-3} d^{-1} on day 493 which rose to 2,300 mg L^{-1} on day 532 as shown in Figure 6-2 (a). pH dropped corresponding to IA/PA ratio increase reflecting the severe VFA accumulation. All parameters indicated that Co reached a critical level at 0.01 mg kg^{-1} FM and was deficient to maintain optimal process performance at OLR 4.0 kg VS m^{-3} d^{-1}.

At that point, Co was re-introduced to this digester initially at the strength of 0.06 mg kg^{-1} FM on day 532, and its dosing was incrementally increased to 0.08, 0.12, 0.24 and maintained at 0.25 mg kg^{-1} FM until the end of run (day 572) as shown in Figure 6-2 (a). This was in attempt to bring the digester performance back to normal range by reducing VFA concentration. The range of Co dosing was based on the recommended concentration which could maintain process stability for moderate loading from the previous researches which was around 0.01 - 0.25 mg Co kg^{-1} FM (Pobeheim *et al.*, 2010; Demirel and Scherer, 2011; Banks *et al.*, 2012; Gustavsson *et al.*, 2013a).

During the period of Co dosage increase, total VFA further increased from around 2,300 up to higher than 11,000 mg L^{-1} which 6,100 mg L^{-1} acetic acid was the predominant followed later by 1,600 mg L^{-1} propionic acid, 1,200 mg L^{-1} iso-valeric and 750 mg L^{-1} n-butyric. VFA did not show decline except several sharply dropped especially acetic acid following each dosage increased. Ceased feeding had to be adopted on day 543 to lower the loading. Fluctuations of VFA might be caused by different strength and frequency of Co supplementation.

VFA accumulation still existed, process performance could not be recovered to the normal operation at OLR 4.0 kg VS m^{-3} d^{-1} with this recommended concentration of 0.25 mg Co kg^{-1} FM by the end of experiment (day 572). This observation proved that at low loadings of 3.0 and 3.5 kg VS m^{-3} d^{-1} in this experiment, Co appeared to be sufficient to supply the needs of the enzymes in the system; but it is clear and in agreement with Banks *et al.* (2012) that at the higher loading rate at 4.0 kg VS m^{-3} d^{-1}, Co become limiting, although this Co limited phenomenon had not been observed until day 532.

It was hypothesised that with adequate Co dosage, VFA accumulation could be resolved rapidly. These results indicated that Co supplementation stimulated VFA degradation, but insufficient

strength could not ensure their complete and timely degradation until a sufficient strength reached. The unrecoverable performance could be explained by this way. Insufficient Co addition was unlikely to stimulate methanogenesis for VFA degradation, but instead enhanced its accumulation. Sudden excessive Co supplementation strongly stimulated VFA accumulation to a great extent, which did inhibit recovery process. In some cases it could induce severe VFA accumulation and failure of digester when it is added to VFA-laden digesters. In this study, non-reversible accumulation of propionic acid accounted for a large proportional of total VFA caused by Co washed-out, mainly because propionic acid oxidation and the following methanogenesis need Co-depending enzymes for their reactions. Propionic acid oxidation produces mixed products of acetate, CO_2, H_2 and formate, which take part in reactions of methane productions (Muller *et al.*, 2010). Insufficient Co supplementation was unable to solve the issue of balancing propionic acid production and degradation.

Optimum Co dosing strength was previously studied. He (2016) suggested that Co supplementation to food waste digester running at OLR 3.0 - 4.0 kg VS m^{-3} d^{-1} should be around 0.3 - 0.5 mg kg^{-1} FM coupled with Se 0.2 mg kg^{-1} FM which is the sufficient strength and could make balancing between propionic acid oxidation and propionic acid degradation. In the same study, Co dosing strength up to 0.5 mg kg^{-1} FM was proposed to stimulate more propionic degradation rate. The demand for TEs is also thought to be dependent on the turnover of metabolites which in turn depends on the organic loading on the system: for example in food waste digestion a demand for Co above the baseline availability in food waste itself is only found when the organic loading exceeds about 5.0 kg VS m^{-3} d^{-1} (Banks *et al.*, 2012).

Results from the current study, suggested that at OLR 3.5 kg VS m^{-3} d^{-1}, this digester could operate at a critical Co concentration of 0.01 mg kg^{-1} FM when it was supplemented with Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM. This level of Co dosing, however, was not sustainable and not sufficient in case of instability initiated for instance by increasing organic loading. From this result, however, it appeared that the recovery strength of Co dosage required was much higher compared with its critical concentration and the recommended TE concentration required for stable process control, especially at high level of VFA.

Digester's parameters

Co washed-out digester operated normally for 492 days under OLR 3.0 - 3.5 kg VS m^{-3} d^{-1}. From Figure 6-3, this digester showed relatively consistent SMP, whereas VMP increased reflecting the OLR increased. Digester showed a good performance (with SMP 0.45 ± 0.02 m^3 CH_4 kg^{-1} VS_{added} d^{-1} and VMP 1.34 ± 0.06 m^3 CH_4 m^{-3} digestate d^{-1} for OLR 3.0 kg VS m^{-3} d^{-1} (day 176 - 450) and SMP 0.45 ± 0.03 m^3 CH_4 kg^{-1} VS_{added} d^{-1} and VMP 1.56 ± 0.09 m^3 CH_4 m^{-3} digestate d^{-1} for OLR 3.5 kg VS m^{-3} d^{-1} (day 451 - 492). Both SMP and VMP showed no apparent influence caused by

Co washing out, indicating sufficient TE addition at the strength of Ni 1.0, Fe 10, Se 0.1 and Co 0.01 mg kg^{-1} FM.

After loading increased from 3.5 to 4.0 kg VS m^{-3} d^{-1} had been applied on day 493, VFA accumulation corresponded to the IA/PA ratio rose to around 1.3 (Figure 6-4). pH dropped from 7.71 to 7.35 (Figure 6-4) reflecting the change in the ratio of IA/PA. When VFA appeared after OLR increased to 4.0 kg VS m^{-3} d^{-1}, gas production showed slight increase for a while but dropped sharply after rapid VFA accumulation resulted in drop in VMP and SMP (Figure 6-3). Both VMP and SMP appeared gas peaks even once dropped to 0.67 m^3 CH_4 m^{-3} d^{-1}, with 0.17 m^3 CH_4 kg^{-1} VS d^{-1} respectively.

By the end of run process performance could not be recovered to normal operation. Based on gas production observation, it was suggested that when OLR increased, gas production decrease indicated that TE supplementation at the strength of Ni 1.0, Fe 10, Se 0.1 and Co 0.25 mg kg^{-1} FM was insufficient. Co strength seemed to be limiting due to loss of performance under OLR 4.0 kg VS $m^{-3}d^{-1}$. Methane percentage changed and remained around 58 ± 0.1 % during stable stage but declined to 41% under unstable condition. TAN concentration increased after increased OLR from 3.0 to 4.0 kg VS m^{-3} d^{-1} was adopted from 2,500 to 3,700 mg kg^{-1} FM (Figure 6-4). Digesters' monitoring parameters during stable digestion at OLR 3.0 kg VS $m^{-3}d^{-1}$ were given in Table 6-3.

6.3.2.2 **Ni washed-out digester (A2)**

6.3.2.2.1 **Critical Ni concentration**

There were no significant differences in digesters' stability, as seen in Figure 6-3 - Figure 6-5, were noticed when running at OLR 3.0 kg VS m^{-3} d^{-1} (day 176 - 450) for Ni washed-out digester. This result suggested that the strength of Co 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM supplementation and Ni 0.03 mg kg^{-1} FM, equal to the baseline concentration from the feed as shown in Figure 6-2 (b) was sufficient to maintain process stability.

After loading increase from 3.0 to 3.5 kg VS m^{-3} d^{-1} was applied on day 451, the successful continuous operation could not be achieved in this digester. It was observed that an earlier and more rapid VFA accumulation appeared in Ni washed-out digester than Co washed-out digester. These results are in agreement with Gustavsson *et al.* (2013a) who indicated that Co is more available than Ni also Co took longer washing-out time to wash out to the critical concentration. The deficiency of Ni at this OLR resulted in process instability. The total VFA increased from below 500 mg L^{-1} on day 451 to 4,600 mg L^{-1} on day 479 (Figure 6-2 (b) at the point where the

total Ni concentration of 0.03 mg kg^{-1} FM, equal to the baseline concentration from the feed as shown in Figure 6-2 (b).

From this result, critical Ni concentration at OLR 3.0 - 3.5 kg VS m^{-3} d^{-1} was determined to be 0.03 mg kg^{-1} FM; to be more accurate, 0.03 mg Ni kg^{-1} FM was critical when supplementation at the strength of Co 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM for digester running at OLR 3.0 kg VS m^{-3} d^{-1}, after loading increased to 3.5 kg VS m^{-3} d^{-1}, VFA accumulated immediately.

6.3.2.2.2 **Effect of Ni on VFA degradation and methane production during its washed out and re-dosed period**

Stability indicator – VFA

From Figure 6-2 (b), rapid VFA accumulation on day 479 (with 3,500 mg L^{-1} acetic acid and 700 mg L^{-1} propionic) occurred after OLR increased from 3.0 to 3.5 kg VS m^{-3} d^{-1} on day 451. pH dropped corresponding to IA/PA ratio increase reflecting the severe VFA accumulation. All monitoring parameters indicated that Ni reached a critical level at 0.03 mg kg^{-1} FM during that stage.

At that point, Ni was re-dosed to this digester initially at the strength of 0.07 mg kg^{-1} FM on day 479, and its dosing was incrementally increased to 0.10, 0.15, 0.18, 0.26, 0.30, 0.39, 0.49, 0.82 and maintained constant at 0.84 mg kg^{-1} FM until the end of run (day 572) as shown in Figure 6-2 (b). This was in attempt to recover the digester performance by reducing VFA concentration. VFA further increased from around 4,700 mg L^{-1} higher up to 12,000 mg L^{-1} on day 526. During the period of dosage increase, VFA did not show decline except several sharply dropped following each dosage increased. Temporary cease of feeding had to be adopted during the recovery period to lower the loading which could help in reducing acetic acid, however, propionic acid still increased. Building up of predominantly single VFA, such as acetic, propionic and iso-valeric acid to 6,600, 2,500 and 900 mg L^{-1} respectively was observed.

Successive increase the Ni dosing in this digester was applied between day 526 to day 572 taking the concentration to the strength of around 0.6 - 0.8 mg Ni kg^{-1} FM. This together with an interval feeding could bring the digester stability back under control. After that, organic loading was immediately raised back and maintained at 3.5 kg VS m^{-3} d^{-1} until the end of trial. Since sufficient high strength Ni was added in time, eventually, VFA that had accumulated was consumed to lower than 300 mg L^{-1} and converted to methane completely in the following 32 days. This indicated that Ni supplementation stimulated VFA degradation, but insufficient strength could not ensure its complete and timely degradation until a sufficient strength was reached. During the VFA increase period, acetic acid accounted for the major proportion followed by propionic acid.

It is noteworthy there was no further VFA accumulation after Ni addition reached 0.6 mg kg^{-1} FM, acetic acid quickly decreased when Ni strength was increased to 0.6 - 0.8 mg kg^{-1} FM whereas propionic acid showed obvious degradation after acetic acid had been already consumed. This response suggested that at a loading of 3.5 kg VS $m^{-3}d^{-1}$, other TE was presenting in adequate strength to maintain stable digestion. The digester operated at OLR 3.5 kg VS $m^{-3}d^{-1}$ and the rest of VFA species existed at very low levels throughout the experiment.

From data obtained, it is clear that the Ni strength of around 0.6-0.8 mg kg^{-1} FM for OLR 3.5 kg VS m^{-3} d^{-1} was sufficient to recover digester performance by significantly reducing VFA concentration from 12,000 mg L^{-1} to below 300 mg L^{-1} and could maintain stable operation at the strength of Co 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM supplementation. These results are in agreement with those obtained from Pobeheim *et al.* (2011) who reported that process stability of maize silage digestion was recovered up to an OLR of 4.3 kg VS m^{-3} d^{-1} by gradually increasing Ni concentration to 0.6 mg kg^{-1} FM, however, 0.88 mg kg^{-1} FM did not further enhance biogas performance. Evranos and Demirel (2014) noted that the highest methane yield of 0.429 m^3 CH_4 kg^{-1} VS_{added} was obtained by dosing Ni 0.5 in combination of Co 0.5 and Mo 0.25 mg kg^{-1} for mono-digestion of maize silage. In addition, typically reported stimulatory concentration for Ni range should be between 0.05 - 0.6 mg kg^{-1} FM (Takashima, Speece and Parkin, 1990; Demirel and Scherer, 2011; Schattauer *et al.*, 2011; Ortner *et al.*, 2015).

Results from the current study, suggested that at OLR 3.0 kg VS m^{-3} d^{-1}, the digester could perform normally at a critical total Ni concentration of 0.03 mg kg^{-1} FM when it was supplemented at the strength of Co 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM. This minimum concentration, however, was not sustainable and not sufficient if instability initiated for instance by organic loading increase. Therefore, the optimum Ni dosing strength was 0.6 - 0.8 mg kg^{-1} FM for loading 3.5 kg VS m^{-3} d^{-1} according to this experiment which did bring the digester performance to the normal operation and could maintain process stability. The test demonstrated that after long - term washed out, TE deficiency appeared earlier at higher OLR.

Digester's parameters

The Ni washed-out digester operated normally for 450 days under OLR 3.0 kg VS m^{-3} d^{-1} with SMP 0.44 ± 0.02 m^3 CH_4 kg^{-1} VS_{added} d^{-1} and VMP 1.33 ± 0.06 m^3 CH_4 m^{-3} digestate d^{-1} as shown in Figure 6-3 which is not significantly different from baseline digestion (Table 6-3). Both SMP and VMP maintained stable without the appearance caused by Ni washing out, indicating sufficient TE addition at the strength of Co 1.0, Fe 10, Se 0.1 and Ni 0.03 mg kg^{-1} FM.

VFA accumulation after loading increased from 3.0 to 3.5 kg VS m^{-3} d^{-1} on day 451, corresponding to the IA/PA ratio rise to above 2.1 (Figure 6-4). pH dropped from 7.60 to 7.03 (Figure 6-4)

reflecting the change in the ratio of IA/PA. These resulted in lower stable operation and lower specific methane yield (Figure 6-3). When VFA appeared after increased OLR, gas production showed slight increase but dropped sharply after rapid VFA accumulation, VMP even once dropped to 0.14 m^3 CH_4 m^{-3} d^{-1}, with 0.08 m^3 CH_4 kg^{-1} VS d^{-1} SMP (Figure 6-3). During a short interval feeding and digester recovered period from day 526 to day 572, both VMP and SMP increased significantly. OLR was switched back to OLR 3.5 kg VS m^{-3} d^{-1}, VMP and SMP appeared gas peaks followed by decline, reaching stable production finally.

The SMP and VMP during the trial period are presented in Figure 6-3. The digester showed relatively consistent SMP, whereas VMP increased reflecting the OLR increase. This increase was caused by more complete substrate conversion after the product induced feedback inhibition was released after VFA decrease. Based on gas production observation, it was suggested that with sufficient TE supplementation at the strength of Co 1.0, Ni 0.8, Fe 10 and Se 0.1 mg kg^{-1} FM when increased OLR, no gas production decrease was observed after stable operation was reached, also the remained elements seemed not to be limiting due to no loss of performance under OLR 3.5 kg VS m^{-3} d^{-1}. Methane percentage changed and remained around 58-59 % during stable operation but declined to even 37% (Figure 6-3) in restored period, however, increased to normal range after process recovery. TAN concentration increased from 2,500 to 3,600 mg kg^{-1} FM (Figure 6-4) after increased OLR from 3.0 to 3.5 kg VS m^{-3} d^{-1} was adopted. Digesters' monitoring parameters during stable digestion at OLR 3.0 kg VS m^{-3} d^{-1} were given in Table 6-3.

6.3.2.3 **Fe washed out digester (A3)**

6.3.2.3.1 **OLR 3.0 kg VS m^{-3} d^{-1}**

Critical Fe concentration and the effect of Fe on VFA degradation and methane production during its washed out and re-dosed

As can be seen in Figure 6-2 (c), although Co, Ni and Se washed-out digesters still could maintain stable operation without VFA accumulation for more than 8 retention times after TE washed-out (day 176 - 450) at OLR 3.0 kg VS m^{-3} d^{-1}, the successful continuous operation could not be achieved in Fe washed - out digester after its supplementation stopped for 2 HRTs. This findings was in agreement with Schmidt *et al.* (2014) who found that an impact of Fe deficiency was observed after Fe depletion for 2 HRTs and indicated that depletion of Fe seems to influence not only methanogens but propionate oxidizing bacteria as well. According to both groups have hydrogenases which can contain Fe and Ni, however, the requirement of Fe is higher than others and therefore Fe deficiency is having a faster and greater impact compared to Ni deficiency (Worm *et al.*, 2009).

Fe washed-out digester showed unstable performance, this changes indicated an acidification of the process due to Fe shortage. The deficiency of Fe led to a rapid VFAs accumulation from day 229 (Figure 6-2 (c), principally acetic and propionic acid, which was observed at above 3,600 mg L^{-1} on day 243 when the Fe concentration reached around 4.0 - 5.0 mg kg^{-1} FM. These resulted in around 30% reducing in biogas yield, along with pH dropped from 7.63 to 7.49 and IA/PA ratio raised to 0.67 as can be seen in Figure 6-4. Due to a fast VFA accumulation occurred in this digester, consequently, Fe was re-introduced to this digester from day 239 in attempt to recover process stability by reducing VFA concentration. After Fe was incrementally re-dosed from 4.0 to 7.0 mg Fe kg^{-1} FM on day 302, process performance could be recovered to the normal operation. After that, Fe was then washed out again from day 309 to establish its critical total Fe concentration, which was confirmed to be around 4.0 - 5.0 mg kg^{-1} FM.

To be more specific, at OLR 3.0 kg VS m^{-3} d^{-1}, supplementation at the strength of Co 1.0, Ni 1.0 and Se 0.1 mg kg^{-1} FM, Fe seemed to be critical when its concentration was less than 5.0 mg kg^{-1} FM. Re-dosing Fe did decrease VFA concentration from almost 3,800 (on day 357) to less than 100 mg L^{-1} (day 371). VFA was converted to methane completely as presented in Figure 6-2 (c) and the digester continued to perform well for the following 80 days in the presence of Fe at the strength of 5.8 mg kg^{-1} FM before OLR increase was applied.

The critical total Fe concentration results match those observed in earlier studies that the required concentration of Fe for CSTR type of mesophilic AD of food waste operated at 1.0 - 4.0 kg VS m^{-3} d^{-1} was 5.0 mg kg^{-1} FM (Zhang *et al.*, 2015) at the strength of Se 0.2, Co 1.0 and Ni 1.0 mg kg^{-1} FM and the required Fe concentration for AD of organic solid waste operated at 3.0 - 3.5 kg TS m^{-3} d^{-1} was 5.2 mg kg^{-1} FM at the strength of Ni 0.04 and Co 0.16 mg kg^{-1} FM (Uemura, 2010).

Digesters' parameters

The successful continuous operation could not be achieved in Fe washed-out digester after its supplementation stopped for 2 HRTs under OLR 3.0 kg VS m^{-3} d^{-1}. VFA accumulation corresponded to the IA/PA ratio rise to above 0.65 (Figure 6-4). pH dropped from 7.63 to 7.34 (Figure 6-4) reflecting the change in the ratio of IA/PA. These resulted in lower stable operation and lower specific methane yield (Figure 6-3). Gas production dropped sharply after rapid VFA accumulation, VMP even once dropped to 0.93 m^3 CH_4 m^{-3} d^{-1}, with SMP 0.31 m^3 CH_4 kg^{-1} VS d^{-1} (Figure 6-3). During the digester recovery period, both VMP and SMP increased significantly, appeared gas peaks followed by decline, eventually reached stable production as seen in Figure 6-3. This SMP and VMP increase was caused by more complete substrate conversion after the product induced feedback inhibition was released after VFA decrease, however changed to normal range after process recovery with SMP 0.44 ± 0.02 m^3 CH_4 kg^{-1} VS_{added} d^{-1} and VMP 1.32 ± 0.03 m^3 CH_4 m^{-3} digestate d^{-1} as shown in Figure 6-3 and Table 6-3.

Based on gas production observation, it was suggested that with sufficient TE supplementation at the strength of Co 1.0, Ni 1.0, Fe 5.8 and Se 0.1 mg kg^{-1} FM, no gas production decreases were observed after operation been recovered, also the remained elements seemed not to be limiting due to no loss of performance under OLR 3.0 kg VS m^{-3} d^{-1}. Methane percentage changed and remained around 58 ± 1.0 % during stable operation but declined to 52% (Figure 6-3) in restored period, however, increased to normal range after process recovery. TAN concentration was maintained at 2,614 ± 128 mg L^{-1} (Figure 6-4). Digesters' monitoring parameters during stable digestion at OLR 3.0 kg VS m^{-3} d^{-1} were given in Table 6-3.

6.3.2.3.2 **OLR higher than 3.0 kg VS m^{-3} d^{-1}**

6.3.2.3.2.1 Critical Fe concentration

There was no significant differences in digesters' stability (Figure 6-3 - Figure 6-5) was noticed when running at OLR 3.0 kg VS m^{-3} d^{-1} (day 176 - 450) and 3.5 kg VS m^{-3} d^{-1} (day 451 - 492) when supplementation at the strength of Co 1.0, Ni 1.0, Fe 6.2 and Se 0.1 mg kg^{-1} FM. The VFA were maintained at relative low level without accumulation. This result suggested that supplementation with this strength was sufficient to maintain process stability.

From day 493, this digester was run with OLR 4.0 kg VS m^{-3} d^{-1}. VFA accumulated corresponding to increase OLR, lower stable operation was observed within 27 days. VFA concentration increased sharply (Figure 6-2 (c)), reflecting the deficiency of Fe in digester. From this result, critical Fe concentration at OLR 3.5 - 4.0 kg VS m^{-3} d^{-1} were determined to be 6.2 mg kg^{-1} FM. To be more specific, at OLR 3.5 kg VS m^{-3} d^{-1}, Fe was critical when its concentration less than 6.2 mg Fe kg^{-1} FM with the strength of Co 1.0, Ni 1.0 and Se 0.1 mg kg^{-1} FM supplementation, and after loading increased to 4.0 kg VS m^{-3} d^{-1}, VFA accumulated immediately.

6.3.2.3.2.2 Effect of Fe on VFA degradation and methane production during its washed out and re-dosed period

Stability indicator – VFA

VFA accumulation occurred in the Fe washed-out digester after increased OLR from 3.5 to 4 kg VS m^{-3} d^{-1} on day 493 which rose to 1,900 mg L^{-1} on day 519 as shown in Figure 6-2 (c). pH dropped corresponding to IA/PA ratio increase reflecting the severe VFA accumulation. All parameters indicated that Fe reached a critical level at 6.2 mg kg^{-1} FM and was deficient to maintain optimal process performance at OLR 4.0 kg VS m^{-3} d^{-1}.

At that point, Fe was re-introduced to this digester initially at strength of 7.14 mg kg^{-1} FM on day 519 and its dosing was incrementally increased to 7.95, 10.08, 11.02, 16.45 and maintained at

around above 20 mg kg^{-1} FM till the end of trial (572 days). This was in attempt to recover the digester performance by reducing VFA concentration.

During the period of dosage increase (day 519 - 572), fluctuations of VFA was observed which might be caused by the different strength and frequency of Fe supplementation. Total VFA did not show decline, except several sharply dropped especially acetic acid on day 523, 537 and 547 following each dosage increased and ceased feeding had to be adopted several days during recovery period to lower the loading. Total VFA further increased from 1,900 mg L^{-1} (day 519) and fluctuated around 12,000 mg L^{-1} (day 548). The build-up of propionate can be seen from day 504 and become the predominant VFA which showed severe increase up to 8,600 mg L^{-1} followed later by 2,200 mg L^{-1} iso-valeric and 1,300 mg L^{-1} iso-butyric by the end of trail (day 536 to day 572). It seems that the threshold of inhibition as indicated by increasing propionic acid concentration, which was reported to be at 2,000 mg L^{-1} (Ma *et al.*, 2009), was exceeded in this digester. The stop of substrate addition has only resulted in a short-term improvement of the process stability but has not led to a significant reduction of the propionic acid concentration. With this recommended Fe concentration dosing, however, these findings together with other parameters indicating process instability. Fe addition did not show the result in process recovery from un-balanced process operation by the end of run.

In this study, non-reversible accumulation of propionic acid accounted of total VFA caused by Fe washed-out, mainly because propionic acid oxidation and the following methanogenesis need Fe-depending enzymes for their reactions. Propionic acid oxidation produces mixed products of acetate, CO_2, H_2 and formate, which take part in reactions of methane productions (Muller *et al.*, 2010). These experimental results supported Schmidt *et al.* (2014) who indicated that depletion of Fe seems to influence not only methanogens but propionate oxidizing bacteria as well. According to both groups have hydrogenases which can contain Fe and Ni (Worm *et al.*, 2009). Similar findings were reported by Osuna *et al.* (2003a) whereby TE (Ni, Co, Fe, Se, Mo, Cu, Zn, and Mn) depletion resulted in propionate accumulation. Then, insufficient Fe supplementation was unable to solve the issue of balancing propionic acid production and degradation.

The results indicated that TE addition, to a large extent, cannot initiate the VFA consumption process in digester with high VFA concentrations. It appeared that after a digester has been subjected to accumulation of VFA for a period of time, the onset of VFA degradation depends on other factors in addition to those of TE concentrations. Once the VFA degradation process has started the supplementation of a specific TE or multicomponent TE matrix can, however, accelerate the VFA consumption rate. As such strategy for stable digestion should focus on the prevention of initial VFA accumulation in the digester by TE supplementation, rather than the recovery of a severely VFA-laden digester. This result supported by the very stable operation without VFA accumulation

for more than 540 days in Se washed-out digester (A4) supplementation at with strength of TE supplementation at Co 1.0, Ni 1.0, Fe 10 and Se 0.05 mg kg^{-1} FM at the same OLR.

Results from the current study, suggested that at OLR 3.5 kg VS m^{-3} d^{-1}, this digester operated at a critical Fe concentration of 6.2 mg kg^{-1} FM when it was supplemented with Co 1.0, Ni 1.0 and Se 0.1 mg kg^{-1} FM. This level of Fe dosing, however, was not sustainable and not sufficient in case of instability initiated for instance by increasing organic loading. It was further complicated by the changes in loading which could have exerted a higher Fe demand. From this and the previous results as discussed for the unrecovered process stability in Co wash-out digester, it confirmed that the recovery strength of Fe dosage required was much higher compared with its critical concentration and the recommended TE concentration required for stable process control, especially at high level of VFA.

Digesters' parameters

Fe washed out digester operated at OLR 3.5 - 4.0 kg VS m^{-3} d^{-1}

Fe washed-out digester operated normally after it had been restored from process instability at OLR 3.0 kg VS m^{-3} d^{-1}. OLR was further increased to 3.5 kg VS m^{-3} d^{-1} on day 451. This digester operated normally during day 451 to day 492 and showed relatively consistent SMP, whereas VMP increased reflecting the OLR increased (with SMP 0.45 ± 0.02 m^3 CH_4 kg^{-1} VS_{added} d^{-1} and VMP 1.56 ± 0.08 m^3 CH_4 m^{-3} digestate d^{-1}). Both SMB and VMB maintained stable indicating sufficient TE addition with the strength of Co 1.0, Ni 1.0, Fe 6.2 and Se 0.1 mg kg^{-1} FM.

After loading increased from 3.5 to 4.0 kg VS m^{-3} d^{-1} had been applied on day 493, VFA accumulation corresponded to the IA/PA ratio rose to around 1.92 on day 537 (Figure 6-4). pH dropped from 7.70 to 7.22 (Figure 6-4) reflecting the change in the ratio of IA/PA. These resulted in lower stable operation and lower specific methane yield (Figure 6-3). When VFA appeared after OLR increased to 4.0 kg VS m^{-3} d^{-1}, gas production showed short increase but dropped sharply after rapid VFA accumulation.

By the end of run process performance could not be recovered to normal operation. Based on gas production observation, it was suggested that when OLR increased, gas production decreased indicated that TE supplementation at the strength of Ni 1.0, Co 10, Fe 20 and Se 0.1 mg kg^{-1} FM was insufficient. Fe strength was limiting due to loss of performance under OLR 4.0 kg VS $m^{-3}d^{-1}$. Methane percentage changed and remained around 58 - 59 % during stable operation but declined to even 40% (Figure 6-3) in restored period, however, increased to normal range after process recovery. TAN concentration increased from 2,500 to 3,700 mg kg^{-1} FM (Figure 6-4) after increased OLR from 3.0 to 4.0 kg VS m^{-3} d^{-1} was adopted. Digesters' monitoring parameters during stable digestion at OLR 3.0 kg VS m^{-3} d^{-1} were given in Table 6-3.

6.3.2.4 **Se washed-out digester (A4)**

6.3.2.4.1 **Critical Se concentration**

Digester with Se washed-out showed a good performance (Figure 6-3 - Figure 6-5) and continued to maintain stable operation without VFA accumulation for 526 days (Figure 6-2 (d)) after running at OLR of 3.0 kg VS m^{-3} d^{-1} (day 176 - 450), 3.5 kg VS m^{-3} d^{-1} (day 451 - 492) and 4.0 kg VS m^{-3} d^{-1} (day 493 - 526). This digester took longer washing out time to wash Se out to the critical concentration and had higher tolerance to Se deficiency. This result suggested that supplementation at the strength of Co 1.0, Ni 1.0 and Fe 10 mg kg^{-1} FM together with Se at the its baseline level as shown in Figure 6-2 (d), was sufficient to maintain process stability. The baseline Se concentration were 0.04, 0.05, 0.05 and 0.06 mg kg^{-1} FM for OLR 3.0, 3.5, 4.0 and 4.5 kg VS m^{-3} d^{-1}, respectively.

Increase in OLR from 4.0 to 4.5 kg VS m^{-3} d^{-1} was further applied on day 527 after Se had been washed-out for 350 days (> 10 retention times). The results showed that the successful continuous operation could not be achieved, VFA concentration showed dramatic increase from below 200 to around 7,400 mg L^{-1} in 17 days, corresponding to increase in OLR, Unstable operation and instability in monitoring process parameters were observed (Figure 6-3 - Figure 6-5). All parameters indicated that Se reached a critical level at 0.06 mg kg^{-1} FM and was deficient to maintain optimal process performance at OLR 4.5 kg VS m^{-3} d^{-1}.

From Figure 6-2 (d), critical Se concentration at OLR 4.0 - 4.5 kg VS m^{-3} d^{-1} was determined to be 0.06 mg kg^{-1} FM; to be more accurate, 0.06 mg Se kg^{-1} FM was critical with supplementation at the strength of Co 1.0, Ni 1.0 and Fe 10 mg kg^{-1} FM for digester running at OLR 4.0 kg VS m^{-3} d^{-1}, and after loading increased to 4.5 kg VS m^{-3} d^{-1}, VFA accumulated immediately.

Critical concentration of Se were previously tested (Banks *et al.*, 2012). In that experiment 0.16 mg Se kg^{-1} FM were demonstrated to be critical at a moderate loading 3.0 kg VS m^{-3} d^{-1}, which is almost three fold higher than this study. The differences might be because in that study Co and Se were washed out together, whereas only Se was examined in this digester for this study. He (2016) also indicated that when Co was supplied in sufficient quantity at the strength of 1.0 mg kg^{-1} FM, Se was critical when its concentration was less than 0.2 mg kg^{-1} at OLR 2.5 kg VS m^{-3} d^{-1}.

6.3.2.4.2 **Effect of Se on VFA degradation and methane production during its washed out and re-dosed period**

Stability indicator – VFA

An immediate accumulation in VFA occurred after OLR increase from 4.0 to 4.5 kg VS m^{-3} d^{-1} on day 527 which rose from around 200 mg L^{-1} up to above 7,400 mg L^{-1} on day 543 with most of the increase as results of acetic acid accumulation (6,000 mg L^{-1}) with the same increasing rate as total VFA. Corresponding to increase OLR, pH decrease from 7.59 to 7.27 (Figure 6-4) accompanied with IA/PA ratio rise from 0.4 to 1.1 (Figure 6-4). All parameters indicated that Se reached a critical level at 0.06 mg kg^{-1} FM and was deficient to maintain optimal process performance at OLR 4.5 kg VS m^{-3} d^{-1}. At the low loadings of 3.0 - 4.0 kg VS m^{-3} d^{-1}, Se appeared to be sufficient for stable operation; but it is clear that at the higher loading rate at 4.5 kg VS m^{-3} d^{-1}, Se become limiting, although this Se limited phenomenon had not been observed until day 540. This indicated that Se was insufficient to form key enzyme complexes Then, such accumulating in acedic might be attributed to the shortage of Se. This was in agreement with Worm *et al.* (2009) who found that the lack of Se supplementation limited the metabolic activity of Se-containing enzyme systems leading to acidification.

A rapid VFA accumulation occurred after increased OLR from 4.0 to 4.5 kg VS m^{-3} d^{-1}. At that point, Se was re-dosed to digester at strength of 0.14 mg kg^{-1} FM on day 543. The Se dosing was further increased and maintained at the concentration of 0.27 mg kg^{-1} FM until day 572. This was in attempt to recover digester performance by reducing its VFA level. The range of Se dosing was based on the recommended concentration which could maintain process stability from the previous reports which was around 0.2 mg Se kg^{-1} FM with the strength of Fe 5.0, Co 1.0 and Ni 1.0 mg kg^{-1} FM supplementations. This strength is enough for stable mode for moderate loading up to 5.0 kg VS m^{-3} d^{-1} (Banks *et al.*, 2012) and OLR 1.0 - 5.5 kg VS m^{-3} d^{-1} (Zhang *et al.*, 2015) in food waste mesophilic digester, representing a significant increase in process performance and operational stability. The required Se at this level supported by the results from He, 2016 who proved that when Co was supplied at the strength of 1.0 mg kg^{-1} FM, Se was critical for food waste digestion running at OLR 2.5 kg VS m^{-3} d^{-1}.

After this event, process operation could not bring back to normal range by the end of the experiment (day 572). Total VFA further increased from around 7,400 to 11,500 mg L^{-1} on day 548 as seen in Figure 6-2 (d), pH dropped to 7.2 accompanied with IA/PA ratio raised to 1.8 (Figure 6-4). During the digester recovery period, building up of single VFA, such as acetic, propionic, iso-valeric, iso-butyric and n-butyric acid was increased to around 7,600, 1,100, 700, 600 and 1,200 mg L^{-1} respectively (Figure 6-2 (d)) was observed. The VFA profile showed that initially acetic acid was predominant, with propionic acid and longer chain length VFA were at low concentrations as

expected. From day 548, total VFA quickly decreased from 11,500 to 5,600 mg L^{-1} with Se supplementation at the strength of 0.27 mg kg^{-1} FM.

The results are in agreement with Zhang *et al.* (2015) who demonstrated that Se was necessary in promoting the recovery of the acidification system. A couple sharp drop following increased dosage and temporary ceased feeding had to be adopted to lower the loading which could help in reducing VFA by decreasing acetic, iso-valeric, iso-butyric and n-butyric acid, however, propionic acid still showed dramatic increase higher up to 5,300 mg L^{-1} on day 572. It is noteworthy that there was no obvious accumulation of acetate, 7,600 mg L^{-1} of acetic acid was completely degraded, then, its accumulation does not appear to be inhibited. The build-up accumulation of propionic acid by the end of run might had been caused by long-term absence of Se which was also reported by Worm *et al.* (2009). It seems that the threshold of inhibition as indicated by increasing propionic acid concentration, which was reported to be at 2,000 mg L^{-1} (Ma *et al.*, 2009), was exceeded in this digester. The stop of substrate addition has only resulted in a short-term improvement of the process stability but has not led to a significant reduction of the propionic acid concentration. With this recommended Se concentration dosing, however, these findings together with other parameters indicating process instability. Se addition did not show the result in process recovery from un-balanced process operation by the end of run.

The accumulation of propionic was presumed to occur because of a deficiency Se required for synthesis of the enzymes needed in syntrophic hydrogenotrophic methane production. In particularly, it has been recognised, however, that Se has been reported as important in formate oxidation because of the requirement in the enzyme formate dehydrogenase (FDH) (Self, 2011) to convert formic acid to methane by the H_2 and CO_2 route. Then, the accumulation of formic acid will result in propionic acid not being degraded in the system. Propionic acid oxidation produced mixed products of acetate, CO_2, H_2 and formate which take part in reactions of methane productions (Muller *et al.*, 2010). It has been accepted that the enzymes required for propionic acid oxidation may themselves require Se (Muller *et al.*, 2010; Worm *et al.*, 2011). Both propionic acid oxidation and methanogenesis need enzymes for their reactions. Therefore, without sufficient TE supplementation, this was unable to balance propionic acid oxidation and to stimulate propionic acid degradation. Similar findings were reported by Osuna *et al.* (2003a) whereby TE depletion resulted in propionate accumulation.

Results from the current study suggested that at OLR 4.0 kg VS m^{-3} d^{-1}, digester could perform normally at a critical total Se concentration of 0.06 mg kg^{-1} FM when there were supplemented at the strength of Co 1.0, Ni 1.0 and Fe 10 mg kg^{-1} FM. This minimum concentration, however, was not sustainable and not sufficient in case of instability initiated for instance by organic loading increase. But it was further complicated by the changes in loading which could have exerted a higher

Se demand, also the test demonstrated that after long-term washed out, TE deficiency appeared earlier in higher OLR digester. From this and the previous results as discussed for the unrecovered process stability in Co and Fe wash-out digesters, it could be confirmed that the recovery strength of Se dosage required was much higher compared with its critical concentration and the recommended TE concentration required for stable process control, especially at high level of VFA.

Digester's parameters

Se washed-out digester operated normally for 526 days under OLR 3.0 - 4.0 kg VS m^{-3} d^{-1}. This digester showed relatively consistent SMP, whereas VMP increased reflecting the OLR increased (with SMP 0.45 ± 0.02 m^3 CH_4 kg^{-1} VS_{added} d^{-1} and VMP 1.34 ± 0.06 m^3 CH_4 m^{-3} digestate d^{-1} for OLR 3.0 kg VS m^{-3} d^{-1} (day 0 - 450), SMP 0.44 ± 0.02 m^3 CH_4 kg^{-1} VS_{added} d^{-1} and VMP 1.56 ± 0.07 m^3 CH_4 m^{-3} digestate d^{-1} for OLR 3.5 kg VS m^{-3} d^{-1} (day 451 - 492) and SMP 0.43 ± 0.02 m^3 CH_4 kg^{-1} VS_{added} d^{-1} and VMP 1.71 ± 0.11 m^3 CH_4 m^{-3} digestate d^{-1} for OLR 4.0 kg VS m^{-3} d^{-1} (day 493 - 526). Both SMP and VMP showed no apparent influence caused by Se washing out, indicating sufficient TE addition at the strength of Co 1.0, Ni 1.0, Fe 10 and Se 0.06 mg kg^{-1} FM.

VFA accumulation after loading increased from 4.0 to 4.5 kg VS m^{-3} d^{-1} on day 527, corresponded to the IA/PA ratio sharply rose to around 1.8 (Figure 6-4). pH dropped from 7.59 to 7.22 (Figure 6-4) reflecting the change in the ratio of IA/PA. When VFA appeared after OLR increased to 4.5 kg VS m^{-3} d^{-1}, gas production showed short increase but dropped sharply after rapid VFA accumulation resulted in VMP and SMP dropped (Figure 6-3). During a short interval feeding and digester recovered period from day 543 to day 572, both VMP and SMP increased. OLR was switched back to OLR 4.5 kg VS m^{-3} d^{-1}, both VMP and SMP appeared gas peaks followed by decline, reaching stable production. This SMP and VMP increase was caused by more complete substrate conversion after the product induced feedback inhibition was released after total VFA decrease.

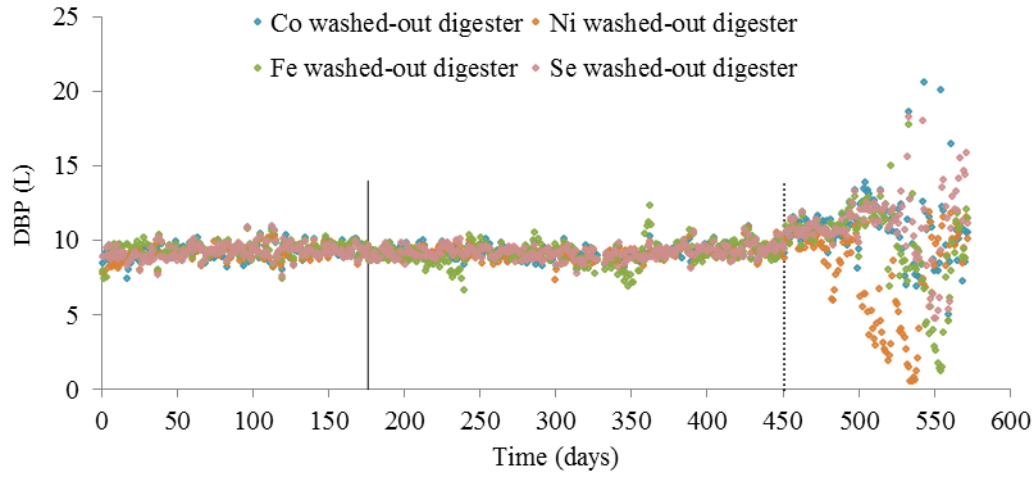
By the end of run process performance could not be recovered to normal operation. Based on gas production observation, it was suggested that when increased OLR, gas production decreased indicated that insufficient TE supplementation at the strength of Ni 1.0, Co 10, Fe 10 and Se 0.27 mg kg^{-1} FM. Se strength seemed to be limiting due to loss of performance under OLR 4.5 kg VS $m^{-3}d^{-1}$. Methane percentage changed and remained around 58-59 % during stable operation but declined to even 40% (Figure 6-3) in restored period, however, increased to normal range after process recovery. TAN concentration increased from 2,500 to 3,800 mg kg^{-1} FM (Figure 6-4) after OLR increased from 3.0 to 4.5 kg VS m^{-3} d^{-1} was adopted. Digesters' monitoring parameters during stable digestion at OLR 3.0 kg VS $m^{-3}d^{-1}$ were given in Table 6-3.

General parameters and biogas performance profiles of TE washed-out digesters were shown in Figure 6-3 - Figure 6-5. Average performance and monitoring parameters for optimal digestion at OLR 3.0 kg VS m^{-3} d^{-1} during day 0 - 450 were shown in Table 6-3.

Table 6-3 Digester efficiency and monitoring parameters during stable digestion (day 0 - 450)

Parameter	Unit	TE washed out digester			
		Co	Ni	Fe	Se
SBP	m^3 biogas kg^{-1} VS_{added} d^{-1}	0.77±0.04	0.76±0.04	0.77±0.05	0.77±0.04
SMP	m^3 CH_4 kg^{-1} VS_{added} d^{-1}	0.45±0.02	0.44±0.02	0.45±0.03	0.45±0.02
VBP	STP m^3 m^{-3} digestate d^{-1}	2.31±0.12	2.29±0.12	2.32±0.16	2.31±0.11
VMP	STP m^3 m^{-3} digestate d^{-1}	1.35±0.07	1.33±0.07	1.35±0.09	1.36±0.07
DBP	L	10.1±0.5	9.4±0.5	10.2±0.6	10.1±0.5
CH_4	%	58.3±0.7	58.1±0.9	58.1±1.1	58.7±0.8
TAN	mg N kg^{-1} FM	2,582±86	2,585±77	2,639±95	2,594±109
FAN	mg N kg^{-1} FM	110±14	112±16	111±15	108±12
TS	% of FM	1.59±0.05	1.59±0.05	1.57±0.08	1.55±0.05
VS	% of FM	1.31±0.05	1.30±0.05	1.31±0.05	1.28±0.05
VS	% TS	82.4±0.50	82.0±0.50	82.9±0.90	82.5±0.60
VSD	%	88.4±2.1	89.2±2.6	87.7±3.2	88.7±2.2
pH	-	7.59±0.06	7.60±0.06	7.58±0.06	7.59±0.05
TA	mg $CaCO_3$ kg^{-1} FM	9,451±270	9,422±252	9,428±450	9,394±362
PA	mg $CaCO_3$ kg^{-1} FM	7,194±307	7,128±311	7,051±574	7,130±354
IA	mg $CaCO_3$ kg^{-1} FM	2,256±173	2,294±183	2,377±242	2,260±222
IA/PA		0.32±0.03	0.32±0.04	0.35±0.07	0.32±0.04
Total VFA	mg L^{-1}	< 100	< 100	< 100	< 100

Note: The numbers shown in table are the mean ± standard deviation



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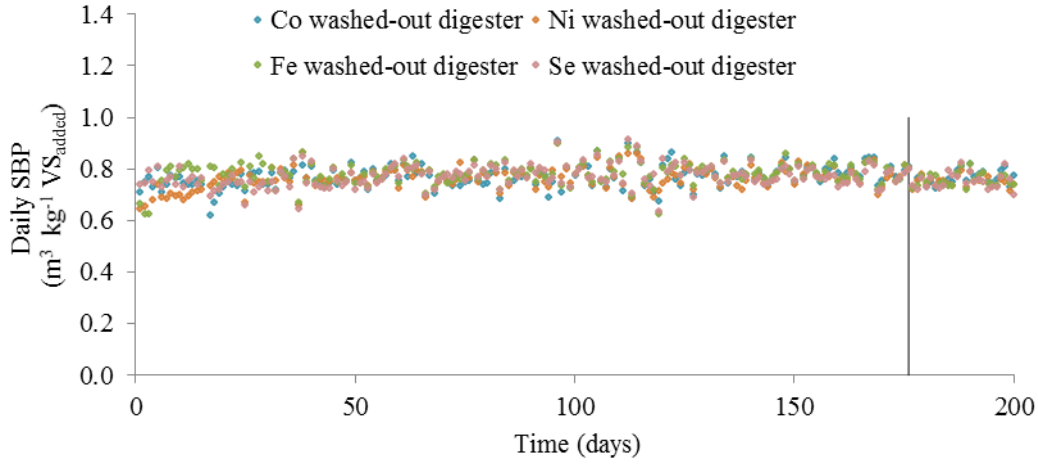

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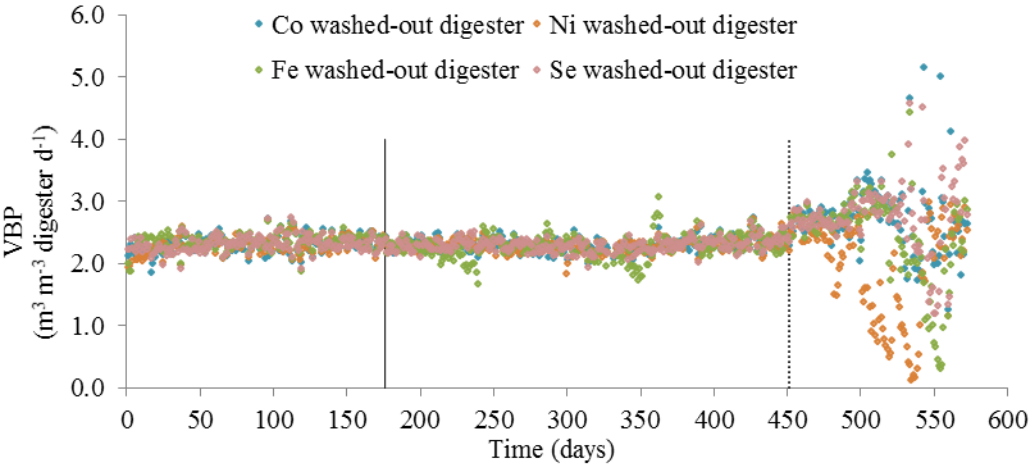
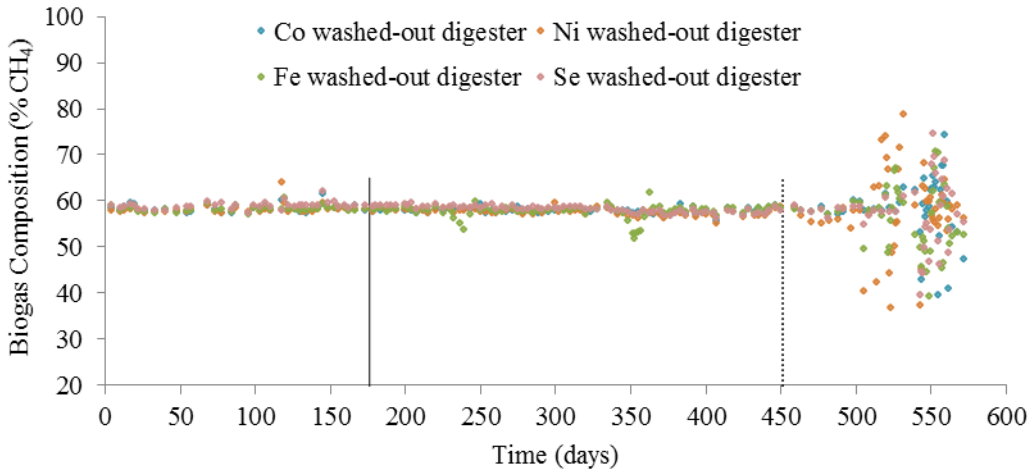


(c)

Figure 6-3 (a) DBP, (b) SBP, (c) VBP, (d) % CH_4, (e) SMP and (f) VMP for the whole experiment. The vertical solid line denoted the initial TE washing out point (day 176) and vertical dashed line is the point where OLR increasing has been applied to digesters (day 451)



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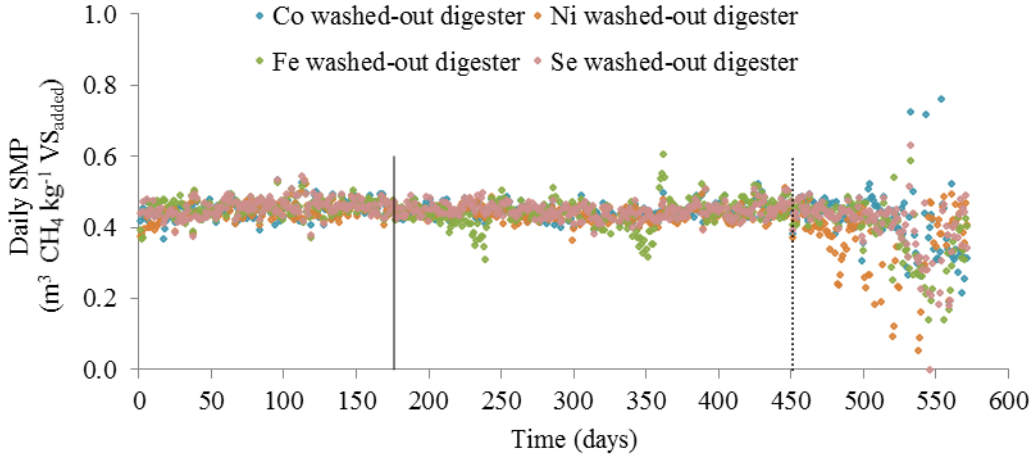

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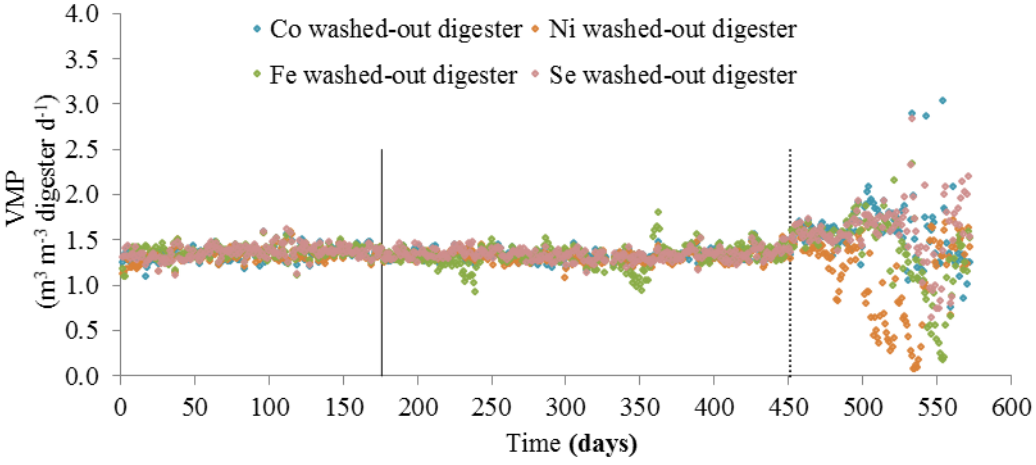
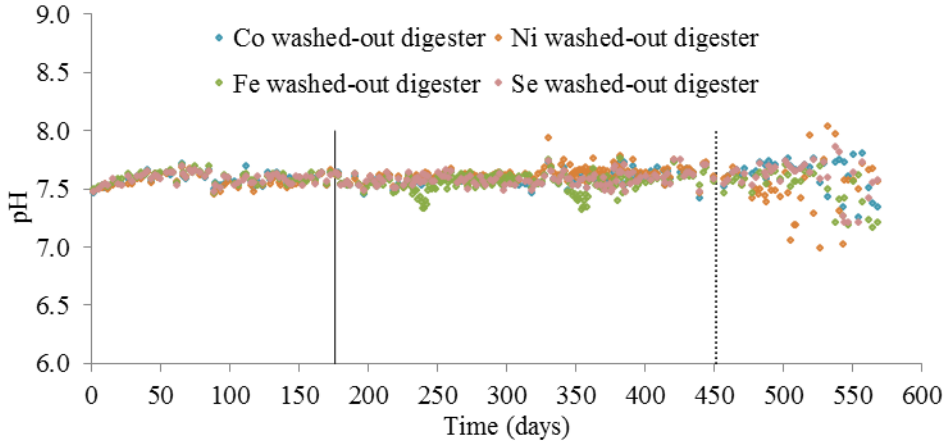


(f)

Figure 6-3 (a) DBP, (b) SBP, (c) VBP, (d) % CH_4, (e) SMP and (f) VMP for the whole experiment. The vertical solid line denoted the initial TE washing out point (day 176) and vertical dashed line is the point where OLR increasing has been applied to digesters (day 451) (continued)



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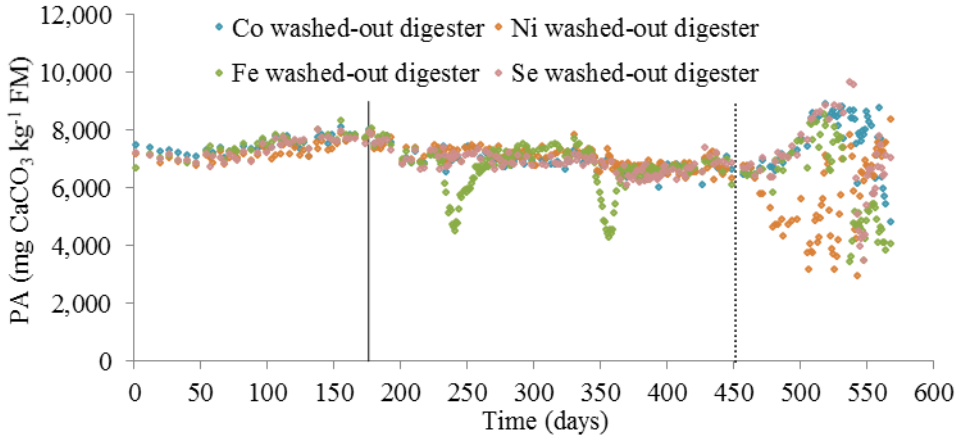

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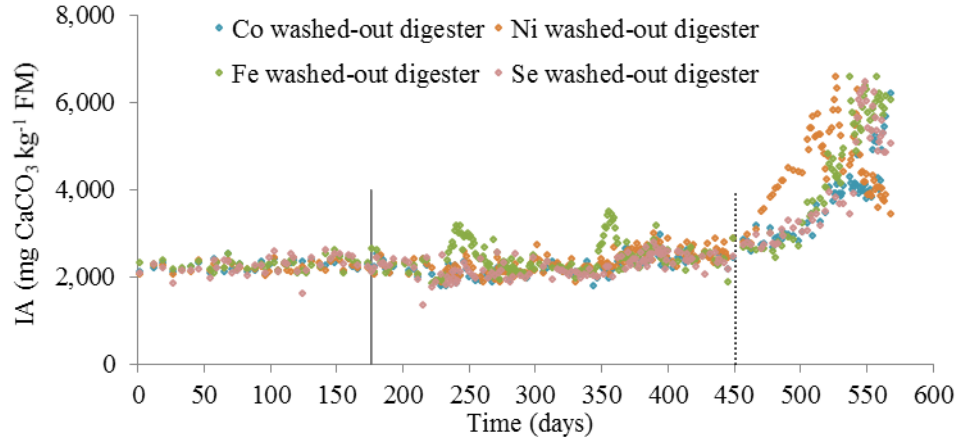
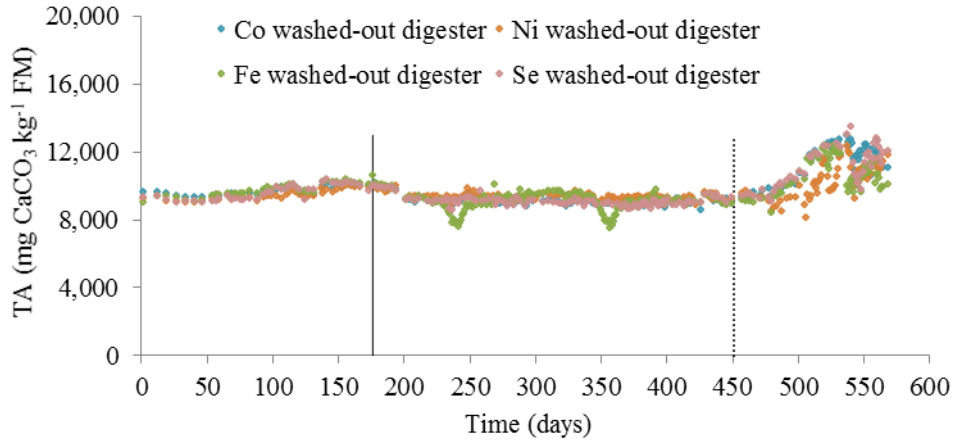


(c)

Figure 6-4 (a) pH, (b) PA, (c) IA, (d) TA, (e) IA/PA ratio, (f) TAN and (g) FAN for the whole experiment. The vertical solid line denoted the initial TE washing out point (day 176) and vertical dashed line is the point where OLR increasing has been applied to digesters (day 451)



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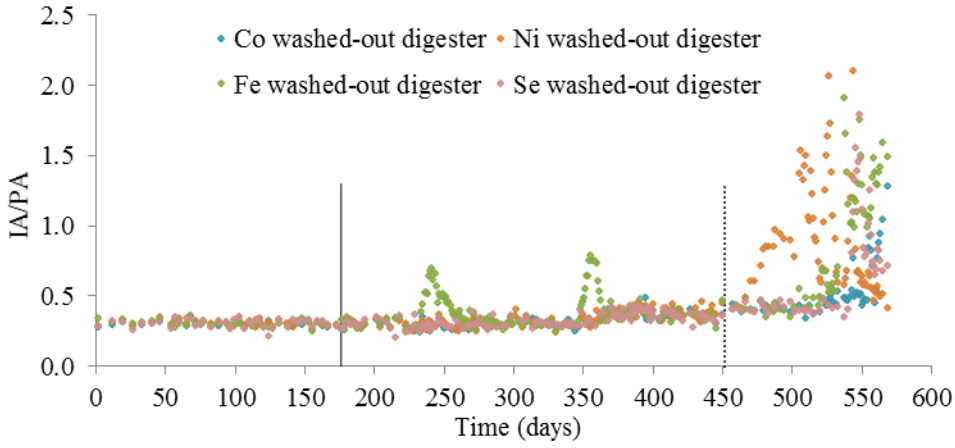

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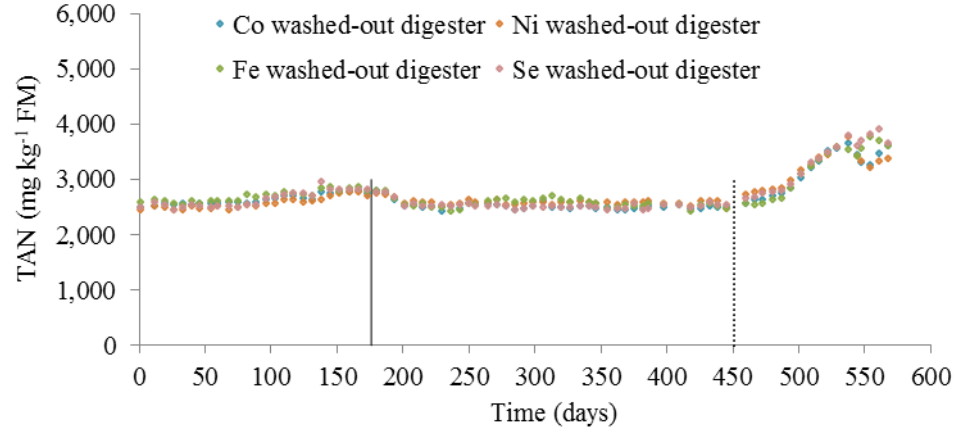
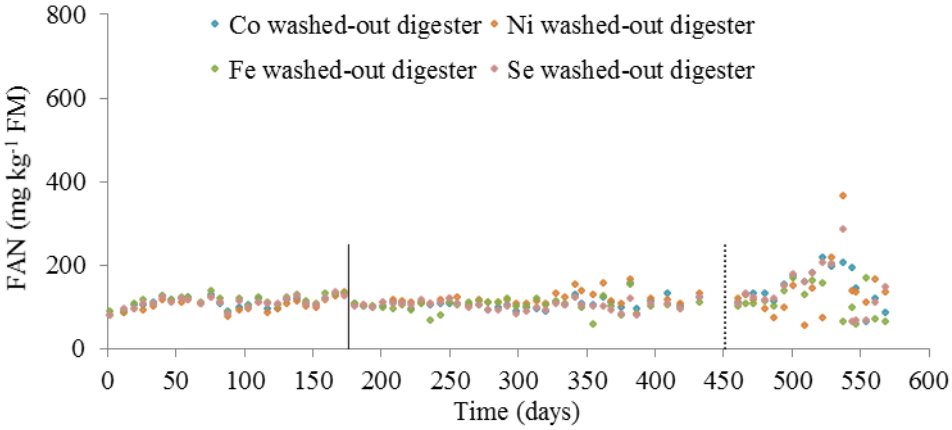


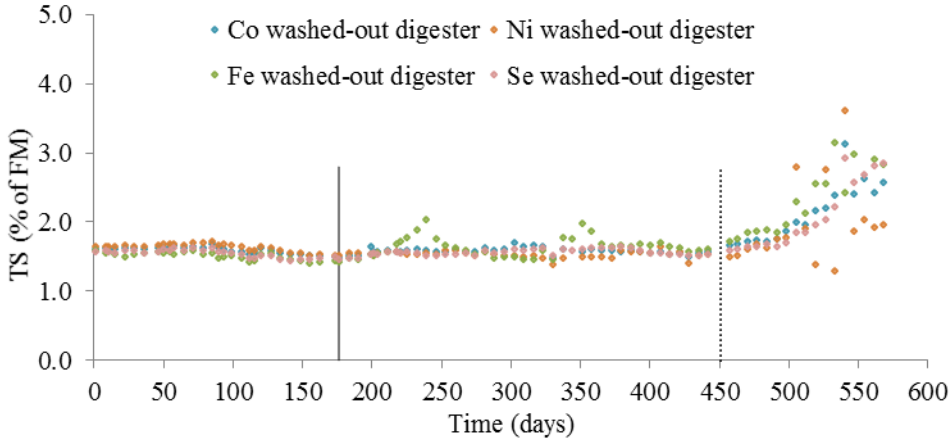
(f)

Figure 6-4 (a) pH, (b) PA, (c) IA, (d) TA, (e) IA/PA ratio, (f) TAN and (g) FAN for the whole experiment. The vertical solid line denoted the initial TE washing out point (day 176) and vertical dashed line is the point where OLR increasing has been applied to digesters (day 451) (continued)

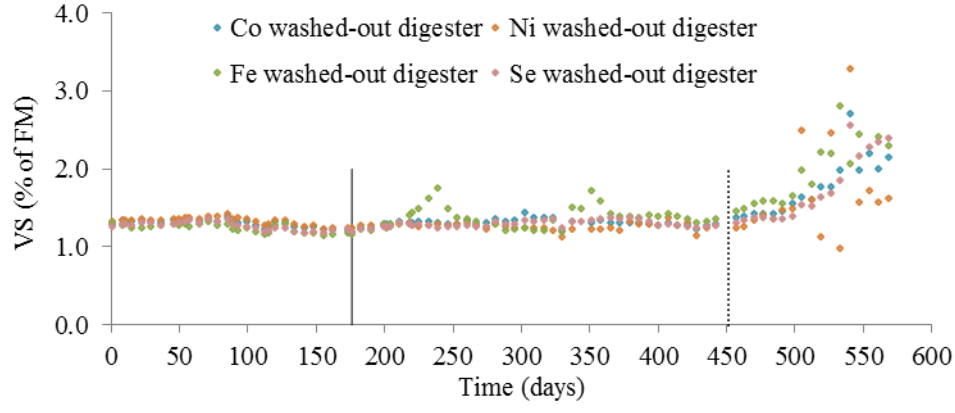


(g)

Figure 6-4 (a) pH, (b) PA, (c) IA, (d) TA, (e) IA/PA ratio, (f) TAN and (g) FAN for the whole experiment. The vertical solid line denoted the initial TE washing out point (day 176) and vertical dashed line is the point where OLR increasing has been applied to digesters (day 451) (continued)



(a)



(b)

Figure 6-5 (a) TS % of FM, (b) VS % FM, (c) VS as % of TS and (d) % VSD for the whole experiment. The vertical solid line denoted the initial TE washing out point (day 176) and vertical dashed line is the point where OLR increasing has been applied to digesters (day 451)

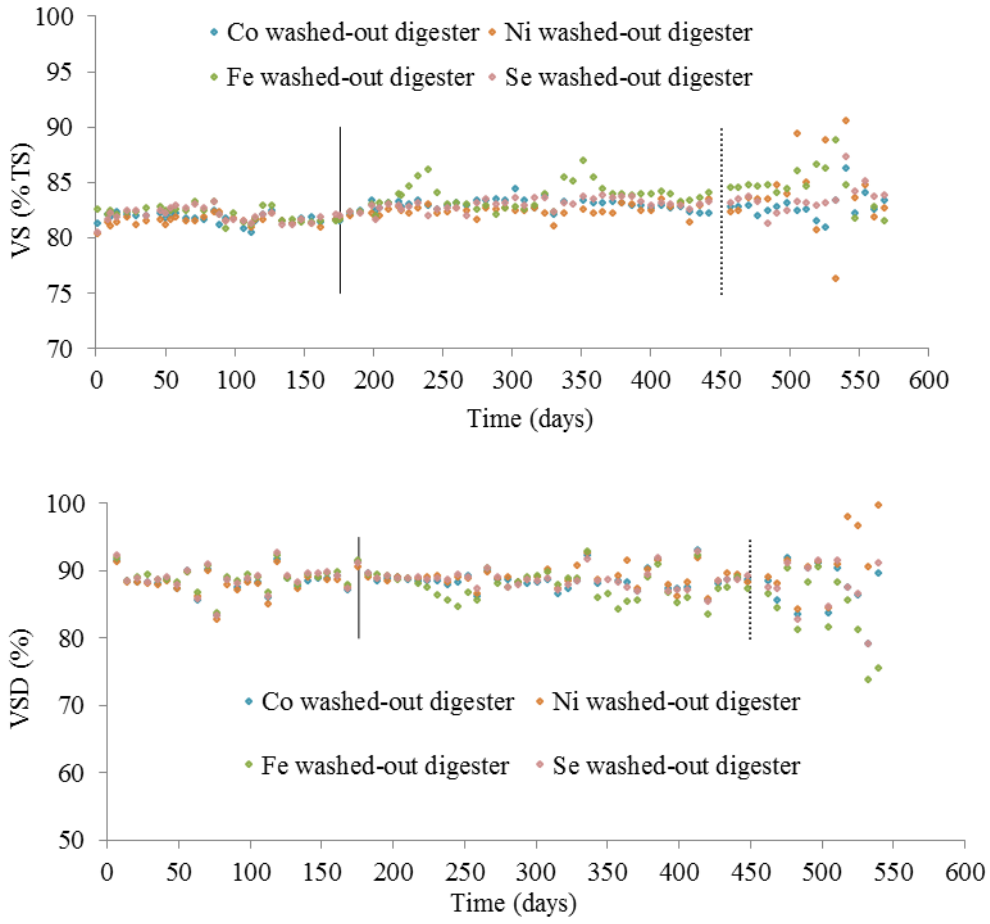


Figure 6-5 (a) TS % of FM, (b) VS % FM, (c) VS as % of TS and (d) % VSD for the whole experiment. The vertical solid line denoted the initial TE washing out point (day 176) and vertical dashed line is the point where OLR increasing has been applied to digesters (day 451) (continued)

Further discussion

TE supplementation is usually used to enhance VFA degradation and digestion stability, especially when VFA has accumulated in the digesters (Lindorfer, Ramhold and Frauz, 2012; Ortner *et al.*, 2014a; Wall *et al.*, 2014; Wei *et al.*, 2014; Zhang *et al.*, 2015). These results indicate, however, that the effect of TE may come into force in all 4 stages simultaneously. Although the TE addition stimulates the microbial activities for each stage if dosed at the right strength, the overall effect may not be preferable in some situations, e.g. when the production of VFA is faster than their degradation by methanogens. This is especially true when TE is applied to VFA-laden digesters. There are several possible reasons for this: 1) the acid-producing bacteria may have higher TE uptake rate and/or lower half-saturation constant compared to methanogens; 2) certain TE are particularly beneficial for bacteria, although not useful for methanogens; 3) the acid-producing bacteria may have a shorter doubling time overall than that of the methanogenic community; and 4) the acid-producing bacteria may be less stressed under VFA-laden conditions and therefore recover more promptly than methanogens.

This issue has been identified by a few other studies. For example, the optimal TE supplementation time was investigated by Yu *et al.* (2015) using Fe in thermophilic batch digesters for wasted activated sludge treatment. The study found that the timing of Fe addition was essential with regard to balancing the process between hydrolysis-acidification and methanogenesis: an earlier supplementation was inhibitory to methanogens due to the inhibitory effect of VFA, whereas a delayed supplementation could not control the VFA accumulation by methanogenesis.

It would be much less complicated if TE were only required by methanogenesis. If this was the case, the overall effect would be very straightforward: methane production and substrate utilisation rates would increase. This is because TE supplementation helped stimulate the conversion of acetate and hydrogen to methane and therefore reduces the product-induced feedback inhibition to acetogenesis, which in turn helps acidogenesis and hydrolysis. In this case, however, it is very difficult to distinguish the direct effect of TE on acidification or the increased acidification caused by the disappearance of feedback-induced inhibition. However, due to the effect of TE on acetogenesis, acidogenesis and hydrolysis, the overall result could become uncertain, as it is affected by a range of factors including digester condition and operating arrangements. This is especially true when TE is added to VFA-laden digesters.

Results from four washed-out digesters confirmed that when methanogenic activities were inhibited, supplementation of TE still functioned on VFA production. This knowledge has practical significance regarding TE supplementation strategy when digesters have been laden with VFA and methanogens are prone to that inhibitory effect resulted in recovery process. It showed that supplementation of Co and Se induced severe VFA accumulation and digestion failure when they were added to VFA-laden digesters. TE supplementation strategy for VFA degradation therefore should be applied with caution. The strength of Co, Fe and Se become limiting by the end of experiment at high level of VFA, however, the recovery strength were not identified in this study due to time constraint.

6.4 Concluding remarks

According to the results obtained from this set of experiments, it could be concluded that

1. The impact of Fe deficiency appeared earlier at OLR 3.0 kg VS m^{-3} d^{-1} while Ni, Co and Se seemed to affect the process at later stages under the higher OLR at 3.0, 3.5 and 4.0 kg VS m^{-3} d^{-1}, respectively.

2. Critical Co, Ni, Fe and Se concentrations were determined when the rest of the TE existed in sufficient quantity (Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM):

2.1. Co concentration became critical at 0.01 mg kg^{-1} FM, equal to the baseline concentration from the feed at OLR 3.5 kg VS $m^{-3}d^{-1}$.

2.2. Ni was critical at 0.03 mg kg^{-1} FM, equal to the baseline concentration from the feed at OLR 3.0 kg VS m^{-3} d^{-1}. Ni at the strength of 0.6 - 0.8 mg kg^{-1} FM is recommended as the minimal strength to maintain stability and performance efficiency at OLR 3.5 kg VS m^{-3} d^{-1}.

2.3. Fe was critical at 5.0 and 6.2 mg kg^{-1} FM at OLR of 3.0 and 3.5 kg VS m^{-3} d^{-1}, respectively.

2.4. Se was critical at 0.06 mg kg^{-1} FM, equal to the baseline concentration from the feed at OLR 4.0 kg VS m^{-3} d^{-1}.

3. This critical (minimum) concentration, however, was not sustainable and not sufficient in case of instability initiated for instance by organic loading increase. A sufficient safety factor, for instance 3-5 folds of its critical concentrations should be applied if these critical concentrations are to be used for developing the TE supplementation strategies to prevent initial VFA accumulation. However, the proper safety factor need to be clarified through further studies as suggested in Chapter 9.

4. Recovery and continuing stable operation, however, required much higher TE strength compared with their critical concentrations at high level of VFA.

5. After long-term washing out, TE deficiency appeared earlier in higher OLR of 4 tested digesters.

Chapter 7: **Dynamic changes of Co, Ni, Fe and Se distribution profiles in anaerobic digester over the course of TE washing out process**

7.1 Introduction

The previous research experiment, as described in Chapter 5 indicated that supplementation of Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM was confirmed to be sufficient for maintaining long-term stable performance at OLR 3.0 kg VS m^{-3} d^{-1}. Although the significant beneficial of the quantification required and effect of TEs on digestion stability was identified. That study, however, left some research questions for further clarification. The system operated for more than 18 HRTs and clearly showed that the requirement for TE was uncoupled from the hydraulic characteristics of the digester, suggesting that TE presented in different chemical species and their bioavailability is a critical element in their function and is, to a certain degree, independent of washout as the results showed that nutrients are not lost from the system simply as a hydraulic function. In order to identify the dynamic changes of Co, Ni, Fe and Se distribution in anaerobic digestion under different operational conditions over a range of main fractions (i.e. in liquid, organically bound, precipitated with sulphide, and in microbial biomass) when their total concentration were gradually decreased in digesters over time, this experiment was conducted.

The determination of total TE concentration was considered at a starting point to evaluate the effect of TE deficiency on anaerobic digestion processes. However, it is commonly accepted that total TE concentration is a poor indicator of the elemental fraction available to microorganisms. The different recommended TE concentrations required for stable operation in anaerobic bioreactor has been reviewed and reported based on total TE content (Schattauer *et al.*, 2011; Thanh *et al.*, 2016), however, the chemical form of the elements was not considered, which obviously has an important impact on TE bioavailability (van Hullebusch *et al.*, 2016). This experiment continued the work described in Chapter 5 and in parallel with the work carried out, as described in Chapter 6 to quantify the critical concentrations of Co, Ni, Fe and Se to maintain stable digestion at moderate OLR by TE washing out experiment.

To achieve this, four objectives were set:

1. To study the dynamic changes of Co, Ni, Fe and Se distribution in different chemical forms under different operational conditions (e.g. when TE are deficient or sufficient) at OLR of 3.0 kg VS m^{-3} d^{-1} over the course of TE washing out process by carrying out sequential extraction (SE);

2. To relate the TE concentration to its potential availability at the point of VFA accumulation;

3. When digesters have been restored to stability by addition of the TE which can be used in reducing VFA levels, then, to see if this can be explained by the fractionation of the TE between the various forms.

7.2 Experimental method

7.2.1 Methodology for sequential extraction for metals fractionation

This set of experiment was carried out by the four TE washed-out digesters (A1 - A4) in parallel to the work in Chapter 6. Methodology for process operation as described in Chapter 6 (section 6.2.1 and 6.2.2). These digesters were initially operated at OLR of 3.0 kg VS m^{-3} d^{-1} with sufficient TE supplementation at the strength of Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM to establish the digestion baseline (day 0 - 175). Samples from: Co (as named A1), Ni (as named A2), Fe (as named A3), and Se (as named A4) washed-out digesters were taken to analyse the total Co, Ni, Fe and Se concentrations and also their sequentially extracted elements.

During TE washing out phase when digesters were operated at previous OLR used (day 176 - 450), sequential extraction (SE) for elemental fractionation was performed in order to determine the forms of these TE and to study the changes in dynamic distribution of elements of interest at frequent time intervals over whole period in comparing with when the digesters had been supplemented with sufficient TE. The Co, Ni, Fe and Se distribution profiles in these digesters were also identified.

The details of sequential extraction method, quality control measures and applied analytical protocol in this present study are extensively described in section 3.5.10 in Chapter 3.

7.3 Results and discussion

7.3.1 The presence of TE in different digestate fractionaltions

The occurrence of certain elements in a specific fractionation depended on the chemical nature and the specific composition of substrate which consist of the complexes of organic and inorganic matter which highly influence their speciation (Gustavsson *et al.*, 2013a). They undergo complex physicochemical reactions and may be present as free ion, complex bound or as precipitates depending on pH, or on the presence of sulphur compounds or organic macromolecules (van Hullebusch *et al.*, 2016). The TE presented in the system, from anaerobic inoculum, substrate and

supplemented TE, represented by 4 fractionations of their occurrence. The distribution among these 4 fractions responds to its availability.

TE supplemented to anaerobic digesters could be participated in an array of reactions such as precipitation, sorption onto surfaces of particulates and biomass, microbial uptake and release of metal-containing microbial products (Zhang *et al.*, 2004; Fermoso *et al.*, 2010; Shakeri Yekta *et al.*, 2014a). Although adsorption seems to be a very fast process, it may be slower than complexation and precipitation. In high ionic strength systems such as anaerobic reactors, the diffusivity of ions may be slowed, increasing the chance of a metal reacting with a precipitating or chelating agent before adsorbing to the biomass (Aquino and Stuckey, 2007). It seems that adsorption plays an important role in metal uptake in biological systems. It is possible that the adsorption sites located on the cell surface, such as those associated with $Na_4P_2O_7$ extracts, act as the main receptor sites of not only free metals, but also Me-SMP complexes, as is believed for metal transport mediated by metallophores (Nies, 1999; Andrews, Robinson and Rodriguez-Quinones, 2003).

In general, Co, Ni and Fe are presented as cations (free metals) which are able to precipitate with anionic compounds such as sulphides (S^{2-}) (Jong and Parry, 2003; La *et al.*, 2003; Ortner *et al.*, 2015), carbonate (CO_3^{2-}) and phosphate (PO_4^{3-}) (van Hullebusch, Zandvoort and Lens, 2003) and the formation of inorganic and organic complexes, which regarded as difficult to take up, with matters in bulk digester liquid (Callander and Barford, 1983a;b; Jansen, 2004; van der Veen, Fermoso and Lens, 2007) or binding with carbohydrate and protein components of organic chelators of microbial origin, the so-called soluble microbial products (SMP) or extracellular polymeric substance (EPS) (Kuo and Parkin, 1996; Barber and Stuckey, 2000; Gonzalez-Gil *et al.*, 2003; Osuna *et al.*, 2003a; Patidar and Tare, 2006; Aquino and Stuckey, 2007). Due to the their positive charges, they also could be adsorbed with negative charged groups which commonly located onto microbial cell or particle surfaces, such as phenolic and carboxylic groups.

It is generally assumed that free metal ions is regarded as easily accessible and readily available for microorganisms to be taken up as it was reported to be the biologically active form (Osuna *et al.*, 2003a) and the highest potential bioavailable species which commonly used to predict TE bioavailability (Worms *et al.*, 2006). Bartacek *et al.* (2012) indicated that the response of the system is significantly related to free metals concentrations which presented at low strength in liquid fraction. The bioavailability of TEs for metabolic pathways of the anaerobic bacteria is in most cases not related to the total amount measured in the medium since only a fraction is present in solution as free metal (Pobeheim *et al.*, 2010). The uptake of metals by microorganisms is assumed to proceed mainly via the transport of free metal ions across the cell membrane (Zandvoort *et al.*, 2006). Worms *et al.* (2006) reported that not only free metals but certain soluble

metal complexes can also be taken up by microorganisms. As a result, metals associated to liquid phase are defined as the most bioavailable form. It is reasonable to believe that the chemical processes can obstruct the anaerobic process by reducing the free metals concentration or other bioavailable form in solution to extremely low values. Then the mobility and availability of TEs becomes an important aspects. From above reasons, these metals could be distributed and found in all fractionations which depending on their concentrations, physicochemical conditions and process operational condition.

In contrast, Se is mainly present as a highly soluble anion (selenite Se (IV), SeO_3^{2-}) which neither forms any precipitates nor adsorbs onto surfaces which are commonly negative charged. A variety of microorganisms in anaerobic digesters have the ability to reduce selenite oxyanions to insoluble Se(0) (direct exzymatic reduction) and some bacteria can directly use oxyanions of selenium (*i.e.* selenite or selenate) as terminal electron acceptors in dissimilatory reduction and also reduce and incorporate Se into organic compounds (e.g., selenoproteins) (assimilatory reduction) (Hockin and Gadd, 2003). Sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) are taken inside the cell by membrane transporters called the sulfate permeates (Aguilar-Barajas *et al.,* 2011). Sulfur in sulfate and in thiosulfate are in the +VI and +IV oxidation state, thus, they belong to the group +VI (selenate) and +IV (selenite) of oxyanions (Markovich, 2001). Therefore, the related oxyanions both selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) could be transported inside the cell by the same type of carriers due to their similar structural characteristics (Aguilar-Barajas *et al.,* 2011).

7.3.2 **TE distribution characteristic during baseline digestion (day 0 - 175)**

It needs pointing out that the sequential extraction (SE) method applied for metals (Co, Ni and Fe) fractionations in this study were done without standard reference materials used to have quality assurance. However, to evaluate the extraction efficiency and determine reliability of the developed SE method used, the total Co, Ni, Fe (and Se) concentrations from the sum of four analysed fractions during SE was compared with the experimentally measured total TE concentrations determined without fractionation (the acid digested sample). The assessment of hydrochloric-nitric acid digestion efficiency for total elements concentrations as described in section 3.5.10.1 was estimated using the spiked samples (standard metals solution) to quantify percentage of the recovery rate. In addition, the other spiked samples were prepared to identify possible matrix interferences and machine detection limit in different matrixes were analysed for quality control of the elements analysis for SE method used.

As presented in Table 7-1, these 2 group of values were in the range of ~ 20 % of differences (83 - 117 %), indicating a good agreement and confirming the correctness of the used methodology and reliability of the obtained results. The differences between the total metals (and Se) extraction

during the SE and the hydrochloric - nitric acid digestions were likely due to inhomogeneity of the sample (Gustavsson *et al.*, 2013b). Table 7-1 showed percentage of recovery for each element during digestion baseline when these four TE washed-out digesters were supplemented with sufficient TE.

Table 7-1 Total TEs concentration (mg kg^{-1} FM) and TE recovery rate (%) on day 175

Digester	Co	Ni	Fe	Se
A1, Co washed-out	1.06 and 105.3	1.38 and 105.4	14.32 and 101.1	0.12 and 104.7
A2, Ni washed-out	1.11 and 106.4	1.07 and 96.4	13.66 and 101.1	-
A3, Fe washed-out	1.07 and 102.7	1.10 and 116.9	13.63 and 99.9	0.16 and 82.68
A4, Se washed-out	1.12 and 101.9	1.32 and 106.1	13.00 and 113.1	-

Gustavsson *et al.* (2013b) evaluated the nitric acid digestion efficiency for Co, Ni, and Fe by digestion of a reference standard material (CRM 029 - 050, RTC) with known Co, Ni, and Fe concentration and reported that 90 % of Fe, 102 % of Co, and 92 % of Ni (mean values of triplicate samples) were extracted from the reference material. Results from the same study reported that the recovery rate of Fe were 88 and 74 %, 85 and 79 % for Co, and while 119 and 91 % for Ni in the two reactors. The differences in metals concentrations between 2 digesters because of the difference organic loading rates and TS contents of the reactors. Two methods (nitric acid digestion and SE) showed reasonable agreement in the levels of Fe, Co, and Ni recovered with a relative standard deviation of around ± 25 % which is in a good agreement to the present study.

The fractions specified by the SE method provide information regarding the metals chemical binding forms. The distribution of Co, Ni, Fe and Se in TE washed-out digesters during digestion baseline (day 0 - 175) were shown in Figure 7-1 to Figure 7-4, respectively. The elemental determinations presented in pie charts were provided in mg kg^{-1} FM. The experimental results showed that when the digesters were supplemented with sufficient TE indicated by a very stable digestion, as described in section 6.3.1, mostly around 97 - 99 % of Co, Ni, Fe and Se presented in extracellular fractions which consist of organic matter bound fraction, precipitation as sulphide fraction and leaving in liquid fraction. Only around 1 - 3 % was found in microbial biomass.

One of the most beneficial of the applied SE method used is, it was allowed discriminating element distribution between the organic matter fraction and sulphide fraction (van Hullebusch *et al.*, 2016). The previous researched studies used procedure originally proposed from Tessier, Campbell and Bisson (1979). It was implemented on anaerobically treated sludge, revealing that organic matter and sulphide fractions are the most important carriers of metals in these matrixes (Angelidis and Gibbs, 1989), however, TE bound to this fraction are simultaneously extracted;

therefore, no information regarding the contribution of each phase in TE binding is provided (van Hullebusch *et al.*, 2016).

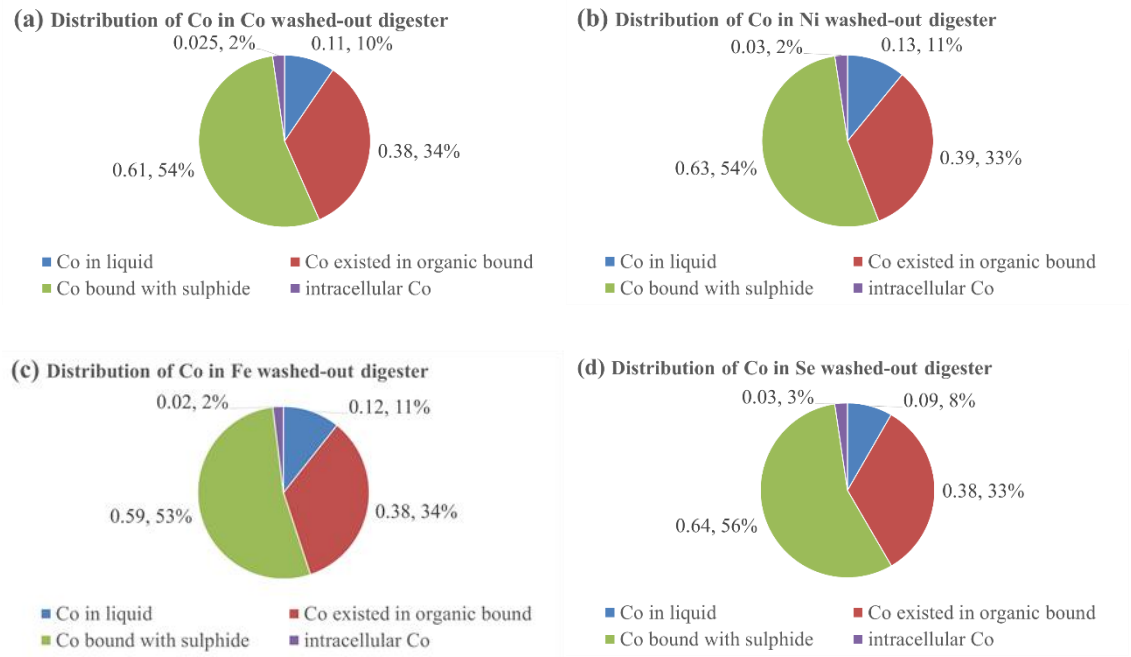


Figure 7-1 (a) - (d) Co distribution in Co, Ni, Fe and Se washed-out digesters, respectively during digestion baseline at OLR of 3.0 kg VS m^{-3} d^{-1} (day 175)

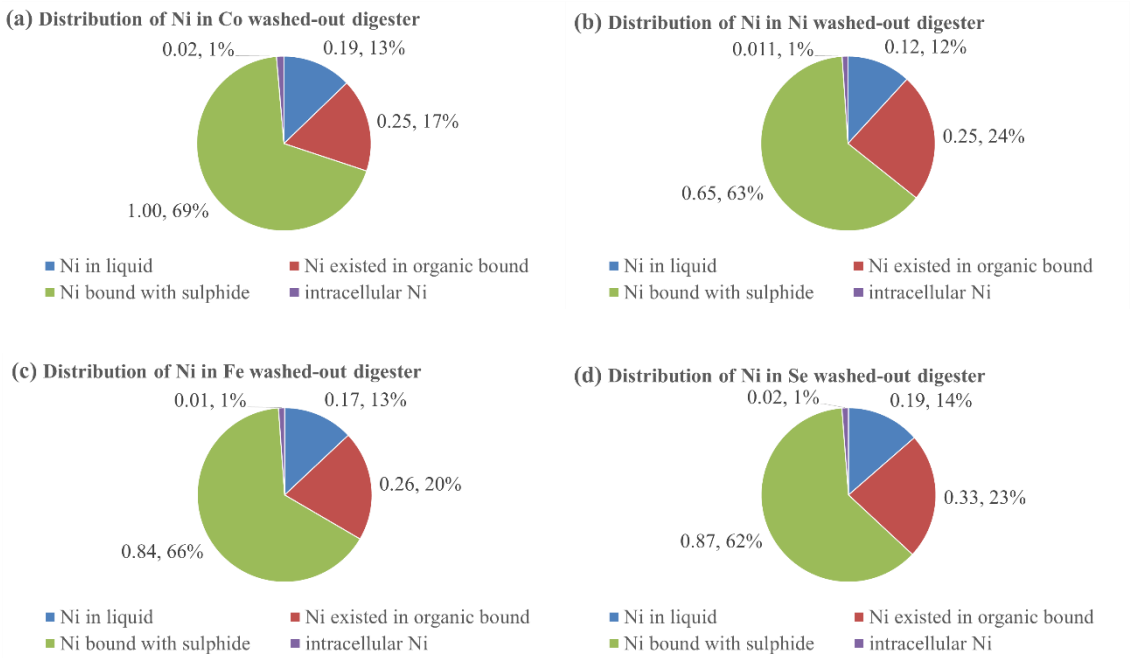


Figure 7-2 (a) - (d) Ni distribution in Co, Ni, Fe and Se washed-out digesters, respectively during digestion baseline at OLR of 3.0 kg VS m^{-3} d^{-1} (day 175)

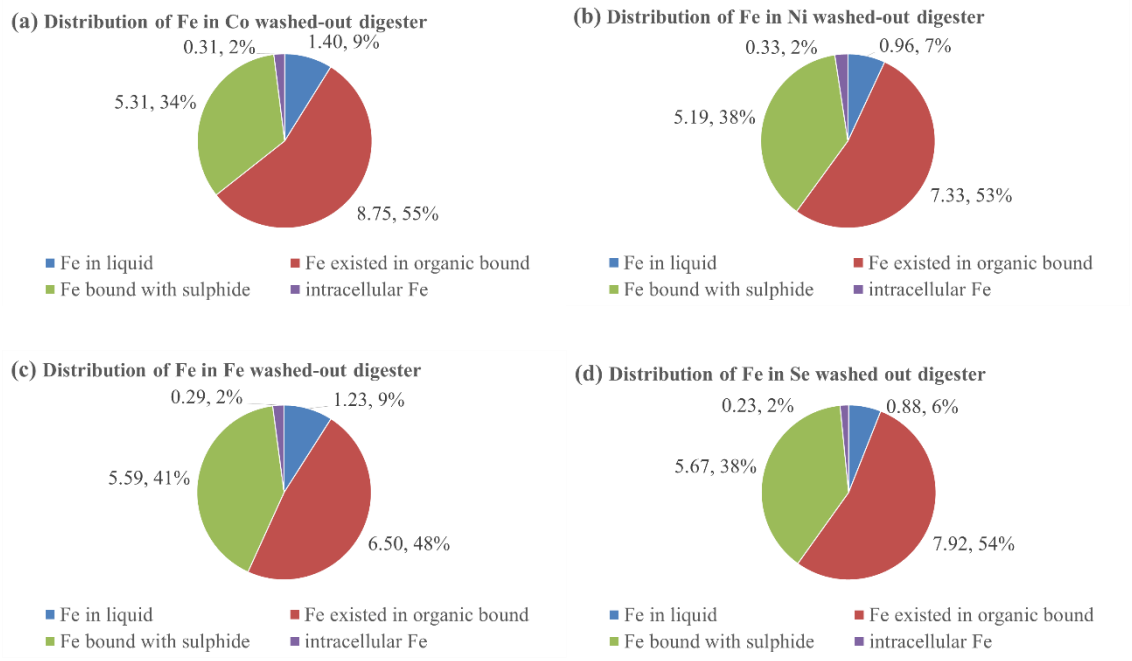


Figure 7-3 (a) - (d) Fe distribution in Co, Ni, Fe and Se washed-out digesters, respectively during digestion baseline at OLR of 3.0 kg VS m^{-3} d^{-1} (day 175)

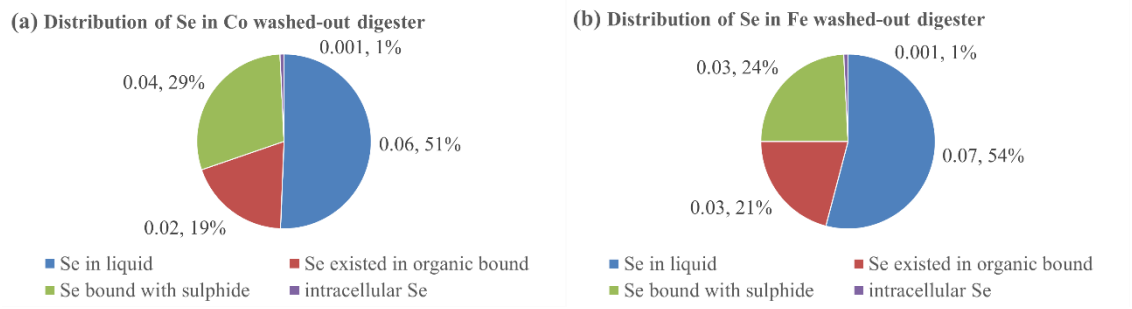


Figure 7-4 (a) and (b) Se distribution in Co and Fe washed-out digesters during digestion baseline at OLR of 3.0 kg VS m^{-3} d^{-1} (day 175)

Results from this study clearly showed that the highest proportion of Co and Ni in digesate was found in sulphide fraction while Fe was mainly observed in organic matter bound fraction. Co (> 50 %) and Ni (> 60 %) mostly stayed in sulphide form (HNO_3 extract) follow by binding with organic ligand at around 30 and 20 % for Co and Ni, respectively. Fe (~ 50 %) showed high affinity for organic compounds. It was more strongly presented in organic matter bound fraction ($Na_4P_2O_7$ extract) followed by precipitated as sulphide (> 30 %). This was in agreement with Aquino and Stuckey (2007) who found that most of the Fe was more strongly adsorbed onto biomass with excreted organic matter or precipitated as sulphide salt.

In other words, from this result, it indicated that more than 80 % of Co, Ni and Fe presented in organic matter/sulphide fractions. These results confirmed those found in the prior studies that

Co- and Ni- sulphide species was predominantly (Shakeri Yekta *et al.*, 2014a; Yekta *et al.*, 2017) in solid phase and also in agreement with other researches who reported that 80 - 90 % of Co and 100 % of Ni were completely associated to the organic matter/sulphide fraction from sulfur-rich stillage fed CSTRs (Gustavsson *et al.*, 2013a; Gustavsson *et al.*, 2013b). In this respect, van der Veen, Fermoso and Lens (2007) also found that sulphide was the dominating bounding form of Co and Ni and has noted that more than 70 % Co and Ni presented in organic/sulphide fraction (oxidizable form) extracted from methanol grown anaerobic granular sludge which increased together with the total content during reactor operation. Dąbrowska (2012) also supported the fact that high concentration of Ni was mainly observed in the organic/sulphide fraction from sewage sludge after both thermophilic and mesophilic digestion processes. Gustavsson *et al.* (2013b) indicated that the high percentage of metals associated with organic and sulphide indicated a very low bioavailability of this metals.

In this study, as shown in Figure 7-1 - Figure 7-4, the experimental results showed that around 10 - 15 % of the total Co, Ni and Fe concentrations were presented in liquid (water-soluble) fraction which reached a maximum value of 0.11, 0.12 and 1.39 mg kg^{-1} FM at total metals strength of 1.06, 1.07 and 13.63 mg kg^{-1} FM for Co, Ni and Fe, respectively. The higher elements presented in water - soluble fraction indicated that the potential bioavailability of elements was high (Gustavsson *et al.*, 2013b). Then, regarding the recent results, a considerable occurred amount of Ni (~ 13 %) in the more water - soluble fraction compared to Co (~ 10 %) and Fe (~ 7.8 %) suggested that Ni was considered to be higher potential bioavailability compared to Co and Fe in investigated digesters.

The high solubility of Co, Ni and Fe in TEs washed-out digesters may be explained by complexation with soluble sulphides or organic ligands in solution. This is because metals in liquid phase comprised of different species such as free metal ions, certain soluble organic metal complexes (Callander and Barford, 1983b;a; Jansen, 2004), metals complexes with soluble microbial products (SMP) (Barber and Stuckey, 2000; Gonzalez-Gil *et al.*, 2003; Patidar and Tare, 2006; Aquino and Stuckey, 2007) or labile metal complexes with inorganic ligands such as phosphate and carbonate (Yekta *et al.*, 2017). These results are consistent with the earlier studies which showed that 10 - 20 of total Co concentration was in sludge liquid phase as dissolved form (Gustavsson *et al.*, 2013a). Pinto-Ibieta *et al.* (2016) reported a strong linear relationship between dissolved and total Co concentration and suggested that dissolved Co is more accurately related with the biological response in comparison with total Co concentration. The same study found that dissolved Co around 0.02 - 0.04 mg kg^{-1} can be considered as an optimum for dissolved Co and gave the highest methane yield coefficient and methane production rate values. Co in liquid fraction may be associated with organic ligand e.g. vitamin B_{12}, i.e. Co-corrinoids. B_{12}-enzymes which are the biological active form of Co in biogas process (Thauer, 1998) and are known to

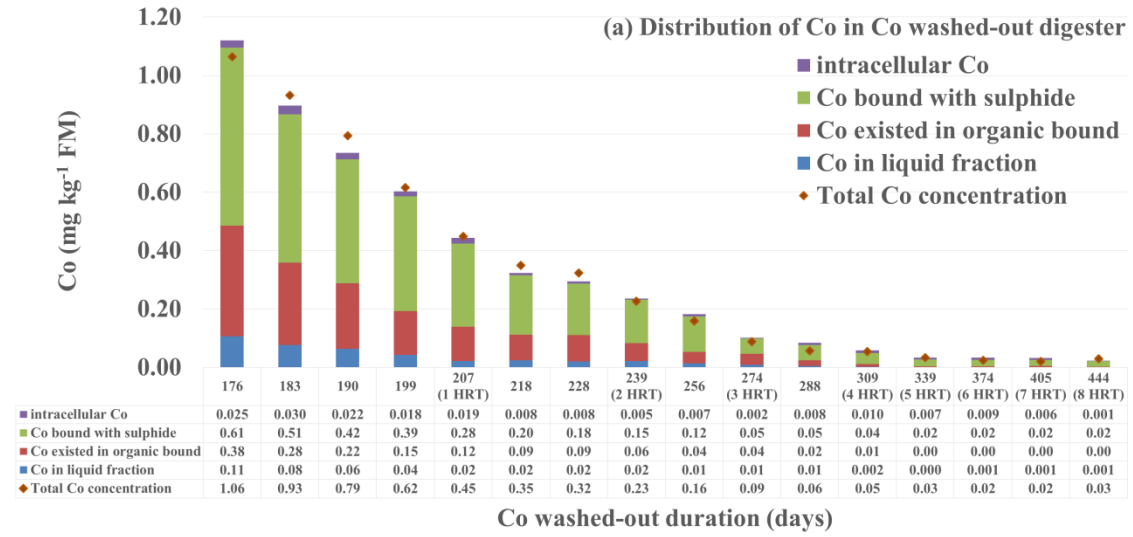
play a crucial role in methanogenesis, methanogens are able to excrete B_{12}-compounds into solution (Mazumder *et al.*, 1987; Zhang *et al.*, 2004). Consequently, dissolved Co may be related to extracellular B_{12}. There was also a possibility that Co presented in liquid phase is likely related to a release of Co-containing biomolecules by microorganisms, the vitamin B_{12} is of intracellular origin and becomes released as a result of cell lyses during sample treatment, e.g. centrifugation of the reactor liquid (Gustavsson *et al.*, 2013a). The formation and release of vitamin B_{12} into methanogenic slurries observed by Zhang *et al.* (2004) further supported this idea.

In contrast, Se mostly appeared in liquid fraction as highly soluble oxyanion (selenite Se (IV), SeO_3^{2-}), reached a maximum value of 0.07 at total Se strength of 0.14 mg kg^{-1} FM which was around 50 % follow by complexed with sulphide (~ 25 - 30 %) and binding with organic ligand around 20 %.

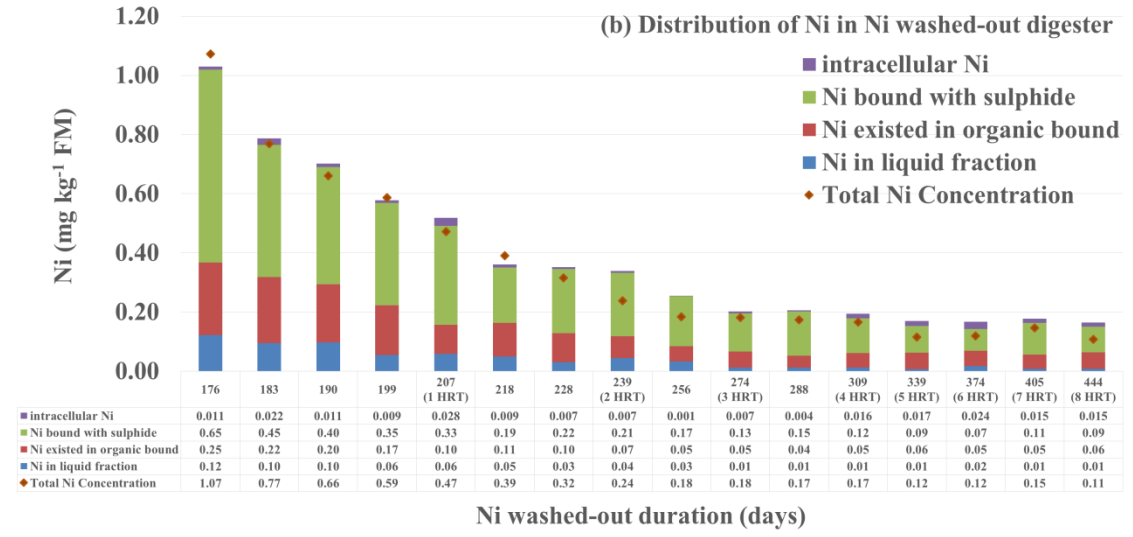
7.3.3 The dynamic changes of TE distribution in different digestate fractionations during their washed-out phase (day 176 - 450) at OLR of 3.0 kg VS m^{-3} d^{-1}

In this study total element concentration has been defined as the summation of the supplemented concentration and the baseline concentration of elements including the concentration of elements from inoculum. The plotted experimentally measured total Co, Ni, Fe and Se concentration profiles in comparing with the total elemental concentrations from sequential extraction were shown in Figure 7-5 (a) - (d). These could be confirmed that the recovery rates of Co, Ni, Fe and Se, which calculated by the sum of those elements from four analysed fractions, were between 80 and 120% of total elements concentration in fresh digestate from acid digestion determined without fractionation, underlining the validity of the methodology used of the obtained results and indicating a reasonable agreement and showed a good agreement with Gustavsson *et al.* (2013b) who reported the recovery rate of Co, Ni and Fe with a relative standard deviation of ± 25 %. The outcome of Co, Ni, Fe and Se distribution profiles in their washed-out digesters by chemical fractionation analysis using SE method were shown in Figure 7-5 (a) - (d).

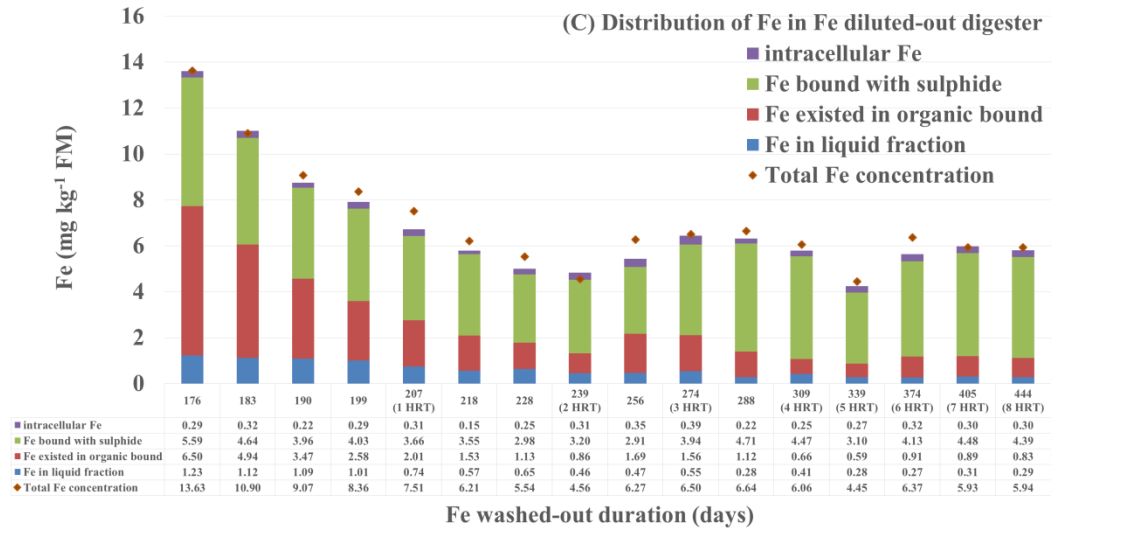
From TE distribution profiles as shown in Figure 7-5 (a) - (d), it appeared that the higher total Co, Ni, Fe and Se concentrations were reflected by the higher of their content presented in extracellular fractions. It was observed that elements found in microbial biomass were consistent for the whole process of TE washing out with the strength of Co 0.01 - 0.03, Ni 0.01 - 0.03, Fe 0.2 - 0.3 and Se 0.001 - 0.002 mg kg^{-1} FM in Co, Ni, Fe and Se washed-out digesters, respectively while elements distributed in extracellular fractions were varied over liquid, organic matter bound and precipitated with sulphide fractions.



	176	183	190	199	207 (1 HRT)	218	228	239 (2 HRT)	256	274 (3 HRT)	288	309 (4 HRT)	339 (5 HRT)	374 (6 HRT)	405 (7 HRT)	444 (8 HRT)
intracellular Co	0.025	0.030	0.022	0.018	0.019	0.008	0.008	0.005	0.007	0.002	0.008	0.010	0.007	0.009	0.006	0.001
Co bound with sulphide	0.61	0.51	0.42	0.39	0.28	0.20	0.18	0.15	0.12	0.05	0.05	0.04	0.02	0.02	0.02	0.02
Co existed in organic bound	0.38	0.28	0.22	0.15	0.12	0.09	0.09	0.06	0.04	0.04	0.02	0.01	0.00	0.00	0.00	0.00
Co in liquid fraction	0.11	0.08	0.06	0.04	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.002	0.000	0.001	0.001	0.001
Total Co concentration	1.06	0.93	0.79	0.62	0.45	0.35	0.32	0.23	0.16	0.09	0.06	0.05	0.03	0.02	0.02	0.03

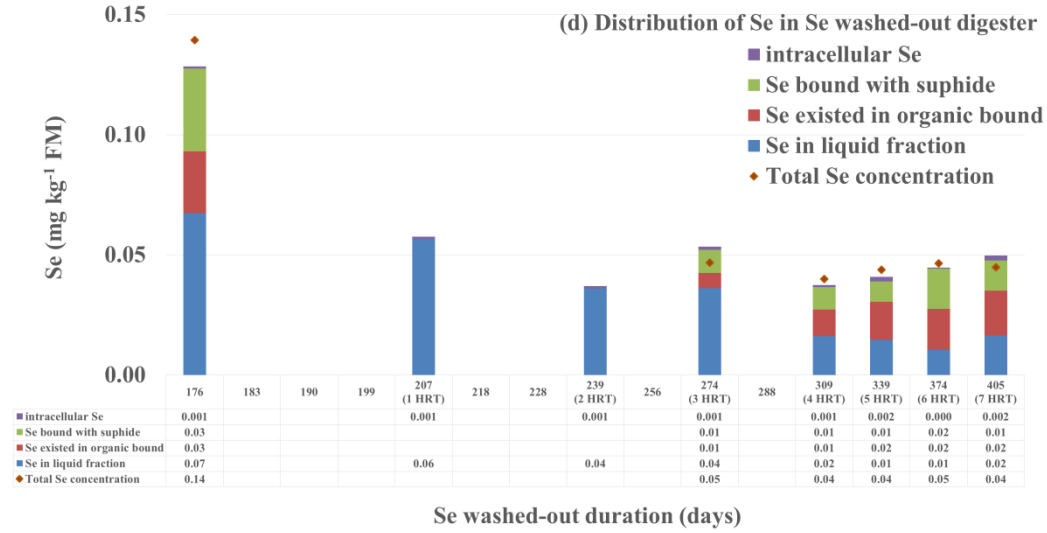


	176	183	190	199	207 (1 HRT)	218	228	239 (2 HRT)	256	274 (3 HRT)	288	309 (4 HRT)	339 (5 HRT)	374 (6 HRT)	405 (7 HRT)	444 (8 HRT)
intracellular Ni	0.011	0.022	0.011	0.009	0.028	0.009	0.007	0.007	0.001	0.007	0.004	0.016	0.017	0.024	0.015	0.015
Ni bound with sulphide	0.65	0.45	0.40	0.35	0.33	0.19	0.22	0.21	0.17	0.13	0.15	0.12	0.09	0.07	0.11	0.09
Ni existed in organic bound	0.25	0.22	0.20	0.17	0.10	0.11	0.10	0.07	0.05	0.05	0.04	0.05	0.06	0.05	0.05	0.06
Ni in liquid fraction	0.12	0.10	0.10	0.06	0.06	0.05	0.03	0.04	0.03	0.01	0.01	0.01	0.01	0.02	0.01	0.01
Total Ni Concentration	1.07	0.77	0.66	0.59	0.47	0.39	0.32	0.24	0.18	0.18	0.17	0.17	0.12	0.12	0.15	0.11



	176	183	190	199	207 (1 HRT)	218	228	239 (2 HRT)	256	274 (3 HRT)	288	309 (4 HRT)	339 (5 HRT)	374 (6 HRT)	405 (7 HRT)	444 (8 HRT)
intracellular Fe	0.29	0.32	0.22	0.29	0.31	0.15	0.25	0.31	0.35	0.39	0.22	0.25	0.27	0.32	0.30	0.30
Fe bound with sulphide	5.59	4.64	3.96	4.03	3.66	3.55	2.98	3.20	2.91	3.94	4.71	4.47	3.10	4.13	4.48	4.39
Fe existed in organic bound	6.50	4.94	3.47	2.58	2.01	1.53	1.13	0.86	1.69	1.56	1.12	0.66	0.59	0.91	0.89	0.83
Fe in liquid fraction	1.23	1.12	1.09	1.01	0.74	0.57	0.65	0.46	0.47	0.55	0.28	0.41	0.28	0.27	0.31	0.29
Total Fe concentration	13.63	10.90	9.07	8.36	7.51	6.21	5.54	4.56	6.27	6.50	6.64	6.06	4.45	6.37	5.93	5.94

Figure 7-5 The changes of TE distribution profiles by sequential extraction analysis during TE washed-out experiment (a) Co, (b) Ni, (c) Fe and (d) Se washed-out digesters



	176	183	190	199	207 (1 HRT)	218	228	239 (2 HRT)	256	274 (3 HRT)	288	309 (4 HRT)	339 (5 HRT)	374 (6 HRT)	405 (7 HRT)
intracellular Se	0.001				0.001			0.001		0.001		0.001	0.002	0.000	0.002
Se bound with suphide	0.03									0.01		0.01	0.01	0.02	0.01
Se existed in organic bound	0.03									0.01		0.01	0.02	0.02	0.02
Se in liquid fraction	0.07				0.06			0.04		0.04		0.02	0.01	0.01	0.02
Total Se concentration	0.14									0.05		0.04	0.04	0.05	0.04

Figure 7-5 The changes of TE distribution profiles by sequential extraction analysis during TE washed-out experiment (a) Co, (b) Ni, (c) Fe and (d) Se washed-out digesters (continued)

Co, Ni and Se those associated with sulphide, presented in organic bound and stayed in liquid fraction gradually dropped during TE washing out stage corresponded to the reducing of their total concentrations, except Fe in sulphide fraction. The aforementioned trend of significant decrease with time at a similar rate as total element concentrations was observed for extracellular fractions in all elements over the course: in liquid (Co: 0.11 - 0.001, Ni: 0.12 - 0.01, Fe: 1.23 - 0.29 and Se: 0.07 - 0.02 mg kg^{-1} FM), in organically bound (Co: 0.38 - 0.002, Ni: 0.25 - 0.06, Fe: 6.50 - 0.86 and Se: 0.03 - 0.02 mg kg^{-1} FM) and in sulphide fraction (Co: 0.61 - 0.002, Ni: 0.65 - 0.09, Fe: 5.59 - 3.20 and Se: 0.03 - 0.01 mg kg^{-1} FM) in Co, Ni, Fe and Se washed-out digesters, respectively.

The important finding was that the amount of Co, Ni, Fe and Se found in microbial biomass fraction could be kept relatively stable for metabolic activities over the course of TE washing out process, although, the elements presented in extracellular fractions were washed-out gradually at similar rate apart from Fe in sulphide fraction as shown in Figure 7-5 (c). The Fe concentration in sulphide bound fraction remained relatively constant over the course, and was significantly at the same level as the Fe concentration in feedstock at around 2.78 mg kg^{-1} FM. This results implied that sulphide was the preferred ligand and had high affinity for binding with Fe over the organic matter fractions. Several studies reported that this affected the bioavailability of elements (Callander and Barford, 1983a;b; Rinzema and Lettinga, 1988; Morse and Arakaki, 1993; Shen, Kosaric and Blaszczyk, 1993; Gonzalez-Gil *et al.*, 2003; Rickard and Luther, 2006; Jansen, Gonzalez-Gil and van Leeuwen, 2007). Sulphide fraction, however, could compete with microbial biomass fraction for Fe. This caused the VFA accumulation when the total Fe concentration in Fe washed-out digester was still high at the strength of 4.0 - 5.0 mg kg^{-1} FM

which was determined to be the critical for OLR used (as described in section 6.3.2.3.1). This confirmed the previous research that the formation of metal-sulphides is often considered to be the most important aspect with regard to metal speciation and limitation for the availability of TE (van der Veen, Fermoso and Lens, 2007; Schmidt *et al.*, 2014). Therefore, the anaerobic digestion and microbial growth is dependent on the availability and optimal supply of nutrients. These results are in agreement with those obtained by Aquino and Stuckey (2007), who found that although adsorption on to biomass surface seems to be a very fast process which plays a significant role in metal uptake in biological systems, however, it might be slower than precipitation and complexation.

As seen in Figure 7-5 (a) and (b), it could be observed that there was no clear different between Co and Ni with their organic and sulphide fractions which implies equal affinity for binding with Co and Ni for these two fractions, however, in this case both sulphide and organic fractions did not compete with intracellular fraction for Co and Ni for the whole period of TE washed-out stage. Sulphide dominated the bonding form distribution of Co and Ni in anaerobic system which has been reported to have a strong influence on metals bioavailability because of the very low solubility products constant (K_{sp}) of CoS and NiS. (Jansen, 2004) and Jansen, Gonzalez-Gil and van Leeuwen (2007), however, proposed that in most cases the dissolution rates of Co and Ni sulphides do not limiting to methanogenic activity in anaerobic wastewater treatment and also suggested that these complexes may act as metals sources of Ni and Co for the methanogens. Co and Ni sulphide precipitation are not inhibit microbial uptake in anaerobic bioreactor by Gustavsson *et al.* (2013b) further supporting this idea. In addition, the total Co and Ni concentration in digesters by the end of this experiment were Co 0.03 and Ni 0.11 mg kg^{-1} FM which is higher than their critical strength at Co 0.01 and Ni 0.03 mg kg^{-1} FM (as describe in section 6.3.2.1.1 and 6.3.2.2.1). Consequently, the very stable process operation of for Co and Ni washed-out digesters for the whole period could be supported by these reasons as discussed.

During the course of TE washing at OLR 3.0 kg VS m^{-3} d^{-1}, Co, Ni and Se washed-out digesters performed normally with no noticeable process instability. Digesters were operated without loss of performance, biogas production and methane did not show significant differences, and no VFA accumulation was observed also digesters' efficiency and others monitoring parameters were similar to baseline scenario (as described in section 6.3.1 and 6.3.2 in Chapter 6). Results from the current study could be indicated that the higher total TE concentrations or the higher TE content in extracellular fractions did not result in better process stability. There was no improvement in methane production observed during TE washing out process. This finding was different from the previous studies who suggested that the higher concentration of elements observed in water soluble fraction (dissolved, complex bound or free ions), which was represented

the bioavailability fractions, showed the better process performance (Ortner *et al.*, 2015) for anaerobic digestion of slaughterhouse waste.

From this and the previous results (Chapter 6), it was reasonable to believe that the decrease of total element concentrations corresponded to only a decrease of out-of-cell fractions, however, elements presented in intracellular fraction is the most important fraction which influenced digester performance, considering the consistent TE concentrations in microbial cells. As a resulted, the use of supplemented total TE concentrations as a control parameter to assess the digester performance is hindered by the involved physicochemical processes, which could entail a wrong estimate of the TE requirement (Pinto-Ibieta *et al.*, 2016).

The important observation was that the concentration of elements in microbial cell fraction when there was sufficient elements presented in digester was roughly equal to their critical concentrations or minimum threshold to sustain stable digestion before VFA start to accumulate (as shown in Chapter 6). The results showed an obvious correlation between elements in intracellular fraction and the stable process performance. These results provided further support for the hypothesis that the chemical fractionation of key elements in microbial biomass influences metabolic activities and significantly influence process stability rather than total element concentrations. For TE washing-out experiment, it could be pointed out that the microbes can just recycle TE in their cells; that state of TE is perfect and therefore can maintain their metabolic activities for a prolonged period until the TE concentration inside was washed by hydraulic action to a level which is too low. This fact must be borne in mind considering that, the critical TE concentration which have been measured in washing out experiment should be the effective TE concentration which should belong to microbe-incorporated TE and thus have strong impact on process stability.

7.3.4 **The interaction between TE concentration in each fractionation and their total concentrations under stable operation**

The relationship between total element concentrations versus the concentration of elements in each fractionation during TE washed-out period at OLR of 3.0 kg VS m^{-3} d^{-1} under stable operation were plotted as shown in Figure 7-6.

The obtained results as shown in Figure 7-6 indicated that there was a significant positive correlation between total Co, Ni, Fe and Se concentrations and concentrations of those elements in extracellular fractions. It can be seen from the correlation analysis that there was a clear trend of increasing of their concentration in all extracellular fractions corresponding to the total element concentrations. Co and Ni strongly related with sulphide form and existed in organic bound following by leaving in liquid fraction, while Fe presented in organic matter bound dominantly

following by complexed with sulphide and dissolved in liquid fraction. In Contrast, Se more strongly stayed in liquid fraction than presented in other extracellular fractions. The most important finding from the current study was that there was no significant increase of Co, Ni, Fe and Se in microbial biomass associated with their total element concentrations under stable operational condition during the period of TE washed-out stage, the intracellular elements could be kept consistent which served adequate amount to activate enzymes essential for the microorganisms involved in anaerobic digestion system.

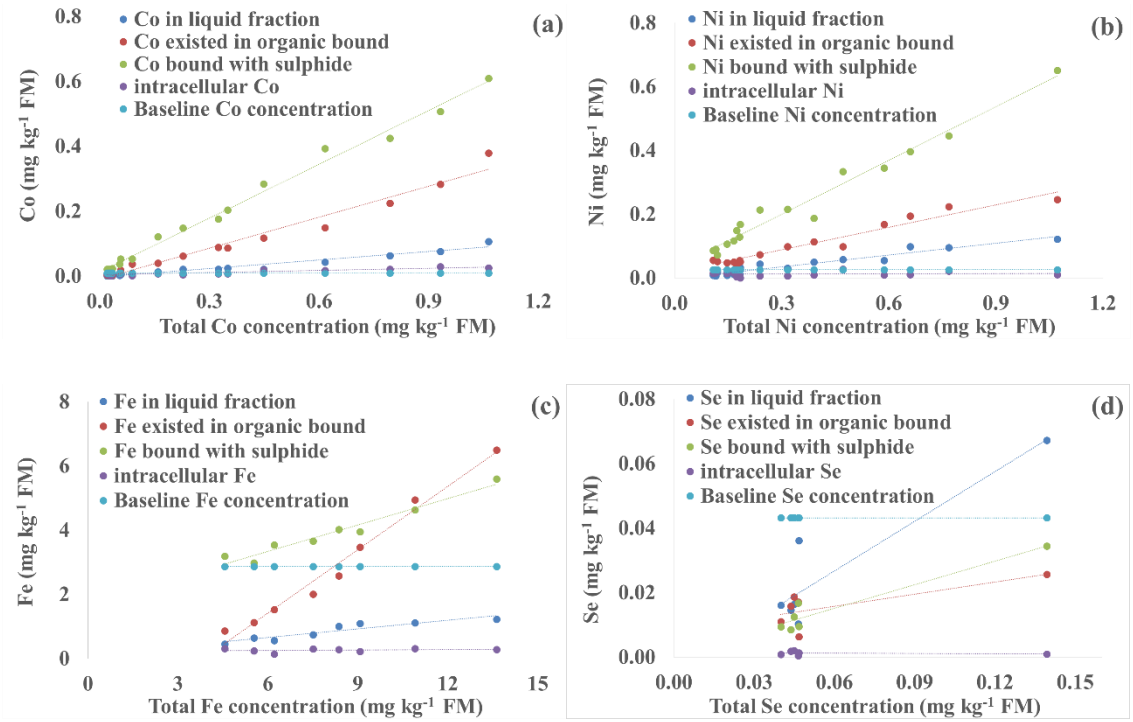


Figure 7-6 Concentration of Co, Ni, Fe and Se in each fractionation and their total element concentrations during TE washed-out period under stable operation, (a) - (d) Co, Ni, Fe and Se washed-out digesters, respectively

7.4 Concluding remarks

Based on the analyses, the following conclusions have been drawn:

1. Microbial biomass maintained relatively stable amount of Co and Ni for metabolic activities although all the extracellular TE fractions were washed - out gradually.

2. Sulphide fraction competed with intracellular fraction for Fe. This caused the accumulation of volatile fatty acids when the total Fe concentration was still high.

3. The fact that TE availability to certain extent decoupled from simple hydraulic washing out effect was explained by the high affinity of microbial biomass for TE in this experiment.

Chapter 8: **The effect of OLR on the requirement of Co, Ni and Fe at constant HRT**

8.1 Introduction

Conducted in parallel with the work described in Chapter 6 and 7, this experiment was aimed to investigate the effect of OLR on the requirement for Co, Ni and Fe supplementation for stable mesophilic anaerobic digestion at constant HRT of 33.3 days. This research was designed according to the adjustable nature of the model substrate used. Apart from its ready biodegradability, another important feature of this model substrate was that OLR was able to decouple from HRT by adjusting the organic matter to water ratio. This allowed the investigation on the effect of OLR on TE requirement without the interference of HRT. This work cannot be done with real-world organic waste as in that case the increase of OLR will associate with the decrease of HRT and therefore the wash-out of microbes. As a result, the microbial biomass concentration in the digester is not proportional to OLR, but reflects the combined effect of loading increase and compensation of wash-out (He, 2016). In order to identify if TE requirement was proportional to OLR at constant HRT, this experiment was carried out.

This trial was started at OLR 1.0 and step-wise increased to 1.5, 2.0, 2.5 and 3.0 kg VS m^{-3} d^{-1}. The initial TE dosing was set at the strength of Co 0.03, Ni 0.03 and Fe 0.3 mg kg^{-1} FM, according to the preliminary result obtained in Chapter 4. Their dosing strength was increased later in this study in response to digester performance and loading increase. Se dosing strength, however, was kept constant at 0.1 mg kg^{-1} FM during the course of this experiment; this supplementation strength ensured that Se was sufficient up to an OLR of 3.0 Kg VS m^{-3} d^{-1}, as shown in Chapter 5 and 6. Performance of this set of digesters at different OLR levels was therefore directly related to the dosing strengths of Co, Ni and Fe. A wide range of digestion parameters were measured during this study, but VFA was again monitored intensively as it is the most important and prompt one to indicate the stability of digesters and therefore the effect of trace elements deficiency; SMP was used to evaluate the substrate conversion efficiency and VMP was employed to indicate the digestion productivity.

To achieve the aim of this research, five objectives were set as below:

1. To ensure all four digesters were operated stably and identically at the initial OLR with the designed initial dosing strength of Co, Ni and Fe;

2. To increase OLR of digesters in a sequential manner whilst maintaining the same TE dose until a point where VFA accumulation occurred;

3. To respond to VFA increase by additional TE supplementation, with an increment of 0.03, 0.03 and 0.3 mg kg^{-1} FM for Co, Ni and Fe, respectively:

4. To identify the minimum TE supplementation strength for each OLR tested;

5. To investigate the effect of dosing ratio of Co, Ni and Fe on their supplementation effectiveness.

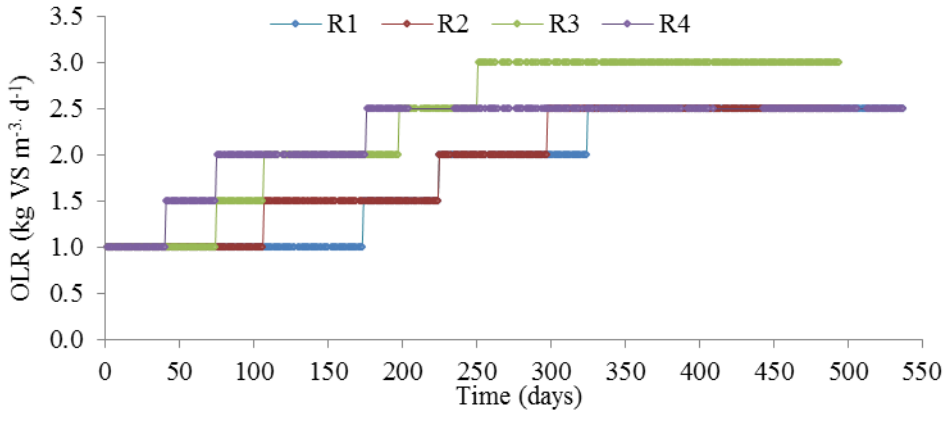
8.2 Experimental method

The start-up of four 5-L digesters for this study was described in section 4.3 Chapter 4. These digesters were operated in a semi-continuous mode and started at OLR of 1.0 kg VS m^{-3} d^{-1} with HRT of 33.3 days by diluting down the feed material. As mentioned before, Co, Ni and Fe solutions were initially added to all digesters at the strength of 0.03, 0.03 and 0.3 mg kg^{-1} FM, respectively, according to the preliminary result obtained in Chapter 4 which indicated that without TE supplementation, digestion could not overcome the instability issue caused by VFA accumulation. Digester could not perform stably over OLR of 1.0 kg VS m^{-3} d^{-1} with the strenght of Co and Ni to their baseline concentration from the feed. Therefore, it is practical to using these amount as an optimised formulation when TE increase need to be applied. In addition, all digesters received Se dosage at a consistent strength of 0.1 mg kg^{-1} FM throughout the experiment. The purpose of the Se supplementation was to prevent Se deficiency to be the limiting factor of the digesters' operation in this test where the effect of OLR on the requirement for Co, Ni and Fe supplementations would be determined.

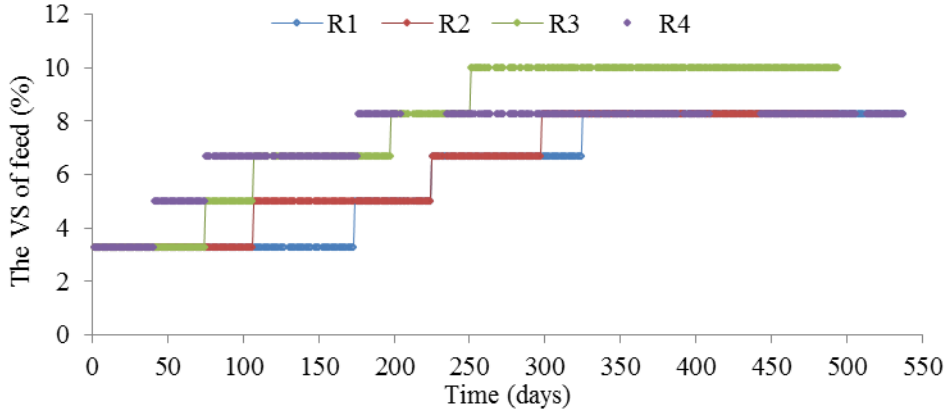
The experimental plan was to raise the loadings incrementally to give OLRs of 1.0, 1.5, 2.0, 2.5 and 3.0 kg VS m^{-3} d^{-1} in these four digesters under conditions of constant HRT of 33.3 days by adjusting feed material concentration, equivalent to the VS of feed at 3.3, 5, 6.7, 8.3 and 10%, respectively. Figure 8-1 shows the entire operational progress of the 4 digesters in terms of OLR and VS of feed. After the initial 40 days of operation at OLR of 1.0 kg VS m^{-3} d^{-1} for all digesters, the loading increase was conducted incrementally for digester R4, while other digesters were still running at a loading of 1.0 kg VS m^{-3} d^{-1}. After another 33 days (1 HRT), the OLR of R4 and R3 was increased to 2.0 and 1.5 kg VS m^{-3} d^{-1}, respectively with the rest two digesters still at 1.0 kg VS m^{-3} d^{-1}. The loading increase during the rest of the experiment did not always happen exactly at the end of a HRT due to time required to respond VFA accumulation by TE supplementation adjustment.

The digester performance was evaluated by daily measurements of biogas and CH_4 production, routine analysis of VSD, pH, alkalinity, TAN and VFA concentrations. Details of digesters construction, substrate used, inoculum properties and TE solutions were described in section 3.1,

3.2, 3.3 and 3.4 of Chapter 3. The dosing strength of Co, Ni and Fe during the 536 days of operation and organic loading applied to each digester were summarised in Table 8-1.



(a)



(b)

Figure 8-1 Application of OLR and the VS of feed changes through the trial in R1 - R4

Table 8-1 TE supplementations and OLR applied to the digester R1 - R4 throughout the trial.

Digester	OLR kgVS m^{-3} d^{-1} (day)	Supplements added (mg kg^{-1} FM)			Co, Ni and Fe	Mass ratio of Co + Ni to Fe
		Co	Ni	Fe		
R4	1.0 (1 - 40)	**0.03**	**0.03**	**0.3**	**1:1:10**	**1:5**
	1.5 (41 - 74)	0.03	0.03	0.3	1:1:10	1:5
	2.0 (75 - 76)	0.03	0.03	0.3	1:1:10	1:5
	2.0 (77 - 116)	0.06	0.06	0.6	1:1:10	1:5
	2.0 (117 - 175)	**0.06**	**0.09**	**0.9**	**0.67:1:10**	**1:6**
	2.5 (176 - 197)	0.06	0.09	0.9	0.67:1:10	1:6
	2.5 (198 - 206)	0.09	0.09	0.9	1:1:10	1:5
	Feed ceased (207 - 233)	0.12	0.12	1.2	1:1:10	1:5
	2.5 (234 - 297)	0.12	0.12	1.2	1:1:10	1:5
	2.5 (298 - 329)	0.15	0.15	1.5	1:1:10	1:5
	2.5 (330 - 343)	0.18	0.18	1.8	1:1:10	1:5
	2.5 (344 - 350)	0.21	0.21	2.1	1:1:10	1:5
	2.5 (351 - 378)	0.24	0.24	2.4	1:1:10	1:5
	2.5 (379 - 406)	0.27	0.27	2.7	1:1:10	1:5
	2.5 (407 - 439)	0.3	0.3	3.0	1:1:10	1:5
	2.5 (440 - 536)	1.0	1.0	10	1:1:10	1:5
R3	1.0 (1 - 78)	**0.03**	**0.03**	**0.3**	1:1:10	**1:5**
	1.5 (79 - 106)	**0.06**	**0.06**	**0.6**	1:1:10	**1:5**
	2.0 (107 - 116)	0.06	0.06	0.6	1:1:10	1:5
	2.0 (117 - 136)	0.06	0.09	0.6	1:1.5:10	1:4
	2.0 (137 - 175)	0.09	0.09	0.6	1.5:1.5:10	1:3.3
	2.0 (176 - 197)	**0.09**	**0.09**	**0.9**	1:1:10	**1:5**
	2.5 (198 - 206)	0.09	0.09	0.9	1:1:10	1:5
	2.5 (207 - 219)	0.12	0.12	0.9	1.33:1.33:10	1:3.75
	2.5 (220 - 249)	**0.12**	**0.12**	**1.2**	1:1:10	**1:5**
	3.0 (250 - 260)	0.12	0.12	1.2	1:1:10	1:5
	3.0 (261 - 273)	0.15	0.15	1.2	1.25:1.25:10	1:4

Digester	OLR kgVS m^{-3} d^{-1} (day)	Supplements added (mg kg^{-1} FM) Co	Ni	Fe	Co, Ni and Fe	Mass ratio of Co + Ni to Fe
	3.0 (274 - 297)	0.15	0.15	1.5	1:1:10	1:5
	3.0 (298 - 331)	0.18	0.18	1.8	1:1:10	1:5
	3.0 (332 - 343)	0.21	0.21	2.1	1:1:10	1:5
	3.0 (344 - 350)	0.21	0.21	2.4	0.875:0.875:10	1:5.7
	3.0 (351 - 378)	0.24	0.24	2.4	1:1:10	1:5
	3.0 (379 - 406)	0.27	0.27	2.7	1:1:10	1:5
	3.0 (407 - 427)	0.3	0.3	3.0	1:1:10	1:5
	3.0 (428 - 439)	0.33	0.33	3.3	1:1:10	1:5
	3.0 (440 - 494)	1.0	1.0	10	1:1:10	1:5
R2	1.0 (1 - 106)	**0.03**	**0.03**	**0.3**	1:1:10	**1:5**
	1.5 (107 - 141)	0.03	0.03	0.3	1:1:10	1:5
	1.5 (142 - 174)	0.03	0.06	0.3	1:2:10	1:3.33
	1.5 (175 - 206)	0.06	0.06	0.3	2:2:10	1:2.5
	1.5 (207 - 224)	**0.06**	**0.06**	**0.6**	1:1:10	**1:5**
	2.0 (225 - 231)	0.06	0.06	0.6	1:1:10	1:5
	2.0 (232 - 296)	**0.06**	**0.09**	**0.9**	0.67:1:10	**1:6**
	2.5 (297 - 343)	0.06	0.09	0.9	0.67:1:10	1:6
	2.5 (344 - 350)	0.09	0.09	0.9	1:1:10	1:5
	2.5 (351 - 385)	0.12	0.12	1.2	1:1:10	1:5
	2.5 (386 - 427)	0.15	0.15	1.5	1:1:10	1:5
	2.5 (428 - 439)	0.18	0.18	1.8	1:1:10	1:5
	2.5 (440 - 494)	1.0	1.0	10	1:1:10	1:5
R1	1.0 (1 - 141)	0.03	0.03	0.3	1:1:10	1:5
	1.0 (142 - 173)	**0.03**	**0.06**	**0.6**	0.5:1:10	**1:6.67**
	1.5 (174 - 197)	0.03	0.06	0.6	0.5:1:10	1.6.67
	1.5 (198 - 224)	**0.06**	**0.06**	**0.6**	1:1:10	**1:5**
	2.0 (225 - 238)	0.06	0.06	0.6	1:1:10	1:5
	2.0 (239 - 297)	0.09	0.09	0.6	1.5:1.5:10	1:3.33
	2.0 (298 - 324)	**0.09**	**0.09**	**0.9**	1:1:10	**1:5**

Digester	OLR kgVS m^{-3} d^{-1} (day)	Supplements added (mg kg^{-1} FM)			Co, Ni and Fe	Mass ratio of Co + Ni to Fe
		Co	Ni	Fe		
	2.5 (325 - 368)	0.09	0.09	0.9	1:1:10	1:5
	2.5 (369 - 439)	0.12	0.12	1.2	1:1:10	1:5
	2.5 (440 - 536)	1.0	1.0	10	1:1:10	1:5

Note: 1. The bold numbers shown are the minimum requirement of TE dosing strength for each OLR, which also shows the optimum ratio of Co, Ni and Fe in supplementation mixture; 2. The OLR shown in table in units of kg VS m^{-3} d^{-1}

8.3 Results and discussion

8.3.1 **Baseline performance and stability assessment (day 0 - 40)**

According to SMP and VFA data shown in Figure 8-2, all four digesters (R1 - R4) performed in the same manner during the first 40 days at OLR 1.0 kg VS m^{-3} d^{-1} with TE supplementation at the strength of Co 0.03; Ni 0.03 and Fe 0.3 mg kg^{-1} FM. This provided an identical start point for the operational changes on day 41 when digesters were started to diverge from each other in terms of OLR and TE addition. The SBP was around 0.75 ± 0.05 m^3 biogas kg^{-1} VS added (SMP 0.45 ± 0.03 m^3 CH_4 kg^{-1} VS_{added}), methane content was around 59.9 ± 1.0 %, Average VFA was below 300 mg L^{-1} (248 ± 19), pH was around 7.5 ± 0.0, which was comparable to the performance of 100-L digester when it was operated stably as seen in section 4.3 Chapter 4.

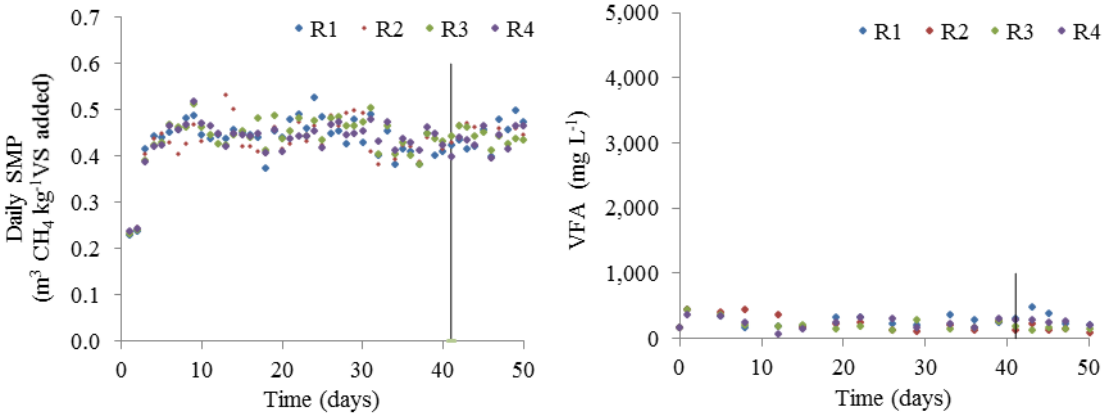


Figure 8-2 SMP and VFA profiles during the first 40 days of operation. The vertical solid line denoted the initial OLR increasing was applied (day 41)

8.3.2 **Effect of OLR on the requirement of Co, Ni and Fe at constant HRT**

8.3.2.1 **Digester R4**

The operation of digester R4 is described first because the OLR increase was firstly applied to this digester. From Figure 8-3, after loading increase from 1.0 to 1.5 kg VS m^{-3} d^{-1} on day 41 with the TE addition at the initial strength at Co 0.03; Ni 0.03 and Fe 0.3 mg kg^{-1} FM, the successful continuous operation could not be achieved in this digester. VFA increased sharply to around 1,200 mg L^{-1} on day 74. This result suggested that this supplementation strength was deficient to maintain process stability at OLR 1.5 kg VS m^{-3} d^{-1}.

OLR was further increased to 2.0 kg VS m^{-3} d^{-1} on day 75 as planned. TE supplementation was doubled on day 77 to the strength of Co 0.06; Ni 0.06 and Fe 0.6 mg kg^{-1} FM; this temporarily reduced VFA level from 2,200 mg L^{-1} to less than 200 mg L^{-1} by day 91. Stable operational condition, however, was not be achieved, as VFA immediately rose again to 2,900 mg L^{-1} with acetic acid was the dominant VFA. Extra supplementation of Ni and Fe was applied from day 117 to make the dosing strength change to Co 0.06, Ni 0.09 and Fe 0.9 mg kg^{-1} FM. Following this action, VFA reduced rapidly and maintained at low level for the following 59 days.

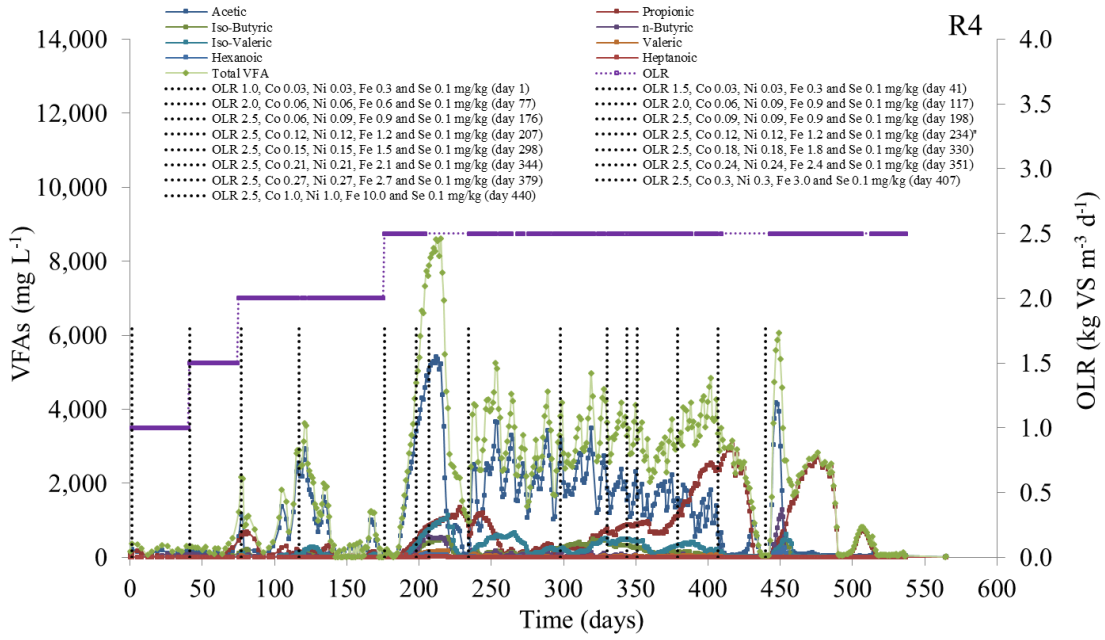


Figure 8-3 VFA profiles and the operation scheme of R4 over this loading experiment

Then afterward, OLR was increased to 2.5 kg VS m^{-3} d^{-1} on day 176 with the TE supplementation at the previously dosed. VFA concentration dramatically increased with acetic acid was the dominant specie from day 185 to above 4,700 mg L^{-1} on day 198 leading to digester failure. The pH dropped to around 6.5 and the methane content was 37 % on day 205. Various attempts were

made in order to recover digester by reducing its VFA level. Feeding was stopped from day 207 to 233 to lower the loading and maintain pH. TE supplementation strength was also raised to Co 0.12, Ni 0.12 and Fe 1.2 mg kg^{-1} FM from day 207. Following this action, total VFA further increased up to higher than 8,500 mg L^{-1} in a week, however, reduced rapidly to around 1,000 mg L^{-1}. OLR was switched back to 2.5 kg VS m^{-3} d^{-1} on day 234 which made VFA immediately rose again.

The loading increase during the rest of the experiment did not apply exactly at the end of a HRT due to time required to respond VFA accumulation by TE supplementation adjustment. During digester recovery period, the dosing was incrementally increased until the strength at Co 0.27, Ni 0.27 and Fe 2.7 mg kg^{-1} FM was reached on day 379. VFA did not show decline but fluctuations which might be caused by different strength and frequency of TE supplementation. Acetic acid showed several sharp drop following each dosage increased. To maintain pH a phase of intermittent feeding was adopted, which could not stop performance deterioration neither: the pH dropped to around 7.0 and the methane content of biogas was less than 52%. Fluctuations of VFA might be caused by different strength and frequency of TE supplementation. VFA concentration fluctuated around 2,000 - 5,000 mg L^{-1}, all these attempts failed. The VFA profile showed that initially acetic acid was the dominant VFA, noticeably however, propionic gradually increased higher up to 2,400 mg L^{-1} on day 407. Extra dose of TE was applied to make the dosing strength change to Co 0.3, Ni 0.3 and Fe 3.0 mg kg^{-1} FM together with ceased substrate addition from day 409 to day 439. Following this action, VFA significantly reduced from 3,300 mg L^{-1} to less than 300 mg L^{-1} within 26 days.

As seen in the period of TE dosage increase in Figure 8-3, process performance could not be recovered to the normal operation at OLR 2.5 kg VS m^{-3} d^{-1}. The unrecoverable process performance was clearly supported the previous results in Chapter 6 that TE supplementation stimulated VFA degradation, but insufficient strength could not ensure their complete and timely degradation until a sufficient strength reached. Inadequate TE supply caused massive process imbalances in term of high VFA accumulation and low biogas production and methane yield. Insufficient TE addition was unlikely to stimulate methanogenesis for VFA degradation, but instead enhanced its accumulation. Sudden excessive TE supplementation strongly stimulated VFA accumulation to a great extent, which did inhibit recovery process. In some cases it could induce severe VFA accumulation and failure of digester when it is added to VFA-laden digesters. Another important issue was that the timing of TE addition was essential with regard to balancing the process between hydrolysis-acidification and methanogenesis: an earlier supplementation was inhibitory to methanogens due to the inhibitory effect of VFA, whereas a delayed supplementation could not control the VFA accumulation by methanogenesis (Yu *et al.*, 2015).

From this and the previous results as discussed in Chapter 6, the unrecovered process stability in R4 confirmed that the recovery strength of TE dosage was required much higher level than their critical strength as described in Chapter 6 and also higher than those existed in digester for maintaining stability and performance efficiency especially at high level of VFA.

R4 was struggling from TE deficiency issue for 264 days (day 176 to day 440). In order to accelerate enrichment of TE, the TE dosing strength was changed to Co 1.0, Ni 1.0 and Fe 10 mg kg^{-1} FM on day 440 in attempt to achieve stability. Resumed feeding was applied from day 442. Following this action, total VFA sharply declined from above 6,000 to around 2,000 mg L^{-1}. Although propionic was promoted to concentration of 2,500 mg L^{-1}, however, it was reduced completely within 3 weeks. Ceased feeding had been adopted several days to lower the loading. No obvious accumulation of VFA was observed afterwards. Process operation could be recovered and stable operation maintained for around 2 HRTs before the reactor was being terminated. This result confirmed the key finding from the previous results (Chapter 5 and section 6.3.1 Chapter 6) that these TE dosing strength was sufficient for stable and optimal digester performance.

8.3.2.2 **Digester R3**

VFA profiles and the operation scheme for R3 over this loading experiment was shown in Figure 8-4. There was no noticeable digester instability observed in R3 for the first 78 days (>2 HRTs). It had been operated normally at OLR 1.0 kg VS m^{-3} d^{-1}. This result provided the supported evidence for R4 indicating that the TE supplementation was sufficient at strength of Co 0.03, Ni 0.03 and Fe 0.3 mg kg^{-1} FM.

The OLR was increased to 1.5 kg VS m^{-3} d^{-1} on day 79 as planned. VFA showed a slight increase to about 500 mg L^{-1}.The TE dosing strength was changed to Co 0.06, Ni 0.06 and Fe 0.6 mg kg^{-1} FM which could keep process performance under stable condition.

Further OLR increase to 2.0 kg VS m^{-3} d^{-1} was then applied on day 107 by supplementation with the TE range as previously used. VFA sharply increased to 2,100 mg L^{-1} on day 117. At that point, additional Ni was applied to 0.09 mg kg^{-1} FM, this temporarily reduced VFA. Stable operational condition, however, was not be achieved, as VFA immediately increased again higher up to 2,600 mg L^{-1}. Extra Co supplementation was applied to make the dosing strength change to Co 0.09, Ni 0.09 and Fe 0.6 mg kg^{-1} FM from day 137, VFA continued to increase to almost 5,000 mg L^{-1} on day 147. Following this action, acetic acid dropped from 3,200 mg L^{-1} to low level within 17 days with the same VFA reducing rate. VFA dropped to 1,850 mg L^{-1} by day 164, however, propionic was promoted to 2,200 mg L^{-1} on day 176. In attempt to recover the digester, then, Fe was applied to make the TE dosing changed to Co 0.09, Ni 0.09 and Fe 0.9 mg kg^{-1} FM from day 176. Eventually, total VFA dropped from 2,700 mg L^{-1} to less than 300 mg L^{-1} within 10 days. R3 can

be recovered to the normal operation and maintained stable operation for the following 12 days before increasing OLR was applied. This stage indicated that this TE supplementation strength was sufficient at OLR 2.0 kg VS m^{-3} d^{-1}.

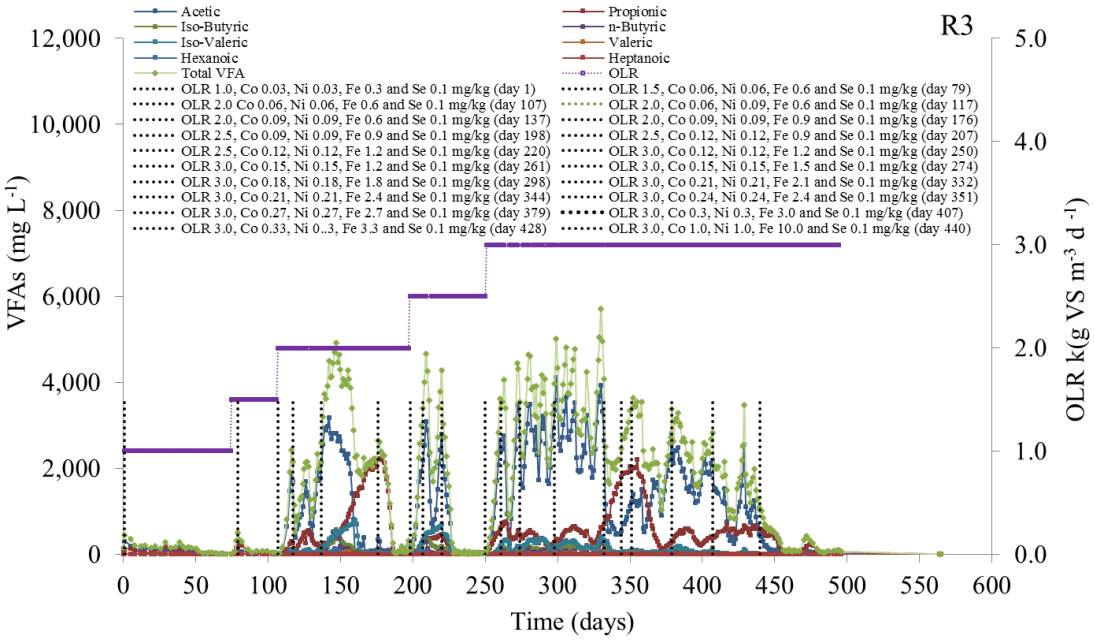


Figure 8-4 VFA profiles and the operation scheme for R3 over this loading experiment

This TE dosing strength was adopted with the higher OLR at 2.5 kg VS m^{-3} d^{-1} on day 198. VFA was dramatically increased to around 3,400 mg L^{-1} on day 207. At that point, extra Co and Ni dosing was applied which temporarily reduced VFA level. Stable operational condition, however, was not be achieved, as VFA immediately rose again to 4,300 mg L^{-1} on day 220. The strength of Fe was raised to 1.2 mg kg^{-1} FM to make TE dosage changed to Co 0.12, Ni 0.12 and Fe 1.2 mg kg^{-1} FM from day 220. Total VFA dropped to less than 300 mg L^{-1} in a week. R3 performed stable for about 1 HRT before further increasing in OLR was applied. This TE dosing range was proven to be sufficient for digestion running at OLR 2.5 kg VS m^{-3} d^{-1}.

Thereafter, OLR was increased to 3.0 kg VS m^{-3} d^{-1} on day 250 by initially maintaining TE supplementation as previously used, VFA accumulated immediately. VFA concentration dramatically increased to above 3,400 mg L^{-1} with acetic acid was the dominant VFA. Additional supplementation of Co and Ni was applied from day 261 to make the dosing strength change to Co 0.15, Ni 0.15 and Fe 1.2 mg kg^{-1} FM. Following this action, temporarily reduced VFA level to around 1,000 mg L^{-1} by day 267. Stable operational condition, however, was not be achieved, as VFA immediately rose again higher to about 3,600 mg L^{-1} on day 274. During digester recovered period, various attempts were made to recover process stability by reducing VFA level. Extra Fe dosing was applied to a strength of 1.5 mg kg^{-1} FM. To maintain pH a phase of intermittent feeding was adopted, which could not stop performance deterioration either: the pH

dropped to around 7.06 and the methane content of biogas was less than 49.3 %. VFA did not show decline but fluctuations which might be caused by different strength and frequency of TE supplementation. Acetic acid showed several sharp drop following each dosage increased. The TE dosing strength was further changed to Co 0.21, Ni 0.21 and Fe 2.1 mg kg^{-1} FM on day 332. Following this action, VFA accumulation still existed but reduced gradually and fluctuated around 1,000-3,500 mg L^{-1}. From day 344, the TE dosage was incrementally increased until the TE dosage reached the strength of Co 0.33, Ni 0.33 and Fe 3.3 mg kg^{-1} FM on day 428 with continuous feeding. The total VFA with 60% accounted for propionic acid dropped to around 1,000 mg L^{-1} by day 439.

As seen in the period of TE dosage increase during digester recovered period, process performance could not be recovered to the normal operation at OLR 3.0 kg VS m^{-3} d^{-1}. The unrecoverable process performance in digester R3 clearly confirmed the previous results which appeared in R4 and in TE washed-out digesters (Chapter 6). The recovery strength of TE dosage required much higher levels than their critical strength, as described in Chapter 6, and also higher than TE concentrations existed in digester for maintain stability and performance efficiency especially at high level of VFA.

R3 was faced the TE deficiency issue for 189 days (day 250 - 439) at OLR 3.0 kg VS m^{-3} d^{-1}. Then, the TE dosing strength was applied to make the dosing strength change to Co 1.0, Ni 1.0 and Fe 10 mg kg^{-1} FM in attempt to achieve stability. Following this action, there was a clear evidence, showing enhanced and faster reduction in VFA level when TE sufficient amount was adopted. VFA was completely consumed and process operation could be recovered and maintained stable for around 2 HRTs before being terminated. This result supported the key finding from Chapter 5, section 6.3.1 Chapter 6 and confirmed the recovery performance of R4 by the end of trial. It appeared that TE supplementation stimulated VFA degradation, but insufficient strength could not ensure its complete and timely degradation until a sufficient strength reached as above aforementioned.

8.3.2.3 **Digester R2**

From Figure 8-5, R2 had been operated normally at OLR 1.0 kg VS m^{-3} d^{-1} with no noticeable of digester instability observed for the first 106 days (> 3 HRTs). This result provided the supported evidence for R4 and R3 indicating that the supplementation strength of Co 0.03, Ni 0.03 and Fe 0.3 mg kg^{-1} FM was sufficient for the OLR used.

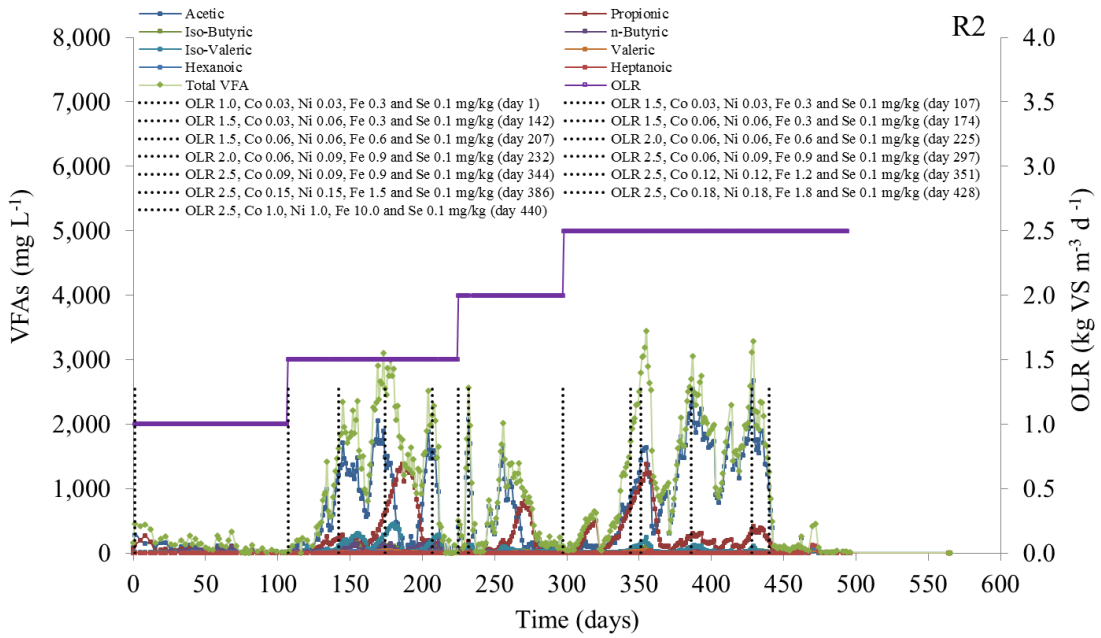


Figure 8-5 VFA profiles and the operation scheme for R2 over this loading experiment

OLR was increased to 1.5 kg VS m^{-3} d^{-1} on day 107 with the previous TE dosing range. Stable process performance could not be achieved, VFA started to increase from day 124 to 1,600 mg L^{-1} on day 142. At that point, extra Ni was applied to the strength of 0.06 mg kg^{-1} FM which reduced total VFA to 1,000 mg L^{-1} by day 161. Stable operational condition, however, was not be achieved, as VFA immediately increased again to 2,600 mg L^{-1} on day 174. Additional Co was applied to the strength of 0.06 mg kg^{-1} FM. Following this action, total VFA dropped with the same rate as propionic increase. VFA reduced from above 2,500 mg L^{-1} to 1,500 mg L^{-1} while propionate started to accumulate higher up to 1,300 mg L^{-1}on day 190. In attempt to recover digester operation, extra Fe was applied to make the TE dosing strength change to Co 0.06, Ni 0.06 and Fe 0.6 mg kg^{-1} FM on day 207. Digester operation could be brought back under control, VFA sharply decreased from 2,700 mg L^{-1} to low level within 10 days. This result supported R3 suggesting that this TE supplementation used was sufficient to maintain stable operation at OLR 1.5 kg VS m^{-3} d^{-1}.

OLR was further increased to 2.0 kg VS m^{-3} d^{-1} on day 225 by TE dosing strength as previously used. This resulted in VFA accumulating which started from 500 mg L^{-1} on day 225 higher up to the above 2,600 mg L^{-1} within a week. At that point, extra Ni and Fe were adopted to make the TE dosing strength change to Co 0.06, Ni 0.09 and Fe 0.9 mg kg^{-1} FM, which temporary reduced VFA within 14 days. Stable operational condition, however, was not be achieved, as VFA immediately rose again to 2,000 mg L^{-1} on day 256. Thereafter, VFA dropped to less than 300 mg L^{-1} by day 278 and maintained at low level for the following 20 days before increase OLR was

applied. This supported the previous result from R4 that this TE addition strength was sufficient to maintain stability at OLR 2.0 kg VS m^{-3} d^{-1}.

Then afterwards, OLR was increased from 2.0 to 2.5 kg VS m^{-3} d^{-1} on day 297 with TE dosage range as earlier OLR used. VFA rose to 1,700 mg L^{-1} on day 344. At that point, Co was applied to make TE dosing strength change to Co 0.09, Ni 0.09 and Fe 0.9 mg kg^{-1} FM. Unfortunately, there was no sign of VFA decreasing, then, the TE supplementation strength were raised to Co 0.12, Ni 0.12 and Fe 1.2 mg kg^{-1} FM on day 351. Following this action, VFA dropped from 3,400 mg L^{-1} on day 355 to around 900 mg L^{-1} by day 364. This temporarily reduced VFA, stable operational condition, however, was not be achieved, as VFA immediately increased again to 2,700 mg L^{-1} on day 386 which clearly reflected TE deficiency issue. The TE dosing strength was changed to Co 0.15, Ni 0.15 and Fe 1.5 mg kg^{-1} FM from day 386 which could reduce VFA level but fluctuated around 1,000-3,000 mg L^{-1} for 42 days with no trend of digester recovery. On day 428, the TE supplementation strength was changed again to Co 0.18, Ni 0.18 and Fe 1.8 mg kg^{-1} FM, VFA still existed at 1,500 mg L^{-1} with 400 mg L^{-1} propionic acid was the predominant specie.

As seen in Figure 8-5, the period of TE dosage increase during digester recovery, process performance could not be recovered to the normal operation at OLR 2.5 kg VS m^{-3} d^{-1}. The unrecoverable process performance in digester R2 was clearly confirmed the previous results which appeared in R4 and R3 in the same study, and in TE washed-out digesters (Chapter 6) as above aforementioned. The recovery strength of TE dosage was required much higher level than their critical strength, as described in Chapter 6, and also higher than those existed in digester for maintain stability and performance efficiency especially at high level of VFA.

As discussed above, the TE deficiency issue occurred in R2 from day 297 to day 439 (142 days) at OLR 2.5 kg VS m^{-3} d^{-1}. At that point, the TE dosage strength was changed to Co 1.0, Ni 1.0 and Fe 10 mg kg^{-1} FM in attempt to achieve stability. VFA was suddenly dropped from 1,500 mg L^{-1} to less than 200 mg L^{-1} in a week and process operation recovered and maintain stable for 2 HRTs before being terminated. There was a clear evidence, showing enhanced and faster reduction in VFA when TE sufficient amount was applied. This result provided a supported evidence for Chapter 5, section 6.3.1 Chapter 6 and the recovery performance of R4 and R3 by the end of trial confirming that this dosing strength was sufficient for stable and optimal digester performance.

8.3.2.4 **Digester R1**

R1 had been performed normally at OLR 1.0 kg VS m^{-3} d^{-1} with no noticeable digester instability for the first 119 days (> 3 HRTs). Stable process performance was not be achieved, VFA sharply increased to 1,400 mg L^{-1} on day 127. Feeding was ceased for 2 days which temporarily reduced

VFA to less than 100 mg L^{-1}, however, VFA with acetic acid was the predominant VFA which increased to 1,400 mg L^{-1} on day 142. This demonstrated that R1 faced TE shortage problem at the strength of Co 0.03, Ni 0.03 and Fe 0.3 mg kg^{-1} FM. To respond to the accumulation of VFA, additional dosing of Ni and Fe was applied to make the dosing strength change to Co 0.03, Ni 0.06 and Fe 0.6 mg kg^{-1} FM on day 142. VFA further increased higher up to 1,900 mg L^{-1} on day 150. VFA, eventually, reduced to less than 100 mg L^{-1} in 23 days. It could be operated normally before increasing OLR. According to the result, this illustrated that this TE dosing was sufficient to maintain stable operation at 1.0 kg VS m^{-3} d^{-1} for longer-term operation (4 HRT).

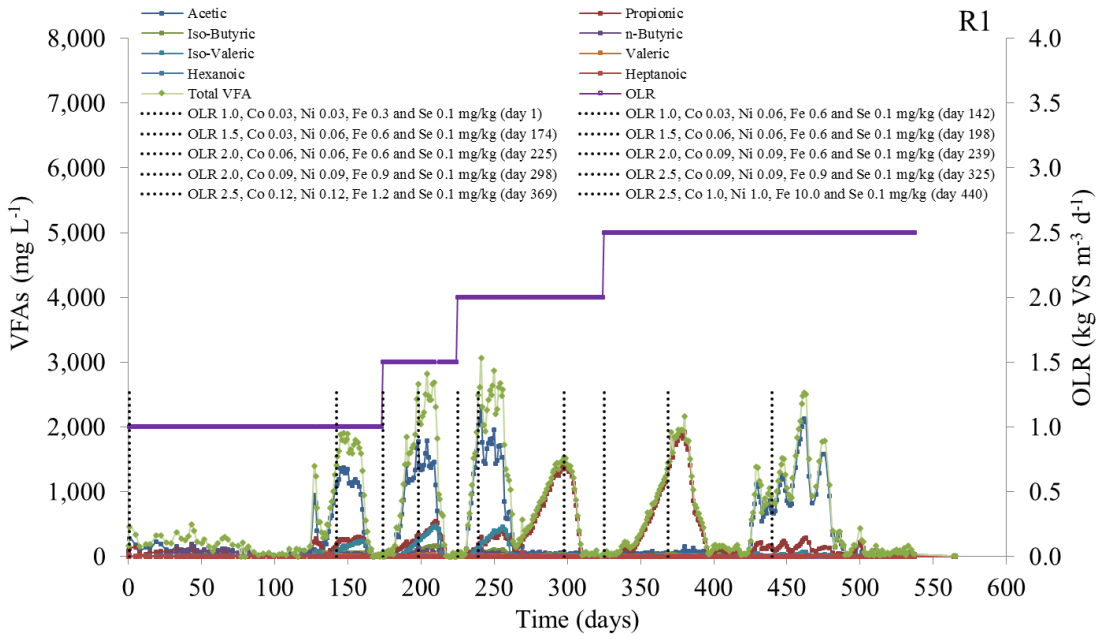


Figure 8-6 VFA profiles and the operation scheme for R1 over this loading experiment

A new OLR at 1.5 kg VS m^{-3} d^{-1} was adopted on day 174 supplemented with the previous TE dosage range. VFA rose up to 2,700 mg L^{-1} on day 198. At that point, additional Co supplementation was applied to make the TE dosing strength change to Co 0.06, Ni 0.06 and Fe 0.6 mg kg^{-1} FM in attempt to reduce VFA level. VFA dropped from 2,800 mg L^{-1} on day 204 to less than 200 mg L^{-1} within 12 days. Result from this stage provided a supported evidence for R3 and R2 indicating that TE dosing at this strength was sufficient to maintain stable operation at 1.5 kg VS m^{-3} d^{-1}.

After a period of time, OLR was increased from 1.5 to 2.0 kg VS m^{-3} d^{-1} on day 225 with the same TE supplementation strength. This resulted in dramatic increase in VFA to 2,500 mg L^{-1} in the following 2 weeks. Both Co and Ni dosing was applied to make TE dosing strength change to of Co 0.09, Ni 0.09 and Fe 0.6 mg kg^{-1} FM on day 239. From this point, it seemed the digester could be recovered, VFA decreased from above 3,100 mg L^{-1} on day 241 to less than 300 mg L^{-1} by day 263. Stable operational condition, however, was not be achieved, as VFA with 95 % propionic

acid immediately increased again to around 1,500 mg L^{-1} on day 298. In attempt to recover digester stability to normal operation, extra Fe was dosed to make TE supplementation change to Co 0.09, Ni 0.09 and Fe 0.9 mg kg^{-1} FM. Following this action, VFA reduced from 1,500 mg L^{-1} to less than 200 mg L^{-1} by day 309. A successful recovering process stability was achieved in two weeks. R1 could be performed normally for another 16 days before an increase in OLR was further applied. This result provided a supported evidence for R3 indicating that the TE dosing strength was sufficient to maintain stable operation at 2.0 kg VS m^{-3} d^{-1}.

OLR was further raised to 2.5 kg VS m^{-3} d^{-1} on day 325. VFA started to increase from day 337 higher up to 1,400 mg L^{-1} on day 369. At that point, to respond to the accumulation in VFA, the TE dosing strength was changed to Co 0.12, Ni 0.12 and Fe 1.2 mg kg^{-1} FM. This resulted in decease in VFA from 2,100 mg L^{-1}, of which propionate was the predominant specie, on day 380 to less than 200 mg L^{-1} by day 395. R1 performed normally for the following 30 days, stable operational condition, however, was not be achieved, as VFA sharply increased again on day 429 to around 1,400 mg L^{-1}. This demonstrated this TE dosage strength was deficient to recover process back to stable operation at OLR of 2.5 kg VS m^{-3} d^{-1}.

As seen in Figure 8-6, the period of TE dosage increase during digester recovery, process performance was not be recovered to the normal operation at OLR 2.5 kg VS m^{-3} d^{-1}. The unrecoverable process performance in digester R1 clearly confirmed the previous results which appeared in R4, R3 and R2 in the same study, and in TE washed-out digesters (Chapter 6) as above aforementioned. It appeared that the recovery strength of TE dosage was required much higher level than their critical strength as described in Chapter 6 and also higher than TE concentrations existed in digester for maintain stability and performance efficiency especially at high level of VFA.

The TE deficiency issue also occurred in R1 from day 325 to day 439 (114 days) at OLR 2.5 kg VS m^{-3} d^{-1}. At that point, the TE dosage strength was changed to Co 1.0, Ni 1.0 and Fe 10 mg kg^{-1} FM in attempt to achieve stability. Sudden excessive TE supplementation strongly stimulated VFA accumulation to a great extent, total VFA with acetic acid was the predominant specie, further increasing from 800 mg L^{-1} on day 440 higher up to 2500 mg L^{-1} on day 463. Eventually, VFA dropped down to less than 300 mg L^{-1} by day 487. Process operation recovered and maintain stable for around 2 HRTs before being terminated. This result provided a supported evidence for Chapter 5, section 6.3.1 Chapter 6 and confirming the issues associated with digester R4, R3 and R2 by the end of trial that this TE dosing strength was sufficient for stable and optimal digester performance.

Further discussion

It is worth to note that, after VFA started to appear in this set of experiment, results demonstrated that the critical TE levels were much higher, when this experiment started from low OLR to high OLR, than results obtained from washing-out experiment at OLR 3.0 kg VS m^{-3} d^{-1} (Chapter 6).

Results indicated that the minimum TE requirement is relevant to OLR at constant HRT. This experiment, the microbial biomass concentration in the digester is a proportional to OLR. When OLR increases, the concentration of microbial biomass increases accordingly if HRT is fixed, and microbes may have to take more TE from their environment. But when TE concentration in their environment is low, there are two problems: 1) the concentration gradient or energy required to find and carry TE to the inside of the cells; 2) the availability/speciation of TE in their environment. For washing-out experiment (Chapter 6 and 7), as it has been pointed out that the microbes can just recycle TE in their cells; that state of TE is perfect and therefore can maintain their metabolic activities for a prolonged period until the TE concentration inside was diluted by hydraulic action to a level which is too low. So essentially, the critical TE concentration that was measured in washing out experiment should be the effective TE concentration which should belong to microbe-incorporated TE, but the total TE concentration used to control this set of experiment should be much higher than that of the effective TE. The minimum requirement of TE dosing strength for each OLR tested were summarise in Table 8-1.

8.3.3 Ratio of Co, Ni and Fe in supplementation mixture

Apart from the individual dosing strength of each TE, the ratio of these TE in supplementation mixture also needs to be optimised in order to achieve stable and efficient digestion at a range of OLR. During this experiment, there was 7 mass ratios (1:2.5, 1:3.33, 1:3.75, 1:4, 1:5, 1:6 and 1:6.67) of Co + Ni to Fe in supplementation mixture had been tested as shown in Table 8-1 to determine the optimal ratio of Co + Ni to Fe as shown in Table 8-2.

As described in section 8.3.2, a ratio of 1:2.5, 1:3.33, 1:3.75 or 1:4 could not restore the process stability even when the total quantity of each TE seemed sufficient (e.g. day 175 - day 206 in R2, day 239 - day 297 in R1, day 207 - day 219 in R3 and day 117 - day 136 in R3). However, digester operation could be recovered from severe VFA accumulation and maintained stably when the TE supplementation was made at a ratio of 1:5, 1:6 and 1:6.67 (e.g. day 176 - day 197 in R3, day 117 - day 175 in R4, day 142 - day 173 in R1). This implied that dosing ratio between Co + Ni and Fe make a clear different in process recovering, and the optimal ratio of Co + Ni and Fe in supplementation mixture based on this study was in range of 1:5 - 1:6.67.

Table 8-2 The recommended mass ratio of Co, Ni to Fe for each OLR

Digester	OLR kgVS m^{-3} d^{-1} (day)	Supplements added (mg kg^{-1} FM)			Co, Ni and Fe	Mass ratio of Co + Ni to Fe
		Co	Ni	Fe		
R4	1.0 (1 - 40)	0.03	0.03	0.3	1:1:10	1:5
	2.0 (117 - 175)	0.06	0.09	0.9	0.67:1:10	1:6
R3	1.0 (1 - 78)	0.03	0.03	0.3	1:1:10	1:5
	1.5 (79 - 106)	0.06	0.06	0.6	1:1:10	1:5
	2.0 (176 - 197)	0.09	0.09	0.9	1:1:10	1:5
	2.5 (220 - 249)	0.12	0.12	1.2	1:1:10	1:5
R2	1.0 (1 - 106)	0.03	0.03	0.3	1:1:10	1:5
	1.5 (207 - 224)	0.06	0.06	0.6	1:1:10	1:5
	2.0 (232 - 296)	0.06	0.09	0.9	0.67:1:10	1:6
R1	1.0 (142 - 173)	0.03	0.06	0.6	0.5:1:10	1:6.67
	1.5 (198 - 224)	0.06	0.06	0.6	1:1:10	1:5
	2.0 (298 - 324)	0.09	0.09	0.9	1:1:10	1:5
R4	2.5 (440 - 536)	1.0	1.0	10	1:1:10	1:5
R3	3.0 (440 - 491)					
R2	2.5 (440 - 494)					
R1	2.5 (440 - 536)					

As discussed in Chapter 2, sulphide was the preferred ligand and had high affinity for binding with Co, Ni and Fe which have been suggested to affect their bioavailability (Gonzalez-Gil *et al.*, 2003; Jansen, Gonzalez-Gil and van Leeuwen, 2007) due to their very low solubility products (Callander and Barford, 1983a;b; Rinzema and Lettinga, 1988; Rickard and Luther, 2006). As a result, the higher ratio of Co, Ni to Fe gave rise to disturbance and function decline probably due to less amount of available Fe. This test demonstrated that not only the total strength but the optimal ratio of a mix of Co, Ni and Fe in supplementation mixture played a significant role on digester stability.

8.3.4 **General parameters and biogas performance indicator**

The performance profiles for R1 - R4 over this loading experiment were shown from Figure 8-7 - Figure 8-12. During stable operation, pH remained practically constant at 7.4 ± 0.1 (Figure 8-7) closed to optimum range for mesophilic methanagenic activity (Wheatley, 1990).

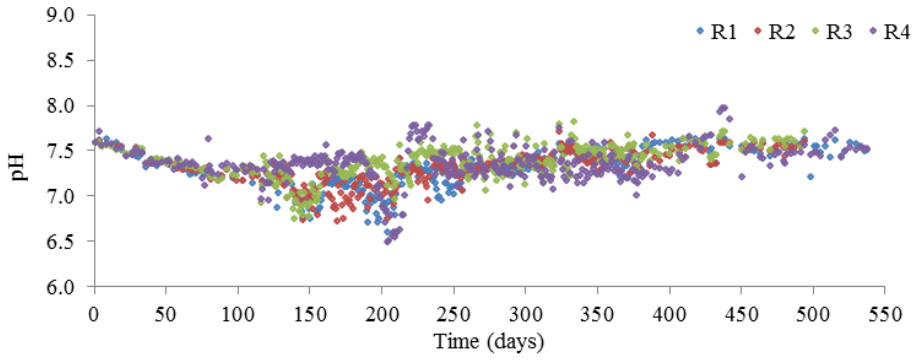
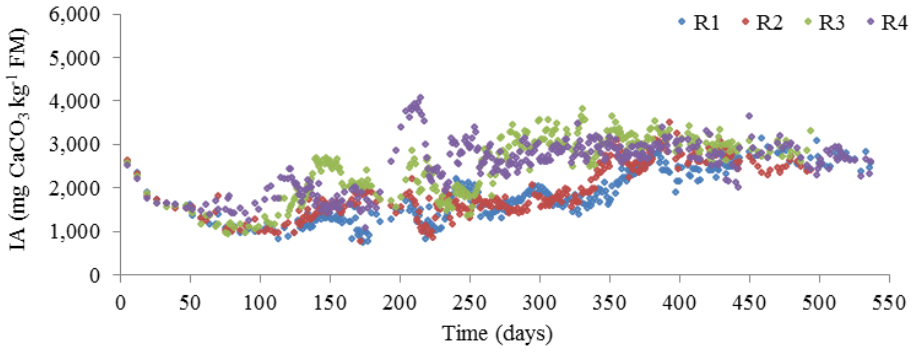
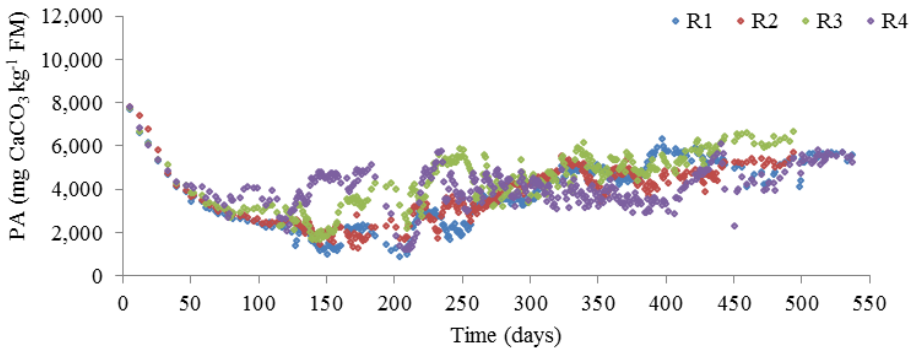


Figure 8-7 pH of digesters R1 - R4 for the whole experiment

The IA/PA was below 0.3 ± 0.1 (Figure 8-8) in the range of optimal operation (Ripley, Boyle and Converse, 1986). VSD was above 93.1 ± 1.0 % (Figure 8-9) and no VFA accumulation was detected. The concentration of each volatile fatty acids (VFA) monitored never exceeded 300 mg L^{-1} (Figure 8-3 - Figure 8-6).



(a)



(b)

Figure 8-8 IA (a), PA (b), (c) TA and (d) IA/PA of digesters R1 - R4 for the whole experiment

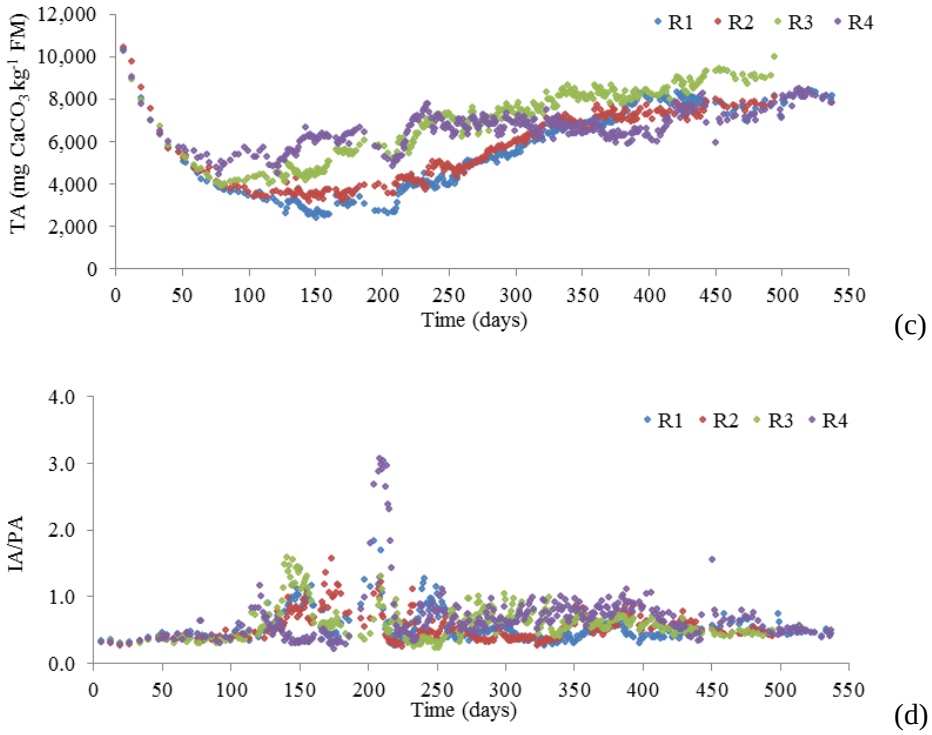


Figure 8-8 IA (a), PA (b), (c) TA and (d) IA/PA of digesters R1 - R4 for the whole experiment (continued)

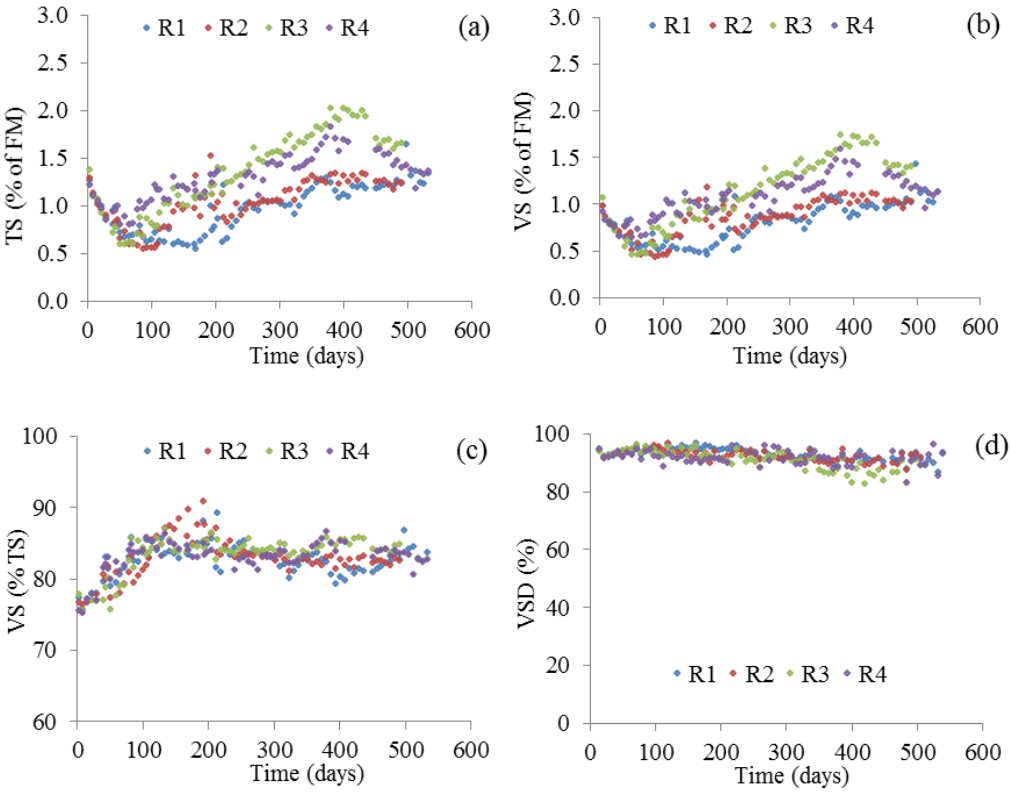


Figure 8-9 (a) TS % of FM, (b) VS % of FM, (c) VS as a % of TS and (d) % VSD of digester R1 - R4 for whole experiment

Figure 8-10 showed TAN and FAN concentrations in digesters R1 - R4. The TAN were initially started around 2,800 mg kg^{-1} FM. These digesters were stabilised with the new inoculum at its new HRT of 33.3 days for 40 days before increasing OLR was applied. With the VS of 10 - 3.3 % in the feed, the TAN and FAN concentrations in well-performed digesters was around 800 - 2,500 mg kg^{-1} FM and below 200 mg kg^{-1} FM depending on OLR applied. It could be indicated that TAN and FAN over the course of operation were well below the inhibitory level which is around > 3,000 - 4,000 mg TAN kg^{-1} FM and 800 mg FAN kg^{-1} FM (Rajagopal, Massé and Singh, 2013).

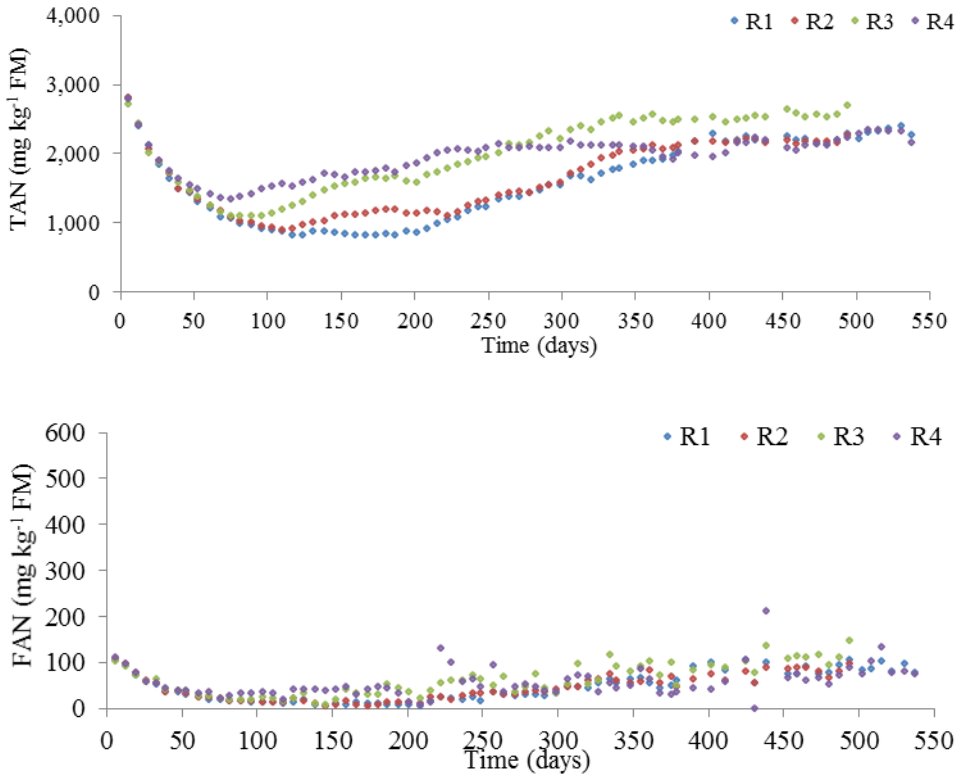


Figure 8-10 TAN and FAN of digesters R1 - R4 for the whole experiment

The organic solids being converted to biogas at a methane content of around 58% ± 2% through testing period (Figure 8-11). These digesters showed relatively consistent SMP, whereas VMP increased reflecting the OLR increased. Under stable operation, results demonstrated that biogas production and methane yield were stable with the specific CH_4 production (SMP) was 0.44 m^3 CH_4 kg^{-1} VS_{added} (0.75 m^3 biogas kg^{-1} VS_{added}) at OLR 1.0 - 3.0 kg VS m^{-3} d^{-1} (Figure 8-13). The average VMP for stable process performance were 0.44, 0.66, 0.88, 1.10 and 1.28 STP m^3 m^{-3} digestate d^{-1} for OLR 1.0, 1.5, 2.0, 2.5 and 3.0 kg VS m^{-3} d^{-1}, respectively (Figure 8-14).

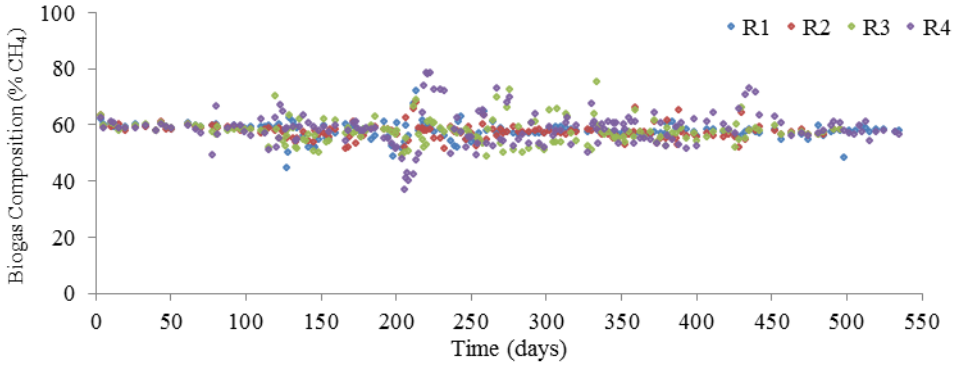


Figure 8-11 Biogas composition in digester R1 - R4 for whole experiment

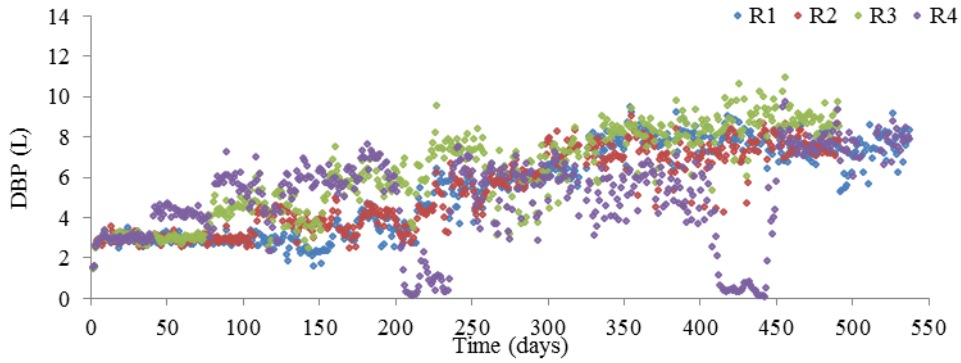


Figure 8-12 Daily Biogas Production (DBP) in digester R1 - R4 for whole experiment

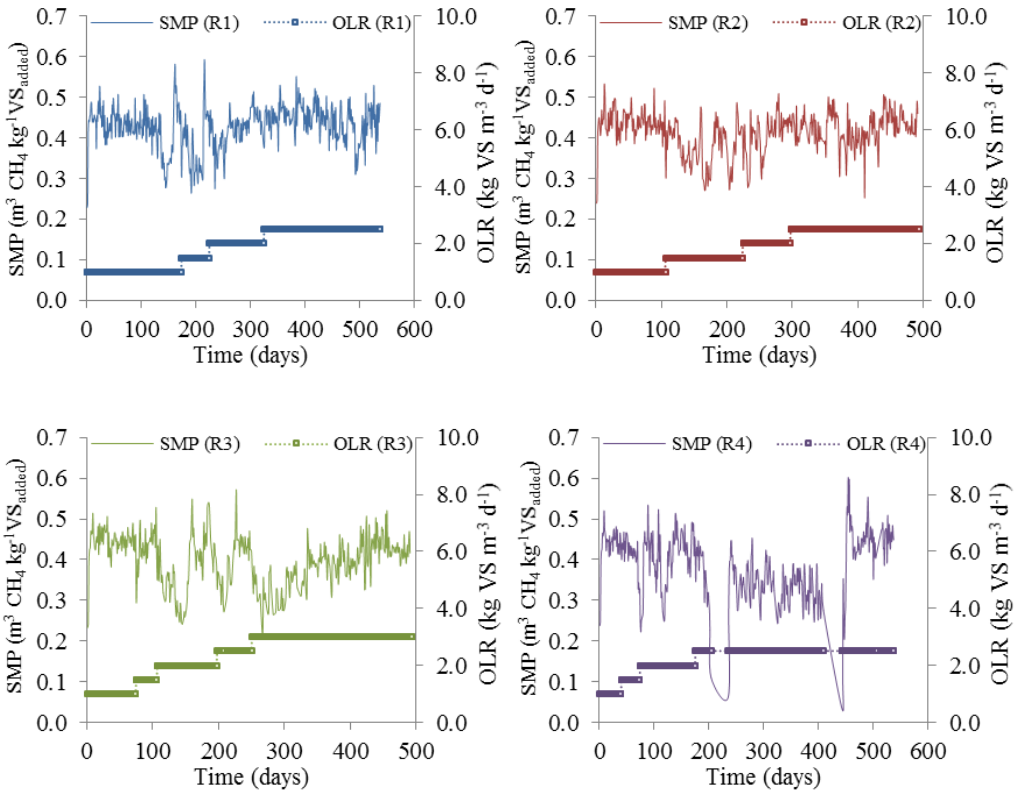


Figure 8-13 Production performance in terms of SMP in digester R1 - R4 for whole experiment

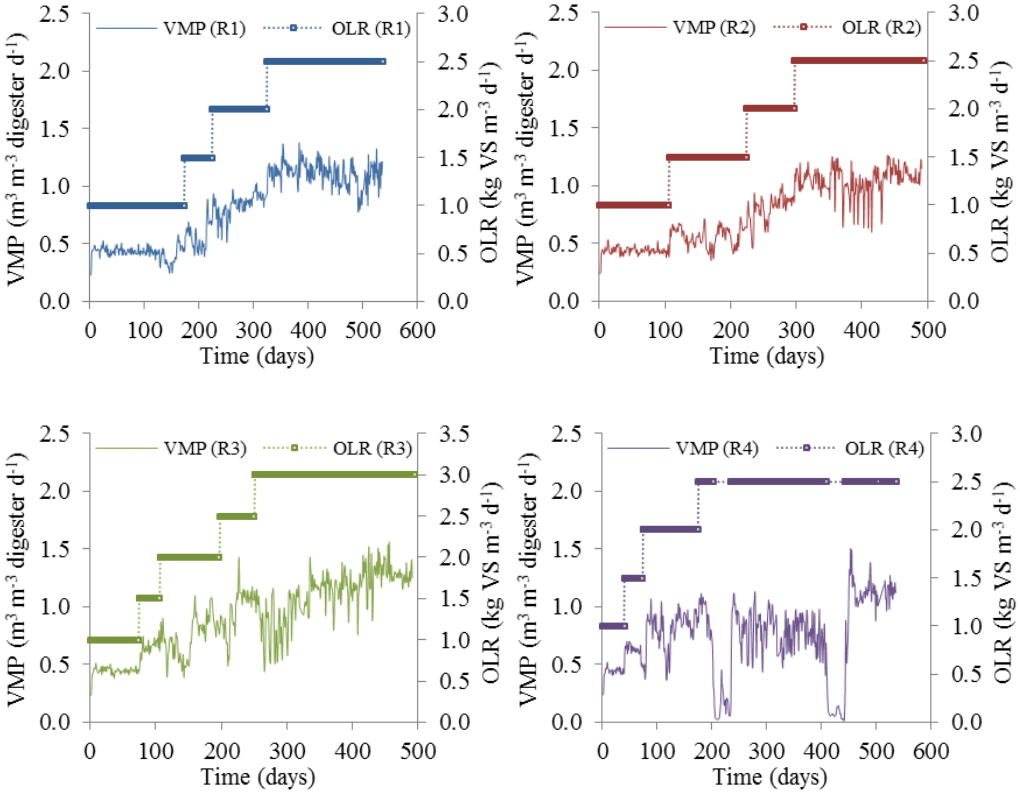


Figure 8-14 Production performance in terms of VMP in digester R1 - R4 for whole experiment

8.3.5 **Co, Ni and Fe distribution under TE deficient and TE sufficient conditions**

Digestate samples were taken from R1 - R4 on day 440 to investigate the Co, Ni and Fe distribution in different fractionations under TE deficient condition. This was done before TE dosing was changed to the strength of Co 1.0, Ni 1.0 and Fe 10 mg kg^{-1} FM supplementation, in comparing with the TE sufficient condition on day 466 for R2 and R3 and on day 498 for R1 and R4.

The total concentration of Co, Ni and Fe were measured in parallel with fractionation analysis. Sequential extraction was performed to determine their distribution in different forms. To evaluate the reliability of the method which was developed in this study, the total strength of Co, Ni and Fe from the sum of four analysed fractions was calculated and compared with the experimentally measured total strength of them determined without fractionation. Table 8-3 showed percentage of recovery for each element under TE sufficient and TE deficient condition. These 2 group of values were in the range of ~ 20 % of differences (80 - 105 %), indicating a good agreement and confirming the correctness of the used methodology and reliability of the obtained results. Besides, the experimentally results confirmed that the strength of Se in digesters R1-R4 were

around 0.13, 0.14, 0.15 and 0.15 mg kg^{-1} FM on day 440. Figure 8-15 - Figure 8-17 illustrated the outcome of the chemical fractionation of Co, Ni and Fe.

The comparison of TE distribution under TE sufficient and TE deficient condition both the relative distribution in different fractionations and absolute values were shown in Figure 8-15- Figure 8-17. It can be seen from chemical fractionation analysis that there was a clear increasing in absolute amount of Co, Ni and Fe both in extracellular fractions and intracellular fraction corresponding to the increasing of total strengths. Under the same dosage rate, it could be observed that the amount of Co, Ni and Fe found in each fractionations were relative similar.

Table 8-3 Total Co, Ni and Fe concentration (mg kg^{-1} FM) and their recovery rate (%) under TE deficient and TE sufficient condition

Digester	Conditions					
	TE deficient			TE sufficient		
	Co	Ni	Fe	Co	Ni	Fe
R1	0.12 (87)	0.12 (80)	1.18 (84)	1.08 (99)	1.05 (99)	11.85 (97)
R2	0.20 (92)	0.21 (104)	1.93 (94)	1.09 (102)	1.06 (105)	12.63 (99)
R3	0.36 (103)	0.35 (102)	3.28 (96)	1.09 (104)	1.05 (99)	12.30 (101)
R4	0.34 (100)	0.33 (93)	3.27 (94)	1.04 (97)	1.01 (95)	12.71 (102)

It appeared that microbes could take more TE for their metabolic activities when TE concentration in their environment is higher. The experimental results showed that when the digesters were supplemented with sufficient Co, Ni, Fe and Se indicated by a very stable digestion (day 440 onwards), mostly around 97 - 98% of them presented in extracellular fractions which consist of organic matter bound fraction, precipitation as sulphide fraction and leaving in liquid fraction. Only around 2 - 3 % was found in microbial biomass.

Co (53 - 54 %) and Ni (61 - 67 %) mostly stayed in sulphide form (HNO_3 extract) follow by binding with organic ligand around 32 - 36 % and 21 - 27 %, respectively. A different behavior was noticed for Fe, 48 - 51 % was more strongly in organically bound form ($Na_4P_2O_7$ extract) and follow by precipitated as a sulphide salt (37 - 40 %). In this trial, around 9 - 13 % of the total strength of Co, Ni and Fe was presented in liquid fraction. This finding was in agreement with those observed in Chapter 7 when digesters receiving sufficient TE supplementation during baseline digestion.

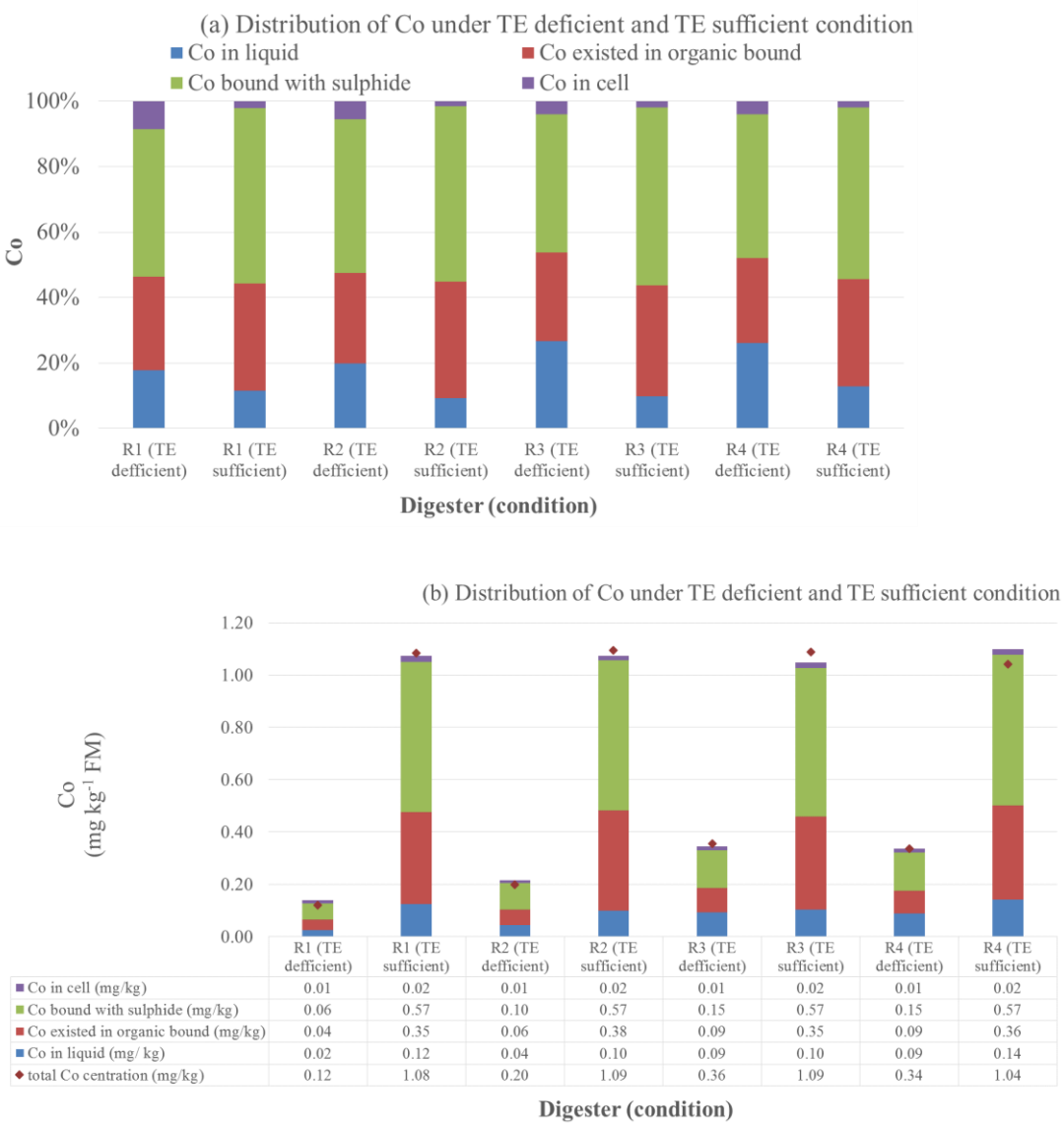
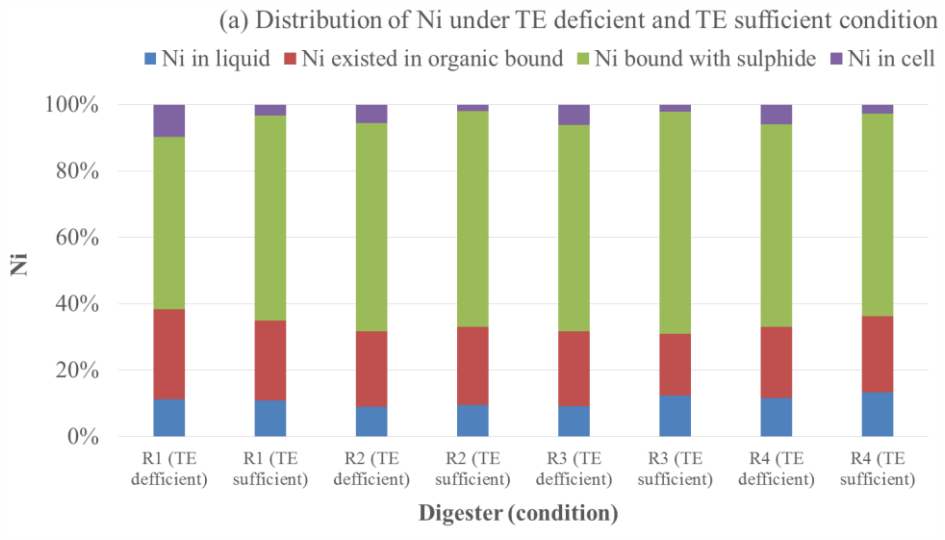
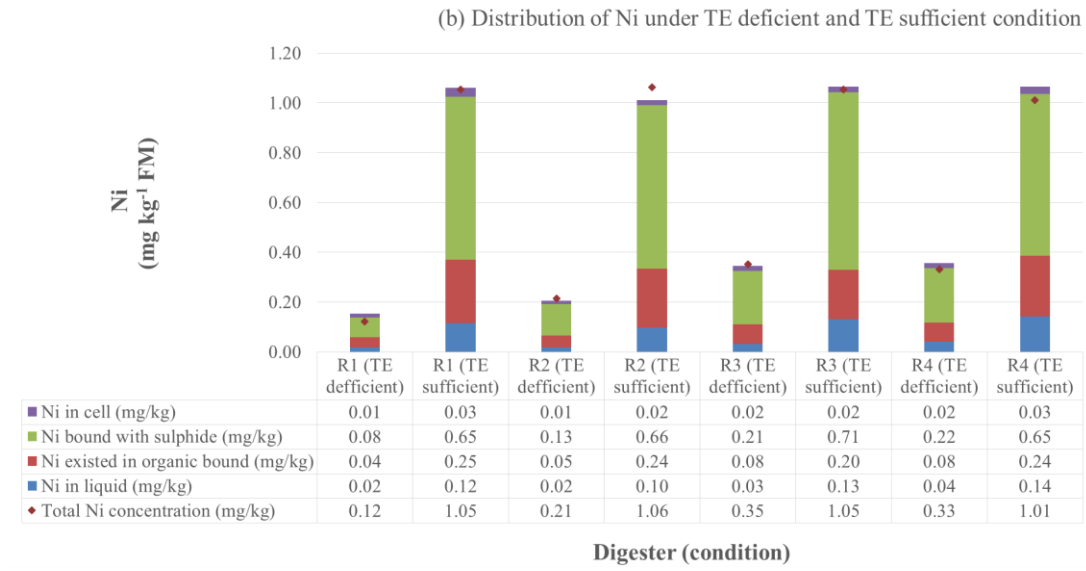


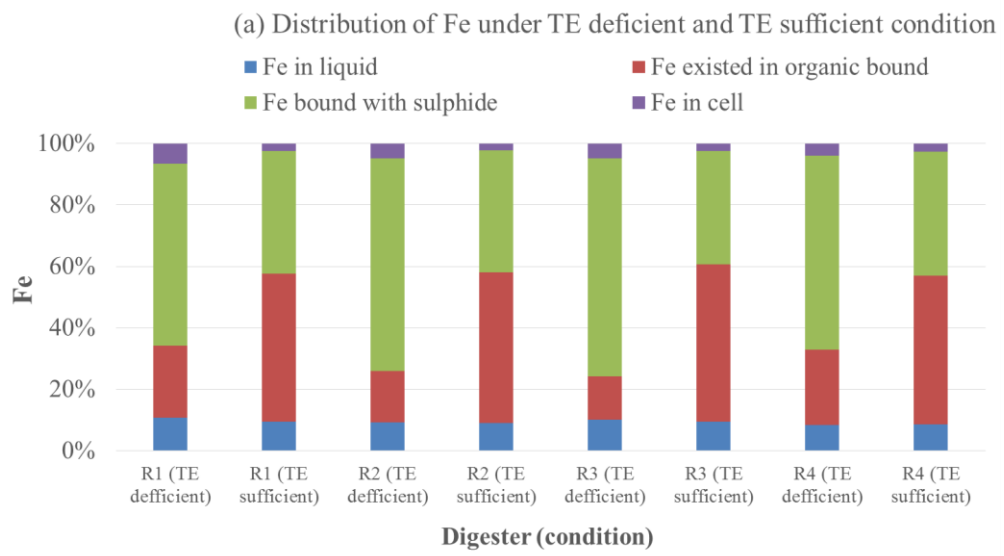
Figure 8-15 Comparison for distribution of Co under TE deficient and TE sufficient condition (a) relative distribution in different fractions (b) absolute value





	R1 (TE defficient)	R1 (TE sufficient)	R2 (TE defficient)	R2 (TE sufficient)	R3 (TE defficient)	R3 (TE sufficient)	R4 (TE defficient)	R4 (TE sufficient)
Ni in cell (mg/kg)	0.01	0.03	0.01	0.02	0.02	0.02	0.02	0.03
Ni bound with sulphide (mg/kg)	0.08	0.65	0.13	0.66	0.21	0.71	0.22	0.65
Ni existed in organic bound (mg/kg)	0.04	0.25	0.05	0.24	0.08	0.20	0.08	0.24
Ni in liquid (mg/kg)	0.02	0.12	0.02	0.10	0.03	0.13	0.04	0.14
Total Ni concentration (mg/kg)	0.12	1.05	0.21	1.06	0.35	1.05	0.33	1.01

Figure 8-16 Comparison for distribution of Ni under TE deficient and TE sufficient condition (a) relative distribution in different fractions (b) absolute value



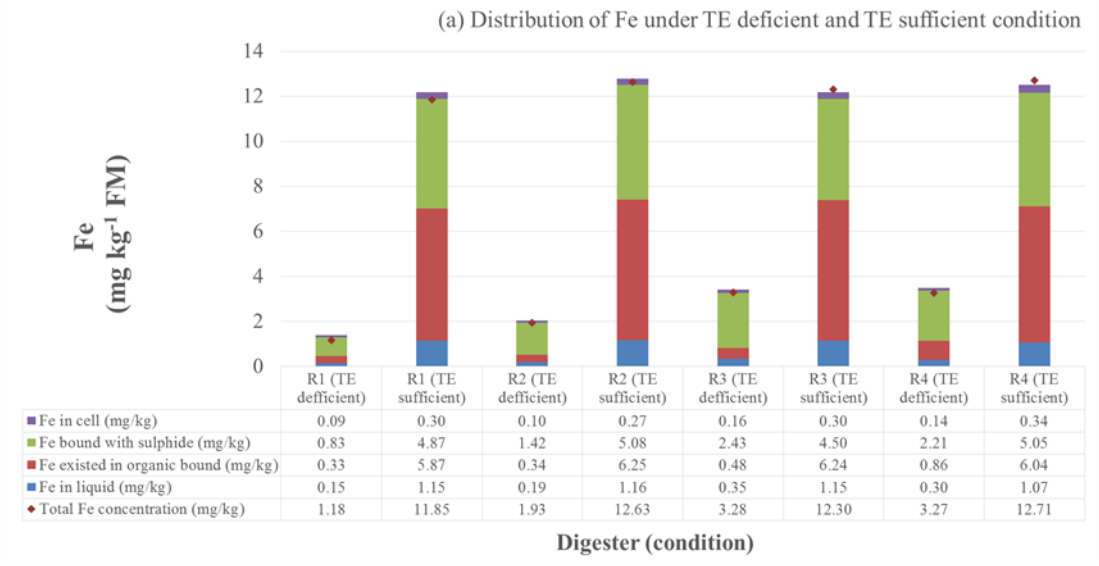


Figure 8-17 Comparison for distribution of Fe under TE deficient and TE sufficient condition (a) relative distribution in different fractions (b) absolute value

8.4 Concluding remarks

According to the results obtained from this set of experiments, it could be concluded that

1. During the OLR increase experiment, the critical TE levels (defined as the concentration when VFA start to appear) appeared to be much higher than those obtained from washing-out experiment (Chapter 6).

2. When OLR increases, the concentration of microbial biomass increases accordingly if HRT is fixed, and microbes may have to take more TE from their environment. But when TE concentration in their environment is low, there are two problems: 1) the concentration gradient or energy required to find and carry TE to the inside of the cells; 2) the availability/speciation of TE in their environment. Therefore, the total TE concentration used to control this set of experiment should be much higher than that of the effective TE which should belong to microbe-incorporated TE.

3. The minimum Co, Ni and Fe requirement relevant to OLR when Se was supplied in sufficient quantity are quantified as follows;

- OLR 1.0 kg VS m^{-3} d^{-1}, a mix of Co 0.03, Ni 0.06 and Fe 0.6 mg kg^{-1} FM
- OLR 1.5 kg VS m^{-3} d^{-1}, a mix of Co 0.06, Ni 0.06 and Fe 0.6 mg kg^{-1} FM
- OLR 2.0 kg VS m^{-3} d^{-1}, a mix of Co 0.09, Ni 0.09 and Fe 0.9 mg kg^{-1} FM
- OLR 2.5 kg VS m^{-3} d^{-1}, a mix of Co 0.12, Ni 0.12 and Fe 1.2 mg kg^{-1} FM

4. Not only the total strength, but the optimal ratio of a mix of Co, Ni and Fe in supplementation mixture played a significant role on digester stability. The ratio of Co + Ni and Fe in supplementation mixture in range of 1:5 - 1:6.67 was found optimal to bring digester process back to normal and ensure stable performance. Higher ratio (1:2.5, 1:3.33, 1:3.75 and 1:4) gave rise to disturbance and function decline probably due to less amount of available Fe.

Chapter 9: **Conclusions and Further work**

9.1 Conclusions

9.1.1 **Digester substrate and anaerobic digestion of model substrate in 100-L CSTR digester without TE supplementation**

1. The low nutrient contents of the model substrate indicated the suitability of substrate used for this research. TKN concentration in feed was unlikely to induce inhibitory effect and therefore this would not become an interfering issue when investigating the TE effect on AD. Another reason for the use of the model substrate is that it is readily biodegradable.

2. The results obtained this CSTR experiment indicated that without TE supplementation, digestion could not overcome the instability issue caused by VFA accumulation. Digester could not perform stably over OLR of 1.0 kg VS m^{-3} d^{-1}.

3. This trial successfully provided the acclimated inoculum with low TE content to conduct 5-L scale CSTR laboratory experiments (Chapter 8, R1 - R4).

9.1.2 **Trace element requirement for long-term mesophilic digestion at OLR 3.0 kg VS m^{-3} d^{-1}**

1. Single element supplementation of Co or Ni was unable to prevent VFA accumulation when baseline TE concentrations were Co 0.01, Ni 0.03, Fe 2.88, Se 0.04 and Mo 0.06 mg kg^{-1} FM.

2. The digester with both Co and Ni supplementation could not maintain long-term stable performance either, but its VFA accumulation appeared later than the control digester (without TE) or digesters with single TE dosing (i.e. Ni alone and Co alone).

3. Fe showed antagonistic effect when supplemented with either Co or Ni and reduced their availability, whilst at the same time it proved to be an essential TE. When supplemented with a mix of Co, Ni and Fe digesters operated well for 400 days but showed VFA accumulation after 12 HRTs.

4. Se was also found to be essential for long-term stable operation of this model substrate.

5. Digester supplemented with Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM was confirmed to be sufficient for stable and optimal digester performance at OLR of 3.0 kg VS m^{-3} d^{-1}.

6. Other TE provided by model substrate was sufficient and there was no evidence that any of them was required in concentration greater than their baseline concentration over 18 HRTs (630 days).

7. The system was operated for more than 18 HRTs and clearly showed that the requirement for TE was uncoupled from the hydraulic characteristics of the digester, suggesting that the chemical species and bioavailability of the TEs is a critical element in their function and is, to a certain degree, independent of washout as the results showed that nutrients are not lost from the system simply as a hydraulic function.

9.1.3 **Effect of Co, Ni, Fe and Se on stabilisation of mesophilic digestion at moderate OLR of 3.0 - 4.5 kg VS m^{-3} d^{-1}: their critical concentrations determination by washing out experiment**

1. The impact of Fe deficiency appeared earlier at OLR 3.0 kg VS m^{-3} d^{-1} while Ni, Co and Se seemed to affect the process at later stages under the higher OLR at 3.0, 3.5 and 4.0 kg VS m^{-3} d^{-1}, respectively.

2. Critical Co, Ni, Fe and Se concentrations were determined when the rest of the TE existed in sufficient quantity (Co 1.0, Ni 1.0, Fe 10 and Se 0.1 mg kg^{-1} FM):

2.1Co concentration became critical at 0.01 mg kg^{-1} FM, equal to the baseline concentration from the feed at OLR 3.5 kg VS $m^{-3}d^{-1}$.

2.2 Ni was critical at 0.03 mg kg^{-1} FM, equal to the baseline concentration from the feed at OLR 3.0 kg VS m^{-3} d^{-1}. Ni at the strength of 0.6 - 0.8 mg kg^{-1} FM is recommended as the minimal strength to maintain stability and performance efficiency at OLR 3.5 kg VS m^{-3} d^{-1}.

2.3 Fe was critical at 5.0 and 6.2 mg kg^{-1} FM at OLR of 3.0 and 3.5 kg VS m^{-3} d^{-1}, respectively.

2.4 Se was critical at 0.06 mg kg^{-1} FM, equal to the baseline concentration from the feed at OLR 4.0 kg VS m^{-3} d^{-1}.

3. This critical (minimum) concentration, however, was not sustainable and not sufficient in case of instability initiated for instance by organic loading increase. A sufficient safety factor, for instance 3-5 folds of its critical concentrations should be applied if these critical concentrations are to be used for developing the TE supplementation strategies to prevent initial VFA accumulation. However, the proper safety factor should be clarified through further studies as suggested in further work.

4. Recovery and continuing stable operation, however, required much higher TE strength compared with their critical concentrations at high level of VFA.

5. After long-term washing out, TE deficiency appeared earlier in higher OLR of 4 tested digesters.

9.1.4 **Dynamic changes of Co, Ni, Fe and Se distribution profiles in anaerobic digester over the course of TE washing out process**

1. Microbial biomass maintained relatively stable amount of Co and Ni for metabolic activities although all the extracellular TE fractions were washed - out gradually.

2. Sulphide fraction competed with intracellular fraction for Fe. This caused the accumulation of volatile fatty acids when the total Fe concentration was still high.

3. The fact that TE availability to certain extent decoupled from simple hydraulic washing out effect was explained by the high affinity of microbial biomass for TE in this experiment.

9.1.5 **The effect of OLR on the requirement of Co, Ni and Fe at constant HRT**

1. During the OLR increase experiment, the critical TE levels (defined as the concentration when VFA start to appear) appeared to be much higher than those obtained from washing-out experiment (Chapter 6).

2. When OLR increases, the concentration of microbial biomass increases accordingly if HRT is fixed, and microbes may have to take more TE from their environment. But when TE concentration in their environment is low, there are two problems: 1) the concentration gradient or energy required to find and carry TE to the inside of the cells; 2) the availability/speciation of TE in their environment. Therefore, the total TE concentration used to control this set of experiment should be much higher than that of the effective TE which should belong to microbe-incorporated TE.

3. The minimum Co, Ni and Fe requirement relevant to OLR when Se was supplied in sufficient quantity are quantified as follows;

- OLR 1.0 kg VS m^{-3} d^{-1}, a mix of Co 0.03, Ni 0.06 and Fe 0.6 mg kg^{-1} FM
- OLR 1.5 kg VS m^{-3} d^{-1}, a mix of Co 0.06, Ni 0.06 and Fe 0.6 mg kg^{-1} FM
- OLR 2.0 kg VS m^{-3} d^{-1}, a mix of Co 0.09, Ni 0.09 and Fe 0.9 mg kg^{-1} FM
- OLR 2.5 kg VS m^{-3} d^{-1}, a mix of Co 0.12, Ni 0.12 and Fe 1.2 mg kg^{-1} FM

4. Not only the total strength, but the optimal ratio of a mix of Co, Ni and Fe in supplementation mixture played a significant role on digester stability. The ratio of Co + Ni and Fe in supplementation mixture in range of 1:5 - 1:6.67 was found optimal to bring digester process back to normal and ensure stable performance. Higher ratio (1:2.5, 1:3.33, 1:3.75 and 1:4) gave rise to disturbance and function decline probably due to less amount of available Fe.

9.1.6 The practical application of TE and supplementation regimes in AD

TEs are dosed to AD to improve the biogas production process e.g. by maintaining process stability, to boost biological activity, to increase rate of methane production and to allow for application of higher organic loading rate (OLRs) or treatment capacity. Little is understood regarding the specific requirement of elements, the concentrations and dosing strategies best suited to sustained supplementation and stable performance in anaerobic biotechnologies. Since the requirements of TEs and the responses to TEs supplementation for maintaining stable process performance depended on several factors including the substrates type, source of inoculum, operating conditions of AD, etc. Therefore, the composition of TEs in substrates and inoculum need to be determined before setting up the AD process and during digestion process, particularly when the nature of substrates changes, in order to estimate the background level of TEs concentrations.

This study provides information regarding the new insight on optimising essential TE supplementation to AD, considering the availability of TE for microbial activities, which is very important to obtain efficient and long-term stable biogas processes. The understanding of the requirement and distribution of TEs in mesophilic AD is important for TEs dosage strategies to the biogas process, which have implication for the downstream effect on recipients. The experimental data showed that supplementation of TEs (Co, Ni, Fe and Se), at a suitable concentration, is critical for maintaining operational stability and digestion efficiency. For long-term operation of the AD process was feasible providing careful attention was paid to supplementation of the feedstock material with TEs so as to maintain the sufficient bioavailable concentration within the digester, in order to reduce the costs of TEs dosing, to mininise the introduction of TEs into the environment and to optimize the biological activity. Results revealed that supplementation with other additional TE species other than Co, Ni, Fe and Se under moderate OLR of 3.0 - 4.5 kg VS m^{-3} d^{-1} was not regarded as necessary. Therefore, concerning to environment and economic aspects, application of full 11 elements supplementation containing other TEs is not harmful, but not essential.

The critical (minimum) essential TE concentrations was quantified, however, these were not sustainable and not sufficient in case of instability initiated for instance by organic loading increase. A sufficient safety factor, for instance 3 - 5 folds of its critical concentrations should be applied if these critical concentrations are to be used for developing the TE supplementation strategies to prevent initial VFA accumulation. However, the proper safety factor should be clarified through further studies as suggested for further work. The TE requirement for digesters fed with the same type of substrate was not fixed. Higher TE strength were needed at higher OLR due to the increased microbial biomass density.

TE supplementation had a two - way effect on AD. Results indicated that TE addition, to a large extent, cannot initiate the VFA consumption process in digester with high VFA concentrations and methanogenic activity had already been inhibited. It appeared that after a digester has been subjected to accumulation of VFA for a period of time, the onset of VFA degradation depends on other factors in addition to those of TE concentrations. Once the VFA degradation process has started the supplementation of a specific TE or multicomponent TE matrix can, however, accelerate the VFA consumption rate. Then, further study on the timing of TE addition which relative to VFA levels need to be taken into consideration to seek a balance between effect of VFA production and VFA consumption. As such strategy for stable digestion should focus on the prevention of initial VFA accumulation in the digester by TE supplementation, rather than the recovery of a severely VFA - laden digester.

9.2 Further work

1. Further work on TE supplementation to recover process stability from VFA accumulation needs be carried out in detail. This research raised the issue that the state of digester needs to be taken into consideration when applying TE supplementation strategy to seek a balance between TE effect on VFA-production and VFA - consuming as well as the order their respond to TE addition. As TE addition showed a stimulation effect on VFA production, careful investigation on the impact of TE supplementation on VFA production is needed, to ensure a beneficial effect is achieved after TE addition.

2. More research also needs to be done to distinguish the critical TE concentration identified via wash-out experiment and their minimal concentration required to maintain stable and efficient digestion. It was shown in this research that the requirement of TE strength for maintaining stable operation is much higher compared with their critical concentrations in wash-out process and the recommended concentrations at high concentration of VFA. Therefore, the scale of margin required or the safety factor levels should be clarified through further studies if TE supplementation strategies are calculated based on critical TE strengths.

3. Studies on microbial community dynamics with changes in trace element concentrations e.g. compare the point of VFA accumulation (TE deficiency) with stable process performance (TE sufficiency).

4. Studies on the effectiveness of TE formulations on the market.

5. Studies on BMP tests on real substrates using the optimal TE formulation suggested in the thesis. This would provide a measure of comparison of the efficiency of the digesters with the theoretical value expected.

6. The correlation between bioavailable TE concentrations (intracellular TE and water soluble TE) and digestion performance e.g. total VFA levels should be further investigated.

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