EVALUATION OF TRACE METAL SPECIATION AND BIOAVAILABILITY IN ANAEROBIC SYSTEMS

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Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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        Date   Student Name

This thesis is dedicated to

my dear parents and my beloved sister

for being so supportive and encouraging

throughout all these years

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Table of Contents

Table Captions

Figure Captions

Abbreviations

AD	Anaerobic Digestion
BCR	Bureau Communautaire de Reference
CH_4	Methane
CO_2	Carbon Dioxide
EDDS	Ethylenediamine-N,N'-Disuccinic Acid
EDTA	EthyleneDiamineTetraacetic Acid
EPS	Extracellular Polymeric Substances
HRT	Hydraulic Retention Time
MP-AES	Microwave Plasma – Atomic Emission Spectroscopy
NTA	NitriloTriacetic Acid
OLR	Organic Loading Rate
SAMBR	Submerged Anaerobic Membrane Bioreactor
SMP	Soluble Microbial Product
SRT	Solids Retention Time
TM	Trace Metal
UASB	Up-flow Anaerobic Sludge Blanket
UHPLC	Ultra-High Performance Liquid Chromatography
VFA	Volatile Fatty Acid

Abstract

The importance of trace metals in anaerobic reactors has already been acknowledged in the literature, however, their bioavailability for microbial uptake which is governed by their speciation, has been poorly understood. There was a lack of information about the interaction between trace metals and organic/inorganic ligands and the operational parameters that affect their speciation in an anaerobic bioreactor. Therefore, the main objectives of this thesis were to investigate the speciation and bioavailability of trace metals in submerged anaerobic membrane bioreactors (SAMBRs) under the following conditions: in the presence of sulfide; with the addition of biodegradable chelating agent EDDS; under nutrient deficiency; under organic shock loads; and under changes in operating parameters i.e. pH, hydraulic retention time (HRT), and solids retention time (SRT). The results showed that acetoclastic methanogens were more constrained by Fe^{2+} limitations compared to other trophic groups, while an organic shock resulted in leaching of Fe^{2+} from the residual to organically bound and soluble forms, and the precipitation of Fe^{2+} as a sulfide salt seemed to reduce its bioavailability to acetoclastic methanogens. Biodegradable chelating agent EDDS when dosed as a metal complex with Fe^{2+} was able to compete with sulfide and keep the Fe^{2+} in a soluble form as an Fe-EDDS complex, hence improving the performance of the SAMBR. Characterization of different EDDS forms by Ultra-Fast Liquid Chromatography (UHPLC) showed the presence of free EDDS and Fe-EDDS complex, and chromatographic analyses confirmed the increase in the Fe soluble fraction. Organic shocks (glucose spikes) applied to the SAMBR resulted in leaching of Fe from residual to soluble and organically bound forms. The supplementation of Fe helped to remove the VFAs accumulated during the organic shocks. SAMBR performance was significantly affected by the low pH (6) and HRT (2 h), but it was still able to achieve high treatment efficiencies at low SRTs (25 d). The reduction in these parameters all resulted in a decrease in

metal content in the SAMBR, and the degree of trace metal loss from the SAMBR varied between metals as it depends on the sensitivity of the trace metals towards the changes, and also their affinity to anaerobic sludge. In all changes of each operating parameter, the trend in speciation of the trace metals generally tended to a shift towards highly bioavailable fractions.

Chapter 1

Introduction

The growing concern about pollution of the environment, especially the aqueous environment is bigger than ever, with more man-made environmental disasters occurring every year. Due to these concerns, discharge standards and legislation for wastewater treatment is becoming tougher and more stringent over time. This not only leads to a dramatic increase in the costs of wastewater treatment for both the municipal and industrial sectors, but also concomitantly results in an increase in the amount of sludge produced from these treatment processes.

The treatment processes for both domestic and industrial wastewaters are primarily carried out using cost effective biological means rather than physical-chemical methods. The heart of biological wastewater treatment is the secondary treatment reactors which remove most of the organic (COD/BOD), much of the N, S, and P (usually), and reduce pathogens. Biological treatment processes can be categorized into two major types, aerobic and anaerobic degradation, depending on whether molecular oxygen is required or not for bacterial growth. Microorganisms in aerobic treatment convert organic matter in the wastewater into carbon dioxide, water, and new biomass in the presence of air. On the other hand, anaerobic treatment processes involve the conversion of the contaminants without oxygen into methane, inorganic products including carbon dioxide and ammonia, and new biomass.

However, in the past, due to historical reasons as well as a number of misunderstandings, aerobic treatment is preferred over anaerobic treatment especially in larger treatment plants. These misunderstandings about the anaerobic processes include the perception that it cannot treat low-strength wastewater (<1000 mg/L COD); has a low tolerance for inhibitory compounds;

cannot be operated at low temperatures (<35ºC) and low HRTs (< 5 – 10 days); and has poor removal efficiencies.

However, aerobic treatment has its drawbacks including: bulking sludges (which can cause catastrophic failure of the treatment processes); large energy consumption; emission of greenhouse gases such as nitrous oxide (N_2O); and large amounts of waste activated sludge produced (which has to be treated properly before disposal, usually by anaerobic digestion). In addition to these, the utilization of oxygen in aerobic treatment contributes significantly to the plant cost by requiring substantial equipment for aeration, and very high operating and maintenance costs. It is estimated that at a conventional treatment plant, about 40% of the total electricity consumed is for the aeration units (Matsushige et al., 1990).

To address those environmental concerns with respect to energy, solids generation, greenhouse gas emissions, and resource recovery, there has been a major shift towards the use of anaerobic treatment in the last 50 years as it possesses numerous advantages compared to aerobic treatment. The first advantage is the lower yields of biomass under anaerobic conditions, hence far less sludge is produced, resulting in lower costs for waste solids disposal. In anaerobic treatment there is no requirement for aeration, and hence capital as well as operation and maintenance costs are significantly lower. Methane is also one of the products from the conversion of organic matter by the anaerobic microorganisms, which is a source of renewable energy.

Every living cell including microorganisms rely on only a number of elements that can be divided into macronutrients (C, H, N, O, P, S), major cations (K, Mg, Ca), and the so-called micronutrients including trace metals (TMs) (Fe, Co, Ni, Zn, Mn, Mo, Se, etc) (Merchant & Helmann, 2012). Anaerobic bacteria are just like other microorganisms that require TMs as essential components of cofactors and enzymes, however, the supplementation of TMs to enhance metabolism has not been well understood. In practice, to fulfill these nutrient requirements TMs are often supplemented in sufficient

quantities to support microbial growth, and hence ensure the optimal performance of the treatment processes. On the other hand, TMs should only be dosed below the inhibitory or toxic level as an excessive metal content in the sludge will inhibit the performance of the anaerobic systems, cause harm to the environment, and minimize the quality and applicability of anaerobic products in agriculture, e.g. digestate used as fertilizer. However, the supplemented TMs are not always necessarily bioavailable for the microorganisms to take up (Zitomer et al., 2008).

The concept of bioavailability of metals in anaerobic digestion has been poorly understood up until now. Total metal concentration is generally used to evaluate the effects of metal stimulation and toxicity in anaerobic digestion. However, it has been demonstrated that the measurement of total metal concentration is not sufficient to determine its bioavailability (Hsu & Lo, 2001; Amir et al., 2005). TM bioavailability depends on their speciation, which in turn is controlled by many factors, including the presence of organic/inorganic ligands, the operating conditions of anaerobic reactors, and the bioreactor configuration (Oleszkiewicz & Sharma, 1990; He et al., 2009).

In terms of the design of anaerobic bioreactors, the most basic system is the completely mixed process such as continuous flow stirred-tank reactor (CSTR), which has been used extensively in many municipal treatment plants. However, it can only achieve high volumetric loading rates with quite concentrated waste streams with a biodegradable chemical oxygen demand (COD) level between 8,000 and 50,000 mg/L. The anaerobic contact process, an analogy to the aerobic activated sludge system, has been applied to many applications but one problem is the biomass lost to the effluent due to bubble generation and attachment in the settling tank. The popular up-flow anaerobic sludge blanket reactor (UASB) developed by Lettinga et al. (1980), is able to separate solids from the outward-flowing wastewater through the formation of granules. However, serious problems that occur are the formation of granules that float and the lack of granule formation, both of which result in loss of

biomass from the system (Rittmann & McCarty, 2001). Therefore, the major challenge for engineers was to be able to accommodate long resident times of the slow growing anaerobic microorganisms, especially the methanogens, which can be done by separating completely the hydraulic retention time (HRT) from the solids retention time (SRT). This led to the development of novel submerged anaerobic membrane bioreactors (SAMBRs) which evolved from aerobic MBRs. SAMBRs are able to retain the slow growing bacteria completely in the reactors, allowing them to grow and persist under unfavorable conditions, e.g. high salinity (Stuckey, 2012). They have been shown to be able to operate at very low HRTs of 3 h, but still can achieve very high COD removals of around 98% (Hu & Stuckey, 2006). Therefore, in the near future the most rational approach to treating organic wastewaters is to use a SAMBR as a stand-alone unit which is capable of treating the wastewater directly, with much less sludge produced. However, the performance of these reactors like other anaerobic systems, can be severely limited by operating under sub-optimal conditions, e.g. when they are limited by TMs in the feed.

Hence, the overall aim of this study was to explore the speciation and bioavailability of trace metals in a Submerged Anaerobic Membrane Bioreactor (SAMBR) under different operating conditions, in the presence of different types of ligands, and to investigate different dosing strategies to improve the bioavailability of the trace metals, and hence the reactor performance.

This thesis is organized as follows:

Chapter 2 provides a comprehensive review of the basics of the most essential TMs in anaerobic digestion, with the main focus on TM speciation and bioavailability in anaerobic digestion including the current state of art of

analytical techniques. The objectives of this study are then outlined in this chapter.

Chapter 3 presents the details of the general experimental procedures and analytical methods for all the following chapters.

Chapter 4 studies the deficiency and bioavailability of Fe^{2+} during organic overloading conditions, and shows how Fe^{2+} supplementation can help to improve the degradation of accumulated VFAs.

Chapter 5 investigates the effects of sulfide on the speciation of Fe^{2+} and the dosing of biodegradable chelating agent EDDS to improve the bioavailability of Fe^{2+} in a batch reactor.

Chapter 6 determines the dosing strategy of the EDDS and Fe^{2+} as they are added into a SAMBR to improve the bioavailability of Fe^{2+} and hence the reactor performance.

Chapter 7 examines the deficiency of several TMs in a SAMBR and evaluates different dosing strategies of TMs to improve the reactor performance.

Chapter 8 investigates the effects of pH, hydraulic retention time (HRT), solids retention time (SRT) changes on reactor performance.

Chapter 9 provides a conclusive discussion highlighting the outcome of research study and recommendations for future research based on these findings.

Chapter 2

Literature Review

Note: this chapter was published in *Biotechnology Advances*, 2016, volume 34, pages 122-136.

2.1   Introduction

The purpose of this chapter is to provide a critical review of the fundamentals and applications of the main themes discussed in this thesis. A concise explanation of the importance of trace metals (TMs), their limitations, requirements and dosing strategies in anaerobic digestion are outlined. This is then followed by a detailed review on the speciation of TMs, which plays an important part in this research. The effects of pH, redox potential, and hydraulic retention time (HRT), on TM speciation in anaerobic digestion are explained. The interaction between TMs in the liquid and solid phases is described in order to explain their bioavailability in anaerobic digestion. The use of different types of chelating agents to improve the TM bioavailability is also discussed in detail. Finally, the development and application of analytical techniques currently used to investigate the speciation and bioavailability of TMs in anaerobic digestion is also presented. The chapter concludes by summarizing these areas thus leading to detailed objectives of this study.

2.2   Importance of trace metals in anaerobic digestion

Biological treatment of wastewaters, including anaerobic treatment, requires not only organic matter, but also essential nutrients for the growth of microorganisms. Some types of wastewater contain a sufficient amount of these nutrients, e.g. swine wastewater (Cestonaro do Amaral et al., 2014). However,

other types of wastewater might require metal supplementation in order to avoid nutrient deficiency, e.g. methanol wastewater (Fermoso et al., 2008a), maize silage (Evranos & Demirel, 2014), and wheat stillage (Schmidt et al., 2014).

The metal nutrients can be classified based on their concentration in the cells into major cations (K, Mg, Ca), and micronutrients (Mn, Fe, Co, Cu, Mo, Ni, Se, W) (Merchant & Helmann, 2012). Metal concentration in the cells range from 10^{-7} to 10^{-3} M for the major cations, and from 10^{-6} to 10^{-15} M for the micronutrients (Williams & Fraústo da Silva, 2000). The essential micronutrients known as TMs are constituents of cofactors in enzyme systems, and the function of these TMs and their associated enzymes in anaerobic reactions have been summarized by a number of researchers (Jarrell & Kalmokoff, 1988; Oleszkiewicz & Sharma, 1990; Schattauer et al., 2011).

TMs considered the most essential in anaerobic digestion are the "transition metals" i.e. Fe, Ni, and Co (Oleszkiewicz & Sharma, 1990), and their effects on anaerobic digestion have been studied extensively in the literature (Hoban & Van Den Berg, 1979; Sharma & Singh, 2001; Pobeheim et al., 2011; Qiang et al., 2012; Shakeri Yekta et al., 2012; Gustavsson et al., 2013a). The importance of Fe depends on its redox properties, and its engagement in energy metabolism (Takashima & Speece, 1989). Fe is utilized in the transport system of the methanogenic bacteria for the conversion of CO_2 to CH_4, and functions both as an electron acceptor and donor (Vintiloiu et al., 2013). Fe also acts as a binding component in sulfide precipitation as it is often supplemented into anaerobic reactors, not only to precipitate the sulfide formed, but also to control the level of hydrogen sulfide in the biogas (Gustavsson et al., 2013a). A corrinoid such as vitamin B_{12} contains the Co ion, and can bind to the coenzyme methylase which catalyzes methane formation in both acetoclastic methanogens and hydrogenotrophic bacteria (Schonheit et al., 1979; Kida et al., 2001). Ni was initially considered as not being essential for bacterial growth (Diekert et al., 1981). However, F_{430}, a low molecular weight coenzyme

discovered in extracts from *Methanobacterium thermoautotrophicum*, was shown to contain substantial amounts of Ni (Speece et al., 1983; Thauer, 1998; Kida et al., 2001). This coenzyme (F_{430}) is contained within the Methyl-coenzyme M reductase enzyme, which reduces methyl coenzyme M to methane in all methanogenic pathways (Friedmann et al., 1990).

Mn acts as an electron acceptor in anaerobic respiration processes (Langenhoff et al., 1997), while Zn takes part in the functioning of enzymes involved in methanogenesis such as coenzyme M methyltransferase (Sauer & Thauer, 2000), and Zn, Cu, and Ni have all been found in a single hydrogenase (Jarrell & Kalmokoff, 1988). There is limited information available in the literature on the positive effects of Zn and Cu on methanogens, but Zn was found in remarkably high concentrations (50-630 ppm) in 10 methanogenic bacteria, whereas Cu was only present in some species (Scherer et al., 1983).

Other TMs such as W and Mo are also found in enzymes, such as formate dehydrogenase (FDH), which catalyzes formate production by propionate oxidizers (Dong et al., 1994; Fermoso et al., 2009; Banks et al., 2012), and some methanogenic bacteria contain W and Mo-containing enzymes for the same purpose (Nies, 1999). Methanobacterium thermoautotrophicum contains two formylmethanofuran dehydrogenase iso-enzymes, a W containing form and a Mo containing form; the Mo enzyme is synthesized only when Mo is present in the growth medium, while the W enzyme is synthesized when either W or Mo is available. If the growth medium contains Mo, the W enzyme will contain Mo rather than W (Hochheimer et al., 1995). All W-containing enzymes have been found in bacteria and archaea, mostly those with an anaerobic metabolism (Nies, 1999).

Although Se is not strictly a TM, it is a very important trace element for anaerobic digestion systems. Early work from Jones and Stadtman (1977) found a direct relationship between the stimulatory effect of selenite on growth, and increased levels of formate dehydrogenase activity in *Methanococcus vannielii*, a strict anaerobic microorganism. The lack of Se in the influent to an anaerobic

treatment process can result in growth limitations for some methanogens, a decrease in microbial activity, and ultimately process failure (Chasteen & Bentley, 2003; Lebuhn et al., 2008; Worm et al., 2009). Therefore, in this review, although the main focus is on the common transition metals i.e. Fe, Co, Ni, Cu, Mn, Mo, and W, other non-metallic elements like Se will also be discussed.

2.3   Trace metal limitation in anaerobic digestion

Although the requirement for TMs is extremely low, a deficiency in essential TMs can cause the enzymes and coenzymes required for metabolism not to be properly synthesized. This severely limits the growth of microorganisms, and leads to process instability and consequently process failure (Parkin & Owen, 1986; Rajeshwari et al., 2000). The effects of essential TM depletion on anaerobic digestion have been well reported in the literature (Zandvoort et al., 2002; Osuna et al., 2003; Zandvoort et al., 2003; Paulo et al., 2004; Zandvoort et al., 2006b; Fermoso et al., 2008a; Fermoso et al., 2008b).

Gonzalez-Gil et al. (1999) found that when Ni and Co were added continuously at a rate of 0.05 to 0.2 μmol/h to a full-scale expanded granular bed reactor treating wastewater containing methanol and formaldehyde, the temporary inhibition phase was not observed, and the rate of methane production increased exponentially. They then hypothesized that these effects were due to micronutrient limitations.

The deficiency of Fe and Ni during anaerobic digestion of wheat stillage resulted in a rapid accumulation of VFAs (Schmidt et al., 2014). The exclusion of Co, Zn and Ni from the methanol-based feed of an UASB reactor induced lower specific methanogenic activity (SMA), and the accumulation of VFAs (Paulo et al., 2004; Fermoso et al., 2008a; Fermoso et al., 2008b).

Mo, W, and Se all seemed to have a long-term effect on methanogenic activity (Worm et al., 2009; Schmidt et al., 2014), and a deficiency of Se was found to be the reason for the accumulation of propionic acid during the

anaerobic digestion of food waste operating at high ammonia concentrations (Banks et al., 2012).

Among different trophic groups in anaerobic digestion, methanogens are the most sensitive to TM deficiency, and hence less effective in competing for these TMs compared to acid-formers (Goodwin et al., 1990). This is due to their slow growth rates and more stringent growth requirements. White and Stuckey (2000) proposed that to prevent limitations in methanogenic activity, the acetate utilization rates required per gram of VSS should be used to estimate whether nutrient supplementation may be required. The threshold value appeared to be 0.25 gAcetate/gVSS.d.

Organic shock loading can cause temporary nutrient deficiencies to anaerobic bacteria, which then lowers the performance of anaerobic digestion. Hawkes et al. (1992) demonstrated that during an 8 h organic shock load, the effluent COD level in a metal dosed UASB reactor increased linearly from 1500 mg/L to around 3500 mg/L, while the effluent COD level in a non-metal dosed UASB reactor increased at a much higher rate from 1500 mg/L to around 8500 mg/L. At an organic loading rate of 17.4 $kgCOD/m^3$.d, Voolapalli and Stuckey (1999) found that under nutrient deficiency homoacetogenic organisms dominated, whereas methanogenic growth was limited.

2.4   Trace metal stimulation in anaerobic digestion

In the literature there are a number of reviews on the stimulatory effect of TM supplementation in anaerobic digestion (Oleszkiewicz & Sharma, 1990; Zandvoort et al., 2006a; Fermoso et al., 2008b; Demirel & Scherer, 2011; Chen et al., 2014b). The required concentrations for the essential metals, i.e. Fe, Ni, Co, Zn, Mo, Se, and W that show stimulatory effects on anaerobic digestion systems are summarized in Table 2.1 in Section 2.5 (see Trace metal requirements in anaerobic digestion). As can be seen in Table 2.1, several factors including reactor type, substrate, organic loading rate (OLR), and

operating temperature can influence the actual TM requirement that has stimulatory effects in anaerobic digestion systems.

TM supplementation is one method to increase VFA utilization, and the inclusion of Fe was found to have a stimulatory effect on acetate utilization by methanogens (Speece, 1962). When Espinosa et al. (1995) increased the organic loading on a UASB reactor treating cane molasses stillage from 5 to 21.5 kgCOD/m^3.d, an accumulation of VFAs, principally propionic acid, was observed. The addition of Fe (100 mg/L), Ni (15 mg/L), Co (10 mg/L), and Mo (0.2 mg/L) reduced the level of acetic acid and propionic acid significantly (1100 to 158 mg/L and 5291 mg/L to 251 mg/L, respectively). Lin et al. (1998) carried out a systematic study on the effect of TM supplementation on the anaerobic degradation of butyric acid. The results showed that the stimulatory effects were in the following order: $Cu^{2+} < Fe^{3+} < Zn^{2+} < Ni^{2+} < Mn^{2+}$, and the normal- and iso-HBu degradation activities of the methanogens increased by 14-25% and 17-43%, respectively. Kim et al. (2002) reported that the supplementation of Ca, Fe, Co, and Ni to a thermophilic non-mixed reactor was required in order to achieve a high conversion of propionate at high concentrations of VFAs. Fe addition of 10 μM almost doubled the methanogenic activity of the sludge in a UASB reactor in the conversion of methanol (Zandvoort et al., 2003). After addition of Fe, Ni, and Co, the propionate and acetate utilization rates in thermophilic and mesophilic digesters increased as much as 50% and 35%, respectively (Zitomer et al., 2008).

Supplementation of Co and Ni has been demonstrated to increase biogas production during the anaerobic digestion of organic matter such as sulfur-rich stillage (Gustavsson et al., 2013a), while Ni alone has been found to increase methane production to 10 gAcetate/gVSS.d (Speece et al., 1983). Co addition to a UASB increased methane production by a factor of three, stimulating both acetogens and methanogens. On the other hand, a combined supplementation of Ni and Co was found to increase methane production from methanol conversion by methanogens (Zitomer et al., 2008).

Patel et al. (1993) determined the effect of FeCl$_3$, NiCl$_2$, CoCl$_2$, CuCl$_2$, and ZnCl$_2$, on the anaerobic digestion of water hyacinth-cattle dung, and found that among the salts studied, the addition of FeCl$_3$ increased gas production more than 60% with high methane content.

Guiot et al. (1995) investigated shock loading in a multiplate anaerobic reactor treating cheese whey effluent at 68h HRT and a 9.5 kgCOD/m^3.d OLR, and they found a substantial reduction in the biomass-specific activity for acetate (5.3 mmol/gVSS.d). This indicated that metal supplementation was important for the acetoclastic methanogens, and also for the other methanogens (formate- and hydrogen-consumers) and acidogens.

2.5   Trace metal requirements in anaerobic digestion

The optimum and range of TMs in anaerobic digestion have been reported in a number of studies, and Table 2.1 provides a summary of the minimum required concentrations for the essential TMs, including Fe, Ni, Co, Mo, Se, and W. The TM requirement is normally determined by the depletion approach, in which the reactor is operated with one element depleted while still augmenting the other elements (Fermoso et al., 2008a; Schmidt et al., 2014). The reported concentration of TMs required during anaerobic digestion differs significantly, depending on the substrate type, operating temperature (mesophilic or thermophilic), digestion operating mode (mono or co-digestion), and also type of methanogens, which in turn leads to a diversity of biochemical processes involved in metal dynamics.

Table 2.1   Required concentration of essential metals for anaerobic digestion systems.

Metal	Required concentration	Reactor	Substrate	OLR	Operating temperature	Reference
Fe	0.20 mg/gCOD removed	Methane fermenter	Glucose	2 gCOD/L.d	Mesophilic	(Takashima et al., 2011)
	0.45 mg/gCOD removed	Methane fermenter	Glucose	2 gCOD/L.d	Thermophilic	(Takashima et al., 2011)
	0.276 mg/gCOD removed	Methane fermenter	High-solid food waste	1.9 – 6.3 gCOD/L.d	Thermophilic	(Qiang et al., 2013)
	0.43-0.66 mg/L	UASB, ABR, HABR	Synthetic sucrose based	1.62 – 1.8 gCOD/L.d for UASB 2.16 – 2.4 gCOD/L.d for ABR, HABR	35°C	(Patidar & Tare, 2008)
	5.0 mg/L	Semi- continuous digester	Food waste	1 – 4 gVS/L.d	37 ± 1°C	(Zhang et al., 2015a)
	0.55 mg/L	UASB	Methanol	2.6 – 7.8 gCOD/L.d	30°C	(Zandvoort et al., 2003)
	1233 mg/L	Semi- continuous digester	Wheat stillage	10 gCOD/L.d	38°C	(Schmidt et al., 2014)

	400 mg/L	CSTR	Mixture of OFMSW and slaughterhouse waste	5.14 gVS/L.d	38°C	(Moestedt et al., 2015)
	5.2 mg/L	CSTR	Organic solid waste	3.0 – 3.5 gTS/L.d	Mesophilic	(Uemura, 2009)
	10 mg/L	UASB	Distillery effluent	5.9 gCOD/L.d	35°C	(Sharma & Singh, 2001)
Ni	0.6 mg/kg	Semi- continuous anaerobic fermenter	Maize silage	3 gODM/L.d	35°C	(Pobeheim et al., 2011)
	0.0063 mg/gCOD removed	Methane fermenter	Glucose	2 gCOD/L.d	Mesophilic	(Takashima et al., 2011)
	0.049 mg/gCOD removed	Methane fermenter	Glucose	2 gCOD/L.d	Thermophilic	(Takashima et al., 2011)
	0.00496 mg/gCOD removed	Methane fermenter	High-solid food waste	1.9 – 6.3 gCOD/L.d	Thermophilic	(Qiang et al., 2013)
	100 mg/kgTS	CSTR	Food waste	1.5 – 6 gVS/L.d	Mesophilic	(Facchin et al., 2013)
	1.0 mg/L	Semi- continuous digester	Food waste	1 – 4 gVS/L.d	37 ± 1°C	(Zhang et al., 2015a)

0.04 mg/L	CSTR	Organic solid waste	3.0 – 3.5 gTS/L.d	Mesophilic	(Uemura, 2009)
0.029 mg/L	Anaerobic granular sludge bioreactors	Methanol	5 – 15 gCOD/L.d	30°C	(Fermoso et al., 2008b)
0.5 mg/L	Batch anaerobic mono-digester	Maize silage	N/A	37°C	(Evranos & Demirel, 2014)
0.1 mg/L	Semi-continuously fed biogas tank reactors	Grain stillage	4 – 4.6 gCOD/L.d	37°C	(Gustavsson et al., 2013a)
0.1 mg/L	UASB	Distillery effluent	5.9 gCOD/L.d	35°C	(Sharma & Singh, 2001)
0.29-0.4 mg/L	UASB, ABR, HABR	Synthetic sucrose based	1.62 – 1.8 gCOD/L.d for UASB 2.16 – 2.4 gCOD/L.d for ABR, HABR	35°C	(Patidar & Tare, 2008)
6.1 mg/kg	Semi- continuous digester	Wheat stillage	10 g/L.d	38°C	(Schmidt et al., 2014)
0.5 mg/kg	CSTR	Mixture of OFMSW and slaughterhouse waste	5.14 gVS/L.d	38°C	(Moestedt et al., 2015)

Co	0.05 mg/kgFM	Semi- continuous anaerobic fermenter	Maize silage	3 gODM/L.d	35ºC	(Pobeheim et al., 2011)
	0.017 mg/gCOD removed	Methane fermenter	Glucose	2 gCOD/L.d	Mesophilic	(Takashima et al., 2011)
	0.054 mg/gCOD removed	Methane fermenter	Glucose	2 gCOD/L.d	Thermophilic	(Takashima et al., 2011)
	0.00443 mg/gCOD removed	Methane fermenter	High-solid food waste	1.9 – 6.3 gCOD/L.d	Thermophilic	(Qiang et al., 2013)
	100 mg/kgTSfed	CSTR	Food waste	1.5 – 6 gVS/L.d	Mesophilic	(Facchin et al., 2013)
	0.22 mg/kgTSfed	Semi- continuous digester	Food waste	5 gVS/L.d	36 ± 1ºC	(Banks et al., 2012)
	0.16 mg/L	CSTR	Organic solid waste	3.0 – 3.5 gTS/L.d	37ºC	(Uemura, 2009)
	0.5 mg/L	Batch anaerobic mono- digester	Maize silage	N/A	37ºC	(Evranos & Demirel, 2014)

			1.62 – 1.8 gCOD/L.d		
0.13-0.29 mg/L	UASB, ABR, HABR	Synthetic sucrose based	for UASB 2.16 – 2.4 gCOD/L.d for ABR, HABR	35ºC	(Patidar & Tare, 2008)
0.1 - 0.3 mg/L	Semi-continuously fed biogas tank reactors	Grain stillage	4 – 4.6 gCOD/L.d	37ºC	(Gustavsson et al., 2013a)
0.5 mg/L	UASB	Distillery effluent	5.9 gCOD/L.d	35ºC	(Sharma & Singh, 2001)
1.0 mg/L	Semi- continuous digester	Food waste	1 – 4 gVS/L.d	37 ± 1ºC	(Zhang et al., 2015a)
1.82 mg/L	UASB	Methanol	2.6 – 7.8 gCOD/L.d	30ºC	(Zandvoort et al., 2004)
2.5 mg/kg	Semi- continuous digester	Wheat stillage	10 gCOD/L.d	38ºC	(Schmidt et al., 2014)
0.5 mg/kg	CSTR	Mixture of OFMSW and slaughterhouse waste	5.14 gVS/L.d	38ºC	(Moestedt et al., 2015)

Zn	0.049 mg/gCOD removed	Methane fermenter	Glucose	2 gCOD/L.d	Mesophilic	(Takashima et al., 2011)
	0.24 mg/gCOD removed	Methane fermenter	Glucose	2 gCOD/L.d	Thermophilic	(Takashima et al., 2011)
	0.15-0.19 mg/L	UASB, ABR, HABR	Synthetic sucrose based	1.62 – 1.8 gCOD/L.d for UASB 2.16 – 2.4 gCOD/L.d for ABR, HABR	35°C	(Patidar & Tare, 2008)
Mo	6 mg/kgTSfed	CSTR	Food waste	1.5 – 6 gVS/L.d	Mesophilic	(Facchin et al., 2013)
	5 mg/kg	Semi- continuous digester	Wheat stillage	10 g/L.d	38°C	(Schmidt et al., 2014)
	0.25 mg/L	Batch anaerobic mono-digester	Maize silage	N/A	37°C	(Evranos & Demirel, 2014)
Se	10 mg/kgTSfed	CSTR	Food waste	1.5 – 6 gVS/L.d	Mesophilic	(Facchin et al., 2013)
	0.16 mg/kgTSfed	Semi- continuous digester	Food waste	5 gVS/L.d	36 ± 1°C	(Banks et al., 2012)

	0.2 mg/L	Semi- continuous digester	Food waste	1 – 4 gVS/L.d	37 ± 1°C	(Zhang et al., 2015a)
W	10 mg/kgTSfed	CSTR	Food waste	1.5 – 6 gVS/L.d	Mesophilic	(Facchin et al., 2013)
	0.5 mg/kg	Semi- continuous digester	Wheat stillage	10 g/L.d	38°C	(Schmidt et al., 2014)

CSTR – Continuously-stirred tank reactor; USAB – Upflow anaerobic sludge blanket reactor; ABR – Anaerobic baffled reactor;

HABR – Hybrid anaerobic baffled reactor; OFMSW – Organic fraction of municipal solid waste; ODM – Organic dry matter

Different substrates have different metal contents, for example wheat stillage has different properties compared to other stillage (e.g. sugar beet, sugar cane, and corn), and it is not suitable for anaerobic digestion due to its high concentration of sulfur, and TM deficiency (Westerholm et al., 2012; Schmidt et al., 2014). Hence, its required concentration for TMs will be higher than for other stillage, while swine wastewater contains a high content of Cu and Zn (Cestonaro do Amaral et al., 2014).

Mesophilic and thermophilic anaerobic systems may also have significantly different nutrient requirements. For example, Takashima et al. (2011) reported the minimum requirements for, Ni, Co, Zn, and Fe in thermophilic glucose fermentation to be 0.40, 0.45, 2.0, and 3.5 mg/L, respectively. These required amounts are ten times more than those required for mesophilic acetate fermentation (Takashima & Speece, 1989), implying a hypothetical decrease in bioavailability or increase in nutrient requirements at thermophilic temperatures. Similarly Uemura (2009) also found that nutrient requirements are higher in thermophilic anaerobic digestion than in mesophilic anaerobic digestion of organic solid waste.

Another factor that can affect metal speciation and bioavailability, and hence the metal dosing strategy, is the reactor configuration. For example, a submerged anaerobic membrane reactor (SAMBR) is different to a conventional up-flow sludge anaerobic sludge blanket reactor (UASB) due to its ability to decouple hydraulic retention time (HRT) and solids retention time (SRT) (Hu, 2004). Stuckey (2012) suggested that the low solids production and solids free effluent in a SAMBR could enable nutrient recycling; therefore, the nutrient requirements for a SAMBR might be less than for an UASB. In addition, Voolapalli and Stuckey (1998) suggested that nutrient limitations might have contributed to greater floc sizes, and bigger flocs may increase the mass transfer resistance of nutrients from the bulk, which could happen in a granular sludge reactor such as UASB. However, there is limited information available in the literature to explain how reactor configuration can influence

metal speciation and bioavailability.

The requirement of TMs has been investigated for many different types of microbial biomass i.e. co-cultures, biofilms, granular sludge, and pure cultures (Schönheit et al., 1979; Koesnandar et al., 1991; Jarvis et al., 1997; Gonzalez-Gil et al., 1999; Basiliko & Yavitt, 2001; Kida et al., 2001). Such variations in the required amounts of TMs are explained by the unique TMs requirement for different methanogenic pathways. In general, Fe, Ni, and Zn requirements are roughly equal for CO_2 reduction, acetoclastic, and methylotrophic pathways. However, Mo and W requirements are higher for CO_2 reduction and methylotrophic pathways, whereas Co requirements are higher for the methylotrophic pathway (Krzycki & Zeikus, 1980; Glass & Orphan, 2012).

2.6   Trace metal supplementation in anaerobic digestion

Although metal deficiency in anaerobic processes is not a rare phenomenon, the significance of TM supplementation is still hugely underestimated (Zandvoort et al., 2006a). In terms of reactor operating modes, Gonzalez-Gil et al. (1999) found that when an anaerobic expanded granular sludge bed (EGSB) reactor was operated in a continuous mode, the amount of Co and Ni required to eliminate the limitations of metal bioavailability was less than that in a batch mode (4 µM compared to 40 µM for both Ni and Co) due to more biological ligands being produced, and the continuous supplementation of nutrients makes sure that free metals are available for biomass to uptake at any time.

In terms of dosing strategies, TMs can be supplemented continuously (Zandvoort et al., 2002), pre-loaded (Zandvoort et al., 2004), or pulsed into anaerobic reactors (Zandvoort et al., 2004). A continuous supplementation of $CoCl_2$ at a low concentration (0.62 µmol/L.d) to a methanol-fed UASB reactor only increased the SMA up to 215 mgCOD-CH_4/gVSS.d (Zandvoort et al., 2002). Pre-loading of 1mM $CoCl_2$ for 24h was also shown to overcome Co limitations in the methanol UASB reactor, but a significant amount of Co

escaped into the effluent (Zandvoort et al., 2004). Fermoso et al. (2008c) found that pulse dosing of a low quantity of $CoCl_2$ (5 μmol/L), a compromise between continuous and pre-loading dosing, also prevented metal limitations, without supplementing Co continuously, and had around 90% Co retention.

TMs can be supplemented individually, or in combination, to anaerobic digesters. Evranos and Demirel (2014) found that during the anaerobic mono-digestion of maize silage, the maximum methane yields of 429 $mLCH_4/gVSS$ added was achieved when Mo, Co, and Ni were supplemented at the same time at concentrations of 0.25, 0.5 and 0.5 mg/L, respectively.

EDTA is known to complex and reduce the bioavailability of TMs (Aquino & Stuckey, 2007). However, EDTA has been used for phytoremediation of heavy metal contaminated soils to improve the bioavailability and plant metal uptake in the soil (Paulo et al., 2004). The use of EDTA in anaerobic reactors to improve metal bioavailability can be different depending on reactor configuration, and hence metal retention capability. For example, EDTA used as a ligand for Co dosing to UASB reactors has been investigated by Fermoso et al. (2008c), and they found that when supplemented as Co-EDTA^{2-} only 8% of the Co supplemented was retained, compared to 90% retention when it was supplemented as $CoCl_2$, making EDTA unsuitable for Co dosing. This is due to the high solubility of Co-EDTA^{2-} added to a continuous UASB system which resulted in Co wash-out. However, in two batch experiments, Vintiloiu et al. (2013) found that Ni complexed with EDTA prior to supplementation can significantly enhance its bioavailability. In a batch system, the EDTA may act as a chelating agent, buffering free ion concentration, which can then be taken up by microorganisms.

The requirement and dosing strategy for TMs also depends on the portion of the supplemented TM that is bioavailable to the microorganisms for uptake. Although metals are available in the reactor at high total concentrations, the stimulating effects of these metals can still be observed, indicating that the metals can be present in a non-bioavailable form (Zandvoort et al., 2006a). For

instance, added Ni (10 µM) increased the biogas production from anaerobic digestion of chicken manure when nickel was already present in the effluent (253 µM) prior to "extra" Ni being supplemented (Williams et al., 1986).

Therefore, in order to reduce the amount of TMs required and maximize the methanogenic activity, it is important to understand how their speciation affects their bioavailability.

2.7   Trace metal speciation in anaerobic digestion

The total metal content in anaerobic cultures is not sufficient to indicate their bioavailability and toxicity (Oleszkiewicz & Sharma, 1990; Amir et al., 2005). It is now apparent that the chemical speciation of TMs must be assessed and then considered in order to be able to predict bioavailability. Soluble metals supplied from the feed can be transformed into other forms, including; adsorbed metals, metal-carbonate, -phosphate, -sulfide precipitates in order of their decreasing bioavailability.

Among these fractions, the precipitation of metal-sulfide is considered to be the most critical process in anaerobic digestion. Sulfide in an anaerobic digester originates from sulfide, sulfate, and sulfur-containing organic compounds in the feed (Callander & Barford, 1983a). There are many studies which confirm the influence of sulfide formation/dissolution on TM distribution in anaerobic digestion (Gonzalez-Gil et al., 1999; van der Veen et al., 2007; Gustavsson et al., 2013b). The speciation and bioavailability of TMs are strongly influenced by the sulfide chemistry (van der Veen et al., 2007); the effects of sulfide on metal bioavailability will be discussed in more detail in Section 2.8.2.

Anaerobic digestion of biological phosphorus removal sludge was found to result in the extensive precipitation of Ca and Mg, markedly reducing the soluble and easily exchangeable fractions of these metals. However, unexpectedly, the enrichment of P actually increased the soluble concentrations of Cu, Zn, Cr, Ni, and Co. This phenomenon was related to the increasing

concentrations of soluble organic matter being released into the liquid phase being available for complexation with TMs (Carliell Marquet, 2002).

Different metals have different bio-uptake processes due to different kinetic and equilibrium processes. For example, it was found that Fe^{2+} solubility was controlled by FeS precipitation/dissolution, whereas Co^{2+} solubility was controlled by the formation of microbial compounds. The presence of one metal can also have an impact on the speciation and thus the bioavailability of another; high Fe concentrations in an anaerobic digester can promote co-precipitation, adsorption, and ion substitution of Co and Ni on FeS (Gustavsson et al., 2013a; Shakeri Yekta et al., 2014). The presence of K in molasses stillage was found to be responsible for the removal of exchangeable forms of essential TMs such as Ni^{2+}, Zn^{2+}, and Cu^{2+} from a UASB sludge matrix (Ilangovan, 1993). The potassium salt, KNO_3, can cause the displacement of metal ions in the sediment/sludge matrix bound by electrostatic attraction (Mulligan et al., 2009).

2.7.1 The effects of operating parameters on trace metal speciation in anaerobic digestion

The bioavailability of metals for microbial uptake and growth is dependent on metal speciation, which is controlled by the operating parameters i.e. pH, redox-potential, and hydraulic retention time (HRT). Dong et al. (2013) studied changes in metal speciation during the high-solids anaerobic digestion of sewage sludge. There was a strong correlation between heavy metal speciation and changes in several operating parameters, including VS/TS, pH, and VFA concentration. Due to the complex aqueous chemistry and syntrophic biological processes in anaerobic systems, changes in these operating parameters will affect the distribution and bioavailability of TMs.

2.7.1.1 pH effects

Medium pH can have dramatic impacts on solution-phase metal concentration. Specifically, in media that contains phosphate as a buffer, a

minor change in pH can affect metal solubility, and hence metal bioavailability by several orders of magnitude (Sandrin & Maier, 2003). In general, at high pHs metals have a tendency to form insoluble metal phosphates and carbonates (Rensing & Maier, 2003), whereas at low pHs the initial leaching of metals from the sludge occurs, and hence their solubility increases (Zandvoort et al., 2005; Gonzalez-Gil et al., 2012). Most of the studies in the literature focus on the effect of pH reduction on metal bioavailability.

Marcato et al. (2009) studied the effect of pH on the bioavailability of Zn and Cu in anaerobically digested and raw pig slurry. They demonstrated a gradual dissolution of Zn from the slurry into solution when the pH decreased below about 6.0, until dissolved Zn accounted for 40% of the total Zn at pH 2.7. Also, Zn was much more sensitive to pH variations than Cu as anaerobic sludge had a very strong affinity for Cu. At neutral pH, van Hullebusch et al. (2005b) reported that Co and Ni were not only found in the organic/sulfide fractions, but also in significant amounts in the residual fraction of granular anaerobic sludge. However, Gonzalez-Gil et al. (2012) found that at low pH (5), this residual fraction was absent and considerable amounts of Ni and Co were present in the bioavailable fractions (exchangeable and carbonates).

Zandvoort et al. (2005) also determined the effect of pH shocks on TM dynamics in USAB reactors, and found that the pH shocks did not affect the total metal concentration in the sludge significantly. However, a 30 d pH shock (pH 5) resulted in a shift of Co, Ni, and Fe from the residual to the organic/sulfides fraction, which can be due to the adsorption and co-precipitation of Ni and Co to FeS. At shorter pH shocks (pH 5 for 4 days), significant losses of Co (18%) and Fe (29%) with the effluent from the organic/sulfide fraction were observed, and this was likely to be due to the dissolution of Fe sulfide and a concomitant release of Co.

Lopes et al. (2008) studied the influence of low pH on nutrient dynamics in anaerobic sludge granules, and found that reducing the pH led to increased solubilisation of metals from the sludge, with Fe and Mn leached most

extensively. Therefore, a change in pH can affect metal speciation as reducing the pH can lead to dissolution/leaching of metals, and hence improve metal bioavailability.

2.7.1.2 Redox potential effects

Under reducing conditions metal speciation in sediments and anaerobic samples is different. Generally, for anaerobic samples, under anaerobic conditions i.e. redox potential <-300 mV and neutral pH, TMs are more difficult to mobilize and mainly precipitate as sulfides and carbonates (Marcato et al., 2009). On the other hand, for sediment samples the redox gradients due to transitions from oxic to suboxic and anoxic conditions result in changes in metal speciation and the dominance of Fe-Mn oxide. Therefore, all of the studies on the effects of redox potential on metal speciation are carried out for sediment samples. Tack et al. (1996) confirmed that the redox potential can have a significant effect on metal speciation in a reduced dredged sediment. They found a higher solubility of Cu (10-90 mg/kgTS) at higher redox potentials (+60 to +370 mV) at a pH between 1 and 2; in comparison, the solubility of Cu was nearly zero at -220 to -150 mV. However, Okbah et al. (2008) found that when the redox potential reduced from 77 to -133 and -170 mV in two sediment samples, a significant amount of the Fe-Mn oxide fraction transformed to the water-soluble and exchangeable fractions, suggesting that reducing conditions were more favorable for metals bound to these fractions.

2.7.1.3 Hydraulic retention time effects

As discussed in Section 2.6, the metal retention capability is dependent on reactor configuration which in turn is coupled to the hydraulic retention time (HRT). Cestonaro do Amaral et al. (2014) studied the distribution of Cu and Zn in swine wastewater during anaerobic digestion over different HRTs. They found that when the HRT was dropped from 17.86 d to 5.32 d, the metal retention capacity of the bioreactors reduced. While other metal fractions

remained the same or decreased, the less bioavailable oxidizable fraction comprised of metals associated with organic matter or sulfide increased, indicating that the metals became less bioavailable to anaerobic microorganisms. This finding is important for the operation of anaerobic reactors; for example, under hydraulic shock loads when the HRT is reduced, a higher dose of TMs is required to compensate for the fraction of the metals which become less bioavailable.

2.8 Trace metal bioavailability in anaerobic digestion

Although there has been a lot of work on metal speciation in natural systems, the linkage between metal speciation and metal bioavailability in anaerobic reactors has not been well understood. There are very few and limited review studies on the TM bioavailability in anaerobic reactors (Worms et al., 2006; Zandvoort et al., 2006a).

There is still much confusion about TM bioavailability. Although the total metal content which may be provided should be sufficient, the metals may not be present in a bioavailable form that can be utilized by the microorganisms (Zitomer et al., 2008). Even full bioavailability does not mean that the required metals are all utilized by the bacterial population (Oleszkiewicz & Sharma, 1990). The bioavailability of TMs in bioreactors depends on the complex interactions between metals in the solid and liquid phases in the bioreactors. Transport of free metal ions through the cell membrane is assumed to be the main mechanism for metal uptake by microorganisms. On the other hand, before the essential metals can make contact with the biomass, they are subjected to a number of biochemical processes in the liquid phase such as sulfide precipitation and the formation of inorganic and organic complexes (Zandvoort et al., 2006a). Therefore, it is essential to understand the interaction of metals with biomass and the liquid phase.

2.8.1   Metal-biomass interaction

Most microorganisms can form biofilms, and the TMs present in anaerobic digestion will interact with these biofilms through physical, chemical, or biological processes; van Hullebusch et al. (2003) provided an extensive review on the mechanisms and analytical tools able to study the fate of metals within biofilms. The distribution, immobilization, and remobilization of metals depend on the sorption properties of the biofilms, determined by many factors including pH, redox potential, and concentration of ligands present within the biofilm matrix. Sheng et al. (2008) carried out an investigation on metal-bacteria adhesion using two anaerobic sulfate-reducing bacteria under different parameters, i.e. metal nutrients, ionic strength, and pH of the solution. The bacteria–metal adhesion force measured by atomic force microscopy (AFM) decreased in the presence of TMs in solution, and increased at a stronger ionic strength in solution. The bacteria–metal adhesion force reached its maximum value when the pH of the solution approached the isoelectric point of the bacteria, i.e. at the zero-point charge.

In another comprehensive review, Worms et al. (2006) provided a different approach to studying metal-biomass interactions by emphasizing the importance of the chemical, biological, and physical processes occurring at and near the biological interface in controlling TM bioavailability through shifts in the limiting bio-uptake fluxes. Examples are biological (transport across the membrane), chemical (dissociation kinetics of metal complexes), and physical (diffusion) processes. These processes are not entirely independent, and can limit TM internalization; microorganisms can adapt by modifying the rate-limiting flux to prevent both TM deficiency and toxicity.

Therefore, by considering all of these physical, chemical and biological processes occurring near the biological interface, or within the biofilm matrix, the predictions of metal speciation and bioavailability will become more accurate.

2.8.2 Metal-liquid phase interaction

Sulfide is important due to the formation of dissolved metal complexes, which are very strong and poorly soluble. It has been shown that the bioavailability of TMs in anaerobic digestion is mostly controlled by sulfide formation/dissolution (Gonzalez-Gil et al., 1999; van der Veen et al., 2007). Since metal sulfides have a very low solubility product, this results in a remarkably low free metal ion concentration, and hence they are expected to be non-bioavailable to anaerobic microorganisms (Callander & Barford, 1983a; Callander & Barford, 1983b; Martell & Smith, 1989). Barber and Stuckey (2000) found that sulfide precipitation reduced TM bioavailability in the following order (most deficient first): Fe > Co > Ni. However, Jansen et al. (2007) found that methanogenic activity was related to the dissolved metal concentration, and this was replenished by a rapid dissolution of freshly formed metal sulfides. Gustavsson et al. (2013a) studied the bioavailability of Co and Ni during the anaerobic digestion of sulfur-rich stillage, and found that 100% of Ni and 80-90% of Co was in the organic matter/sulfides fraction. However, Ni still had significant stimulatory effects, which is in agreement with the assumption that Ni-sulfide can become a storage of and source for Ni as hypothesized by Jansen et al. (2007). Limitations in methanogenic activity tend to occur in the case of stabilization of the precipitates due to co-precipitation or ageing, decreasing the rate of dissolution. However, in the dynamic anaerobic environment, it is expected that less stable amorphous metal-sulfide phases will dominate, and concomitantly higher dissolution rates will occur (Jansen et al., 2007).

Other important inorganic ligands such as PO_4^{3-} and CO_3^{2-} are widely present in the liquid phase of anaerobic reactors, and their concentrations may vary substantially in practice (Bartacek et al., 2008a). Although the complexes of these inorganic ligands are less strong than the metal sulfide complexes, the importance of carbonate and phosphate complexes on metal bioavailability has been demonstrated in a number of studies on anaerobic media (Bartacek et al.,

2008a; Fermoso et al., 2009; Gustavsson et al., 2013a). Gustavsson et al. (2013a) found that when Co was added to anaerobic reactors treating a sulfur-rich stillage, the dissolved concentration of Co measured was much higher than what was estimated from the equilibrium constant, which may be explained by the complexation of Co with sulfide or organic ligands like SMPs as discussed by Jansen et al. (2007). The role of SMPs as a chelating agent on the bioavailability of TMs will be discussed in Section 2.8.3.1.

2.8.3 The effects of chelating agents on trace metal bioavailability in anaerobic digestion

The bioavailability of TMs not only relies on the total metal concentration and the environmental conditions influencing speciation as discussed above, but also on their precipitation as sulfide, carbonate, and phosphate salts; chelation/complexation with organic and inorganic ligands including those synthesized or released by microorganisms to facilitate metal uptake (siderophores), and the kinetics of precipitation and complexation reactions. In this section, the effects of both natural chelating agents i.e. soluble microbial products (SMPs), extracellular polymeric substances (EPS), yeast extract, and synthetic chelating agents i.e. nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA) on TM bioavailability will be discussed in detail.

2.8.3.1 Soluble microbial products (SMPs)

It is well known that bacteria excrete a range of organic compounds known as SMPs into solution due to biomass decay and substrate metabolism (normally with biomass growth) (Barker & Stuckey, 1999). There are several factors that cause SMP production, and nutrient deficiency and metal toxicity are two of these (Barber & Stuckey, 2000; Aquino & Stuckey, 2003; Aquino & Stuckey, 2004).

SMPs have chelating capacities due to the functional groups found in

them, including carboxylates, hydroxyls, phenols, sulfhydryls (-SH), and amines (Kuo & Parkin, 1996). These chelating groups can act as ligands, and complex most of the common metals present in wastewaters and sludges (e.g. Cu, Fe, Ni, and Zn). A methodology was developed by Callander and Barford (1983a) to assess each of the individual effects of precipitation, chelation, and bioavailability of TMs in anaerobic digestion. In a later study, Callander and Barford (1983b) found a significant difference between the measured soluble metal ion Fe^{2+} and the calculated concentration based on equilibrium and solubility product constants, suggesting that natural chelating agents such as SMPs formed in the anaerobic digester play an important role in making metal ions available to microorganisms. The complexation of metals with SMPs in anaerobic reactors such as the upflow anaerobic sludge blanket bioreactor (UASB), anaerobic baffled reactor (ABR), and hybrid anaerobic baffled reactor (HABR) have been shown to play a significant role in ensuring soluble metal bioavailability. Patidar and Tare (2008) studied the effects of sulfide toxicity on SMP production and metal bioavailability in three anaerobic reactors; a UASB, ABR, and HABR. The results showed that with an increasing sulfide concentration in the three reactors (up to 90 mg/L as free H_2S), the anaerobic microorganisms also increase SMP production to maintain higher soluble concentrations of TMs through complexation, and reduce the effect of sulfide toxicity. Kuo and Parkin (1996) found that 300 mg/L of SMP in an anaerobic system can chelate up to 0.75 mM of Ni.

The affinity for complexation with SMPs is not the same for different metals. Barber and Stuckey (2000) studied the bioavailability of TMs in an anaerobic baffled reactor (ABR) and found that the affinity for complexation of the metals is in the following order: Ni > Co >> Fe.

2.8.3.2 Extracellular polymeric substances

According to a definition by Pal and Paul (2008), extracellular polymeric substances (EPS) of microbial origin are a complex mixture of biopolymers

comprising nucleic acids, polysaccharides, proteins, lipids, humic substances, uronic acids, etc. EPS formation results from bacterial secretions, shredding of cell lysates, cell surface materials and adsorption of organic constituents from the environment. Regardless of origin, EPS can be loosely bound to the cell surface, or bacteria may be embedded in the EPS. FTIR analysis confirmed the presence of ionisable functional groups such as phosphate, carboxyl, and hydroxyl/amino groups in the EPS samples from an anaerobic membrane bioreactor (AnMBR), and these are related to proton binding sites for metals (Liu et al., 2015). Metals have been reported to have a different binding capacity with EPS from anaerobic granular sludge (Artola et al., 1997; d'Abzac et al., 2010). There are a number of EPS extraction protocols that can be applied to study metal-EPS interactions. d'Abzac et al. (2010) compared four chemical and four physical extraction protocols from anaerobic granular sludges. The results showed that the chemical protocols resulted in contamination of the samples by the chemical extracting agents, which modify the binding properties of the EPS extracts. On the other hand, the composition of the physically extracted EPS samples is similar and would be preferred as less detrimental methods to investigated proton and metal binding properties. However, the physical schemes require more advanced analysis to examine unwanted macromolecules disruption that can have an effect on the metal binding properties. Recently, Bourven et al. (2015) developed a method using semi-preparative and analytical chromatographic columns that allow for separation of the EPS fraction according to its MW distribution. This separation allows researchers to study both the EPS composition as well as its metal-EPS binding capability.

2.8.3.3 Yeast extract

Gonzalez-Gil et al. (2003) found that yeast extract can increase the bioavailability of Ni and Co dramatically; this was due to the formation of strong organic complexes with the yeast extract, which facilitates the

dissolution of metal sulfides. However, direct supplementation of yeast extract to full-scale anaerobic reactors may not be an economical practice (Hu et al., 2008a).

2.8.3.4 Nitrilotriacetic acid and ethylenediaminetetraacetic acid

Synthetic chelating agents such as nitrilotriacetic acid (NTA) and ethylenediaminetetraacetic acid (EDTA) are sometimes included in nutrient solutions to prevent TM precipitation (Callander & Barford, 1983a; Grčman et al., 2001). When the concentration of these chelating agents and the stability constants of their complexation with metals are high enough, metal ion concentrations can be reduced to levels below those corresponding to the solubility products of S^{2-}, CO_3^{2-}, and PO_4^{3-} to prevent precipitation (Callander & Barford, 1983a).

Hu et al. (2008a) studied the effect of NTA on the bioavailability of Ni during the methane fermentation of a sulfate-containing wastewater. They found that when the sulfide concentration doubled from 0.25 to 0.5 and 1 mM, by adding 10 μM NTA methane production increased by 10, 30 and 48%, respectively. In addition, the sulfide and carbonate fraction of Ni in the biomass dropped by 58.0 and 28.8%. This data showed that the NTA complexed Ni, preventing Ni from precipitating with the sulfide, promoted Ni internalization, and hence enhancing the bioavailability of Ni for methanogens. However, the effect of NTA on the bioavailability of other essential TMs such as Co, Zn, and Mn is unknown.

The role of an EDTA metal complex in metal bioavailability in anaerobic digestion is very interesting. As already discussed in Section 2.6, the effect of an EDTA metal complex on metal bioavailability depends on the reactor configuration, i.e. a batch or continuous system. In addition, the concentration of the EDTA metal complex can also have different effects on metal bioavailability. Bartacek et al. (2012) found that if injected in high amounts, the complexation of Co with EDTA ($[CoEDTA]^{2-}$) resulted in lower toxicity than

the free Co (i.e. CoCl$_2$) in methanogenic granular sludge, which was the same finding obtained with Ni (Bartacek et al., 2010). This phenomenon was explained by the strong binding of metals to EDTA, and this complex cannot cross bacterial cell walls (Worms et al., 2006), hence making the metals less bioavailable to microorganisms. On the other hand, in the same study, when small amounts of [CoEDTA]$^{-2}$ were injected into batch bottles it stimulated the methanogenic activity of a Co limited granular sludge by increasing the SMA value (mg COD-CH$_4$/gVSS.d) by 233%, whereas the SMA did not increase at all after the first CoCl$_2$ pulse addition. Bartacek et al. (2012) suggested this was due to the fast metal transport by the complex (minutes to tens of minutes), compared to the non-complexed free species (tens of minutes up to hours). Therefore, the complexed metal species can penetrate more quickly into the whole volume of the anaerobic granule. On the other hand, the free metal species precipitate with the matrix, and hence can accumulate inside the granule to a very high concentration and can be kept in the granular sludge for a longer time.

Aquino and Stuckey (2007) studied the effects of both natural chelating agents (i.e. SMPs) and synthetic chelating agents (i.e. NTA, EDTA, and citrate) on the bioavailability and toxicity of Fe and Cu on methanogenesis. Their results showed that 1 mM of EDTA complexed the metals, making them unavailable to methanogens, and hence reduced the methane production rate. SMPs are usually excreted to scavenge metal nutrients, and the addition of SMPs increased Cu bioavailability through the direct uptake of Cu-SMPs complexes. Cu toxicity was reduced by adding stoichiometric amounts of NTA to complex it, making most of the Cu soluble.

2.9 Techniques to investigate metal speciation and bioavailability in anaerobic digestion

2.9.1 Sequential extraction schemes

The need to understand metal distribution (speciation) in order to predict the mobility and bioavailability of trace metals (TMs) during anaerobic digestion has led to the development of a large number of experimental schemes designed to fractionate metals into specific forms (Lester et al., 1983). Based on the metal fractionation, the mobility of TMs can be evaluated and then combined with information on the activity of anaerobic microorganisms to determine the bioavailability of TMs. Single and sequential extraction schemes have been developed and broadly implemented to determine which chemical forms the TMs are present in and, indirectly, of their potential bioavailability. However, these schemes have been criticized 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 parameters such as temperature, pH, extracting time, reagent concentration, stirring system, the ratio of solid mass to volume of extractants; and so on (Förstner, 1993; Hursthouse, 2001; Gleyzes et al., 2002; Hsu et al., 2015).

Filgueiras et al. (2002) provided a very comprehensive review on different sequential extraction schemes used to fractionate metals in different environmental samples, e.g. soil, sediment, sewage sludge, and fly ash. The main differences between them were the extractant reagents, extraction conditions, and number of fractions. Given the wide range of schemes used, The Commission of the European Communities Bureau of Reference (known as BCR), now the Standards, Measurement and Testing Programme, began a programme in 1987 in an attempt to harmonize the methodology used in different schemes (Ure et al., 1993). The BCR scheme extracts metals into three fractions, i.e. exchangeable, Fe and Mn oxides, and organic matter and sulfides. The modified BCR scheme (Sahuquillo et al., 1999) has been shown to extract

the Fe-based components of the reducible fraction (Mossop & Davidson, 2003). The original and modified BCR has been used to fractionate metals in anaerobic sludge in many studies (Quevauviller et al., 1994; Rauret et al., 2000; Virkutyte et al., 2005; Alonso et al., 2006; Fuentes et al., 2008; Dąbrowska & Rosińska, 2012).

The Tessier scheme (Tessier et al., 1979) is one of the most widely applied schemes, and the original and modified Tessier scheme (Osuna et al., 2004) has been applied to sewage sludge (Legret, 1993; Perez Cid et al., 2002; Lasheen & Ammar, 2009; Dong et al., 2013) and anaerobic granular sludge (Osuna et al., 2004; van Hullebusch et al., 2005a). The Tessier scheme partitions metals into three fractions i.e. exchangeable, carbonates, and organic matter. 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.

The third popular scheme was developed by Stover et al. (1976), and fractionates metals into five different fractions i.e. soluble/exchangeable, adsorbed, organically bound, carbonate, and sulfide fractions. This scheme was then modified by Lake et al. (1985) and used in many studies on metal extraction for anaerobic sludges (Rudd et al., 1988; Carliell & Wheatley, 1997; Carliell Marquet, 2002; Aquino & Stuckey, 2007; Hu et al., 2008a).

For comparison, van Hullebusch et al. (2005b) applied these three popular schemes to determine the fractionation of Ni, Co, Cu, Mn, Fe, and Zn in anaerobic granular sludge from UASB reactors. The results showed that the modified Tessier scheme seemed to be the most applicable scheme to determine the metal fractionation in anaerobic granular sludge. Although the BCR scheme gave similar trends and good reproducibility, it seemed to be limited to anaerobic matrixes as the extraction step for the reducible fraction can leak the TMs out to the organic/sulfides fraction, and hence may overestimate the recovery of the oxide fraction. In addition, Fe and Mn oxides are not commonly

found in anaerobic sludge (van der Veen et al., 2007). However, van der Veen et al. (2007) found that the oxidizable or so-called organic/sulfide fraction of the modified Tessier scheme does not represent metal sulfides well as a significant amount of Fe was extracted in the residual fraction with unknown composition, although there was an excessive amount of sulfide in the sludge. Shakeri Yekta et al. (2012) suggested that the organic/sulfide extraction step dissolved FeS as 24-27% of Fe remained unextracted but it was dissolved by the acid volatile sulfide (AVS) extraction step. The transformation of Fe from a sulfide mineral was likely due to the formation of a secondary phase of Fe i.e. jarosite. This change in Fe and S speciation might lead to inaccurate results for the speciation of other TMs i.e. Co and Ni, known to associate with this Fe phase. Because of the higher number of extraction steps, the Stover scheme gave a poorer total recovery, especially the sulfur from the sulfide fraction compared to the Tessier and BCR schemes (van Hullebusch et al., 2005b). The reagents used in this scheme were not absolutely selective for a single form; EDTA extracted more than 90% of the precipitates of Cu, Pb, and Zn carbonates, but only 68% of the Cd carbonate (Lester et al., 1983). Furthermore, this scheme was also found to lack in selectivity for Ni, and hence was not sensitive enough to detect small changes in metal speciation that might be significant with respect to digester performance (Carliell Marquet, 2002).

Other less popular schemes include the Sposito procedure (Sposito et al., 1982) used by (McGrath and Cegarra (1992); Pichtel and Anderson (1997); Amir et al. (2005)), and the scheme developed by Qiao and Ho (1997) used by (Hsu and Lo (2001); Song and Greenway (2004); Nomeda et al. (2008)).

All of the above schemes were carried out in a batch procedure, but (Wisotzky and Cremer (2003a); Wisotzky and Cremer (2003b)) developed a sequential extraction method in columns, which reduces the extraction time, guarantees anaerobic conditions and improves the precision of the extraction procedure. Sample preparation for all sequential extraction schemes is very similar; the sludge sample is centrifuged and the pellets are dried in aluminum

pans at 105ºC for at least 24 h (van der Veen et al., 2007; Ortner et al., 2014) and then stored in a desiccator at room temperature. Dried samples are ground in a porcelain mortar and then sieved through a stainless steel sieve with a nominal size of 0.4 mm. The powder samples are then stored in polyethylene bags in a desiccator until use in subsequent extraction steps. However, pre-treatment by oven drying and milling is not recommended due to the time required, and an artificial shifting in metal speciation towards insoluble fractions (Rudd et al., 1988; Ortner et al., 2014).

For the determination of total metal content in sludge and residual fraction after the sequential extraction procedure, acid digestion is the most common method applied to digest the sample. The popular procedures for acid digestion include: total digestion with concentrated nitric acid (65%) e.g. EPA 3051 procedure (5 ml HNO_3 for 0.5 gTSS) (EPA, 1994) used by Liu and Sun (2013); concentrated nitric acid (65%) combined with concentrated hydrogen chloride (37%) e.g. *aqua regia* procedure (7.5 mL HCl: 2.5 mL HNO_3 for 0.5 gTSS) developed in an European Standard (Standard, 2000) and used by (Virkutyte et al. (2005); Long et al. (2009); Bartacek et al. (2012); Dąbrowska and Rosińska (2012)); and nitric acid combined with hydrogen chloride and hydrogen fluoride (5 mL HNO_3: 4 mL HCl: 1 mL HF for 0.5 gTSS) (Alonso et al., 2006). However, HF is less common as it is hazardous and can degrade glassware and adulterate analytical results. The *aqua regia* procedure was found to be very effective compared to the EPA method 3051, especially for metals deposited in a silicate matrix (e.g. Ni, Cr, Pb) (Florian et al., 1998). The *aqua regia* procedure has been applied in the most popular sequential extraction schemes (e.g. BCR, Tessier, and Stover) to determine the total metal content and residual fraction.

Most of the current studies use a microwave-assisted acid digestion technique to determine the metal content in sediments, sludges, and soils (Mossop & Davidson, 2003; van Hullebusch et al., 2005b). The use of closed pressurized vessels together with microwave heating under temperature

programming allows for a very short decomposition time for different matrices, and an increase in accuracy (Florian et al., 1998).

Autoclave digestion is another technique developed for Nordic countries and validated in the European project 'Horizontal' on the basis of Nordic standards for the digestion of sludge and sediment samples. The Swedish Standard Method (SS-028311) also extracts metals from the sludge with 7 M HNO_3 in an autoclave (at $120^\circ C$ for 30 min). This method has been used in several studies on metal speciation in anaerobic reactors (Shakeri Yekta et al., 2012; Gustavsson et al., 2013b). Other less common techniques used to digest soil and sludge samples include a digestion block with a mixture of HCl and HNO_3 (8 mL: 2 mL) for 0.5g sample for 1h at $105^\circ C$ (McGrath & Cunliffe, 1985), and in a water bath with a mixture of HNO_3, HF, $HClO_4$, HCl in (4:1:1:1) ratio, respectively, for 3 h (Lasheen & Ammar, 2009).

After digestion the sample is filtered, made up to an appropriate volume with deionized water, and measured by atomic absorption spectrometry (AAS), flame or inductively coupled plasma-atomic emission spectrometry (ICP-AES), or inductively coupled plasma–mass spectrometry (ICP-MS), or microwave plasma atomic emission spectrometry (MP-AES).

2.9.2 Techniques to determine free metal ion concentration

The stimulation of TMs cannot be assessed only from the total metal content in the liquid phase, but requires the assessment of metal speciation, especially free metal concentration (Bartacek et al., 2008a). However, this approach is only valid when the equilibrium state is established and the physical-chemical processes (precipitation, sorption, chemical speciation, etc) are faster than the parallel biological processes (metal uptake and methane production) (Bartacek et al., 2010). To measure the amount of 'free' metal ion in aqueous solutions, there are a number of available techniques. The Wageningen Donnan Membrane Technique (WDMT) was developed by Temminghoff et al. (2000) in order to study Co speciation in anaerobic granular sludge. One

drawback of this technique is the long data acquisition time (4 d to establish equilibrium, 5 d for WDMT measurement) (Bartacek et al., 2008b).

Diffusive gradients in thin film (DGT) and diffusional equilibrium in thin film (DET) are among the most promising in situ techniques for measuring aqueous metal species for bioavailability assessment purposes (Meylan et al., 2004; van der Veeken et al., 2008; Yezek et al., 2008; Liu et al., 2013; Chen et al., 2014a). They are capable of measuring labile species of metals, such as free metal ions, inorganic complexes, and a small portion of organic complexes (Li et al., 2005; Ren et al., 2015). However, similar to ISE, the use of DGT/DET in wastewater has been rare (Buzier et al., 2006).

2.9.3   Techniques to determine sulfur speciation

It has been shown that the TM speciation and bioavailability in anaerobic digestion processes strongly depends on the chemistry of sulfur (S) (Jansen et al., 2007; van der Veen et al., 2007; Fermoso et al., 2009), and there are a number of analytical techniques to determine changes in S speciation. Sulfur K-edge X-ray absorption near-edge spectroscopy (XANES) is an effective tool to identify and quantify many different S compounds, their binding forms, and oxidation states in a sample, which are useful to determine the fate of S species in anaerobic digestion (Shakeri Yekta et al., 2012).

Acid volatile sulfide (AVS) analysis is a classic technique to measure the sulfide fraction reactive metals in anoxic samples, based on volatilization of the reduced S bound in mineral fractions, mainly FeS (Jong & Parry, 2004; van der Veen et al., 2007); 1 M HCl is used as the extracting reagent. Rickard and Morse (2005) carried out a comprehensive review of AVS extraction which discussed the following drawbacks of the AVS-SEM technique: (1) very different dissolved and solid sulfide components may be making largely varying contributions to evolved H_2S, and (2) minor variations in the extraction method for AVS can result in different results for the same samples.

2.9.4 Chromatographic and spectroscopic techniques

Chromatographic techniques such as Ion Chromatography - Conductivity Detector (IC-CD), Gas Chromatography - Flame Ionization Detector (GC-FID) and spectroscopic techniques such as X-ray Diffraction Spectroscopy (XRD), Fourier Transform Infrared Spectroscopy (FTIR), and X-ray absorption near edge structure (XANES) spectroscopy have also been used to study the speciation of metals in anaerobic sludge (Lenz et al., 2006a; Zhu et al., 2014).

2.9.5 Chemical equilibrium modelling

Chemical equilibrium models have been developed to predict metal speciation, and hence metal bioavailability in environmental systems, although the environmental systems are always dynamic and rarely at equilibrium (Worms et al., 2006). The geochemical equilibrium models such as Visual MINTEQ, Visual MINTEQA2 and PHREEQC have the capability to predict metal speciation by estimating precipitation, equilibrium aqueous speciation and dissolution of minerals, adsorption, complexation, solid phase saturation states, etc at thermodynamic equilibrium (Virkutyte et al., 2005; Bartacek et al., 2008a; Ortner et al., 2014). By comparing the predictions with the actual metal distribution measured experimentally, i.e. sequential extraction, it is possible to observe the changes in metal speciation caused by the biomass and natural and microbial chelators, hence, the mechanisms by which biomass take up essential metals could be deduced (Aquino & Stuckey, 2007).

2.10 Summary of literature review

- Reactor configuration is considered as a key design criterion that determines the retention capacity of trace metals in a bioreactor. Most of the studies on trace metal speciation and bioavailability in the literature were carried out for anaerobic digesters. Anaerobic membrane bioreactors (AnMBRs) are becoming very popular due to their complete biomass retention, high efficiencies of COD removal, and separation of HRT and

SRT. However, there is little research available on the fate of trace metals in the AnMBRs;

- Fe^{2+} is often dosed to reduce the sulfide level in anaerobic digesters, however, the bioavailability of metal sulfides is still debated in the literature. Furthermore, there has been no research on the effect sulfide has on the bioavailability of Fe^{2+} in a continuous system like an AnMBR;

- Conventional chelating agents such as EDTA and NTA have shown to be able to improve the bioavailability of trace metals in the presence of sulfide, but they have very low biodegradability. To date, a very limited number of studies have been carried out to determine if biodegradable chelating agents such as EDDS can be alternatives;

- Chelating agents have been dosed to anaerobic reactors either as a metal complex or simultaneously with the trace metals, but which dosing strategy is more suitable depends on not only the added concentrations of both chelating agents and the metals, but also the reactor configuration. So far, there has no quantitative analytical technique available to determine the speciation of the chelating agents i.e. EDDS in anaerobic systems in order to evaluate the effectiveness of their dosing strategy;

- Many studies in the literature have shown the effects of the increase in organic loading rate and the metal deficiency on the performance of AnMBRs. However, the significance of the accumulated trace metals in seed sludge has not been emphasized in these conditions. There is an essential need to determine what is the most effective dosing strategy for trace metals and how these metals transform over time;

- The changes in operating parameters such as pH, HRT, and SRT are also known to affect the performance of AnMBRs. There is little information available to understand their impacts on the speciation and bioavailability of trace metals and how they correlate with the reactor performance.

2.11 Research objectives

Based on the literature review above, the aim of this project was to evaluate the speciation and improve the bioavailability of trace metals in a SAMBR. The specific objectives were:

- To investigate the effects of Fe^{2+} and its bioavailability for controlling VFAs during organic shock loads in batch reactors and a submerged anaerobic membrane bioreactor (SAMBR);

- To assess the effect of sulfide on the speciation and bioavailability of Fe^{2+} in a batch setup and in a SAMBR;

- To determine the dosing strategies for EDDS to improve the bioavailability of Fe^{2+} in the presence of sulfide in a SAMBR;

- To determine the possibility of pulse dosing of TMs to improve the performance of a SAMBR under trace metal deficiency;

- To study the effect of changes in pH, hydraulic retention time (HRT), and solids retention time (SRT) on the speciation and bioavailability of trace metals in a SAMBR.

Chapter 3

Experimental Methodology

3.1   Introduction

This chapter presents a description of the submerged anaerobic membrane bioreactor (SAMBR) system, experimental setups, followed by details of seed sludge, synthetic feed and the nutrients used for the SAMBR. It also describes the sample preparation procedures and analytical methods for the fractionation techniques employed to extract the trace metals (TMs) in the anaerobic systems studied, as well as the chromatographic analysis to quantify the chelating agent. The specific experimental procedures will be described in the following chapters.

3.2   Set-up of Submerged Anaerobic Membrane Bioreactor system

Figures 3.1 and 3.2 illustrate the operation and experimental set-up of the submerged anaerobic membrane bioreactor (SAMBR) system used for this study (Kunacheva et al., 2017). The SAMBR was designed by Hu and Stuckey (2006), and has been used in a number of previous studies (Akram & Stuckey, 2008; Vyrides et al., 2010; Trzcinski et al., 2011; Zhou et al., 2016). The lab-scale SAMBR was made from polymethyl methacrylate (Plexiglas) and had an effective volume of 3.2 L. A microfiltration flat sheet membrane (222 mm (W) × 315 mm (L) × 6 mm (T)) as shown in Figure 3.3, was made of chlorinated polyethylene by Kubota with a surface area of 0.116 m^2 and a maximum pore size of 0.4 and average of 0.2 μm was used.

The reactor was divided into two different zones by a baffle which helped to direct the liquid flow upwards past the membrane, and then downwards

through the biogas sparger. The membrane was submerged into the reactor and the reactor was placed in a water bath which maintained the temperature at 35 ± 1°C. Biogas in the headspace of the reactor was re-circulated through a stainless steel tube diffuser with four holes which generated coarse bubbles in order to mix the biomass in the reactor and scour the surface of the membrane to prevent membrane fouling. A vacuum pump (B100 SEC, Charles Austin) was used to recycle biogas from the headspace back to the diffuser, and the sparging rate was controlled by a gas flowmeter (RMA-22-SSV, Dwyer) at 4-5 L per minute (LPM).

An online data logging system was set up to continuously monitor pH, ORP, and temperature using a pH/ORP probe (Mettler Toledo, M300) and transmembrane pressures (TMP) using a digital pressure gauge (Ashcroft). A pressure transmitter (PMP 1400, RS components) and a turbine type flowmeter (Model 101-3) were provided to transmit pressure and flow signals to a personal computer (PC). This arrangement was followed by peristaltic pumps which operated at uniform speed to provide a constant reflux through the membrane. Part of the effluent removed through the membrane was recycled back to the reactor, while the remaining liquid was discharged from the system as the effluent stream. The HRT was controlled using another pump on the effluent stream after the flux pump.

3.3   Cultivation of anaerobic sludge for the SAMBRs

Seed sludge for anaerobic reactors was obtained from a municipal wastewater treatment plant digesters (Ulu Pandan Water Reclamation Plant, Singapore), and cultivated in 5L continuously mixed laboratory batch reactors for more than 3 months. The seed reactors were fed once a week in fill-and-draw mode (7 d HRT), and the feed was based on that used by Ketheesan et al. (2016), consisted of glucose (333 mgCOD/L), peptone (99 mgCOD/L), meat extract (33 mgCOD/L), sodium bicarbonate (812.5 mg/L), K_2HPO_4 (10 mg/L), and essential metal nutrients including $CoCl_2.6H_2O$ (0.29 mg/L), $FeCl_2.4H_2O$

(1.96 mg/L), $MnCl_2.4H_2O$ (0.09 mg/L), $Na_2MoO_4.2H_2O$ (0.04 mg/L), and $NiCl_2.6H_2O$ (0.05 mg/L).
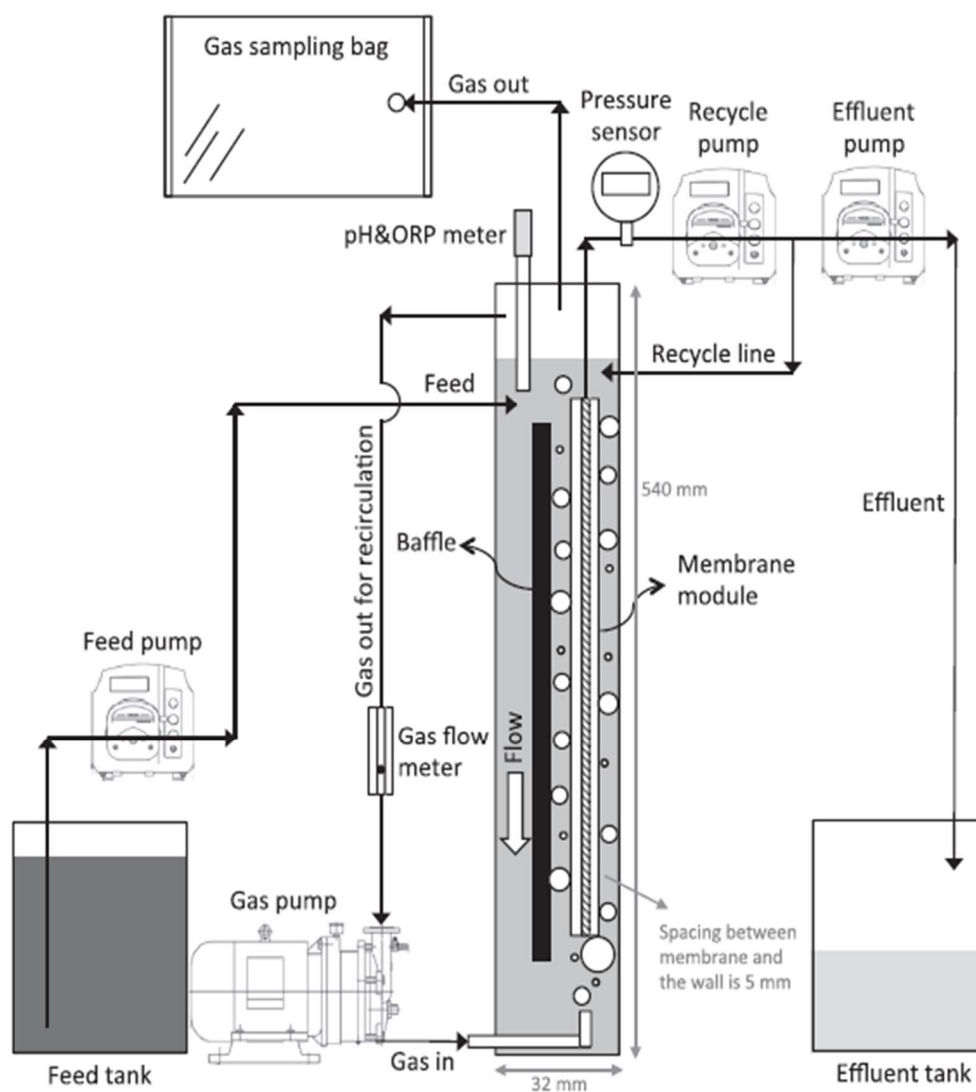
Figure 3.1   Schematic diagram of the SAMBR system (Kunacheva et al., 2017).

Figure 3.2   Experimental setup of the SAMBR system used in this study.

Figure 3.3   The flat sheet membrane used in the SAMBRs.

3.4 Feed and nutrients for the SAMBRs

A carbohydrate-protein substrate was supplemented with metal nutrients to serve as the synthetic wastewater. The SAMBRs wastewater was maintained at approximately 500 ± 20 mgCOD/L, and was based on that used by Akram and Stuckey (2008). The composition of the feed media is as shown in Table 3.1. Feed was prepared by adding peptone, meat extract, K_2HPO_4, $NaHCO_3$ and nutrients to tap water, and autoclaving at 121^oC for 30 min. They were mixed together after cooling to room temperature, before connecting the feed bottle to the pumps. Eight times concentrated feed was prepared to avoid daily preparation which was laborious and time consuming. The feed was diluted 80 times by adding tap water before entering the reactor.

Table 3.1   Composition of concentrated feed for SAMBR experiments (40 gCOD/L).

Carbon source and macro-nutrients (g/L)		Metal nutrients (g/L)	
Glucose	26.70	$FeCl_2.4H_2O$	0.157
Peptone	7.99	$CoCl_2.6H_2O$	0.0238
Meat extract	2.68	$NiCl_2.6H_2O$	0.0045
K_2HPO_4	0.80	$MnCl_2.4H_2O$	0.0075
$NaHCO_3$	65	$Na_2MoO_4.2H_2O$	0.00375
		$ZnCl_2$	0.0031

3.5 Analytical techniques

3.5.1 General sample preparation

A large quantity of samples was generated over the various periods of reactor operation, and a wide variety of parameters were measured.

Consequently, the data presented at any point was obtained from a replicate analysis of each liquid sample. The definition of soluble for this study was any material passing through 0.45 μm filters, hence prior to any analysis all samples were first centrifuged and then filtered to remove any suspended solids and ensure a clear supernatant, to facilitate accurate analysis. Most samples were centrifuged at 12,000 rpm (model 5810 R, Eppendorf) for 10 min and filtered through disposable 0.45 μm syringe filters (Membrane Solutions) except the samples for VFAs and EDDS measurement which were filtered through disposable 0.2 μm syringe filters (PALL, Acrodisc®).When larger volumes of sample (i.e. > 100 mL) were handled a bigger centrifuge was used, and in this case samples were centrifuged at 4000 rpm (model 5810 R, Eppendorf) for 20 min, and then filtered through 0.45 μm glass fiber filter paper (Advantec) using a vacuum apparatus.

3.5.2   pH

pH was measured offline using a pH meter (Mettler Toledo, M300), and online in the SAMBRs using a pH probe (Mettler Toledo, M300) calibrated with buffer solutions of pH 7 and pH 4. Values obtained for pH were accurate to within ± 0.02.

3.5.3   Chemical Oxygen Demand (COD)

The measurement of COD was based on the 'closed reflux, colorimetric method', described in section 5220-D of APHA (1992), which requires the preparation of digestion solution and sulphuric acid reagent. Digestion solution for high range COD was first prepared by adding 10.216 g of $K_2Cr_2O_7$, previously dried at 103°C, 167 mL of concentrated H_2SO_4 and 33.3 g of $HgSO_4$ to 500 mL of distilled water. The mixture was then left to cool to room temperature before diluting to 1000 mL. The digestion solution for low range COD was prepared the same as for high range but only used 1.022 g of

$K_2Cr_2O_7$. The sulphuric acid reagent was prepared by adding Ag_2SO_4 technical grade powder to concentrated H_2SO_4 at the rate of 5.5 g Ag_2SO_4/kg H_2SO_4.

The COD protocol consisted of adding, in this order, 2.5 mL of sample/diluted sample, 1.5 mL of digestion solution and 3.5 mL of sulphuric acid reagent into COD tubes. The tubes were tightly sealed and mixed properly. The mixtures were then refluxed in a COD reflux reactor (Model ECO 16, VELP Scientifica) at 150ºC for 2 h. After cooling the samples were analyzed on a Shimadzu UV/Vis scanning spectrophotometer (UV-2600) (triplicate) at a wavelength of 600 nm for high range COD and 420 nm for low range COD. Potassium hydrogen phthalate (KHP) was used to prepare standard solutions in the range 10 – 1000 mg/L. The detection limit was 5 mg/L and the standard deviation based on triplicates was within ±5%.

3.5.4 Total and volatile suspended solids (TSS/VSS)

TSS and VSS were used to estimate the biomass concentration in the anaerobic systems. A 5 mL sample (triplicate) was added to a pre-weighed foil tray and left overnight in an oven pre-set at 105ºC (Memmert, Model UM500) to allow for the evaporation of water. The dried sample was left to cool in a desiccator after which the resulting weight was recorded to calculate the TSS value. The tray was then placed in a furnace (Model P330, Nabertherm) at 550ºC for 1 h to allow for the combustion and evaporation of the organic fraction. After cooling in a desiccator the final weight of the tray was recorded, and a calculation to determine the VSS was then performed as given in Standard Methods (APHA, 1992). The coefficient of variance for 5 identical samples was within ±5%.

3.5.5 Volatile Fatty Acids (VFAs)

VFAs (C1-C5) were determined using a Shimadzu Prominence ultra-fast liquid chromatography (UFLC, SPD-20AD) with a UV diode array detector (DAD, SPD-M20A) at 210 nm using an Aminex® HPX-87H (300 × 7.8 mm)

column. Analysis time was 25 min for each sample operating under isocratic and isothermal conditions with a sample volume of 20 μL (triplicate); the column was maintained at 55ºC, and the eluent was 0.005M H_2SO_4 at a flow rate of 0.8 mL/min. Before analysis samples were centrifuged at 12000 rpm for 20 min and filtered through 0.2 μm filters.

A total of seven VFAs including formic acid, acetic acid, propionic acid, butyric acid, and valeric acid were quantified with this method. The detection limit was 3 mg/L, and the coefficient of variance (COV = SD/average value) of ten identical samples was within ± 2%.

3.5.6   Biogas production and composition

Biogas volume from the SAMBRs was measured with a gas-sampling bag using a gas pump with a flow meter, while biogas volume for the serum bottles was measured using calibrated wetted glass syringes. The composition of biogas (methane, oxygen, nitrogen, and carbon dioxide) and gas phase hydrogen (triplicate for the batch bottles) was determined using a Shimadzu GC-TCD (2010 Plus) fitted with an Agilent J&W Column (60 m × 0.53 mm × 30 μm) and VICI Valcoplot column (15 m × 0.53 mm × 20 μm). The carrier gas was Helium set at a flow rate of 156 mL/min, column temperature was 35ºC, detector temperature was 100ºC, and injector temperature was 40ºC. The coefficient of variance (CV) for biogas composition of ten identical samples was within ± 3%. The methane yields from the batch and SAMBR experiment were defined as the volume of methane (mLCH$_4$) produced by unit mass of active biomass (gVSS) per unit time (d) i.e. mLCH$_4$/gVSS.d, which has often been used on anaerobic digestion to determine the activity of anaerobic bacteria (Cho et al., 2005; Souto et al., 2010; Hussain & Dubey, 2015).

3.5.7   Biochemical Methane Potential (BMP) analysis

The bioassay analysis was conducted in serum bottles using the technique developed by Owen et al. (1979), with the composition of the biomedia being

given in Table 3.2. Seed sludge was transferred to 50 mL tubes and centrifuged at 4000 rpm for 20 min at 4°C and the supernatant discarded. Biomedia was added to each centrifuge tube to mix with the solid pellets. The contents of the tubes were then transferred into serum bottles under anaerobic conditions while continuously flushing the bottles with nitrogen at a flow rate of approximately 0.5 L/min. Concentrated substrate and buffer ($NaHCO_3$) was then added to each serum bottle before capping it with leak proof Teflon seals. All serum bottles were equilibrated at 35°C, and zeroed 15 min after substrate addition.

Methanogenic activity ($mLCH_4/gVSS.d$) was detected by measuring the composition and amount of biogas produced over time in bottles using GC-TCD and calibrated wetted glass syringes, as described in Section 3.5.6. Triplicate samples were carried out for each set of experiments, and the COV in this case was ± 3%.

Table 3.2   Chemical composition of biomedia used in BMP bottles (Owen et al., 1979).

	Volume of stock solution	Amount of stock solution for 1.8 L biomedia	Compounds	Conc. in stock solution (g/L)	Final conc. in medium (mg/L)
S1	100 mL	1.8 mL	Resazurin	1.0	1.0
S2	100 mL	5.4 mL	$(NH_4)_2HPO_4$	26.7	80.1
S3	1000 mL	27 mL	Trace Elements solution:		
			$CaCl_2 \cdot 2H_2O$	16.7	250.5
			NH_4Cl	26.6	399
			$MgCl_2 \cdot 6H_2O$	120	1800
			KCl	86.7	1300
			$MnCl_2 \cdot 4H_2O$	1.33	19.95
			$CoCl_2 \cdot 6H_2O$	2	30

			H_3BO_3	0.38	5.7
			$CuCl_2 \cdot 2H_2O$	0.18	2.7
			Na_2MoO_4	0.17	2.55
			$ZnCl_2$	0.14	2.1
S4	50 mL	1.8 mL	$FeCl_2 \cdot 4H_2O$	370	370
S5	50 mL	1.8 mL	$Na_2S \cdot 9H_2O$	500	500
S6	1000 mL	18 mL	Vitamin Solution:		
			Biotin (vitamin H)	0.002	0.02
			Folic acid (dihydrate)	0.002	0.02
			Pyridoxine hydrochloride (vitaminB6)	0.01	0.1
			Riboflavine (vitamin B2)	0.005	0.05
			Thiamine (vitamin B1)	0.005	0.05
			Nicotinic acid	0.005	0.05
			Pantothenic acid	0.005	0.05
			Cyanocobalamine (vitamin B12)	0.0001	0.001
			p-aminobenzoic acid	0.005	0.05
			lipoic acid (thioctic acid)	0.005	0.05
S7			$NaHCO_3$		4667

3.5.8 Metal extraction

The BCR sequential extraction procedure proposed by Ure et al. (1993), and then modified by Sahuquillo et al. (1999), was used to determine metal speciation; a description of the BCR procedure is given in Table 3.3. The extraction used CH_3COOH, $NH_2OH\text{-}HCl$, H_2O_2 and CH_3COONH_4 to sequentially fractionate metals into three fractions: soluble/exchangeable, Fe and Mn oxides, organic matter and sulfide, respectively. The pseudo-total metal content in the sludge, and the residual fraction after sequential extraction, was

obtained by digesting with *aqua regia* solution (HCl:HNO$_3$ 3:1 v/v) in a microwave digester (Anton Paar, Multiwave 300).

Table 3.3   Operating conditions required in the revised BCR sequential extraction method (van Hullebusch et al., 2005b).

Fraction	Extracting Agent	Extraction conditions	
		Shaking time[a]	Temperature
F1. Exchangeable + water and acid soluble	40 mL CH$_3$COOH (0.11 M, pH = 7)	16 h	20°C
F2. Iron and manganese oxides	40 mL NH$_2$OH–HCl (0.5 M, pH = 1.5)	16 h	20°C
F3. Organic matter and sulfides	20mL H$_2$O$_2$ (30%, pH = 2), 50mL CH$_3$COONH$_4$ (1M, pH = 2)	1, 2, 16 h	20, 85, 20°C
F4. Residual	10 mL demineralised water, 10 mL aqua regia (HCl:HNO$_3$, 3:1)	26 min	Microwave-oven[b]

[a] Shaking was applied at 30 rpm.

[b] Extraction of the residual fraction in the microwave was equal to the pseudo-total extraction method.

3.5.9   Metal analysis

The concentrations of metals in the liquid phase were measured by using microwave plasma atomic emission spectrometry (MP-AES; AGILENT model 4200) (triplicate). The liquid fractions for metal analysis were first centrifuged at 10,000 rpm for 10 min, and then filtered through 0.45 µm disposable syringe filters (Membrane Solutions) to remove any residual solids prior to MP-AES

analysis. The detection limit was 0.01 mg/L, and the COV of ten identical samples was within ±1%. The metal content measured in this study was expressed as µg of metal/gTSS, which has also been used in most of studies in the literature as TSS is often used as a parameter for the study of metal sorption onto anaerobic sludge (Osuna et al., 2004; van Hullebusch et al., 2005a; van Hullebusch et al., 2006; Fermoso et al., 2010).

3.5.10   Measurement of EDDS and Fe-EDDS complex

The separation of EDDS and Fe-EDDS complex was carried out on a Shimadzu UHPLC system, equipped with a SPD-M20A Photo Diode Array Detector. The method developed by Narola et al (2011) to determine the disodium EDTA in meropenum drug substance was adapted and modified in this study to determine the EDDS and Fe-EDDS complex in the test reactors.

The analytical column used in this study was a Kinetex 2.6 µm XB-C18 column (150 × 4.6 mm). The mobile phase was prepared by dissolving 4.1 mg of sodium acetate trihydrate, 0.64 mg Tetrabutyl ammonium bromide, adjusting the pH to 4.0 with concentrated glacial acetic acid, and then making up the volume to 1L with deionized water. The flow rate was 0.8 mL/min, and the retention time was 7.5 min, while the column temperature was maintained at 25°C. The detector UV wavelength was 243 nm, and the injection volume was 20 µL.

Standards for EDDS were prepared from a commercial EDDS solution (Enviomet C140), which was provided by Innospec (USA). Standards for the Fe-EDDS complex were prepared by mixing EDDS with Fe^{2+} as $FeCl_2.4H_2O$ in excess overnight in the dark to prevent photo-degradation (Tandy et al., 2005). Calibrations for EDDS and Fe-EDDS complex were performed with 5 standards for each, and the R^2 value was 0.997 and 0.998, respectively. The coefficient of variance (CV) of ten identical samples was within ±3%. The samples were filtered through 0.2 µm before measuring in the Ultra-High Performance Liquid Chromatography (UHPLC) in triplicates.

3.5.11 Sulfide analysis

Soluble sulfide was analysed following the HACH spectrophotometric Methylene Blue Method (Method 8131) (triplicate); the detection limit was 5 µg/L S^{2-} and the COV of ten identical samples was within ±5%.

3.5.12 Statistical analysis

The significance of experimental results was determined by the independent sample t-test when necessary. Statistical significance was set at $p < 0.05$.

Chapter 4

Iron deficiency and bioavailability in anaerobic batch and submerged membrane bioreactors during organic shock loads

Outline:

4.1 Introduction

4.2 Experimental and analytical methods

4.3 Results and discussion

4.4 Conclusions

Note: this chapter was published in *Bioresource Technology*, 2016, volume 211, pages 136-145.

4.1 Introduction

The dependency of anaerobic digestion (AD) on metal nutrients, including Fe^{2+}, has been mentioned in the literature review. The role of Fe^{2+} in anaerobic digestion processes depends on its redox properties, and its engagement in energy metabolism (Takashima & Speece, 1989). It has been reported that the stimulating effect of Fe^{2+} is attributed to its ability to improve acetate utilization by methanogens (Speece, 1962), and its role in the transport system of methanogens for the conversion of CO_2 to CH_4 (Vintiloiu et al., 2013). Therefore, Fe^{2+} can play an important role in improving the degradation of VFAs (Zandvoort et al., 2003; Zitomer et al., 2008).

The occurrence of organic shock loading (sudden changes in feed strength) is of prime importance in AD as this can result in process instability, including a drop in pH, and a drop in COD removal and accumulation of volatile fatty acids (VFAs) that can ultimately lead to AD process failure (Akram & Stuckey, 2008). Hawkes et al. (1992) demonstrated that effluent COD levels in a metal supplemented UASB reactor showed a linear response to the duration of organic shock loading, while the performance of a non-metal supplemented reactor reduced substantially, with the accumulation of propionate. In the literature, some other studies have also reported that the utilization of propionate (Espinosa et al., 1995; Zitomer et al., 2008), acetate (Osuna et al., 2003) and butyrate (Lin et al., 1998) as substrate for mesophilic/thermophilic anaerobic bioreactors improved with the addition of metal nutrients such as Fe^{2+}, Ni, Co and Mo. However, none of the previous studies have specifically looked at the significance of Fe^{2+} bioavailability during organic shock loads.

As discussed in the literature, operating conditions can have a significant impact on the speciation and bioavailability of TMs in anaerobic bioreactors, and during organic shock loads operating conditions change from steady state conditions. There has been not much attention given to the understanding of the relationship between metal dynamics and bioavailability during organic shock loads, which is essential for the early determination of metal nutrient limitations, and hence managing these reactors more efficiently.

Therefore, considering the essential roles of Fe^{2+} in anaerobic digestion processes, the scope of the experiments in this chapter was limited to the speciation and bioavailability of Fe^{2+} during organic overloading conditions in a submerged anaerobic membrane bioreactor (SAMBR), and how the supplementation of Fe^{2+} can improve the removal of accumulated VFAs during the shock loads.

4.2 Experimental and analytical methods

4.2.1 Stages 1-4

Stages 1-4 were performed in 125 mL serum bottles (100 mL working volume) in batch/semi-batch mode. Since the seed reactors were fed once every three days, the seed sludge for the batch/semi-batch tests were obtained on Day 3, just before feeding the seed reactors. The initial biomass was set to 3 gTSS/L in all the serum bottle reactors; the bottles were then placed in a shaker at 35ºC, and the pH of the media adjusted to 7.0 ± 0.1 at the start using diluted NaOH or HCl. All tests were run in triplicate (CV = $\pm 5\%$).

Stage 1:

The seed sludge obtained from seed reactor two (SR2) was centrifuged, and culture media added with sodium bicarbonate (2,000 mg/L) and K_2HPO_4 (80 mg/L).

Stage 2-4:

Seed sludge obtained from SR1 was centrifuged and culture media added as follows: sodium bicarbonate (2,000 mg/L), K_2HPO_4 (80 mg/L), $CoCl_2.6H_2O$ (2.38 mg/L), $FeCl_2.4H_2O$ (15.8 mg/L), $MnCl_2. 4H_2O$ (0.75 mg/L), $Na_2MoO_4.2H_2O$ (0.75 mg/L), $NiCl_2.6H_2O$ (0.9 mg/L), and $MgCl_2$ (2 mg/L). In order to test the deficiency of Fe^{2+}, all other selected metals were supplemented into each test reactor except Fe^{2+}.

4.2.2 Stage 5

Stage 5 studies were in a continuously fed SAMBR which was operated at 35ºC, and steady state was maintained at 8 h HRT with a synthetic feed strength of 500 mg COD/L. The SRT (solids retention time) was set at 150 d, and the biomass ranged from $4 - 4.1$ gVSS/L during the test period. The organic shock loadings were simulated by glucose spikes equivalent to 1 gCOD/L and 2 gCOD/L total concentrations in the SAMBR.

4.2.3 Metal extraction

Sequential metal extraction for this experiment was carried out using the procedure proposed by Stover et al. (1976), and then modified by Lake et al. (1985), to determine the metal concentration in dewatered sludge. The extraction used KNO_3, KF, $Na_4P_2O_7$, EDTA, and HNO_3 to sequentially fractionate metals into soluble/exchangeable, adsorbed, organic bound, carbonate, and sulfide forms, respectively. The EDTA extractable fraction can also represent the metals precipitated as metal phosphates (Carliell & Wheatley, 1997). The residual fraction was obtained by digesting the residual sludge with HNO_3 at 80ºC in heating blocks.

4.3 Results and Discussion

4.3.1 Stage 1: The effects of organic shock loading on the speciation of Fe

The objective of Stage 1 experiments was to examine the dynamics of Fe speciation under different organic shock loads using the seed grown under Fe-sufficient conditions, and sequentially extract it to determine the availability of TMs in solid matrices. There are a number of methods proposed to sequentially extract TMs from anaerobic sludge (Stover et al., 1976; Tessier et al., 1979; Lake et al., 1985; Osuna et al., 2004). However, the scheme proposed by Stover et al. (1976) and modified by Lake et al. (1985) was used in this study since metals bound to Fe/Mn oxides in Tessier's scheme, and the organic/sulfide fraction of the modified Tessier's scheme (Osuna et al., 2004) do not clearly differentiate between metal sulfide fractions commonly found in AD.

The trend of VFA accumulation, pH changes, and the dynamics of Fe speciation (mgFe/gTSS) are shown in Figures 4.1-4.4 when the reactors were subjected to glucose spikes (organic shocks) equivalent to 1 gCOD/L and 3 gCOD/L final concentration in the reactor. As can be seen in all cases, Fe initially accumulated (7.95 ± 0.05) in the seed sludge as sulfides, carbonates/phosphates and non-defined forms (residuals) due to a prolonged

supply of Fe^{2+} in the feed. Generally, residuals are assumed to contain minerals that may hold Fe within their crystalline structure (Tessier et al., 1979). Ortner et al. (2014) also pointed out that non-bioavailable forms of Fe were the highest in anaerobic sludge (30% to 70%) compared to other metals such as Co, Cu, Mo, Ni and Zn. In the current study, a rapid accumulation of VFAs and a subsequent pH drop resulted in the mobilization of Fe^{2+} from sulfide forms to "relatively bioavailable forms'' such as soluble and organically bound Fe with the 1 gCOD/L shock. With the 3 gCOD/L shock the reactor pH dropped to 5.5, and almost 9% of Fe in the residual form leached out to soluble and organically bound forms, and at the end of 48 hours this was equivalent to a dose of about 4.5 mg/L $FeCl_2.4H_2O$, although no external Fe^{2+} was dosed initially. Adding acid to sewage sludge can lead to an exchange of protons (from the acid) based on the following reaction: FeS (s) $+ H^+ = HS^- + Fe^{2+}$ ($logK_{sp}=3.0$) (Stylianou et al., 2007). Gonzalez-Gil et al. (2012) showed that the residual fraction was almost absent at low pH (5), and large amounts of Ni and Co were translocated to exchangeable and carbonate fractions in anaerobic sludge. Thus, Stage 1 studies clearly demonstrated that the accumulation of Fe in the seed sludge should be accounted for by supplementing Fe^{2+} during organic shock loads.
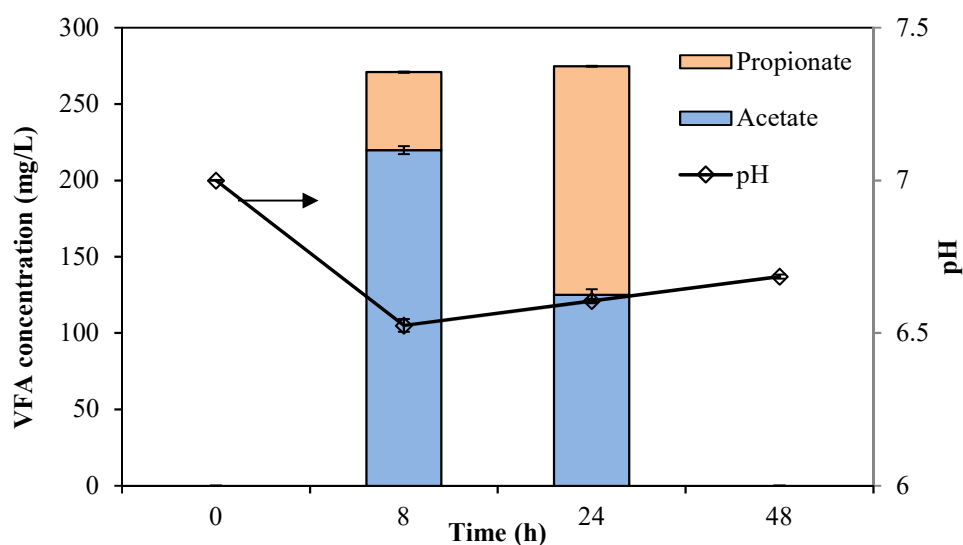
Figure 4.1　　VFA production at 1g COD shock in Stage 1.
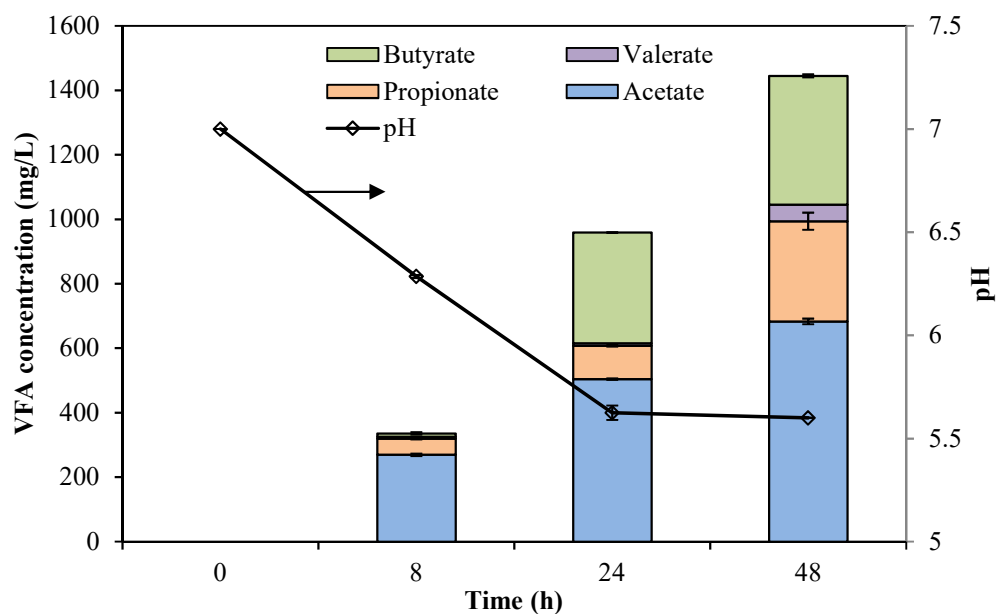
Figure 4.2   VFA production at 3g COD shock in Stage 1.
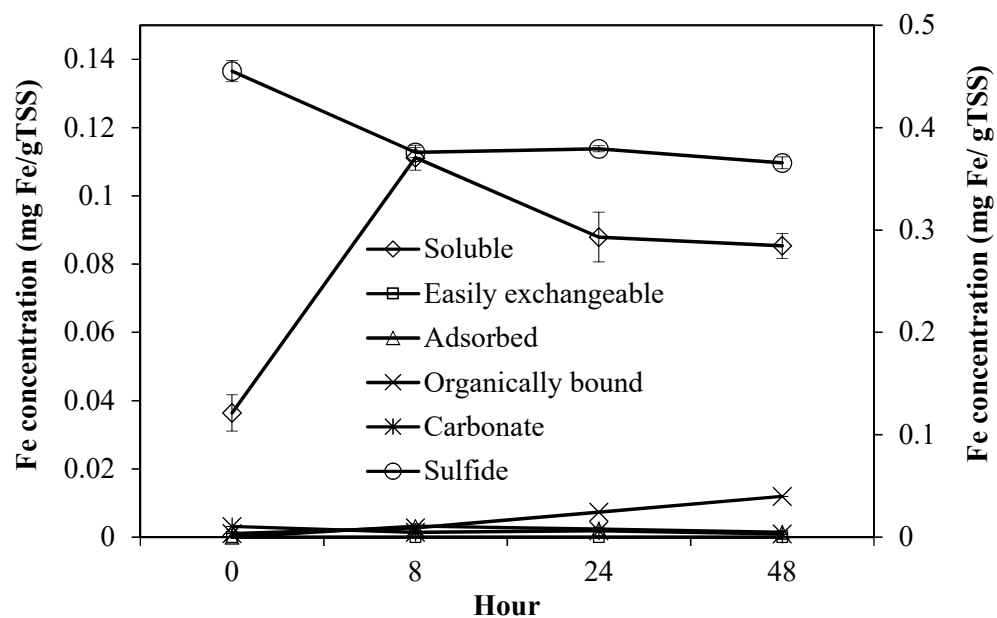
Figure 4.3   Fe speciation at 1 gCOD shock in Stage 1.
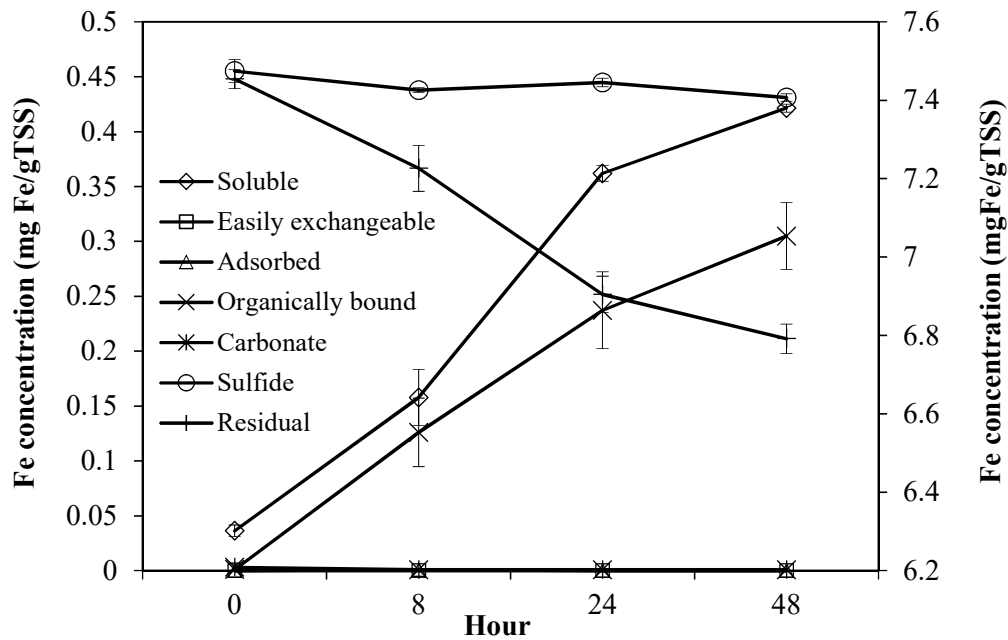
Figure 4.4   Fe speciation at 3 gCOD shock in Stage 1.

4.3.2   Stage 2: Effects of Fe deficiency on the responses to shock loading

In Stage 2, the responses of batch reactors to organic shocks were tested under Fe-sufficient (15.8 mg/L as $FeCl_2.4H_2O$) and Fe-deficient (no Fe supplementation) conditions using seed grown under Fe-deficient conditions. Figures 4.5, 4.6, and Figure 4.7 show their responses in terms of VFAs, and rate of methane production, respectively, when subjected to glucose spikes of 1.5 gCOD/L and 3 gCOD/L. It can be seen that Fe deficiency had a statistically significant effect on VFA accumulation in the cases of 1.5 gCOD/L ($p < 0.05$), 3 gCOD/L ($p < 0.05$), as well as methane production in both cases. While total VFA concentration was <20 mg/L in the case of 1.5 gCOD/L, a doubling in organic load (3 gCOD/L) resulted in an almost 15-fold increase in total VFAs, irrespective of whether the seed was Fe limited; propionate and acetate accumulated predominantly with the 3 gCOD/L shock.
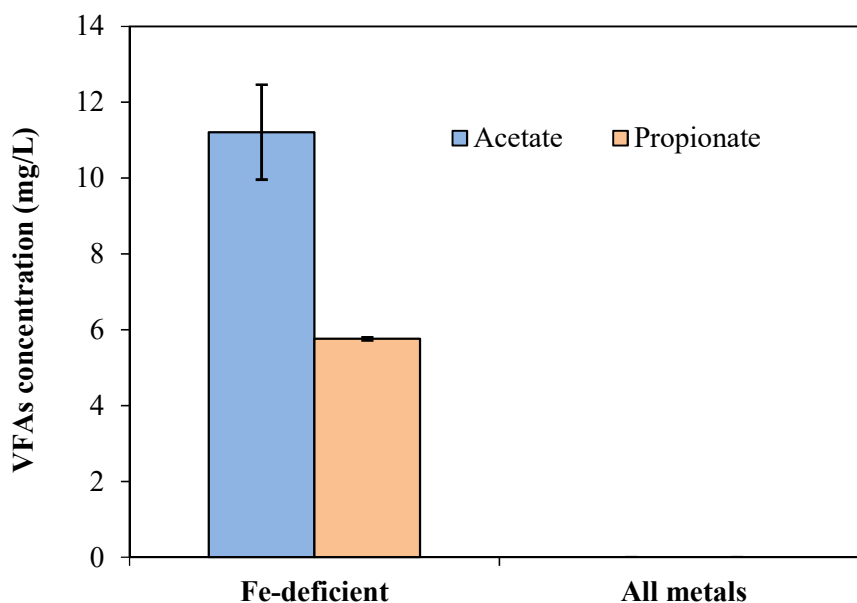
Figure 4.5   Effects of Fe deficiency on VFA accumulation at 1.5 gCOD/L
glucose concentration (100 hours) (Stage 2).
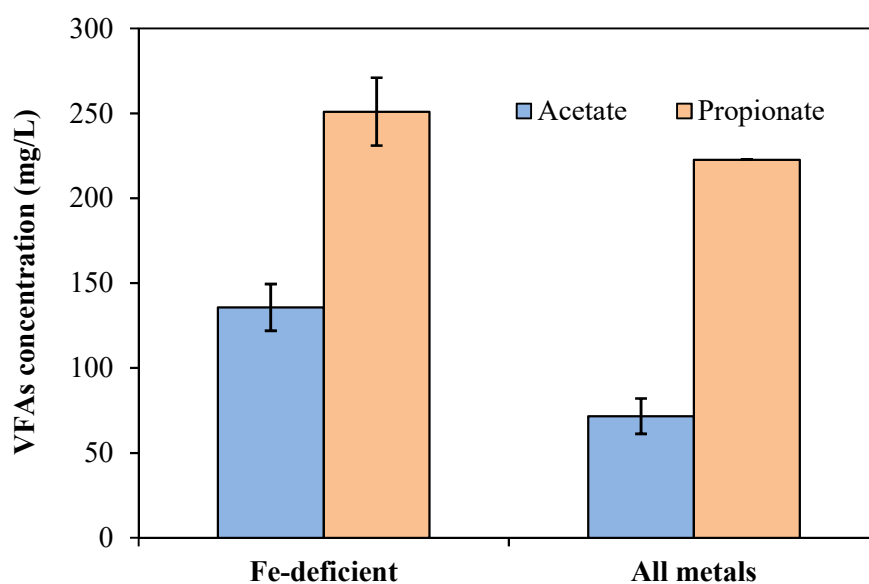
Figure 4.6   Effects of Fe deficiency on VFA accumulation at 3 gCOD/L
glucose concentration (100 hours) (Stage 2).
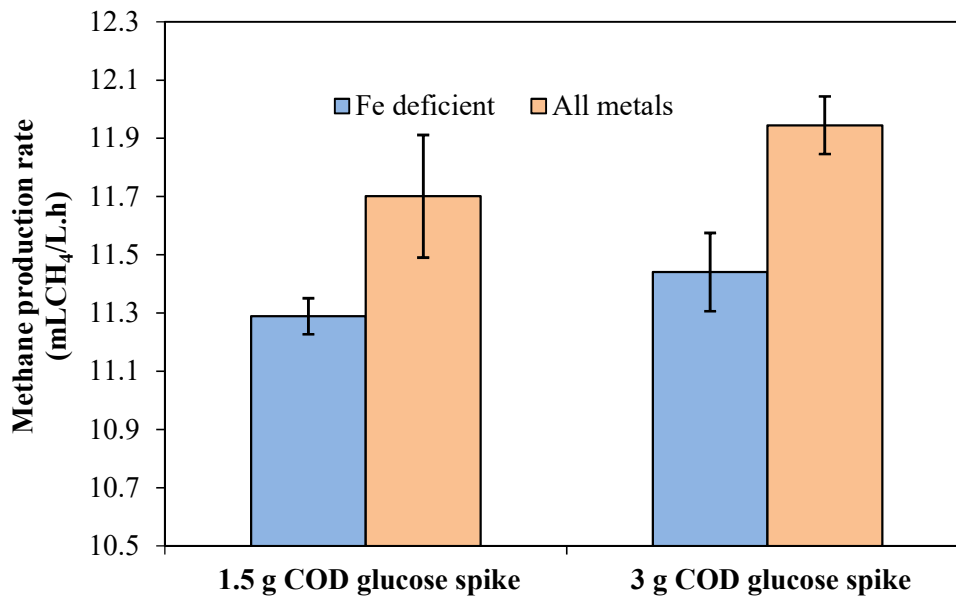
Figure 4.7   Effects of Fe deficiency on methane production at 1.5 gCOD/L and 3 gCOD/L glucose concentration (100 hours) (Stage 2).

It is clear from the literature that kinetic differences between acidogenic/acetogenic bacteria and slow growing methanogenic archaea will result in the accumulation of methanogenic precursors such as VFAs, H_2, and CO_2 at higher organic overloads (Leitão et al., 2006). When hydrogenotrophic methanogens cannot keep up with the high acetate/hydrogen production at higher organic loadings, butyrate and propionate are preferably produced by the acidogens as a feed-back mechanism to reduce hydrogen pressure in the system (Ketheesan & Stuckey, 2015). With the 3 gCOD/L shock, kinetic differences and thermodynamic considerations can significantly influence the accumulation of VFAs, irrespective of Fe supplementation. However, higher VFA accumulation, and a lower rate of methane production, in Fe deficient reactors signify the synergetic effects of Fe limitations with 3 gCOD/L. It could be postulated that there was competition for TMs amongst fermentative bacteria, acetogens and methanogens, assumingly due to the difference in microbial growth rates. This competition can create temporary nutrient limitations, and furthermore, the activity of propionate oxidizing bacteria, acetoclastic

methanogens, and probably hydrogenotrophic methanogens can potentially be affected by Fe deficiency and retard the degradation of acetate and propionate during shock loading. The supplementation of Fe^{2+} with other metals such as Ni and Co has been shown to accelerate the degradation of propionate and acetate in several studies; Zitomer et al. (2008) observed a 50% and 35% increase in propionate and acetate utilization rates, respectively, after supplementation of Ni, Co and Fe into thermophilic digesters. Furthermore, Uemura (2009) observed process failure due to severe acidification in mesophilic digesters, and the system only recovered after the addition of nutrients such as Ni, Co and Fe.

4.3.3 Stage 3: Effects of Fe dosage on the degradation of VFAs

Stage 2 studies confirmed the effects of Fe deficiency on the degradation of acetate and propionate during organic shock loads. However, Stage 2 studies did not give any clear idea about the optimum dosage of Fe^{2+}, and its effects. Since propionate to methane conversion in AD involves several pathways such as propionate to acetate conversion by non-methanogenic propionate oxidizing bacteria, and acetate to methane conversion by acetoclastic methanogens, propionate was selected as a model substrate in Stage 3 to determine the optimum dose of Fe^{2+} in the test reactors. As shown in Figure 4.8, different doses of Fe^{2+} (0 – 100 mg/L) were simultaneously spiked into propionate loaded reactors (1.5 gCOD/L) to evaluate their effects on methane production. A statistically significant ($p < 0.05$) improvement in the kinetics of propionate to methane conversion was observed with a Fe^{2+} concentration of $10 - 30$ mg/L, although 10 mg/L (equivalent to 3.34 $mgFe^{2+}/gTSS$) was found to be optimum. However, Fe^{2+} concentrations above 50 mg/L inhibited propionate conversion rates: metals such as Fe disrupt enzyme function and structure by binding with thiol and other groups on proteins, or by replacing naturally occurring metals in enzyme prosthetic groups (Chen et al., 2008).
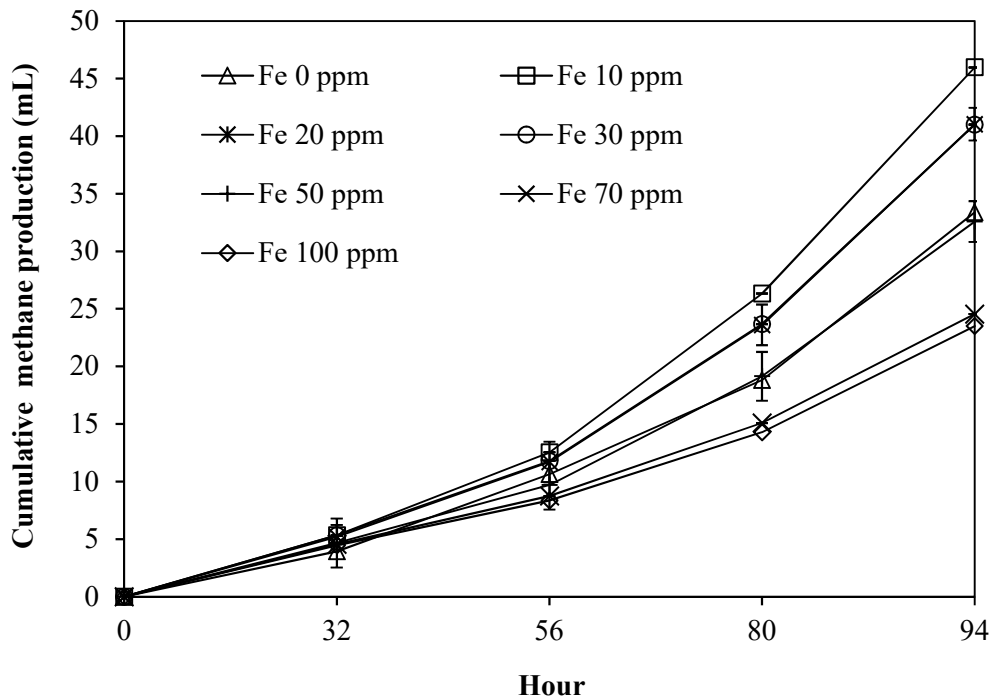
Figure 4.8   Effects of different doses of Fe^{2+} on biogas production in batch reactors (Stage 3).

Figures 4.9 and 4.10 show Fe speciation in the batch reactors at the end of Stage 3; the soluble Fe concentration was extremely low (< 0.01 mg/L) in all cases, irrespective of its initial concentration. About 70−80% of the Fe^{2+} added precipitated as sulfides, leaving a lower proportion of the Fe bound to organics and carbonates in the Fe^{2+} supplemented reactors. The initial sludge contained 1.68 $mgFe^{2+}$/gTSS, in which 43% of the Fe was available as sulfides. Callander and Barford (1983a) showed that Fe and other metals including Ni and Co can also precipitate as sulfides as long as the total concentration of such metals does not exceed the total sulfide (H_2S + HS^- + S^{2-}) level; otherwise, such metals can be precipitated as carbonates (not for Ni). Espinosa et al. (1995) reported that about 80% of the added metals were retained by the sludge in a laboratory UASB treating molasses stillage, and Fe and Ni were mainly precipitated as carbonates and sulfides.
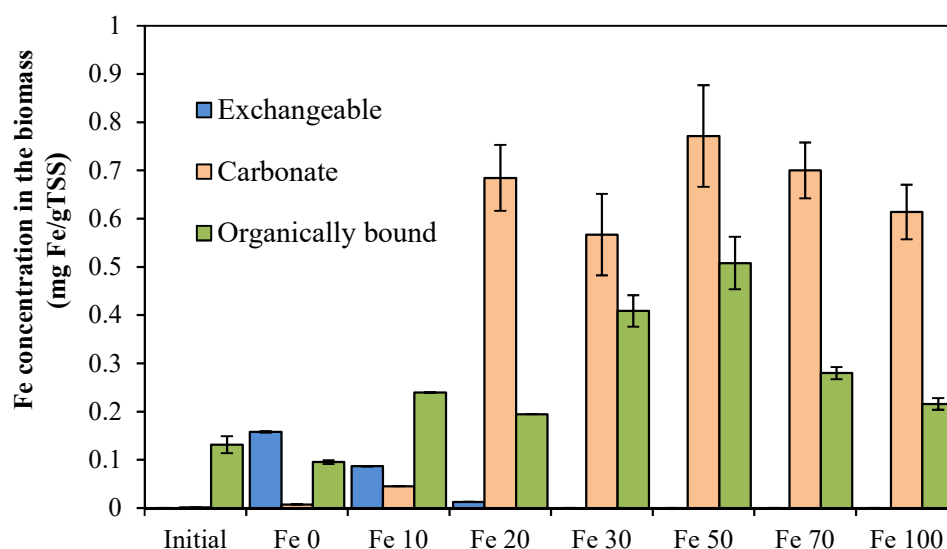
Figure 4.9   Speciation of Fe into "relatively bioavailable forms'' (Stage 3) at the end of the batch run.
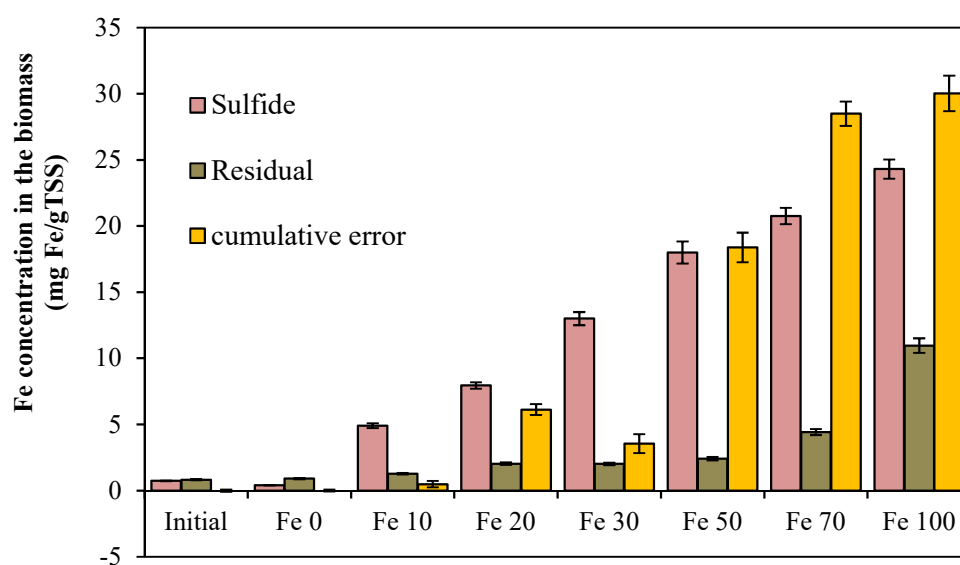
Figure 4.10   Speciation of Fe in its "relatively non-bioavailable forms'' (Stage 3) at the end of the batch run.

Another interesting observation from Stage 3 was the easily exchangeable Fe fraction available in Fe 0 mg/L, 10 mg/L and 20 mg/L which is considered

to be highly mobile/bioavailable compared to other forms. While the easily exchangeable fraction accounted for almost 23% of the total available Fe with Fe 0 mg/L, it only represented 0.15% of total Fe with 20 mg/L. Considering the speciation of Fe in the initial sludge and Fe 0 mg/L, it is probable that the Fe available in the form of organically bound fraction can be converted to easily exchangeable fractions under Fe limited conditions due to the chemical equilibria, while increased Fe^{2+} supplementation does not mean that more Fe will be in an easily exchangeable form.

The level of dissolved sulfide is also important in anaerobic reactors as sulphate entering can be reduced to sulfide by sulphate reducing bacteria (SRB). While SRB may compete with methanogens for available substrates such as acetate/propionate, sulfide can be toxic to anaerobic microorganisms at elevated concentrations (Chen et al., 2008): however, dissolved sulfide concentrations in our study only ranged between 3 – 4 mg/L, which was far below the toxicity level. The addition of metals such as Fe, Co, and Ni could lead to the precipitation of metal sulfides. However, in the present study higher methane production with 10 mg/L Fe compared to 0 mg/L Fe suggests that this improvement was due to Fe^{2+} supplementation rather than the reduction in sulfide levels.

It can be seen from Figures 4.9 and 4.10 that the Fe toxicity in the cases of 50, 70 and 100 mg/L Fe^{2+} supplementation is attributable to the availability of Fe in sulfide and residual forms. Thus, it can be assumed that Fe available in the sulfide/residual forms can also be bioavailable to microorganisms to cause inhibition, although the results do not clearly distinguish between the role of the residual and sulfide fractions. Notably, a high proportion of the residual fraction, and cumulative errors (indicating the discrepancy between the total Fe^{2+} input and the Fe extracted using the sequential extraction method) in the mass balance of Fe at higher Fe^{2+} dosages (> 50 mg/L) indicates the complexity of sequential extraction at high Fe^{2+} dosages. In fact, a lower level of soluble Fe, and discrepancies in mass balances at higher Fe^{2+} loading in Stage 3 studies

suggested that Fe could have been lost during the filtration steps as Fe can bind to colloidal particles which are produced during organic shock loading (Aquino & Stuckey, 2007).

The speciation of metal nutrients in anaerobic reactors is complex and often exhibits non ideal behavior, thus theoretical predictions of equilibrium concentrations often fail to correlate well with actual measurements (Ortner et al., 2014). Callander and Barford (1983a) found that the theoretical estimation of soluble Fe was 100 fold lower than the one measured in the reactor. One possible explanation for this was that Fe could be partially bound to complexing agents such as siderophores which are excreted by microbes to enhance the solubility and uptake of Fe ions; thus ionic equilibria is not reflected by the real situation in reactors (Ortner et al., 2014).

4.3.4 Stage 4: The effects of Fe^{2+} dose on the degradation kinetics of acetate, propionate and butyrate

In Stage 4, the effects of Fe^{2+} deficiency were tested against different VFAs such as acetate, propionate and butyrate to obtain more insight into the effects of Fe^{2+} limitations on different trophic groups in AD. Hence, batch reactors were subjected to two consecutive organic over loads (equivalent to final concentrations of 1 and 3 gCOD/L) within a 100 hour interval, and Fe^{2+} equivalent to 10 mg/L (equivalent to 3.34 $mgFe^{2+}/gTSS$) was spiked simultaneously with 1 gCOD/L. The rate of methane production was then observed in both cases (Figures 4.8) for different substrates such as acetate, propionate and butyrate. It can be seen that a longer lag period was observed in the case of methane evolution from butyrate degradation, although the average rate of methane production from acetate and butyrate at the end of 60 h was not statistically significant (30.3±0.62 and 29±1.48 $mLCH_4/gVSS.d$, respectively) in Fe-sufficient conditions. Theoretically, one mole of butyrate is converted to two moles of acetate and hydrogen, and the accumulation of end products such as acetate and H_2 could thermodynamically inhibit butyrate degradation (de

Bok et al., 2004). These factors could have presumably delayed methane production from butyrate. In terms of Fe limitation, the stimulatory effect of Fe^{2+} was only statistically significant in the case of acetate indicate that the acetoclastic methanogens were more constrained by Fe limitations compared to the other trophic groups.

Figure 4.11 and 4.12 shows the average rate of methane production from acetate, propionate and butyrate with 3 gCOD/L. While Fe^{2+} supplementation in the acetate loaded batch reactor resulted in a 2.1–3.9 fold increase in the rate of methane production, the rate of methane production from propionate only increased by 1.2–1.5 fold, compared to the non-Fe supplemented reactors, within the first 60 h. However, the stimulatory effect of Fe^{2+} supplementation on butyrate to methane conversion was not statistically significant (108±1.9 mLCH$_4$/gVSS.d without Fe; 104±4 mLCH$_4$/gVSS.d with Fe) at the end of 30 h. This could be due to thermodynamic considerations as butyrate is converted to acetate, and this may be rate limiting the overall conversion of butyrate to methane. Osuna et al. (2003) also reported that the continuous addition of metals (Co, Fe, Ni, Mn, Mo and Se) to the influent stimulated the degradation of acetate and propionate compared to the sludge grown under nutrient deprived conditions. However, metal nutrient limitations did not seem to stimulate the conversion of butyrate to methane in that case. It can be seen from Figure 4.11 and 4.12 that the rate of methane production from propionate and butyrate improved significantly in the case of 3 gCOD/L, compared to 1 gCOD/L, with or without Fe^{2+} addition. In previous work, successive additions of propionate, butyrate and acetate were tested in an anaerobic reactor to evaluate its methanogenic activity, and it was observed that the degradation time of all the VFAs in the second feeding was shorter than in the first feeding (Öztürk, 1991). Hence, adaptation of specific substrate-degrading bacteria/archaea could have possibly shortened the degradation time of VFAs. Ultimately, the population of archaea present in the test culture determines the kinetics of methane

production, and specific methanogenic activity tests (SMA) give a reasonable
measure of this.
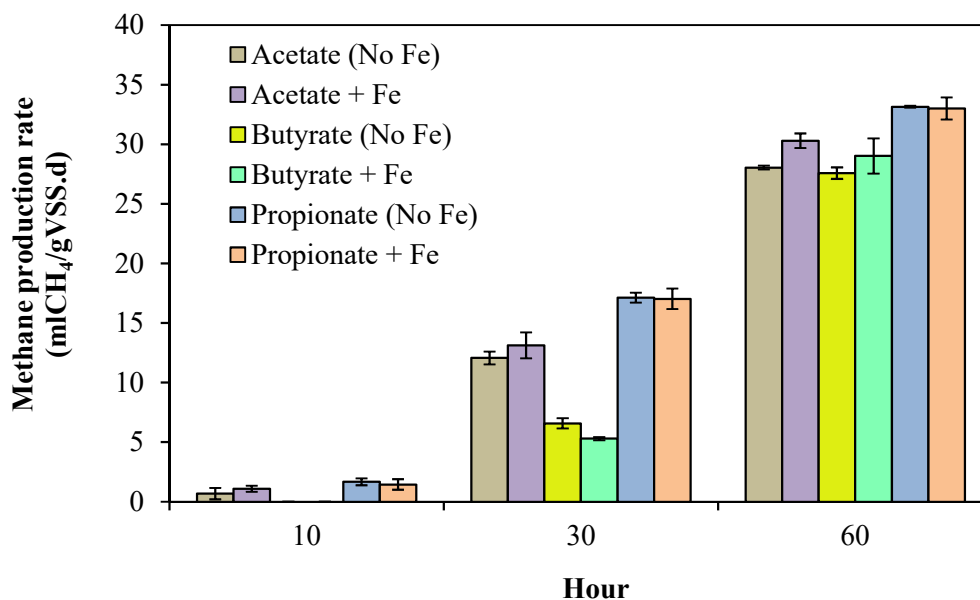
Figure 4.11   Effects of Fe^{2+} on the rate of methane production from different
substrates at 1 gCOD/L (Stage 4).
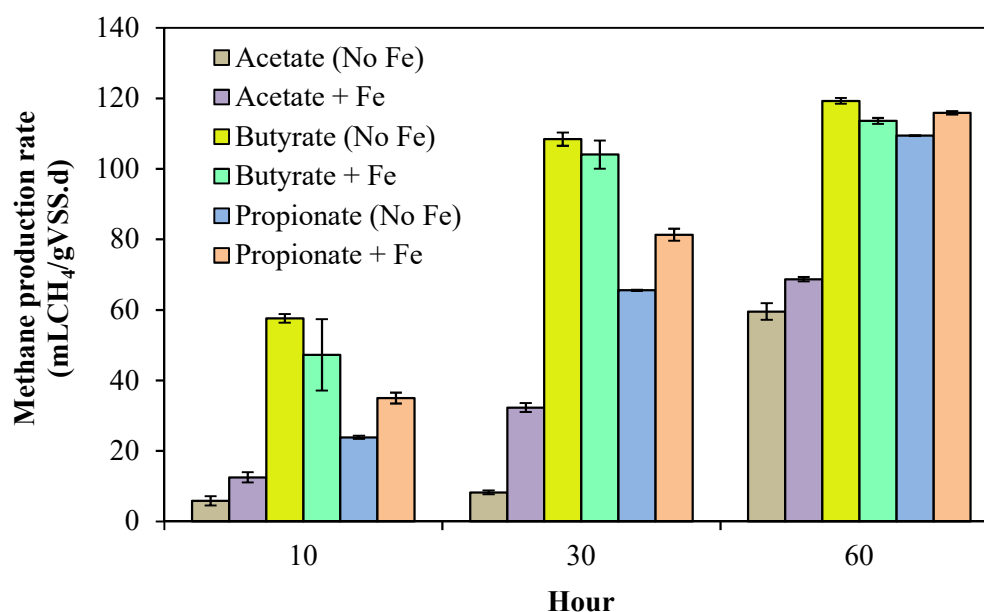
Figure 4.12   Effects of Fe^{2+} on the rate of methane production from different
substrates at 3 gCOD/L (Stage 4).

96

It has been documented that the majority of anaerobic propionate-oxidizing bacteria use the methyl-malonyl-CoA pathway, yielding acetate, CO_2 and H_2 (de Bok et al., 2004). Therefore, propionate to methane conversion involves the activity of propionate oxidizing bacteria as well as the hydrogenotrophic and acetoclastic methanogens. However, acetate can be directly converted into methane by acetoclastic methanogens; therefore, it could be postulated that acetoclastic methanogens were more constrained by Fe limitations compared to the other trophic groups. In a previous study, Schmidt et al. (2014) reported that Fe deficiency affected the activities of both methanogenic archaea and propionate oxidizing bacteria in an anaerobic system.

Figure 4.13 shows the various physical/chemical forms of Fe in the acetate and propionate reactors at the end of the experiment, and suggests that the additional Fe was mainly precipitated either as sulfides, or bound to organics. Notably, only a lower proportion of Fe (< 0.01 mgFe/gTSS) was precipitated as carbonate/phosphate bound-Fe (EDTA fraction). Hence, it could be postulated that the Fe in sulfide and organically bound forms can also be bioavailable to methanogens, although there has been considerable disagreement about the bioavailability of metal sulfides in AD. Zehnder (1983) suggested that the availability of Fe to methanogens could be restricted when sulfide was present. In contrast, Gustavsson et al. (2013b) reported that the precipitation of Co and Ni as sulfides did not inhibit the microbial uptake of Co and Ni. Our Stage 4 studies also revealed that the soluble Fe concentration in the reactor, as well as the Fe in easily exchangeable and adsorbed forms in the sludge, were almost zero at the end of the batch test. Gustavsson et al. (2013b) suggested that the soluble/bioavailable intermediates are labile and short-lived, and therefore difficult to detect.
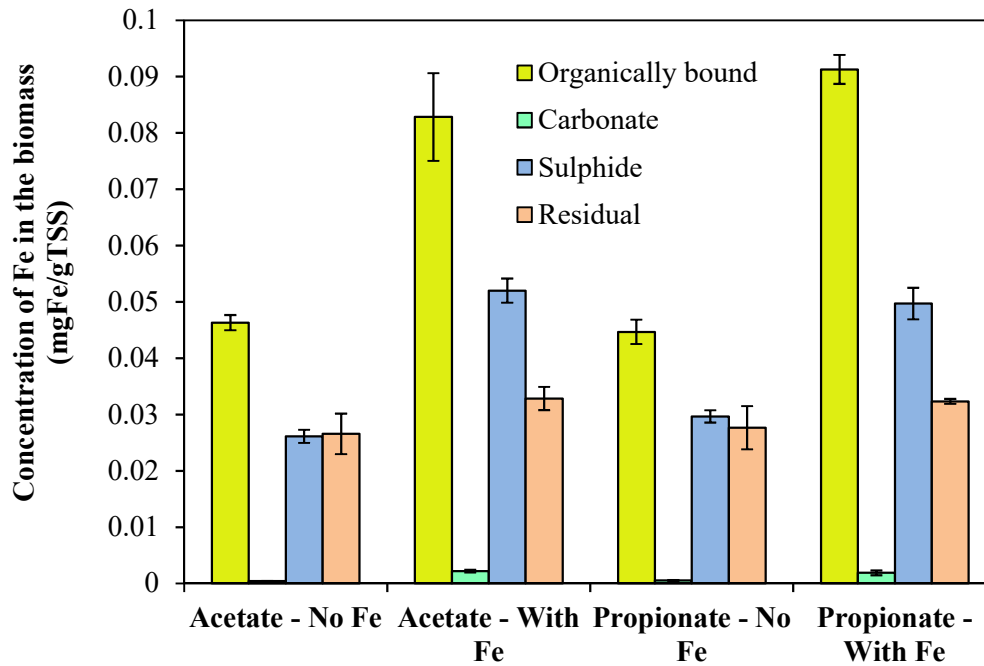
Figure 4.13   The distribution characteristics of Fe in batch reactors (Stage 4).

4.3.5   Stage 5: The effects of Fe supplementation in a SAMBR

The objective of Stage 5 was to demonstrate the effects of Fe^{2+} supplementation in a continuously fed SAMBR during organic shock load conditions. Table 4.1 shows the steady state performance of the SAMBR at different HRTs; this was initially set at 48 h for two weeks, and then reduced to 8 h step-wise over two months. The reactor was stable (low VFAs) at each HRT before it was reduced to ensure a smooth transition from 48 h HRT to 8 h HRT; thus VFA accumulation was almost negligible in all cases, and the pH ranged between 6.9 to 7.1. Table 4.2 shows the speciation of Fe in the SAMBR at steady state (8 h HRT) and a solid retention time (SRT) of 150 d; the bioavailability of Fe in the soluble form reflects the continuous feeding of Fe^{2+} in the influent, and Fe was still present in both sulfide and residual forms in the biomass. Prior to any organic shock, the reactor was maintained at steady state conditions (8 h HRT and 500 mg/L feed strength). Considering the metal-retaining ability of anaerobic sludge, the SAMBR response to organic shocks

was first tested without Fe supplementation, followed by tests with supplementation. With supplementation, Fe^{2+} was spiked immediately after the organic shock and the effluent was stopped for 5 min to prevent the immediate loss of soluble Fe in the effluent. Tables 4.3 and 4.4 show the SAMBR responses during glucose shocks of 1 g and 2 gCOD, with and without Fe^{2+} supplementation.

Table 4.1   Steady state performance of the SAMBR at different HRTs.

HRT (h)	OLR (gCOD/ L.d)	COD removal (%)	VFA (mg/L)
48	0.25	94	-
36	0.33	94	-
24	0.5	96	-
12	1.0	96	2-3
10	1.2	97	2-3
8	1.5	97	2-3

Table 4.2   Speciation of Fe in anaerobic sludge in the SAMBR.

Form	Concentration (mgFe/gTSS)
Soluble (reactor)	0.55±0.05
Exchangeable	0
Adsorbed	0
Carbonate	0.06±0.02
Sulfide	0.47±0.05
Organically bound	0.03±0.02
Residual	0.35±0.01

It can be seen that acetic and propionic acids mainly accumulated in all cases (Table 4.3 and 4.4), with a concomitant drop in pH. A rapid reduction in VFAs, and maintaining the pH within favourable limits are essential strategies to recover reactor performance. As shown in Tables 4.3 and 4.4, the effects of Fe^{2+} supplementation were statistically significant (COV for VFA measurements was $\pm 2\%$) in the case of 1 gCOD compared to 2 gCOD for the rapid removal of VFAs. In the first case, almost 50% of the acetate was reduced by Fe^{2+} supplementation in the first hour, and completely removed within two hours. However, a slower response was observed in the case of propionic acid, and only 60% of the accumulated propionic acid was eliminated by Fe^{2+} supplementation in 2 h. In anaerobic environments propionate is oxidized to acetate, bicarbonate and H_2/formate, and its degradation by acetogenic bacteria is thermodynamically unfavourable ($\Delta G^{\circ} > 0$), and only becomes possible when these products are removed by methanogens (de Bok et al., 2004). In a previous study, Espinosa et al. (1995) reported that the addition of Fe, Ni, Co and Mo to the influent reduced the level of propionic acid from 5,210 mg/L to 626 mg/L in a laboratory scale UASB reactor fed with cane molasses stillage, but this took 12 d.

Table 4.3   Responses of the SAMBR to a glucose spike of 1 gCOD.

Case	glucose spike (as gCOD)	Fe dosage (as mg/L in SAMBR)	Time (h)	VFA (mg/L)			pH
				Acetate	Propionate	Others	
Case 1	1g	0	0	0	0	0	7.02
			1	36.9	46.9	3.56	6.7
			2	24.3	23.2	13.9	6.83
Case 2	1g	10	0	0	0	0	7.01
			1	19.1	48.2	11.5	6.86
			2	0	9.6	5.50	6.85

Table 4.4   Responses of the SAMBR to a glucose spike of 2 gCOD.

Case	glucose spike (as gCOD)	Fe dosage (as mg/L in SAMBR)	Time (h)	VFA (mg/L)				pH
				Acetate	Propionate	Butyrate	others	
Case 1	2g	0	0	0	0	0		7.18
			0.5	134	134	5.8		6.48
			1	62	112	4.4		6.66
Case 2	2g	10	0	0	0	0		6.9
			0.5	105	121	7.4		6.62
			1	60.2	120.4	4		6.64

When the SAMBR was subjected to a spike of 2 gCOD, reductions in acetic acid (from 134 mg/L to 105 mg/L) and propionic acids (from 134 mg/L to 121 mg/L) were observed in the first 30 min after Fe^{2+} supplementation, however, no statistically significant improvements were observed after 1 hr. These results showed that propionate accumulation was significant compared to acetate in the case of 2 gCOD/L. Notably, the pH dropped to 6.48 and 6.62 in both Fe-deficient and Fe^{2+} supplemented conditions (in 30 min), respectively, although the 30 µm flocs in the SAMBR would ameliorate this effect through mass transfer considerations. However, it is possible that the limiting effects of Fe on the degradation of VFAs could have been overwhelmed by an unfavourable pH drop.

Understanding metal dynamics and speciation is more complicated in continuous bioreactors compared with batch reactors. An "optimum" dose of metals should be continuously fed to the reactor to ensure maximum substrate conversion, and to prevent disturbances in reactor performance, however, continuously supplying metal nutrients can lead to an accumulation of metals by the biomass. Long SRTs could result in a substantial accumulation of metal

salts, mainly as sulfides and residuals (Stage 5), on the other hand, the metal content of the sludge will decrease due to leaching if the supply of metals to the reactor is stopped. Zandvoort et al. (2006a) demonstrated that metal loss from the biomass (especially Fe, Co and Ni) was proportional to their initial content in the sludge when their supply was halted. They suggested that part of the observed decline could be attributed to a "dilution effect" as a result of bacterial growth which has to be supported by metals available in the inoculum sludge. Another study by Zandvoort et al. (2004) showed that both preloading (supplementation of cobalt to sludge prior to feeding), and in situ (supplementation of cobalt to feed) loading was effective in achieving increased reactor performance of a methanol fed UASB reactor operating under cobalt limitations. However, they found that in situ dosing needed substantially lower amounts of cobalt, while resulting in significantly smaller losses of cobalt in the effluent. Thus understanding the dynamics of Fe in anaerobic sludge and the timely addition of Fe^{2+} during organic shock loads could enable quicker recovery of reactor performance through more rapid VFA removal.

4.4   Conclusions

With seed sludge grown under Fe-sufficient conditions, an organic shock resulted in leaching of Fe from the residual to organically bound and soluble forms. With Fe-deficient seed sludge, addition of Fe^{2+} to an acetate loaded batch reactor resulted in a 2.1–3.9-fold increase in the rate of methane production, while from propionate methane increased 1.2–1.5 fold compared to the non-Fe supplemented reactors. Accumulation of Fe in the seed sludge should be accounted for by supplementing Fe^{2+} during organic shock loads.

Chapter 5

Effect of Ethylenediamine-N,N'-disuccinic acid (EDDS) on the speciation and bioavailability of Fe^{2+} in the presence of sulfide

Outline:

5.1 Introduction

5.2 Experimental and analytical methods

5.3 Results and discussion

5.4 Conclusions

Note: this chapter was published in *Bioresource Technology*, 2017, volume 229, pages 169-179.

5.1   Introduction

As discussed in the literature review, precipitation of the metal sulfide plays a very important role in the fate of TMs in anaerobic digestion as it can limit TM bioavailability for microbial uptake, and hence increase the required dosage of TMs.

To prevent the precipitation of TMs with sulfide in anaerobic bioreactors it is possible to add chelating agents, which will complex with the TMs and keep them in a soluble form. It has been shown that the TM bioavailability in anaerobic digestion can be improved by adding natural chelating agents e.g. soluble microbial products (SMPs) (Aquino & Stuckey, 2007) or synthetic chelating agents e.g. Ethylene diaminetetraacetic acid (EDTA) and

Nitrilotriacetic acid (NTA) (Fermoso et al., 2008c; Hu et al., 2008a; Bartacek et al., 2012; Vintiloiu et al., 2013). Although EDTA and NTA have higher complexing capabilities with TMs than SMPs, these materials are poorly biodegradable and could pose a threat to the environment when ultimately discharged in the effluent (Tandy et al., 2004). Alternatively, readily biodegradable chelating agents such as Ethylenediamine-N,N'-disuccinic acid (EDDS), Imino disuccinic acid (IDS), and Glutamic acid diacetic acid (GLDA) can be used; however, there is limited research looking at the viability of using such chelating agents in anaerobic systems.

Due to its biodegradability, EDDS has been an alternative for traditional chelating agents in several commercial products e.g. industrial detergents. In environmental applications, EDDS has been used for induced phytoextraction and soil washing to remove heavy metals such as Pb, Zn, Cu, and Cd (Kos & Leštan, 2003; Zhao et al., 2010; Satyro et al., 2014; Ferraro et al., 2015; Beiyuan et al., 2016). However, to date there is only one study that has demonstrated the use of EDDS to increase metal bioavailability in anaerobic digestion (Zhang et al., 2015b). In this study, the optimum dose of EDDS was not justified, and the effect of EDDS addition was only determined on a combination of TMs i.e. Fe, Co, and Ni, rather than on any individual TM.

Therefore, the objective of this study was to evaluate the effect of sulfide on the bioavailability of Fe^{2+}, and to determine how supplying the biodegradable chelating agent EDDS could improve Fe^{2+} bioavailability, and hence increase the methane yields in anaerobic digestion. The effect of EDDS on Fe^{2+} bioavailability in the presence of sulfide was first investigated in a model media, and then extended to an actual anaerobic digestion process. In order to give deeper insights into the relationship between metal speciation and metal complexation, the applicability of the Community Bureau of Reference (BCR) extraction method was systematically assessed in dried/wet sludge conditions, and a new UHPLC method was developed for the determination of free-EDDS and Fe-EDDS complex.

5.2   Experimental and analytical methods

5.2.1   Metal extraction of dried and wet samples with the BCR extraction method

This experiment was aimed at comparing the speciation and recovery of metals from dried and wet anaerobic sludge samples with the BCR method. For dried samples, the sludge was centrifuged at 4,000 rpm for 15 min and then the pellets were dried in aluminium pans at 105°C overnight. Dried pellets were then ground in a porcelain mortar to reduce particle size, and then stored in a desiccator at room temperature. During the extraction step, 0.6 g of dried sample was placed into a 50 mL centrifuge tube. For wet samples, a volume of anaerobic sludge equivalent to 0.5 gTSS was centrifuged at 4,000 rpm for 15 min in a 50 mL centrifuge tube. The sequential extraction for both dried and wet sludge was carried out in quadruplicate, and the concentration of metals from each extraction step was measured using MP-AES.

5.2.2   Effects of metal nutrient supplementation on anaerobic digestion

This experiment was aimed at comparing the effects of selected metal nutrients on methane production with anaerobic sludge. The "Control" set did not have substrate added or metal nutrients, while the "No Metal" set contained only substrate, but the test reactors were supplemented with a group of metal nutrients that were characterized by similar chemical properties, i.e. set 1: Fe + Co + Ni (transitional metals), set 2: Cu + Mn + Zn (other TMs), and set 3: Ca + Mg (macronutrients). The substrate was glucose, the F/M ratio was fixed at 1:1 (2 gCOD as glucose to 2 gVSS), and NaHCO$_3$ was added as a buffer.

The experiment was carried out in a batch automatic methane potential test system (AMPTS II, Bioprocess Control AB, Sweden) in triplicate. The tests were performed in standard 500 mL bottles, and the working volume of each test reactor was 400 mL. The reactors were flushed with nitrogen gas passing through tubing to ensure anaerobic conditions prior to start-up, and the

temperature was controlled at 35°C. Stirrers in all the reactors were set at 60 s on and 30 s off at 46 rpm during the whole experiment. Metal speciation was assessed using the BCR method with wet samples.

The overall batch tests were carried out in phases; during Phase 1 the metal nutrients were added at the concentrations in the nutrient recipe (Table 5.1) used by Hu (2004) and Souto et al. (2010). During Phases 2 and 3, the same dose of substrate was injected again into the test reactors, while the metal concentrations were doubled and trebled from the initial concentrations, respectively, in order to magnify the effects of metal supplementation on methane production.

Table 5.1   Metal concentrations supplemented to test reactors.

		Reactor concentration (mg/L)		
		Phase 1	Phase 2	Phase 3
Common	KH_2PO_4	390	NA	NA
	K_2HPO_4	90	NA	NA
	NH_4Cl	300	NA	NA
	$Na_2S.9H_2O$	30	NA	NA
Set 1	$FeCl_2.4H_2O$	1.2	2.4	3.6
	$NiCl_2.6H_2O$	0.03	0.06	0.09
	$CoCl_2.6H_2O$	1.2	2.4	3.6
Set 2	$ZnCl_2$	0.06	0.12	0.18
	$CuCl_2.2H_2O$	0.018	0.036	0.054
	$MnCl_2.4H_2O$	0.3	0.6	0.9
Set 3	$CaCl.2H_2O$	60	120	180
	$MgCl_2.6H_2O$	60	120	180

NA – Not Added

The supplemented concentrations are for the whole compounds

5.2.3 Determination of optimal concentration for Fe^{2+} in anaerobic digestion

The aim of this study was to evaluate the speciation and bioavailability of Fe^{2+} in anaerobic digestion. The experiment was performed in batch reactors with 60 mL serum bottles (45 mL working volume). The initial biomass was 3g TSS/L in all test reactors, and different Fe^{2+} concentrations were evaluated i.e. 5, 10, 20, 30, and 50 mg/L as $FeCl_2.4H_2O$. Glucose (1.5 gCOD/L) and $NaHCO_3$ (2 g/L) were added as substrate and buffer, respectively. The pH of all test bottles was adjusted to 7.0 ±0.1 using diluted NaOH or HCl, and the bottles were then incubated in a shaker at 35ºC and 170 rpm.

5.2.4 Biodegradation of EDDS in anaerobic digestion

Since EDDS is a readily biodegradable chelating agent, it can be a source of carbon for anaerobic microorganisms, and hence can contribute to methane production. Therefore, in order to determine if the methane production from EDDS biodegradation was significant or not, a batch experiment was carried out in 60 mL serum bottles (45 mL working volume). A range of EDDS concentrations were tested, i.e. 5.24, 13.09, and 52.36 mg/L, which were equivalent to the molar ratio to Fe^{2+} optimal concentration, i.e. 0.1:1, 0.25:1, and 1:1. Glucose (1.5 gCOD/L) and $NaHCO_3$ (2 g/L) were added to all bottles as substrate and buffer, respectively, while EDDS was not added to the "Control" set.

5.2.5 Effect of EDDS on Fe^{2+} concentration in the presence of sulfide in model media

The aim of this study was to evaluate the effect of EDDS on Fe^{2+} in the presence of sulfide in a model media. The model media was prepared by centrifuging anaerobic sludge from a seed reactor and then filtering the supernatant through a 0.45 μm filter. The experiment was carried out in 125 mL serum bottles with a 90 mL working volume. Fe^{2+} was added at the optimal

concentration of 10 mg/L (3.34 $mgFe^{2+}$/gTSS), and EDDS was pre-mixed with Fe^{2+} at different molar ratios i.e. 0.1:1, 0.25:1, 0.5:1, 0.75:1, and 1:1 overnight in the dark to prevent photo-degradation. Sulfide was added to all the test reactors at 8 mg/L as $Na_2S.9H_2O$, which is below its toxicity level (Mountfort & Asher, 1979), and acetate/acetic acid solution was added to provide buffering capacity. The pH of all test bottles was then adjusted to 7.0 ±0.1 at the start of the test using diluted HCl, and the bottles were flushed with nitrogen for 2 minutes to ensure anaerobic conditions. Sampling was carried out immediately at the beginning, and six times within 40 h.

5.2.6 Effect of EDDS on Fe^{2+} bioavailability in the presence of sulfide in anaerobic digestion

The experiment was performed in a batch setup with 60 mL serum bottles (45 mL working volume). The experimental conditions of this experiment were the same as for the experiments in Section 2.8.4 i.e. 10 mg/L Fe^{2+} (3.34 $mgFe^{2+}$/gTSS); EDDS:Fe molar ratios 0.1:1, 0.25:1, 0.5:1, 0.75:1, and 1:1; 1.5 gCOD/L glucose; 2 g/L $NaHCO_3$, except anaerobic sludge was used instead of model media. Sulfide was added at 8 mg/L as $Na_2S.9H_2O$ into all bottles except the "No S" set. The bottles were flushed with nitrogen for 2 minutes to ensure anaerobic conditions, and BCR sequential chemical extraction was carried out at the end of the experiment.

5.3 Results and Discussion

5.3.1 Metal extraction of dried and wet samples with the BCR extraction method

Sequential extraction is one of the important techniques which has been used extensively to determine the speciation of metals in different environmental samples. However, most of the studies in the literature involved extraction using dried samples, which are normally ground and sieved to

achieve a homogeneous size. Rudd et al. (1988) and Ortner et al. (2014) compared the extraction of dried and wet samples using the Stover and Tessier methods, and found that dried samples not only required more preparation time, but also could cause an artificial shifting in metal speciation, e.g. the formation of metal oxides, although more reproducible results were obtained than with wet samples.

Figure 5.1 compares the extraction efficiencies of the most common metals present in anaerobic digesters, i.e. Fe, Ni, Co, Cu, Zn, Mn, Ca, and Mg for both dried and wet samples. The extraction efficiency (%) denotes the sum of the metal concentrations from all fractions over the pseudo-total concentration in µg/gTSS (Table 5.2). The results show that the BCR method exhibits good recovery for all the metals for both dried and wet sample i.e. 89.0% to 113.5%, with the exception of Co and Cu for the dried sample i.e. 71.0% and 40.5%, respectively. The findings from this experiment indicated that the extraction of metals using the BCR method with wet samples could not only produce comparable but also better extraction efficiencies than a dried sample. While the use of wet samples for metal extraction reduced the time required for sample preparation, it also eliminated the possibility of artificial altering the metal speciation. The later advantage is very important in the study of metal speciation and bioavailability as some of the TMs, particularly Fe^{2+}, seems to be very sensitive to oxidation during the drying process (Ortner et al., 2014), and hence could possibly overestimate the fraction of Fe-Mn oxides. Therefore, wet sample extraction was found to be more suitable for the study of metal speciation and bioavailability in anaerobic digestion, and was used throughout this study.
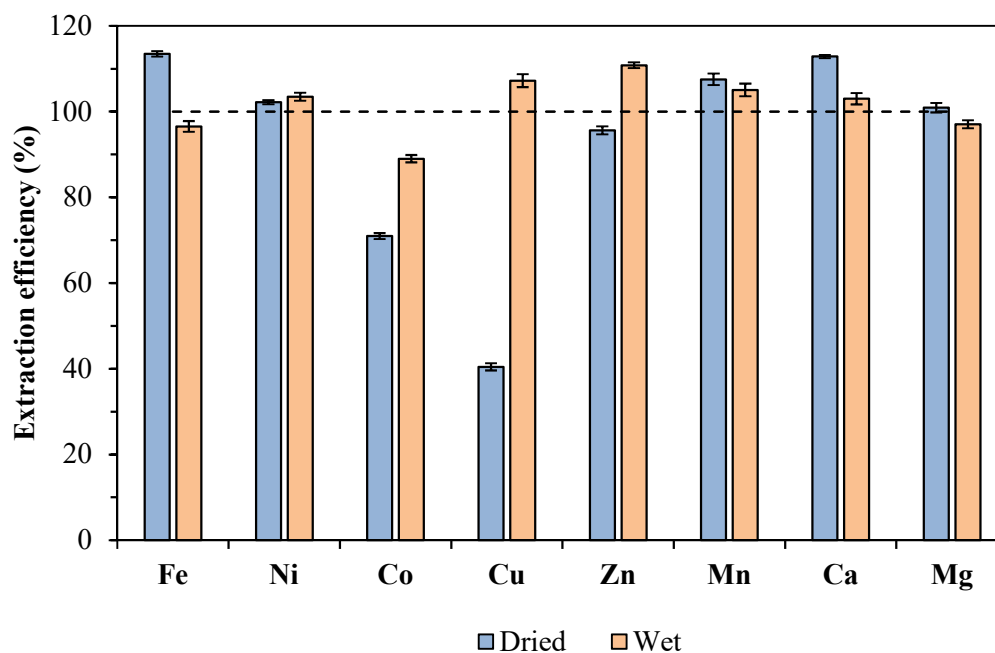
Figure 5.1   Extraction recovery as a percentage (%) for metals in dried and wet samples (n = 3).

Table 5.2   Extraction efficiency (%) for each metal (n = 3).

Metal	Extraction efficiency (%)	
	Dried sample	Liquid sample
Fe	113.5 ± 0.6	96.6 ± 1.2
Ni	102.2 ± 0.5	103.5 ± 0.9
Co	71.0 ± 0.7	89.0 ± 0.9
Cu	40.5 ± 0.8	107.2 ± 1.5
Zn	95.6 ± 0.9	110.8 ± 0.7
Mn	107.5 ± 1.3	105.1 ± 0.5
Ca	112.9 ± 0.4	103.0 ± 1.3
Mg	100.9 ± 1.1	97.1 ± 0.9

5.3.2 Effects of metal nutrient supplementation on methane production in anaerobic digestion

The methane production rate ($mLCH_4$/d) for each set was determined from the slope of the cumulative methane production graph from time zero to the plateau region. From Figure 5.2 it can be seen that the Ca + Mg addition set had the highest rate of methane production in all three phases i.e. 58.9 $mLCH_4$/d (Phase 1), 64.8 $mLCH_4$/d (Phase 2), and 59.2 $mLCH_4$/d (Phase 3) (Table 5.3) compared to other sets from each phase, with the P values less than 0.05. Thus, the results confirm the importance of macro-nutrients in anaerobic digestion (White & Stuckey, 2000; Merchant & Helmann, 2012; Romero-Güiza et al., 2016). The set with the second highest rate of methane production was the Fe + Ni + Co set i.e. 40.2, 40.6, and 41.7 $mLCH_4$/d, in phases 1, 2 and 3 respectively. These transitional metals are considered the most essential TMs in anaerobic digestion (Oleszkiewicz & Sharma, 1990). The methane production rate for the last metal set Cu + Zn + Mn was not statistically different to the No Metal set.

For the Ca + Mg set, the maximum methane production rate was observed in Phase 2 i.e. 64.8 $mLCH_4$/d when the concentrations were doubled i.e. 180 mg/L as $CaCl.2H_2O$ and $MgCl_2.6H_2O$. Accordingly, the time required to reach the maximum rate of methane production (Max Time) was also the shortest in Phase 2 i.e. 64 h (Table 5.3); this indicated that the optimal concentrations for Ca and Mg could be from 180 to 250 mg/L (Owen et al., 1979).

For the Fe + Ni + Co set, when their concentrations increased the methane production rate also increased (statistically significantly) from 40.2 $mLCH_4$/d to 41.7 $mLCH_4$/d. Accordingly, the maximum time (Max Time) to peak gas production decreased from 115 h to 96 h (Table 5.3). Therefore, the optimal concentrations for Fe, Ni, and Co might be even higher than the dosage in Phase 3 i.e. 3.6, 0.09, and 3.6 mg/L, respectively. For the Cu + Zn + Mn set, the trend was in contrast to the Fe + Ni + Co set as the methane production rate decreased from 38.3 $mLCH_4$/d to 32.6 $mLCH_4$/d when the dosage increased.

The Max Time increased from 121 h to 123 h (Table 5.3) which showed some inhibitory effects of Cu, Zn, or Mn above their optimal concentrations. The overall results confirm that the rate of methane production was co-limited by one/several metals, and that the systematic addition of limiting metals could improve the rate of methane production and yields.
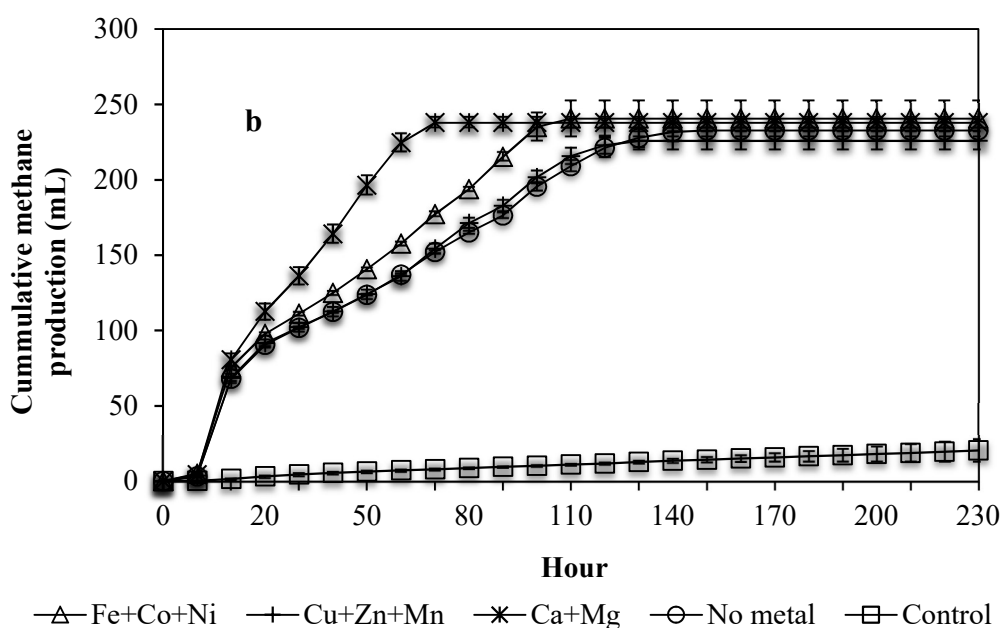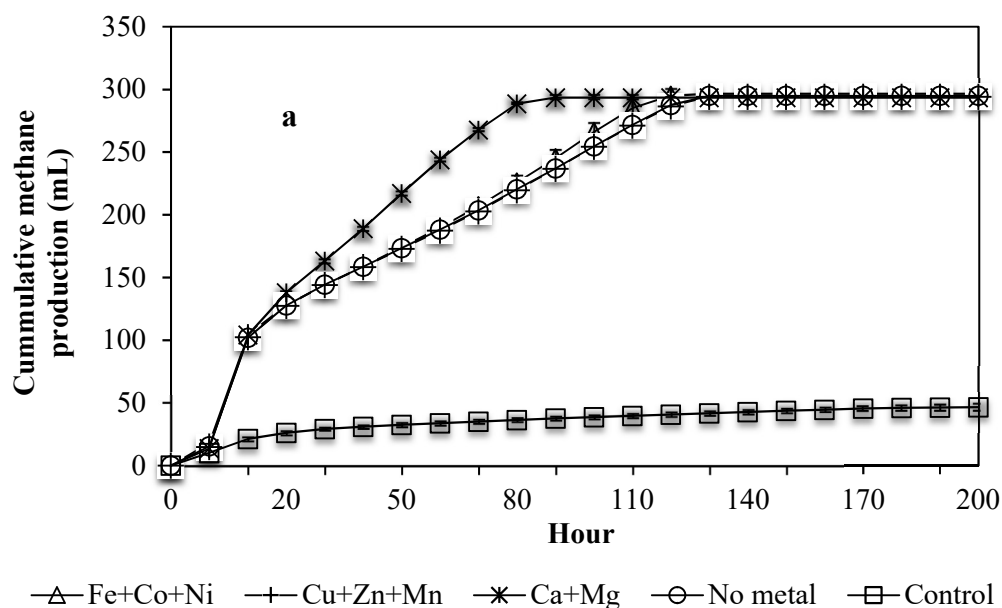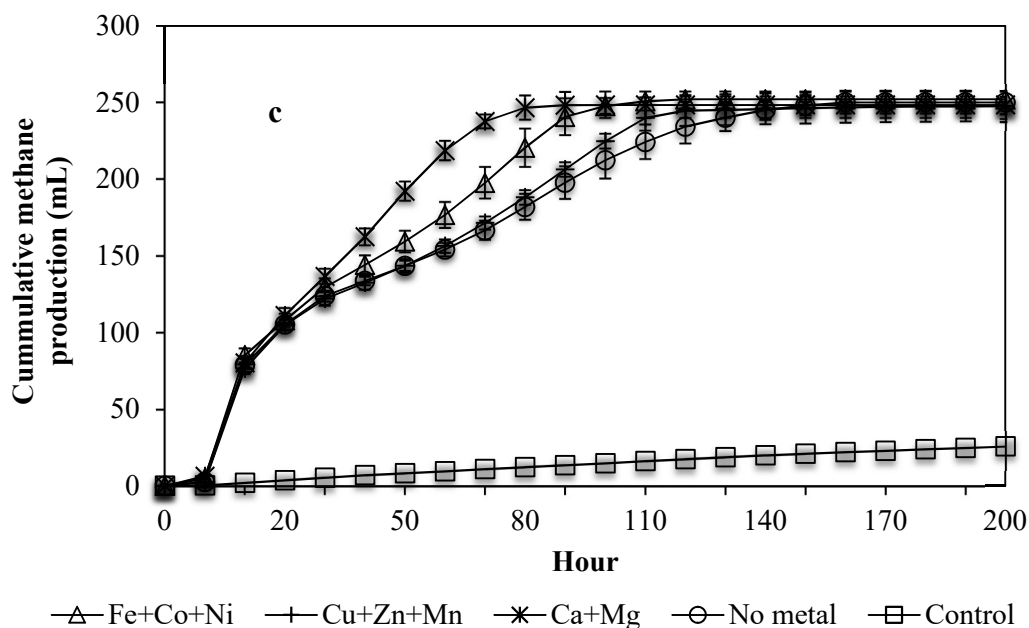
Figure 5.2   Cumulative methane production (mL) (a) Phase 1, (b) Phase 2,
(c) Phase 3 (n = 3).

Table 5.3   Methane production rate (mLCH₄/d) and time to reach maximum
methane production rate (Max Time h) (n = 3).

	Fe + Ni + Co		Cu + Zn + Mn		Ca + Mg		No Metal	
	$mLCH_4/$ d	Max Time (h)	$mLCH_4/$ d	Max Time (h)	$mLCH_4$ /d	Max Time (h)	$mLCH_4$ /d	Max Time (h)
Phase 1	40.2	115	38.3	121	58.9	83	38.4	122
Phase 2	40.6	104	32.7	123	64.8	64	31.0	134
Phase 3	41.7	96	32.6	123	59.2	75	27.7	143

At the end of Phase 3, chemical sequential extraction using the BCR method with wet samples was carried out to fractionate the metals remaining in each test bottle. Figure 5.3 (a-h) shows the fractionation, while Figure 5.4 compares the percentage of the sum of Soluble (Sol) and Exchangeable (F1) fractions to the total content for all the metals. The Soluble and Exchangeable fractions are considered the most bioavailable fractions for microbial uptake. It can be seen that the metal speciation in the most bioavailable fraction correlated well with the methane production rate in each of the metal groups. The Ca + Mg set had the highest percentage, i.e. 81.5% (Ca) and 81.9% (Mg), following by the Fe + Co + Ni set i.e. 12.6% (Fe), 45.8% (Co) and 49.6% (Ni), and Cu + Mn + Zn set i.e. 1.3% (Cu), 12.3% (Mn) and 58.1% (Zn). This similar trend between metal speciation and methane production rate showed that the availability of metals in the more bioavailable fractions led to a higher metal uptake, and hence a higher methane production rate.

(d) Cu

(e) Zn

(h) Mg

Sol - Soluble, F1 - Exchangeable, F2 - Fe/Mn oxides,
F3 - Organic matter, F4 - Residual
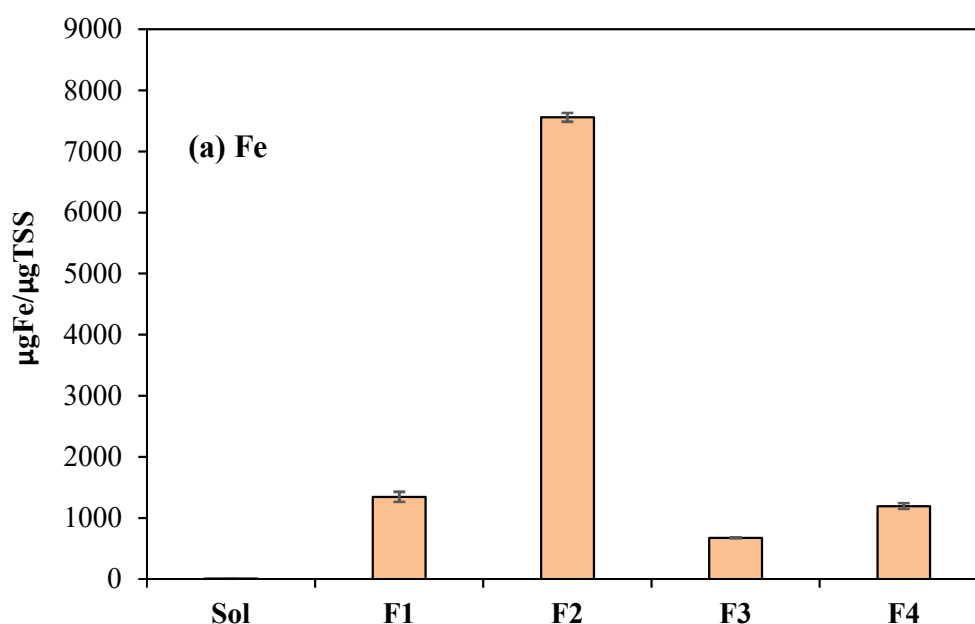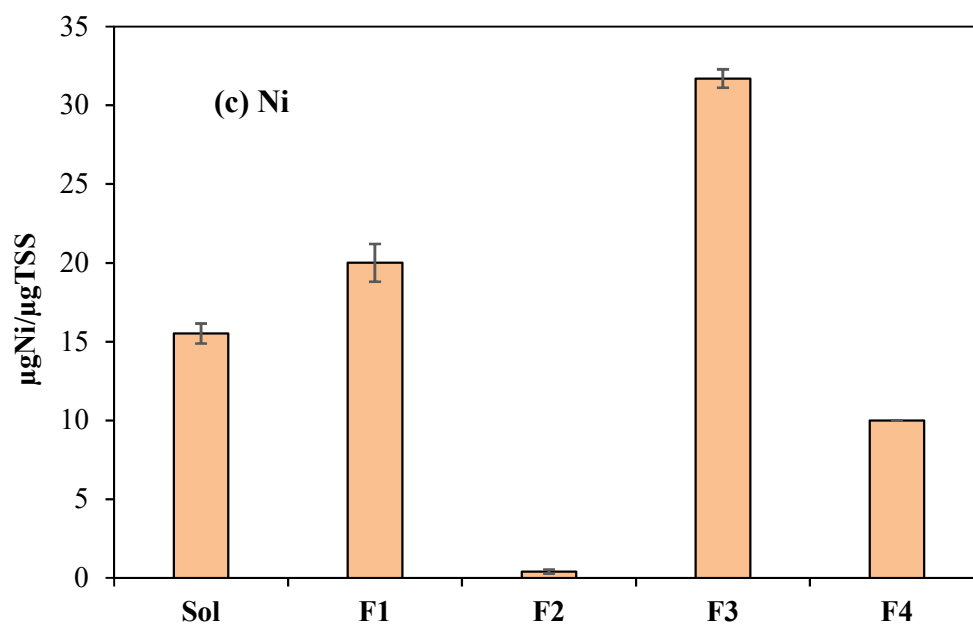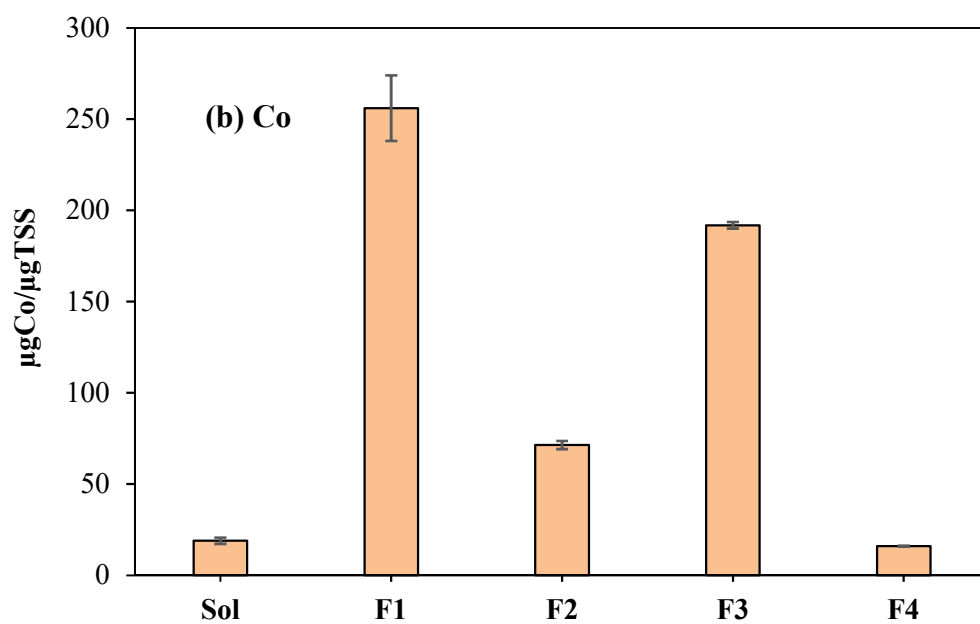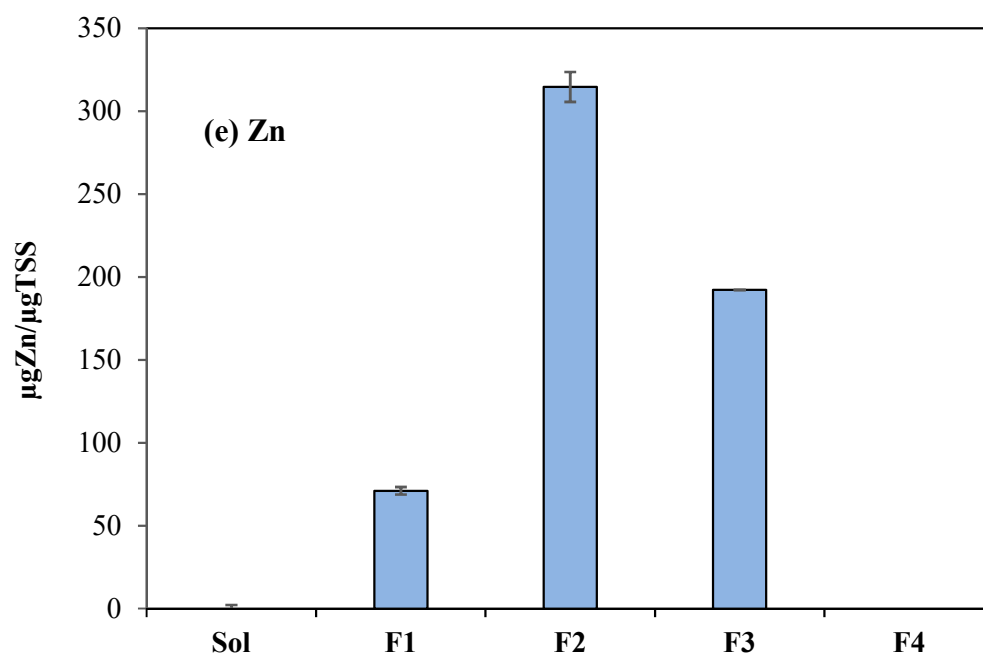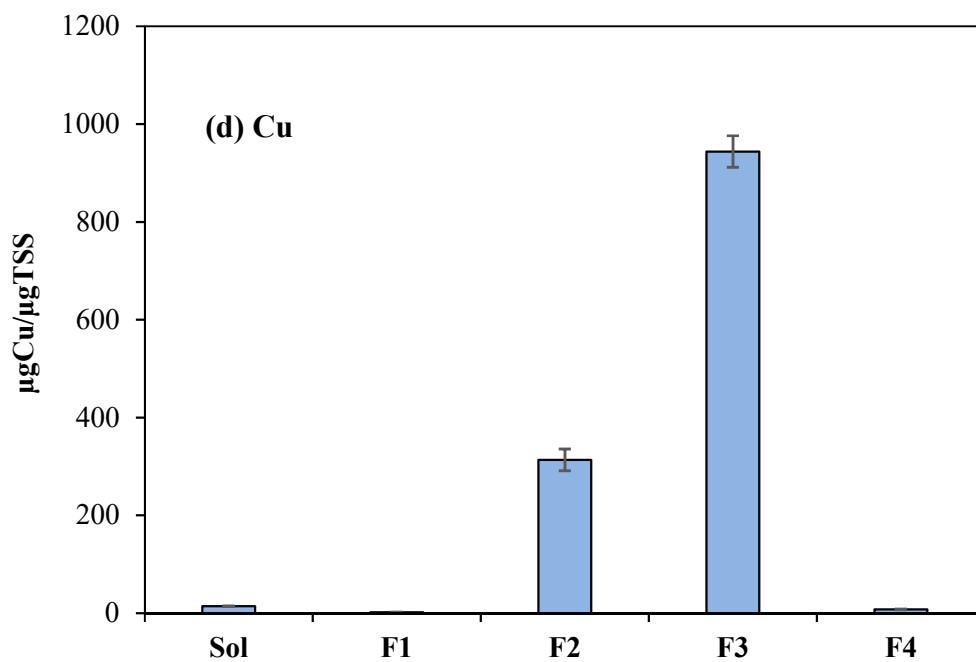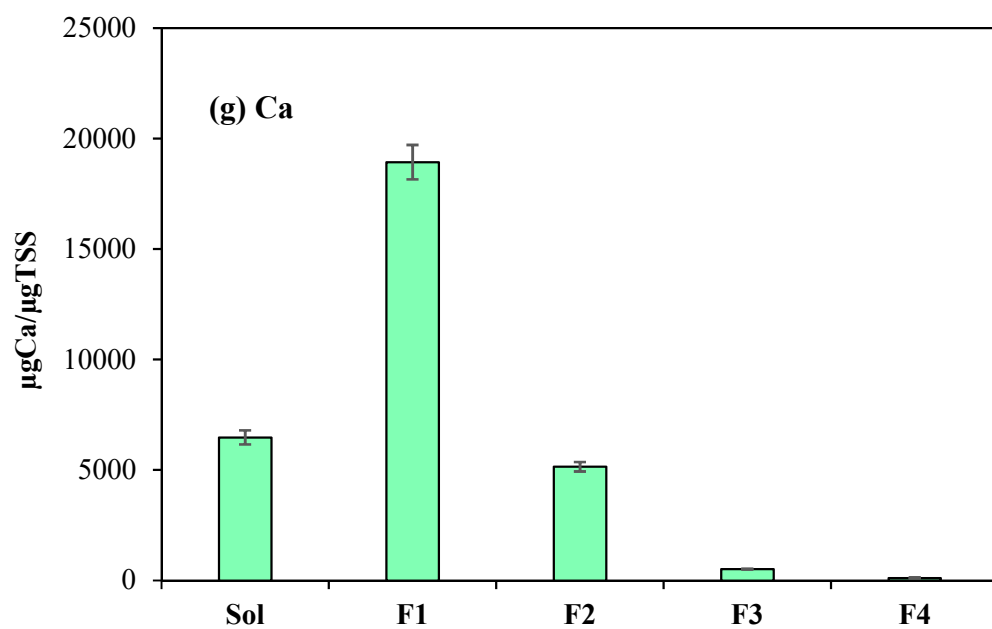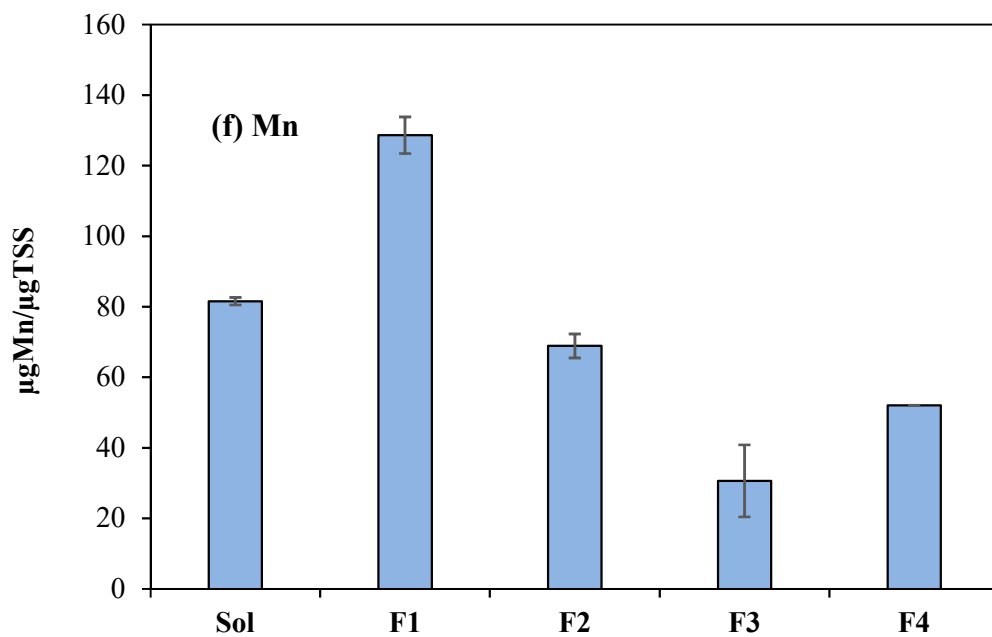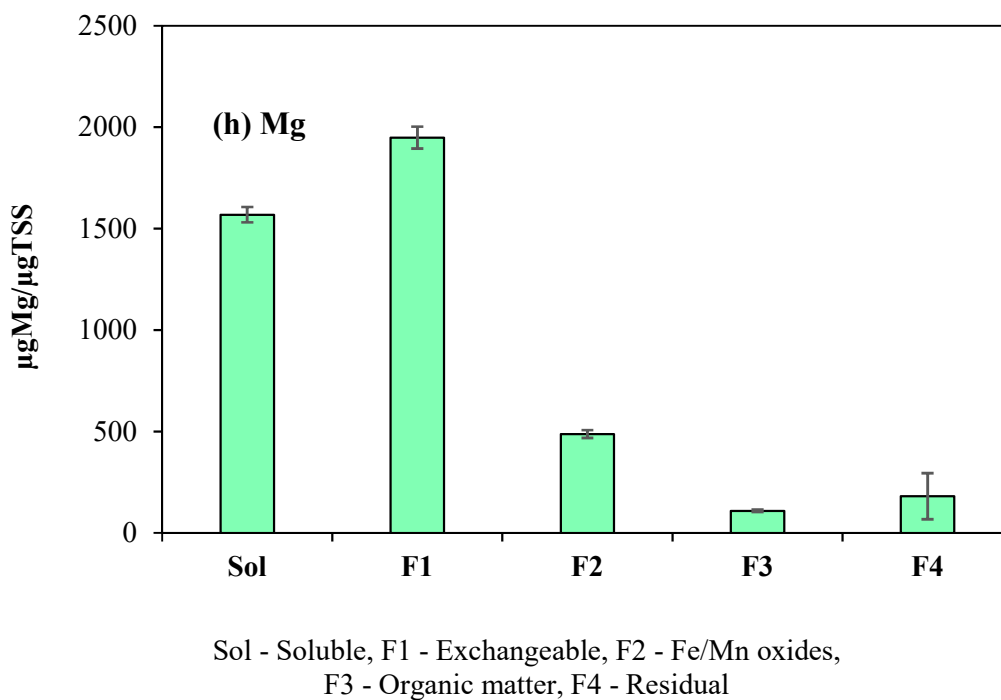
Figure 5.3   Sequential extraction for each metal (a-h) at the end of Phase 3
using the BCR method (n = 3).
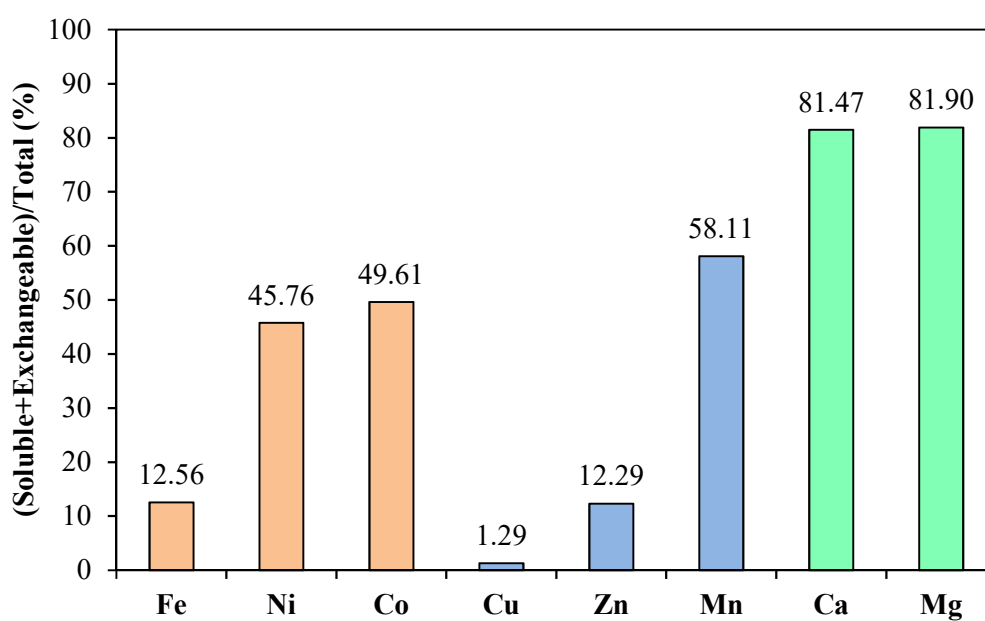
Figure 5.4   Percentage of (Soluble + Exchangeable fraction)/Total metal
content (n = 3).

5.3.3 Determination of optimal concentration for Fe^{2+} in anaerobic digestion

Iron (Fe) is one of the most important TMs in anaerobic digestion, together with Co and Ni. The importance of Fe^{2+} in anaerobic digestion depends on its redox properties, and its involvement in energy metabolism (Takashima & Speece, 1989). The stimulating effect of Fe^{2+} is attributed to its ability to improve acetate utilization by methanogens (Speece, 1962), and its role in the transport system of methanogens for the conversion of CO_2 to CH_4 (Vintiloiu et al., 2013). Therefore, Fe^{2+} plays an important role in improving the degradation of VFAs (Zandvoort et al., 2003; Zitomer et al., 2008; Ketheesan et al., 2016). Since the reported optimal concentration for TMs including Fe^{2+} varies significantly between studies, which could be due to different experimental conditions i.e. substrate type, seed sludge source, and temperature (Thanh et al., 2016), the aim of this experiment was to determine the optimal concentration for Fe^{2+} required for the seed sludge from anaerobic bioreactors in this study.

The seed sludge used for this experiment was grown in Fe^{2+}- deficient conditions, and the level of Fe^{2+} in the seed sludge was found to be 0.08 $mgFe^{2+}/gTSS$, which was well below the lowest added Fe^{2+} concentration i.e. 1.53 $mgFe^{2+}/gTSS$ (5 mg/L Fe^{2+}). Figure 5.5 shows the methane yields ($mLCH_4/gVSS$) at different Fe^{2+} concentrations. The results show that the supplementation of Fe^{2+} at different concentrations had different effects on methane production; at low concentrations supplementation of Fe^{2+} stimulated methane production. Methane yields increased compared to the control set (no Fe added) (65.5 $mLCH_4/gVSS$) when Fe^{2+} concentration increased from 5 to 10 then 20 mg/L, but it reached the maximum at 10 mg/L (68.5 $mLCH_4/gVSS$). A statistical analysis (t-test) indicated that the increase in methane yields was statistically significant at 10 mg/L Fe^{2+} concentration. However, at higher Fe^{2+} concentrations, i.e. 30 and 50 mg/L, a slight inhibitory effect on the methanogens was observed as the methane yields i.e. 62.8 $mLCH_4/gVSS$ (50 mg/L Fe^{2+}) dropped significantly compared to the control set i.e. 65.5

$mLCH_4/gVSS$. It is believed that when the free ion concentration of a metal exceeds some threshold value, possible inhibiting mechanisms could disrupt enzyme function and structure by binding with thiol and other groups on protein molecules, or by replacing natural metals in enzyme prosthetic groups (Chen et al., 2014b).
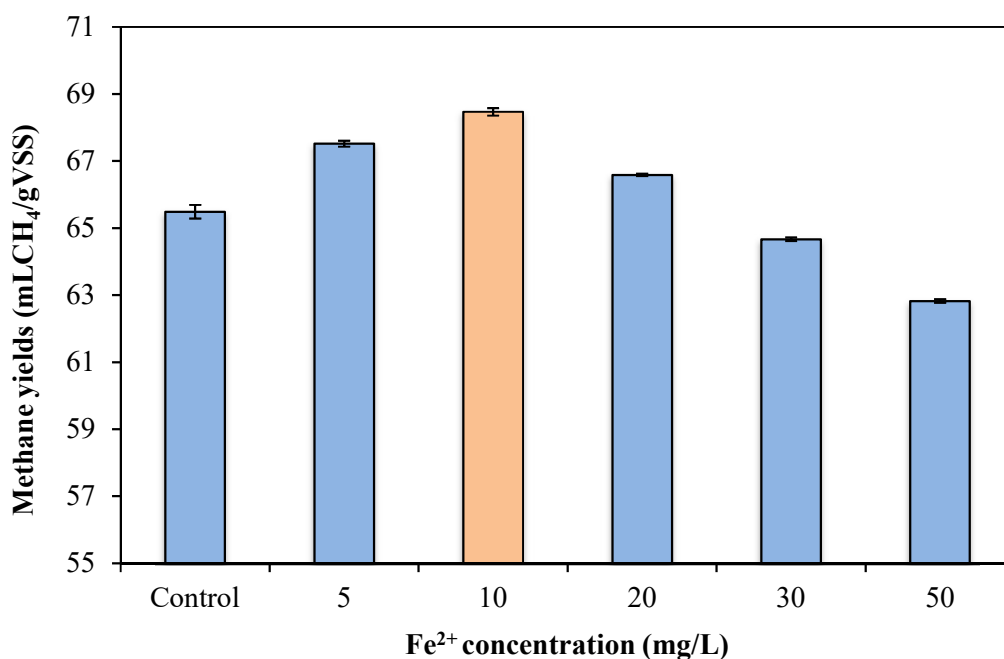
Figure 5.5   Methane yields ($mLCH_4/gVSS$) at different Fe^{2+} concentrations (n = 3).

The results from this experiment showed that the optimal concentration for Fe^{2+} in anaerobic digestion was 10 mg/L (3.34 $mgFe^{2+}/gTSS$), and this was in agreement with the findings of Sharma and Singh (2001). As mentioned in the literature review (Table 2.1), the reported optimal concentration for Fe^{2+} varies significantly, i.e. 0.55 mg/L (Zandvoort et al., 2003) to 100 mg/L (Zhang et al., 2015b), and these differences depend on the substrate type, operating temperature (mesophilic or thermophilic), digestion operating mode (mono or

co-digestion), and also type of methanogens. Therefore, the optimal concentration for TMs can only be reported and applied for a specific study.

5.3.4 Biodegradation of EDDS in anaerobic digestion

As can be seen in Figure 5.6, when the EDDS concentration increased from 0.1:1 to 0.25:1, and then to 1:1 EDDS:Fe^{2+} molar ratio at 10 mg/L Fe^{2+}, the methane yields decreased from 93.7 mLCH$_4$/gVSS to 91.1 mLCH$_4$/gVSS, and to 90.4 mLCH$_4$/gVSS, respectively, after 106 h. However, the methane yields observed from all of the EDDS concentrations were not significantly different compared to the control set (no EDDS) (88.9 mLCH$_4$/gVSS) with a P value of 0.068, 0.106, and 0.359 for the 0.1:1, 0.25:1, and 1:1 molar ratios, respectively. In another independent experiment, the time required for a similar amount of EDDS at a 1:1 molar ratio to 10 mg/L Fe^{2+} (3.34 mgFe^{2+}/gTSS) to be completely biodegraded in anaerobic cultures was found to be around 7 days (168 h), which is longer than the duration of this experiment (106 h). Therefore, it can be concluded that within 106 h, when EDDS was added in a 1:1 EDDS: Fe^{2+} molar ratio to the methane bioassay, the contribution of its biodegradation to methane production was not statistically significant.
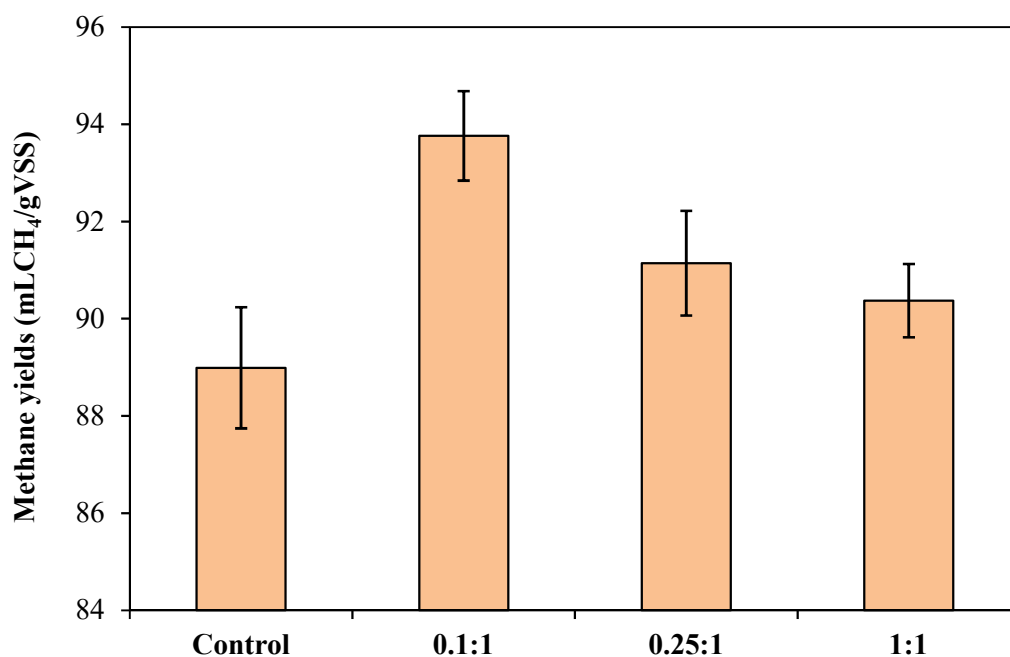
Figure 5.6   Methane yields (mLCH$_4$/gVSS) at different EDDS:Fe molar ratios (n = 3).

5.3.5   Effect of EDDS on Fe^{2+} concentration in the presence of sulfide in model media

Figure 5.7 shows the remaining soluble Fe^{2+} concentration without EDDS and with EDDS at different molar ratios to 10 mg/L Fe^{2+} (3.34 mgFe^{2+}/gTSS). The results confirmed that at time zero (immediately after pH adjustment), the soluble Fe^{2+} concentration in all sets was reduced significantly as the Fe^{2+} was precipitated as FeS. As expected, without EDDS soluble Fe^{2+} was zero within the first 2 h, however, as the EDDS:Fe molar ratio increased, the Fe^{2+} concentration remaining in the soluble form also increased from 0.09 mg/L to 3.32 mg/L. In other words, the addition of EDDS was able to keep the Fe^{2+} in a soluble form up to 33.6% at a 1:1 molar ratio in the presence of 8 mg/L S^{2-}. The remaining soluble Fe^{2+} concentration at all molar ratios seemed to be constant after 10 h. The results from this experiment showed that the addition of EDDS was able to keep Fe^{2+} in a soluble form as the Fe-EDDS complex, even in the presence of sulfide.
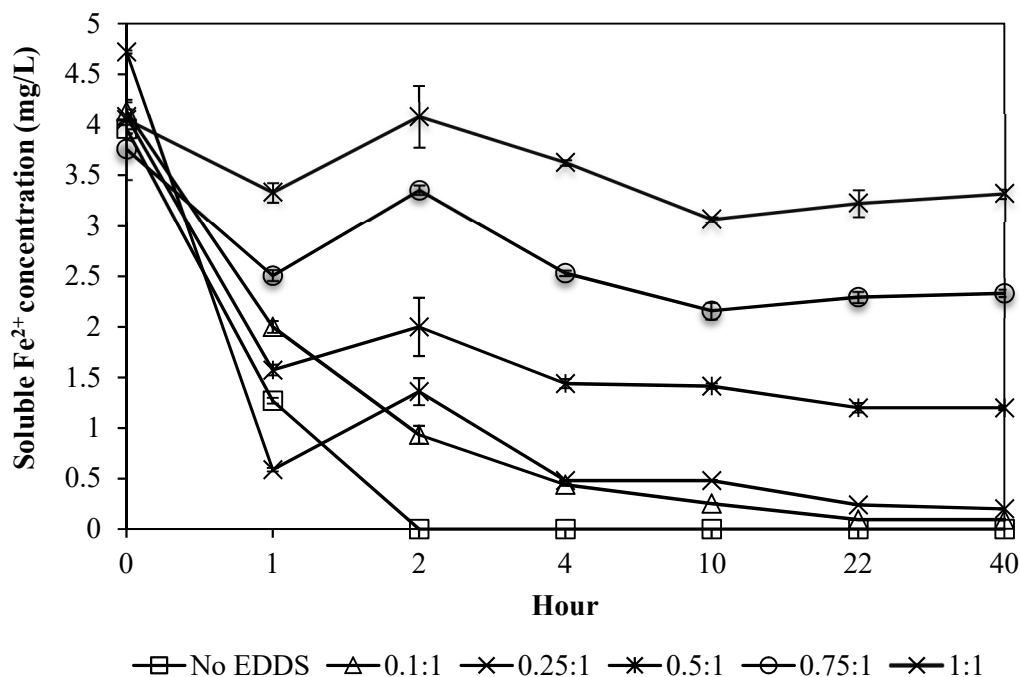
Figure 5.7   Soluble Fe^{2+} concentration in the presence of 0.25 mM S^{2-} at different EDDS:Fe molar ratios with 10 mg/L Fe^{2+} (n = 3).

5.3.6   Effect of EDDS on Fe^{2+} bioavailability in anaerobic digestion

From Figure 5.8 it can be seen without sulfide (No S set), the addition of Fe increased the methane yields from 129.7 mLCH4/gVSS (Control set) to 135.6 mLCH4/gVSS. However, in the presence of sulfide and without EDDS (No EDDS set), there was no statistically significant increase in methane yields observed between this set and the Control. When EDDS was added and its concentration increased (Fe:EDDS molar ratio increased), the methane yields also increased from 135.5 mLCH4/gVSS at 0.1:1 to a maximum of 146.9 mLCH4/gVSS at a 1:1 molar ratio. At the 1:1 molar ratio, methane yields (mLCH4/gVSS) increased by 13.3%, 11.8%, and 8.33%, compared to the Control, No EDDS, and No S set, respectively. In addition, the rate of methane production (mLCH4/gVSS.d) was increased by 11.2%, 10.4%, and 7.2%, compared to the Control, No EDDS, and No S set, respectively. As discussed in Section 3.4, within 106 h. the contribution of methane production from the

degradation of EDDS was not statistically significant, and this experiment was carried out within 100 h; hence, the increase in methane yields and methane production rate at the 1:1 molar ratio was undoubtedly from the positive effect of EDDS on the bioavailability of Fe^{2+}.

Sequential extraction revealed good recovery for Fe^{2+} in all sets, i.e. 93.2% to 106.3%. Figure 5.9 and Table 5.4 show the fraction and the concentration of Fe from each extraction step. It can be seen that Fe^{2+} was not present in the soluble fraction in all three Control sets when EDDS was not added. For the Control 3 set (with S and no EDDS), the sum of two fractions Sol and F1, was lower than the other two Control sets (20.6% compared to 25.9% and 25.0%). Furthermore, the least bioavailable fraction (F4) for the Control 3 set was higher than the other Control sets (11.9% compared to 2.08% and 8.9%). This was presumably due to the presence of the sulfide that led to the precipitation of Fe^{2+}, hence reducing its solubility and bioavailability.
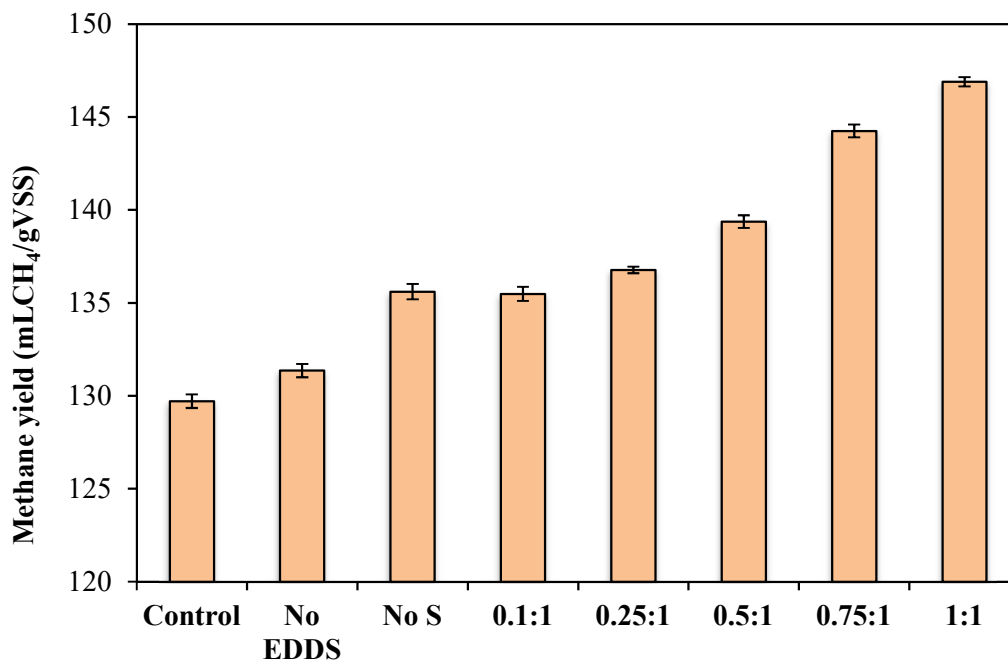
Sulfide was added to all bottles except the "No S set".

Figure 5.8   Methane yields (mLCH4/gVSS) at different EDDS:Fe molar ratios (n = 3).

On the other hand, with the addition of EDDS, the soluble fraction of Fe^{2+} increased with an increase in the EDDS:Fe molar ratio i.e. from 0.07% at 0.1:1 to 1.73% at 1:1. Similarly, the sum of the Sol and F1 fractions also increased from 22.3% at 0.1:1, to the maximum of 29.8% at a 1:1 molar ratio. In addition, the residual fraction F4 showed a decreasing trend; 10.8% at a 0.1:1 molar ratio, to 6.94% at a 1:1 molar ratio. These findings show that EDDS kept Fe^{2+} in the bioavailable fraction (soluble and exchangeable) as an Fe-EDDS complex, and prevented Fe^{2+} becoming less bioavailable.
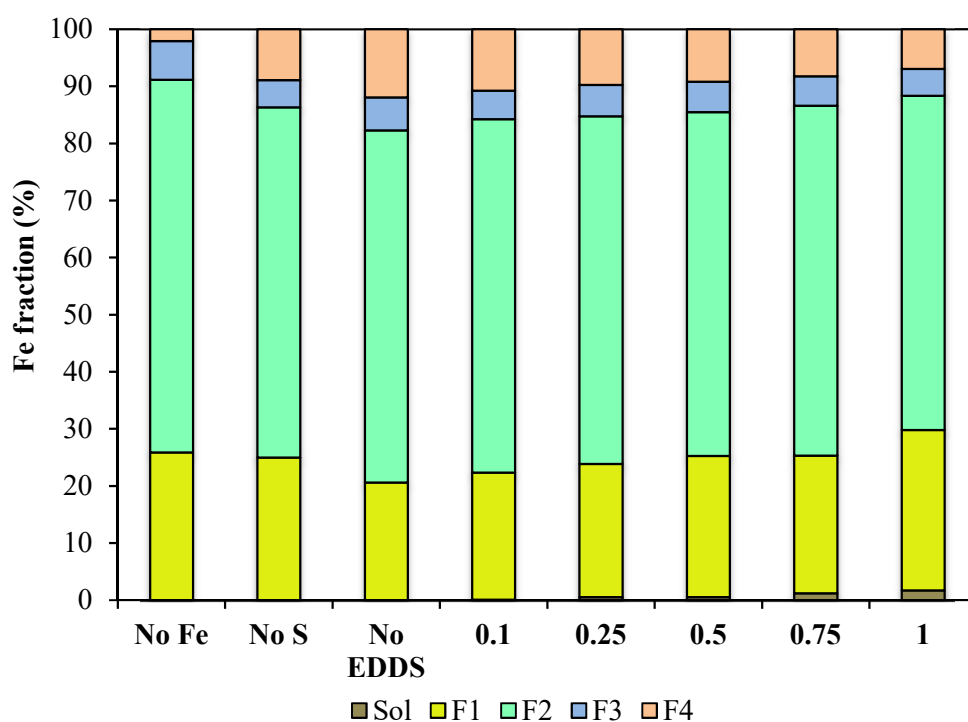
Sol - Soluble, F1 - Exchangeable, F2 - Fe/Mn oxides,

F3 - Organic matter, F4 - Residual

Figure 5.9   Fe fractions from sequential extraction (n = 3).

Table 5.4   Fe^{2+} concentration (μgFe^{2+}/gTSS) from sequential extraction at
the end of experiment (n =3).

	Sol	F1	F2	F3	F4	Sum of fractions	Total by acid digestion
No Fe	0.0	3135.5	7910.1	820.1	252.5	12118.2	12820.4
No S	0.0	3323.7	8166.1	637.5	1185.2	13312.5	13948.5
No EDDS	0.0	2847.9	8527.4	796.7	1650.4	13822.5	13965.4
0.1:1	9.7	3099.6	8624.6	688.4	1502.5	13924.8	13129.7
0.25:1	70.6	3209.6	8366.1	758.0	1342.2	13746.6	14021.5
0.5:1	70.7	3358.8	8169.0	725.7	1247.8	13571.9	13795.8
0.75:1	169.1	3361.2	8533.2	724.2	1147.4	13935.1	13110.2
1:1	236.1	3839.9	7999.5	646.1	948.5	13670.0	14205.2

Sol - Soluble, F1 - Exchangeable, F2 - Fe/Mn oxides,

F3 - Organic matter, F4 - Residual

The measurement of free EDDS and the Fe-EDDS complex (Table 5.5) by UHPLC confirmed that the increase in the molar ratio of EDDS resulted in an increase in the concentration of both free EDDS and Fe-EDDS complex. The total concentration of EDDS remaining in the soluble form increased from 48.9% at a 0.1:1 molar ratio, to 80.4% at a 1:1 molar ratio. This result supports the findings on the increase in methane yields, and the increase in Fe^{2+} in the soluble fraction when the molar ratio increased. This was due to the increase in the amount of the Fe-EDDS complex which was bioavailable for microbial uptake. Therefore, it can be concluded that a higher dose of EDDS was able to compete with sulfide and keep the Fe^{2+} in a soluble form as the Fe-EDDS complex, resulting in a higher methane yields.

Table 5.5   Measurement of free EDDS and Fe-EDDS complex (mg/L) at different molar ratios in UHPLC (n = 3).

	0.1:1	0.25:1	0.5:1	0.75:1	1:1
Free EDDS	1.1	1.8	3.0	5.2	6.0
Fe-EDDS complex	3.1	9.9	22.8	36.3	50.4
Total EDDS	6.3	15.7	31.4	47.0	62.7
% of added mass EDDS	48.9	63.3	72.7	77.2	80.4

5.4 Conclusions

Fe^{2+} supplementation stimulated methane yields at 10 mg/L (3.34 mgFe^{2+}/gTSS) in anaerobic digestion, while the presence of 8 mg/L sulfide led to the precipitation of FeS, which limited microbial uptake in the short term. EDDS was found to be able to improve the bioavailability of Fe^{2+} by keeping the metal in soluble/bioavailable form, and enabling the bio-uptake of the Fe-EDDS complex. When EDDS was added at a 1:1 molar ratio to Fe^{2+} at 10 mg/L, the rate of methane production was increased by 11.17%, while the methane yields increased by 13.3%. Due to its superior biodegradability, EDDS can be an excellent alternative to the conventional chelating agents i.e. EDTA and NTA to improve trace metal bioavailability.

Chapter 5: Effect of Ethylenediamine-N,N'-disuccinic acid (EDDS) on
the speciation and bioavailability of Fe^{2+} in the presence of sulfide

Chapter 6

Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe^{2+} in the presence of sulfide in a submerged anaerobic membrane bioreactor

Outline:

6.1 Introduction

6.2 Experimental and analytical methods

6.3 Results and discussion

6.4 Conclusions

Note: this chapter was published in *Chemical Engineering Journal*, 2017, volume 330, pages 175-182.

6.1 Introduction

The importance of Fe^{2+} in the degradation of VFAs and the effect of sulfide on the bioavailability of Fe^{2+} has been discussed in the literature review. The results from Chapter 5 showed that in a batch system, biodegradable chelating agent EDDS was able to improve the bioavailability Fe^{2+} in the presence of sulfide. However, the effect of adding a chelating agent on metal bioavailability is very complex as it depends on many factors, including the type of chelating agent, the added concentration, and the reactor configuration.

Chapter 6: Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe^{2+} in the presence of sulfide in a submerged anaerobic membrane bioreactor

Reactor configuration (batch or continuous) has an important role in determining how the chelating agent should be dosed as it controls the retention of the TMs. Chelating agents can be added either as a complex with the metals (Fermoso et al., 2008c; Bartacek et al., 2012; Vintiloiu et al., 2013), or simultaneously with the metals (Hu et al., 2008a; Hu et al., 2008b). Fermoso et al. (2008c) investigated the use of EDTA as a ligand for Co dosing to UASB reactors. They found that when supplemented as Co-EDTA complex, only 8% of the Co supplemented was retained, whereas 90% retention of Co was achieved when it was supplemented as $CoCl_2$. This is because when added to a continuous UASB system, the highly soluble metal complex Co-EDTA was washed out. However, Vintiloiu et al. (2013) found that when Ni was complexed with EDTA prior to supplementation to a batch system, there was a significant increase in Ni bioavailability. The reason for this in the batch system was that EDTA was able to chelate free ion concentration, which can be taken up by the anaerobic microorganisms. The innovative submerged anaerobic membrane bioreactor (SAMBR) is also a continuous system which is better than the USAB system in terms of biomass retention capability due to the presence of a membrane, and there will be no TMs associated with the biomass escaping into the effluent.

The aim of the experiments in this chapter was, therefore, to evaluate the dynamics of Fe^{2+} and the presence of sulfide on its speciation and bioavailability in a SAMBR. The dosing strategies for EDDS and Fe^{2+}, either as a complex or individual ion, was investigated in batch reactors, and then applied to the SAMBR to determine which strategy was more effective in improving the bioavailability of Fe^{2+} and hence the performance of the SAMBR.

6.2 Experimental and analytical methods

6.2.1 Dosing strategy for EDDS and Fe in the presence of sulfide in a batch reactor

This experiment was aimed at determining whether Fe^{2+} and EDDS are more effective when they are supplemented as a metal-chelator complex (Fe-EDDS), or simultaneously (Fe+EDDS), and was performed in batch reactors using 125 mL serum bottles (100 mL working volume). Seed sludge was withdrawn from parent anaerobic reactor. In all test bottles, the initial biomass and the F/M ratio were set to 3 gVSS/L and 0.5 gCOD/gVSS; glucose (1.5 gCOD/L) and NaHCO$_3$ (2 g/L) were added as substrate and buffer, respectively. Fe^{2+} was supplemented at its optimal concentration, i.e. 10 mg/L as FeCl$_2$.4H$_2$O (3.34 mgFe^{2+}/gTSS), and EDDS was added at the optimal molar ratio to Fe^{2+}, i.e. 1:1 ratio, as determined from previous work (Thanh et al., 2017). In the Fe-EDDS set, the metal complex was prepared by mixing the respective amounts of Fe^{2+} and EDDS overnight in the dark to prevent photo-degradation before adding it to the test bottles; in the Fe+EDDS set, Fe^{2+} and EDDS were added simultaneously to the test bottles. Three control sets were prepared, i.e. Control 1 – substrate only, Control 2 – substrate and EDDS, Control 3 – substrate and Fe^{2+}. Sulfide solution was prepared in an oxygen-free environment, and then added to all bottles at 8 mg/L as Na$_2$S.9H$_2$O, which is below its toxic level (Mountfort & Asher, 1979). The pH of all test bottles was adjusted to 7.0 ±0.1 using diluted NaOH or HCl. The bottles were flushed with nitrogen for 2 min to ensure anaerobic conditions, sealed and incubated in a shaker at 35ºC and 170 rpm. All tests were run in triplicates (CV = ±5%), and carried out over 8 days.

6.2.2 Effect of sulfide on the speciation and bioavailability of Fe^{2+} in a SAMBR

The aim of this experiment was to determine what effects the presence of sulfide could have on the dynamics of speciation, and hence the bioavailability of Fe^{2+} in a SAMBR. The set-up of the SAMBR used for this study was described in detail by Zhou et al. (2016). The SAMBR was treating synthetic wastewater with the feed composition based on that used by Akram and Stuckey (2008). The operating conditions for the SAMBR were set as 500 mgCOD/L feed, 6 h HRT, and 100 d SRT. The pH in the system was maintained in the range of 6.9 and 7.1 using 1M $NaHCO_3$, while the oxidation-reduction potential (ORP) was in the range of -463 ± 4 mV throughout the experiment.

Since the scope of this study was limited to Fe^{2+} only, the feed for the SAMBR contained Fe^{2+} as the only TM to eliminate the possibility of its interaction with other TMs. The experimental design for each stage of the SAMBR is described in Figure 6.1. During the first stage (Stage 1), Fe^{2+} was added to the SAMBR at the optimal concentration of 10 mg/L as $FeCl_2.4H_2O$, which is equivalent to 0.22 mg/gTSS.d. During the second stage (Stage 2), the same level of sulfide as in the batch experiment described above was added to the feed, i.e. 8 mg/L as $Na_2S.9H_2O$. The feed was then sparged with nitrogen and sealed to ensure anaerobic condition. Each stage of this experiment was carried out over 10 days.

6.2.3 Dosing strategy for EDDS and Fe^{2+} in the presence of sulfide in a SAMBR

After Stage 2, the most effective dosing strategy for EDDS and Fe determined from experiment 6.2.1 was applied to the SAMBR in the final stage of this study (Stage 3) (Figure 6.1). The same experimental conditions as experiment 6.2.2 were used, but Fe^{2+} and EDDS were added to the feed either

as a complex (Fe+EDDS), or simultaneously (Fe-EDDS), at a 1:1 molar ratio with the optimal Fe^{2+}concentration, i.e. 10 mg/L (0.22 mg/gTSS.d) (Figure 6.1). The concentrated feed was kept in a cold room, and new feed was freshly prepared every day. The feed bottle was also sparged with nitrogen and covered with aluminum foil to prevent photo-degradation of the EDDS, and the experiment was carried out in 9 days.

Stage 1
- 500 mgCOD/L, 6 h HRT, 100 d SRT
- 10 mg/L FeCl$_2$.4H$_2$O
- 10 days

Stage 2
- 500 mgCOD/L, 6 h HRT, 100 d SRT
- 10 mg/L FeCl$_2$.4H$_2$O + 8 mg/L Na$_2$S.9H$_2$O
- 10 days

Stage 3
- 500 mgCOD/L, 6 h HRT, 100 d SRT
- Fe-EDDS (Fe:EDDS = 1:1, 10 mg/L FeCl$_2$.4H$_2$O) + 8 mg/L Na$_2$S.9H$_2$O
- 9 days
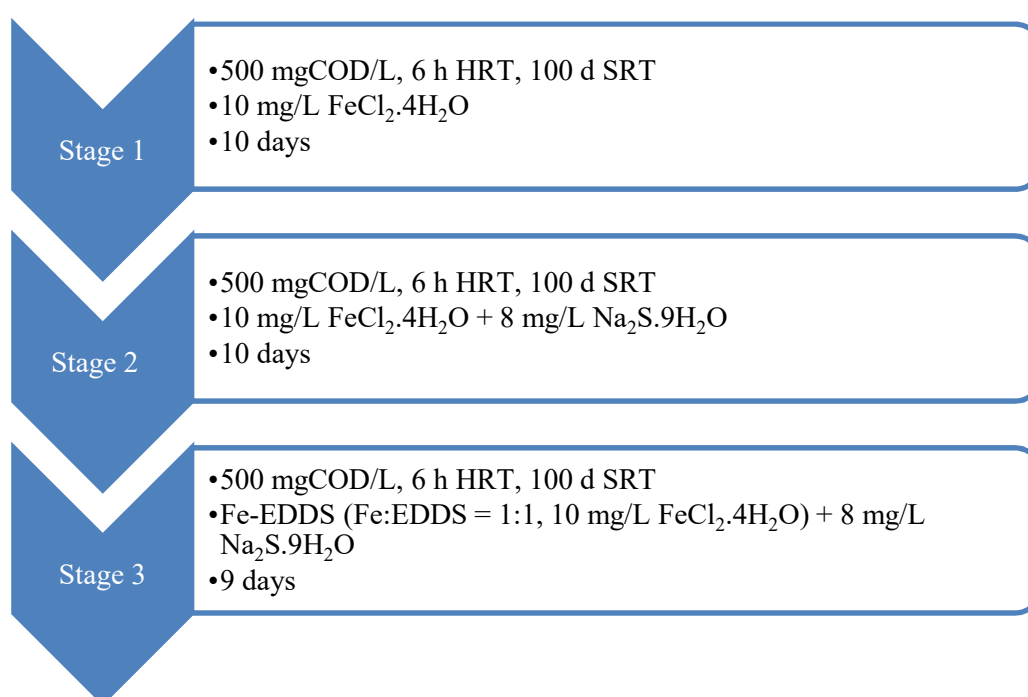
Figure 6.1   Schematic diagram of experimental design for each stage of the SAMBR.

6.3   Results and Discussion

6.3.1   Dosing strategy for EDDS and Fe^{2+} in the presence of sulfide in a batch setup

Figure 6.2 shows that the methane yields from the Control 1 set (substrate only) reached 114.3 mLCH$_4$/gVSS, which was 65.3% of the theoretical

methane production, and the remaining substrate was probably used for biomass generation. In comparison, there was no statistical difference in the methane yields between the other two control sets i.e. Control 2 (substrate and EDDS) (114.3 mLCH$_4$/gVSS) and Control 3 (substrate and Fe) (116.3 mLCH$_4$/gVSS) to Control 1 (p > 0.05). These findings have two implications; firstly, the addition of EDDS to the Control 2 set did not result in any significant improvement in the methane yields compared with those without EDDS. Hence, any increase in methane yields will be undoubtedly from the positive effect of EDDS on the bioavailability of Fe^{2+}, and not from its degradation within the 8 day test. Secondly, the addition of Fe^{2+} to the Control 3 set did not significantly increase the methane yields compared to the Control 1 set (p > 0.05), which indicates that due to the presence of added sulfide in the test bottles, most of the Fe^{2+} probably precipitated as FeS, and hence became less bioavailable for the microorganisms to take up.

The addition of Fe^{2+} and EDDS simultaneously (Fe+EDDS set) stimulated the methanogens as there was a significant increase in methane yields compared to the Control 3 set, i.e. 124.1 mLCH$_4$/gVSS v.s. 116.3 mLCH$_4$/gVSS (p < 0.05). These results were in agreement with findings from a previous study of ours (Thanh et al., 2017), which was carried out under the same experimental conditions with the same seed sludge. However, compared to the Fe+EDDS set, the methane yields from the metal complex set Fe-EDDS was significant higher i.e. 136.5 mLCH$_4$/gVSS (p < 0.05). As a result, compared to the Control 3 set (without EDDS), the methane yields from the Fe-EDDS set also increased significantly by 17.3% (p < 0.05). The results suggested that the addition of EDDS was able to compete with the sulfide to maintain a certain level of Fe^{2+} solubility, and hence improve its bioavailability for microbial uptake. Clearly, the dosing of EDDS as a complex with Fe^{2+} showed a better improvement in methane yields than when it was supplemented simultaneously with Fe^{2+}.

Hydrogen concentration is often an indicator of process stability in anaerobic digestion systems (Ketheesan & Stuckey, 2015). The measurement of hydrogen in the gas phase (average of 3 samples) (Figure 6.3) showed that from time zero to 8.5 hours of the experiment, there was no hydrogen detected in the gas phase. However, after 8.5 hours, the hydrogen concentration was at the same level in all sets (142.3 ppm to 143.8 ppm) ($p > 0.05$). As time increased, the hydrogen concentration in the Fe-EDDS set reduced at a faster rate than the other sets ($p < 0.05$), and reached the lowest value at the end of the experiment (17.0 ppm). This finding indicated that the Fe supplemented as a complex with EDDS was better at stimulating the growth and metabolism of the methanogens than when it was supplemented without EDDS or simultaneously with EDDS, and it effectively enhanced the removal rate of the hydrogen produced by substrate degradation by the fermentative bacteria.

Control 1 – Substrate only, Control 2 – Substrate + EDDS, Control 3 – Substrate + Fe²⁺

Figure 6.2   Methane yields (mLCH₄/gVSS) from different sets in the batch experiment after 8 d (n = 3).
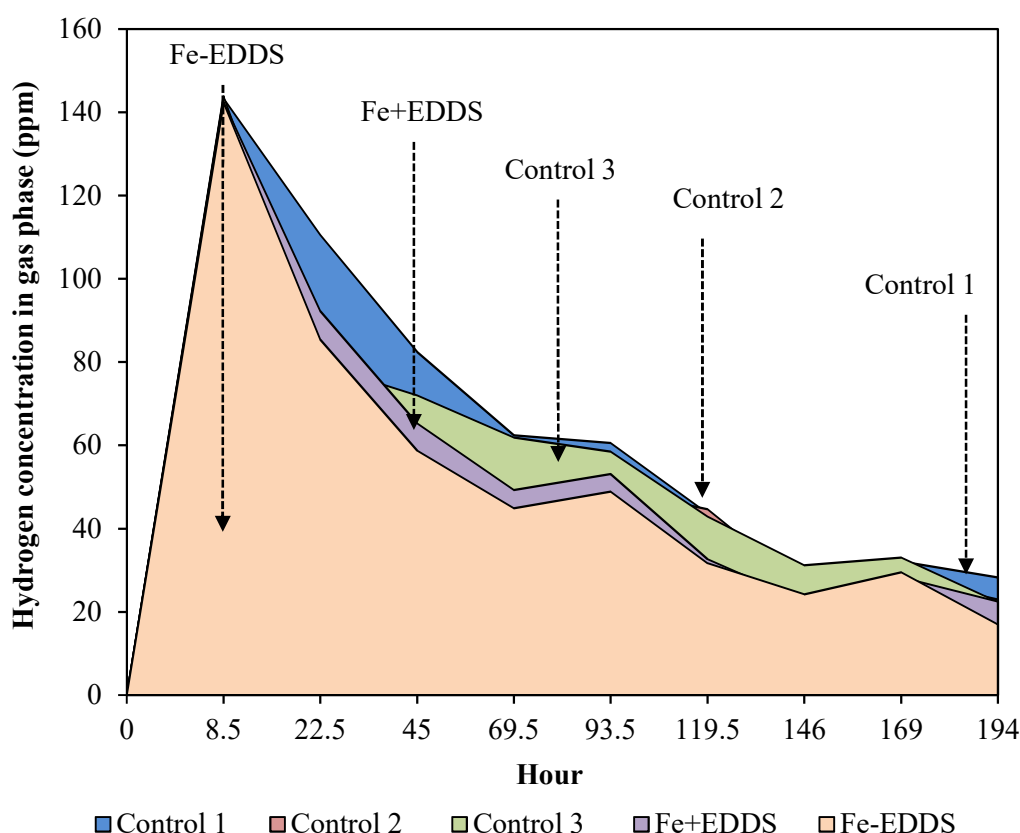
Control 1 – Substrate only, Control 2 – Substrate + EDDS, Control 3 – Substrate + Fe²⁺

Figure 6.3   Hydrogen concentration in the gas phase (ppm) from the batch experiment over 8 d (n = 3).

The measurement of metal content in each fraction from the BCR method at the end of the experiment is given in Table 6.1. The metal extraction showed good recovery for Fe^{2+} in all sets i.e. 95.6% to 104.6%. The distribution of Fe^{2+} between different fractions is illustrated in Figure 6.4. Fe^{2+} was found to be present mainly in the Fe-Mn oxides fraction (F2) i.e. 43.9% to 48.5%. The first fraction of the Control 1 set (substrate only), i.e. Soluble (Sol), was the lowest compared to that of the other sets, i.e. 0.03%, as no Fe^{2+} was added to this set. The Sol fraction for the Control 2 set (substrate and EDDS) was higher than for the Control 3 set (substrate and Fe), i.e. 2.01% and 0.28% ($p < 0.05$),

respectively. The reason for this difference is due to the complexation of EDDS with Fe^{2+} present in the seed sludge, which was found to be around 0.1 mgFe^{2+}/gTSS, and hence there was still a fraction of Fe^{2+} left in the soluble form. The presence of 8 mg/L sulfide in Control 3 set resulted in the precipitation of Fe^{2+} as FeS, and hence this reduced its solubility. The addition of EDDS to the Fe-EDDS and Fe+EDDS sets both increased the soluble fractions to 9.04% and 7.15% ($p < 0.05$), respectively, which shows that EDDS was able to compete with sulfide to keep the Fe^{2+} in a soluble form. The sum of the two most bioavailable fractions in the sequential chemical extraction method (BCR), i.e. Soluble and Exchangeable fraction (Sol+F1), was not statistically different for all the three Control sets, i.e. 29.7%, 29.9%, and 28.7% ($p > 0.05$). However, the addition of EDDS to the Fe-EDDS and Fe+EDDS sets resulted in an increase in this sum, i.e. 38.6% and 34.3% ($p < 0.05$), respectively. In contrast, the sum of the last two fractions which are considered the least bioavailable, i.e. Organic matter/sulfide and Residual (F3+F4) was the lowest for the Fe-EDDS and Fe+EDDS, set i.e. 17.3% and 21.8%, respectively.

These speciation results showed that the addition of EDDS was able to prevent the Fe^{2+} from precipitating with sulfide, and kept it in a highly bioavailable form, i.e. Sol and F1. Dosing of Fe^{2+} as a metal complex with EDDS also resulted in a better retention of Fe^{2+} in these highly bioavailable forms than when they were added simultaneously (Fe+EDDS).

Chapter 6: Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe^{2+} in the presence of sulfide in a submerged anaerobic membrane bioreactor

Table 6.1   Fe content ($\mu gFe^{2+}/gTSS$) from sequential extraction at the end of the batch experiment (n =3).

	Sol	F1	F2	F3	F4	Sum of fractions	Total by acid digestion	Recovery (%)
Control 1	4.2	4061.0	6610.6	1004.5	2025.7	13706.0	13101.7	104.6
Control 2	276.2	3841.5	6594.2	964.8	2078.6	13755.3	14393.6	95.6
Control 3	39.6	4088.0	6968.8	1011.2	2272.1	14379.7	14711.6	97.7
Fe-EDDS	1409.7	4613.1	6870.1	997.8	1706.2	15596.9	15836.7	98..5
Fe+EDDS	1090.9	4133.6	6702.6	1001.9	2324.9	15253.9	14890.6	102.4

Control 1 – Substrate only, Control 2 – Substrate + EDDS, Control 3 – Substrate + Fe^{2+}

Sol - Soluble, F1 - Exchangeable, F2 - Fe/Mn oxides, F3 - Organic matter, F4 – Residual
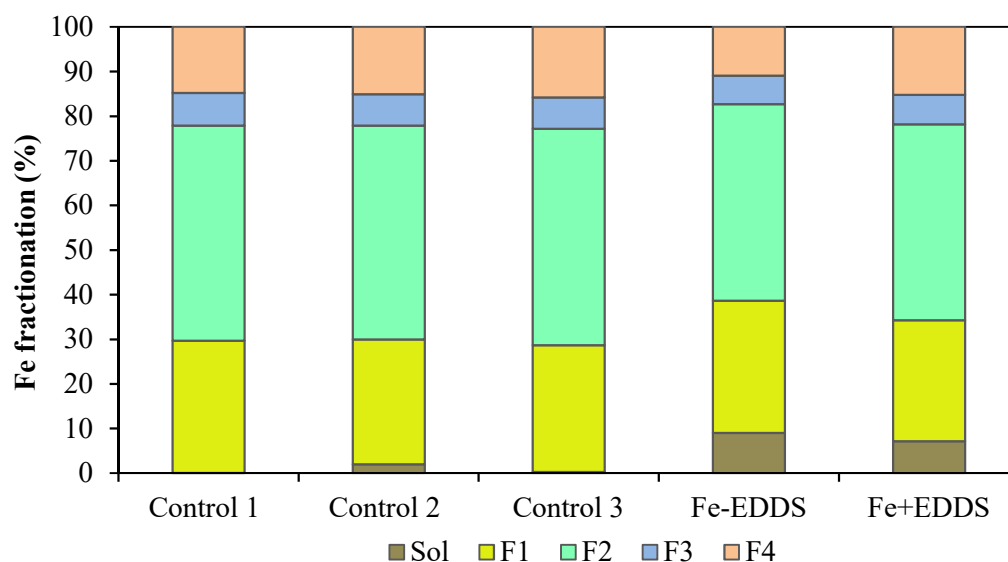
Sol - Soluble, F1 - Exchangeable, F2 - Fe/Mn oxides, F3 - Organic matter, F4 – Residual.

Control 1 – Substrate only, Control 2 – Substrate + EDDS, Control 3 – Substrate + Fe^{2+}

Figure 6.4   Fe fractionation using the BCR method at the end of the batch experiment (8 d) (n = 3).

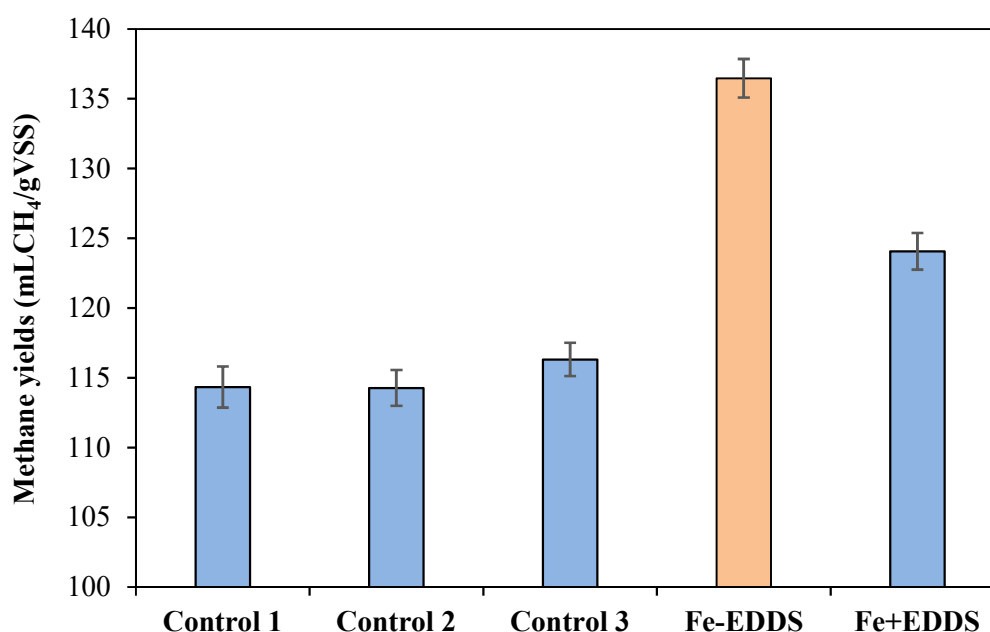# Chapter 6: Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe^{2+} in the presence of sulfide in a submerged anaerobic membrane bioreactor

The measurement of free EDDS and EDDS in the metal complex using UHPLC (Table 6.2) showed that the concentrations of these two EDDS forms in the Fe-EDDS set were statistically higher than those in the Fe+EDDS set i.e. 4.78% and 36.47% compared to 3.96% and 33.58% ($p < 0.05$), respectively. As the result, the total concentration of EDDS remaining in the soluble form for the Fe-EDDS set was higher, i.e. 78.8% compared to 71.7% for the Fe+EDDS set ($p < 0.05$). This result supports the findings on the increase in methane yields, and the increase in highly bioavailable fractions when the EDDS was added. This was due to the increase in the amount of Fe^{2+} present in the Fe-EDDS complex which was (probably) bioavailable for microbial uptake. Furthermore, the amount of EDDS in a free and complex form for the Fe-EDDS set was higher than for the Fe+EDDS set, which explains why Fe^{2+} is better to dose as a complex with EDDS than separately. The result from this experiment was in agreement with previous studies carried out in batch systems (Bartacek et al., 2012; Vintiloiu et al., 2013) that the bioavailability of the TM was increased when it was dosed as a metal complex with a chelating agent. However, these studies were carried out with conventional chelating agents such as EDTA, which has very low biodegradability compared to the biodegradable chelating agent EDDS used in this work. Furthermore, without the analytical technique to determine the fate of the added chelating agent, these studies were not able to explain the mechanisms behind the improvement in trace metal bioavailability. Therefore, the dosing of a novel biodegradable chelating agent EDDS in this experiment showed that it can be an excellent alternative to the conventional chelating agents as it was effective competing with sulfide, and kept the Fe^{2+} soluble so it could be taken up more easily by the microorganisms. It should be noted that the biodegradability of EDDS in an anaerobic system requires further study in order to optimise the dosage frequency as it would be undesirable to discharge effluent containing significant amounts of EDDS into the environment.

Table 6.2   Measurement of free EDDS and Fe-EDDS complex (mg/L) using UHPLC in the batch experiment (n = 3).

	Fe-EDDS	Fe+EDDS
Free EDDS	4.78	3.96
Fe-EDDS complex	36.4	33.6
Total EDDS	41.3	37.5
% of added mass EDDS	78.8	71.7

6.3.2   Effect of sulfide on the speciation and bioavailability of Fe^{2+} in a SAMBR

Before starting this experiment, the SAMBR was operated with a mixture of TMs (Fe, Co, Ni, Zn, Mn, and Mo) for about 3 months, therefore, there were high levels of these TMs, especially Fe^{2+}, in the sludge of the SAMBR (10004 µgFe/gTSS, 837.1 µgCo/gTSS, 658.5 µgNi/gTSS, 1202 µgZn/gTSS, 148.7 µgMn/gTSS, and 124.4 µgMo/gTSS). The accumulation of these TMs could explain why when Fe^{2+} was supplemented as the only metal nutrient to the SAMBR (Stage 1), the reactor was still capable of operating at very high treatment efficiencies for 10 days as the accumulated TMs were still sufficient to support the growth of the anaerobes. The average soluble COD levels inside the reactor and in the effluent (Figure 6.5) were 50.1 mg/L and 8.2 mg/L, respectively, and thus the average COD removal was remarkably high, i.e. 98.4%; the average daily methane yields reached 58.3 mLCH$_4$/gVSS.d (Figure 6.6). However, when sulfide was added to the feed (day 11 - Stage 2), the SAMBR performance dropped slightly, and the average soluble COD inside the reactor and in the effluent (Figure 6.5) increased to 64.8 mg/L and 17.2 mg/L (p < 0.05), respectively. Hence, the corresponding average COD removal in this

stage decreased to 96.6% ($p < 0.05$). The addition of sulfide also showed a negative effect on the average daily methane yield which was reduced by 14.6% to 49.8 mLCH$_4$/gVSS.d ($p < 0.05$) (Figure 6.6); since sulfide was added below its toxic level, it should have not inhibited the anaerobic microorganisms. VFAs (formate and acetate) were found to increase from 1.62 mgCOD/L at Stage 1 to 3.98 mgCOD/L ($p < 0.05$). Therefore, the decrease in SAMBR performance during Stage 2 was almost certainly due to the indirect limiting effects of sulfide on the growth rate of methanogens by reducing the bioavailability of the metals for microbial uptake.
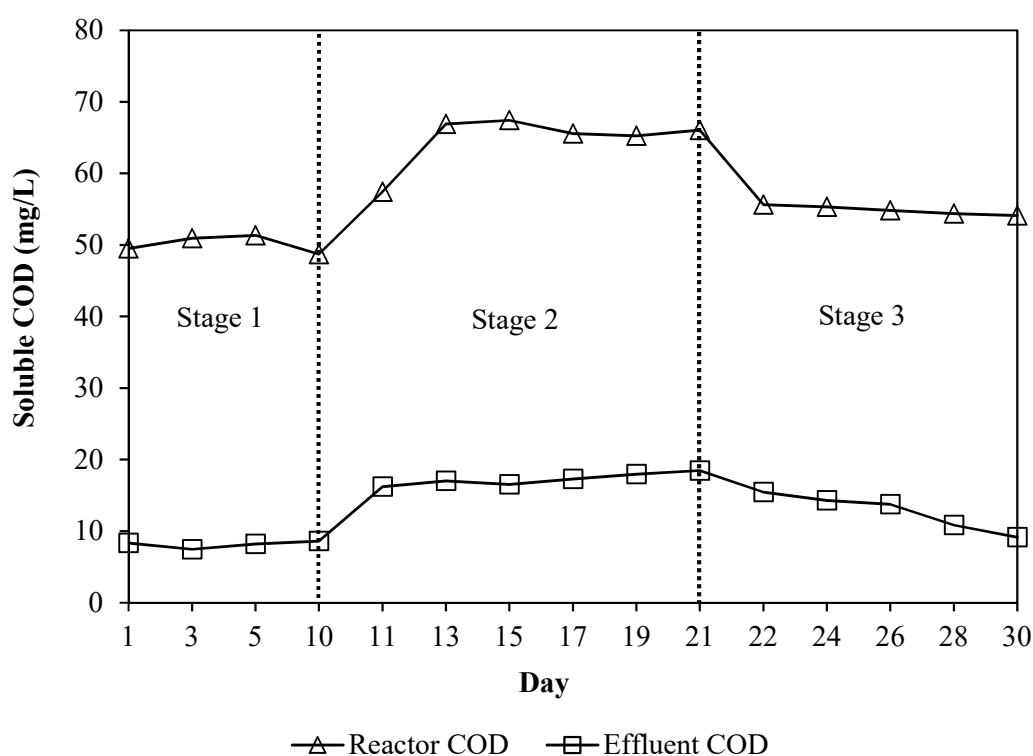
Stage 1 – Fe only, Stage 2 – Fe and S, Stage 3 – Fe, EDDS and S

Figure 6.5   Soluble COD in the SAMBR reactor and effluent at different stages (n = 3).
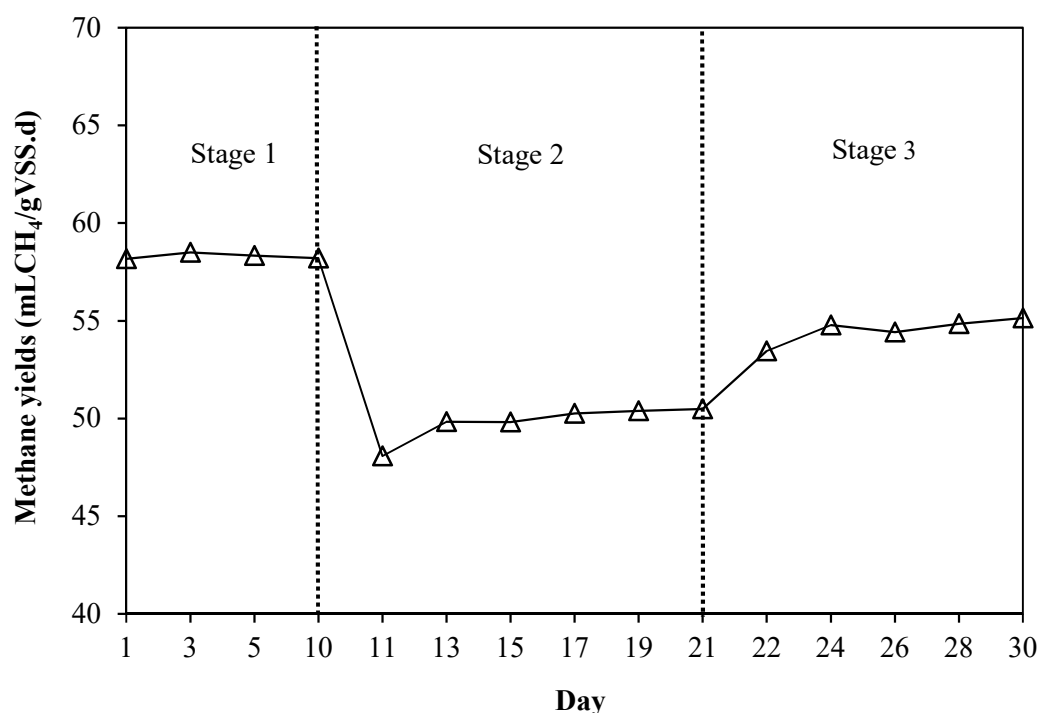
Stage 1 – Fe only, Stage 2 – Fe and S, Stage 3 – Fe, EDDS and S

Figure 6.6   Methane yields of the SAMBR at different stages.

The soluble concentration of Fe^{2+} was measured in the influent and effluent during each stage of the experiment (Figure 6.7). It can be clearly seen that there was difference between the level of Fe^{2+} in the influent and in the effluent, which indicated that Fe^{2+} accumulated in the reactor. The significance of Fe^{2+} accumulation in a SAMBR which is important for the metal dosing, has been discussed in Section 4.3.1. The solids retention time (SRT) therefore needs to be maintained in order to control the level of Fe^{2+} associating with the biomass in the reactor. In addition, the level of Fe^{2+} in the effluent cannot be negligible compared to the level of Fe^{2+} in the influent, hence, dosing optimization is essential to make sure the effluent quality is within the discharge standards.

Chapter 6: Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe²⁺ in the presence of sulfide in a submerged anaerobic membrane bioreactor

The average concentration of soluble Fe^{2+} in the influent in Stage 1 was 29.1 µgFe^{2+}/gTSS. When sulfide was introduced into the feed in Stage 2, the level of soluble Fe^{2+} dropped to an average of 11.4 µgFe^{2+}/gTSS ($p < 0.05$), which means 60.9% of the added Fe^{2+} may precipitate with sulfide as FeS. The average level of soluble Fe^{2+} in the effluent was 25.4 µgFe^{2+}/gTSS in Stage 1, which was then reduced by 72.9% to only 6.9 µgFe^{2+}/gTSS in Stage 2 ($p < 0.05$). This finding clearly shows the effect of sulfide on the solubility of Fe^{2+} as it had already precipitated the Fe in the feed before entering the SAMBR, hence reducing the level of soluble Fe^{2+} in the reactor and also in the effluent.
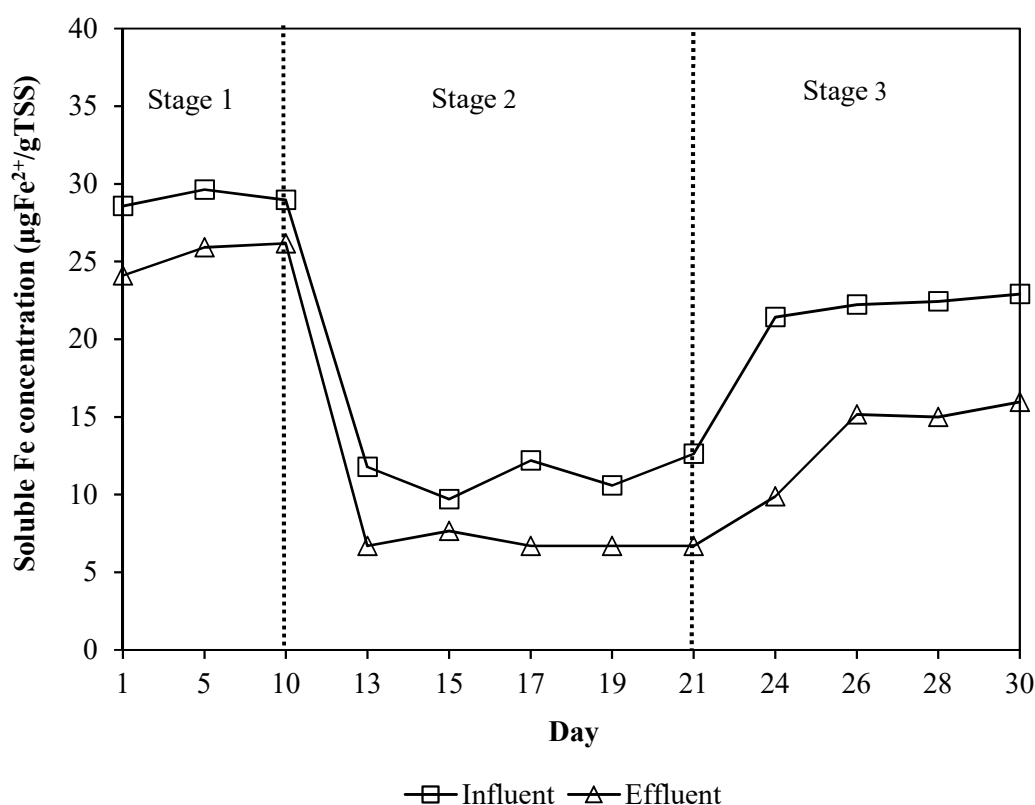
Stage 1 – Fe only, Stage 2 – Fe and S, Stage 3 – Fe, EDDS and S

Figure 6.7   Soluble Fe concentration in the SAMBR influent and effluent at different stages.

Chapter 6: Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe²⁺ in the presence of sulfide in a submerged anaerobic membrane bioreactor

Table A.1 (Appendix) gives the content of Fe^{2+} in each fraction from the BCR extraction, while Figure 6.8 documents the distribution of Fe^{2+} during each stage of the experiment. These results show that without sulfide (Stage 1), Fe^{2+} was mainly present in Fe-Mn oxides fraction (F2) in the SAMBR, i.e. 49.8% of the total Fe^{2+} content. The Exchangeable (F1) and Residual fractions (F4) were the second highest fractions, i.e. 19.8% and 20.6%, respectively. The Soluble (Sol) and Organic matter/sulfide (F3) fractions were the lowest at 0.26% and 9.53%, respectively. During Stage 2, with the addition of sulfide to the feed, there was a significant decrease in the first two fractions which have the highest bioavailability (Sol and F1) to 0.09% and 5.02% ($p < 0.05$). In contrast, the last two fractions (F3 and F4) which are considered to be the least bioavailable fractions, increased to 15.0% and 39.8% ($p < 0.05$).
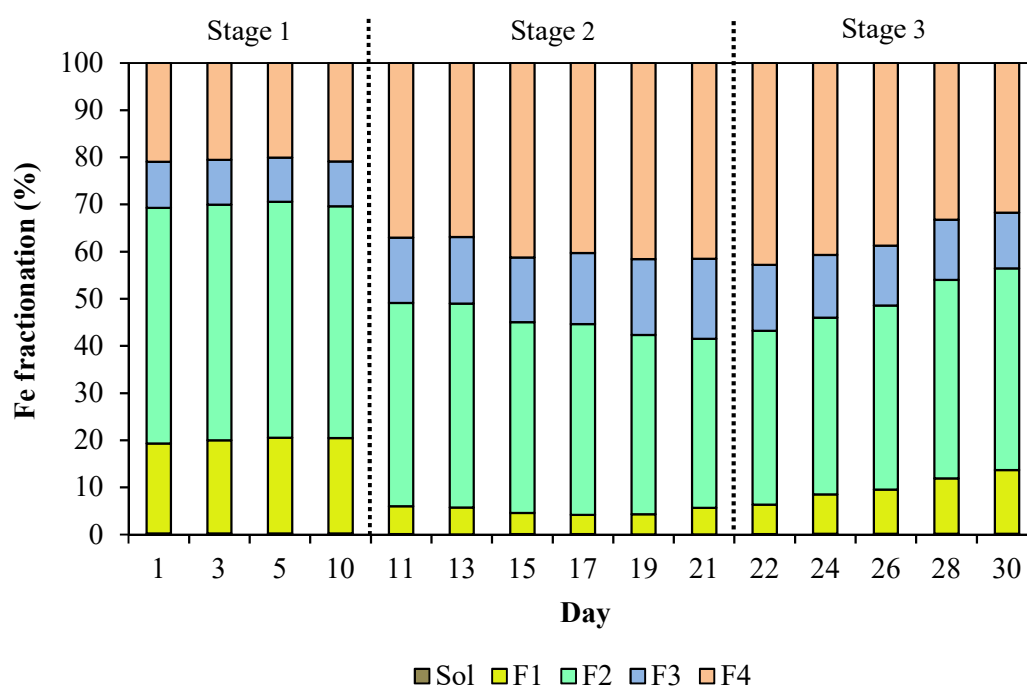
Sol - Soluble, **F1** - Exchangeable, **F2** - Fe/Mn oxides,

F3 - Organic matter, **F4** - Residual

Figure 6.8   Speciation of Fe in the SAMBR at different stages.

The results from this experiment show that in the absence of sulfide, with Fe^{2+} as the only TM, the SAMBR was still able to achieve very high treatment efficiencies. However, in the presence of sulfide in the feed, the precipitation of Fe^{2+} as FeS resulted in a shift in Fe speciation towards less bioavailable fractions, which in turn reduced the performance of the SAMBR.

6.3.3   Dosage of EDDS to improve the bioavailability of Fe^{2+} in the presence of sulfide in a SAMBR

The dosing strategy for Fe^{2+} and EDDS determined from experiment 6.3.1 was applied in Stage 3 of this study, as Fe^{2+} was pre-complexed with EDDS before adding it to the feed for the SAMBR. The introduction of EDDS clearly helped improve the performance of the SAMBR, as the average soluble COD in the reactor and effluent decreased from 64.8 mg/L and 17.2 mg/L (Stage 2) to 54.9 mg/L and 12.7 mg/L (Stage 3) ($p < 0.05$), respectively (Figure 6.5). Accordingly, the average COD removal of the SAMBR increased from 96.6% (Stage 2) to 97.5% (Stage 3) ($p < 0.05$). The average daily methane yields also increased by 9.46% from 49.8 mLCH$_4$/gVSS.d (Stage 2) to 54.5 mLCH$_4$/gVSS.d (Stage 3) ($p < 0.05$), however, this was still slightly below the level before the addition of sulfide and EDDS (Stage 1) i.e. 58.3 mLCH$_4$/gVSS.d (Figure 6.6). The VFA level was also slightly reduced from 3.98 mgCOD/L to 1.92 mgCOD/L ($p < 0.05$).

The measurement of soluble Fe^{2+} concentrations in the feed and effluent during Stage 3 showed the positive effect of EDDS on Fe^{2+} bioavailability when it was added to the feed as an Fe-EDDS complex. From Figure 6.7, it can be seen that the average soluble Fe^{2+} level in the feed and effluent increased from 11.4 μgFe^{2+}/gTSS and 6.9 μgFe^{2+}/gTSS (Stage 2) to 22.2 μgFe^{2+}/gTSS and 14.0 μgFe^{2+}/gTSS (Stage 3) ($p < 0.05$), respectively. However, these levels of Fe^{2+} in Stage 3 are still below those at Stage 1 i.e. 29.1 μgFe^{2+}/gTSS (influent) and 25.4 μgFe^{2+}/gTSS (effluent). These findings show that the

complexation of Fe^{2+} and EDDS in the feed was able to prevent Fe^{2+} from precipitating with the sulfide, hence improving Fe^{2+} solubility.

Results from BCR extraction showed a reverse trend in Fe^{2+} speciation when the Fe^{2+} was added to the feed as a complex with EDDS (Figure 6.8). The most bioavailable fractions of Fe^{2+}, i.e. Soluble (Sol) and Exchangeable (F2), increased slightly from 0.09% and 5.0% in Stage 2 to 0.2% and 9.9% in Stage 3 ($p < 0.05$), respectively, while the dominant fraction of Fe-Mn oxides (F2) was relatively stable, i.e. 40.2% (Stage 2) and 39.6% (Stage 3) ($p > 0.05$). The last two fractions which are considered to be the least bioavailable, i.e. Organic matters/sulfides (F3) and Residual (F4), reduced from 15.0% and 39.8% (Stage 2) to 12.9% and 37.5% (Stage 3) ($p < 0.05$), respectively. The extraction results show that when complexed with EDDS, the speciation of Fe^{2+} was shifted towards more bioavailable fractions, i.e. Soluble and Exchangeable, and hence the bioavailability of Fe^{2+} for microbial uptake was improved. Other TMs (Co, Ni, Zn, Mn and Mo), although not added to the reactor, had accumulated in the seed sludge, and their speciation results showed that the addition of EDDS also slightly increased their soluble fraction, however, there was no statistically significant difference in other fractions from the BCR extraction (data not shown).

A mass balance of soluble EDDS in the reactor determined using UHPLC is shown in Table 6.3. It can be seen that the sum of free EDDS and EDDS in the Fe-EDDS complex was up to about 84% of the total soluble EDDS measured in the reactor. The concentration of free EDDS and Fe-EDDS complex in the influent, reactor, and effluent of the SAMBR is shown in Tables 6.4 and 6.5. Since EDDS was added as a complex to the feed (Fe-EDDS), it was expected that there would be very low concentrations of free EDDS detected. Table 6.4 shows that the concentration of free EDDS in the influent was very low, i.e. 0.04 – 0.07 mg/L, which is about 5% of the total EDDS added to complex with Fe^{2+}. The concentration of free EDDS in the reactor and

effluent, therefore, was also not significant, i.e. $0 - 0.02$ mg/L. The average concentration of EDDS as the Fe-EDDS complex in the influent was 1.13 mg/L, which was 86.2% of the total added EDDS (Table 6.5). The average equivalent concentration of Fe^{2+} in the Fe-EDDS complex in the feed was 0.15 mg/L (9.52 µg/TSS), which is 85.6% of the total soluble Fe^{2+} measured in the feed. In addition, this level of Fe^{2+} present in the complex (0.15 mg/L = 0.05 mg/gTSS.d) was about 25.4% of the total amount of Fe^{2+} added to the feed (10 mg/L = 0.22 mg/gTSS.d). These results could explain why the average soluble concentration of Fe^{2+} in the influent increased in Stage 3 as greater amounts of Fe^{2+} were kept in solution. From the previous studies carried out in a continuous system (Fermoso et al., 2008c), it was expected that the retention of the Fe-EDDS complex by the SAMBR was not very high due to its high solubility, i.e. up to 83% of the complex in the reactor left in the effluent. However, the results of this experiment showed that the effect of complexation on the bioavailability of Fe^{2+} was still significant, as the majority of added EDDS was able to compete with sulfide and keep Fe^{2+} in a soluble form for microbial uptake.

Table 6.3   Mass balance of soluble EDDS in the reactor (mg/L) using UHPLC in the SAMBR at Stage 3 (Fe, EDDS, and S) (n = 3).

Day	22	24	26	28	30
Free EDDS	0.06	0.07	0.04	0.04	0.05
EDDS as Fe-EDDS	0.02	0.03	0.01	0.02	0.02
Total EDDS	0.01	0.02	0.00	0.01	0.01
Recovery (%)	79.24	71.30	76.43	84.35	72.69

Table 6.4   Measurement of free EDDS (mg/L) using UHPLC in the SAMBR at Stage 3 (Fe, EDDS, and S) (n = 3).

Day	22	24	26	28	30
Influent	0.06	0.07	0.04	0.04	0.05
Reactor	0.02	0.03	0.01	0.02	0.02
Effluent	0.01	0.02	0.00	0.01	0.01

Table 6.5   Measurement of Fe-EDDS complex (mg/L) using UHPLC in the SAMBR (Fe, EDDS, and S) at Stage 3 (n = 3).

Day	22	24	26	28	30
Influent	0.84	1.13	1.19	1.17	1.31
Reactor	0.75	0.72	0.78	0.82	0.79
Effluent	0.61	0.59	0.63	0.68	0.65

6.4   Conclusions

The results from this study showed that the presence of sulfide in the feed resulted in a decrease in Fe^{2+} bioavailability as Fe^{2+} speciation was shifted towards the less bioavailable fractions, i.e. organic matter/sulfide and residual, which in turn reduced the performance of the SAMBR. When EDDS and Fe^{2+} were dosed at a 1:1 molar ratio (10 mg/L Fe^{2+}) as a complex, the shift in Fe^{2+} speciation was reversed. When applied to the SAMBR, its performance was improved as both COD removal and daily methane yields increased significantly.

Chapter 7

Effects of trace metal deficiency and supplementary on a submerged anaerobic membrane bioreactor

Outline:

7.1 Introduction

7.2 Experimental and analytical methods

7.3 Results and discussion

7.4 Conclusions

Note: this chapter was published in *Bioresource Technology*, 2017, volume 241, pages 161-170.

7.1 Introduction

The importance of trace metals (TMs) has been emphasized in the literature. In anaerobic treatment processes, some types of wastewater contain a sufficient level of these TMs, such as swine wastewater (Cestonaro do Amaral et al., 2014), whereas TM supplementation is required for other types of wastewater, eg. methanol wastewater (Fermoso et al., 2008a), maize silage (Evranos & Demirel, 2014), and wheat stillage (Schmidt et al., 2014). When TMs are not supplemented for these wastewaters this will result in nutrient deficiency for the anaerobic microorganisms, and this is known to increase the minimum generation time of methanogens, decrease rates of substrate utilization, and can ultimately lead to process failure (Worm et al., 2011).

In term of dosing strategies, TMs can be added to the reactor continuously (Zandvoort et al., 2002), pre-loaded (Zandvoort et al., 2004) or pulsed (Zandvoort et al., 2004; Fermoso et al., 2008c). A continuous addition of $CoCl_2$ at low levels (0.62 µmol/L.d) to a methanol-fed UASB reactor could only increase the specific methanogenic activity (SMA) up to 215 mgCOD-CH_4/gVSS.d (Zandvoort et al., 2002). The pre-loading strategy involved contacting the inoculum for a UASB reactor with a 1mM cobalt solution ($CoCl_2.6H_2O$) for 24 h at 30ºC in a serum bottle in the absence of substrate, and was found to be able to control Co limitations in the methanol-fed UASB reactor, but it resulted in very high levels of Co being lost in the effluent (Zandvoort et al., 2004). Fermoso et al. (2008c) investigated the feasibility of pulse dosing of a low quantity of $CoCl_2$ (5 µmol/L) to a USAB reactor, a compromise strategy between continuous and pre-loading, to control metal limitations. They found that without having to supplement Co continuously, around 90% of the Co was retained in the granular sludge matrix.

The aim of this study was, therefore, to evaluate the effect of TM deficiency on the performance of a SAMBR by excluding the TMs from the influent. Consequently, rapid addition of a mixture of TMs as a pulse to the SAMBR was investigated to determine if this could be an effective dosing strategy to overcome nutrient deficiency. Changes in metal content, speciation and bioavailability over time during the nutrient deficiency and metal dosing period were monitored to evaluate their correlation with changes in SAMBR performance.

7.2 Experimental and analytical methods

7.2.1 Trace metal deficiency

Before starting the nutrient deficiency experiment, the SAMBR was operated at steady state (normal) conditions, i.e. 6 h HRT, 100 d SRT and 500 mgCOD/L feed, with TMs supplemented at concentrations specified in Table

3.1 (Section 3.4) for 76 d. From day 15 onwards, no TMs were added to the feed and the SAMBR was allowed to operate under nutrient deficient conditions for 24 d (day 17 to day 41). Samples were collected during each period to analyse for reactor performance and TM speciation.

7.2.2 Trace metal dosing strategies

The pulse dosing of TMs was carried out in three phases, with the metal concentrations given in Table 7.1. In Phase 1, on day 43 after the nutrient deficiency, TMs were dosed at 5 times of their daily dosing amounts at normal operating conditions to determine if the performance of the SAMBR can be restored. In Phase 2, on day 51, the same amount of TMs in Phase 1 was added again to confirm if a second pulse could have the same effect as the first. In Phase 3, on day 59, a third pulse was added at 10 times the daily loading of TMs to test the limit of anaerobic microorganisms to a high level of TMs. The TM solution was prepared in advance and added directly to the SAMBR; samples were collected regularly during each pulse to analyse for reactor performance and TM speciation.

Table 7.1   Concentrations of TMs for each pulse (mg/L).

Day	43 - 50	51 – 58	59 - 72
	1st pulse (5x)	2nd pulse (5x)	3rd pulse (10x)
Fe	2.75	2.75	5.51
Co	0.44	0.44	0.88
Ni	0.08	0.08	0.17
Zn	0.06	0.06	0.13
Mn	0.13	0.13	0.26
Mo	0.07	0.07	0.14

5x (5 time) and 10x (10 time) the daily metal mass loading at steady state conditions

7.3 Results and Discussion

7.3.1 Trace metal deficiency

7.3.1.1 The performance of the SAMBR

During normal conditions, the continuous addition of TMs for more than 2 months (76 d) clearly resulted in a positive effect on the performance of the SAMBR. Within the sampling period (last 16 d), the SAMBR was able to achieve very high treatment efficiency, i.e. 96.8% COD removal, with the soluble COD concentration in the reactor (supernatant) and effluent being relatively stable with an average value of 59.0 mg/L and 16.0 mg/L, respectively, as shown in Figure 7.1. The difference between the soluble COD level in the reactor and in the effluent was due to the membrane which retained a large amount of dissolved COD by the thin polarisation layer, or cake, on the membrane surface. The flux of the membrane in the SAMBR was kept at 15 L per square meter per hour (LMH) throughout the experiment which was much below the critical flux (24 LMH). The transmembrane pressure (TMP) was measured continuously during the whole experiment and its value was fairly constant ranging from 3.52 kPa to 3.69 kPa, hence, the cake layer formation was relatively stable. Therefore, the changes in COD level and its difference in the reactor and effluent was only due to the effects of different metal dosing stages (deficiency or pulsing).

However, 9 days after the TMs were excluded from the feed (day 25), the soluble COD concentration in the reactor and effluent rose significantly to 79.32 mg/L and 36.28 mg/L ($p < 0.05$), respectively. On day 41 (25 days after TM exclusion), these concentrations increased further to 94.2 mg/L and 50.4 mg/L ($p < 0.05$), which are equivalent to a 59.3% and 233% increase compared to these concentrations under "normal" conditions. As a result, the COD removal dropped, but still maintained a relatively acceptable level, i.e. 89.9% ($p < 0.05$).

The VFA profile in comparison to the soluble COD in the reactor and effluent is shown in Figures 7.2 and 7.3. Similar to the COD profile, there were concentration differences between the reactor VFAs (as COD), and the effluent VFAs (as COD) from the reactors. The cake gel layer, acting as a secondary dynamic membrane, appeared to have made a significant contribution to reducing the VFAs passing through the membrane. It can be seen that with the supplementation of TMs, only very low concentrations of VFAs were detected in the reactor (up to 2 mg COD /L), and none were found in the effluent. This indicates that the amount of TMs added was sufficient to support the growth of the anaerobic microorganisms to convert all the incoming substrate COD into biomass, CH_4 and CO_2.

However, the absence of TMs had a significant effect on the performance of the SAMBR as the soluble COD concentration in the reactor and effluent increased, and the VFAs also increased accordingly. A VFA peak was measured on day 41 in both the reactor and effluent, i.e. 25.5 mg/L as COD (27.0% of reactor COD) ($p < 0.05$) and 4.9 mg/L as COD (9.7% of effluent COD) ($p < 0.05$), respectively. The accumulation of VFAs due to TM deficiency in anaerobic systems has been reported in several studies (Worm et al., 2009; Banks et al., 2012; Schmidt et al., 2014). It is clear from the literature that there are kinetic differences between different groups of anaerobic bacteria, and since the methanogens have very slow growth rates, and strict growth requirements, the fast growing acidogenic and acetogenic bacteria compete more efficiently for the available metal nutrients (White & Stuckey, 2000). Ketheesan et al. (2016) observed the accumulation of VFAs in Fe-deficient anaerobic reactors, and postulated that the competition for TMs by those groups of bacteria can create a temporary nutrient imbalance, which is bound to affect the methanogens primarily, compounding the problem of the accumulation of intermediates, i.e. VFAs.

Figures 7.2 and 7.3 also show that the soluble COD in the reactor and the effluent, respectively, was considerably higher than the VFAs concentration as

COD. This implies that VFAs were not the only contributors to the soluble COD, but also other organic compounds such as soluble microbial products (SMPs). Bacteria are known to excrete SMPs into solution due to biomass decay and substrate metabolism (Barker & Stuckey, 1999), and it has been confirmed that metal nutrient deficiency is one of the factors that result in increased SMP production in anaerobic reactors (Barber & Stuckey, 2000; Aquino & Stuckey, 2003).
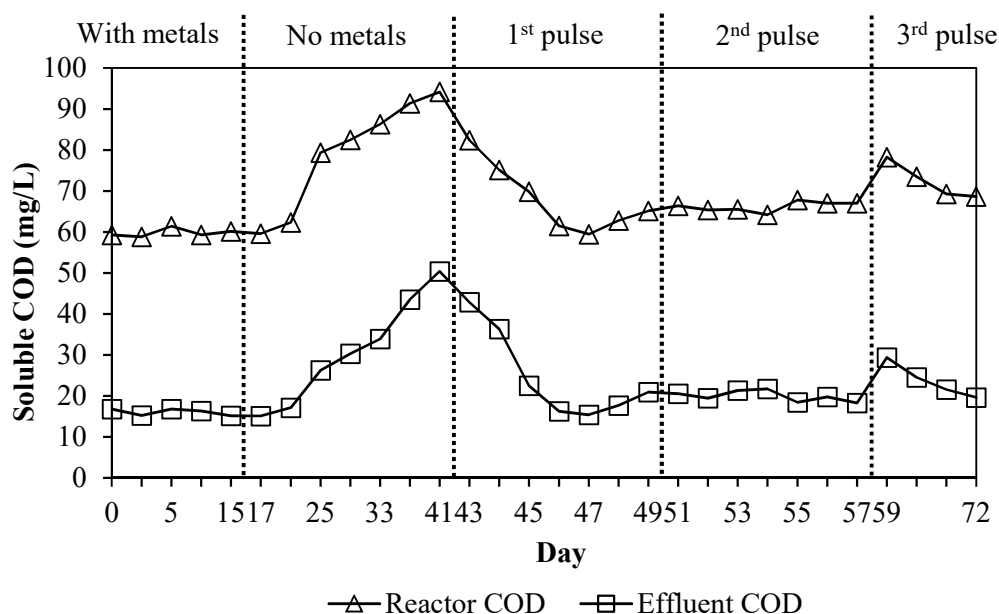
Figure 7.1   Profile of soluble COD in reactor and effluent and the methane yields from the SAMBR (n = 3).
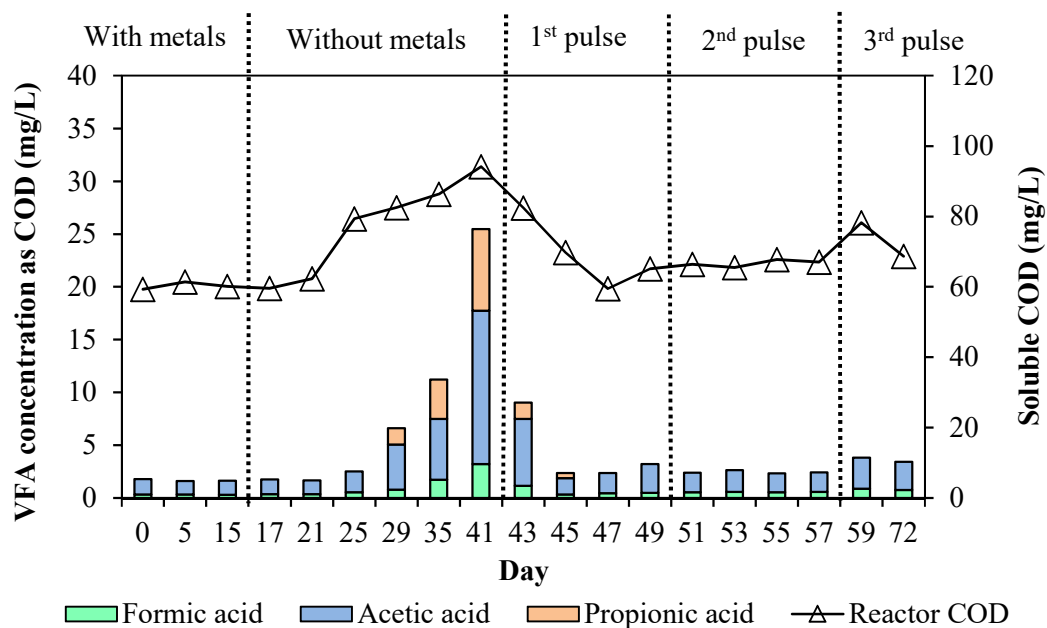
Figure 7.2   VFA concentrations as COD (left), and Reactor COD (right) of
the SAMBR (n = 3).
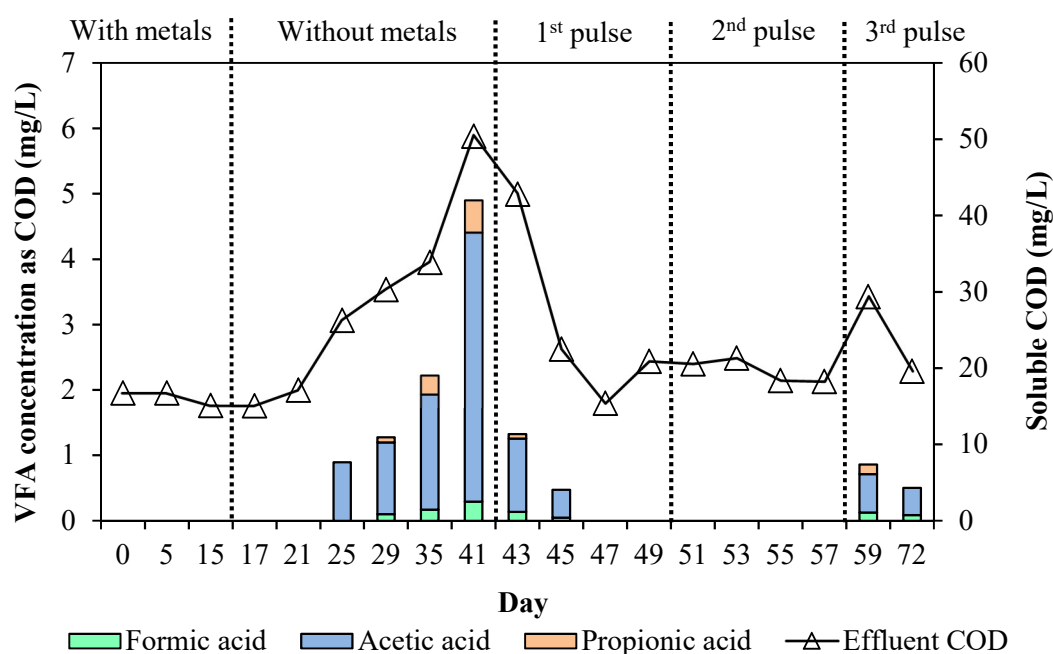
Figure 7.3   VFA concentrations as COD (left), and Effluent COD (right) of
the SAMBR at different phases (n = 3).

Figure 7.4 shows the profile of methane yield during normal TM deficiency and system recovery periods. With the supplementation of TMs, the methane yield from the SAMBR were relative stable at an average of 80.4 mLCH$_4$/gVSS.d (p > 0.05); around 87% of the theoretical yield (92.4 mLCH$_4$/gVSS.d at 35°C) was achieved, with the balance due to cell growth, accumulation of biomass in the reactor, and loss of methane in the effluent which can be up to 17% (Kunacheva et al, 2017). However, the methane yields dropped abruptly by around 38% to 50.5 mLCH$_4$/gVSS.d (p < 0.05) on day 41 due to the absence of TMs in the influent. This result clearly reflected the negative influence of TM deficiency on the methanogens as it limited their growth, and hence reduced their ability to convert the influent COD into biomass, CH$_4$ and CO$_2$.
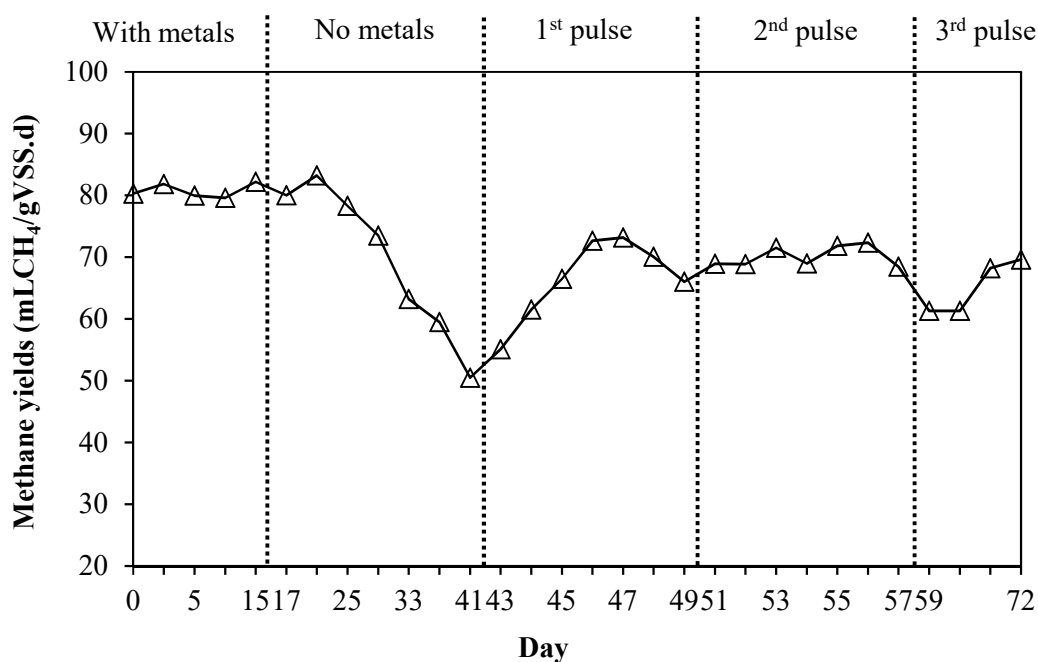
Figure 7.4   Methane yields of the SAMBR at different phases (n = 3).

7.3.1.2   The speciation of trace metals

Table 7.2 shows the content of TMs in the influent, reactor, and effluent during different phases of the study. During the first phase (with metals), the average total metal content (sum of all BCR fractions) in the reactor (µg/gTSS) (in comparison with the metal content in the feed (µg/gTSS)) was in the following order: Fe (0.9%) > Co (0.6%) > Zn (0.1%) > Ni (0.4%) > Mo (0.7%) > Mn (1.7%). These results showed that the level of metal content in the reactor varied between metals, and this was almost certainly due to the different physicochemical properties of the metals, and also their supplemented concentrations in the feed. Since the reactor had been operated with the continuous supplementation of TMs for a long period of time (5 months in total and steady state for 76 days), and with long retention of biomass in the reactor (100 d SRT), this could have resulted in the substantial accumulation of TMs in the reactor in both the cells and as precipitates. The initial content of these metals in the seed sludge at the beginning of this experiment was quite high; the highest was for Fe (6387 µg/gTSS or 114.4 µmol/gTSS) while the lowest was for Mn (157.8 µg/gTSS or 2.9 µmol/gTSS). Compared to the metal content in the sludge, the level of metals added to the feed was very low i.e. from 0.1% for Zn up to 1.7% for Mn, but due to the complexity of anaerobic sludge and its environment, the total metal content in the sludge is not a good indicator of metal bioavailability and mobility (Zandvoort et al., 2006a).

Table 7.2 also shows that the effluent concentration of all TMs was always lower than the influent, which explains the increase in total metal content in the reactor based on a mass balance. The accumulation rate of metals (µg/gTSS.d) is given in Table 7.3, and occurs in the following order: Fe (2.5%) > Zn (25.4%) > Co (2.4%) = Mn (8.1%) > Mo (11.9%) > Ni (4.2%). The percentage is the ratio between the average metal accumulation rate in the reactor (µg/gTSS.d), and the average metal content added in the feed (µg/gTSS.d), and this indicates that Zn and Fe/Co had the highest and lowest affinity towards the biomass in the reactor, respectively.

Table 7.2   Metal content (µg/gTSS) in the influent, reactor, and effluent from the SAMBR at different phases (n = 3).

Fe	With metals			No metals				1st pulse			2nd pulse			3rd pulse		
Day	**0**	**5**	**15**	17	25	33	41	**43**	**45**	**49**	51	53	57	**59**	**65**	**72**
Influent	57.1	59.2	60.5	0.0	0.0	0.0	0.0	290.0	0.0	0.0	285.4	0.0	0.0	570.9	0.0	0.0
Reactor	6386.8	6425.4	6477.3	6401.6	6314.9	6196.4	6072.5	6192.6	6114.8	6080.7	6265.2	6215.9	6192.2	6687.3	6470.8	6266.5
Effluent	25.9	26.2	26.3	26.9	27.3	27.5	29.2	41.3	32.9	29.4	46.8	28.4	24.9	61.8	42.1	28.4

Co	With metals			No metals				1st pulse			2nd pulse			3rd pulse		
Day	**0**	**5**	**15**	17	25	33	41	**43**	**45**	**49**	51	53	57	**59**	**65**	**72**
Influent	9.1	9.5	9.7	0.0	0.0	0.0	0.0	46.4	0.0	0.0	45.7	0.0	0.0	91.4	0.0	0.0
Reactor	1568.5	1572.6	1582.7	1557.0	1504.6	1407.1	1239.1	1262.4	1244.5	1177.1	1215.5	1189.9	1170.0	1249.9	1184.0	1140.8
Effluent	2.4	2.4	2.6	2.7	2.9	3.3	3.8	5.5	4.3	3.2	5.6	4.1	2.8	6.4	4.6	3.4

Ni	With metals			No metals				1st pulse			2nd pulse			3rd pulse		
Day	**0**	**5**	**15**	17	25	33	41	**43**	**45**	**49**	51	53	57	**59**	**65**	**72**
Influent	1.7	1.8	1.8	0.0	0.0	0.0	0.0	8.7	0.0	0.0	8.6	0.0	0.0	17.2	0.0	0.0
Reactor	429.8	431.6	434.8	414.6	391.1	351.9	295.2	301.3	296.4	289.9	296.0	291.4	288.9	301.7	289.1	273.7
Effluent	0.3	0.4	0.6	0.6	0.8	0.9	0.6	1.4	1.1	0.8	1.4	0.9	0.6	2.7	2.1	1.2

Zn	With metals			No metals				1st pulse			2nd pulse			3rd pulse		
Day	**0**	**5**	**15**	17	25	33	41	**43**	**45**	**49**	51	53	57	**59**	**65**	**72**
Influent	1.3	1.4	1.4	0.0	0.0	0.0	0.0	6.7	0.0	0.0	6.6	0.0	0.0	13.2	0.0	0.0
Reactor	1273.1	1286.4	1296.1	1288.6	1254.9	1219.3	1123.8	1129.2	1128.1	1115.5	1121.6	1113.8	1100.9	1111.7	1099.8	1068.2
Effluent	0.2	0.2	0.3	0.4	0.5	0.7	0.8	1.2	0.7	0.5	0.9	0.6	0.4	1.8	1.2	0.5

Mn	With metals			No metals			1st pulse			2nd pulse			3rd pulse		
Day	**0**	**5**	**15**	17	25	33	41	**43**	**45**	**49**	51	53	57	**59**	**65**	**72**
Influent	2.7	2.8	2.8	0.0	0.0	0.0	0.0	13.6	0.0	0.0	13.4	0.0	0.0	26.8	0.0	0.0
Reactor	157.8	160.8	171.4	165.7	146.2	123.1	103.6	107.9	97.9	91.5	101.0	100.5	95.2	103.8	93.8	86.7
Effluent	0.7	0.8	0.8	0.9	1.1	1.2	1.4	2.5	1.8	1.3	2.6	2.1	1.4	3.6	2.8	1.6

Mo	With metals			No metals			1st pulse			2nd pulse			3rd pulse		
Day	**0**	**5**	**15**	17	25	33	41	**43**	**45**	**49**	51	53	57	**59**	**65**	**72**
Influent	1.4	1.5	1.5	0.0	0.0	0.0	0.0	7.3	0.0	0.0	7.2	0.0	0.0	14.3	0.0	0.0
Reactor	216.5	219.2	226.3	219.7	199.6	180.7	141.5	143.7	138.9	132.8	136.6	131.3	129.4	142.0	136.6	130.8
Effluent	0.4	0.5	0.7	0.7	0.9	1.2	1.4	2.3	1.5	1.1	1.5	1.3	0.9	2.9	2.1	1.5

Table 7.3   The accumulation and leaching rate of TMs (µg/gTSS.d) compared to the loading rate of TMs (µg/gTSS.d) at different phases (n = 3)

Metal	Accumulation rate/loading rate (µg/gTSS.d)	Leaching rate/loading rate (µg/gTSS.d)			
	With metals	**No metals**	**1st pulse**	**2nd pulse**	**3rd pulse**
Fe	6.0/235.8 (2.5%)	13.2/0.0	16.0/41.0 (39%)	10.4/41.3 (25%)	30.1/39.9 (75%)
Co	0.9/37.7 (2.4%)	12.7/0.0	12.2/6.6 (185%)	6.5/6.6 (98%)	7.8/6.4 (122%)
Ni	0.3/7.1 (4.2%)	4.8/0.0	1.6/1.2 (133%)	1.0/1.2 (83%)	2.0/1.2 (167%)
Zn	1.5/5.9 (25.4%)	6.6/0.0	2.0/0.9 (222%)	3.0/0.9 (333%)	3.1/0.9 (344%)
Mn	0.9/11.1 (8.1%)	2.5/0.0	2.3/1.9 (121%)	0.8/2.0 (40%)	1.2/1.9 (63%)
Mo	0.7/5.9 (11.9%)	3.1/0.0	1.6/1.0 (160%)	1.0/1.1 (91%)	0.8/1.0 (80%)

The accumulation of TMs, or the metal retention capacity, in an anaerobic reactor depends on their reactions in anaerobic sludge, which involves several mechanisms, namely, complex formation, chelation of metals, ion exchange, adsorption, inorganic micro-precipitation and translocation of TMs into the cells (Zandvoort et al., 2006a). Therefore, it is very difficult to determine the ultimate fate of metals in anaerobic reactors as these mechanisms are complex and compete with each other, and often exhibit non ideal behaviour; hence, theoretical modelling of equilibrium concentrations often fail to correlate well with actual measurements (Ortner et al., 2014). There has been a limited number of studies carried out to determine the adsorption capacity and kinetics of metal precipitation, and these are only for a few TMs such as Co, Ni, and Zn

(van Hullebusch et al., 2004; van Hullebusch et al., 2005a; Hawari & Mulligan, 2006). Between Co and Ni, there was no significant difference in the adsorption capacity of granular sludge (van Hullebusch et al., 2004), while Ni and Zn were found to have a very low affinity for anaerobic sludge sites (Leighton & Forster, 1997; Artola et al., 2000; Wang et al., 2007).

When all TMs were excluded from the feed, the SAMBR was operating under nutrient deficient conditions. The results show that the metal content in the reactor reduced immediately, which was probably due to the leaching of metals from the sludge, but also as a result of some "dilution" due to bacterial growth which has to be supported by the metals available in the inoculum sludge (Zandvoort et al., 2003). In this study, there was no increase in biomass concentration in the reactor during the metal deficient period ($p > 0.05$), and hence TM washout was the only cause of the reduction in the total metal content in the reactor, which increases the metal content in the effluent. The leaching rate of the metal content (μg/gTSS.d) is also presented in Table 7.3, and was found to be of the same order as for the total metal content in the reactor, i.e. Fe > Co > Zn > Ni > Mo > Mn. However, the leaching rate as a percentage of the metal loading rate (μg/gTSS.d) was ordered differently, i.e. Zn > Co > Mo > Ni > Mn > Fe, which was due to the different reactions (complexation, precipitation, adsorption, absorption) of different trace metals in anaerobic digesters.

It can also be seen that the order of the leaching rate (μg/gTSS.d) for all metals was the same as for their total content in the reactor. Zandvoort et al. (2006a) also found that metal leaching from the reactor/biomass (Fe, Co, and Ni) was proportional to their initial concentrations in the sludge when their supplementation was stopped. In another study, Fe and Ni loss was found to greatly exceed the "dilution" effect resulting from biomass growth, despite increases in sludge bed volume (Zandvoort et al., 2003).

Most of the previous studies on the deficiency of TMs were carried in up-flow anaerobic sludge blanket (UASB) reactors. Fermoso et al. (2008a) found

that, in the same period in their study (around 30 days), Co, Ni, Zn, and Fe were lost from the granular sludge in UASB reactors through the effluent at a rate of around 2, 2.8, 17 and 184 μg/gTSS.d, respectively. The leaching rate of these metals in this study was 12.7 (Co), 4.8 (Ni), 6.6 (Zn), and 13.2 (Fe) μg/gTSS.d, which showed that Zn and Fe were better retained in the SAMBRs.

Table A.2 (Appendix) gives the metal content (μg/gTSS) in each fraction from the BCR extraction, while Figure 7.5 show the speciation of TMs in both the liquid and solid phases in the reactor. Generally, during normal operating conditions, TMs were present in a soluble form in extremely low concentrations (<0.18% of the total metal content) in all cases, except for Mn and Mo as their soluble concentrations reached to 18.7% and 5.01%, respectively. However, when the reactor was in a nutrient deficient mode, a reduction in soluble concentration was observed for several TMs i.e. 12.84% to 10.64% (Mn) ($p < 0.05$), and 5.01% to 3.19% (Mo) ($p < 0.05$), and clearly the cells were abstracting as much of the TMs in solution as they ran out internally.

In the solid phase, most of the TMs were present mainly in the organic matter/sulfide fraction (F3) of around 74% (Co), 57% (Ni), 59% (Zn), 25% (Mn), and 61% (Mo). This fraction contributes significantly to the accumulation of TMs in the reactor, as metals are known to be better retained in the sludge due to sulfide precipitation (Gonzalez-Gil et al., 2003). Callander and Barford (1983b) showed that metals such as Co and Ni can be precipitated in their sulfide form as long as the total concentration of these metals does not exceed the total sulfide ($H_2S + HS^- + S^{2-}$) level. Hydrogen sulfide and organic matter including thiol functionalities are known to have a high affinity for Co and Ni (Smith et al., 2002) The exception was for Fe as its Fe-Mn oxide fraction (F2) accounted for around 70% of its total content; van Hullebusch et al. (2005b) also found the highest amount of Fe present in this fraction (up to 34%). One hypothesis is that the formation of ferrous phosphate or ferrous-hydroxyl-phosphate compounds (possible amorphous; as suggested by (Smith & Carliell-

Marquet, 2009)) that would then be readily dissolved by the low pH (2) of the extracting reagent used in the 2nd BCR step.

The BCR method (Table 3.2) was developed to categorize metals into different extraction fractions according to their level of bioavailability; therefore, the first two fractions, i.e. Soluble (Sol) and Exchangeable (F1) are considered as the most bioavailable, while the last two fractions, i.e. Organic matter/sulfide (F3) and Residual (F4), are the least bioavailable. For all the metals, the sum of Sol and F1 was always lower than the sum of F3 and F4 ($p < 0.05$), indicating that under all conditions, most of the TMs in the reactor had a low bioavailability for microbial uptake. The high metal content in the last two fractions was possibly due to the inevitable presence of inorganic ligands in the reactor such as sulfide, phosphate, or carbonate which encourages the precipitation of metals, hence making them become less soluble (Speece, 1996). Since they have low solubility products, this results in their precipitation and hence reduces their free ion concentration (Thanh et al., 2016).

As the total content of TMs in the reactor decreased during nutrient deficiency, their speciation shifted as most of the metals were mobilized from the relatively bioavailable fractions, i.e. Sol and F1, towards less bioavailable fractions i.e. F3 and F4. The increase in the sum of the F3 and F4 fractions was in the following order: Mo (4.23%) > Ni (3.96%) > Fe (2.79%) > Mn (2.16%) > Zn (1.85%) > Co (1.29%), however, only the increases in Mo, Ni, Fe, and Mn were statistically significant ($p < 0.05$). These results indicate that the loss in total metal content in the reactor was coming from the fractions that were highly soluble and easy to wash out from the sludge, i.e. Soluble and Exchangeable, and also because metals were better retained in the last two fractions.

The results from this experiment showed that the omission of TMs from the feed to the SAMBR resulted in a reduction in reactor performance, i.e. a decrease in COD removal and methane yields, and an increase in VFA concentrations. Previous studies on the effect of trace metal deficiency in

anaerobic reactors also showed similar findings, however, they all focussed on individual TMs such as Co (Zandvoort et al., 2002; Zandvoort et al., 2006b), Ni (Fermoso et al., 2008d), and Zn (Fermoso et al., 2008a). In addition, these studies were carried out in UASB reactors, which might not have the same TM retention capacity as the SAMBR used in this study as the presence of a membrane helps to prevent the loss of solids from the reactor. The fate of TMs when they leached out from the reactor was also monitored constantly throughout this study.

The speciation results from BCR extraction showed that the total TMs accumulated in the reactor were depleted during the metal deficiency, and their leaching rates varied due to their different degrees of affinity/reactions in the anaerobic sludge, while their speciation shifted towards less bioavailable fractions. Therefore, a quick dosing strategy was assessed to determine whether this could help the reactor overcome its nutritionally deficient conditions.

Iron (Fe)
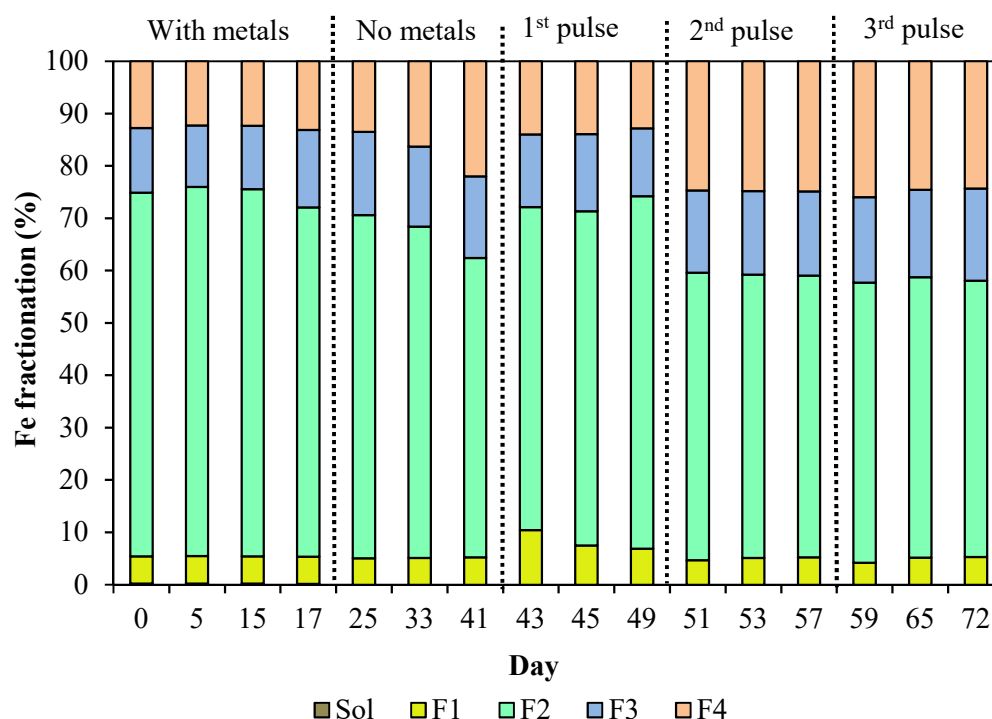
Cobalt (Co)

Nickel (Ni)

Zinc (Zn)

Manganese (Mn)

Molybdenum (Mo)

Sol - Soluble, F1 - Exchangeable, F2 - Fe/Mn oxides,

F3 - Organic matter, F4 - Residual
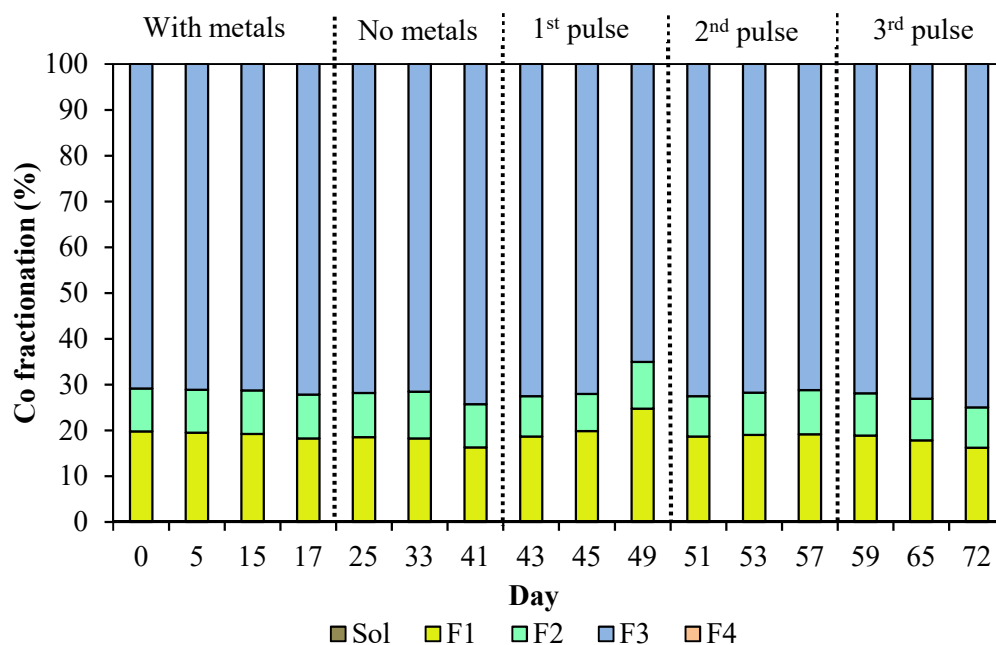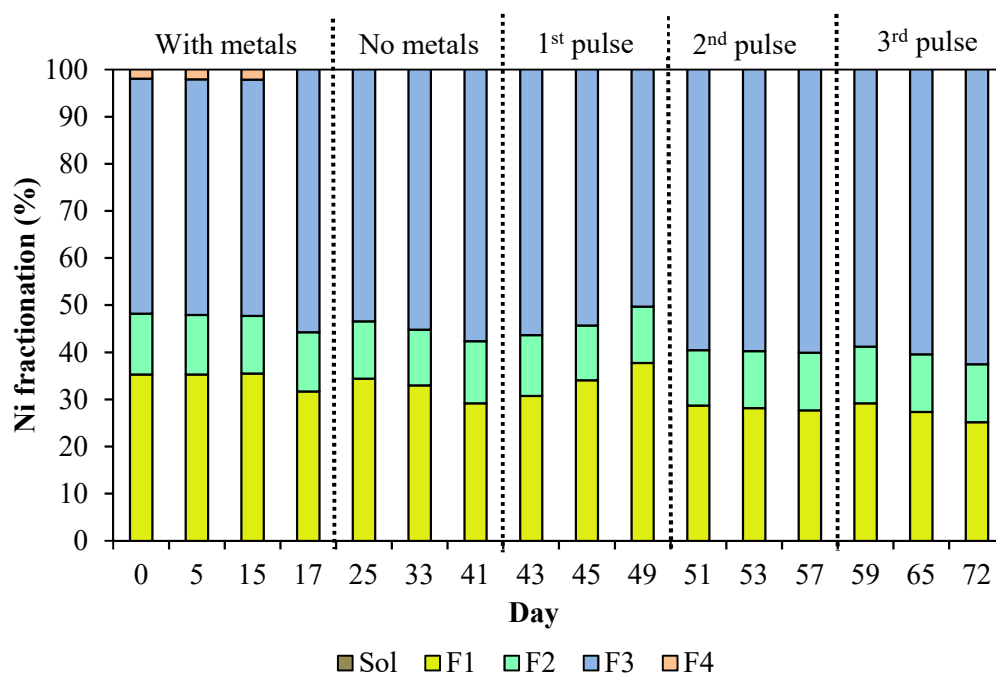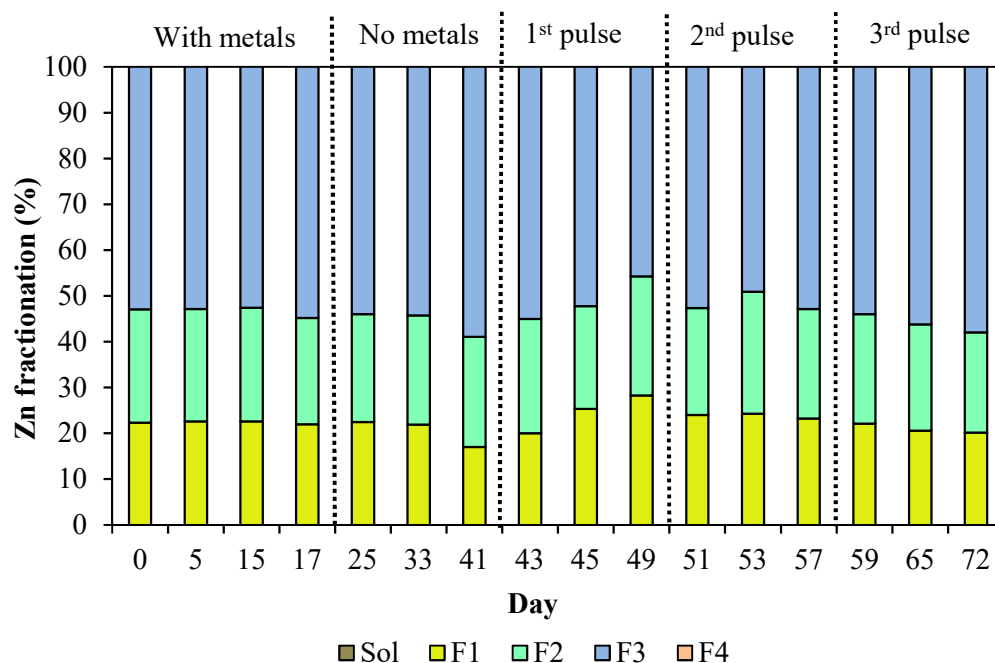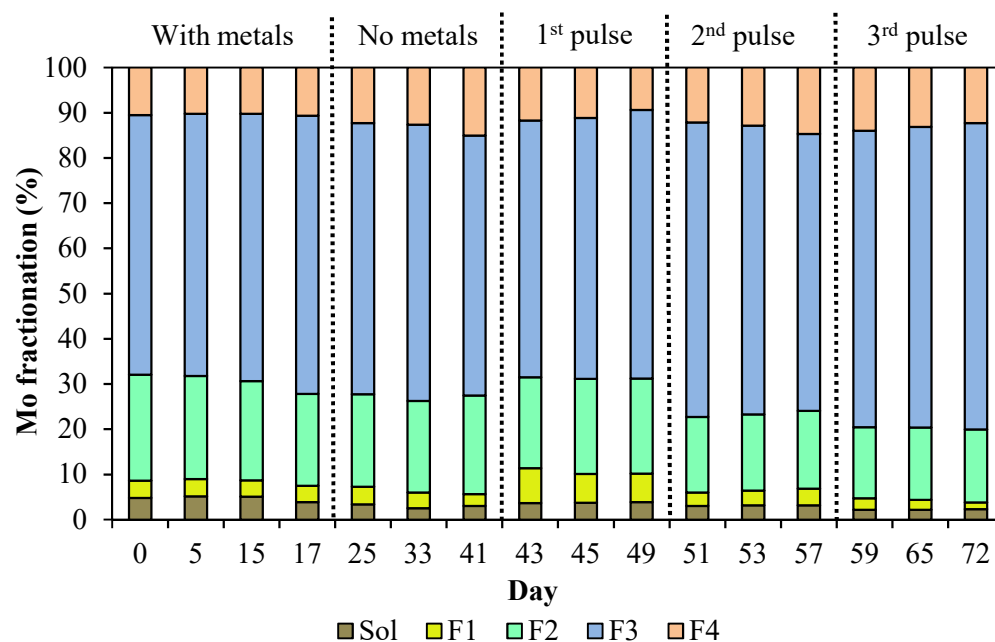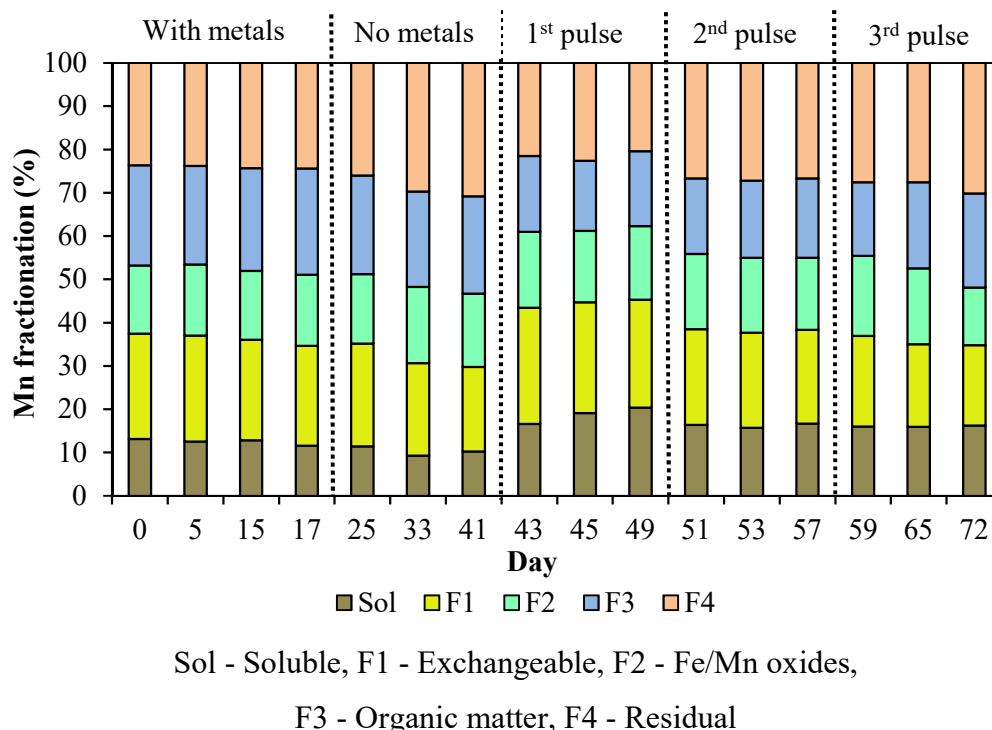
Figure 7.5   Speciation of TMs in the SAMBR at different phases (n = 3).

7.3.2 Trace metal dosing strategies

7.3.2.1   The performance of the SAMBR

Following the first pulse of TMs (from day 43 to 49) after their exclusion from the feed, the performance of the SAMBR improved within 4 days as the COD removal increased substantially from 89.9% to 96.9% ($p < 0.05$) on day 47. The corresponding soluble COD concentrations in the reactor and effluent also reduced to similar levels as normal conditions, i.e. 59.5 mg/L and 15.4 mg/L ($p < 0.05$), respectively (Figure 7.1). The VFA levels in the reactor and effluent also reduced to below 2 mgCOD/L and 0 mgCOD/L ($p < 0.05$) 4 days after the pulse (Figures 7.2 and 7.3), indicating that the added TMs enhanced the degradation of the accumulated VFAs during metal deficiency. Ketheesan et al. (2016) also found the addition of Fe to a Fe-deficient reactor helped to

degrade acetate which was added as a substrate, and resulted in a 2.1-3.9-fold increase in the rate of methane production. The methane yields also increased by around 45% from 50.5 mLCH$_4$/gVSS.d (day 41 – end of no metal phase) to 73.2 mLCH$_4$/gVSS.d ($p < 0.05$) (day 47) (Figure 7.4). However, from day 49 the SAMBR performance reduced slightly as the soluble COD increased slightly to 65.2 mg/L and 20.9 mg/L ($p < 0.05$), which led to a decrease in COD removal to 95.8% (Figure 7.1), and the methane yield also reduced slightly to 66.0 mLCH$_4$/gVSS.d ($p < 0.05$) (Figure 7.4). These results indicated that although the improvement in SAMBR performance was substantial after the pulse addition, the effect of TMs was only temporary as after 9 d the pulsed TMs should have been completely washed out from the reactor, and the reactor returned to the previous metal deficient condition.

A second pulse with the same TM loading was then applied on day 51. However, there was no significant improvement in the SAMBR performance after the first pulse as the average COD removal remained at around 96% ($p > 0.05$) and the methane yield did not change significantly i.e. 68 mLCH$_4$/gVSS.d (day 57) ($p > 0.05$) (Figure 7.4). The main difference between the first and second pulse was the accumulated COD level in the reactor prior to the pulse: in the first pulse 94.16 mgCOD/L accumulated, however, only 65.18 mgCOD/L accumulated in the second pulse. Therefore, the improvement on the SAMBR performance was more significant after the first pulse. This finding was not in agreement with Fermoso et al. (2008c) as they found that the specific methanogenic activity (SMA) of a Co deficient USAB increased even after the second pulse of CoCl$_2$.

The third pulse with 10 times TM loading was applied on day 59. One day after the pulse, the methane yields dropped to only 61.3 mLCH$_4$/gVSS.d ($p < 0.05$) (Figure 7.4), which was 24% below the level under "normal conditions" with TMs in the feed. Due to this low methane yield, the soluble COD in the reactor and effluent increased from 67.1 mg/L and 18.3 mg/L (day 57 – 2nd pulse) to 78.3 mg/L and 29.4 mg/L (day 59 –3rd pulse) ($p < 0.05$), respectively

(Figure 7.1). This led to a reduction in COD removal efficiency from 96.2% (day 57 – 2nd pulse) to 94.1% (day 59 –3rd pulse) ($p < 0.05$). The VFA concentrations in the reactor and effluent rose slightly to 3.4 mgCOD /L and 0.9 mgCOD/L on day 59 ($p < 0.05$), respectively (Figure 7.2 and 7.3). These results indicate that the high concentration of the TMs added in the third pulse might have resulted in some inhibitory effects as the direct instant loading of those metals into the bioreactor caused some immediate stress to the methanogenic bacteria, and hence reduced the performance of the SAMBR.

7.3.2.2   The speciation of trace metals

When TM addition to the feed was stopped, there were no TMs in the feed stream to the SAMBR, and hence it was certain that there was no interference with the metals being dosed. Measurements of TM concentrations in the reactor and effluent after each pulse are shown in Table 7.2. Due to the instant dose, there was a significant increase in the metal content in the reactor and effluent within 24 h after each pulse of TMs ($p < 0.05$). The retention capacity of the TMs in the reactor over 24 h following each pulse varied between each metal, with Co having the highest retention, i.e. 7.0% – 12.3% of the added Co lost in the effluent, while Mn had the lowest, i.e. 20.2% – 31.6% of the added Mn lost in the effluent. Table 7.3 shows the leaching rate of TMs (µg/gTSS.d and µmol/gTSS.d) after each pulse. Similar to the results from experiment 3.1, Fe was not well retained in the reactor and had the highest leaching rate i.e. 10.4 – 30.1 µg/gTSS.d or 18.7 – 53.8 mmol/gTSS.d, whereas Mo had the lowest rate i.e. 0.8 – 1.6 µg/gTSS.d or 1.5 – 2.2 mmol/gTSS.d.

Table A.2 (Appendix) gives the metal content (µg/gTSS) in each fraction from the BCR extraction, while Figure 7.5 shows the speciation of TMs in both the liquid and solid phases in the reactor for each pulse. The data indicates that following the first pulse there was a shift in metal speciation as the TMs were mobilized from the last two fractions F3 and F4 towards the "relatively bioavailable fractions" (Sol and F1) ($p < 0.05$). This increase in the bioavailable

fractions could be due to the complexation of the added TMs with the increased amount of SMPs produced during nutrient deficiency. SMPs are natural chelating agents which are known to play an important role in making metal ions available for microbial uptake by maintaining higher soluble concentrations of TMs through complexation (Patidar & Tare, 2008). On the other hand, the interaction between different types of TMs due to chemical equilibria when they are supplemented together to an anaerobic system also needs to be taken into account in evaluating metal speciation. For example, it was suggest that Co and Ni are able to exchange with Fe^{2+}, preferably with Fe sulfide, as FeS has the highest solubility ($Ksp = 10^{-18}$) compared to NiS ($Ksp = 10^{-27}$) and CoS ($Ksp = 10^{-20}$) (Oleszkiewicz & Sharma, 1990). Osuna et al. (2003) found that the addition of Co to a metal depleted UASB reactor resulted in not only a reduction in the total amount of Fe, but also shifted its speciation from the exchangeable fraction towards the organic fraction. In contrast, following the second pulse of TMs speciation was shifted slightly towards less bioavailable fractions, i.e. F3 and F4 ($p < 0.05$). A more significant shift was observed after the third pulse as a higher percentage of the metal content in the F3 and F4 fractions was observed ($p < 0.05$), as shown in Table A.2 (Appendix).

The speciation results seem to correlate well with the changes in SAMBR performance, as the increase in metal content in the bioavailable fractions (Sol and F1) following the first pulse was able to enhance reactor recover from the nutrient deficit. On the other hand, the reduction in these bioavailable fractions after the second and third pulses seemed to limit the bioavailability of the TMs, and hence did not result in any improvements in SAMBR performance. For the third pulse, the concentration of TMs supplemented was considerably higher, and the increase in less bioavailable forms, i.e. organic matter/sulfide and residuals, could be attributed to the inhibition by TMs as these fractions can also be bioavailable to anaerobic microorganisms, although there has been some argument about whether metal sulfides are bioavailable in AD. Zehnder et al.

(1982) suggested that the presence of sulfide could restrict the availability of Fe to methanogens. However, Gustavsson et al. (2013b) found that the precipitation of Co and Ni with sulfide did not limit the microbial uptake of Co and Ni. Ketheesan et al. (2016) also found that the toxicity of Fe supplemented at 50, 70, and 100 mg/L could be due to the availability of Fe in the dominant sulfide and residual forms.

7.4   Conclusions

This study showed that temporary nutrient limitations caused by the exclusion of TMs from the feed to a SAMBR quite quickly resulted in a significant reduction in the metal content of the sludge, shifting the speciation of TMs towards less bioavailable fractions, i.e. organic matter/sulfide and residual, which resulted in reduced COD removal and methane yields. A dosing strategy by pulsing a mixture of TMs at 5 times of the normal daily metal loading seemed to be effective in controlling the metal deficiency by increasing the metal content of the more bioavailable fractions, i.e. soluble and exchangeable, and hence help the reactor recover to optimal performance within 4 days. However, with higher doses (10x), TM addition was not helpful because the TMs might have inhibited the methanogenic bacteria due to the instant loading of high amounts of TMs into the reactor.

Chapter 8

Effect of operating conditions on speciation and bioavailability of trace metals in submerged anaerobic membrane bioreactors

Outline:

8.1 Introduction

8.2 Experimental and analytical methods

8.3 Results and discussion

8.4 Conclusions

Note: this chapter was published in *Bioresource Technology*, 2017, volume 243, pages 810-819.

8.1   Introduction

The bioavailability of TMs for microbial uptake and growth in anaerobic bioreactors is strongly dependent on metal speciation, and this in turn can be affected by some of the reactor operating parameters such as pH, hydraulic retention time (HRT), and possibly solids retention time (SRT) (Thanh et al., 2016). Changes in pH can not only affect the performance of the anaerobic microorganisms, it can also dramatically influence the fate of TMs in anaerobic digestion, and these effects can be interrelated. A number of studies have been carried out in Up-flow Anaerobic Sludge Bed (UASB) reactors to determine the correlation between TM speciation and changes in pH (van Hullebusch et al., 2005b; Zandvoort et al., 2005; Lopes et al., 2008; Gonzalez-Gil et al., 2012;

Dong et al., 2013). Generally, these studies showed that an increase in pH led to the transformation of TMs from available mobile fractions like their "exchangeable" component, to stable organic or residue forms which reduced their bioavailability. In contrast, a reduction in pH resulted in the dissolution of TMs into solution, shifting them from non-bioavailable fractions (residual and organic/sulfide) towards more bioavailable fractions (exchangeable and carbonates).

Understanding the relationship between HRT and TM speciation in anaerobic processes is very limited. Cestonaro do Amaral et al. (2014) studied the distribution of Cu and Zn in swine wastewater in an up-flow bio-digester; they found that when the HRT was reduced from 17.86 d to 7.57 d, the metal retention capacity of the bioreactors decreased. While other metal fractions remained the same or decreased, the less bioavailable oxidizable fraction comprised of the metals associated with organic matter/sulfide increased, indicating that the metals became less bioavailable to anaerobic microorganisms.

Finally, SRT is another important operating parameter for anaerobic bioreactors, as the SRT needs to be maintained at a certain level so that there is a sufficient active population present in the reactors to enable anaerobic digestion to proceed efficiently. At long SRTs, it is expected that TMs will accumulate, and are present in high levels in the bioreactor, and also that the majority of the metals are associated with the biomass, either on the surface or inside the biomass. Therefore, at short SRTs it is possible that the high daily biomass removal rate will alter the total content of TMs and change their speciation. There has been no study in the literature carried out to determine how the SRT could influence TM speciation in anaerobic bioreactors.

Reactor configuration also plays an important role in the dynamics of TMs as it affects the metal retention capacity of anaerobic bioreactors (Thanh et al., 2016). Most of the studies in metal speciation and bioavailability have been carried out in UASB or conventional CSTR reactors. The anaerobic membrane bioreactor (AnMBR) is one of the most advanced anaerobic reactor designs

available as it offers independent control of the SRT and HRT, which then allows larger volumes of wastewater to be treated on a smaller footprint, and also prevents any loss of biomass into the effluent (Kunacheva et al., 2017).

Based on these considerations, this study was aimed at exploring how the changes in operating parameters in a SAMBR, i.e. pH, HRT, and SRT, can influence the bioavailability of TMs, i.e. Fe, Co, Ni, Zn, Mn, and Mo, by monitoring their speciation over time under a variety of reactor operating conditions.

8.2   Experimental and analytical methods

8.2.1   SAMBRs

Three independent experiments with changes in pH, HRT, and SRT were carried out in three SAMBRs with the same configuration as described in Chapter 3, i.e. SAMBR1, SAMBR2, and SAMBR3, respectively. The steady state (control) conditions for these SAMBRs were defined as 500 mg/L COD, pH 7, 6 h HRT and 100 d SRT.

8.2.2   Changes in pH

SAMBR1 was operated at steady state conditions at neutral pH 7 for 36 d. The pH was subsequently reduced to 6.5 and then 6 by adding diluted HCl to adjust the pH. The reactor was operated at each pH for about 8 d, which constitutes 32 HRTs, and previous work in this lab has showed that steady state occurs in this time (Kunacheva et al., 2017). At the end of the pH 6 run, no further pH adjustment was carried out on the feed, and the SAMBR was allowed to recover until the pH was back to neutral.

8.2.3   Changes in HRT

SAMBR2 was operated under steady state conditions at 6 h HRT for 53 d. The HRT was subsequently reduced from 6 h to 4 h, and then to 2 h by

increasing the speed of the influent pumps, i.e. double and triple, respectively. The membrane flux was also increased accordingly in order to maintain a constant liquid level in the SAMBR, and the reactor was operated at each HRT for 5 d. SRT was maintained at 100 d, whereas, VSS was 10.2 g/L at the start of 6 h HRT. At the end of the 2 h HRT run, the membrane was removed and the cake layer on the membrane surface was carefully collected by rinsing with de-ionized water, and the physical parameters and TM composition in the cake layer were analysed.

8.2.4   Changes in SRT

SAMBR3 was operated under steady state conditions at an SRT of 100 d with the daily removal of 32 mL of sludge for almost 3 months; during this time samples were collected several times to establish baseline conditions. Before the SRT was reduced to 25 d, it was temporarily reduced to 75 d and 50 d for 1 week each to ensure a smooth transition. To maintain 25 d SRT, 128 mL of sludge was removed every day from the reactor for 27 d. This SRT is considered as the optimal operating SRT to achieve minimum normalized production of soluble microbial products (SMPs) in an anaerobic CSTR (Kuo et al., 1996).

8.3   Results and Discussion

8.3.1   Changes in pH

8.3.1.1   Performance of the SAMBR1 at different pHs

As expected, since low pHs of 6.5 and 6 are outside the favoured pH range for anaerobic microorganisms of 6.8 − 7.2 (Rittmann & McCarty, 2001), they caused an upset, especially to the methanogens, and hence destabilized the performance of the SAMBR. As a result, there was a significant increase in the average soluble COD in the reactor and effluent from 65.6 mg/L and 12.6

mg/L (pH 7) to 251.3 mg/L and 145.2 mg/L (pH 6.5) (p < 0.05) and then to 390.7 mg/L and 237.4 mg/L (pH 6) (p < 0.05), respectively (Figure 8.1). The difference between the soluble COD level in the reactor and in the effluent was due to the membrane which retained a large amount of dissolved COD by the thin polarisation layer, or cake, on the membrane surface. The corresponding average COD removal dropped from 97.6% (pH 7) to 74.3% (pH 6.5) (p < 0.05) and then to 52.5% (pH 6) (p < 0.05).
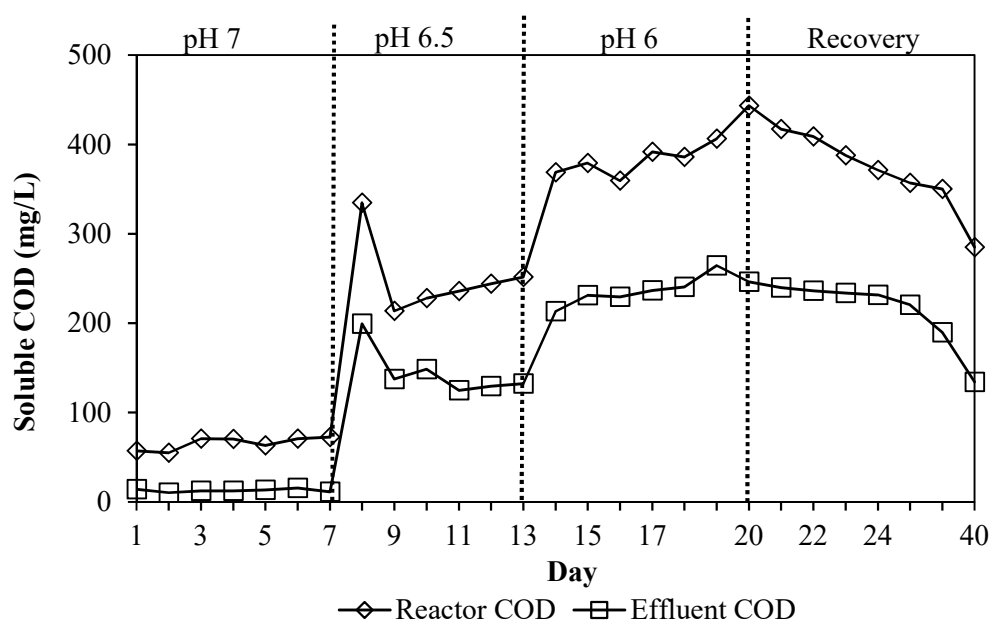
Figure 8.1   Soluble COD in the reactor and effluent of the SAMBR1 at different pHs (n = 3).

The pH reduction also resulted in a proportional increase in VFA concentrations in the reactor and effluent, with acetate as the main VFA accumulated (Figures 8.2 and 8.3). As can be seen that the total VFA concentrations (as COD) in the reactor were considerably lower than the soluble reactor COD. This implies that VFAs are not the only contributor to the soluble COD in the reactor, but there are also SMPs which are released by the bacteria under stressed conditions.
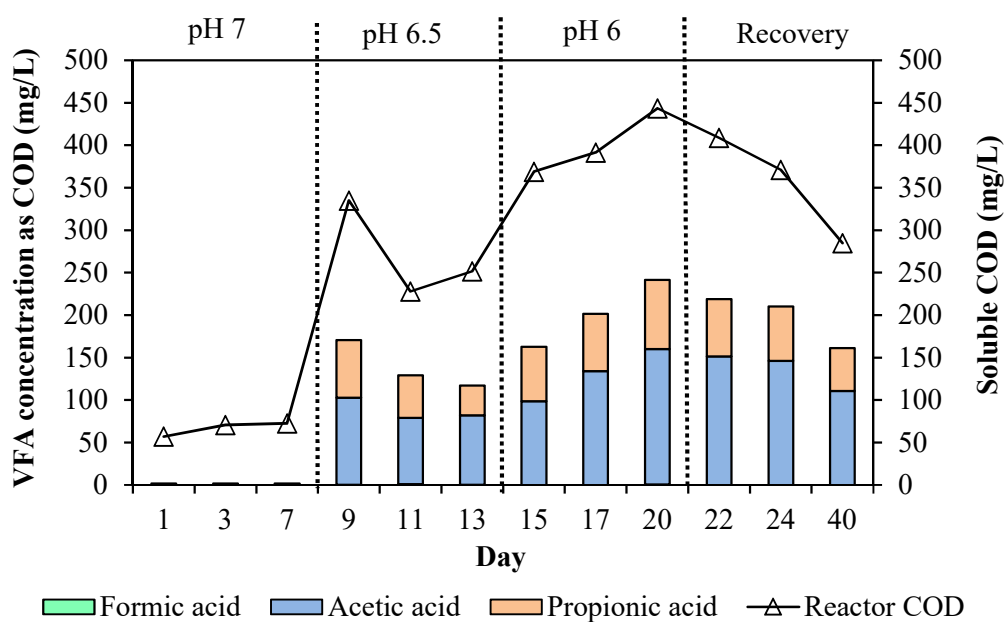
Figure 8.2   VFA concentration as COD with Reactor COD of the SAMBR1 at different pHs (n =3).
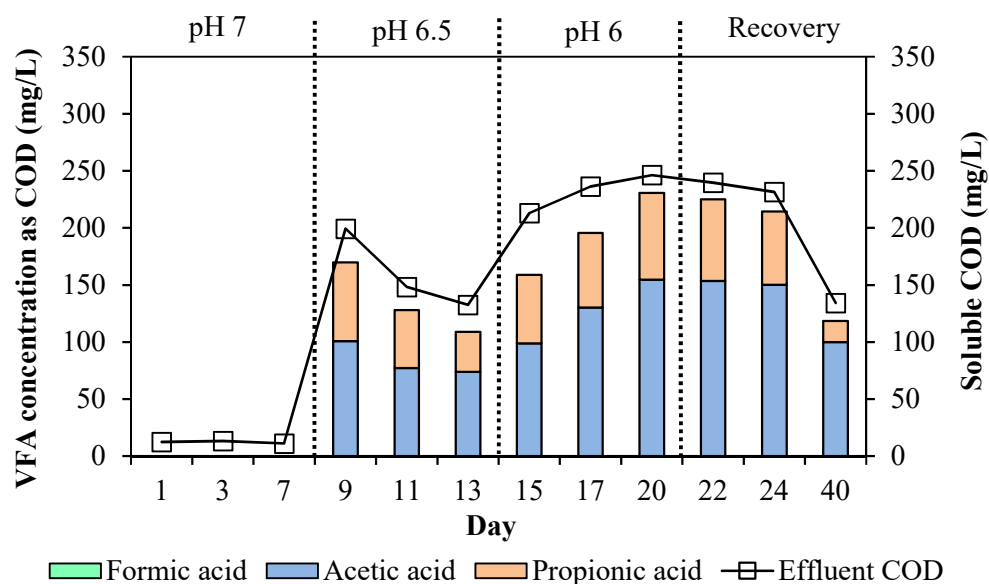
Figure 8.3   VFA concentration as COD with Effluent COD of the SAMBR1 at different pHs (n =3).

The methanogens were seriously affected by the low pHs, but there was no sign of complete inhibition as the average methane yields declined from 59.6 $mLCH_4/gVSS.d$ (pH 7) to 41.7 $mLCH_4/gVSS.d$ (pH 6.5) (p < 0.05) and then to 30.9 $mLCH_4/gVSS.d$ (pH 6) (p < 0.05) (Figure 8.4).
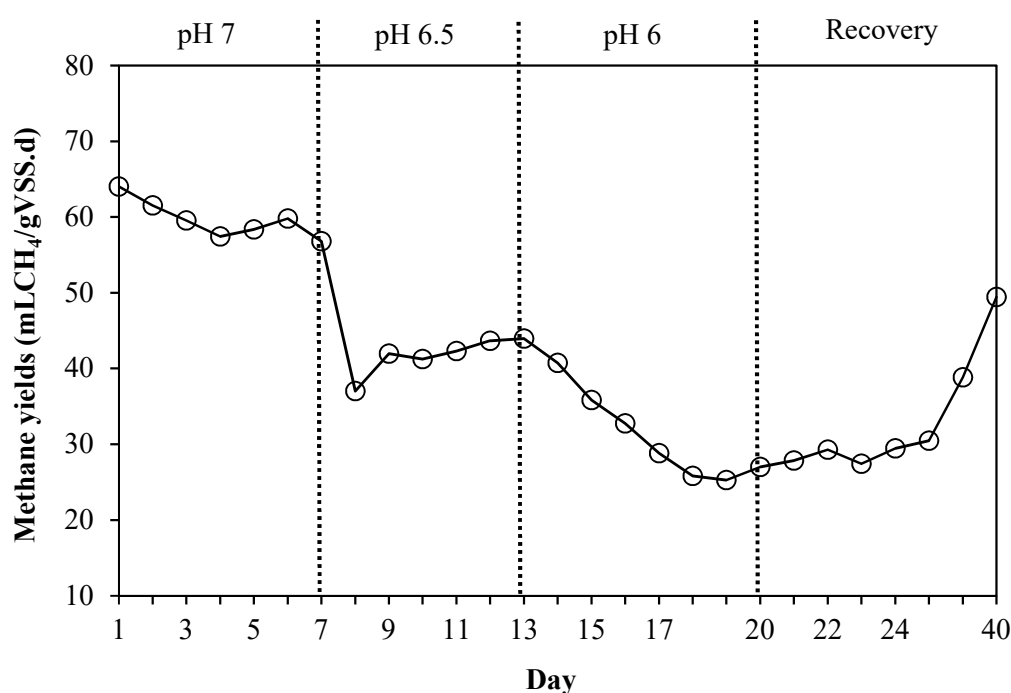
Figure 8.4   Methane yields of the SAMBR1 at different pHs.

The reactor took only 2 d for the pH to return to its favoured pH for methanogens, i.e. pH 6.8, but much longer to return to neutral (pH 7) i.e. 12 d. Accordingly, recovery in SAMBR COD removal was very slow (Figure 8.1), and the methane yield only increased to 73.2% and 49.4 $mLCH_4/gVSS.d$ (p < 0.05), respectively, 20 d after the end of pH 6 (Figure 8.4). In methanol fed granular sludge bioreactors, Zandvoort et al. (2005) also found that the pH shock (7 to 5) significantly affected reactor performance as after 2 d the methanol and VFA concentrations in the effluent increased to 1418 mgCOD/L and 186 mgCOD/L, respectively. However, the shock was performed for a

much shorter period (30 h), and hence the bioreactors were able to recover very quickly, i.e. no methanol or VFAs were present in the effluent 3 d after the pH was restored to 7.

8.3.1.2 Dynamic changes in trace metal speciation at different pHs

Total content of trace metals - The total content (μg/gTSS) of each TM in the SAMBR at pH 7 is shown in Table 8.1. Due to the long period of operation with the continuous supplementation of TMs, the total metal content in the reactor was high, i.e. from 147.9 μg/gTSS for Mo up to 11256.1 μg/gTSS for Fe, and was in the following order: Fe > Zn > Co > Ni > Mn > Mo. The reduction in pH from 7 to 6.5 and then to 6 caused TMs to be washed out from the sludge, and hence a reduction in the retention capacity of TMs over time in the SAMBR was observed. The order of the leaching rate (μg/gTSS.d) in metal content (Table 1) was slightly different at each pH, i.e. Fe > Ni > Co > Zn > Mn > Mo (pH 6.5) and Fe > Zn > Ni > Co > Mn > Mo (pH 6). Lopes et al. (2008) also found a high degree of leaching for Fe, Ni, and Co from acidifying sulfate reducing granular sludge at low pHs (6, 5, and 4). When the pH returned to neutral (7), there was a significant increase in metal content for all TMs ($p < 0.05$) compared to the level at the end of pH 6 i.e. from 2.2% (Mn) to 14.28% (Co). This was due to the increase in pH which stopped the leaching of TMs from the sludge. Zandvoort et al. (2005) also found that immediately following the pH shock (pH 7 to 5 for 30 h) the effluent concentration of Fe, Co, and Ni from UASB reactors quickly returned to values found prior to the shock.

Table 8.1   Total content (µg/gTSS) of TMs as an average of samples collected at each pH and leaching rate (µg/gTSS.d) of TMs determined from total content (µg/gTSS) at the beginning and the end of each pH in the SAMBR1.

Metal	pH							
	7		**6.5**		**6**		**Recovery**	
	Metal content (µg/g)	Metal leaching rate (µg/g.d)	Metal content (µg/g)	Metal leaching rate (µg/g.d)	Metal content (µg/g)	Metal leaching rate (µg/g.d)	Metal content (µg/g)	Metal leaching rate (µg/g.d)
Fe	11256.1	21.7	8825.0	486.2	6895.9	385.8	7062.3	-33.3
Co	902.8	-0.2	806.7	19.2	760.5	9.2	869.1	-21.7
Ni	686.9	-0.7	588.5	19.7	528.1	12.1	544.2	-3.2
Zn	1226.6	0.6	1144.0	16.5	1057.9	17.2	1108.1	-10.0
Mn	161.9	0.5	155.5	1.3	139.8	3.1	142.9	-0.6
Mo	147.9	0.4	142.3	1.1	127.0	3.1	144.6	-3.5

Speciation of trace metals - Table A.3 (Appendix) shows the measurements, and Figure 8.5 shows the distribution of each TM in different fractions from the BCR extraction method. In the liquid phase, generally, soluble TMs were relatively low (from 0.0% up to 2.64%), which was also reported by Oleszkiewicz and Sharma (1990) and Roussel and Carliell-Marquet (2016), except for Mn which reached as high as 17.2%. As expected, the lower pH caused a higher leaching rate of TMs from the sludge, and a higher metal solubility (Zandvoort et al., 2005; Lopes et al., 2008; Gonzalez-Gil et al., 2012). This resulted in an increase in the average soluble fraction as the pH was reduced from 7 to 6.5 then to 6 i.e. Fe (0.15% – 0.77% – 2.09%) ($p < 0.05$), Ni (1.39% – 1.95% – 2.64%) ($p < 0.05$), Mn (12.05% – 14.4% – 17.17%) ($p < 0.05$), Mo (1.34% – 2.02% – 2.44%) ($p < 0.05$), except for Co and Zn which

was not found in the soluble fraction at any pH value, and this finding was in agreement with Marcato et al. (2009).

Iron (Fe)
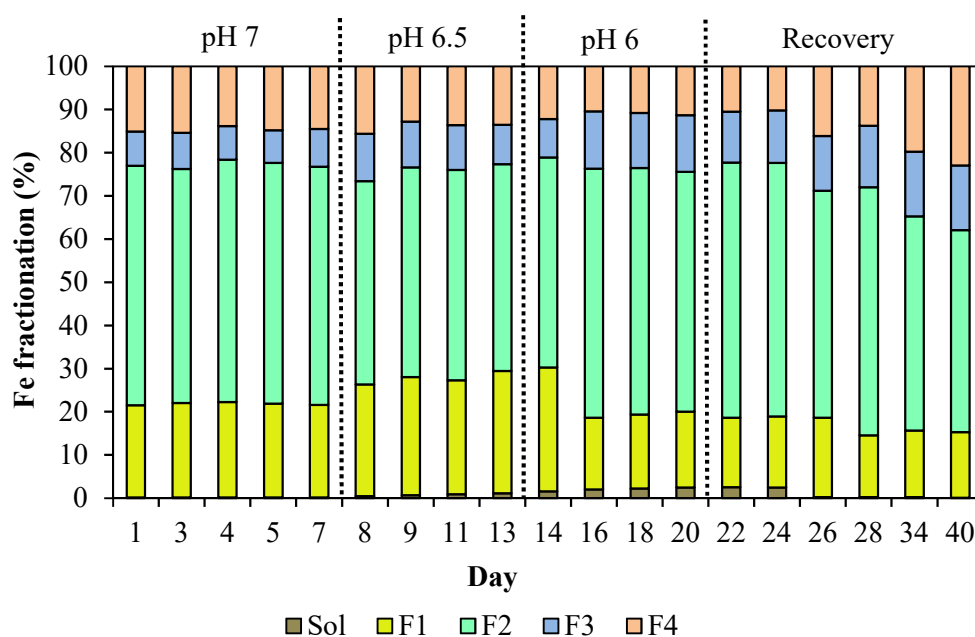
Cobalt (Co)
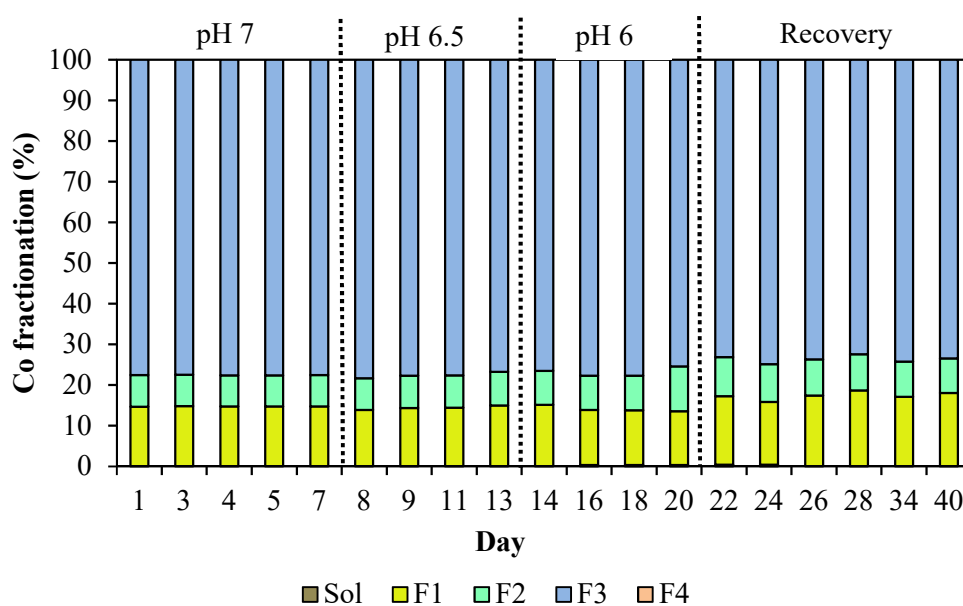
Nickel (Ni)
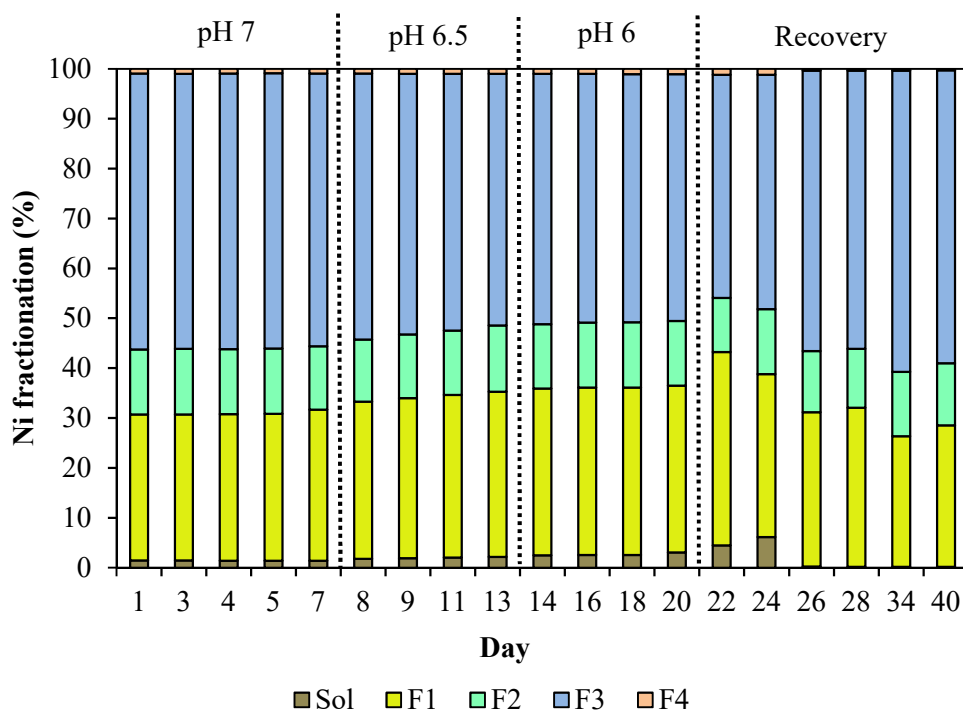
Zinc (Zn)
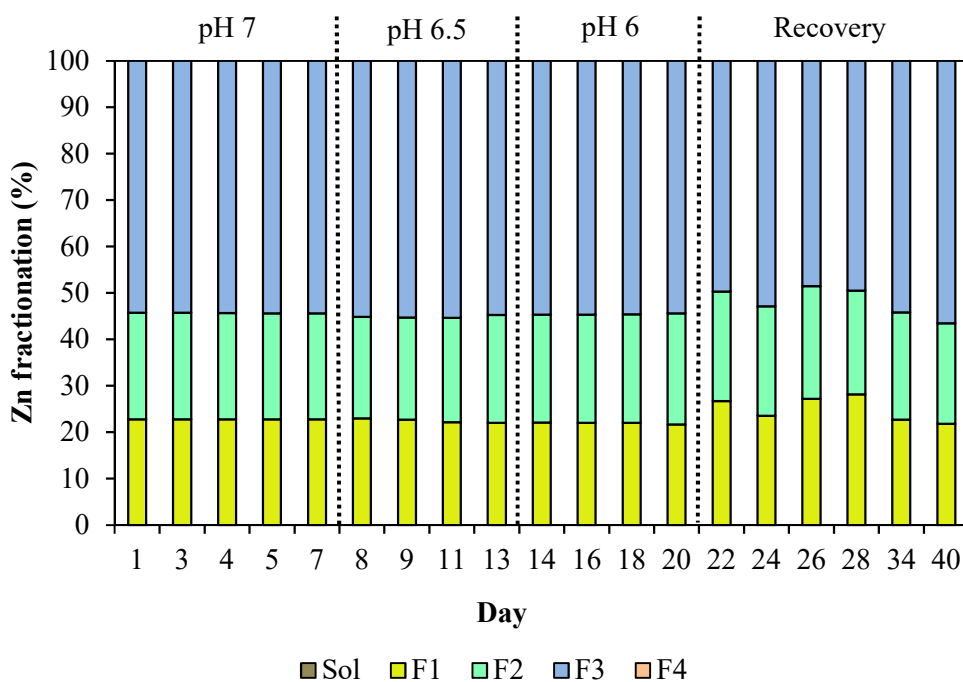
Manganese (Mn)
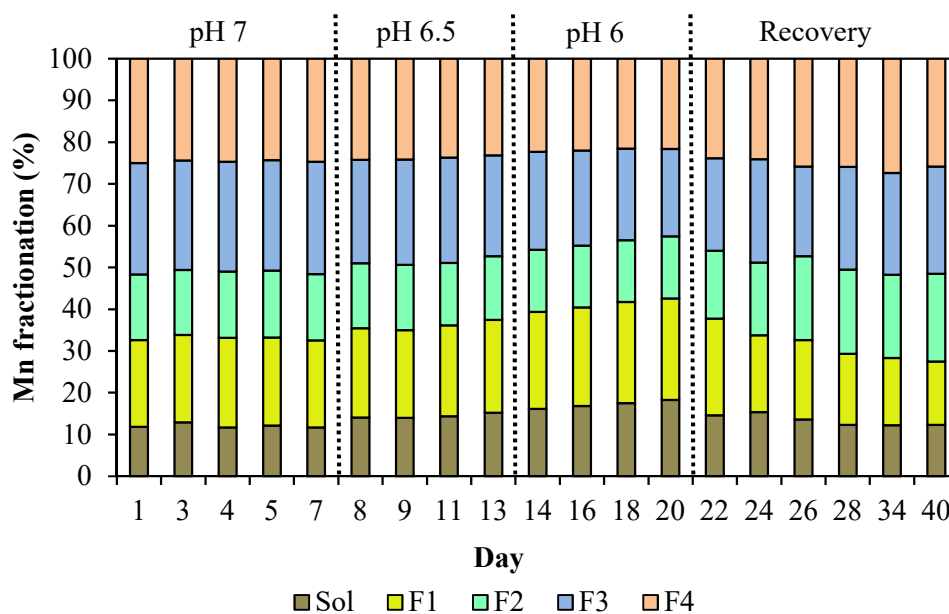
Molybdenum (Mo)
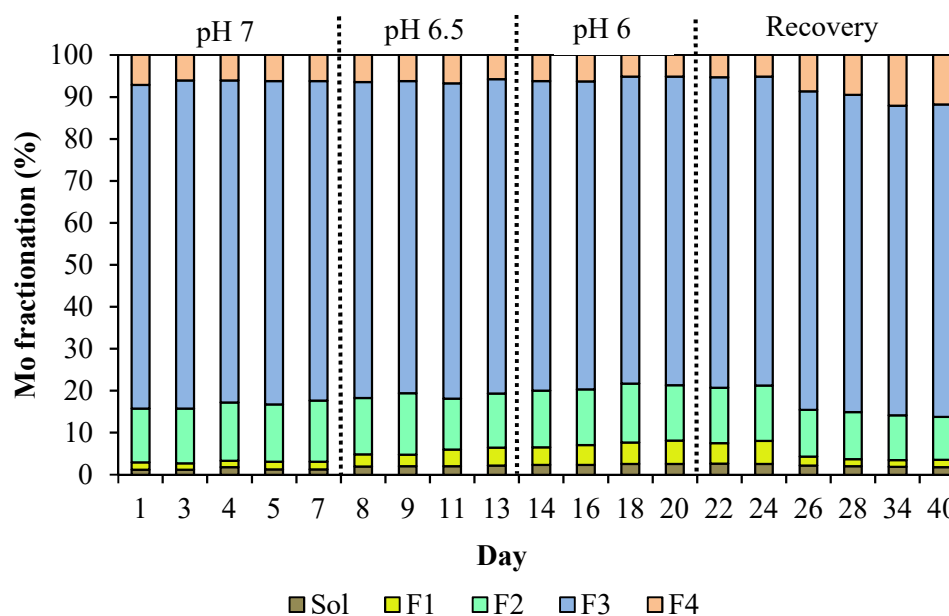
Sol - Soluble, **F1** - Exchangeable, **F2** - Fe/Mn oxides,

F3 - Organic matter, **F4** - Residual

Figure 8.5   Speciation of TMs in SAMBR1 at different pHs (n = 3).

Chapter 8: Effect of operating conditions on speciation and bioavailability of trace metals in submerged anaerobic membrane bioreactors

In the solid phase, most of the TMs were found to exist mainly in the organic matter/sulfide fraction (F3), which is considered to have low bioavailability, except for Fe where its dominant fraction was the Fe-Mn oxides fraction (F2). These results were similar to the findings from Chapter 7 as precipitation of metals such as Co and Ni with sulfide was the reason for the dominance of the F3 fraction, and dissolution of amorphous Fe by the BCR extracting reagent possibly resulted in the accumulation of Fe in the F2 fraction. The changes in TM speciation at different pH values varied for different TMs, as shown in Figure 8.5.

There was a significant shift in the speciation of Fe as the average for the F1 fraction (Exchangeable) increased from 21.7% (pH 7) to 26.6% (pH 6.5) (p = 0.028) but then reduced to 19.4% (pH 6) (p = 0.021), while the F3 fraction (Organic matter/sulfide) also increased from 8.08% (pH 7) to 10.1% (pH 6.5) (p = 0.482) then to 11.76% (pH 6) (p = 0.478), respectively. In contrast, the F2 fraction (Fe-Mn oxides) reduced from 55.3% (pH 7) to 47.4% (pH 6.5) (p = 0.034) but then increased to 53.5% (pH 6) (p = 0.044), and the F4 fraction (Residual) also reduced from 14.7% (pH 7) to 13.9% (pH 6.5) and further reduced to 11.2% (pH 6), respectively. As the F1 and F2 fractions are considered more bioavailable (total 76.0% pH 7, 75.0% pH 6.5, and 74.7% pH 6) than the F3 and F4 fractions (total 22.8% pH 7, 24.1% pH 6.5, and 23.2% pH 6), these results did not indicate that the speciation of Fe shifted towards higher or lower bioavailability.

In contrast, the change in Co speciation at lower pHs was much less significant. The average F1 fraction was relatively stable at pH 7 and pH 6.5, i.e.14.8% and 14.4% (p = 0.144), respectively, and only slightly reduced to 13.8% at pH 6.5 (p = 0.042). The average F2 fraction also did not vary significantly at pH 7 and pH 6.5, i.e. 7.7% and 8% (p = 0.185), respectively, then it slightly increased to 9.05% at pH 6 (p = 0.032). At all pH values, the F3 fraction was relatively stable i.e. 77.6%, 77.6% and 76.8% (p = 0.203), while no Co was found in the F4 fraction.

Ni showed a significant increase in its bioavailability as the average F1 fraction increased from 29.5% at pH 7 to 32.3% at pH 6.5 (p = 0.041) and then it stayed relatively constant at 33.5% at pH 6 (p = 0.138). On the other hand, the average F3 fraction, which is considered less bioavailable than the F1 fraction, reduced significantly from 55.1% at pH 7 to 51.9% at pH 6.5 (p = 0.028) and then to 49.8% at pH 6 (p = 0.043). However, the average of the remaining two fractions, F2 and F4, did not change significantly as they stayed at the same levels of around 13% and 1% (p = 0.073 and p = 0.09), respectively.

Although the total metal content in the reactor for Zn reduced, no pattern was established for Zn speciation at different pHs as the average Zn content in the F1, F2, and F3 fractions was approximately constant at around 23%, 23%, and 55%, with p values of 0.193, 0.238, and 0.142, respectively. Similar to Co, no Zn was found in the last fraction F4.

The bioavailability of Mn was slightly improved when the pH was reduced. The average of the F1 fraction was 21.0% at pH 7, and it remained almost the same at 21.6% at pH 6.5 (p = 0.181) before increasing to 23.9% at pH 6 (p = 0.031). In contrast, at lower pH values, there was no significant difference in the F2 fraction i.e. 15.8% to 15.4% to 14.9% (p = 0.394), whereas, the last two fractions (F3 and F4) showed a similar declining trend i.e. 26.5% to 24.8% to 22.3% (p = 0.028), and 24.6% to 23.8% to 21.9% (p = 0.031) at pH 7, 6.5 and 6, respectively.

Similar to Mn, the speciation of Mo shifted towards higher bioavailable fractions (F1), which slightly increased from 1.71% to 3.5% to 4.9% (p = 0.043), while the F2 fraction did not change significantly, i.e. 13.6%, 13.3% and 13.5% at pH 7, 6.5 and 6 (p = 0.341), respectively. The average content of Mo in the last two fractions (F3 and F4) reduced as the pH reduced, i.e. 77.1% to 74.9% to 73.5% (p = 0.041) and 6.33% to 6.27% to 5.66% (p = 0.047), respectively.

Chapter 8: Effect of operating conditions on speciation and bioavailability of trace metals in submerged anaerobic membrane bioreactors

The pH of the reactor was allowed to recover after the pH adjustment was stopped. For all TMs, when the pH increased their total content increased accordingly, showing that the metal solubility had been reduced, and the reactor was able to regain its metal retention capacity, i.e. up to 14.3% after pH 6 (Table 8.1). In addition, the speciation of the TMs was reversed towards less bioavailable fractions, i.e. decreasing Soluble and Exchangeable forms, and increasing Organic matter/sulfide and Residuals, as shown in Figure 8.5.

In summary, a reduction in pH resulted in a decrease in total metal content, but an increase in the soluble fraction for all TMs. Fe and Ni underwent the most significant changes in their BCR fractions, while changes in Co, Mn and Mo were less significant. The speciation of Ni, Mn, and Mo shifted towards stable fractions, i.e. Soluble and Exchangeable, which are more bioavailable to the anaerobic microorganisms, whereas, Fe and Co showed no shifting pattern in their speciation. Zn was found not to be sensitive to pH changes, which is in agreement with Lopes et al. (2008). These results showed that the degree of change in metal speciation varied as some metal ions are more sensitive to pH changes than others, and also some metals have stronger affinity towards anaerobic sludge binding sites than others (Leighton & Forster, 1997; Artola et al., 2000; Marcato et al., 2009).

8.3.2   Changes in HRT

8.3.2.1   Performance of the SAMBR2 under different HRTs

Figure 8.6 shows soluble COD concentrations in the reactor and effluent from SAMBR2 at different HRTs. It can be seen that the effluent COD in the SAMBR increased initially with decreasing HRT, but the reactor recovered rapidly from the hydraulic shock within a few days, and became stable again; a similar profile could be seen when the HRT was decreased from 6 h to 4 h and 2 h. It should be pointed out here that theoretically there should be no concentration difference between the reactor and effluent COD during

microfiltration with a pore size of 0.4 μm. However, Figure 8.6 shows that the reactor COD in the SAMBR2 was more than six times higher than the effluent COD at HRT 6 h, five times at 4 h, and 1.5 times at 2 h. This difference in COD concentrations is similar to the findings from the pH experiment which is due to the rejection of soluble COD by the fouling layer on the membrane surface. In addition, Figure 8.7 shows that the state of the SAMBR2 membrane at the end of the HRT experiment as a cake was formed on the surface of the membrane. This cake formation might have occurred almost immediately in the SAMBR after the flux pump speed was increased to accommodate higher influent loading rates. Over the whole experiment, the effluent COD at various HRTs was the highest at 71.2 mg/L at the beginning of 2 h HRT (day 10), indicating that good COD removal was achieved under virtually all conditions. The soluble COD removal efficiencies were consistent with previous studies in anaerobic membrane bioreactors (Hu, 2004; Kunacheva et al., 2017). From Figure 8.6 it can be seen that the removal efficiency decreased with decreasing HRT; it was nearly 99% at an HRT of 6 h, then reduced slightly to 96% at the beginning of 4 h HRT (day 5) ($p < 0.05$), but returned to 99% after 5 days (day 9) ($p < 0.05$), dropped to 88% at the beginning of 2 h HRT (day 10) ($p < 0.05$) but still achieved 93% after 5 days (day 14) ($p < 0.05$). Kunacheva et al. (2017) were also able to achieve 94% COD removal when the HRT of a SAMBR was reduced from 4 h to 2 h for 6 d. These results showed that the tolerance of the SAMBR to changes in HRT is very high as there was no washout of biomass, and the dynamic fouling layer on the membrane acts to reject many of the SMPs as well as solids.

The measurement of VFA concentrations at each HRT (Figure 8.8 and 8.9) showed that at steady state only 0.35 mgCOD/L of VFAs were present in the reactor, and no VFAs were detected in the effluent; this indicates that the incoming COD was almost all degraded to biomass, CH_4 and CO_2. Figures 8.8 and 8.9 also shows that the major VFAs present in the reactor and effluent were formate, acetate, and propionate. When the organic loading rate doubled at 4 h

HRT, VFA concentrations in the reactor increased rapidly to 11.5 mgCOD/L (day 5), but then decreased to 1.63 mgCOD/L ($p < 0.05$) after 5 days (day 9) showing quick recovery of the SAMBR. When the organic loading rate doubled again at 2 h HRT, a VFA peak of 62.9 mgCOD/L was found on the first day (day 10), but after 5 days it reduced to 20.3 mgCOD/L ($p < 0.05$) (day 14). The rapid accumulation of VFAs on the first day of HRT reduction was because the feed glucose was metabolized into acetate very quickly, leading to an accumulation of intermediate VFAs in the reactor. The VFAs in the effluent were only slightly less than those in the reactor, implying that VFA rejection by the membrane was not significant. As the soluble COD present in the reactor and effluent were considerably higher than the VFAs, this indicates that the COD in the reactor and effluent was not only VFAs, but also SMPs produced by the biomass.
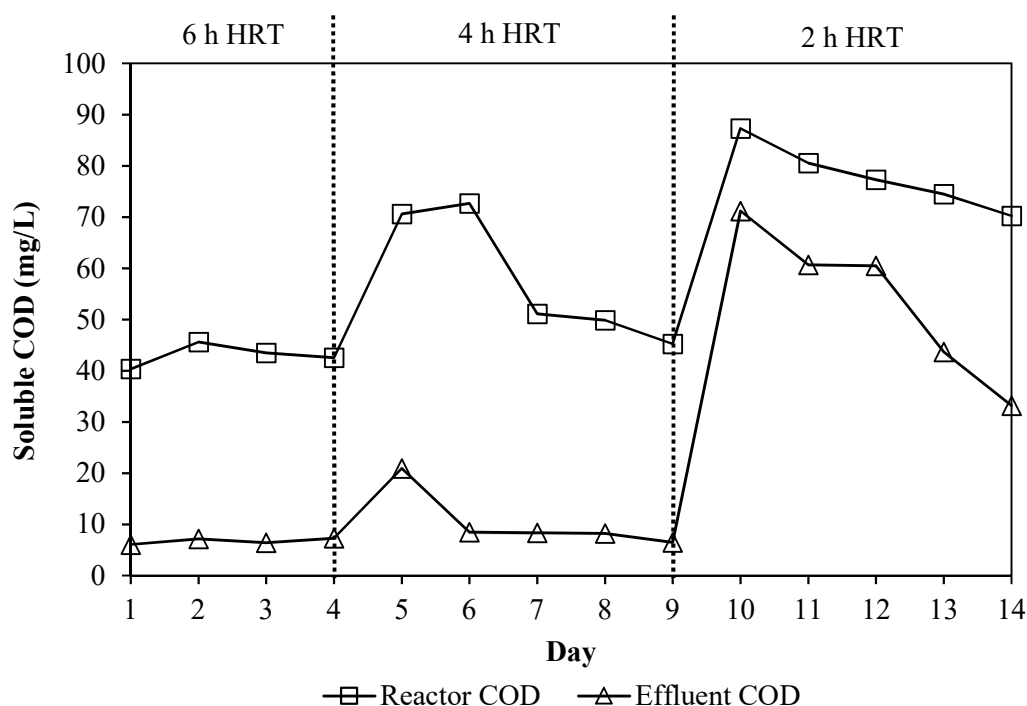
Figure 8.6   Soluble COD in SAMBR2 reactor and effluent at different HRTs (n = 3).

Figure 8.7   Cake layer on the membrane surface of the SAMBR2 at the end of 2 h HRT.
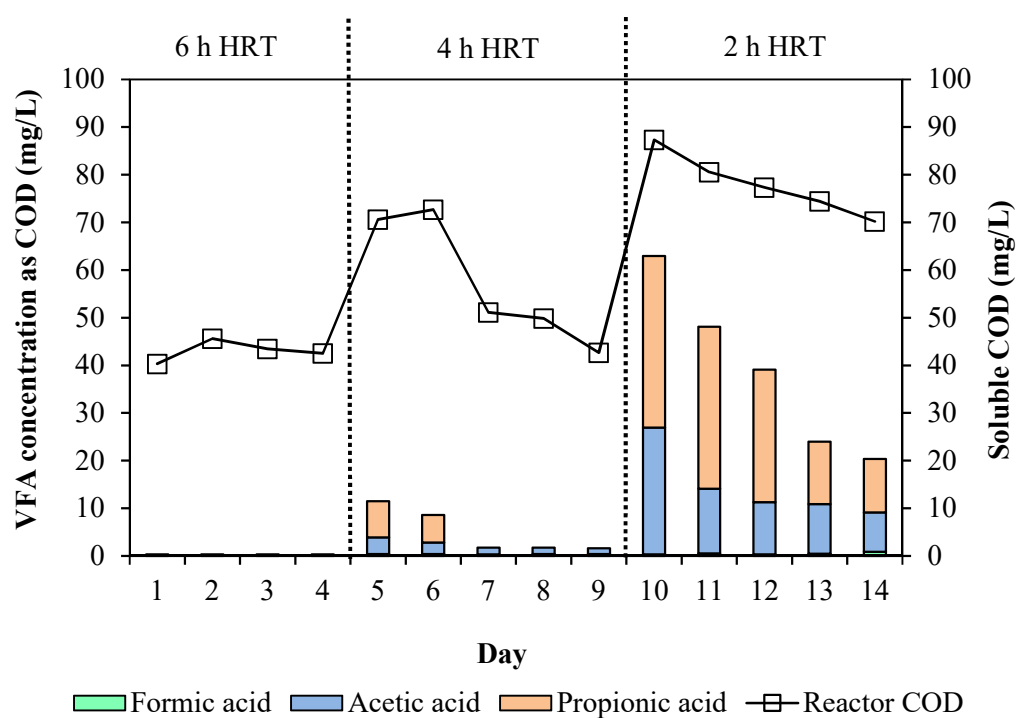
Figure 8.8   VFA concentration as COD with Reactor COD of SAMBR2 at different HRTs (n = 3).
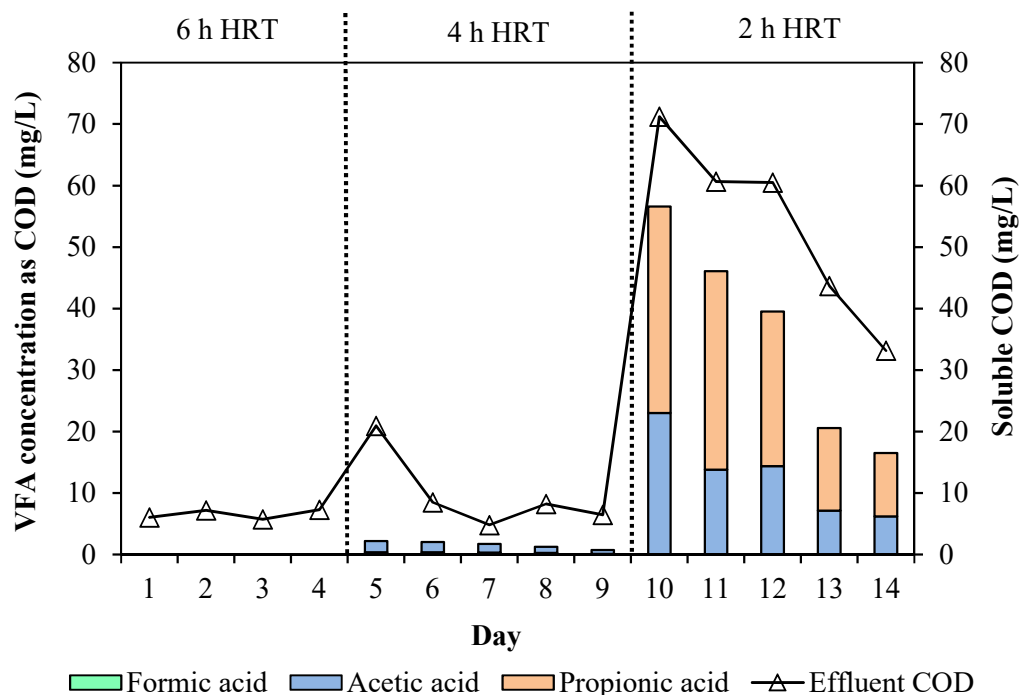
Figure 8.9   VFA concentration as COD with Effluent COD of SAMBR2 at different HRTs (n = 3).

Due to the increase in organic loading rate (OLR) when the HRT was reduced, it was obvious that the methane yields in the SAMBR would increase from steady state (6 h HRT), i.e. 40.6 mLCH$_4$/gVSS.d to 48 mLCH$_4$/gVSS.d ($p < 0.05$) and it further increased to 85.39 mLCH$_4$/gVSS.d ($p < 0.05$) 5 days after 4 h and 2 h HRT, respectively. In order to accommodate the increase in OLR, the biomass concentration in the reactor also increased accordingly. At 6 h HRT the average TSS and VSS of the SAMBR were 11.69 g/L and 10.56 g/L, respectively, and they increased to 13.47 g/L and 12.37 g/L at 4 h HRT ($p < 0.05$), and increased further to 17.15 g/L and 15.9 g/L at 2 h HRT ($p < 0.05$). These results indicate that when the OLR was tripled, the biomass content in the SAMBR almost doubled from its initial levels at 6 h HRT. The average transmembrane pressure (TMP) also increased from 4.12 kPa at 6 h HRT to 9.86 kPa at 4 h HRT ($p < 0.05$) and then to 13.42 kPa at the end of 2 h HRT (p

< 0.05). The flux of the membrane had to be increased from 20 L per square meter per hour (LMH) at 6h HRT to 25 (LMH) at 4h and then to 37 (LMH) to accommodate the increase in feed flowrate into the reactor.

8.3.2.2 Dynamic changes in trace metal speciation under different HRTs

Total content of trace metals - The total content of each TM in the SAMBR at steady state (6 h HRT) was determined, and it was in the following order: Fe > Ni > Co > Zn > Mo > Mn (Table 8.3). The retention capacity of TMs in the SAMBR was reduced for all TMs when the HRT was reduced to 4 h and 2 h. The reduction in total content of TMs after each HRT reduction was the same for both 4 h and 2 h, i.e. Fe > Ni > Co > Zn > Mn > Mo. The 2 h HRT exhibited a higher level of reduction in total content than at 4 h HRT for all TMs, except for Ni and Mo in which the level was almost the same at both HRTs. Cestonaro do Amaral et al. (2014) also found that when the HRT of a digester was reduced from 17.86 d to 5.32 d, the retention capacity of Zn and Cu decreased significantly, which was due to an increase in flow rate, generating greater internal agitation of biomass in the digester dragging solids, and associated TMs, into the effluent. However, in this study, due to the membrane, no solids were lost from the reactor. Nevertheless, the low pH resulting from the hydraulic shock loading (pH 6.78 ±0.05) at 2 h HRT might have caused the TMs to leach out from the SAMBR in the soluble phase.

Table 8.2   Total content (µg/gTSS) of TMs as an average of samples collected at each HRT and leaching rate (µg/gTSS.d) of TMs determined from total content (µg/gTSS) at the beginning and the end of each HRT in the SAMBR2.

| Metal | HRT (day) | | | | | |
| | **6** | | **4** | | **2** | |
	Metal content (µg/g)	Metal leaching rate (µg/g.d)	Metal content (µg/g)	Metal leaching rate (µg/g.d)	Metal content (µg/g)	Metal leaching rate (µg/g.d)
Fe	8487.0	6.6	7487.5	199.9	5393.9	418.7
Co	950.4	-1.7	909.9	8.1	638.8	54.2
Ni	1010.1	1.2	728.3	56.4	443.7	56.9
Zn	880.6	0.9	838.0	8.5	775.6	12.5
Mn	126.0	1.3	111.8	2.9	86.1	5.1
Mo	152.1	0.8	146.0	1.2	137.2	1.8

Speciation of trace metals - The measurement of TM content in each fraction from BCR extraction is given in Table A.4 (Appendix), and the speciation of TMs is shown in Figure 8.10. Similar to the results from the pH experiment, in the liquid phase the soluble concentration for most of the TMs was very low, i.e. up to 2.63%, except for Mn and Mo which reached 16.0% and 28.2%, respectively. When the HRT was reduced, the soluble fraction for Fe, Co, Ni and Zn remained relatively constant, whereas the Mn and Mo soluble levels decreased from 15.6% and 25.6% at 6 h HRT to 12.4% and 12.2% at 2 h HRT ($p < 0.05$), respectively. In the solid phase, again similar to the pH experiment, most of the TMs were found in the organic matter/sulfide

fraction, except for Fe and Mn where their dominant fraction was Fe-Mn oxides and Exchangeable, respectively.

Iron (Fe)
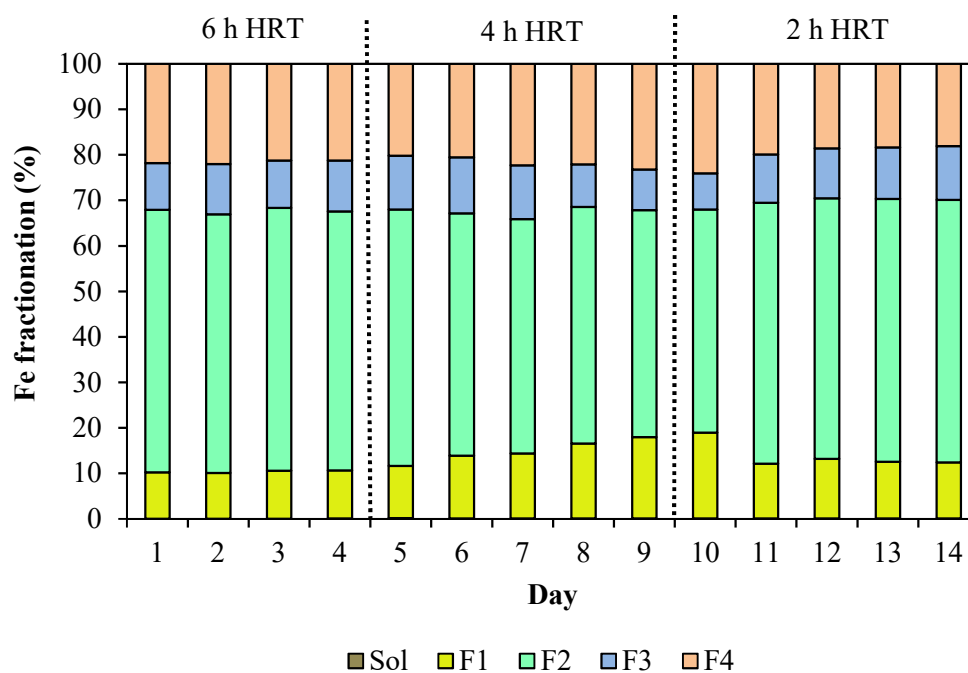
Cobalt (Co)
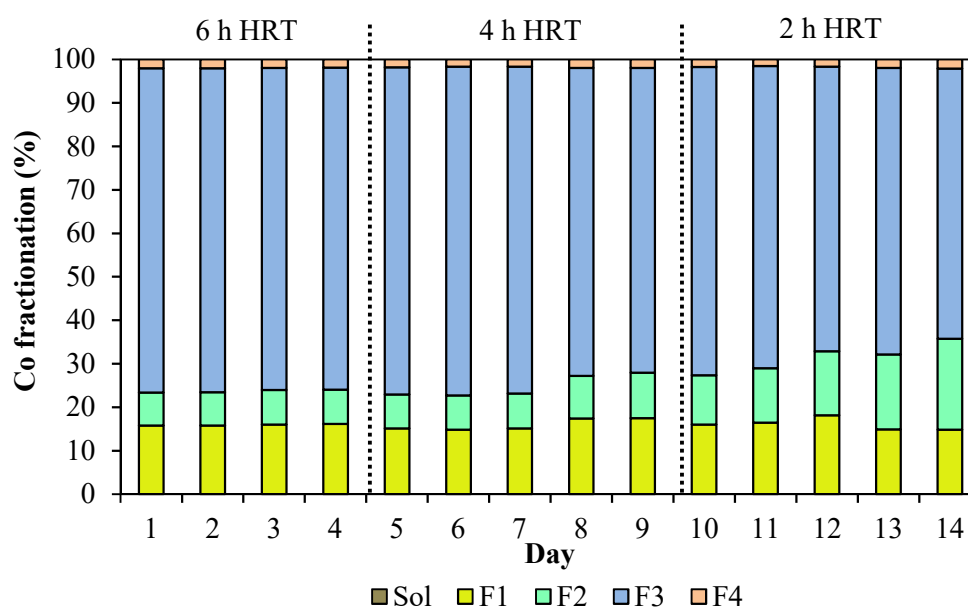
Nickel (Ni)
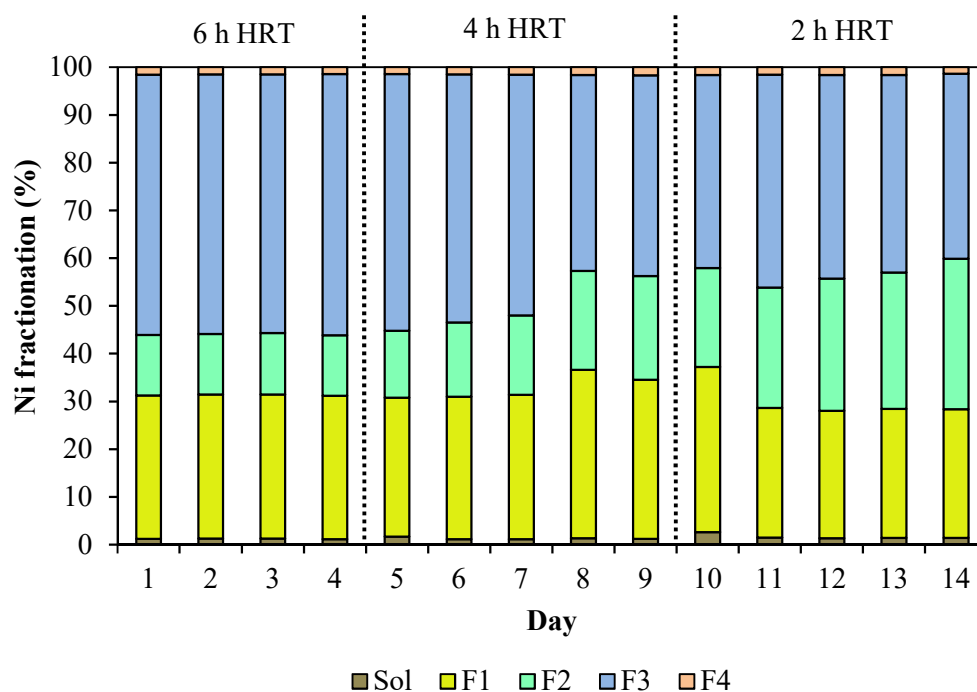
Zinc (Zn)
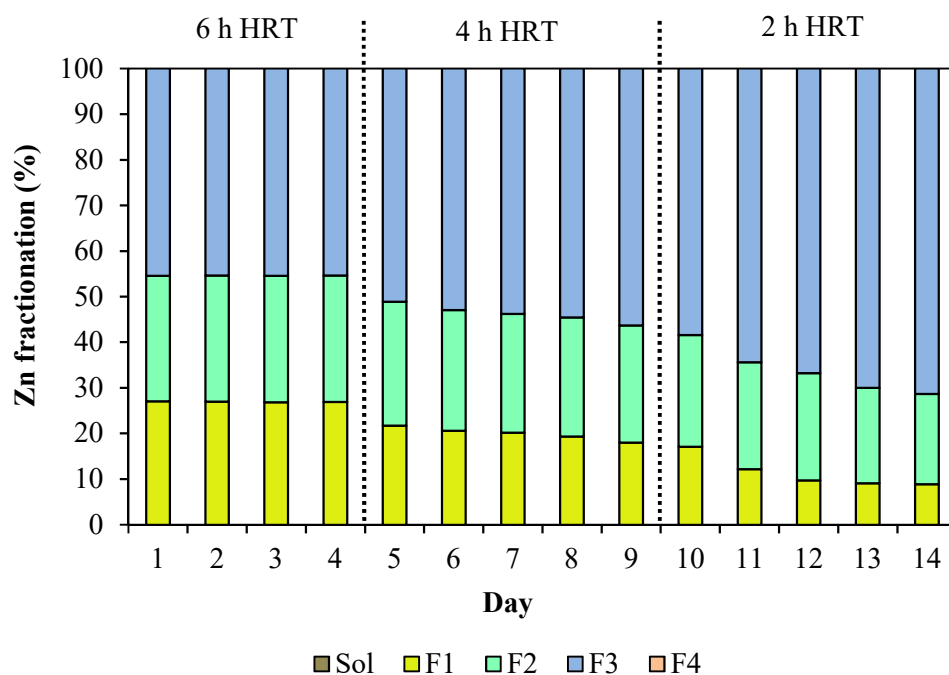
Manganese (Mn)
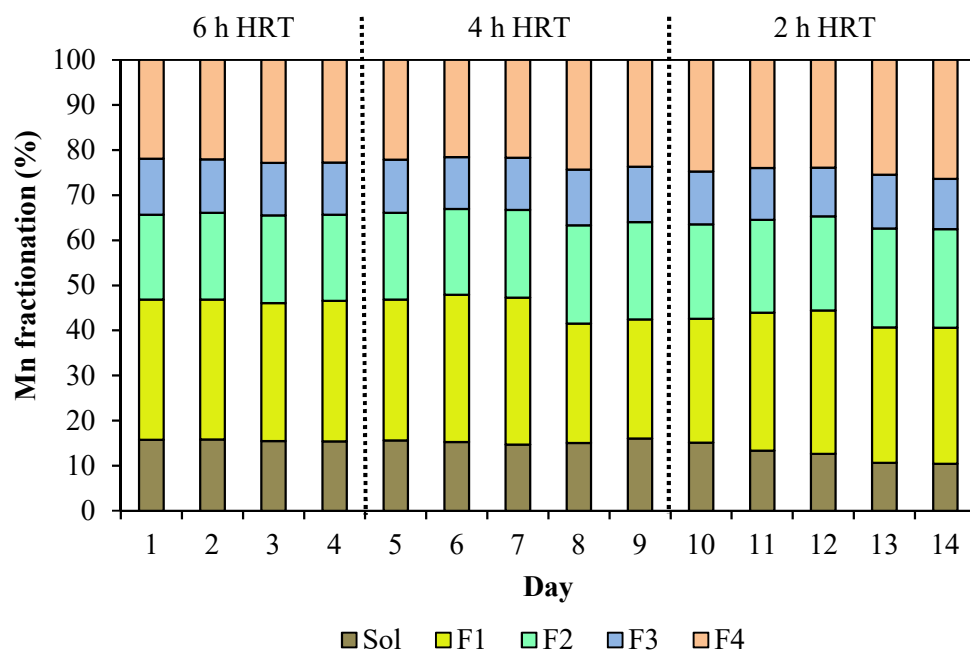
Molybdenum (Mo)
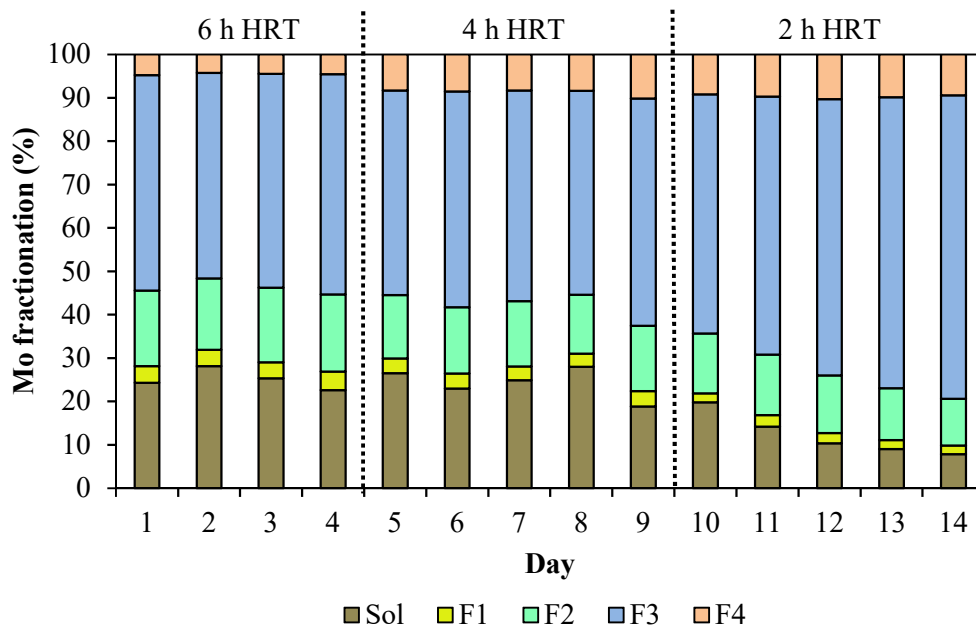
Sol - Soluble, **F1** - Exchangeable, **F2** - Fe/Mn oxides,

F3 - Organic matter, **F4** - Residual

Figure 8.10   Speciation of TMs in the SAMBR2 at different HRTs (n = 3).

The bioavailability of Fe slightly increased as the average F1 fraction increased from 10.3% at 6 h HRT to 14.8% at 4 h HRT (p = 0.038) and remained constant at 13.8% at 2 h HRT (p = 0.067). Similarly, the average of F2 fraction also increased from 52.6% at 6 h HRT to 57.3% at 4 h HRT (p = 0.035), but did not change significantly at 2 h HRT i.e. 55.8% (p = 0.083). On the other hand, the average F3 fraction was relatively stable at different HRTs i.e. 10.7%, 10.9% and 10.5% (p = 0.183), whereas, the last fraction F4 was also at the same level at 6 h and 4 h HRT i.e. 21.6% and 21.7% (p = 0.104), respectively, but then it reduced to 19.8% at 2 h HRT (p = 0.035).

The change in Co speciation was more significant, although the first fraction F1 was the same at different HRTs, i.e. 15.9%, 16.0%, and 16.1% (p = 0.152), the second fraction F2 increased considerably from 7.8% at 6 h HRT to 15.3% at 2 h HRT (p = 0.021). On the other hand, the less bioavailable fractions, i.e. F3, reduced from 74.3% at 6 h HRT to 66.8% at 2 h HRT, and the residual F4 was not statistically different when the HRT was reduced i.e. 2.0% and 1.8% (p = 0.092).

Just like Co, Ni bioavailability improved significantly; the Ni content in the first and the last fractions F1 and F4 stayed relatively constant at around 30% and 1.5% (p = 0.063 and 0.085), respectively. However, the F2 fraction increased significantly from 12.7% at 6 h HRT to 26.7% at 2 h HRT (p = 0.013), whereas, the F3 fraction reduced significantly from 54.5% at 6 h HRT to 41.5% at 2 h HRT (p = 0.021).

In contrast to the TMs above, the bioavailability of Zn reduced as the Zn content in the first two fractions (F1 and F2) both decreased when the HRT was reduced i.e. from 27.0% and 27.6% at 6 h HRT to 11.4% and 22.4% at 2 h HRT (p = 0.024 and 0.041), respectively. In contrast, the F3 fraction increased significantly from 45.4% at 6 h HRT to 66.2% at 2 h HRT (p = 0.034) and no Zn was found in the last fraction F4.

The results from the BCR extraction showed no significant change in the Mn speciation as all of Mn fractions remained the same at all HRTs i.e.

around 30%, 20%, 12%, and 25% (p = 0.059, 0.091, 0.121, and 0.072) for the F1, F2, F3, and F4 fractions, respectively.

Similar to Zn, the first two fractions (F1 and F2) of Mo both reduced from 3.9% and 17.0% at 6 h HRT to 2.2% and 12.8% at 2 h HRT (p = 0.028 and 0.031), respectively. In contrast, the last two fractions F3 and F4 increased significantly from 49.2% and 4.4% at 6 h HRT to 63.0% and 9.7% at 2h HRT (p = 0.013 and 0.041), respectively.

Analysis of TM speciation in the cake layer collected at the end of the 2 h HRT run showed that the dominant fraction for TMs was still organic matter/sulfide fraction (F3), except for Fe which was present mainly in the Fe-Mn oxides fraction (F2). Compared to the speciation of TMs in the biomass from the reactor at steady state (6 h HRT), the TMs in the cake layer were found at higher levels in the last two fractions F3 and F4 (Figure 8.11). The distribution, immobilization, and remobilization of metals depend on the sorption properties of the biofilms, determined by many factors including pH, and in this case, change in pH due to lower HRT might have affected the interaction of TMs with the biofilm in the cake layer (Thanh et al., 2016). This result indicates that the biomass attached to the membrane surface contains significant amounts of TMs in poorly bioavailable forms. These fractions have very low mobilities, and hence they have a higher resistance to the dissolution of TMs under low HRTs. It was found that TMs in the form of metal oxides/precipitates were often the origin of inorganic fouling (Gkotsis et al., 2014), and they coupled with organic foulants to enhance the formation of cake layer on the membrane surface (Gao et al., 2011; Zhou et al., 2015).
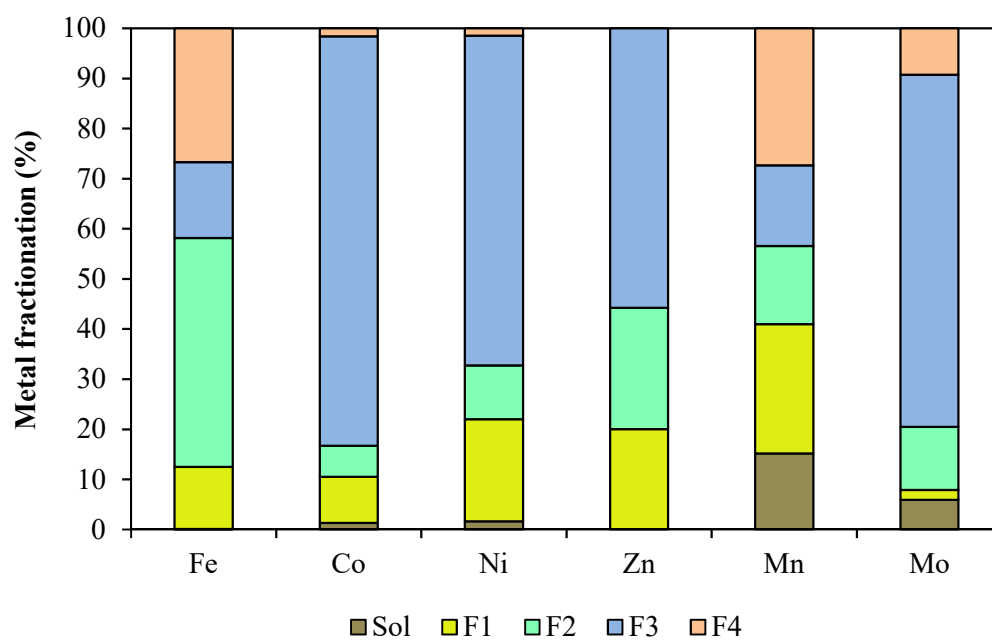
Sol - Soluble, F1 - Exchangeable, F2 - Fe/Mn oxides,

F3 - Organic matter, F4 - Residual

Figure 8.11   Speciation of TMs in the cake layer of the SAMBR2 at the end of 2 h HRT experiment (n = 3).

In summary, the SAMBR was still able to achieve good treatment efficiencies when the HRT was reduced from 6 h to 2 h. In terms of TM speciation, lowering the HRT resulted in the loss of TMs from the SAMBR, especially at 2 h HRT, although there was no significant increase in the soluble fraction. The speciation of Fe, Co, and Ni was shifted towards highly bioavailable fractions, whereas Zn, Mn, and Mo became less bioavailable at lower HRTs. Hence TM bioavailability changed in complex ways as the HRT decreased, and since Fe, Co and Ni are essential for methanogens (Thanh et al., 2016), changes in their speciation may have had a positive impact on methanogenesis.

8.3.3   Changes in SRT

8.3.3.1   Performance of the SAMBR under different SRTs

Figure 8.12 showed that the average effluent COD at different SRTs was fairly constant at around 13 mg/L, and the COD removal was always more than 97%. The average COD in the reactor was 76.3 mg/L at the "steady state" SRT of 100 d, but reduced to 68.6 mg/L at 75 d SRT and 62.5 mg/L at 50 d SRT ($p < 0.05$), and then 53.5 mg/L at 25 d SRT ($p < 0.05$).
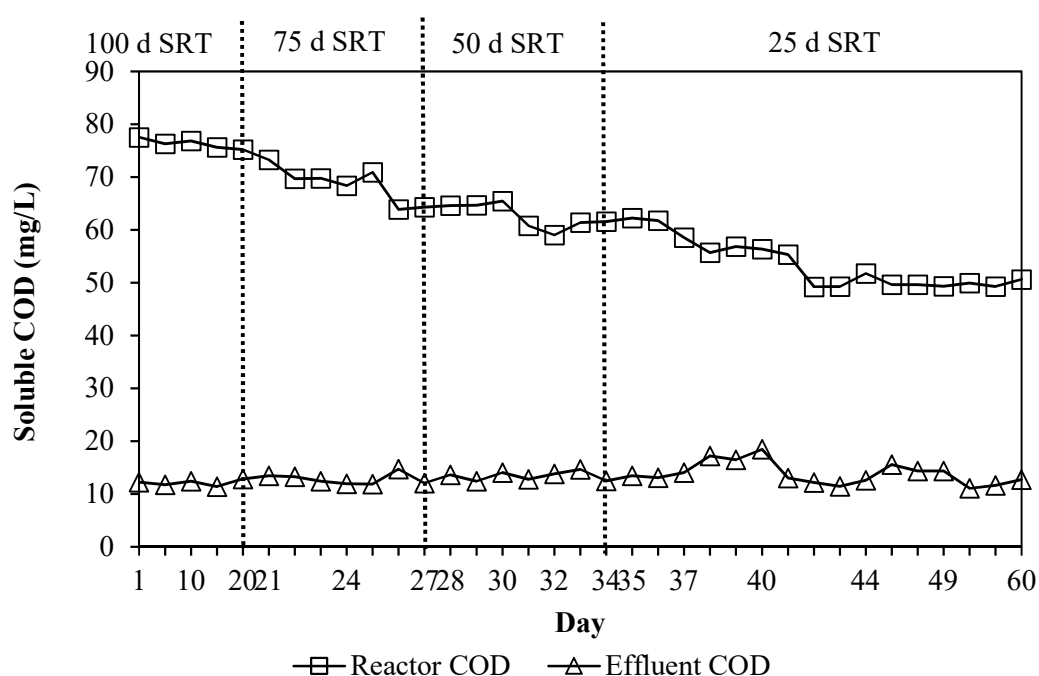
Figure 8.12   Soluble COD in the SAMBR3 reactor and effluent at different SRTs (n = 3).

It is known that a long SRT will result in an accumulation of inactive biomass which does not contribute to the degradation of the substrate. On the other hand, at low SRTs microorganisms will be lost from the reactor. A rule of thumb for the design of anaerobic digestion systems is that the SRT should be at least twice the generation time of the methanogens (Dohányos & Zábranská, 2001). In this study, although a significant amount of biomass was removed

daily within 27 days (day 34 to day 60), 25 d was still above the minimum SRT (10 d) (Weimer, 1998). The maximum substrate utilization rate was calculated using the following equation:

$$K_{max} = \frac{-\mu_{max}X}{Y}$$

Where:

K_{max} is the maximum substrate utilization rate (gCOD/L.d)

μ_{max} is the maximum specific growth rate (0.27/d) (Wong et al., 2014)

X is the biomass concentration (5.95 gVSS/d at 25 d HRT)

Y is the yield coefficient (0.357 gCOD/gVSS) (Wong et al., 2014)

The calculated K_{max} value was 4.42 gCOD/L.d, which is more than double the substrate load in the influent i.e. 2 gCOD/L.d. In addition, the maximum OLR on the reactor is 0.75 gCOD/gVSS.d, which is also much higher than the applied OLR at 25 d HRT. These calculations show that the amount of substrate loaded into the SAMBR at the lowest SRT, i.e. 25 d was still below its maximum capacity. As the SRT decreased, the growth rate of anaerobic microorganisms increased due to the increase in OLR, hence, there was still a sufficient amount of active microorganisms in the reactor to consume the same amount of substrate, and hence to maintain the high level of treatment efficiency.

Reducing the SRT resulted in a reduction in the average biogas production rate from 100.7 mL/gVSS.d at 100 d SRT to 81.3 mL/gVSS.d ($p < 0.05$). The methane yields were also found to reduce from 65.2 mLCH$_4$/gVSS.d at 100 d SRT to 60.8 mLCH$_4$/gVSS.d at 75 d SRT and to 57.6 mLCH$_4$/gVSS.d at 50 d SRT ($p < 0.05$), then finally to 56.6 mLCH$_4$/gVSS.d at 25 d SRT ($p < 0.05$) (Figure 8.13). Therefore, the methane yields dropped by 13.3% when the SRT reduced from 100 d to 25 d. This was possible due to the reduction in biomass content in the reactor, as reflected by the decrease in VSS values from

9.3 g/L at 100 d SRT to 6.8 g/L at 25 d SRT ($p < 0.05$). SRT is known to significantly affect the substrate-utilization rate (the utilization rate for acetate or dissolved hydrogen gas for methanogens) in an anaerobic MBR (AnMBR) since the endogenous decay term become less significant with decreasing SRT (Yeo & Lee, 2013). At shorter SRT, the amount of substrate for hydrolyzing/fermenting bacteria could be higher than for methanogens, which could possibly result in a decrease in methane production rate.
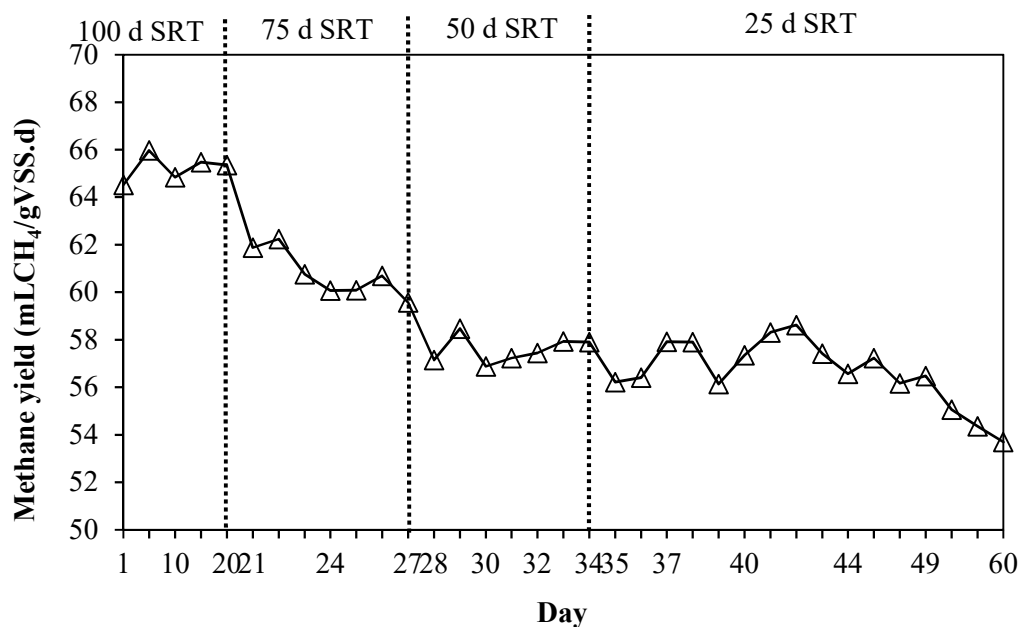
Figure 8.13   Methane yields in the SAMBR3 at different SRTs.

8.3.3.2 Dynamic changes in trace metal speciation under different SRTs

Total content of trace metals - The total content of TMs at all SRTs was found to be in the following order: Fe > Co > Zn > Ni > Mo > Mn (Table 8.5). As the SRT was reduced, a greater amount of biomass was removed daily, and if the biomass removal rate is higher than the biomass growth rate, it will result in a decrease in biomass content in the SAMBR. With the same daily TM loading, as the SRT reduces it is expected that the total content of TMs in the SAMBR will also reduce due their adsorption onto the solids; hence, the TM

content will be depleted. The depletion rate was different for each TM and was in the following order: Fe > Ni > Co > Zn > Mn > Mo (Table 8.5). This difference could be attributed to the level of accumulation and affinity of a TM for the biomass in the SAMBR, as discussed in the above experiments. Among the TMs, the depletion rate was the highest for Fe, i.e. 32.3% and the lowest for Mn i.e. 0.2% when the SRT was reduced to 25 d.

Table 8.3   Total content (µg/gTSS) of TMs as an average of samples collected at each SRT and leaching rate (µg/gTSS.d) of TMs determined from total content (µg/gTSS) at the beginning and the end of each SRT in the SAMBR3.

| Metal	SRT (day)							
	100		**75**		**50**		**25**	
	Metal content (µg/g)	Metal leaching rate (µg/g.d)	Metal content (µg/g)	Metal leaching rate (µg/g.d)	Metal content (µg/g)	Metal leaching rate (µg/g.d)	Metal content (µg/g)	Metal leaching rate (µg/g.d)
Fe	7048.2	28.8	6652.7	56.5	6298.9	50.5	5491.1	32.3
Co	1124.3	0.7	1088.3	5.1	1031.8	8.1	1018.5	0.5
Ni	873.5	1.8	824.1	7.1	772.2	7.4	640.3	5.3
Zn	911.6	0.1	892.1	2.8	887.1	0.7	832.8	2.2
Mn	139.4	0.0	134.0	0.8	125.6	1.2	120.5	0.2
Mo	194.5	-0.7	192.5	0.3	186.2	0.9	174.8	0.5

Speciation of trace metals - The measurement of TMs in each fraction using BCR extraction is given in Table A.5 (Appendix), and the speciation of TMs at different SRTs is shown in Figure 8.14. In the liquid phase, similar to the previous experiments on pH and HRT, very low levels of TMs were present in the soluble fraction, i.e. up to 2.2%, except for Mn and Mo which

were up to 14.7%. This fraction showed a decreasing trend as the SRT reduced to 25 d for all TMs.

Iron (Fe)
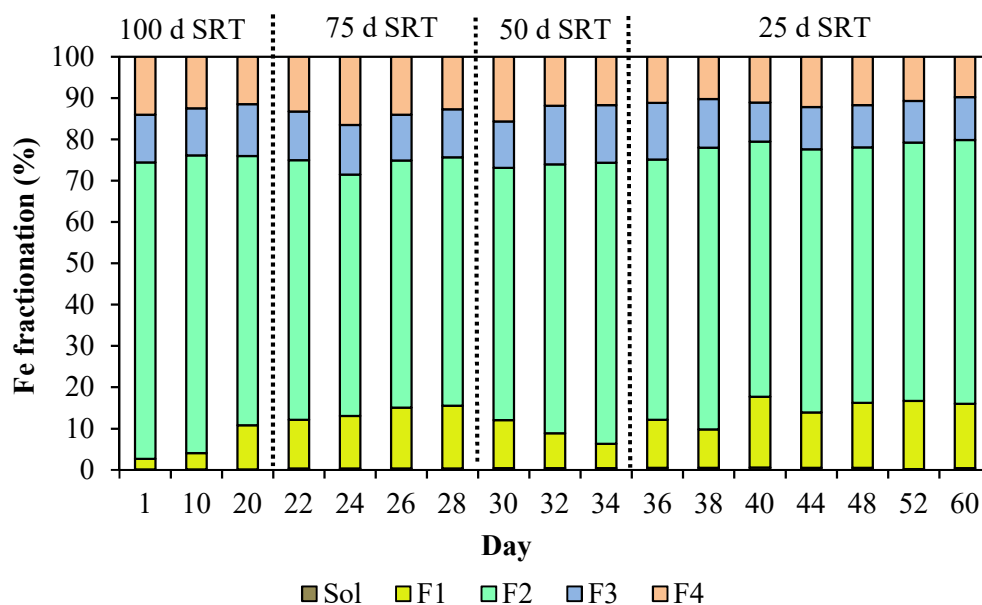
Cobalt (Co)

Nickel (Ni)

Zinc (Zn)

Manganese (Mn)
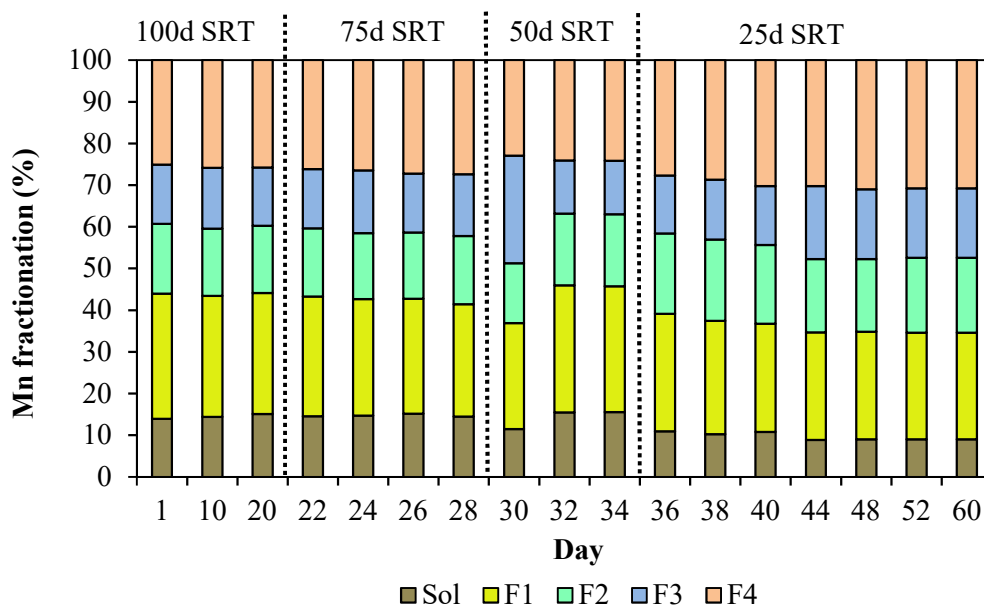
Molybdenum (Mo)
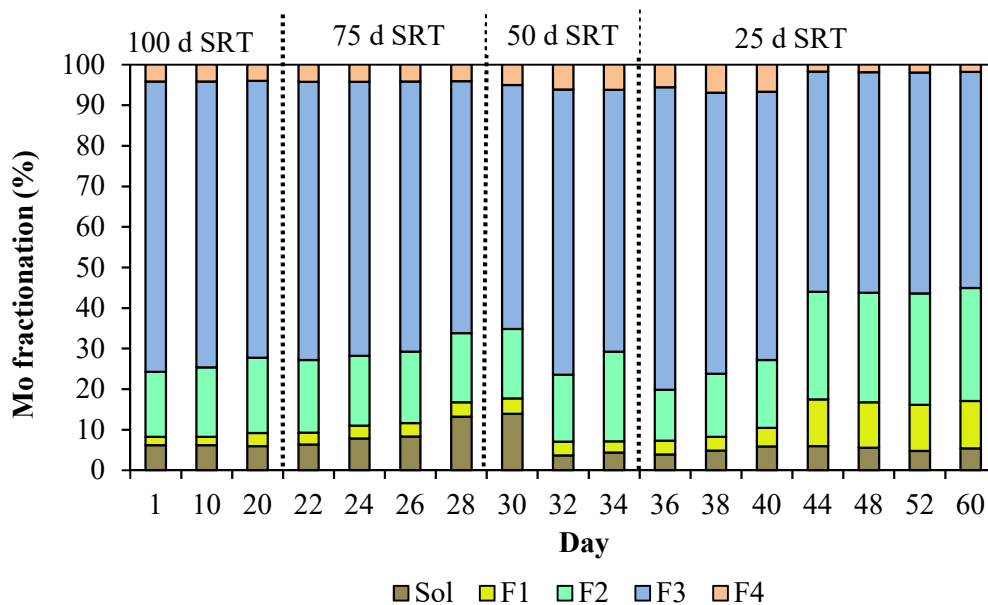
Sol - Soluble, **F1** - Exchangeable, **F2** - Fe/Mn oxides,

F3 - Organic matter, **F4** - Residual

Figure 8.14   Soluble COD in the SAMBR3 reactor and effluent at different

SRTs (n = 3).

In the solid phase, generally the dominant fraction for most of the TMs was still organic matter/sulfide, except for Fe as up to 69.7% was found in the Fe-Mn oxides fraction, which was the same as the above experiments. The first fraction (Exchangeable F1), the second most bioavailable fraction, was found to increase significantly ($p < 0.05$) for all TMs, i.e. up to around 10% (Zn), except for Ni and Mn. The second fraction Fe-Mn oxides (F2) was relatively stable during the changes in SRT ($p > 0.05$), except for Fe which reduced from 69.7% to 63.6% ($p < 0.05$). In contrast to the first fraction, the level of TMs presented in the last two fractions with the lowest bioavailability, i.e. organic matter/sulfide and residual, reduced significantly ($p < 0.05$), except for Ni and Mn.

Therefore, it can be concluded that the reduction in SRT from 100 d to 25 d reduced the total content of TMs due to the high removal of biomass. However, the speciation results showed that TMs were mobilized towards more bioavailable fractions, except for Ni and Mn. These results could be used to explain the findings on the performance of the SAMBR as the TMs became more bioavailable to support the growth of anaerobic microorganisms which had to consume a higher substrate loading.

8.4 Conclusions

The results clearly showed the strong effect of low pH, HRT, and SRT values on TM leaching/accumulation dynamics and speciation in SAMBRs. There were two similar trends when these operating parameters changed. Firstly, the total content in the SAMBRs of all TMs was reduced significantly. Secondly, their speciation tended to shift towards highly bioavailable fractions, i.e. Soluble and Exchangeable, except for Fe, Co, and Zn (pH reduced from 7 to 6.5 to 6); except for Zn, Mn, and Mo (HRT reduced from 6 h to 4 h to 2 h); and except for Ni and Mn (SRT reduced from 100 d to 25 d). Knowing which metals are retained in the reactor or lost in the effluent, and whether their speciation is shifted towards higher or lower bioavailability under each applied

condition, a specific dosing strategy can be developed to make sure that there is a sufficient amount of TMs present in the bioavailable fraction in the SAMBR for microbial uptake.

Chapter 9

Conclusions and Future Work

Outline:

9.1 Introduction

9.2 Concluding discussion

9.3 Conclusions

9.4 Recommendations for future work

9.1 Introduction

The extent to which this work has accomplished the initial objectives outlined in Section 2.11 is analyzed in this chapter. In addition, the importance of the findings, and the conclusions which can be drawn from this study, are presented. Finally, the areas of research that could be further explored, and the knowledge gaps that need to be investigated in more detail are highlighted in Future Work.

9.2 Concluding discussion

The main aims of this study were:

- to investigate the effect of Fe^{2+} supplementation on the degradation of accumulated VFAs during organic shock loads in the SAMBR;
- to understand the influence of the addition of biodegradable chelating agent EDDS, and determining its dosing strategy to improve the bioavailability of Fe^{2+} in the presence of sulfide in the SAMBR;

- to observe the changes in pH, HRT, and SRT on the speciation of trace metals in the SAMBR; and, to investigate the pulse doing of trace metals to improve the performance of the SAMBR during trace metal deficiency.

Based on the experimental findings, the following points can be made:

9.2.1 Iron deficiency and bioavailability in anaerobic batch and submerged membrane bioreactors during organic shock loads

- When the batch reactors were subjected to a glucose spike of 1 gCOD/L, a rapid accumulation of VFAs and a subsequent pH drop resulted in the mobilization of Fe^{2+} from their sulfide forms to "relatively bioavailable forms'' such as soluble and organically bound Fe. With the 3 gCOD/L shock, pH 5 in the reactor resulted in almost 9% of Fe^{2+} in the residual form leaching out to soluble and organically bound forms, although no external Fe^{2+} was dosed initially;

- With the 3 gCOD/L shock, kinetic differences and thermodynamic considerations resulted in an almost 15-fold increase in total VFAs, irrespective of Fe supplementation. A lower rate of methane production in Fe deficient reactors signify the synergetic effects of Fe limitations with 3 gCOD/L. The competition for TMs amongst fermentative bacteria, acetogens and methanogens seemed to create temporary nutrient limitations, which retarded the degradation of acetate and propionate during shock loading;

- The addition of 10 mg/L Fe^{2+} (equivalent to 3.34 mgFe^{2+}/gTSS) was found to be the optimal concentration for Fe^{2+} to improve the kinetics of propionate to methane conversion. The inhibition of Fe^{2+} concentrations above 50 mg/L was attributable to the availability of Fe^{2+} in less mobile forms as they can also be bioavailable to microorganisms. Among the VFAs, the stimulatory effect of Fe^{2+} was statistically significant for the

degradation of acetate, as the methane production rate increased by 2.1–3.9 fold within the first 60 h compared to the non-Fe supplemented reactors;

- The effects of 10 mg/L Fe^{2+} (equivalent to 3.34 mgFe^{2+}/gTSS) supplementation to a SAMBR were statistically significant in the case of the 1 gCOD glucose shock compared to 2 gCOD for the rapid removal of VFAs as almost 50% of the acetic acid was reduced in the first hour, and completely removed within 2 h. Propionic acid was removed at slower rate as only 60% of the accumulated propionic acid was eliminated within in 2 h.

9.2.2 Effect of Ethylenediamine-N,N'-disuccinic acid (EDDS) on the speciation and bioavailability of Fe^{2+} in the presence of sulfide

- The BCR extraction method exhibits good recovery for most of the metals for both dried and wet samples i.e. 89.0% to 113.5%. However, the extraction of metals from wet samples not only produced comparable results, but also better extraction efficiencies than a dried sample. The use of wet samples for metal extraction reduced the time required for sample preparation, and it also eliminated the possibility of artificial altering the metal speciation;

- At their optimal concentrations, macronutrients Ca and Mg exhibited the highest increase in methane production rate i.e. 64.8 mLCH_4/d, whereas trace metals Fe, Co, and Ni came second with up to 41.7 mLCH_4/d, and the methane production rate was the lowest for Cu, Zn, and Mn i.e. 38.3 mLCH_4/d;

- In a model media (no biomass), in the presence of 8 mg/L sulfide, the remaining soluble concentration of 10 mg/L Fe^{2+} (equivalent to 3.34 mgFe^{2+}/gTSS) was reduced to zero within the first 2 hours as the Fe^{2+} was precipitated as FeS. When EDDS was added at 1:1 molar ratio to Fe^{2+}, the soluble Fe^{2+} concentration increased to 3.32 mg/L (33.6% increase). With biomass, there was no significant difference in methane yields when the

same amount of sulfide was added. However, in the presence of EDDS at 1:1 molar ratio to Fe^{2+}, the methane yields increased by 13.3% and the methane production rate also increased by 11.2%. The speciation of Fe^{2+} was shifted towards higher bioavailable fractions, which indicates that EDDS was able to complex with the Fe^{2+} and preventing it from precipitating with sulfide;

- Determining the concentration of free EDDS and Fe-EDDS complex using HPLC was able to confirm the increase in soluble concentration of Fe^{2+} as high amount of Fe-EDDS complex was present in the solution, which was then bioavailable for microbial uptake.

9.2.3 Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe^{2+} in the presence of sulfide in a submerged anaerobic membrane bioreactor

- In the presence of sulfide, the dosing of EDDS as a complex with Fe^{2+} resulted in greater improvement in the bioavailability of Fe^{2+} than when it was supplemented simultaneously with Fe^{2+} as it prevented the precipitation of Fe^{2+} with sulfide. As the result, the methane yields and production rate from the Fe-EDDS increased significantly by 17.3% and 9.9%, respectively. It also effectively removed the hydrogen produced by substrate degradation by the fermentative bacteria;

- The addition of EDDS as a metal complex (Fe-EDDS) or simultaneously (Fe+EDDS) both increased the soluble Fe fraction to 9.0% and 7.2%, respectively. However, the Fe-EDDS addition resulted in a better retention of Fe^{2+} in the highly bioavailable forms (soluble and exchangeable) than Fe+EDDS;

- The measurement of free EDDS and Fe-EDDS complex by UHPLC showed that these two EDDS forms in the Fe-EDDS set were statistically higher than those in the Fe+EDDS set, i.e. 4.8% and 36.5% compared to 4.0% and 33.6%, respectively;

- There was a significant amount of trace metals, including Fe^{2+}, accumulated in the seed sludge of the SAMBR prior to the experiment. These accumulated metals seemed to be sufficient to support the growth of anaerobic microorganisms as the reactor was still capable of achieving very high treatment efficiencies for 10 d when only Fe^{2+} was added to the feed;

- The presence of 8 mg/L of sulfide clearly reduced the solubility of Fe^{2+} as it had already precipitated the Fe^{2+} in the feed before entering the SAMBR, thereby reducing the level of soluble Fe^{2+} in the reactor for microbial uptake;

- The precipitation of Fe^{2+} as FeS resulted in a shift in Fe^{2+} speciation towards less bioavailable fractions, which in turn reduced the performance of the SAMBR as the average soluble COD inside the reactor and in the effluent increased to 64.8 mg/L and 17.2 mg/L, respectively. The corresponding average COD removal reduced to 96.6%, and the average daily methane yields were reduced by 14.6% to 49.8 mLCH$_4$/gVSS.d;

- The dosing of EDDS as a metal complex to the SAMBR clearly helped to improve the performance of the SAMBR as the COD removal increased to 97.5%, and the methane yields also increased to 54.5 mLCH$_4$/gVSS.d;

- Measurements from UHPLC showed that when pre-complexed with EDDS, 86.2% of Fe was retained in solution as an Fe-EDDS complex. The speciation of Fe^{2+} in the SAMBR was then shifted towards more bioavailable fractions, i.e. Soluble and Exchangeable, showing that the addition of EDDS was able to improve the bioavailability of Fe^{2+} for microbial uptake.

9.2.4 Trace metal deficiency and dosing strategies in a submerged anaerobic membrane bioreactor

- When trace metals were excluded from the feed, significant accumulation of VFAs in the SAMBR was observed due to the kinetic differences

between different groups of anaerobic bacteria which might have created temporary nutrient imbalances;

- Organic compounds such as soluble microbial products (SMPs) might have contributed to the increase in soluble COD in the reactor and the effluent as they were released by the bacteria to scavenge the available metal nutrients in solution;

- A continuous supply of trace metals to the SAMBR resulted in substantial metal accumulation in the following order: Fe > Zn > Co = Mn > Mo > Ni. Most of the trace metals accumulated in the low bioavailable form, i.e. Organic matter/sulfide, as they were better retained in the reactor as sulfide precipitates;

- During metal deficient conditions, the metal content in the reactor was reduced due to the leaching of metals in the following order: Fe > Co > Zn > Ni > Mo > Mn. The distribution of metals was shifted from relatively bioavailable fractions, i.e. Soluble and Exchangeable, towards less bioavailable fractions i.e. Organic matter/sulfide and residual;

- A pulse of trace metals at the five-times daily loading into the SAMBR improved reactor performance temporarily within 9 d by removing the VFAs accumulated from the metal deficient period. The trace metals added were mobilized towards bioavailable fractions. Pulses of the ten-times daily loading did not result in any improvement in the reactor performance, and the bioavailability of trace metals, but also might have inhibited the methanogenic microorganisms.
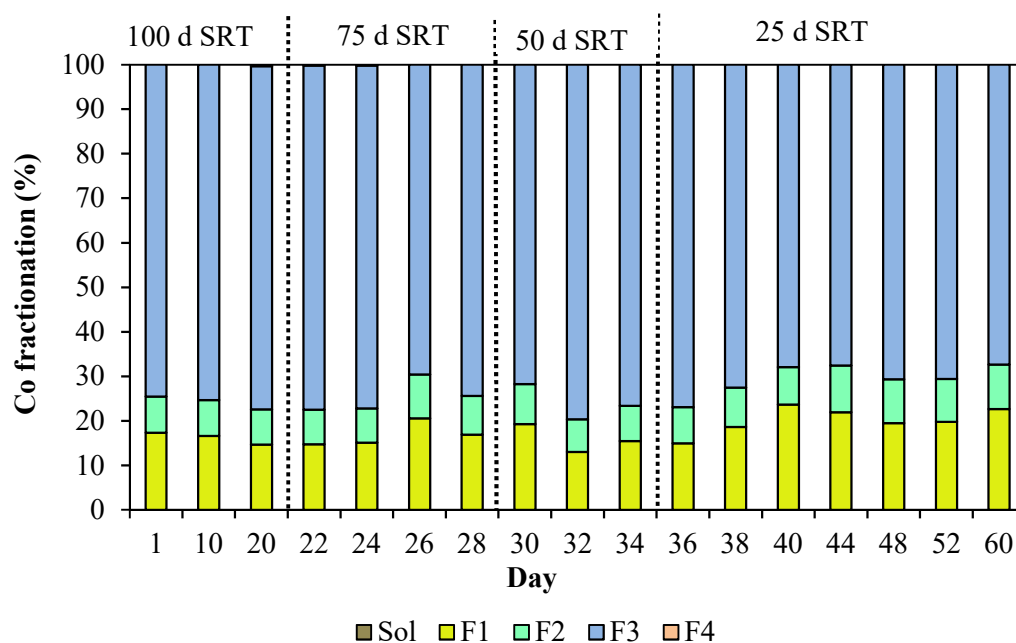
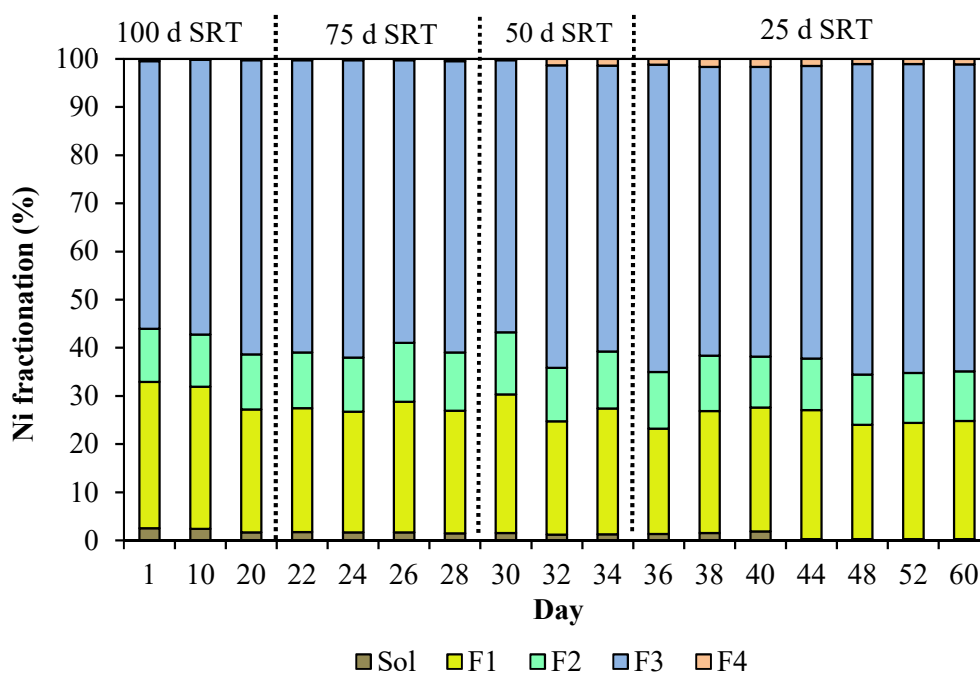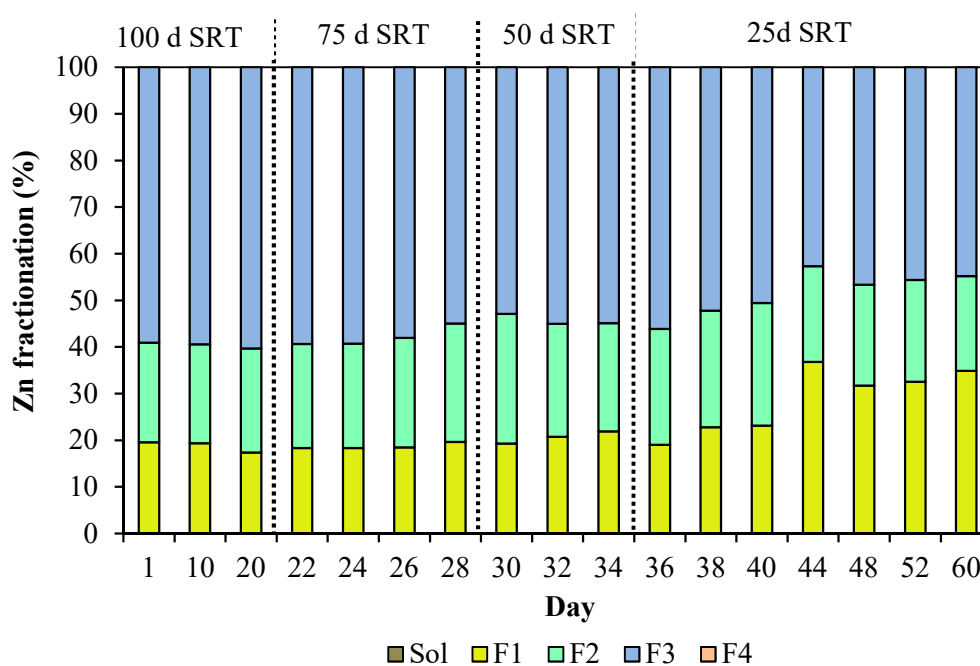### 9.2.5 Effect of operating conditions on speciation and bioavailability of trace metals in submerged anaerobic membrane bioreactors

- Low pHs of 6.5 and 6 reduced the SAMBR performance significantly as they seriously affected the methanogens. It took 12 d for the pH to return to neutral, but the COD removal increased only very slowly i.e. to 73.2% after 20 d;

- The reduction in pH resulted in an increase in soluble metal concentration and leaching of metals from the reactor in the following order: Fe > Ni > Co > Zn > Mn > Mo (pH 6.5) and Fe > Zn > Ni > Co > Mn > Mo (pH 6). Fe and Ni underwent the most significant changes in each BCR fraction;

- On the other hand, changes in Co, Mn and Mo contents were less significant. The speciation of Ni, Mn, and Mo shifted towards bioavailable fractions, i.e. Soluble and Exchangeable, whereas, Fe and Co showed no patterns in their speciation, and Zn was found not to be sensitive to the pH changes;

- Higher organic loading rates (OLR) at low HRTs of 4 h and 2 h resulted in the rapid accumulation of VFAs, but the SAMBR was able to recover very quickly. However, higher methane yields were obtained due to the increase in biomass content in the reactor;

- High leaching rates of trace metals from the SAMBR occurred when the HRT was reduced in the following order: Fe > Ni > Co > Zn > Mn > Mo, due to the low pH effect which increased the solubility of trace metals. The speciation of Fe, Co, and Ni was shifted towards highly bioavailable fractions, whereas Zn, Mn, and Mo became less bioavailable at lower HRTs;

- A reduction in SRT from 100 d to 25 d did not affect the performance of the SAMBR as COD removal was still more than 97%, although the methane yields were reduced by 13.3%. This was possibly due to the reduction of substrate utilization rate for methanogens at shorter SRT;

- A reduction in SRT from 100 d to 25 d resulted in a higher biomass removal rate than biomass growth rate, which consequently reduced the biomass content in the SAMBR in the following order: Fe > Ni > Co > Zn > Mn > Mo. However, the speciation results showed that trace metals were mobilized towards more bioavailable fractions, except for Ni and Mn.

9.3 Conclusions

Based on the results obtained from this study, the following conclusions can be drawn:

1) The accumulation of trace metals in the seed sludge was very significant due to the prolong continuous supply of trace metals, hence, it needs to be taken into account for an effective metal dosing strategy;

2) Acetoclastic methanogens were more constrained by Fe^{2+} limitations compared to the other trophic groups. An organic shock resulted in leaching of Fe^{2+} from the residual to organically bound and soluble forms while the precipitation of Fe^{2+} as sulfide seemed to reduce its bioavailability to acetoclastic methanogens;

3) Supplementation of 10 mg/L Fe^{2+} (equivalent to 3.34 mgFe^{2+}/gTSS) helped to reduce significantly and quickly the accumulation of VFAs during organic shocks (1 and 2 gCOD glucose spike) in the SAMBR;

4) The presence of 8 mg/L sulfide indirectly limited the growth of methanogens as it precipitated the Fe^{2+} hence reduced the Fe^{2+} bioavailability for microbial uptake;

5) Biodegradable chelating agent EDDS when dosed at 1:1 molar ratio to Fe^{2+} at 10 mg/L (equivalent to 3.34 mgFe^{2+}/gTSS) was able to compete with sulfide at 8 mg/L and to keep the Fe^{2+} in a soluble form as Fe-EDDS complex, which was bioavailable for the microorganism, hence resulting in a higher methane yields;

6) When EDDS and Fe^{2+} were dosed at a 1:1 molar ratio (10 mg/L Fe^{2+} or 0.22 mg/gTSS.d) as a metal complex into the SAMBR, the Fe^{2+} speciation was reversed to highly bioavailable fractions, and hence the SAMBR performance was improved as the both COD removal and methane yields increased;

7) The performance of the SAMBR was significantly affected by the reduction in pH (from 7 to 6.5 and 6) as the low pHs were out of favoured range for the methanogens and the reduction in HRT (from 6 h to 4 h and 2

h) due to the hydraulic shock loads; however, the SAMBR was able to sustain a low SRT 25 d as the corresponding OLR was still below the maximum OLR of the SAMBR;

8) The degrees of accumulation and leaching of trace metals in the SAMBR at different operating conditions (pH, HRT, and SRT) varied between metals as they depend on the sensitivity of the trace metals towards the changes and also their affinity to anaerobic sludge sites;

9) The reduction of pH resulted in a decrease in total metal content in the SAMBR, but an increase in the soluble fraction for all TMs. Trace metal speciation was shifted towards highly bioavailable fractions for Ni, Mn, and Mo;

10) Lowering the HRT also resulted in the loss of metals from the SAMBR, but no significant increase in the soluble fraction. The bioavailability of essential trace metals i.e. Fe, Co, Ni was improved as their speciation was shifted towards highly bioavailable fractions, and hence the changes in HRT in the short term may have had a positive impact on methanogenesis;

11) The high removal of biomass in the SAMBR at low SRT 25 d also reduced the metal content in the reactor, and trace metals became more bioavailable except for Ni and Mn.

9.4 Recommendations for future work

Based on the findings of this research and issues raised during the conduct of this research, the following recommendations for future study are suggested:

1) Trace metals such as Fe, Ni, and Co have been studied extensively in the literature; however, other metals such as the macro-nutrients i.e. Ca and Mg and other trace metals i.e. W and Se are also essential to the optimal operation of anaerobic digestion. Hence, further research is required in order to determine the relationship between their speciation and bioavailability in anaerobic digestion. In addition, the effects of one trace

metal on the speciation and bioavailability of another during co-precipitation and adsorption are also important to understand;

2) EDDS has shown that due to its superior biodegradability, it can be an excellent replacement for the conventional chelating agents EDTA and NTA to improve the bioavailability of Fe. However, the biodegradability of EDDS during anaerobic digestion needs to be carefully evaluated. The application of EDDS should also be extended to other trace metals i.e. Co and Ni, although competition between different trace metals for complexation with EDDS needs to be investigated as it might reduce the influence of EDDS dosing on the improvement of anaerobic reactor performance. Other new generation biodegradable chelating agents with higher complexing ability such as Glutamic acid diacetic acid (GLDA) and Iminodisuccinic acid (IDS) could be investigated to compare with the results with EDDS;

3) Better characterization and quantification of natural chelating agents SMPs, and further development of the EPS extraction procedure should be carried out in order to determine how important their roles are in trace metal complexing and binding properties in anaerobic reactors;

4) Metal sulfides such as NiS have been shown to become a storage of and source for trace metals. To further understand which factors influence the bioavailability of metal sulfides inside the bioreactor, it is necessary to determine their speciation in terms of organic/inorganic sulfides, possible interaction with other metal sulfides, and the degree of crystallinity of the metal-sulfide precipitates using Extended X-ray absorption fine structure (EXAFS) and XRD (X-ray diffraction);

5) Methanogens including acetoclastic methanogens and hydrogenotrophic methanogens are thought to be the most sensitive microbial group in anaerobic digestion processes to changes in trace metal speciation and bioavailability. The use of microbial community analysis such as DNA extraction, pyrosequencing, and terminal restriction fragment length

polymorphism (T-RFLP) fingerprint (Wintsche et al., 2016) to monitor the dynamics of bacterial and methanogenic communities will be useful to explain the changes in trace metal speciation and bioavailability on anaerobic trophic groups. Much greater insight, and more data, on the microbial ecophysiology of trace metals will be needed to support this goal;

6) It would be useful to harmonize sequential extraction schemes for data comparability, and the design of new and simple schemes based on the existing ones with shorter extraction times. Advanced techniques include XANES, DGT, and DMT need to be further developed and adapted to better classify metal speciation, and hence to predict metal bioavailability in anaerobic reactors. The use of analytical techniques with higher accuracy such as ICP-MS and optical microfiber sensor (Ji et al., 2016) to detect trace metals at ppb level will help to better observe the changes in metal concentration in the effluent from the reactor.

Publications

Chapter 2	Literature review

Thanh, P.M., Ketheesan, B., Yan, Z., Stuckey, D. 2016. *Trace metal speciation and bioavailability in anaerobic digestion: A review*. Biotechnology Advances 34, 122-136.

Chapter 4   Iron deficiency and bioavailability in anaerobic batch and submerged membrane bioreactors during organic shock loads

Ketheesan, B., Thanh, P.M., Stuckey, D.C. 2016. *Iron deficiency and bioavailability in anaerobic batch and submerged membrane bioreactors (SAMBR) during organic shock loads*. Bioresource Technology 211, 136-145.

Chapter 5   Effect of Ethylenediamine-N,N'-disuccinic acid (EDDS) on the speciation and bioavailability of Fe^{2+} in the presence of sulfide

Thanh, P.M., Ketheesan, B., Yan, Z., Stuckey, D. 2017. *Effect of Ethylenediamine-N,N'-disuccinic acid (EDDS) on the speciation and bioavailability of Fe^{2+} in the presence of sulfide in anaerobic digestion*. Bioresource Technology 229, 169-179.

Chapter 6   Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe^{2+} in the presence of sulfide in a submerged anaerobic membrane bioreactor

Thanh, P.M., Ketheesan, B., Stuckey, D.C., Zhou, Y. 2017. *Dosing of Ethylenediamine-N,N'-disuccinic acid (EDDS) to improve the bioavailability of Fe^{2+} in the presence of sulfide in a submerged anaerobic membrane bioreactor*. Chemical Engineering Journal 330, 175-182.

Chapter 7   Effects of trace metal deficiency and supplementary on a submerged anaerobic membrane bioreactor

Thanh, P.M., Ketheesan, B., Stuckey, D.C., Zhou, Y. 2017. *Effects of trace metal deficiency and supplementation on a submerged anaerobic membrane bioreactor.* Bioresource Technology 241, 161-170.

Chapter 8   Effect of operating conditions on speciation and bioavailability of trace metals in submerged anaerobic membrane bioreactors

Thanh, P.M., Ketheesan, B., Zhou, Y., Stuckey, D.C. 2017. *Effect of operating conditions on speciation and bioavailability of trace metals in submerged anaerobic membrane bioreactors.* Bioresource Technology 243, 810-819.

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Appendix

Table A.1   Fe content (µg/gTSS) from sequential extraction in the SAMBR (n = 3).

	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	25.9	1912.9	5016.5	977.8	2105.4	10038.5
	3	25.9	2014.8	5081.5	971.1	2090.7	10184.0
Stage 1	5	28.0	2109.3	5195.3	971.7	2084.1	10388.6
	10	27.6	2057.1	4994.9	966.6	2125.3	10171.7
	11	9.7	594.2	4328.5	1382.9	3719.8	10035.0
	13	8.8	564.8	4326.5	1415.3	3692.0	10007.4
	15	9.3	462.1	4115.4	1398.2	4196.3	10181.4
Stage 2	17	9.6	419.1	4101.9	1530.5	4088.0	10149.2
	19	8.7	437.0	3900.8	1651.1	4273.1	10270.6
	21	8.7	592.2	3771.4	1792.4	4374.8	10539.4

	22	11.7	649.6	3806.4	1444.4	4429.5	10341.5
	24	18.0	912.8	4083.4	1454.5	4439.8	10908.5
Stage 3	26	16.8	965.1	4017.2	1309.8	3987.6	10296.6
	28	16.9	1222.1	4352.1	1326.3	3440.4	10357.7
	30	18.2	1379.1	4370.5	1202.4	3243.6	10213.8

Table A.2   Metal content (µg/gTSS) in each fraction from BCR extraction method (n = 3).

Fe	Day	Sol	F1	F2	F3	F4	Sum of fractions
With metals	0	11.4	332.1	4438.3	788.3	816.6	6386.8
	5	11.2	337.6	4533.1	754.6	788.8	6425.4
	15	11.6	339.3	4541.0	785.4	800.0	6477.3
No metals	17	10.4	329.3	4275.0	946.5	840.4	6401.6
	25	0.0	317.9	4140.8	1002.7	853.6	6314.9
	33	0.0	316.6	3921.1	946.1	1012.6	6196.4
	41	0.0	317.0	3471.3	948.0	1336.2	6072.5
1st pulse	43	0.0	643.7	3824.7	858.9	865.3	6192.6
	45	0.0	458.9	3904.7	899.1	852.2	6114.8
	49	0.0	418.7	4093.8	789.0	779.3	6080.7
2nd pulse	51	0.0	293.3	3438.3	985.4	1548.2	6265.2
	53	0.0	317.4	3365.3	992.2	1541.1	6215.9
	57	0.0	323.8	3331.7	995.9	1540.7	6192.2

	59	0.0	278.8	3577.7	1095.6	1735.2	6687.3
3rd pulse	65	0.0	333.3	3465.2	1082.2	1590.0	6470.8
	72	0.0	329.1	3309.5	1104.1	1523.6	6266.5

Co	Day	Sol	F1	F2	F3	F4	Sum of fractions
	0	0.0	310.4	146.6	1111.5	0.0	1568.5
With metals	5	0.0	306.7	147.2	1118.7	0.0	1572.6
	15	0.0	304.6	150.3	1127.7	0.0	1582.7
	17	0.0	284.6	148.9	1123.5	0.0	1557.0
	25	0.0	278.8	145.3	1080.6	0.0	1504.6
No metals	33	0.0	256.6	144.0	1006.6	0.0	1407.1
	41	0.0	202.1	116.5	920.5	0.0	1239.1
	43	0.0	235.3	111.6	915.5	0.0	1262.4
1st pulse	45	0.0	246.9	101.6	896.1	0.0	1244.5

	49	0.0	291.7	119.7	765.7	0.0	1177.1
	51	0.0	226.9	106.7	881.9	0.0	1215.5
2nd pulse	53	0.0	226.3	110.0	853.6	0.0	1189.9
	57	0.0	224.3	112.7	832.9	0.0	1170.0
	59	0.0	235.8	115.5	898.6	0.0	1249.9
3rd pulse	65	0.0	211.4	107.0	865.5	0.0	1184.0
	72	0.0	185.4	100.0	855.4	0.0	1140.8

Ni	Day	Sol	F1	F2	F3	F4	Sum of fractions
	0	0.0	151.8	55.4	214.2	8.3	429.8
With metals	5	0.0	152.2	54.5	215.9	9.0	431.6
	15	0.0	154.3	53.2	218.1	9.2	434.8

	17	0.0	131.4	52.1	231.1	0	414.6
No metals	25	0.0	134.6	47.5	208.9	0	391.1
	33	0.0	116.0	41.7	194.1	0	351.9
	41	0.0	86.2	38.8	170.2	0	295.2
	43	0.0	92.6	38.9	169.7	0	301.3
1st pulse	45	0.0	101.0	34.4	160.9	0	296.4
	49	0.0	109.3	34.7	145.9	0	289.9
	51	0.0	85.0	34.7	176.3	0	296.0
2nd pulse	53	0.0	82.1	35.3	174.1	0	291.4
	57	0.0	79.9	35.4	173.5	0	288.9
	59	0.0	88.1	36.3	177.3	0	301.7
3rd pulse	65	0.0	79.1	35.3	174.6	0	289.1
	72	0.0	68.8	33.7	171.2	0	273.7

Zn	Day	Sol	F1	F2	F3	F4	Sum of fractions
With metals	0	0.0	284.5	314.5	674.1	0	1273.1
	5	0.0	290.4	315.7	680.2	0	1286.4
	15	0.0	293.1	321.4	681.6	0	1296.1
No metals	17	0.0	283.0	299.5	706.1	0	1288.6
	25	0.0	282.1	295.5	677.2	0	1254.9
	33	0.0	267.4	290.3	661.6	0	1219.3
	41	0.0	191.0	270.7	662.1	0	1123.8
1st pulse	43	0.0	225.8	282.1	621.3	0	1129.2
	45	0.0	285.9	252.6	589.6	0	1128.1
	49	0.0	315.5	289.6	510.4	0	1115.5
2nd pulse	51	0.0	269.4	261.7	590.5	0	1121.6
	53	0.0	270.5	296.8	546.4	0	1113.8
	57	0.0	256.1	263.0	581.9	0	1100.9
3rd pulse	59	0.0	245.6	265.8	600.3	0	1111.7

	65	0.0	225.9	255.2	618.7	0	1099.8
	72	0.0	215.6	233.7	619.0	0	1068.2

Mn	Day	Sol	F1	F2	F3	F4	Sum of fractions
	0	20.7	38.3	24.9	36.5	37.3	157.8
With metals	5	20.2	39.3	26.4	36.7	38.2	160.8
	15	22.0	39.9	27.2	40.8	41.6	171.4
	17	19.1	38.3	27.1	40.7	40.4	165.7
No metals	25	16.8	34.6	23.5	33.4	38.0	146.2
	33	11.4	26.3	21.7	27.1	36.6	123.1
	41	10.6	20.2	17.6	23.3	31.9	103.6
	43	17.9	28.9	18.9	18.9	23.2	107.9
1st pulse	45	18.8	25.0	16.1	15.9	22.1	97.9
	49	18.7	22.8	15.5	15.8	18.7	91.5

	51	16.6	22.3	17.6	17.6	26.9	101.0
2nd pulse	53	15.8	22.1	17.4	17.9	27.4	100.5
	57	15.9	20.6	15.9	17.5	25.4	95.2
	59	16.6	21.8	19.2	17.6	28.6	103.8
3rd pulse	65	14.9	17.9	16.4	18.7	25.9	93.8
	72	14.1	16.1	11.6	18.8	26.1	86.7

Mo	Day	Sol	F1	F2	F3	F4	Sum of fractions
	0	10.4	8.3	50.8	124.2	22.8	216.5
With metals	5	11.2	8.4	50.0	127.1	22.5	219.2
	15	11.6	8.1	49.7	133.8	23.1	226.3
	17	8.5	8.0	44.7	135.1	23.4	219.7
No metals	25	6.7	7.8	40.8	119.7	24.6	199.6
	33	4.6	6.3	36.6	110.4	22.9	180.7

	41	4.3	3.7	30.9	81.4	21.3	141.5
	43	5.3	11.1	28.9	81.6	16.8	143.7
1st pulse	45	5.2	8.9	29.2	80.2	15.5	138.9
	49	5.2	8.3	28.0	78.9	12.4	132.8
	51	4.1	4.1	22.8	89.0	16.6	136.6
2nd pulse	53	4.2	4.2	22.1	83.9	16.8	131.3
	57	4.1	4.8	22.2	79.2	19.0	129.4
	59	3.1	3.6	22.3	93.1	19.8	142.0
3rd pulse	65	3.0	3.0	21.9	90.8	17.9	136.6
	72	3.0	2.0	21.1	88.6	16.1	130.8

Table A.3   Metal content (µg/gTSS) in each fraction from BCR extraction method at different pHs (n = 3).

Fe	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	16.9	2407.0	6239.1	896.1	1696.8	11255.9
	3	16.0	2445.6	6062.0	938.9	1722.2	11184.7
pH 7	4	16.7	2560.2	6498.1	901.2	1602.1	11578.3
	5	17.8	2428.2	6219.6	842.8	1649.2	11157.6
	7	16.4	2384.4	6125.5	965.1	1612.5	11103.8
	8	38.1	2333.2	4237.2	987.1	1407.7	9003.2
	9	57.5	2418.1	4294.5	937.2	1131.7	8839.0
pH 6.5	11	79.2	2349.8	4333.3	922.1	1211.6	8896.0
	13	96.5	2423.3	4105.0	777.1	1159.8	8561.7
	14	127.8	2356.2	3986.8	730.6	996.5	8197.9
	16	137.5	1115.5	3878.1	893.2	701.8	6726.1
pH 6	18	147.7	1110.7	3711.8	827.6	700.9	6498.7
	20	152.9	1081.9	3419.4	810.3	696.3	6160.8

	22	161.1	1015.2	3726.1	740.5	663.3	6306.2
	24	157.0	1042.7	3719.9	775.2	643.6	6338.3
pH	26	17.4	1376.6	3922.8	948.9	1204.3	7470.1
Recovery	28	14.2	1022.8	4099.5	1014.2	978.7	7129.4
	34	18.3	1121.2	3613.9	1095.2	1436.4	7285.0
	40	5.7	1192.4	3671.4	1173.5	1801.9	7844.9

Co	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	0.0	132.3	69.9	699.6	0.0	901.7
	3	0.0	133.9	69.4	699.9	0.0	903.2
pH 7	4	0.0	133.0	69.1	700.1	0.0	902.2
	5	0.0	133.3	69.2	701.2	0.0	903.7
	7	0.0	133.3	69.3	700.7	0.0	903.3

	8	0.0	116.6	65.4	658.5	0.0	840.5
pH 6.5	9	0.0	116.1	64.3	628.1	0.0	808.5
	11	0.0	115.9	64.3	624.8	0.0	805.0
	13	0.0	115.5	63.9	593.4	0.0	772.8
	14	0.0	116.4	63.7	587.3	0.0	767.4
pH 6	16	2.7	101.4	63.6	583.8	0.0	751.5
	18	2.8	101.4	63.6	585.7	0.0	753.5
	20	2.8	101.9	84.7	580.2	0.0	769.6
	22	2.5	100.4	57.0	436.2	0.0	596.1
	24	2.7	101.8	61.3	493.4	0.0	659.2
pH Recovery	26	0.0	159.8	81.0	674.7	0.0	915.5
	28	0.0	176.6	84.3	685.5	0.0	946.4
	34	0.0	174.5	88.9	758.7	0.0	1022.1
	40	0.0	194.3	91.4	789.4	0.0	1075.1

Ni	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	9.7	201.1	89.8	379.8	6.5	686.8
	3	10.0	201.1	90.6	379.0	6.7	687.4
pH 7	4	9.4	201.0	89.5	378.4	6.3	684.7
	5	9.2	201.5	89.2	377.2	6.2	683.3
	7	9.4	209.9	88.0	378.5	6.3	692.1
	8	11.2	199.3	78.6	337.2	6.1	632.4
	9	11.1	191.0	75.9	310.8	6.0	594.7
pH 6.5	11	11.6	185.5	73.4	293.0	5.8	569.3
	13	11.9	184.9	74.0	281.4	5.5	557.6
	14	13.3	181.0	69.5	271.6	5.3	540.6
	16	13.6	179.1	69.5	266.3	5.5	534.0
pH 6	18	13.1	175.2	68.2	259.5	5.6	521.6
	20	15.7	172.7	66.7	255.6	5.6	516.2

	22	18.6	161.2	45.1	185.8	5.1	415.7
	24	26.7	143.1	56.9	205.6	5.3	437.6
pH	26	1.1	180.4	71.7	327.6	2.2	583.0
Recovery	28	1.0	184.3	68.0	322.5	2.0	577.8
	34	1.0	156.7	76.9	361.5	2.0	598.2
	40	1.0	185.2	81.4	383.3	1.9	652.9

Zn	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	0.00	279.6	281.7	666.5	0.0	1227.8
	3	0.00	280.0	282.2	667.4	0.0	1229.6
pH 7	4	0.00	279.6	281.2	666.2	0.0	1227.0
	5	0.00	279.0	280.0	665.8	0.0	1224.7
	7	0.00	279.2	279.2	665.5	0.0	1223.8
pH 6.5	8	0.00	272.3	258.6	653.0	0.0	1184.0

	9	0.00	265.3	257.3	646.2	0.0	1168.8
	11	0.00	250.7	254.1	626.2	0.0	1131.0
	13	0.00	240.2	253.9	598.1	0.0	1092.1
	14	0.00	237.2	249.6	586.9	0.0	1073.7
	16	0.00	235.0	248.6	583.6	0.0	1067.3
pH 6	18	0.00	233.2	247.7	578.9	0.0	1059.7
	20	0.00	223.6	246.3	560.9	0.0	1030.8
	22	0.00	221.5	195.8	412.0	0.0	829.3
	24	0.00	233.8	233.8	524.3	0.0	991.9
pH	26	0.00	326.6	291.8	582.7	0.0	1201.2
Recovery	28	0.00	334.5	265.0	587.6	0.0	1187.1
	34	0.00	273.6	278.8	653.0	0.0	1205.4
	40	0.00	269.5	266.7	697.4	0.0	1233.6

Mn	Day	Sol	F1	F2	F3	F4	Sum of fractions
pH 7	1	19.4	33.9	25.8	43.5	40.9	163.4
	3	21.1	34.4	25.6	43.1	40.0	164.2
	4	18.8	34.6	25.7	42.4	39.8	161.3
	5	19.5	33.8	25.6	42.4	39.0	160.4
	7	18.8	33.3	25.5	43.1	39.6	160.3
pH 6.5	8	22.3	34.0	24.8	39.3	38.5	159.0
	9	22.1	33.2	24.6	39.8	38.2	157.9
	11	22.2	33.8	23.2	39.1	36.7	155.1
	13	22.8	33.3	22.8	36.2	34.7	149.9
pH 6	14	23.0	33.2	21.2	33.5	31.9	142.8
	16	23.6	33.2	20.9	32.0	30.9	140.7
	18	24.3	33.6	20.6	30.5	29.9	138.9
	20	25.0	33.3	20.4	28.6	29.6	136.9

	22	18.6	29.5	20.7	28.3	30.4	127.4
	24	20.4	24.4	23.1	32.9	32.0	132.9
pH	26	20.7	28.8	30.4	32.6	39.1	151.6
Recovery	28	18.3	25.4	29.9	36.7	38.6	148.9
	34	18.3	24.0	29.8	36.4	40.9	149.4
	40	18.1	22.4	31.0	37.7	38.1	147.3

Mo	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	1.8	2.7	19.1	115.3	10.7	149.6
	3	1.7	2.1	18.1	108.8	8.4	139.1
pH 7	4	2.8	2.3	21.3	117.5	9.3	153.1
	5	1.9	2.8	20.6	116.1	9.3	150.7
	7	1.8	2.7	21.4	111.9	9.1	146.9
pH 6.5	8	2.7	4.0	18.6	103.8	8.8	137.8

	9	2.9	4.1	21.5	109.1	9.1	146.8
	11	2.9	5.8	17.4	108.5	9.7	144.2
	13	3.0	6.0	18.1	105.3	8.0	140.5
	14	3.0	5.6	17.7	97.1	8.1	131.6
	16	3.1	6.3	17.7	97.8	8.3	133.2
pH 6	18	3.1	6.2	16.9	88.3	6.2	120.6
	20	3.1	6.8	16.2	90.2	6.3	122.6
	22	3.3	6.1	16.7	93.2	6.7	126.0
	24	3.2	7.0	16.7	93.0	6.5	126.3
pH	26	3.3	3.3	16.8	114.7	13.0	151.1
Recovery	28	3.0	2.5	16.8	112.9	14.2	149.5
	34	2.9	2.4	16.3	112.7	18.4	152.8
	40	2.9	2.9	16.7	120.7	19.0	162.1

Table A.4   Metal content (μg/gTSS) in each fraction from BCR extraction method at different HRTs (n = 3).

Fe	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	6.2	861.7	4896.7	868.8	1849.3	8482.7
6 h HRT	2	6.1	845.9	4803.9	929.2	1859.7	8444.8
	3	5.9	904.2	4947.0	893.4	1820.3	8571.0
	4	6.6	893.0	4810.7	943.3	1795.9	8449.5
	5	16.0	909.2	4466.8	937.8	1603.2	7933.0
	6	6.6	1028.7	3980.5	916.8	1533.8	7466.5
4 h HRT	7	6.5	1080.4	3886.5	897.1	1681.5	7552.0
	8	6.6	1218.4	3848.9	692.2	1635.3	7401.4
	9	6.5	1266.9	3535.3	632.7	1643.2	7084.5
	10	6.8	1309.5	3398.3	552.5	1668.9	6936.0
2 h HRT	11	4.3	665.1	3158.1	586.2	1096.0	5509.8
	12	4.6	674.1	2953.0	564.1	957.9	5153.7
	13	4.5	614.1	2843.7	559.1	903.7	4925.0

	14	4.1	547.2	2565.9	523.7	804.1	4445.0

Co	Day	Sol	F1	F2	F3	F4	Sum of fractions
6 h HRT	1	0.0	148.9	71.4	703.1	19.5	942.9
	2	0.0	149.8	72.7	708.1	19.0	949.7
	3	0.0	153.4	75.8	709.6	18.6	957.5
	4	0.0	153.5	75.3	704.5	18.1	951.4
4 h HRT	5	0.0	148.0	76.4	736.7	17.6	978.7
	6	0.0	144.1	76.1	734.9	16.2	971.3
	7	0.0	145.1	77.1	721.0	16.0	959.2
	8	0.0	146.0	81.6	593.4	16.2	837.1
	9	0.0	140.6	83.8	562.9	15.8	803.1
2 h HRT	10	0.0	123.3	87.2	545.7	13.5	769.7
	11	0.0	119.9	91.4	507.0	11.0	729.3

	12	0.0	115.3	93.4	415.3	10.4	634.3
	13	0.0	86.2	99.2	380.8	11.3	577.5
	14	0.0	71.5	101.0	300.3	10.3	483.1

Ni	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	12.4	303.2	128.1	551.3	16.0	1011.0
6 h HRT	2	13.0	304.8	128.1	549.9	15.6	1011.4
	3	12.7	305.5	130.5	549.0	15.3	1013.0
	4	11.5	301.6	127.2	549.8	14.8	1004.9
	5	15.2	263.2	127.2	486.6	12.8	905.0
	6	8.8	235.3	122.4	409.4	11.8	787.7
4 h HRT	7	8.7	225.5	124.0	376.5	11.6	746.4
	8	8.1	217.6	127.6	252.8	10.3	616.5
	9	7.2	195.3	127.3	246.1	10.1	586.1

	10	14.9	195.3	116.9	228.0	9.5	564.5
	11	6.7	124.8	115.9	204.5	7.3	459.3
2 h HRT	12	5.8	111.8	115.9	178.8	6.9	419.2
	13	5.6	110.4	116.6	168.8	6.8	408.2
	14	5.1	99.0	115.9	142.2	5.1	367.3

Zn	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	0.0	238.5	242.5	400.6	0.0	881.5
	2	0.0	237.7	244.2	400.1	0.0	881.9
6 h HRT	3	0.0	236.9	244.1	400.7	0.0	881.7
	4	0.0	236.2	242.8	398.1	0.0	877.2
	5	0.0	186.4	233.6	439.1	0.0	859.1
4 h HRT	6	0.0	171.7	221.0	441.3	0.0	833.9
	7	0.0	167.3	216.0	446.5	0.0	829.7

	8	0.0	161.8	219.1	457.0	0.0	837.9
	9	0.0	149.3	212.9	467.4	0.0	829.6
	10	0.0	138.9	199.0	474.1	0.0	811.9
	11	0.0	90.8	175.2	480.9	0.0	746.9
2 h HRT	12	0.0	72.9	176.7	501.4	0.0	751.0
	13	0.0	70.4	161.7	540.9	0.0	773.0
	14	0.0	70.8	157.4	567.0	0.0	795.2

Mn	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	20.4	40.3	24.4	16.1	28.4	129.5
6 h HRT	2	19.9	39.0	24.2	14.9	27.7	125.8
	3	19.5	38.6	24.6	14.6	28.8	126.1
	4	18.9	38.3	23.5	14.2	28.0	122.8
4 h HRT	5	19.2	38.4	23.6	14.5	27.2	122.9

	6	17.6	37.9	22.1	13.3	25.0	115.9
	7	16.7	37.1	22.2	13.2	24.7	113.9
	8	15.4	27.2	22.4	12.7	25.0	102.8
	9	16.5	27.3	22.3	12.7	24.5	103.3
	10	14.9	27.0	20.6	11.6	24.3	98.4
	11	12.2	28.1	19.0	10.6	22.0	91.9
2 h HRT	12	11.0	27.7	18.2	9.4	20.7	87.0
	13	8.5	23.9	17.5	9.5	20.3	79.6
	14	7.7	22.3	16.2	8.2	19.5	73.8

Mo	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	36.3	5.8	26.2	74.4	7.1	149.7
6 h HRT	2	45.9	6.1	26.8	77.2	6.9	162.9
	3	38.1	5.5	25.8	74.0	6.8	150.3

	4	32.9	6.2	25.9	73.9	6.6	145.5
	5	40.8	5.2	22.4	72.5	12.8	153.7
	6	31.6	4.8	21.0	68.4	11.8	137.5
4 h HRT	7	34.9	4.4	21.1	68.0	11.6	140.0
	8	44.1	4.8	21.3	74.0	13.2	157.4
	9	26.6	5.0	21.2	74.0	14.4	141.3
	10	29.1	3.0	20.3	80.8	13.5	146.7
	11	19.6	3.7	19.3	82.0	13.5	138.0
2 h HRT	12	13.8	3.2	17.9	85.2	13.8	133.9
	13	12.4	2.8	16.3	91.8	13.5	136.9
	14	10.3	2.6	14.1	91.3	12.3	130.6

Table A.5   Metal content (µg/gTSS) in each fraction from BCR extraction method at different SRTs (n = 3).

Fe	Day	Sol	F1	F2	F3	F4	Sum of fractions
100 d SRT	1	8.7	187.0	5215.0	835.9	1021.7	7268.2
	10	9.1	285.4	5175.8	815.5	897.7	7183.5
	20	9.0	713.9	4364.7	837.4	767.9	6692.9
75 d SRT	22	24.3	755.6	4050.9	759.2	851.6	6441.7
	24	25.1	844.9	3898.1	802.1	1100.0	6670.2
	26	25.4	1010.1	4118.0	762.2	966.7	6882.3
	28	25.9	1000.5	3978.8	769.8	841.7	6616.7
50 d SRT	30	26.5	729.1	3839.2	706.3	983.3	6284.4
	32	26.5	530.2	4092.1	892.3	745.0	6286.0
	34	28.0	370.4	4305.4	880.8	741.9	6326.5
25 d SRT	36	29.8	697.2	3769.6	818.1	669.6	5984.4
	38	32.1	564.2	4142.0	715.9	619.8	6073.9
	40	32.9	1009.3	3637.1	555.5	651.4	5886.3

	44	28.1	736.7	3503.9	560.4	668.8	5497.9
	48	25.4	834.1	3276.2	540.1	619.0	5294.8
	52	9.3	849.6	3219.4	518.6	548.8	5145.7
	60	20.0	710.0	2905.7	473.6	445.7	4555.0

Co	Day	Sol	F1	F2	F3	F4	Sum of fractions
100 d SRT	1	0.0	194.2	91.8	837.3	0.0	1123.3
	10	0.0	189.4	91.9	860.0	0.0	1141.3
	20	0.0	162.2	87.6	854.6	4.0	1108.3
75 d SRT	22	0.0	157.9	83.2	829.1	1.9	1072.1
	24	0.0	169.6	87.0	866.4	1.9	1124.9
	26	0.0	206.9	98.9	700.7	0.0	1006.5
	28	0.0	194.3	100.0	855.3	0.0	1149.6
50 d	30	0.0	190.5	89.4	709.8	0.0	989.7

SRT	32	0.0	136.0	76.7	833.2	0.0	1045.9
	34	0.0	164.0	83.3	812.6	0.0	1059.9
	36	0.0	150.3	81.8	772.2	0.0	1004.3
	38	0.0	189.5	89.5	738.4	0.0	1017.4
	40	0.0	243.6	86.4	699.3	0.0	1029.3
25 d SRT	44	0.0	228.9	110.2	705.9	0.0	1044.9
	48	0.0	200.8	101.6	728.0	0.0	1030.4
	52	0.0	207.8	101.6	741.9	0.0	1051.2
	60	0.0	215.7	95.0	641.1	0.0	951.8

Ni	Day	Sol	F1	F2	F3	F4	Sum of fractions
	1	22.2	269.1	97.6	492.5	3.9	885.3
100 d SRT	10	21.2	261.6	96.5	505.2	2.0	886.5
	20	13.9	216.9	97.0	519.0	2.0	848.9

	22	14.0	205.6	92.5	485.2	1.9	799.2
75 d SRT	24	13.5	205.8	92.3	506.4	1.9	819.9
	26	13.8	227.0	102.1	491.1	2.1	836.1
	28	12.4	214.0	102.1	508.5	4.1	841.2
	30	11.6	215.9	96.3	423.5	2.1	749.5
50 d SRT	32	9.5	185.2	87.3	494.8	10.6	787.4
	34	9.7	203.8	92.5	463.2	10.8	779.8
	36	9.9	159.1	86.2	465.2	8.8	729.3
	38	11.1	185.2	84.0	438.0	12.3	730.6
	40	12.9	179.3	73.6	418.8	11.4	695.9
25 d SRT	44	1.6	169.5	68.0	384.0	9.4	632.4
	48	1.6	141.3	61.9	383.7	6.3	594.8
	52	1.6	141.1	60.5	375.0	6.2	584.3
	60	1.4	126.4	52.9	328.6	5.7	515.0

Zn	Day	Sol	F1	F2	F3	F4	Sum of fractions
100 d SRT	1	0.0	176.8	193.2	534.3	0.0	904.3
	10	0.0	179.8	197.0	551.4	0.0	928.2
	20	0.0	156.7	201.5	544.0	0.0	902.2
75 d SRT	22	0.0	153.7	187.9	498.5	0.0	840.1
	24	0.0	158.5	193.2	512.2	0.0	863.9
	26	0.0	177.8	225.9	559.3	0.0	963.0
	28	0.0	177.2	229.0	495.1	0.0	901.3
50 d SRT	30	0.0	172.5	248.1	472.8	0.0	893.4
	32	0.0	183.1	213.8	485.6	0.0	882.4
	34	0.0	194.1	205.4	486.0	0.0	885.5
25 d SRT	36	0.0	164.6	214.9	485.1	0.0	864.6
	38	0.0	199.4	217.9	456.2	0.0	873.5
	40	0.0	194.3	220.7	424.8	0.0	839.8
	44	0.0	307.0	171.1	355.7	0.0	833.8

	Day	Sol	F1	F2	F3	F4	Sum of fractions
	48	0.0	261.9	178.6	385.7	0.0	826.2
	52	0.0	268.2	180.6	376.2	0.0	825.0
	60	0.0	267.1	155.7	343.6	0.0	766.4

Mn	Day	Sol	F1	F2	F3	F4	Sum of fractions
100 d SRT	1	19.3	41.5	23.2	19.7	34.8	138.5
	10	20.2	40.9	22.7	20.6	36.4	140.8
	20	20.9	40.3	22.4	19.4	35.8	138.8
75 d SRT	22	18.7	36.9	21.0	18.2	33.6	128.5
	24	19.3	36.7	20.8	19.7	34.8	131.3
	26	21.2	38.6	22.2	19.7	38.1	139.8
	28	19.7	36.8	22.3	20.2	37.3	136.3
50 d SRT	30	14.8	32.8	18.5	33.3	29.6	129.1
	32	19.0	37.6	21.2	15.7	29.6	123.1

	34	19.4	37.6	21.5	16.0	30.1	124.6
	36	12.2	31.5	21.5	15.5	30.9	111.6
	38	12.3	32.7	23.5	17.3	34.6	120.4
25 d SRT	40	14.3	34.3	25.0	18.8	40.0	132.3
	44	10.9	32.0	21.9	21.7	37.5	124.0
	48	11.1	31.7	21.4	20.6	38.1	123.0
	52	10.9	31.0	21.7	20.2	37.2	120.9
	60	10.0	28.6	20.0	18.6	34.3	111.4

Mo	Day	Sol	F1	F2	F3	F4	Sum of fractions
100 d SRT	1	11.6	3.9	30.0	134.2	7.7	187.3
	10	12.1	4.0	33.3	137.6	8.1	195.2
	20	11.9	6.5	37.3	137.4	8.0	201.1
75 d	22	11.2	5.1	31.8	121.4	7.5	177.0

		Sol	F1	F2	F3	F4	
SRT	24	14.5	5.8	31.9	125.1	7.7	185.0
	26	16.9	6.9	36.0	136.1	8.5	204.4
	28	26.9	7.3	34.7	126.4	8.3	203.6
50 d SRT	30	29.6	7.9	36.5	127.8	10.6	212.4
	32	6.3	5.8	28.6	121.8	10.6	173.1
	34	7.5	4.8	38.2	111.7	10.8	173.0
25 d SRT	36	7.7	6.6	24.9	147.2	11.0	197.5
	38	8.6	6.2	27.8	124.2	12.3	179.2
	40	10.0	7.9	28.6	113.0	11.4	170.9
	44	10.9	21.1	48.4	99.4	3.1	183.0
	48	9.5	19.0	46.0	92.7	3.2	170.4
	52	7.8	18.6	45.0	89.1	3.1	163.6
	60	8.6	18.6	44.3	84.6	2.9	158.9

Sol – Soluble, F1 – Exchangeable, F2 – Fe-Mn oxides,

F3 – Organic matter and sulfides, F4 – Residual