The role of cobalt and nickel in biogas production from anaerobic digestion of acetate

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Dedication

This thesis is written in loving memory of my late brother

Lentswe Ditalelo,

and in

Gratitude to my sister

Dianah Ditalelo

ABSTRACT

While the individual need for Ni and Co in anaerobic digesters has been established, together with the biochemistry underpinning such need; their co-requisite in anaerobic digestion of acetate has not been extensively studied. In addition, the balance between the catalytic and toxic concentrations of Ni and Co in anaerobic acetate digesters is not well documented.

The aim of this study was to examine and evaluate the effects of individual and combined dosage of Ni and Co on biogas production as well as on methanogenic population balance of a mesophilic (37°C) anaerobic acetate digester so as to determine their catalytic and toxic concentrations.

A laboratory experimental regime was developed, this involved running of four identical 5 L semi-Continuous Stirred Tank Reactors (CSTR) operated at a Hydraulic Retention Time (HRT) of 10 days and feed acetate at a loading rate of 1.8 g L^{-1} d^{-1}. The four digesters were labelled as: D1, D2, D3 and D4 and were dosed with, Ni, Ni and Co, Co and no Ni and Co respectively. Quantitative Polymerase Chain Reaction (qPCR) was used to examine the evolution of methanogenic population in the digesters.

The results showed that individual and combined dosage of Ni and Co led to production of significantly more methane/biogas compared to the control digester; thus indicating the importance of supplementation of Ni and Co in achieving high levels of anaerobic digestion of acetate. The combined dosage of Ni and Co was found to lead to production of more biogas than their individual dosage, while the percentage of methane in the biogas was constant at 64% regardless of the dosage.

When co-dosed, the increase in biogas production due to the dosage of Ni and Co were delineated, and were found to be additive. This implied that Ni and Co were co-required for anaerobic digestion of acetate.

Methanosarcinales were the dominant order of methanogens in all digesters during the early days of their operation, with the family Methanosaetaceae more abundant than Methanosarcinaceae. This was credited to these families' differential abundance in the seed sludge used. In all digesters Methanosaetaceae reduced with digestion time, while Methanosarcinaceae increased.

The increase of the nominal concentrations of Ni and Co from 0.275 µM (0.0162 mg/L) and 0.199 µM (0.012 mg/L) respectively, to their common concentration of 14.924 µM (Ni =0.867 mg/L; Co=879 mg/L) due to their daily dosage was found to be stimulatory/catalytic to biogas production from the anaerobic digestions system used. Nevertheless the relative positions of these concentrations on the dose-response curve could not be determined due to the observed time dependent reduction in biogas production by all digesters. This reduction in biogas production with digestion time was postulated to be caused by the observed decline in Methanosaetaceae population with digestion time as was determined by the 16s rRNA analysis.

It was found that when Ni and Co were co-dosed to a large amount (of at least a combined concentration of 31.50-504.00 µM in all digesters' seed sludge) they led to inhibition of biogas production. The concentration of volatile solids was also found to influence the concentration of Ni and Co that led to inhibition of biogas production.

In this research the co-requisite of Ni and Co for improved mesophilic anaerobic digestion of acetate has been established. This co-requisite was found to be in line with the biochemical roles of Ni and Co in anaerobic digestion of acetate.

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To those who I didn't call back, am sorry about that…….

LIST OF ABBREVIATIONS AND SYMBOLS

AD	Anaerobic Digestion
ASBR	Anaerobic Sludge Bed Reactor
BLM	Biological Ligand Model
BPP	Biogas Production Potential
CHP	Combined Heat and Power
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EGSB	Expanded Granular Sludge Bed
EPA	Environmental Protection Agency
ESEM	Environmental Electron Scanning Microscope
ESM	Electron Scanning Microscope
F_{430}	Coenzyme F_{430}
GFAAS	Graphite Furnace Atomic Absorption Spectroscopy
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
ID	Internal Diameter
L*org*	Organic Loading Rate
M(int)	Internalised metal ion
NIMBY	Not In My Back Yard
NOEL	No Observable Effect Level
PDMS	poly-dimethylsiloxane
q PCR	quantitative Polymerase Chain Reaction
RO	Reverse Osmosis
RSD	Relative Standard Deviation
SBP	Specific Biogas Production
SBR	Sludge Bed Reactor
SRT	Solid Retention Time
TAN	Total Ammonia Nitrogen
TE	Trace Elements
TS	Total Solids
UASB	Up flow Anaerobic Sludge Bed Reactor
UK	United Kingdom
VFA	Volatile Fatty Acids
V_{max}	Maximum Biogas Production
VS	Volatile Solids
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

KEY ELEMENTS AND COMPOUNDS

ATP	Adenosine Triphosphate

CH_3-B_{12}	Methyl-vitamin B_{12}
CH_3COOH	Acetic acid
CH_3-S-CoM	Methyl Coenzyme M
CH_4	Methane
Co	Cobalt
CO_2	Carbon dioxide
CoM-S-S-CoB	Heterodisulphide product of CH_3-S-CoM and H-S-CoB
H-S-CoB	Coenzyme B
Ni	Nickel
Vit_{B12}	Vitamin B_{12}

NOTATIONS

θ	Hydraulic Retention Time
θ_{min}	Minimum Hydraulic Retention Time
k	Monod maximum specific uptake rate (kg substrate COD kg biomass^{-1} day^{-1})
K_s	Half saturation value (kg substrate COD m^{-3})
Y	Yield of biomass on substrate (Kg biomass kg substrate^{-1} day^{-1})
μ	Specific growth rate, d^{-1}
μ_{max}	Maximum specific growth rate, d^{-1}
D_{opt}	Maximum dilution rate or optimum dilution rate
M^{2+}	Moles of divalent metal ions (Ni and Co)
M^{Z+}	Metal ion

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CHAPTER 1. INTRODUCTION

Most of the methane (approximately 70%) produced in many anaerobic digesters is believed to come from the precursor acetate through the process of acetate cleavage performed by acetoclastic methanogens (Zeikus, 1977, McCarty, 1964). Majority of anaerobic digester failures are caused by accumulation of acetate/acetic acid that is generated through acetogenesis; this is a process whereby other volatile fatty acids (VFAs) are converted to acetic acid by acetogenic bacteria (Gerardi, 2003, Parkin and Owen, 1986). Therefore successful anaerobic digestion of acetate or acetic acid can prevent failure of many anaerobic digesters.

Industries that generate acetate as a waste by product such as Sasol through its Fischer-Tropsch process face problems associated with its handling and disposal. Successful anaerobic digestion of acetate can alleviate problems associated with its handling and disposal, as well as leading to energy recovery (Mathir, 2013). During Sasol's Fischer-Tropsch reaction, coal is gasified in the presence of oxygen and steam at 1200°C and 3 MPa to generate methane gas, as waste by products, short chain organic acids, a large part being acetate, are also produced (Mathir, 2013). Disposal of these short chain organic acids presents a challenge for Sasol (Mathir, 2013).

In the case of a WasteWater Treatment Plant (WWTP) operation, there is economic interest in obtaining as much energy as possible in the form of methane gas from an anaerobic sewage sludge digester. Combustion of methane gas in a Combined Heat and Power Plant (CHP) produce both electric and thermal (heat) energy. The produced electricity can be exported to the general electrical grid, or sold locally where the WWTP is situated. This export or sale of power to the grid can lower electricity tariffs to communities, which in turn can have an impact of reducing the extent to which communities or residents object to location of waste

management facility in their vicinity; a phenomenon normally acronymed NIMBY; for Not In My Back Yard.

The main attractiveness of anaerobic digestion (AD) systems in waste treatment compared to aerobic digestion is due to the former's capacity to sustain high organic loading rates as well as producing approximately 20% less biomass compared to aerobic digestion (Batstone et al., 2002, Dinsdale et al., 2000). In anaerobic treatment of wastewater there is no use of external energy for aeration as compared to aerobic treatment; in addition to this, during anaerobic digestion, 95% of the energy from the organic matter (waste) is retained in methane compared 60% of the energy which is retained in biomass during aerobic treatment (OpenCourseWare, 2014). The obtainment of energy rich methane as well as the low energy requirement for operation of anaerobic wastewater treatment systems make them have a low carbon foot print compared to aerobic digestion system. The recovery of energy from combustion of methane gas allows the anaerobic digestion system to be energy neutral or positive, this offset the energy (which in many cases is from fossil fuels) that could otherwise be drawn from the grid hence further reducing the carbon foot print of the anaerobic digestion system compared to aerobic treatment system. Figure 1.1 shows the interconnection of various units of a WWTP and where anaerobic digestion is situated.

Considering the importance and high contribution of acetate to methane production, it is of paramount importance to find ways of making its anaerobic digestion as successful as possible. One such way has been supplementation of metal ions to an anaerobic digester, this is exemplified by a burgeoning market of micronutrient sold in the anaerobic digestion industry, e.g Omex Environmental Ltd, Norfolk, United Kingdom (Takashima et al., 2011). Despite this, little is known about the role of metal ions in conversion of acetate to methane/biogas gas (acetotrophic methanogenic route) and on how this conversion can be

improved. Dosing of micronutrients to digesters is currently largely done in a non-selective manner, i.e., dosing is not specific to a digester.

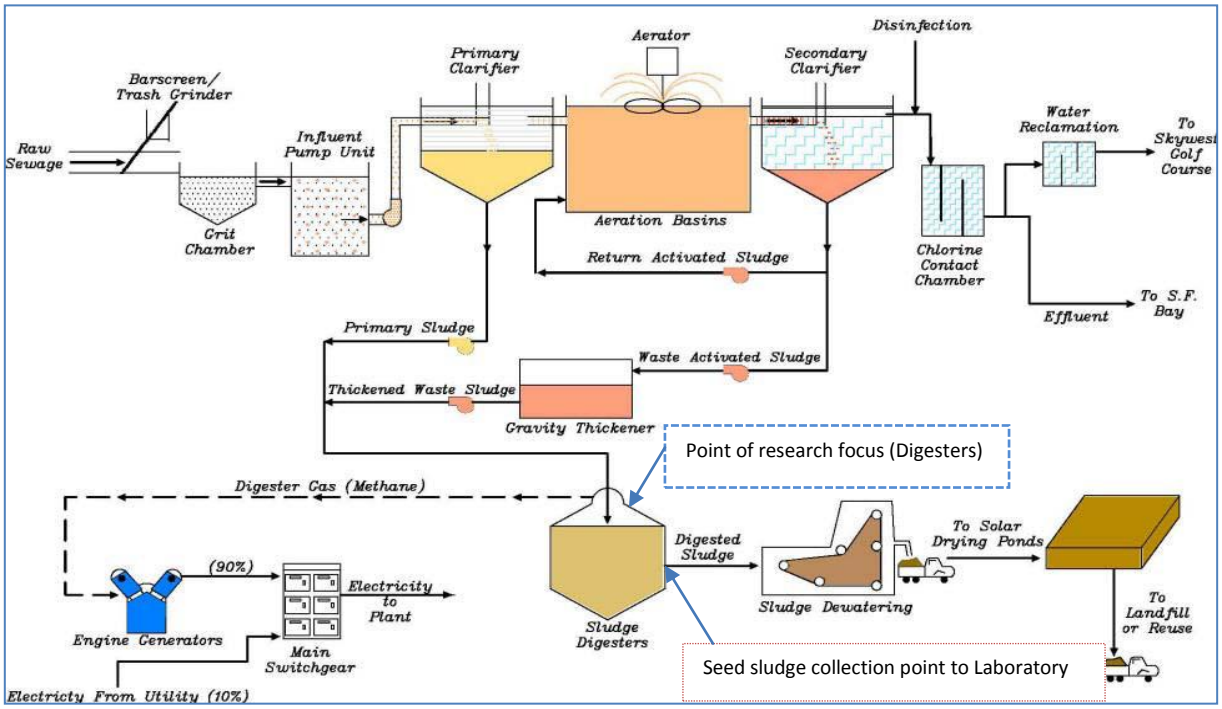


Figure 1.1 Interconnection of various units of a typical WWTP (Templeton, 2009).

1.1 Research Aim

This study aims to examine and evaluate through laboratory experiments, the effects of dosage of Ni and Co on biogas production as well as methanogenic community balance of a laboratory scale mesophilic (37°C) anaerobic acetate digester. For the anaerobic digestion system employed, the boundary concentrations of these elements' catalytic and toxic concentrations were also to be determined.

1.1.1 *Specific research objectives*

I. To review the literature to find the biochemical and empirically observed role and effects, respectively, of dosage of Ni and Co to anaerobic digesters with the aim of improving their efficiency/biogas production. This will also reveal the associated knowledge gaps in this field as well as informing the research hypothesis.

II. To develop a test or experimental regime for laboratory scale anaerobic acetate digesters for examining the effects of their dosage with individual and a combination of Ni and Co at mesophilic (37°C) temperatures, focusing mainly on their biogas/methane production.

III. To determine, using laboratory scale digesters, the effects of their dosage with individual and a combination of Ni and Co in terms of their concentration, on the rate of mesophilic (37°C) anaerobic acetate digestion to biogas.

IV. To determine through experiments, the effects of individual and combined dosage of Ni and Co in terms of their concentration on methanogenic population balance (abundance and diversity) of mesophilic (37°C) laboratory scale anaerobic acetate digesters using quantitative polymerise chain reaction (qPCR).

V. From the experimental regime employed, to define and account for potential links between metal ion (Ni and Co) availability and methanogenic population balance of digesters, and the implication of such on biogas/ methane yield.

VI. To determine the concentration of Ni and Co that is inhibitory to biogas production of the anaerobic digestion system employed.

From the review of the literature, and also in line with the aims and objectives of this research, the following research hypothesis (1.2) was developed.

1.2 Research hypothesis

Dosage of individual metal ions; Ni and Co, will lead to increased rate of anaerobic acetate biodegradation to biogas, with their co-dosage having a synergistic or an additive effect, but there is a threshold for their effectiveness and toxicity. This hypothesis is presented graphically in Figure 1.2.

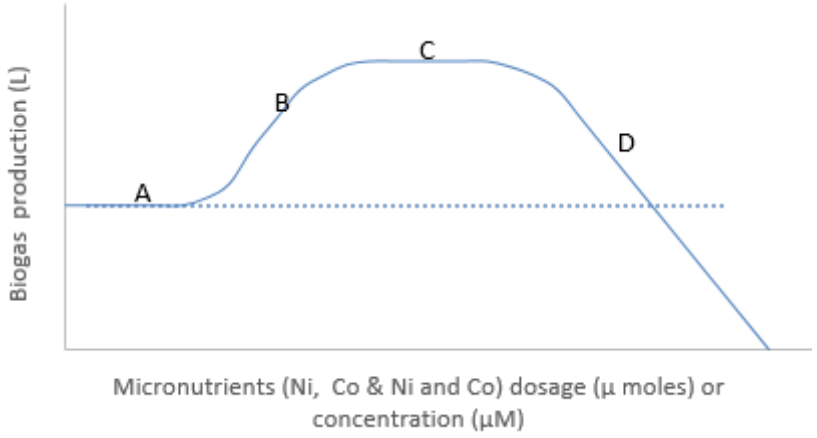


Figure 1.2. Hypothetical dose response curve for essential micronutrients supplementation to an anaerobic bioreactor (A)= NOEL, (No observable effect level) or limiting concentration; (B)= Effective/stimulatory concentration; (C)= Saturation or excess concentration; (D) =Toxic concentration.

The hypothetical dose response curve suggested in Figure 1.2 is conceived as the depiction of the result of change in biological reaction with change in micronutrient concentration. Biogas production rate is used as an indicative parameter or measure of the rate of biological reaction, specifically rate of methanogenesis as a function of dosed micronutrients and their resultant concentration. Because biogas production rate is measured as an indicative parameter of the rate of biological reaction, it is anticipated that there will be a dose or concentration of micronutrients that will cause a certain level of increase in biological reaction, but the result of that increase in biological reaction would not be translated into a detectable or measurable change in biogas production, hence the depiction of the No Observable Effect Level (NOEL) in the hypothetical dose response relationship (region A),

Figure 1.2. For example, in theory if one methanogenic cell up-take enough Ni and Co, its methanogenensity is expected to increase, but practically the result of that increased methanogenensity as increased biogas production is not expected to be detectable. A similar principle is applied when micronutrients concentration surpasses their optimum concentration, the decrease in the rate of their biological reaction as depicted in Figure 2.10 (zone of decreasing stimulation) is not immediately expected to be translated into the observable decrease in the overall biogas production, as depicted by region C of the hypothetical dose response curve. Most of the principles applied in conceptualisation of the hypothetical dose-response relation are borrowed from the field of toxicology, both stimulatory concentration to biogas production and toxic concentration depends on the response of the same organism. Several definitions of the NOEL have been used depending on the test being performed, the Environmental Protection Agency (EPA) defines NOEL as 'the highest concentration of a toxicant to which organisms are exposed in a full life cycle or partial life cycle (short time) test, that causes no observable adverse effects on them (i.e. the highest concentration of a toxicant at which the values of the observed responses are not statistically different from the controls). This value is used along with other factors to determine toxicity limits in effluent disposal permits (USEPA, 1994). A similar principle that is used to judge/determine toxicity can be applied to activation concentration as both principles relies on the substance/ion interacting with microbial cell; resulting in that cell expressing a biological response, which is normally the quantified parameter (e.g. biogas production).

1.3 Thesis presentation

This section outlines the presentation and connection between different sections of this thesis. Chapter 1 introduces the subject of anaerobic digestion, outlines the motivation for the study as well as providing the scope of the research. Chapter 2 entails a review of the literature relevant to the biochemistry of anaerobic digestion in the context of Ni and Co supplementation to a digester. This set the background for the scientific legitimacy of the hypothesis. Previous studies in which micronutrients were dosed to digesters are reviewed. This allows the biochemical roles of Ni and Co in methanogenesis to be linked to empirical/practical observations. From the review of the literature a gap in scientific knowledge as pertaining to anaerobic digestion of acetate is revealed and presented in section 2.8. Materials and methods used to test the study's hypothesis are presented in Chapter 3. Presentation of the experimental findings, their interpretation and discussion as well as implication is done in Chapter 4. This Chapter is composed of two parts: Part one and part two. Part one deals with the catalytic side of the dose response curve, while part two covers the toxicity side. Chapter 4 ends with the general overview of the project in light of the dynamics of anaerobic digestion system employed in this research. Conclusions from this research as well as recommendations for further research are presented in Chapter 5.

CHAPTER 2. LITERATURE REVIEW

This chapter sets the background based on both biochemical and empirical observations for the research hypothesis that co-presence/dosage of Ni and Co in an anaerobic acetate digester will increase biogas production from that digester. Different stages of anaerobic digestion leading to production of methane are discussed; this will justify the need to improve conversion of acetic acid/acetate to biogas. The biochemistry of methanogenesis from acetate together with the physiology of methanogens are also discussed. The role of Ni and Co in the biochemistry of methanogenesis is also examined, hence justifying the need to supplement these elements in an anaerobic digester. Performance of digesters that had been supplemented with various micronutrients not necessarily limited to Ni and Co is reviewed and discussed while drawing inference on what this potentially mean for dosage of Ni and Co to anaerobic acetate digesters.

2.1 Stages of anaerobic digestion process.

This part will outline the various stages of the anaerobic digestion process up to the point of generation of methane. This will show how the anaerobic digestion of acetate fit into the wider scope of anaerobic digestion process, as well as exhibiting why it is important to the anaerobic digestion process as a whole.

There are conventionally three stages involved in anaerobic digestion of complex organic polymers to methane and carbon dioxide (Gerardi, 2003). These stages, in order of their occurrence are; Hydrolysis, Acidogenesis and Methanogenesis (Gerardi, 2003). In some cases a fourth stage of acetogenesis (acetate production) is presented as a separate stage as in

Parkin and Owen (1986) while in other studies as in Gerardi (2003) acetogenesis is presented as part of the acid generation stage, as its end product is acetic acid which is part of the Volatile Fatty Acids (VFA). In this study acetogenesis was included as part of acidogenesis. As anaerobic digestion stages are conducted by different groups of microorganisms requiring different environmental growth conditions, it is crucial to maintain a constant 'working velocity' for each group so as to achieve a constant continuity of the AD process. It is important to recognise that conversion of large insoluble or soluble polymeric substances to small soluble molecules does not result in reduction of the organic strength or organic content of the substrate being digested. It is only through methanogenesis stage that the carbon content of the substrate being digested is reduced when methane (CH_4) and carbon dioxide (CO_2) are given off as gases. This implies that for reduction of organic strength of the waste to be achieved, methanogenesis stage has to be driven to completion or near completion. This shows the importance of interdependence of the three stages involved in anaerobic digestion (Gerardi, 2003). Despite the importance of methanogenesis stage it is the most common 'bottle neck' in the anaerobic digestion process.

It is important to maintain favourable conditions relating to, but not limited to, the following factors as outlined by Parkin and Owen (1986) : optimum retention time, adequate mixing, proper pH, adequate nutrient concentration, absence of toxic materials as well as proper feed characteristics in order to have a healthy digester. This research is focused on provision of proper micronutrients at suitable concentration.

2.1.1 *Individual stages of anaerobic digestion*

The following is a brief review of the literature on each stage involved in anaerobic digestion, mostly based on the digestion of domestic sewage sludge.

2.1.2 *Hydrolysis stage.*

In this stage, large insoluble organic polymeric substances are broken down into their constituent molecules that can be assimilated by bacterial cells for metabolism (Gerardi, 2003, Parkin and Owen, 1986). This is accomplished by extracellular hydrolytic enzymes secreted by specific bacteria (Parkin and Owen, 1986). Without efficient hydrolysis, the whole anaerobic digestion process will be limited in case of sewage sludge digestion as most of the organics are polymeric in nature. Therefore conditions suitable for effective hydrolysis have to be maintained for effective waste stabilisation to eventually be achieved. These conditions include, effective mixing, suitable temperature and pH, amongst others. The hydrolyzation of cellulose to glucose molecules by the hydrolytic bacterium *Cellulomonas* through its secretion of the enzyme cellulase is an example of hydrolysis in anaerobic digestion (Gerardi, 2003). This then allows glucose to be assimilated into microbial cells for metabolism.

2.1.3 *Acidogenesis stage.*

In this stage, individual monomers (constituents of polymers) produced during hydrolysis stage are fermented into long chain organic acids and short chain VFAs (Gerardi, 2003, Parkin and Owen, 1986). Table 2.1 gives major organic acids and other products produced during acid production stage (acidogenesis) in an anaerobic digester. This is accomplished by a large number of facultative as well as obligate anaerobes present in the human digestive system in case of domestic sewage sludge digestion. Accumulation of VFA's, mainly acetic acid is the common cause of digesters going 'sour' or failing mainly as a result of reduction in their pH.

Of the mentioned acids and non-acids in Table 2.1, acetic acid is the most important for the stability of anaerobic digestion system. This is because it is the most abundantly produced in addition to being the most common precursor for methane production in most of anaerobic digesters.

Table 2.1. Some of the products of acidogenesis stage, adapted with modification from Gerardi (2003).

Name	Chemical formula
Methanol	CH_3OH
Ethanol	CH_3CH_2OH
Propanol	$CH_3CH_2CH_2OH$
Butanol	$CH_3(CH_2)_2CH_2OH$
Formic acid	HCOOH
Acetic acid	CH_3COOH
Butyric acid	$CH_3(CH_2)_2COOH$
Caproic acid	$CH_3(CH_2)_4COOH$
Lactic acid	$CH_3CHOHCOOH$
Propionic acid	CH_3CH_2COOH
Succinic acid	$HOOCCH_2CH_2COOH$

2.1.3.1 Acetogenesis

Being the most abundant component of VFAs produced during acidogenesis stage (Parkin and Owen, 1986, McCarty, 1964), it is important to outline how acetic acid is produced in a typical anaerobic digester. This will also show or justify its inclusion as part of acidogenesis in this study.

Majority of the various VFAs produced during acid production (acidogenesis) are converted to acetic acid by acid forming bacteria (Gerardi, 2003). This acetic acid can then be converted to acetate thorough various reactions in the digester. This makes acetate the most important and most abundant of all the volatile fatty acids. The majority (approximately 72%) of the methane produced from most of anaerobic digesters is believed to come from the precursor acetate (Gerardi, 2003, McCarty, 1964). The accumulation of acetic acid or acetate in a digester will lead to a reduction in digester pH, this lowering of digester pH normally culminate in inhibition of the digestion process, hence digester failure or collapse. This is why

it is crucial to ensure that acetate or acetic acid is mineralized to methane in order to maintain process stability.

It is also possible to convert carbon dioxide directly to acetic acid (Parkin and Owen, 1986). The rise of hydrogen partial pressure to above 10^{-4} atm reportedly lead to inhibition of acetoclastic methanogenesis (Parkin and Owen, 1986); in this case if acetoclastic methanogenesis has been the main route of acetate removal, accumulation of acetic acid will occur, which will result in lowering of digester pH which will in turn stifle the anaerobic digestion process, hence digester failure. This will be particularly true in digesters that are dominated by Methanosaetaceae, as this family can only metabolise acetate. An increase in hydrogen partial pleasure can induce an increase in the rate of hydrogenotrophic methanogenesis (Schink, 1997, Lovley and Ferry, 1985).

There is at times a syntrophic association between hydrogen producing bacteria and hydrogenotrophic methanogens in a digester (Parkin and Owen, 1986). This symbiotically prevents halting of the digestion process due to high hydrogen partial pressure in digesters where acetate splitting is the main, but not only acetate's digestion route.

2.1.4 *Methanogenesis*

During this last phase, methane (CH_4) and carbon dioxide (CO_2) are produced, and waste is stabilised as its carbon content is reduced. This is achievable because of the insolubility of methane gas which is given off from digesters, thereby reducing the organic strength of the waste (Parkin and Owen, 1986). Some of the carbon dioxide produced in this stage is given off the reactor as gas while some dissolve as bicarbonate-alkalinity, depending on system equilibrium (Parkin and Owen, 1986). Methane production through splitting of acetic acid, a process known as acetoclastic cleavage (Gerardi, 2003) is defined by:

$$CH_3COOH(aq) \rightarrow CH_4(g) + CO_2(g) \qquad Eq\ (1)$$

Acetic acid degradation through equation (1) is performed by acetoclastic methanogens (Zeikus, 1977). Commonly 30% of methane is many digesters is produced from other pathways including reduction of carbon dioxide with hydrogen accomplished by hydrogenotrophic methanogens (Parkin and Owen, 1986) through:

$$CO_2(aq) + 4H_2\ (aq) \rightarrow CH_4\ (g) + 2H_2O \qquad Eq(2)$$

The occurrence of equation (1) and equation (2) in an anaerobic digester is not mutually exclusive. In some digesters, equation (2) is the dominant methanogenic route depending on microbial ecosystem balance, i.e. availability of acetate oxidising microorganisms and hydrogenotrophic methanogens, as well as micronutrient availability, as was the case in Banks et al. (2012)'s food waste digester.

2.2 Focusing on the anaerobic digestion of acetate.

As acetate is mostly the largest precursor of methane, as well as being the most common cause of digester failure due to its accumulation (Gerardi, 2003) it is important to find ways of improving the ability to digest it. Our improved ability to anaerobically digest acetate open way for its employment as one of the feasible solutions to challenges emanating from handling waste acetate produced by some industries such as the coal gasification industry. In the context of this research, supplementation of Ni and Co to anaerobic digesters was investigated as one of the ways of improving anaerobic digestion of acetate.

Anaerobic digestion of acetate is dominated by two archaea genera, *Methanosarcin*a sp and *Methanosaeta* sp (Batstone et al., 2002). According to Batstone et al. (2002), Methanosarcina outgrow Methanosaeta at acetate concentrations above 1 mM, while below this concentration, the converse is true. On the other hand Mogens et al. (2008) reported that Methanosarcina out

grows Methanosaeta at acetate concentrations above a much lower value of approximately 0.40 mM (≈25 mg COD/L). On average, Methanosarcina sp have a maximum specific growth rate (μ_{max}), (hence generation time) of about five (5) times that of Methanosaeta sp, being (0.71 and 0.12).d^{-1} respectively at high (>> 0.4 mM)) acetate concentration (Mogens et al., 2008). This difference in growth rates of these organisms at high and low substrate concentration is postulated to the difference in their energy requirements for activating for metabolism one mole of acetate. Methanosaeta are said to use two moles of ATP in activation of one mole of acetate at low concentration, while Methanosarcina use one mole of ATP to do the same activation of one mole of acetate at high acetate concentration (Batstone et al., 2002). This leads to their different growth rates as a function of substrate concentrations. Due to their kinetic superiority at low acetate concentration and ability to attach to substrate over Methanosarcina, Methanosaeta normally dominate anaerobic high rate treatment systems based on high solids retention times, such as sludge bed reactor and anaerobic filters (Mogens et al., 2008, Kendall and Boone, 2006). Methanosaeta has been found to be the dominant acetoclastic methanogen in a variety of digesters (Conklin et al., 2006).

2.2.1 *Morphological features of Methanosarcinales.*

This section outlines morphological or physical features of methanogens that are anticipated to be dominant in methane production in this study. These are the members of the genera Methanosaeta and Methanosarcina. Knowing morphological features of relevant methanogens is important as it can aid in their quick and simple, although with low accuracy, identification such as with the use of Electron Scanning Microscope (ESM). Knowledge of the relative abundance of methanogens is important as it allows the reactor to be altered to favour the growth of a desired methanogen.

The order Methanosarcinales is one of the five orders of methanogens, it is composed of two families; Methanosaetaceae and Methanosarcinaceae (Kendall and Boone, 2006), which are

the only organisms known to be capable of acetate splitting into CH_4 and CO_2. Methanosaeta, genus of the family Methanosaetaceae, can only obtain energy through acetate splitting while Methanosarcina (genus of the family Methanosarcinaceae) are capable of reducing carbon dioxide with hydrogen for energy with the concomitant methane production (Mogens et al., 2008, Kendall and Boone, 2006). This metabolic versatility of Methanosarcina makes them more resilient to changes in environmental conditions of a digester more than Methanosaeta.

Methanosarcina sp and Methanosaeta sp are morphologically distinctive from each other, with the former being characterised by coccoid shape, appearing in grape like formation, while Methanosaeta sp have a filamentous shape and appear in spaghetti like form (they appear to be rods joined end to end) (Mogens et al., 2008). It is reported by Mortimer et al. (1981) that colonies/clusters of *Methanosarcina barkeri* of 2-3µm in diameter were observed aggregated into a few to hundreds of units. These distinctive morphological features can be used as a quick way of determining the dominant methanogen in a digester, as well as making inference on digester's performance considering substrate concentration and operating conditions.

2.2.2 *Physiological properties of Methanosarcinales*

Knowledge of methanogens' physiological requirements is crucial as it informs what environmental as well a chemical conditions to maintain in a digester so as to improve their (methanogens) metabolism, hence biogas production. Environmental conditions include parameters like temperature and pH while chemical conditions involve determining which micronutrients are needed for improved metabolism by which methanogens. For the overall well survival and metabolism of organisms, their environmental should be able to support their physiological requirements, hence consideration of physiological properties of Methanosarcinales in this section.

Methanogens are generally known for their fastidious nature, i.e. it is difficult to grow or culture them at high purity level (Mortimer et al., 1981). Despite this, Methanosarcina species

are much of an exception as their metabolic substrates are diverse; these include: acetate, hydrogen/carbon dioxide, methylamines, dimethylamines, trimethylamines, methanol and other forms of methylamines (Mortimer et al., 1981). The diversity of their metabolic substrates implies that they have a high chance of survival hence digester population dominance at times of digester stress (e.g. VFA accumulation). Growth of methanogens is reported to be fastest at mesophilic temperature (37-39)°C on H_2/CO_2, methanol and methylamines as substrates as compared to their growth on acetate (Mortimer et al., 1981).

Methanosarcina sp's ability to utilise hydrogen is crucial as it prevent or reduce chances of its (H_2) accumulation which leads to thermodynamic un-favourability of the acetoclastic cleavage process (Lee and Zinder, 1988). Neutral or near neutral pH is reportedly the most favourable for Methanosarcina growth (Mortimer et al., 1981).

Methanosaeta sp's are known to only metabolise acetate for energy (Mogens et al., 2008, Kendall and Boone, 2006). Given that acetate digesting methanogens have difference kinetic parameters and substrate affinity at different substrate concentrations, it is possible to physically manipulate a digester (acetate or sewage sludge) to select for either Methanosaeta or Methanosarcina dominance. Given their greater substrate utilization rate at high substrate concentration, it is in the interest of a digester operator to select for Methanosarcina especially during digester start-up period as substrate concentration normally will be high. This can be achieved through; keeping solid retention time at around 10 days and increasing the acetate concentration (Conklin et al., 2006) to 1-4 mM. The following engineering approaches can be used to achieve increase in acetate concentration in a typical sewage sludge digester, consequently resulting in Methanosarcina population dominance: feeding only one quadrant of a digester, limited mixing and feeding less frequently but at high organic loading rates (Conklin et al., 2006). A study performed by Conklin et al. (2006) showed that an hourly acetate fed digester enriched for Methanosaeta, while a daily fed one enriched for

Methanosarcina at the same organic loading rate. Methanosarcina dominated digester was found to perform better than Methanosaeta dominated one in terms of their ability to withstand shock loading conditions (Conklin et al., 2006); this is expected considering growth kinetics of Methanosarcina compared to Methanosaeta at normal acetate concentrations (excluding extreme values; too low or too high). To achieve a better digester stability and increased resilience, it is better to select for environmental conditions that favour the dominance of Methanosarcina (Conklin et al., 2006).

Knowledge of the environmental and biochemical conditions favourable to Methanosaeta and Methanosarcina is crucial as it allows a digester operator to select for a desired family based on desired outcome. In terms of acetate digestion, understanding of the relative abundance of Methanosaeta and Methanosarcina is useful as it allows for the anticipation of possible digestion pathways as well as allowing for effective control of the anaerobic digestion system. Crucial digester operation parameters such as maximum organic loading rate (L_{org}) as well as the dominant digestion pathway can be predicted from real time knowledge of methanogenic population dynamics in a digester.

2.3 Energetics of acetate anaerobic digestion

In this section the energetics of each of the pathways leading to methane production from acetate anaerobic digestion are reviewed and discussed. Understanding the dominant pathway of acetate digestion under particular digestion condition (mesophilic or thermophilic) coupled with understanding of the physiology of methanogens allows determination/deduction of the most likely dominant methanogen during acetate digestion. This allows for provision of an operation focused to a desired pathway, hence a desired methanogen.

Despite majority (70%) of methane in most digesters being believed to be produced from acetoclastic cleavage, acetate has not been demonstrated to serve as the only electron donor for methanogenesis in pure culture studies (Zeikus, 1977). This is mainly due to the fastidious nature of methanogens and the general insufficiency of knowledge about their growth and handling. High purity species on *M. barkeri* and *Methanococcus* have shown slow acetate fermentation (digestion) of about 15 weeks (Zeikus, 1977). Pure culture strains of *M. barkeri* and *Methanobacterium thermoautotrophicum*, were shown to convert 1% solution of sodium acetate to methane while growing on a filter sterile phosphate buffer basal medium (PPBM) and H_2/N_2 gas phase (Zeikus et al., 1975). The acetate conversion rate was dependent on the concentration of both acetate, CO_2 and H_2 with the largest amount of CH_4 being produced from CO_2 reduction in the presence of *M. thermoautotrophicum* (Zeikus et al., 1975). It is crucial to recognise that methanogenesis that occurs in pure culture studies is neither congruent nor representative of that which occurs in nature nor in a digester. As such it is important to not fall for the temptation of extrapolating pure culture studies to an anaerobic digester. Given the importance of the acetoclastic cleavage reaction, its energy balance and ability to self-sustain is of important consideration in the field of anaerobic digestion.

Table 2.2. Energy of metabolism of methanogenic bacteria, Adapted from Zeikus (1977).

Reaction	ΔG (Kcal/.mole) reaction
1. $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-32.7
2. $4HCO_2^- + 4H^+ \rightarrow CH_4 + 3CO_2 + 2H_2O$	-34.7
3. $4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-76.4
4. $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-8.6

As per Table 2.2, comparison of the energy released per mole of methane produced shows that reduction of carbon dioxide by hydrogen (equation 1,Table 2.2) produce four (4) times more energy than acetate cleavage (equation 4, Table 2.2) (Zeikus, 1977).

Thermodynamically, the former reaction should be more favourable compared to the latter, but the converse is true in reality, i.e. in a digester and in nature. Table 2.2 suggest that in the world governed by natural competition, a methanogen surviving solely on fermentation of acetate (or acetoclastic cleavage) would be out competed as it would be slow growing based on the above energetics (Zeikus, 1977). The energy yield from hydrolysis of adenosine 5´-triphosphate (ATP) is said to range from 8.2-12.5Kcal/mol at bacterial physiologically conducive conditions (Zeikus, 1977). Incorporation of the normal efficiency of bacterial energy transfer suggested by Decker et al. (1970) to be (30-50)% with energy production from ATP hydrolysis and the energy from equation 4-Table 2.2, which is (8.6 Kcal/mol), renders formation of an energy rich compound via substrate level phosphorylation unlikely let alone sufficient for cell growth (Zeikus, 1977).

To date there is reliance on the observation that majority of acetate is converted to methane via acetoclastic cleavage as opposed to the biochemical account for the sustainability of such reaction in terms of its energetics.

2.3.1 *Acetate oxidation*

In this section digester conditions that favour or lead to oxidation of acetic acid to CO_2 and H_2 with the subsequent reduction of CO_2 by H_2 to CH_4 are highlighted. This is important for informing the digester operator of which acetic acid utilisation route could be dominant under particular operation conditions.

As noted by Garcia (2008) thermodynamic analysis is of vital importance when dealing with energetic balance of syntrophic relationship of microbes, as it determine the feasibility of certain reactions. The potential effects of thermodynamics of acetate oxidation and acetoclastic cleavage are considered in this section, this will inform, together with consideration of presence of absence of inhibitory substances; which digestion pathway will be potentially favoured considering the operation conditions of digesters.

The process and progress of methanol anaerobic digestion is well documented by Fermoso (2008). As acetic acid formation is involved in this process, it is important to present its brief discussion. Three main pathways are possible for anaerobic digestion of methanol (Figure 2.1) adapted from Fermoso (2008) and Zandvoort et al. (2002). These pathways as shown in Figure 2.1 are: (1) conversion to hydrogen and carbon dioxide then to methane by hydrogenotrophic methanogens, (1 through C); (2), direct conversion to methane (methylotrophic methanogenesis) and (3), acetogenesis then acetoclastic cleavage, (3 through A) (Fermoso, 2008).

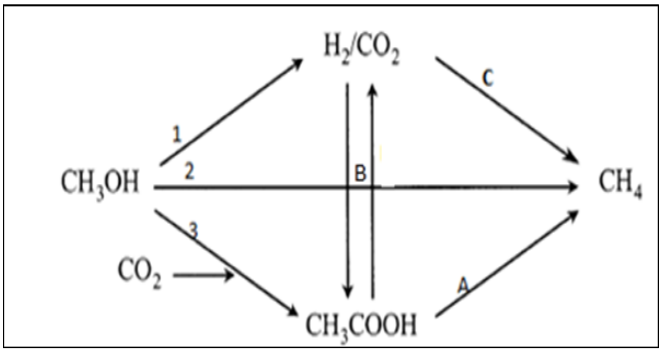


Figure 2.1 Possible pathways for methanol anaerobic degradation, adapted from Fermoso (2008).

Of interest to this research are the paths: A, B and C, extracted into Figure 2.2. This is because the majority of methane (70%) is commonly produced from acetate (Parkin and Owen, 1986), and all other VFAs are converted to acetate with the concomitant production of hydrogen during acetogenesis. This produced hydrogen is also a precursor for methane through its reduction of carbon dioxide. Accumulation of VFAs was observed in methanol and grain stillage digesters devoid of Ni and Co, a large part of these VFAs was acetate (Gustavsson, 2012, Fermoso, 2008). In Gustavsson (2012)'s study it was evident from qPCR data in conjunction with 454-pyrosequencing data that Methanosarcinales, dominated by Methanosaeta sp, was the most abundant order during periods of process stability, low VFA accumulation and high methane production. This occurred when both Ni and Co were dosed to digesters to about 0.1-0.3 mg/L (Gustavsson, 2012). The proportion of pyro-sequences

belonging to Methanoculleus sp (order Methanomicrobiales) increased with increasing reactor instability and VFA accumulation, mainly acetic acid (Gustavsson, 2012); this coincided with decreasing concentration of Co and Ni.

Given that Methanosaeta sp can only use acetate for methanogenesis (Kendall and Boone, 2006) while all Methanomicrobiales (including Methanoculleus sp) can use H_2 and CO_2 as substrate for methanogenesis, and incognisance of the fact that reactor stability coincided with the dominance of Methanosaeta sp while instability (with VFA accumulation) coincided with the dominance of Methanococculeus sp (Gustavsson, 2012), it is most probable that the dominant pathway for methane production was directly from acetate as opposed to its oxidation to CO_2/H_2 with subsequent reduction of CO_2 by H_2 to CH_4.

In Fermoso (2008)'s study, supplementation of 0.5 μM of Co to a Co devoid anaerobic methanol digester boosted acetogenesis without any increase in methanogenesis, while supplementation of 0.5 μM of Ni to an acidified methanol digester (77% of MeoH feed was converted to acetate) led to complete removal of the acetate and no further methanol or acetate accumulation. An increase in Methanosarcina sp was found to accompany stable reactor operation while a reduction in Methanosarcina sp was found to coincide with unstable reactor performance (Fermoso, 2008). As the hydrogen partial pressure was not measured in Fermoso (2008)'s study and only Methanosarcina sp's evolution was examined, it is not possible to unequivocally conclude whether the accumulated acetate was removed through its oxidation route with subsequent CO_2 reduction by H_2 or through its direct cleavage.

Irrespective of the VFA/acetate digestion pathway employed in Gustavsson (2012) and Fermoso (2008)'s studies, it was evident that both Ni and Co were needed for stable digestion and aversion of VFA/acetate accumulation in digesters.

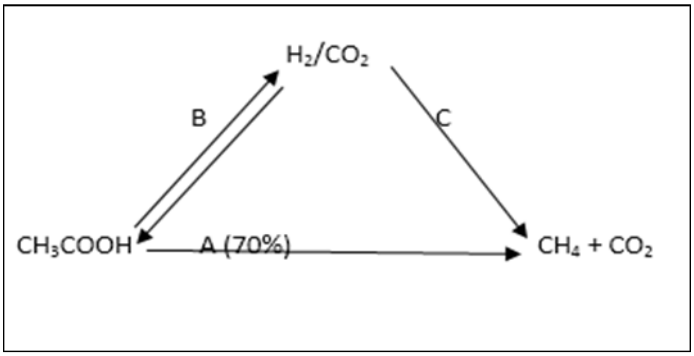


Figure 2.2. Dominant pathways for methane production from acetate.

Environmental conditions which commonly coincide with acetate oxidation to H_2 and CO_2 reported by Karakashev et al. (2006) are; high temperatures (50-60)°C, high VFA concentration as well as high ammonia concentration (Schink, 1997). These conditions are generally unfavourable for acetoclastic methanogens. It is unclear whether stress induced by deficiency of essential micronutrients in an anaerobic acetate digester can induce the acetate oxidation pathway (F. Ishaq 2012, personal communication).

Given that the production of methane from H_2/CO_2 also relies on the production of methyl-Coenzyme M and its subsequent reduction to methane as much as do acetoclastic cleavage (Thauer, 1998), it is unlikely that the absence of Ni and Co would trigger acetate oxidation to H_2/CO_2 and subsequent reduction of CO_2 by H_2 to CH_4. It is also worth noting that the first observation of Ni's catalyses of methanogenesis was on the substrate H_2/CO_2 (Schönheit et al., 1979).

A 2-^{14}C (methyl-group carbon) labelled experiment performed by Karakashev et al. (2006) showed that under the above mentioned acetoclastic methanogens inhibitory conditions, acetate oxidation to CO_2/H_2 and the subsequent reduction of CO_2 by H_2 was the main pathway of methane production. This was also found to be accompanied by low Methanosaetaceae population (Karakashev et al., 2006).

A possible explanation as to why a two-step and two organism syntrophic acetate oxidation to H_2/CO_2 and the subsequent reduction of CO_2 to CH_4 by H_2 is mostly associated with

thermophilic temperatures is suggested by Schink (1997) as being because of the increase in the Gibbs free energy with increasing temperature as per Van Hoff's equation:

$$\Delta G = \Delta H - T\Delta S \qquad Eq\ (3)$$

Where:
ΔG= Change is Gibbs free energy
ΔH=change in enthalpy
ΔS=change in entropy
T=absolute temperature

From equation (3) and Figure 2.3, the free energy change (Gibbs free energy) from acetate conversion to methane and carbon dioxide increases slightly with increasing temperature (Schink, 1997). Possibly, at mesophilic temperatures a smaller amount of energy is given that can support only one organism, thus a single organism acetate cleavage reaction occurs as compared to higher amount of energy that is given off at high (thermophilic) temperatures that can support two organisms and two steps; being acetate oxidation with subsequent CO_2 reduction by H_2 thereby producing CH_4 (Schink, 1997).

The usually observed effect of temperature in anaerobic digestion of substrates rich in Total Ammonia Nitrogen (TAN) such as swine manure is its effect in increasing the amount of free ammonia (NH_3) as was observed by Hansen et al. (1998). Because acetoclastic methanogens are more susceptible to ammonia toxicity than hydrogenotrophic ones (Hansen et al., 1998); they are normally lost when a digester has high ammonia concentration; thus leaving hydrogenotrophic methanogens to dominate the digestion process as was observed by Banks et al. (2012). As such in digesters that are operated at high temperature (thermophilic) digesting TAN rich substrates, acetate oxidation to CO_2 and H_2 with the subsequent methane production form their reaction would be highly plausible.

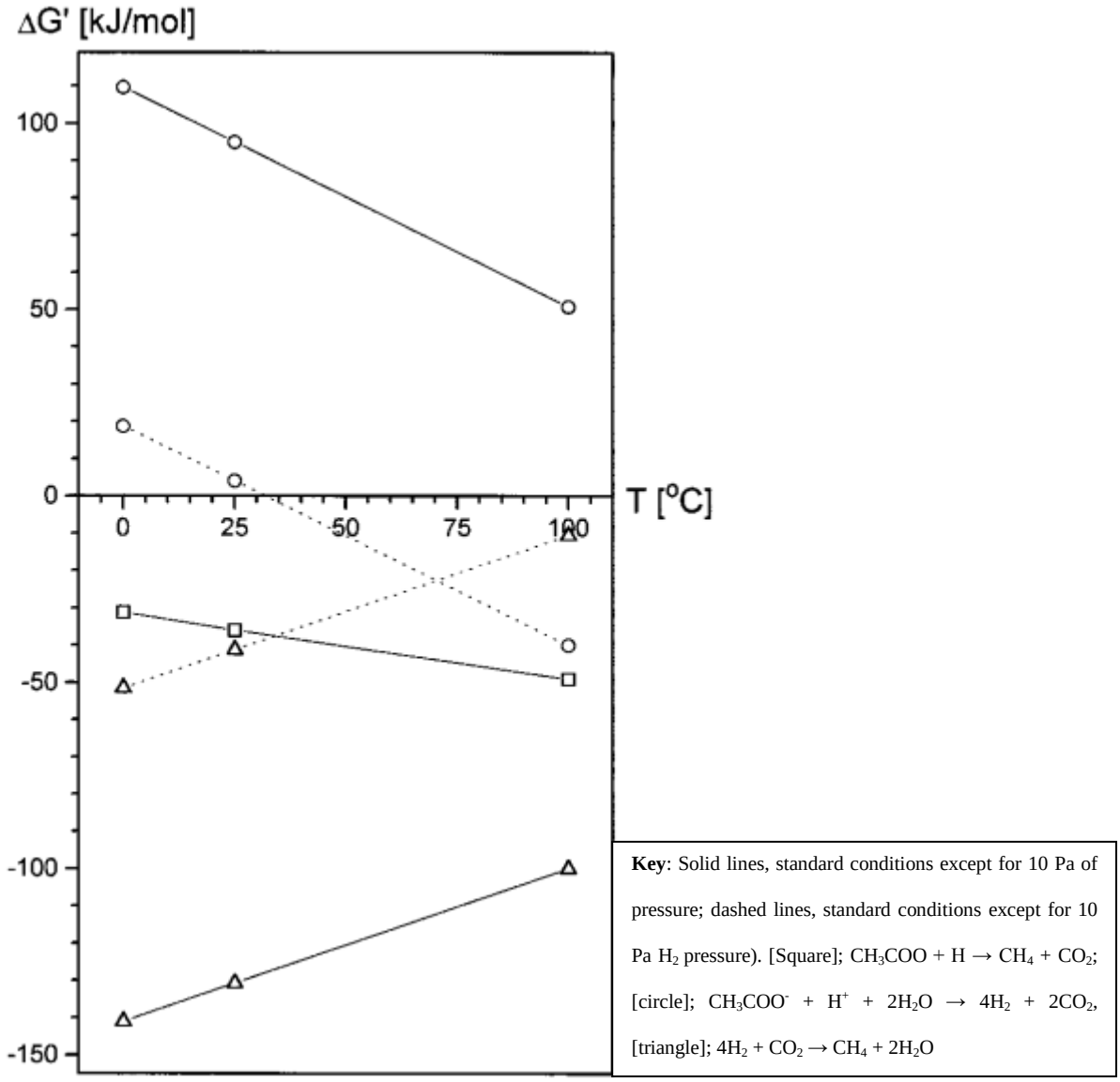


Figure 2.3. Temperature dependence of the free energy change in anaerobic hydrogen and acetate metabolism, calculated from Van't Hoff equation adapted from Schink (1997).

When anaerobic mesophilic acetate digesters are operated in the absence of acetoclastic methanogens inhibitory ammonia; the likely dominant pathway of their acetate or acetic acid removal would be acetoclastic cleavage, (Figure 2.2, path A).

The biochemical basis of the anticipation that co-dosage of Ni and Co in an anaerobic acetate digester will produce additive or synergetic effect in terms of biogas production is presented in section 2.4. This is discussed mainly in terms of acetoclastic cleavage as it is normally the main route of methane production in the absence of Methanosarcinales inhibitors.

2.4 Biochemistry of methanogenesis from acetate (the final step)

This section provides the biochemical basis or foundation to the hypothesis as to why the co-presence or dosage of Ni and Co in an anaerobic acetate digester is expected to exert an additive or synergistic effect with respect to biogas production. First a series of steps in the form of reactions through which the methyl group of acetate goes through up to its gaseous escape as methane is given in Table 2.3. The individual biochemical role played by Ni and by Co as well as their envisaged combined role will be reviewed and discussed.

Methanogenesis is the final step in a series of reactions that lead to production of methane in a conventional anaerobic sewage sludge or other biodegradable organic substrate digester. The production of methane from acetate is catalysed by a variety of enzymes, this lead to production of a variety of intermediates as discussed by Thauer (1998). Table 2.3 gives a series of reactions leading to methane production from acetate as understood to date (Thauer, 1998).

Table 2.3 Reactions and enzymes known to be involved in formation of methane from acetate by Methanosarcinales (Thauer, 1998)

Reaction	Catalysing enzyme
(feed);$CH_3COOH+CoA \rightarrow Acety\text{-}CoA+H_2O$……*eq (a)* *$\Delta G' = +35.7$ k J rnol^{-1}*	*Acetatekinase, phosphotransacetylase or acetate Thiokinase.*
Acetyl-CoA+$H_4SPT \rightarrow CH_3\text{-}H_4SPT+CO_2+CoA+2[H]$.*eq (b)* *$\Delta G' = +41.3$ kJ mol^{-1}*	*Carbon-monoxide dehydrogenase/acetyl-CoA synthase*
$CH_3\text{-}H_4SPT$+H-S-COM→ CH_3-S-CoM+ H_4SPT……*eq (c)* *$\Delta G'$* = -30 kJ mol^{-1}	*Methyl-H_4SPT : Coenzyme-M Methyltransferase.*
CH_3-S-COM+H-S-COB→ COM-S-S-COB+$CH_{4(g)\uparrow}$…*eq (d)* *$\Delta G' = -45$ kJ mol^{-1}*	*Methyl-coenzyme M reductase*
COM-S-S-COB+2[H] → H-S-CoB + H-S-CoM …...*eq (e)* *$\Delta G' = -40$ kJ mol^{-1}*	*Heterodisulphide reductase*

Table 2.3 shows a series of reactions that the acetate's methyl group goes through up to the final gaseous escape of methane (*at equation d).* Methane is given off as gas due to its insolubility in water (CH_4 harvesting from anaerobic digesters is possible due to methane's insolubility in water). The enzyme which catalyses reduction of methyl-Coenzyme M (CH_3-S-CoM) with Coenzyme B (H-S-CoB) producing methane and a heterodisulphide product of coenzyme M and Coenzyme B (CoM-S-S-CoB) is *Methyl-coenzyme M reductase*. This enzyme (*Methyl-coenzyme M reductase)* as well as *Methyl-H_4SPT:coenzyme M methyltransferase* are reported to be produced exclusively by methanogenic archea (Thauer, 1998). This sets the background for exclusive occurrence of methyl-coenzyme M in methanogens; implying that for production of methane to be improved, biochemical activities of methanogenic archaea have to also be improved. It is reported that the reduction of the heterodisulphide (CoM-S-S-CoB) to individual Coenzymes (H-S-CoM and H-S-CoB) is coupled with ATP synthesis which provides energy for growth of methanogens (*equation e*, Table 2.3).

At this point it is important to acknowledge that assuming that measures which precede and lead to production of both CH_3-S-CoM and H-S-CoB are not limiting, increasing the production and biochemical activity of methyl-coenzyme M reductase will result in increasing the rate of methanogenesis from anaerobic digestion of acetate. In terms of sewage sludge digestion, this will increase the rate and possibly extent of waste stabilisation (as acetate is commonly the main VFA produced). As a result the turnaround time of digestion, in turn energy production from an anaerobic digester would be increased.

The roles played by Co and Ni in catalysing the production and utilisation of methyl-coenzyme M respectively, will be reviewed and discussed in section 2.1.4. In terms of industrial applicability, this is the crux of the basis of the anticipated need for co-dosage of Ni and Co to anaerobic digestion systems.

2.4.1 *The role played by Cobalt and Nickel in methanogenesis*

The individual biochemical catalytic roles played by Ni and Co during acetoclastic cleavage (as this is the main anticipated reaction route) is discussed below. Co is believed to catalyse the first three reactions, being; *a*, *b* and *c*, Table 2.3. In short, the supplementation of Co to an acetate digester is expected to lead to an increase in production of methyl-coenzyme M (Thauer, 1998) from the methyl group of acetate, which is subsequently anticipated to lead to an increase in biogas production, assuming that the reduction of CH_3-S-CoM is not rate limiting (reaction *d*, Table 2.3.). This is more so given that methyl-coenzyme M has been identified as a unique intermediate precursor for methanogenesis (Thauer, 1998).

2.4.1.1 Cobalt

Cobalt has been found to be the metallo centre of most cobalamines (Ludwig and Matthews, 1997). The unusual cobalt-carbon (Co-C) bond at the centre of cobalamines produces their unusual chemistry and reactivity (Ludwig and Matthews, 1997). Cynocobalamines (vitamin B_{12}) have been shown to participate in several metabolic processes (Smith, 1958). The first observation of increased rate of a chemical reaction with the supplementation of Vitamin B_{12} (Vit B_{12}) was reported by Helleiner and Wood (1956) cited in Barker et al. (1958). They observed an increased rate of methionine formation from a mixture of homocysteine, serine and ATP in the presence of Vitamin B_{12}. This implied participation of the vitamin in methyl group transfer or formation (Helleiner and Wood (1956) cited in Barker et al. (1958). The first isolation of the then called pseudo Vitamin B_{12} was reported by Barker et al. (1958). This coenzyme was observed to be necessary for isomerisation of glutamate in to β-methylaspartate; an intermediate in the formation of mesaconate by cell extracts of *Clostridium tetanomorphum* (Barker et al., 1958). This reaction involves rearrangement of carbon atoms in glutamate, particularly the formation of methyl group. This coenzyme was

later isolated by Weissbach et al. (1959) who reported the formation of different cobalamines by microbial cells as per the cyclic base of substrate that they were grown on.

The structure of this coenzyme was studied through X-ray analysis (Lenhert and Hodgkin, 1961) and was found to differ from that of cynocobalamines in having an adenine nucleotide bond to Co in place of cyanide. The generic structure of Vit-B_{12} co-factor is presented in Figure 2.4. The methyl analogue of cobamine coenzyme was produced in the laboratory by Guest et al. (1962) in which the methyl group replaced the 5-deoxyadenosyl group part of the cobamine, forming a methyl-cobamine. The incubation of homocysteine with this methyl cobamine led to production of methionine through a slow spontaneous reaction (Guest et al., 1962). This was a crucial observation as it meant that cobamines are involved in methyl group transfer. This led to discovery by Blaylock and Stadtman (1963) in which the biosynthesis of methane from the methyl moiety of methyl-cobalamines was observed. This in turn opened way for a variety of studies assessing the biological or metabolic requirement for Co by methanogens, with the intention of increasing biogas production from digesters.

Wolfe and McBride (1971) discovered and named an intermediate product in biosynthesis of methane Coenzyme M (CoM). Their experiment involved the incubation of a radioactive labelled (^{14}C) methyl-cobalamine ($^{14}CH_3$-B_{12}) with cell extracts (proteins). The products formed were a reduced Vit-B_{12}, and a radioactive methylated intermediate product (Wolfe and McBride, 1971). The dependence of the formation of the methylated co-enzyme on protein extract, pH, temperature and ATP showed to a large extent the enzymatic dependence of the methyl transfer process (Wolfe and McBride, 1971). Methane production was found to be more rapid (3.25 faster) from CH_3-CoM as compared to from reductive demethylation of CH_3-B_{12} (Wolfe and McBride, 1971) as 0.78 µmole of $CH_4.hr^{-1}$ mg of $protein^{-1}$ were produced from CH_3-CoM compared to 0.24 µmoles of $CH_4.hr^{-1}$ mg of $protein^{-1}$ produced from CH_3-B_{12}. This is in line with the view that production of CH_3-S-CoM is a step closer to

methane production. As CH_3-S-CoM production is dependent on Vit $B_{12,}$ the metallo centre of which is Co; this makes a case for Co supplementation to anaerobic acetate digesters.

The amount of radioactively labelled methane was found to be directly proportional to the amount of $^{14}CH_3$-S-CoM added to the reaction mixture, which in turn was found to be directly proportional (up to 0.4 µmoles) to the amount of $^{14}CH_3$-B_{12} added (Wolfe and McBride, 1971). The relationship between $^{14}CH_3$-B_{12} added and $^{14}CH_3$-CoM produced is shown in Figure 2.5. This justifies Co supplementation to anaerobic digesters as it (Co) plays a pivotal role in the activity and activation of methyl transfer proteins, hence having a direct positive impact on the formation of CH_3-S-CoM. This mean that supplementation of Co to anaerobic acetate digester will lead to increased production of CH_3-S-CoM, which will subsequently result in increased biogas production.

The structure (as well as methylation) of Coenzyme M was later elucidated by Taylor and Wolfe (1974) and was found to be 2.2-dithiodiethanesulfonic acid. The active form of this co-factor was found to be 2-mecarptoethanesulfonic acid produced from reduction of 2.2-dithiodiethanesulfonic acid (Taylor and Wolfe, 1974). When 2-mecarptoethanesulfonic acid is methylated, 2-(methylthio)-ethanesulfonic acid is produced, the subsequent demethylation of which produce methane (Taylor and Wolfe, 1974).

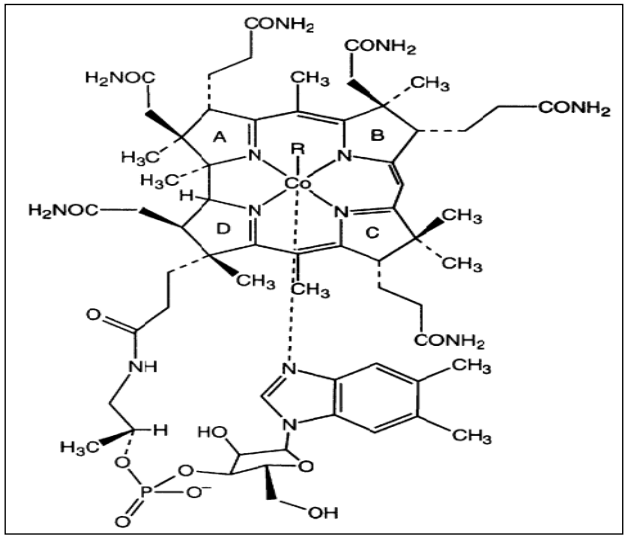


Figure 2.4. Generic molecular structure of vitamin B_{12} co factor involved in methyl transfer, note the central Co atom; figure adapted from Ludwig and Matthews (1997).

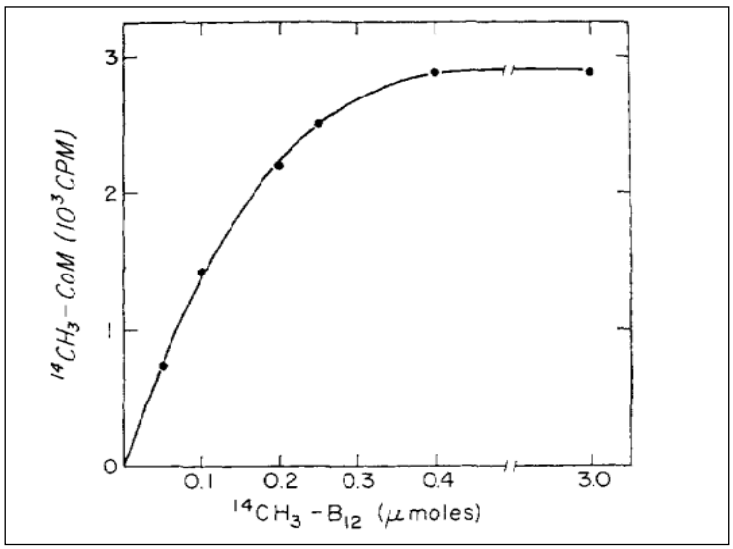


Figure 2.5. The relationship between CH_3-B_{12} added and $^{14}CH_3$-CoM produced adapted from (Wolfe and McBride, 1971).

2.4.1.2 Nickel

Synthesise of F_{430} is reported to be dependent on the presence of Ni (Diekert et al., 1980b), and F_{430} is reported to be the prosthetic group of Methyl-coenzyme M reductase (Ellefson et al., 1982). A prosthetic group is a strongly bonded non-protein component of a holoenzyme that is required for the activation of the enzyme. As the synthesis of the prosthetic group of

the enzyme which is responsible for demethylation (removal of methyl group) of methyl-coenzyme M is dependent of the presence of Ni, while in turn the formation of methyl-coenzyme M is dependent on the presence of Co; the co-presence of these elements is anticipated to have a synergistic or additive effect in biogas production from anaerobic digestion of acetate. This is the biochemical basis of the co-dosage of Ni and Co in this study. It is also worth noting that in view of biogas production, there is an anticipated co-dependence of Co and Ni on the production and subsequent utilisation of CH_3-S-CoM respectively.

It is important to consider properties of methyl-coenzyme M reductase that can be altered so as to increase its activity. One such important feature is its prosthetic group, Coenzyme F_{430}, (Fermoso, 2008, Thauer, 1998). Since co-enzyme F_{430} was first reported by Gunsalus and Wolfe (1978), extensive progress have been made in elucidating its biochemistry (structure and functionality). It was found in a study by Schönheit et al. (1979) that Ni is required for growth of methanogens. The requirement of Co and Mo was also observed, but at a much lower level than Ni, which was dosed at 150 nM compared to 20 nM for Co and Mo (Schönheit et al., 1979). The substrate for this experiment was H_2/CO_2 mixture at 80/20 % respectively. A study by Diekert et al. (1980a) established that Ni is incorporated into the structure of F_{430}. This study involved following ^{63}Ni uptake into the structure of coenzyme F_{430}, this was done by growing *Methanobacterium thermoautotrophicum* cells in a medium supplemented with 2 µmol of ^{63}NiCl/L (Diekert et al., 1980a). An uptake of 1.2 µmol of ^{63}Ni/g of dry cell mass of the dosed 2.0 µmol of ^{63}Ni was found, with 70% of the ^{63}Ni being incorporated in to F_{430} (Diekert et al., 1980a). The dependence of induction of F_{430} synthesis on the presence of Ni was reported by Diekert et al. (1980b) using the same organism *(M.Thermoautophricum*). It was also shown that the amount of F_{430} produced was positively correlated to the amount of Ni added as cells dosed 2.5 µM of Ni produced 28 times more F_{430} compared to those dosed 0.075 µM of Ni (Diekert et al., 1980b). Later it was found through

the work of Ellefson et al. (1982) that F_{430} is the prosthetic group of methyl-coenzyme M reductase and that it is always present in stoichiometry of 1 mol of Ni per mol of F_{430}, and two moles of F_{430} per mole of methyl reductase, implying two active sites in methyl reductase. The structure of coenzyme F_{430} as in Figure 2.6 was elucidated and described by Färber et al. (1991).

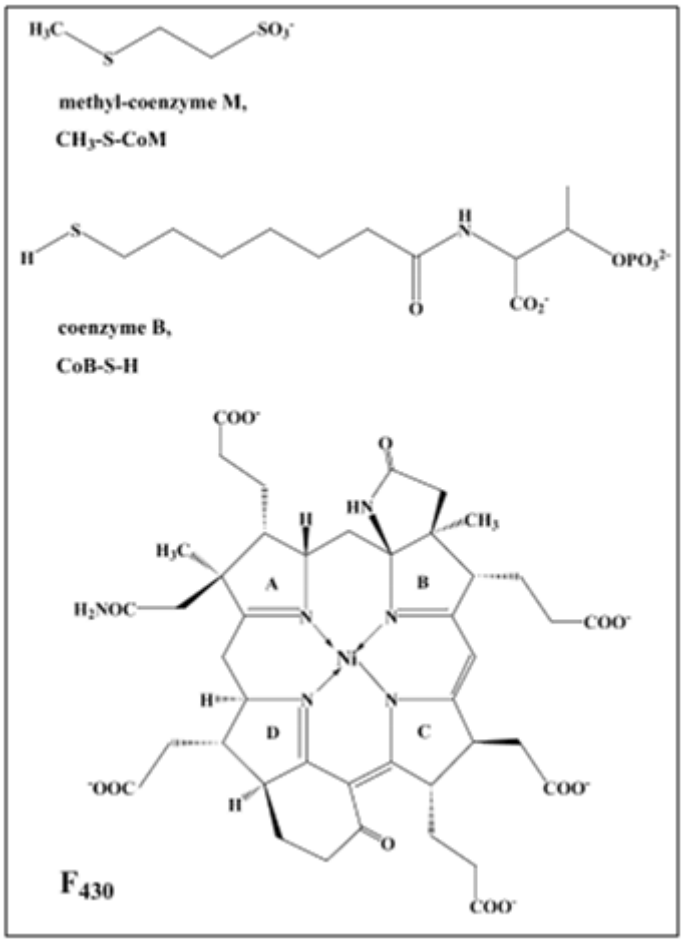
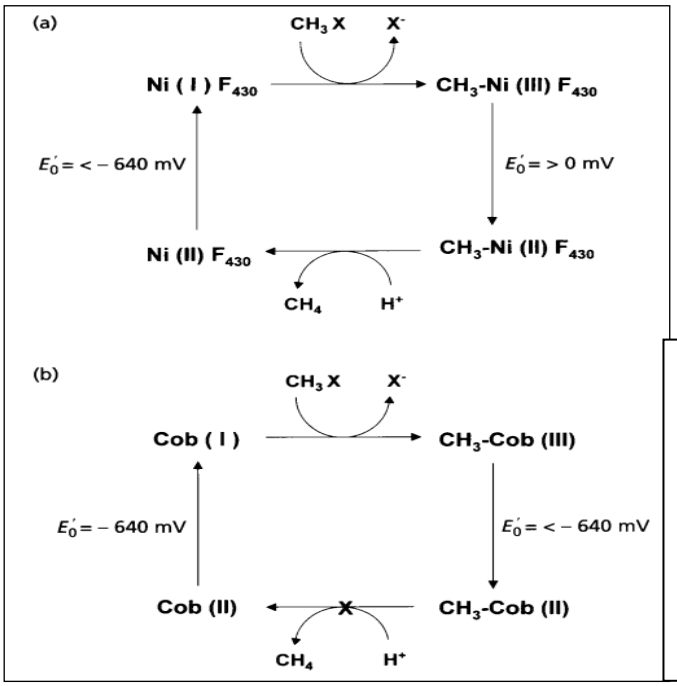


Figure 2.6. Structures of; methyl coenzyme M, coenzyme B, and coenzyme F_{430}, note the position of Ni in F_{430} Pelmenschikov et al. (2002)

Following the above research strides, there has been a marked increase in evaluation of prospects of metal ion especially Ni, Co and Fe supplementation to anaerobic digesters with the aim of increasing their biogas output as well as improving the overall health of a digester. Increasing methane output could be achieved through increasing the rate of *reaction d*, Table 2.3 assuming that formation of CH_3-S-CoM is not limiting (Thauer, 1998).

2.4.2 *Possible catalytic mechanisms of Ni and Co in Methanogenesis ~How Ni and Co work.*

In this section a brief review of the possible catalytic reaction mechanism for both Ni and Co is undertaken. This will provide a vision as to why Ni is deemed a good methyl reducing catalyst while Co is deemed a good methyl transfer catalyst (Thauer, 1998). With this knowledge, it could be possible to separate the biogas production due to Ni catalyses from that of due to Co catalyses when these two elements are co-dosed. Separation of biogas production due to Ni from that of due to Co when these two elements are co-dosed will provide the ability to check/judge whether an additive or synergistic relationship results from the co -dosage of Ni and Co. If affirmed, this will further support the need to co-dose these two elements during operation of anaerobic acetate digesters.



Note: CH_3X, at experimentation represented; methyltosylate, methylhalides or methylsulphonium in the reaction: $CH_3\text{-}X + 2Ni(I)F_{430} + BH \rightarrow CH_4 + 2Ni(II)F_{430} + X^- + B^-$ (Jaun and Pfaltz, 1988, Lin and Jaun, 1992).

Figure 2.7. Properties of coenzyme F_{430} (a) and cobalamine (b); showing why the former is deemed a good methyl reducing catalyst while the latter a good methyl transfer catalyst, adapted from Thauer (1998).

Physical experiments were conducted by Lin and Jaun (1992) as well as Jaun and Pfaltz (1988) to study methane formation at physical level so as to draw inference into biological methanogenesis; as such when inferred to methanogenesis in digesters. CH_3X in Figure 2.7

models CH_3-S-CoM (methyl-coenzyme M) as it is on this compound that Ni(I) in F_{430} of Methyl-coenzyme M reductase is believed to exert a nucleophilic attack on the methyl group which is bonded to sulphur (Thauer, 1998, Ermler et al., 1997).

Ni in F_{430} is normally isolated in Ni(II) oxidation state and it is reported to be reducible to Ni(I) oxidation state upon activation of Methyl-Coenzyme M reductase (Thauer, 1998).

This Ni(I) in F_{430} is believed to be methylated by the methyl group from CH_3-S-CoM to methyl-Ni(III)-F_{430} which is a very unstable or labile compound as such it is easily reduced to methyl-Ni(II)-F_{430} by any electron donor available in solution given the high elelctrophilic nature of Ni(III) and the large redox potential of NI(III)/Ni(II) pair, Figure 2.7(a) (Jaun, 1993). The spontaneous protonolyses of methyl-Ni(II)-F_{430} by H-S-CoB is said to yield methane and Ni (II)-F_{430} (Lin and Jaun, 1991).

Irrespective of the reaction mechanism involved, it is believed that hydrogen for this protonolyses comes from H-S-CoB (Scheller et al., 2010, Pelmenschikov and Siegbahn, 2003, Chen et al., 2012). Goubeaud et al. (1997) showed that methyl-coenzyme M reductase is activated upon reduction of Ni(II)-F_{430} to Ni(I)-F_{430}. In view of its reaction properties, the Ni porphinoid is deemed to be a good methyl group reduction catalyst (Scheller et al., 2010, Thauer, 1998). This is contrary to cobalt which is deemed to be a good methyl group transfer catalyst (Thauer, 1998). Figure 2.7(a) shows thermodynamic favourability of Ni as a methyl reducing catalyst as opposed to Co being a good methyl transfer catalyst (Thauer, 1998). From Figure 2.7, the respective redox potentials show that reduction of methyl-Ni(III)-F_{430} to methyl-Ni(II)-F_{430} is thermodynamically more favoured as compared to the reduction of methyl-Cob(III)-alamin to methyl-Cob(II) alamin, Figure 2.7(b) (Thauer, 1998).

An alternative reaction mechanism is suggested or offered by Pelmenschikov et al. (2002) based on calculations using B3LYP hybrid density functional method and chemical models. In the research by Pelmenschikov et al. (2002) the energy of formation of products was

estimated, and the findings suggested the formation of a methyl radical prior to methane. It is suggested that the Ni(I) of F_{430} breaks the C-S bond of CH_3-S-CoM releasing the methyl radical ($CH_3^{\cdot}$) which in turn gets rapidly quenched by the hydrogen of H-S-CoB, thereby producing methane (Chen et al., 2012, Pelmenschikov and Siegbahn, 2003, Pelmenschikov et al., 2002). The formation of CoB-S-S-CoM is anticipated to occur in the last step with the concomitant reduction of Ni(II) to Ni(I) (Pelmenschikov et al., 2002). The two mechanisms are shown in Figure 2.8 (*a* = CH_3-Ni formation, *b* = CH_3^- formation). Formation of the heterodisulphide product (CoB-S-S-CoM) was also studied by Pelmenschikov and Siegbahn (2003) using density functional theory and was found to be in agreement with that suggested by Pelmenschikov et al. (2002) earlier.

There has not been experimental evidence for the formation of either a methyl radical or a Ni-CH_3^- intermediate (Scheller et al., 2013) as such the biological catalytic mechanism of Ni unlike that of Co is still not fully understood. The formation of an δ-alkane-Ni complex was reported by Scheller et al. (2010) making the formation of an intermediate methyl-Ni product during methanogenesis more plausible.

The theoretical model by Pelmenschikov et al. (2002) and the experimental work of Scheller et al. (2013) both conclude that the breakage of C-S bond in methyl-coenzyme M is the rate limiting step during the reaction of CH_3-S-CoM and H-S-CoB. These authors (Scheller et al., 2013, Pelmenschikov et al., 2002) also concur that C-S bond breakage is caused or induced by Ni in F_{430} in Ni(I) state. These views are crucial from an Environmental Engineer's view point as they imply that the deficiency of Ni in an anaerobic digester would impair the release of methyl group from CH_3-S-CoM; hence biogas production. This can also explain the previously observed prompt VFAs reduction following Ni dosage to a UASB methanol digesters by Fermoso (2008) as well as the increased growth rate of *M. thermoautotrophicum* in the presence of Ni observed by Schönheit et al. (1979).

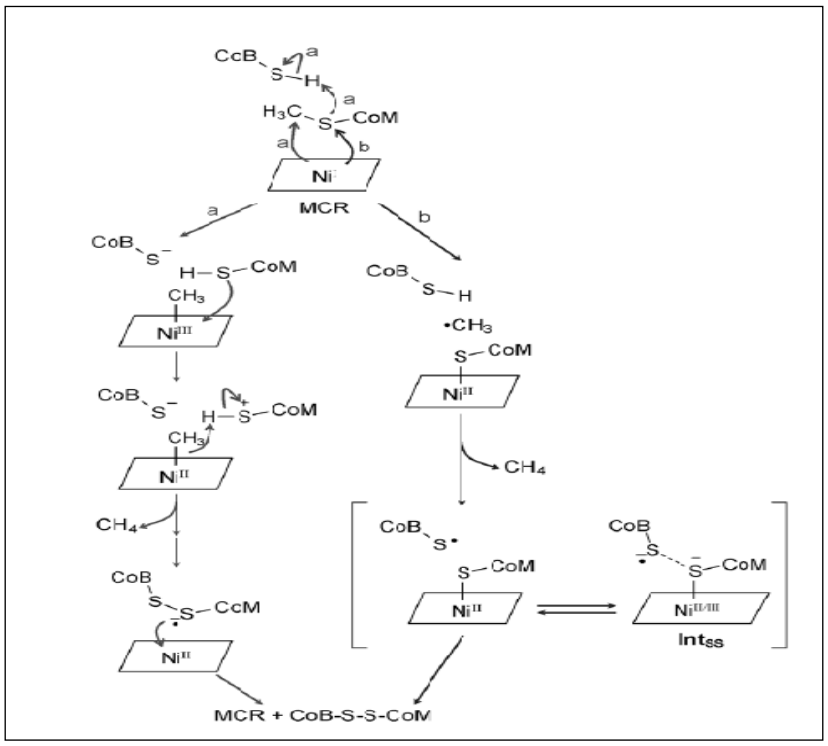


Figure 2.8. Possible reaction mechanisms for the formation of methane ; *a*= CH_3-Ni(III)-F_{430}; *b* = formation of methyl-radical, adapted from Chen et al. (2012).

As enzymes which are involved in methyl transfer are dependent on or are activated by the presence of Co bearing Vit-B_{12} co-factors (Thauer, 1998, Ludwig and Matthews, 1997) it is anticipated that the production of methyl-coenzyme M and subsequently methane, will increase following the dosage of Co; assuming that the availability of Ni does not limit the reduction of CH_3-S-CoM to methane. The dosage of Ni is anticipated to induce and increase the rate of reduction of methyl-coenzyme M to methane by hydrogen of coenzyme B (Scheller et al., 2013, Scheller et al., 2010, Pelmenschikov et al., 2002).

2.4.2.1 Summary of the biochemical case for co-dosage of Ni and Co.

In summary, the biochemical bases for the anticipation that the combined dosage of Ni and Co at stimulatory concentrations to ananeroboc acetate digesters would lead to additive or synegestic effects in terms of their biogas production was shown form the review of the literature. The bases for this anticipation emanate from the interconectivity/interdependence of the catalytic roles of Ni and Co in anaerobic digestion. This interconectivity is

conceptualised pictorically in Figure 2.9. Co is expected to catalyse the production of CH_3-S-CoM, while Ni is expected to catalyse its (CH_3-S-CoM) subsequent reduction to methane, hence their co-dosage is anticipated to lead to production of more biogas than their individual dosage.

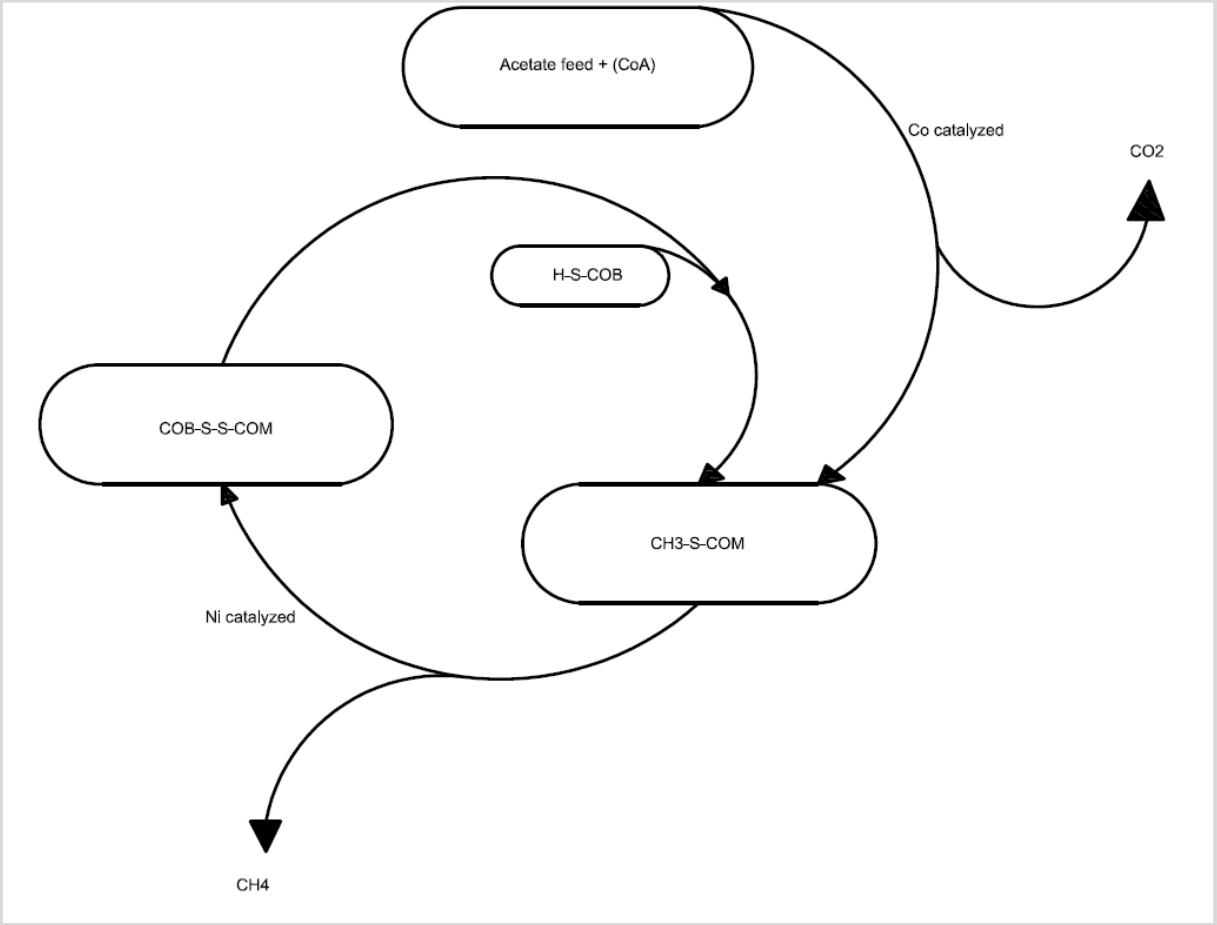


Figure 2.9. Schematic view of catalytic interconnectivity of Ni and Co.

The observations made from digesters following their supplementation with various micronutrients were briefly reviewed and discussed below (section 2.5). Different digester configurations, digesting different substrates, dosed different amounts and combinations of micronutrients are considered in section 2.5. Given the disparity that exists in the anaerobic digestion field, and considering that these digesters had been dosed to improve their efficiency, their consideration provide a general empirical observation on the behaviour of digesters supplemented with micronutrients.

2.5 Some of the observations made from supplementation of trace elements to digesters.

The ability of the dosed micronutrients to exert their effects (alter the biological activity of microorganisms) is affected by several factors. These include but are not limited to; metal ion bioavailability, the type of substrate digested as well as the type of digester used. As such supplementation of trace elements to digesters will catalyse biogas production to different degrees depending on some of these factors. In the work of Fermoso (2008) purposed to optimise metal dosing in granular sludge bed based reactors like the UASB and Expanded Granular Sludge Bed reactors (EGSB), increased methane production was observed on supplementation of 0.5 µM of Ni, while supplementation of 0.5 µM of Co increased production of VFA-mainly acetate. But SMA with acetate as the substrate was increase on Co supplementation to sludge from a Co deprived digester. While this work showed the need to dose metal ions to methanol digesters, co-dependence as well as individual contribution of Ni and Co to biogas production when co-dosed was not investigated. It was demonstrated by Banks et al. (2012) that during digestion of nitrogen rich food waste, approximately 0.2 mg/L of both Se and Co were required to avoid VFA, specifically propionate, accumulation as a result of inhibition of propionate oxidising bacteria or formate reducing hydrogenotrophic methanogens. Se and Co supplementation allowed for higher organic loading rates to be achieved. In contrast to Fermoso (2008)'s observation were supplementation of Co to a Co deprived digester increased acidogenesis from methanol, supplementation of Co increased methane production in Banks et al. (2012)'s food waste digesters. This disparity can be explained by the different microorganisms as well as enzymatic routes involved in these digesters, which calls for the need to specifically identify trace elements that are to be supplemented to each digester.

Methanomethylovorans thermophilia was isolated from a methanol UASB digester and was found to need an optimum Co concentration of 0.5-2.0 µM for methanol digestion by Jiang (2006). A syntrophic methanol degrading co-culture was also enriched from a Co deprived media, this co-culture was found to convert methanol to acetate in the presence of Co, otherwise it converted it to CH_4. The co-culture was composed of homoacetogen and hydrogenotrophic methanogen (Jiang, 2006). The concentration of the micronutrient needed was well established in the study by Jiang (2006) as it was conducted on almost pure culture; while this is necessary, it is not representative of a digester environment which normally contains a variety of microorganisms which can result in various responses to dosage of trace elements. In pure culture studies of *M. barkeri* grown on methanol, it was established that 0.5 µM of Co was stimulatory to its growth, while 5.0 µM was found to be toxic (Jiang, 2006). Exertion of toxicity at the low concentration of 5.0 µM (≈ 0.3 mg/L) could be reflective of the high accessibility of Co in pure culture studies.

A high dilution rate was achieved by Kida et al. (2001) after supplementation of 0.5 mg L^{-1} of Ni and 0.2 mg L^{-1} of Co to a digester treating acetate containing synthetic wastewater with a 100% COD removal efficiency accompanied by high biogas production. The concentration of corrinoids and F_{430} increased with supplementation of Ni and Co, which suggested that biogas was produced through the activities of these corrinoids and F_{430} (Kida et al., 2001). While the need to dose Ni and Co was established, their individual contribution to increased methanogenesis was not, as well as evaluation of their co-dependence. Also the effect of only one concentration of each of these elements was investigated, which is not representative of the variety of concentrations that may naturally exist in a digester. In a study conducted by Speece et al. (1983) purposed to examine the effect of Ni and yeast extract on acetate utilization rate, acetate utilization rate was found to be 2-5 g g VSS^{-1} d^{-1} in the absence of Ni, 10 g $^{-1}$g VSS^{-1} d^{-1} when 0.5 mg/L of Ni was present and 12-15 g g VSS^{-1} d^{-1} in the presence of

both Ni and yeast extract. Although Co was supplied at 4.0 mg/L, its effect on biogas production or acetate utilisation was not studied (Speece et al., 1983). Gustavsson (2012)'s study showed that supplementation of Ni, Co and Fe at 0.5, 0.2 and 0.5 g L^{-1} respectively was necessary to successfully digest wheat stillage at organic loading rate of 0.5 g VS L^{-1} d^{-1}, despite the high sulphite concentration in the fed stillage. It was acknowledged that the high sulphite concentration in wheat stillage might have negatively affected the bioavailability of these micronutrients (Gustavsson, 2012). In a different study by the same author (Gustavsson, 2012) the individual need for Ni and for Co was established in grain stillage digester through omission of each of these elements in separate digesters. A Ni omitted digester failed promptly while that omitted of Co did not fail, but both accumulated VFAs; 80% of the VFAs in a Ni omitted digester were acetate, while in a Co deprived digester acetate accounted for 60% of the VFAs (Gustavsson, 2012). From this study, accumulation of acetate due to lack of Ni and lack of Co was demonstrated. This showed the individual necessity of Co and Ni for aversion of acetate accumulation in grain stillage anaerobic digesters. Gustavsson (2012)'s study also demonstrated the effect of digester environment on the bioavailability of micronutrients dosed to them. Despite Gustavsson (2012) having established the individual need for Ni and Co in grain stillage digesters, their individual contribution to biogas production was not established when they were co-dosed, as well as their co-dependence for biogas production.

The level of progress made to date in understanding anaerobic digestion as well as the role of various micronutrients in its biochemistry have been reported by various researchers, but it is succinctly presented in the autobiographical article by Thauer (2015). This article gives biochemical roles that various micronutrients play in catalysing anaerobic digestion; this in turn gives biochemical legitimacy to the expectation that certain micronutrients will lead to increased biogas production rates. Through experimentation, a variety of researches (some

reviewed above) have shown that dosage of micronutrients in most cases lead to increase in biogas production rates from anaerobic digestion. The combination of both empirical observations and biochemical studies strongly suggests that the co-dosage of Ni and Co to an anaerobic acetate digester will lead to increased biogas production in an additive or synergistic manner; hence the hypothesis of this study.

2.6 Metal ion toxicity at high concentrations

Despite the well-known and documented beneficial effects of supplementation of specific micronutrients to anaerobic digesters in terms of increasing biogas production, the same essential elements are also known for being toxic to microorganisms when in large enough concentration. The effects of increased dosage or presence of micronutrients on the rate of biological reactions has been summarised by McCarty (1964) into three zones as follows: (I); Zone of increasing stimulation, (II); zone of decreasing stimulation and (III); toxicity zone. These various zones are shown in Figure 2.10. The same categorisation applies in an anaerobic digester. A similar versions of Figure 2.10 has also been suggested by Fermoso (2008) to be applicable to dosage of micronutrients to anaerobic digesters.

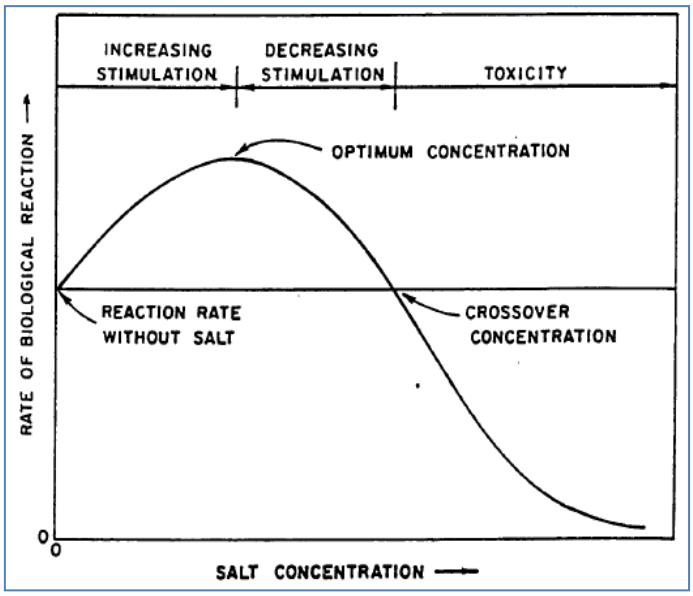


Figure 2.10. Effects of increasing micronutrients to a microbial system (McCarty, 1964).

When micronutrients such as Ni or Co are supplemented to digesters, their concentration in digesters should be maintained at the optimum amount, and should not be at the toxicity concentration as digestion will cease or drastically reduce. There has been great variability in reported toxic concentrations of Ni and Co in anaerobic digestion systems; this is mainly due to the variability that exists within and between digestion systems. Great disparity in levels of toxic concentrations is also observed between anaerobic digesters and aerobic treatment systems. The optimal concentration for Ni and Co on aerobic activated sludge was found to be 10 and 5 mg/L respectively by Gikas (2008) and a synergistic effect was also observed at these stimulatory concentrations. The potent or lethal concentration for these elements was found to lie between 160-320 mg L^{-1}, which Co being more potent than Ni (Gikas, 2008). Various factors affect the toxicity of metal ions in an anaerobic digester, these factors are discussed by Oleszkiewicz and Sharma (1990) and they include: pH, redox potential, precipitation by sulphide, carbonates and phosphates, chelation by both organic and synthetic ligands amongst others. Generally the nature or origin of sludge being digested will affect the biochemistry of a digesters, for examples, if Ni and Co are supplemented to sewage sludge from wastewater that has been treated by iron (Fe) for Chemical Phosphorus Removal (CPR)

it is possible that a higher bioavailability of these elements, hence toxicity at lower concentrations would be observed compared to in a non-Fe dosed wastewater (Smith and Carliell-Marquet, 2009, Oleszkiewicz and Sharma, 1990). This is because the dosed Fe could in addition to phosphorus removal; precipitate the anions that would otherwise render Ni and Co non-bioavailable.

The toxicokinetics (mode of toxicity) of Ni is believed to be through, but not limited to; prolongation of the lag phase of bacterial growth, inducement of abnormal morphological developments in cells, exertion of adverse effects on biochemical processes such as; DNA replication, transcription, translation and enzymatic activity as well as altering of the surface charge of cell (Babich and Stotzky, 1983). Gikas (2008) observed that since the chemistry of Ni and Co are similar, Ni=[Ar]$3d^8 4s^2$; Co=[Ar]$3d^7 s^2$, they also exhibit similar modes of toxicity. These modes of toxicity imply that toxicity of Ni and Co ions is exerted through direct ion and microbial cell interaction. When supplemented to digesters, the total concentration of ions, rather than their individual concentration should be considered in estimation of the point or concentration of onset of toxicity.

Metal ions' potential to exert toxicity is anticipated to vary with the type of substrate being digested amongst other factors; substrates that have a tendency to complex or precipitate metal ions, thus reducing the concentration of the free form of the ions, are expected to reduce the potential of these ions to be uptaken by microorganisms, thus reducing their potential to exert toxicity. The bioavailability of Ni and Co in a sulphate rich grain stillage digester was studied by Gustavsson (2012), considering the sulphates' effect on metal ions precipitation. In view of this, when metal ions are supplemented to a digesters there is a need to define the stimulatory as well as toxic concentration for that particular digester. This could prevent the sudden collapse of a digester due to metal toxicity from ions intended for stimulation of biogas production.

The following section considers factors that affect the bioavailability of metal ions in a digester, as not all of the dosed ions are anticipated to be bioavailable.

2.7 Metal ions bioavailability

For a micronutrient to alter the biochemistry of a cell thus exerting its effect, it has to first enter the microbial cell concerned. The proportion of the metal ion that is able to be used or taken up by the bacteria is generally viewed as being bioavailable (Oleszkiewicz and Sharma, 1990). Environmental conditions in a digester and the total metal ion dosed to a digester affect the bioavailable fraction of the metal ion dosed, these conditions include: pH, redox potential, as well as precipitation, e.g. by sulphides (Oleszkiewicz and Sharma, 1990). The free metal ion that is not complexed is generally viewed as the proportion of the total metal ion concentration that is bioavailable (Fermoso, 2008). This is possibly because the free form of the ion is the most reactive compared to the complexed form and also the free ion normally has a smaller size compared to the compound that it could form when it is complexed. In view of this, digesters that are digesting simple substrates like methanol and acetate are expected to have higher bioavailability of metal ions compared to those digesting complex substrate; such as sludge, grain stillage or food waste. If a digester has high bioavailability of ions, dosing of a relative small amount of the micronutrient could result in increased performance compared to in a digester with low bioavailability. Several models have been developed to estimate the bioavailable concentration of the metal ion in an aquatic system. The most common model being the Biological Ligand Model (BLM) (Fermoso, 2008). The BLM model assumes chemical equilibrium of the aquatic system and also a single point of entry of the metal ion into the cell (Fermoso, 2008).

Despite the assumptions of the BLM, an anaerobic digester, especially a continuously fed one, is hardly ever at equilibrium and cells have been shown to be actively involved in the transport and internalisation of the ions of interest (Worms et al., 2006). The following areas as outlined by Worms et al. (2006) are considered to be integral to bioavailability of ions in an aquatic system;

- The behaviour of metal ions during their transport from the bulk body of the liquid into the cell membrane.
- Then transfer/ entry of the metal ion into the cell across the cell membrane.
- The role played by the microorganism in modifying and influencing the cell up take i.e. active transport vs diffusion.

Figure 2.11, adapted from Worms et al. (2006) with modifications shows the interaction between various physical, biological and chemical processes that affect the uptake and internalisation of metal ion by microorganisms. The BLM model depicts the mechanisms through which microbial cells can take up essential micronutrients, these involves the micronutrients passively diffusing across the cell membrane along their concentration gradient and the active transport of the micronutrient through secretion of binding ligands which aid the cell in the uptake of micronutrient across its membrane normally at gazetted points (Worms et al., 2006). The internalised metal ion (M_{int}) will then be able to affect the biochemical activities of the cell; which can lead to increased acetate metabolism, hence biogas production. When metal ions or other toxicants are in the cell an undesirably high concentration, they are secreted out of the cell by the process of active transport; this is essentially the cell's defence mechanism against toxicity (Worms et al., 2006).

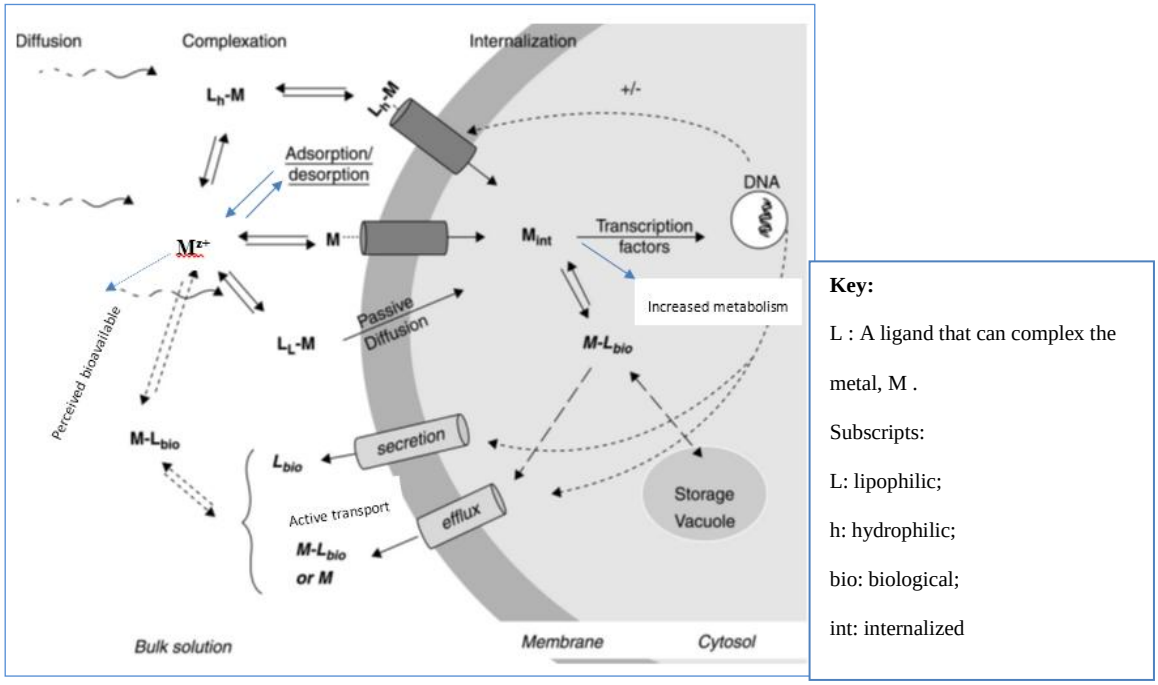


Figure 2.11. Conceptual model of some of the important physicochemical processes leading to and following the uptake of a trace metal ion by an aquatic microorganism , adapted with modifications from (Worms et al., 2006).

The biochemical case for supplementation of Ni and Co in anaerobic acetate digester has been identified (section 2.4.1). This is in line with the various observations made form supplementation of metal ion to digesters (section 2.5). Despite this, gaps in the field of anaerobic digestion of acetate were identified and are presented in section 2.8.

2.8 Knowledge Gap

While the individual need for Ni and Co have been established for various anaerobic digestion systems (Gustavsson, 2012, Fermoso, 2008) digesting different substrates, the effect of their co-dosage at stimulatory concentrations has not yet been investigated, especially in an anaerobic acetate digester.

Although studies have been conducted on stimulatory concentrations of Ni, Co and other metal ions in an anaerobic digester (Banks et al., 2012, Gustavsson, 2012, Zandvoort et al.,

2002, Fermoso, 2008), not much has been reported on the toxic concentrations of these various ions for each of the digestion systems used, i.e. the boundary concentration for supplementation of metal ions of interest have not been extensively reported or established.

The digestion of acetate has not been as extensively studied as the digestion of other substrates such as methanol, hence this study will add to an increasing pool of knowledge on the anaerobic digestion of acetate as it is commonly the main precursor for methane in most of the anaerobic digestion systems.

Studies on ways of improving the anaerobic digestion of acetate or acetic acid will also contribute to informing methods of acidified reactor recovery, i.e. recovery of a digester that has accumulated acetic acid or acetate.

This study seeks to close these gaps by defining different ranges/parts of the hypothetical dose response curve (Figure 1.2 , page-3) for Ni, Co and Ni and Co dosage in laboratory scale mesophilic (37°C) anaerobic acetate digesters.

CHAPTER 3. MATERIALS AND METHODS

This chapter outlines how the experiment was conducted in order to determine various regions of the hypothetical dose response curve (Figure 1.2). The research was divided into two parts:

Part 1: Determination of regions A & B of the hypothetical dose response curve. These regions represent stimulatory concentrations of Ni, Co and Ni and Co necessary for biogas production, region B, as well as their limiting concentration, region A. This part was conducted through digestion of acetate in 5.0 L digesters.

Part 2: Determination of region D of the hypothetical dose response curve for; Ni, Co and Ni and Co dosage. This part was mainly examined through digestion of acetate in 120.0 ml serum bottles seeded with effluent from the 5.0 L acetate digesters. This was carried out off-line to avoid a sudden collapse of digesters due to large concentration of metal ions. From the knowledge or region B and D, region C (excess concentration) could be deduced.

The last section (3.8) of this chapter presents the computation, as well as its basis, for prediction of the theoretical amount of methane that could be obtained from the complete anaerobic digestion of acetic acid into carbon dioxide and methane gases.

3.1 Source of seed biomass

Digested domestic sewage sludge obtained from a Wastewater Treatment Plant (WWTP) in Birmingham, UK, was used to seed all the four 5.0 L digesters. Seed sludge from a WWTP was obtained at the exit point of a sludge digester for dewatering and further processing. The seed sludge was composed of combined primary and secondary digested sludge. A 25.0 L plastic bottle was used to collect sludge to the laboratory. Total Solids (TS) and Total Volatile

Solids (TVS) were determined immediately on arrival to the laboratory as per Standard Methods (Eaton et al., 2005).

3.2 Operation of 5.0 L digesters (part 1)

This section outlines operation of digesters during start-up period, (first five days), during which no effluent was withdrawn from digesters to achieve acclimatisation of microorganisms to acetate and to ensure that all measuring instruments are in working conditions, especially the gas counters. The operation of digesters when fed daily is also outlined.

3.2.1 *Digester start-up period*

The method of setting up and operating of digesters was influenced by that previously employed by Fermoso (2008) with replacement of Up Flow Anaerobic Sludge Blanket Reactor (UASB) with a sime-Continuous Stirred Tank Reactor (CSTR). Four identical plastic (PVC) digesters of 5.0 L working volume with 600.0 ml head space were used.

Digesters' daily acetate concentration of 30 mM (1.8 g/L) was targeted in consideration of the observation made by Ishaq (2012) that 60 mM (3.6 g/L) of an acetate solution made of 99.75% sodium acetate and 0.25% glacial acetic acid was toxic to methane producing microorganisms, possibly due to toxicity of one of the components in the media, likely sodium. With the expectation that not all of the daily fed acetate would be completely utilised, the optimum concentration of 60 mM found by Ishaq (2012) was reduced to the daily targeted concentration of 30 mM (1.8 g/L) in this study.

Five litres (5.0 L) digester volume was selected because this volume allowed low acetate concentration of (30 mM or 1.8 g/L) to be digested while producing a volume of biogas that could be easily measured by the gas measuring device used. A minimum volume of 34.5 mL

could be measured by the gas counters employed. If digesters of a small volume (<< 5 L) were used, a larger acetate concentration was going to be needed in order to produce the minimum amount of biogas volume that could be recorded by the gas counters, which would be approaching the inhibitory acetate concentration of greater than 60 mM observed by Ishaq (2012). A much bigger digester (>> 5 L) in addition to being expensive to run would produce large gas flow rates, which would increase the error of the gas flow counters used. Walker et al. (2009) observed that the counter calibration of the used gas counters in this study had a gas flow rate dependency, with the volume per count increasing with flow rate. In consideration of the inhibitory acetate concentration (>> 60 mM) and the low desired biogas flow rate, 5.0 L digesters were found to be more suitable for this study.

3.2.2 *Selection of suitable acetate feed composition*

Drawing from Ishaq (2012)'s work in which 60 mM (3.6 g/L) of acetate buffer consisting of 99.75 % sodium acetate and 0.25% glacial acetic acid at an optimal pH range of 7 to 7.2 was used as feed in serum bottles; a suitable acetate feed composition was developed in light of continuous feeding of digesters and the possible resulting sodium toxicity, hence there was the need to reduce the proportion of sodium (Na) in the feed.

A suitable feed composition was determined by feeding digesters acetate with progressively reduced Na proportion, Figure 3.1. The used loading rate was 1.8 g acetate. L^{-1}.day $^{-1}$, from day 0 to day 4, then no further feeding was done. Magnesium and calcium are key macronutrients for microorganisms as exhibited by their independent supplementation by Fermoso (2008), hence they were selected to replace Na as acetate ion carriers.

Figure 3.1 shows progressive increase in biogas production from digesters from the daily average of 0.5 L to 1.5 L with progressive reduction of the Na content from 100% to 25% (D2 to D1 & D4). In view of this performance, sodium acetate: calcium acetate and magnesium acetate in mole ratio of: 25:35:40 respectively at a mixture pH of (7.0-7.5) was used as

digester feed. The introduction of magnesium acetate and calcium acetate led to reduction of the concentration of sodium in the digesters while keeping the acetate concentration the same, thereby averting possible sodium toxicity. Potassium hydrogen phosphate (K_2HPO_4) and ammonium chloride (NH_4Cl) were added as part of macronutrients to a concentration of 1.44 mM and 5.23 mM respectively.

After realisation of the suitable acetate feed composition, digesters were operated as follows during the first five days of start up; 18.0 g of acetate (3.6 g/L) composed of; sodium acetate: calcium acetate and magnesium acetate in mole ratio of: 25:35:40 respectively at a pH of (7.0-7.5) was fed to all four digesters. During this first five days, no effluent was withdrawn from digesters. Digesters were seeded with 25.0 g equivalence of Total Volatile Solids (TVS) of digested domestic sewage sludge. The final volume of the mixture (acetate, dilution water and seed sludge) was 5.0 L and was digested at 37.0 ±2 °C in a temperature controlled room. This mixture was made up of (1.0 L seed sludge, 1.0 L acetate feed, and 3.0 L dilution water).

Biogas production from digester during the period of start up is presented in Figure 3.2. The average daily biogas production across all digesters during start up period was 2.5 L/day with a standard deviation of 0.15 L (RSD=6.1%); this shows consistency and reproducibility of performance of digesters when operated under the same condition. The percentage of methane in the produced biogas was constantly 64±1%.

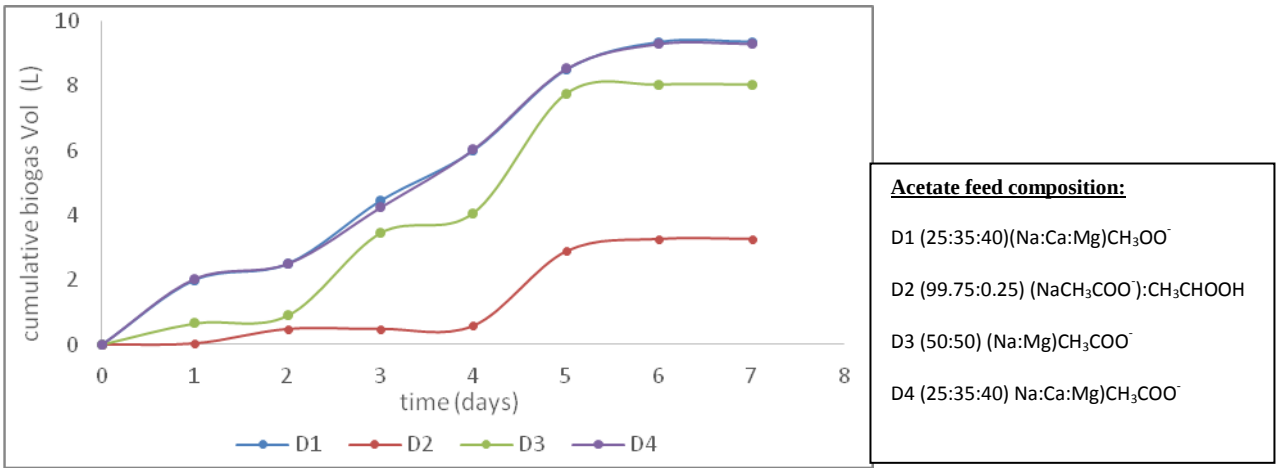


Figure 3.1. Cumulative biogas production from 30 mM of acetate with different composition

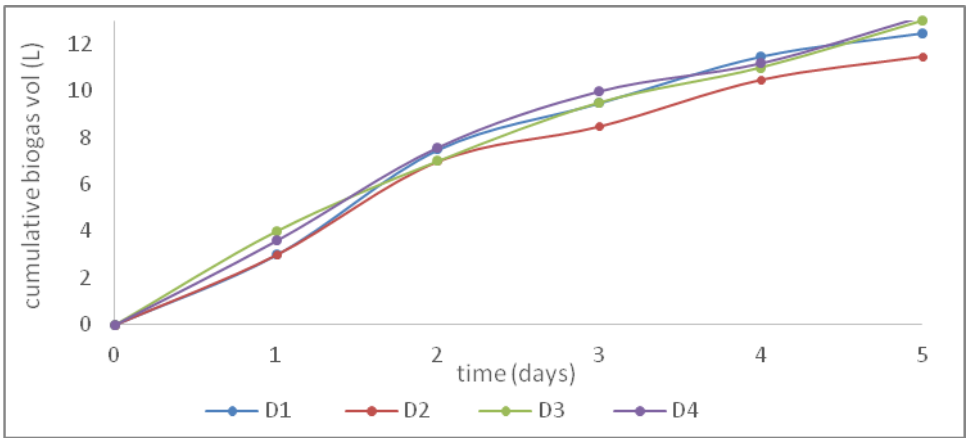


Figure 3.2. Cumulative biogas production from 60 mM of acetate composed of (25:35:40)(Na:Ca:Mg)CH_3OO^- during start up period.

3.2.3 *Operation of digesters when daily fed*

After 5 days of start-up, digesters were fed macro and micronutrients as per a feeding regime and concentration adapted from Fermoso (2008), see Table 3.1.

Digesters were daily fed 9.0 g of acetate at a loading rate of 1.8 g acetate L^{-1} day^{-1}. 500.0 ml of a 300 mM acetate solution was fed to digesters following removal of the effluent of an equivalent volume. Prior to removal of the effluent, digester contents were mixed by vigorous shaking. The acetate solution feed was produced in 8.0 L batch and was autoclaved in Boxer Lab equipment autoclave at 110°C for 2.5-3 hours, then cooled to room temperature. This was done to avert the prior observed growth of unidentified organisms on storage of un-autoclaved medium. This growth was visible as large fibrous structures in the stored medium.

The volume of biogas produced during digestion was recorded cumulatively in 10 minutes intervals using U3-Lab jack connected to a computer equipped with a four cell gas counter program obtained from School of Civil Engineering and Environment, University of Southampton. A schematic view of digester set up is presented in Figure 3.3.

3.3 Schematic of digester set up

The schematic view, with a picture insert, of the digesters set up is presented in Figure 3.3. This shows how the various units of the digester set up were interconnected.

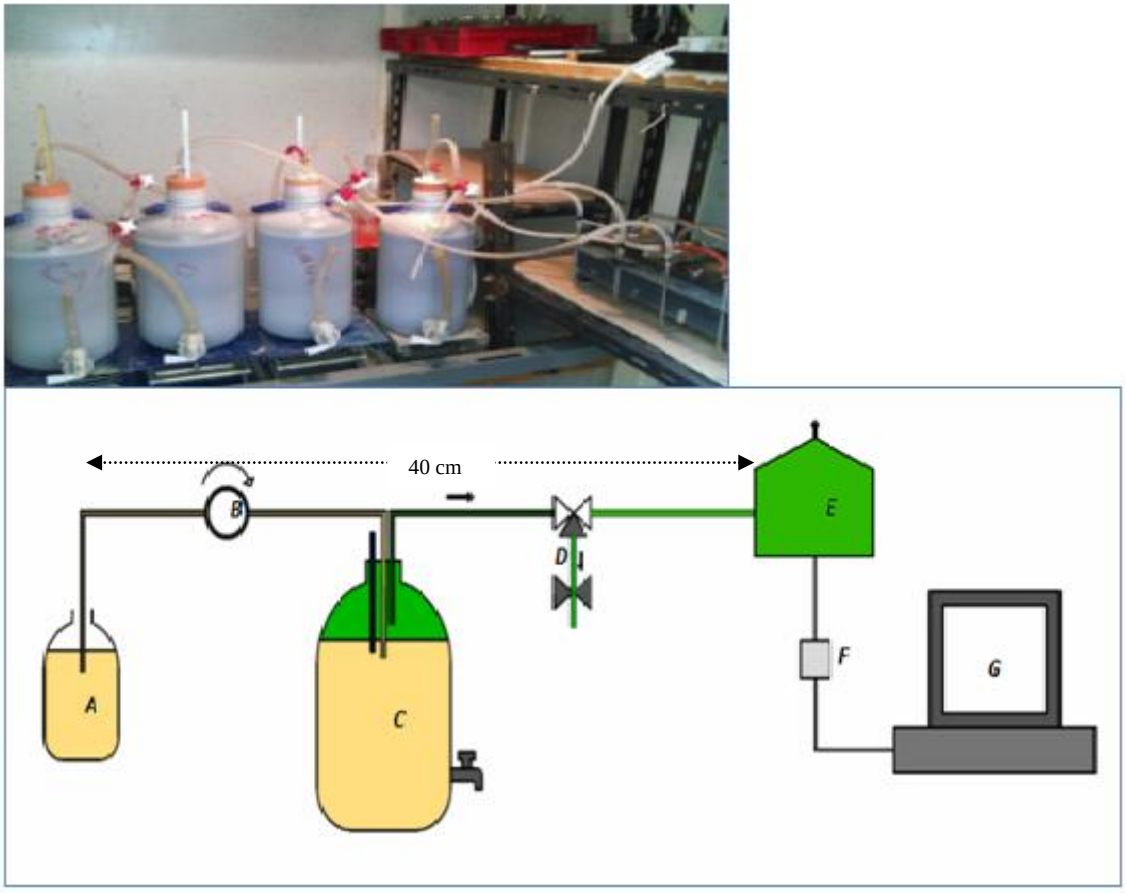


Figure 3.3. Schematic diagram of digesters set up, with photo inserted above.

Schematic diagram explanation;

- *(*A) Acetate tank-for holding acetate solution.
- (B) Pump-for quantitative acetate transfer to digesters.
- (C) Digester-for acetate digestion, note the effluent outlet tap.
- (D) 3 and 2 way flow valve-for directing biogas flow and also a point of percentage methane measurement.
- (E) Biogas counter-recording biogas volume and biogas out let.

- (F) Lab jack-converting analogue to digital data, biogas volume.
- (G) Computer-biogas volume recording.

Table 3.1. Regime used to dose micronutrient to digesters.

Day	Period	Digesters				number of moles of Ni and Co added daily ($X10^{-6}$)
		D1	D2	D3	D4	
0-12	I	0.00	0.00	0.00	0.00	0.00
12-35	II	Ni	Ni&Co	Co	free	2.50
35-60	III	Ni	Ni&Co	Co	free	3.75
60-90	IV	Ni	Ni&Co	Co	free	5.00
90-129	V	Ni	Ni&Co	Co	free	7.50
129-143	VI	Co	free	Ni	Ni	2.50
143-165	VII	Ni&Co	Ni&Co	Ni&Co	Ni&Co	2.50
165-180	VIII	Ni	Ni&Co	Co	free	157.50 then 630.00

All digesters were supplemented with 25.00 μ moles of Fe (II) as $FeCl_2.6H_2O$. Ni was added as $NiCl_2.6H_2O$ and Co was supplied as $CoCl_2.6H_2O$. Desired amounts of Fe, Ni and Co were dosed to the influent at the time of feeding of digesters. Dosing of micronutrients to the stored medium was avoided so as to prevent their possible precipitation no storage.

3.4 Rational for the choice of a Continuous Stirred Tank Reactor

Despite a large part of this research being inspired and informed by the work of Fermoso, (2008) a sime-CSTR is used as opposed to a UASB, or any other reactor. The rational for the use of CSTR is as follow:

(1) Quick turn-around time.

To turn around more that 90% the contents of a CSTR need three times the Hydraulic Retention Time (HRT) of the system. When contrasted with a Sludge Bed Reactor (SBR) which can have Solid Retention Time (SRT) of up to 50 days, a sime-CSTR is a better choice where content turning around is important. This is the case in this research as there is the need

to turn around the contents of the digester so that the background quality of the sludge doesn't influence the downstream performance of digesters, hence affording the opportunity to observe the effect of dosage of micronutrients. The solids of an UASB can be retained for hundreds of days.

(2) The effluent represents contents of the digester.

The effluent of a CSTR represent its contents, as such vital parameters such as pH, solids and metal ions can be measured daily and be related to the health or performance of a digester in real time. This is advantageous as it allows prompt rectification measures to the employed where discrepancies in performance are observed.

(3) Easy of operation.

Overall a CSTR is relatively easy to operate compared to the other digestion systems such as UASB that could have been employed for this study, in addition to this; it is the most common digestion systems available in both academia and industry.

As biogas production data give inference on the quantity and viability of methanogens, the growth kinetics of Methanosarcinales, particularly their maximum specific growth rate (μ_{max}) with relevance to the operation of the sime-CSTR used, mainly the hydraulic retention time were considered in section 3.4.1. Methanosarcinales were considered as they were expected to be the main methane producers.

3.4.1 *The relevance of growth kinetics of Methanosarcinales on the operation of the CSTR used*

The major disadvantage of a CSTR in this research has been its labour intenseness. Given that it was not ideal to examine the effect of micronutrients on biogas production through comparing the biogas production of each digester to itself before and after dosage, i.e., making a retrospective comparism within the same digester; hence a control digester was added. The behaviour of each of the dosed digesters on each day was instead compared to that

of a control digester on the same day. This was important in view of the used hydraulic retention time of 10 days, which is close to the average minimum hydraulic retention time (θ_{min}) of Methanosaeta of 8 days using kinetic parameters given in (Mogens et al., 2008) while parameters in Conklin et al. (2006) gave a θ_{min} of 5 days. This made maintenance of zero rate of change of biomass concentration, especially Methanosaetaceae difficult. The studies in Conklin et al. (2006) were mostly based on digestion of acetate under mesophilic conditions as it was the case in this study.

Table 3.2. Average Kinetic parameters for mesophilic growth of Methanosaeta and Methanosarcina from Conklin et al., (2006)

Parameter	Methanosaeta	Methanosarcina
k (mg COD/mg VSS.d)	10.1 (±16)	12.2 (±5.5)
K_s (mg COD/L)	49 (±19)	280 (±77)
Y (mg VSS/mg COD)	0.019 (±0.002)	0.48 (± 0.032)

The parameters in Table 3.2 were used in Mogens et al. (2008), Conklin et al. (2006) and in this study as explained in Batstone et al. (2002) as follows :

k=Monod maximum specific uptake rate (kg substrate COD kg biomass^{-1} day^{-1})

K_s = Half saturation value (kg substrate COD m^{-3})

Y=Yield of biomass on substrate (Kg biomass kg substrate^{-1} day^{-1})

From Metcalf and Eddy (2003) the value of maximum specific growth rate (μ_{max}) can be calculated from that of *k* and Y as:

$$\mu_{max} = kY \qquad Eq(4)$$

Where: μ_{max} = maximum specific growth rate, d^{-1}.

Chen and Hashimoto (1978) showed that θ_{min} below which washoutof biomass occurs is equal to the reciprocal of μ_{max} as:

$$\theta_{min} = \frac{1}{\mu_{max}} \qquad Eq(5)$$

Knowledge of θ_{min} is important as it informs the digester operator of the maximum dilution rate or optimum dilution (D_{opt}) that is not to be exceeded to avert washout of biomass. Wittrup (2007) and Pirt (1975) presented the estimation of D_{opt} according to:

$$D_{opt} = \mu_{max}\left(1 - \sqrt{\frac{K_s}{K_s + S_o}}\right) \qquad Eq(6)$$

Where: S_o= acetate concentration inside the digester, assuming no usage (S_o= 1.8 g/L).

With the use of equation (6) and the average kinetic parameters of Methanosaeta and Methanosarcina as outlined in Conklin et al. (2006) the optimum dilution rate of 0.203 d^{-1}, which correspond to hydraulic retention time of approximately five days (≈ 5 days as per the computation) was realised. As such the hydraulic retention time that is considerably greater than 5 days was expected to be sufficient for growth of Methanosarcinales.

The process feasibility of a CSTR is very much dependent on the growth rate (μ) of the anaerobic consortia, thus making it fragile in operation (Mogens et al., 2008). Because of the fragile nature of a CSTR and the inability to decouple hydraulic retention time and solid or biomass retention time, it is generally difficult in a CSTR to achieve a state in which the growth of microorganisms is the same across a long period of time i.e. rate of change of microorganisms being zero (Mogens et al., 2008). In anaerobic high rate systems, biomass retention and liquid retention are uncoupled, thus allowing obtainment of high biomass concentration while maintaining long SRT and relatively short HRT; this allows the application of high COD loadings (Mogens et al., 2008). The ability to separately control the HRT and SRT allow the operator of a high rate anaerobic digestion facility to maintain a desired solid or biomass concentration over a long time; hence the performance of the digester over this period can more easily be retrospectively compared. The Anaerobic Contact Process (ACP) and the Anaerobic Sludge Bed Reactors (ASBR), particularly the Upflow Anaerobic

Sludge Bed Reactor (USAB) are examples of those digesters that afford the operator an opportunity to separately control SRT and θ (Mogens et al., 2008) thus easily allowing maintenance of constant biomass concentration.

3.5 Biogas production from acetate digestion in 120 ml serum bottles (part 2)

On days show in Table 3.3, the effluent from each digester was used to conduct biogas production assay at various micronutrients concentrations, also shown in Table 3.3. On these days, 600.0 ml of a well-mixed effluent (instead of 500.0 ml) was obtained from each digester and their biogas production assay with acetate as the substrate at various metal ion concentrations was conducted in triplicates. 100.0 ml of the effluent from each digester was poured into a serum bottle and a butyl rubber stopper placed on (without the vial seal); this was allowed to settle (decant) for about an hour. After a clear supernatant was visible, 80.0 ml of the supernatant was carefully poured out of the serum bottle leaving a residue of 20.0 ml. 20.0 ml of a 300 mM acetate solution was poured into the bottle followed by 60.0 ml of Reverse Osmosis treated water (RO water). This made the final acetate concentration in the serum bottle to be at least 60.0 mM. In this serum bottle a specific metal ion or ion combination was dosed to a desired concentration. No alteration in terms of micronutrients addition was done to the control serum bottle. The above procedure was carried out in triplicates. The average cumulative biogas volume produced in the first four days was taken to represent the Biogas Production Potential (BPP) of each digester at the time of sample obtainment. This was because the highest amount of biogas was produced in the first four days of digestion in serum bottles. The percentage of methane produced was also measured daily.

Table 3.3. Days of incubation of serum bottles at various metal ion concentrations

Day of start of incubation and effluent obtainment	Total metal ion concentration (µM) corrected to (Ni, Co and Ni and Co), (excluding the background conc).
75	504.00
95	126.00
109	63.00
113	31.50
122	15.75

The volume of biogas produced was measured daily using a manometer made of two burettes connected by a silicon rubber tube, Figure 3.4. Piercing of the butyl rubber which was held firmly in place by an aluminium vial seal freed the biogas which in turn displaced water in the manometer, thereby giving a reading of gas volume. This method is described and was used by Ishaq (2012). Note that the manometer measures pressure, which is reflected as volume in millilitres (ml).

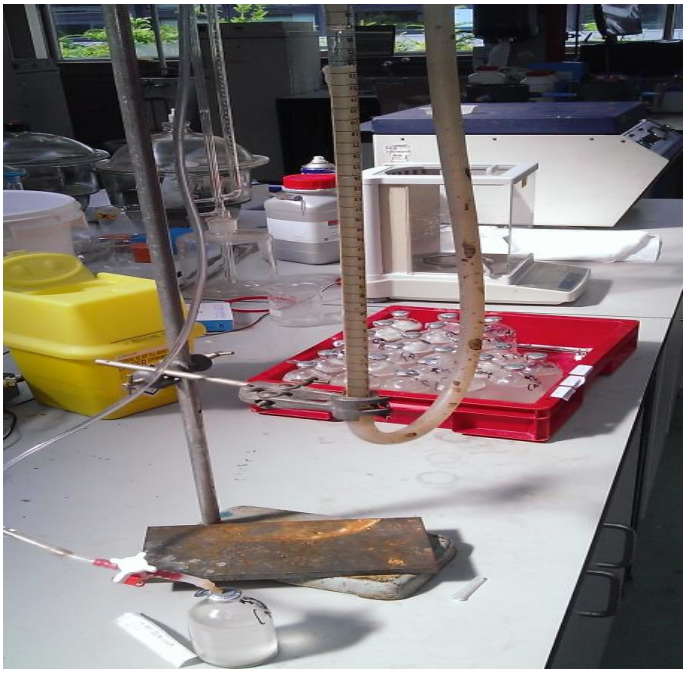


Figure 3.4 Measuring gas volume using manometer.

3.6 Equipment and instruments calibration and optimisation.

The four (4) digesters including connection silicon tubes (Figure 3.3) from VWR, UK were checked for leakage by immersing each one of them in a bucket full of water then pumping air in the digesters using Rana Air (200) fish burble pump. No bubbles formed from any of the four digesters or silicone tubes, thus indicating no visible leakage. The most permeable form of silicone, poly-dimethylsiloxane (PDMS) is reported to have permeability in the range of 1000 Barriers by several researchers (Beeskow-Strauch et al., 2015, Raharjo et al., 2007, Merkel et al., 2000). This permeability was observed by Merkel et al. (2000) at 35 °C and at pressures varying from 1 atmosphere to above 15 atmospheres (atm). The unit of permeability, the Barrer (Stern, 1968) is defined by:

$$[Barrer] = \left[1 \times 10^{-10} \frac{[cm^3]\,(STP) * [cm]}{[cm^2] * [s] * [cm\,Hg]}\right] \quad (7)$$

The permeability of materials can also be expressed under various operation conditions of pressure and temperature. Equation (7) shows that the rate of gas permeation through a membrane, silicone in this case, is directly proportional to the gas permeability coefficient or permeability, the membrane surface area through which the gas permeate, the trans-membrane gas partial pressure difference and is inversely proportional to the thickness of the membrane in use. The partial pressure of an individual gas in a mixture of gases can be calculated from Dalton's gas law of partial pressures as:

$$P_i = P_{total} * \chi_i \quad (8)$$

Where: P_i= mole fraction of the i^{th} gas,

P_{total} =the total pressure of the mixture of gases

χ_i =the mole fraction of the i^{th} component in the total mixture of *n* components.

Assuming that the pressure in silicone tubes used was constant at 1 atm, (considering the small volume of 35.4±2 ml needed to cause tipping of each gas counter trough) the partial

pressure of methane inside the tubes can be estimated using equation 8, since the mole fraction of methane can be obtained from the percentage of methane in biogas which was 64.0±1%. The connection tubes used had an internal diameter (ID) of 5.00 mm, wall thickness of 1.03 mm and the longest tube had a length of 40.00 cm, and this was used to connect D1 to the gas counter as it was the furthest. Using equation (8) and assuming an internal tube pressure of 1 atm i.e. 760 mm Hg, the partial pressure of methane was calculated as 486.4 mm Hg. Assuming the silicon tubes used had a permeability of 1000 Barrers and using the details of the longest tube used, a daily methane outflow rate of ($2.96*10^{-3}$) cm^3/s, or 255.60 cm^3/d was found. This represents 7% of the maximum amount of methane gas that can be obtained when a 100% of 9.0 g of acetic acid is converted to methane gas and carbon dioxide at 37 °C. Since all digesters were connected by the same silicon tubes and methane permeation at maximum biogas production can be only 7%, it is not expected that biogas leakage would cause discrepancy in interpretation and drawing of conclusions from biogas data or experimental results.

3.6.1 *Calibration of Gas counters.*

A four cell gas counter (Figure 3.5) was used to measure the volume of biogas produced. It was composed of four separate troughs immersed in water in each cell (Figure 3.5*b*). These troughs were connected by a circuit bearing a two-way switch through a four pin plug (obtained from Maples, UK). The circuit was in turn connected to a data logger, U3 Lab jack. Biogas produced by each digester was conveyed through a tube to a specific cell in the gas counter. The pressure of biogas caused tipping of troughs, this tipping of troughs in turn complete the circuit at the two-way switch point, which was recorded by the data logger and stored by the computer.

The gas counter was calibrated by pumping air using Rana Air (200) fish burble pump through each of its cells then collecting it (air) at the exit end and recording the volume using

an inverted measuring cylinder, displacement method. The number of tips (counts) was recorded by the computer connected to a lab Jack. Then the volume (ml) was calculated as a product of the number of tips or counts and unit volume (ml) needed to cause each tip. Each cell was calibrated in triplicate then an average was taken as a representative volume. The gas counters' cells were then harmonised. This was done so that the same unit gas volume can cause tipping in each of the cells. Harmonisation of the gas counter cells was done by connecting two cells in series then pumping air through the first cell, then directing the exiting air from the first cell to the entrance of the second cell. The troughs were then adjusted so that they tip almost simultaneously. The third cell was then harmonised with the second, then the fourth with the third in that order. This produced a scenario whereby a standard or fixed volume of biogas caused tipping in all four cells of the gas counter, hence allowing performance, biogas production, of digesters connected to each of them to be compared easily.

Harmony of the cells was checked by pumping air through each cell individually using a pre-calibrated Rana Air (200) fish burble pump, for a fixed time period, after which all gas cells recorded the same number of tips. Each tip was found to be caused by 35.40±2 ml. The fish burble pump's flow rate was calibrated by recording the amount of water it displaces from an inverted measuring cylinder over a fixed period of time.

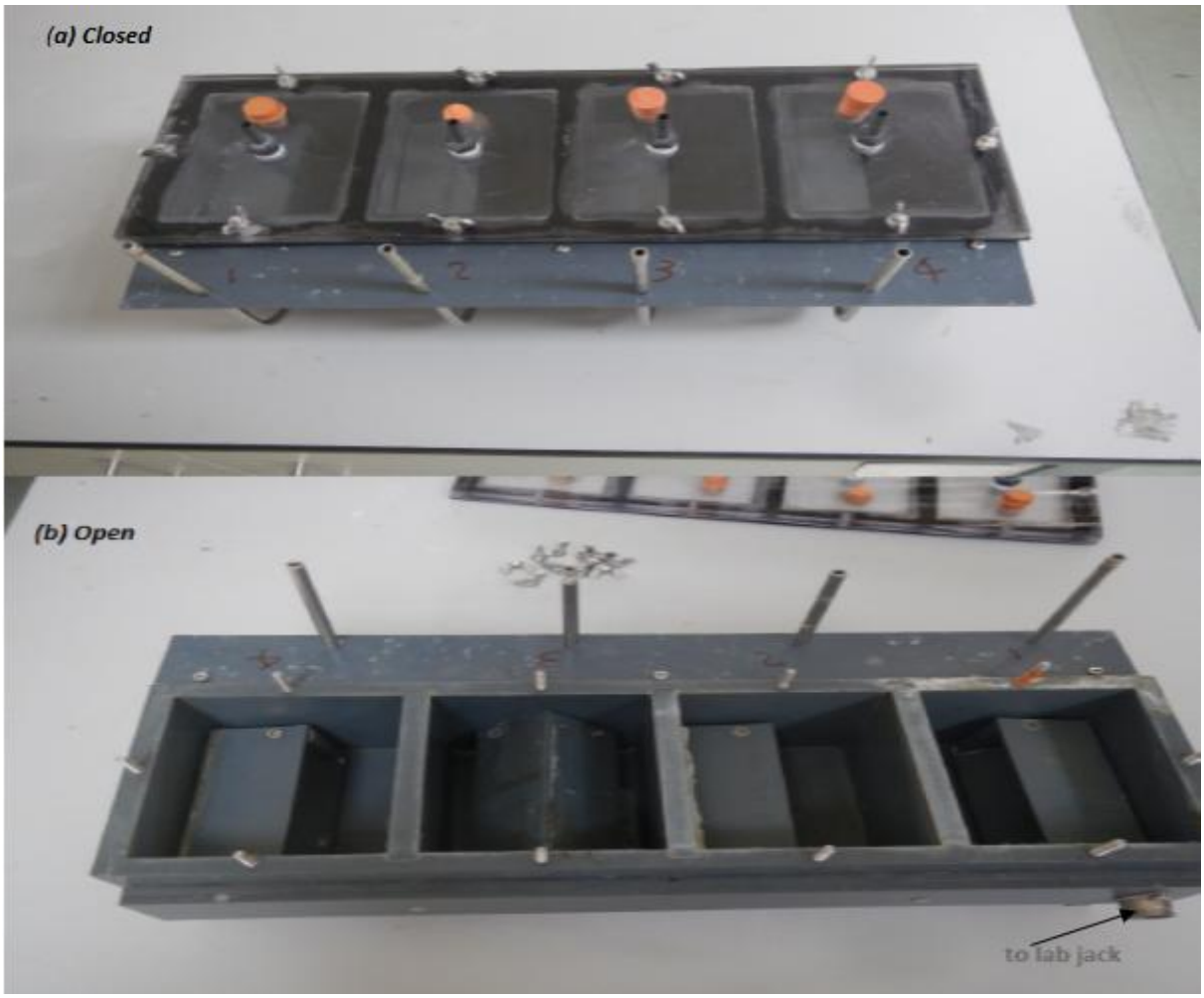


Figure 3.5. Picture of gas counters used, (a) gas counter closed, (b) gas counters open with cell 3 in tipping position.

3.6.2 *Calibration of the Gas Meter used for measuring percentage of methane in biogas*

The percentage of methane in biogas was determined using a gas meter, supplied by Gas Measuring Instrument (Ltd), Scotland. The gas meter used for measuring percentage methane in biogas was calibrated by drawing to full capacity of the micro syringe 1.00 ml of ultra-high purity (N5.5) methane, then removing a desired volume of the drawn methane followed by drawing into the syringe to full capacity (1.00 ml) of air from the laboratory. This air was designated ‘dry’ air. The whole 1.00 ml made of a mixture of pure methane and air was then emptied into the gas meter and the percentage methane content was read from the meter. The percentage of methane read from the gas meter was plotted against the known percentage of methane injected into the gas counter, thus giving a calibration graph of the gas meter, Figure

3.6. This calibration was done from 0% with a 25% step increase in methane content in the syringe up to 100%, Figure 3.6. In cognisance of the water vapour content of biogas, the same calibration was done with replacement of 'dry' air with air drawn over a steaming water bath. This air was designated 'wet' air. This was done to cater for the possible effects of moisture or water vapour on the calibration. Figure 3.6 shows that the wet air gave readings indicating 3% lower methane content than the dry air reading. This is within the acceptable margin of error, more so that not much variation in methane content was expected from acetate digestion. The calibration obtained using 'wet' air was henceforth employed for all the percentage methane readings. The methane gas used for this calibration was obtained from BOC plc, UK.

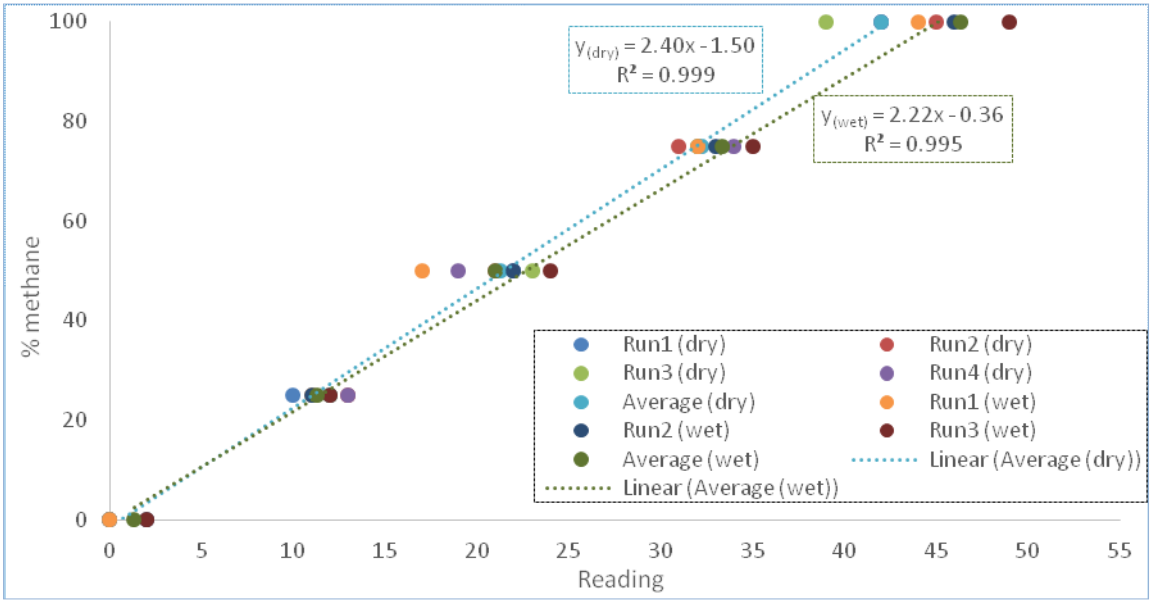


Figure 3.6. Results of gas meter calibration with 'dry' and 'wet' air.

3.7 Analytical measurement of important parameters

It is important to determine various parameters as they are both indicative and reflective of the extent of biological reactions occurring in a digester. These parameters can be physical or

microbial in nature. Although these are presented separately, they are interdependent and influence each other in nature or digester.

3.7.1 *Physical parameters measurement*

A variety of physical conditions of a digester affect its performance or biogas production. Some of the physical parameters measured are outlined below.

Dissolved oxygen (DO), pH, total and volatile solids were measured according to procedures outlined in Eaton et al. (2005). Dissolved oxygen (DO) was measured using Hanna Instrument- DO meter and probe (H1 9146) while pH was measured using Mettler-Toledo pH meter and probe. Sludge metal content was measured using Graphite Furnace Atomic Absorption Spectroscopy (GFAAS).

3.7.1.1 Total and volatile solids measurements

20-40 ml of each digester's effluent was used to measure total and volatile solids content of the reactor according to Eaton et al. (2005). Nickel tins were washed with tap water, tissue tried then dried in the furnace at 505°C for at least one hour. Tins were then cooled in a desiccator for about 1-2 hours, then weighed to obtain their empty mass (Tmass). A known volume (V) of the effluent was poured into a prior weighed tin then heated at 105°C for at least 6 hours (overnight) in the oven. The tins now containing total solids were weighed again (after cooling for 2 hours in a desiccator) to obtain the mass of tin plus that of total solids (Tin + TS) mass. The tins containing total solids were then placed in a furnace at 505°C to burn off the volatile fraction of the total solids for at least 6 hours (overnight). The tins now containing fixed solids were cooled in a desiccator for 2-3 hours, after which their mass was measured together with that of fixed solids (Tin + fixed solids). The digesters' solids content were then calculated as follows:

- Total solids (g/ml) were calculated as; $\frac{(\mathrm{Tin}+\mathrm{Ts})-(\mathrm{Tmass})}{\text{sample Volume}} = \mathrm{TS}$

- Total volatile solids (g/ml) were calculated as; $\frac{(\text{Tin}+\text{TS})-(\text{Tin}+\text{fixed solids})}{\text{sample volume}} = \text{Vs}$

3.7.1.2 Concentration of acetate in effluent

The percentage of acetate in the effluent was measured using High Performance Liquid Chromatography (HPLC) specifically anion HPLC. This gave an indication of what proportion of the acetate in a digester was utilised. Samples were prepared for HPLC analysis by filtration using a 540 whatman filter paper followed by centrifugation to clarity (13000xg, 20°C, 4 mins). The supernatant was collected using vials (approximately 5.0 ml). The anion HPLC separation method and detection used for VFA/acetate measurement was developed by Redwood and Macaskie (2006) and is presented in Table 3.4.

Table 3.4. Conditions of anion chromatography (Redwood and Macaskie, 2006)

Columns	IonPac AS11-HC analytical (4mm) IonPac AG11-HC analytical (4mm)				
Eluent	A deionised water(18MΩ.cm^{1}) B 5 mM NaOH C 100 mM NaOH D 250mM NAOH				
Gradient (%)	Time (min)	A	B	C	D
	−2	60	40	0	0
	0 (inj.)	60	40	0	0
	1	88	12	0	0
	10	88	12	0	0
	20	80	0	20	0
	23	20	0	0	80
	24	20	0	0	80
	24.01	60	40	0	0
	27	60	40	0	0
Flow rate	15 ml/min				
Inj. volume	15µL				
Detection	Suppressed conductivity ,(ASRS) Auto suppression recycle mode				

3.7.1.3 Measurement of sludge metal content

The amount of Ni and Co in seed sludge was measured before and after each dosage period. This was done to establish the background concentration of both Ni and Co in addition to examining the washoutand increase of these ions in those digesters in which they were not dosed or dosed respectively. With knowledge of the evolution of Ni and Co with time, the performance of each digester could be linked to its metal ion content during each dosage period; thus allowing the evaluation of the effect of each dosage (concentration) in terms of biogas production and other important parameters such as microbial evolution. With knowledge of the background concentration of both Ni and Co in the seed sludge and the feeding regime used, their theoretical concentration evolution could be calculated; as such their measured and calculated concentrations complemented each other in evaluating the performance of digesters under their different dosages, hence concentrations.

3.7.1.3.1 Sample preparation

0.5-0.8 g equivalence of total solids of sludge (approximately 20.0 ml) was digested using 8.0 ml of aqua-regia composed of (3:1) V/V of analytical grade HCl/HNO_3. This solution was then heated over a hot plate to reduce its volume to approximately 10.0 ml; then it was allowed to cool to room temperate, after which 30.0 ml of Reverse Osmosis (RO) treated water was added. Another 8.0 ml 3:1 anar grad HCl/HNO_3 (V/V) was then added for the second phase of digestion, followed by evaporation of the solution to approximately 10.0 ml over a hot plate then cooling to room temperature. After cooling, the solution was filtered through 540 whatman filter paper, then re-filtered using a 0.45 µm Polytetrafluoroethylene (PTFE) filter disc into a 50.0 ml volumetric flask, then made up to the mark using RO water. The Ni and Co content of this solution was then determined using Graphite Furnace Atomic Absorption Spectroscopy (GFAAS).The above sample preparation method (with exclusion of

micro filtration) is described in Standard Methods (APHA/AWWA/WEF, 1998) and was employed by Roussel (2013).

3.7.1.3.2 GFAAS operation

For metal analysis a 280 Perkin Elmer Graphite Furnace Atomic Absorption Spectroscopy was used. This machine was equipped with an auto-sampler that dispenses a standard volume (20 µL) of the sample to the furnace for atomisation then quantification. Using purchased standards of Ni and Co (from Sigma Aldrich, UK) the machine was able to automatically generate a calibration graph within a range of 1.00-25.00 ppb for both Ni and Co. Extracted samples (3.7.1.3.1) were placed in vials and analysed according to a program in Table 3.5. Metal concentration measurements were made in triplicates and an average taken automatically by the machine. The average values with a standard deviation of more than 2% of the average were discarded. Samples anticipated to have the lowest concentration of the metal ions of interest were analysed first followed by those anticipated to have a higher concentration, e.g. Ni in D4 was analysed first in a sample obtained on day 129, with the sample obtained on day 12 being the last, while in D2 the converse was true. This was done to reduce chances of contamination between samples obtained of different days as a blank made of Milli-Q water of conductivity 18.2 MΩ cm^{-1} was measured between samples obtained from the same digester on different days. The metal content of the Milli-Q water was constantly below detection. Milli-Q water that was acid digested in the same way as samples was read/analysed between analyses of samples obtained from different digesters, e.g. before changing samples from those of D1 to D2 acid digested Milli-Q water was read. This prevented further chances of cross contamination of samples between digesters.

Prior to atomisation of the analyte, after aspiration the solvents were dried off by heating to 110°C for 20 seconds followed by a 5s temperature ramp to 130°C for 30 seconds. Drying of

the solvent in GFAAS helps to reduce possible interference of the solvent as well as its vapours with analyte atoms or the beam.

Table 3.5. Program used for the operation of GFAAS

Analyte	Excitation wave length(nm)	Atomization Temp °C	Atomization time (s)
Ni	232.0	2300	5
Co	242.5	2400	4

3.7.2 *Microbial parameters measurement*

Parameters related to both identification and quantification of methanogens were assessed so as to relate biogas production to microbial population abundance or dynamics.

3.7.2.1 Imagery

In order to narrow methanogens search, samples obtained on various days were viewed using a Philips XL-30 Environmental Scanning Electron Microscope (ESEM)-FEG to determine morphology of microorganisms in them. Effluent samples were treated with a thin coating of gold in a vacuum chamber to increase their conductivity. A thin coating was used as it results in better scans while preserving morphological integrity of the organisms. Samples were then fixed on a brass disc using a carbon tape. Both carbon or gold coating and sticky carbon tape help to suppress accumulation of surface charge on samples during analysis. Samples were then placed into a microscope vacuum chamber stage/platform and viewed at various magnifications.

The following are preparatory steps for sample viewing using Scanning Electron Microscope (SEM) as used in Metallurgy and Materials, University of Birmingham.

Step 1: Chemical Fixation

- Primary fixation -2.5% Glutaraldehyde in 0.1M Phosphate buffer for 1 hour.

Step 2: Dehydration

- 50% Alcohol - 2 x 15 mins

- 70% Alcohol - 2 x 15 mins
- 90%Alcohol - 2 x 15 mins
- 100% Alcohol - 2 x 15 mins

Step 3: Critical Point Dry

- Alcohol is replaced with liquid CO_2 and then heated up to the critical point to dry the sample.

Step 4: Mount sample on a stub and coat with Gold or Platinum, ready for Viewing.

3.7.2.2 Identification and quantification of methanogens

Identification and quantification of methanogens in each digester was carried out in collaboration with National University of Ireland, Galway (School of Natural Sciences-Microbial Eco-physiology and Eco-Engineering Laboratory) under the supervision of Drs Estefania Porca and Gavin Collins. This was conducted using quantitative Polymerize Chain Reaction (qPCR). Figure 3.7 and Figure 3.8 show the appearance of samples at the time of sampling and at the time of analysis, respectively. Worth noting is the change in colour of the effluent from blackish to whitish as the digester contents are turned over with time in all digesters.

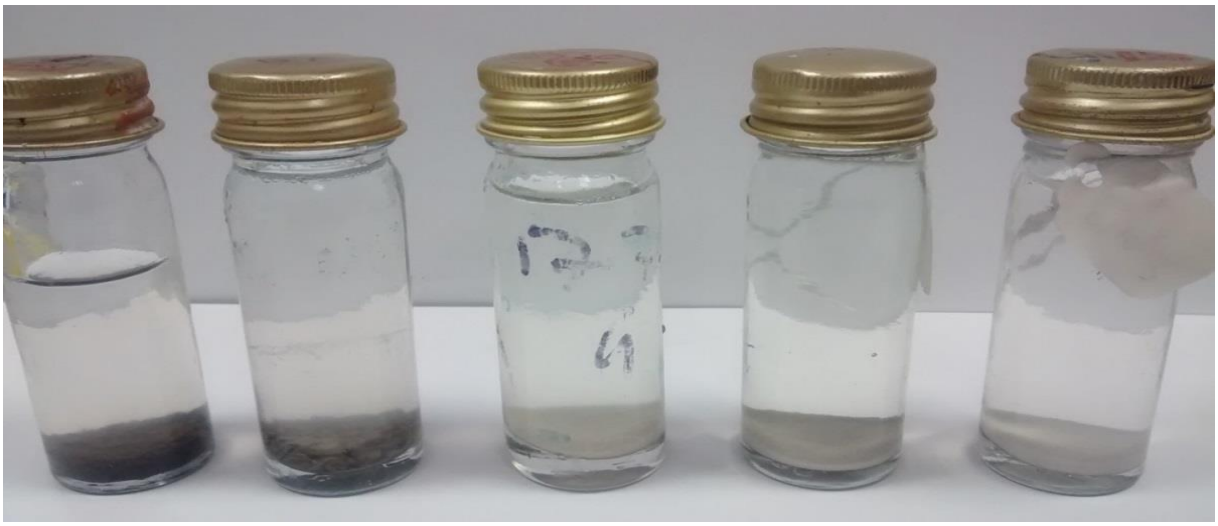


Figure 3.7. Effluent samples just after sampling from D1. L-R, day: 7, 22, 77,122, &158; (note absence of day 129)

Figure 3.8. Effluent samples after thawing ready for analysis from D2. L-R, day: 7, 22, 77,122, 129 &158.

3.7.2.2.1 Genomic DNA extraction

All samples were stored at -20°C until they were processed. The samples were defrosted at room temperature and left to settle for 1 hour. Given the good settling characteristics of the sludge or biomass (Figure 3.7 and Figure 3.8) no prior centrifugation was done to obtain a sludge pellet to use in DNA extraction. After sludge settlement and decantation of the supernatant, 0.05 g equivalence of VS of biomass (or sludge) was weighed and used in a fully automated nucleic acid extractor employing magnetic bead technology (Maxwell 16 DNA Purification Kits; Promega) to extract and purify genomic DNA following manufacturer´s instructions. The DNA was stored at -20°C. The DNA concentration of each sample was measured using the Qubit dsDNA (Invitrogen). These data were used for further calculations, for examples in DNA yield i.e. the DNA obtained or extracted from specific quantity of biomass.

3.7.2.2.1.1 DNA quantification through (q) PCR

All qPCR reactions were performed using 20 µl reaction capillary tubes with the LightCycler 480 Probe Master (Roche Diagnostics). Each capillary tube was separately loaded with 2 µl of DNA diluted 1:10, followed by addition of: 10 µl of Taqman Mastermix (Roche Diagnostics); 1 µl of each of the forward and reverse primers (10 µM); 1 µl of the TaqMan probe corresponding to each primer and probe set (10 µM); and PCR-grade sterile water to a final

volume of 20 µl. A control without the corresponding DNA template was included in every assay for each primer and probe set. All experiments were duplicated (see Table 3.6). A two-step amplification of the target DNA was performed applying the following conditions: an initial incubation at 94°C for 10 min, 45 cycles of denaturation at 94°C for 10 s and simultaneous annealing and extension at 60°C for 30 s, while the annealing and extension for the *Methanomicrobiales*-specific set was performed at 63°C. The transition rate was 20°C/s for all segments in the two-step cycling. The standard curves were performed following the method described byYu et al. (2006).

Table 3.6. Specific primer and probe sets used to detect methanogenic bacteria.

Primer and probes names	Function	Target group	Sequence (5 →3´)	Amplicon size (bp)
MBT857F	F primer		CGWAG GGAAG CTGTT AAGT	
MBT929F	TaqMan	*Methanobacteriales*	AGCAC CACAA CGCGT GGA	343
MBT1196R	R primer		TACCG TCGTC CACTC CTT	
MMB282F	F primer		ATCGR TACGG GTTGT GGG	
MMB749F	TaqMan	*Methanomicrobiales*	TYCGA CAGTG AGGRA CGAAA GCTG	506
MMB832R	R primer		CACCT AACGC RCATH GTTTA C	
MCC495F	F primer		TAAGGGCTGGGCAAGT	
MCC686F	TaqMan	*Methanococcales*	TAGCGGTGRAATGYGTTGATCC	337
MCC832R	R primer		CACCTAGTYCGCARAGTTTA	
Msc380F	F primer		GAAAC CGYGA TAAGG GGA	
Msc492F	TaqMan	*Methanosarcinaceae*	TTAGC AAGGG CCGGG CAA	408
Msc828R	R primer		TAGCG ARCAT CGTTT ACG	
Mst702F	F primer		TAATC CTYGA RGGAC CACCA	
Mst753F	TaqMan	*Methanosaetaceae*	ACGGC AAGGG ACGAA AGCTA GG	164
Mst862R	R primer		CCTAC GGCAC CRACM AC	

3.8 Theoretical methane production from acetic acid

The amount of methane, hence biogas, that could be obtained from conversion of acetic acid was estimated (section 3.8). This is important for comparing periodic biogas production data to theoretical values and drawing inference on the overall health of the digesters.

The amount of methane obtainable from complete oxidation of an organic substrate can be estimated from knowledge of the Chemical Oxygen Demand (COD) of the substrate digested. This is important as COD represents the most important parameter in waste stabilisation, more especially industrial wastewaters (Mogens et al., 2008). From Mogens et al. (2008) the COD of an organic compound of the form $C_nH_aO_b$ can be calculated through:

$$COD_t = \frac{8(4n+a-2b)}{12n+a+16b}\ \mathrm{gCOD/gC_nH_aO_b} \qquad Eq(9)$$

With the use of equation (9), 1.0 gram of acetic acid corresponds to a COD of 1.067 grams.

If the compound of the form $C_nH_aO_b$ is completely biodegradable and is 100% converted to methane and carbon dioxide (with no sludge yield) the theoretical amount of methane and carbon dioxide can be estimated via the Buswell equation; after Buswell and Mueller (1952) as:

$$\mathrm{C_nH_aO_b} + (\mathrm{n} - \mathrm{a}/4 - \mathrm{b}/2)\mathrm{H_2O} \rightarrow \left(\frac{\mathrm{n}}{2} - \frac{\mathrm{a}}{8} + \mathrm{b}/4\right)\mathrm{CO_2} + \left(\frac{\mathrm{n}}{2} + \frac{\mathrm{a}}{8} - \mathrm{b}/4\right)\mathrm{CH_4} \qquad Eq(10)$$

The complete oxidation of one mole of methane gas requires two moles of oxygen, which theoretically translates to 1 kg of COD being converted to 0.35 m^3 of CH_4 at STP (Mogens et al., 2008). With the use of the ideal gas law:

$$\mathrm{PV} = \mathrm{nRT} \qquad Eq(11)$$

Where: P = pressure (atm)

V= gas volume (m^3)

n = number of gas moles

R = universal gas constant, 0.082057 L atm K^{-1} mol^{-1}

T = Temperature in °K

and equation (10) , the theoretical amount of methane (L) expected at 37°C from 9.0 grams of acetate fed per day was calculated to be 0.43 L g acetate $fed^{-1}day^{-1}$. It is important to recognise that this value is only theoretical and assumes that all of the daily fed acetate would be converted to biogas with no sludge yield; hence this value over estimate the amount of methane that can be produced. A considerable amount of the produced methane will also dissolve in both the digester water and the gas counter trough water (Walker et al., 2009).

CHAPTER 4. RESULTS AND DISCUSSION

This chapter presents experimental findings, their interpretation and discussion as well as their implication in this study as a whole. A recast of the aim of the study is provided to aid the reader in connecting it to the findings of the study.

The primary aim of this research was to investigate and define the deficiency, stimulatory, excess and toxic concentration ranges for individual and combined dosage of nickel (Ni) and cobalt (Co) to anaerobic acetate digesters at mesophilic temperatures (37±2 °C). As a subset of this aim, the effect of co-dosage of Ni and Co was also investigated. In order to carry out this investigation, four identical 5.0 L anaerobic acetate fed digesters were ran as explained in Materials and Methods chapter (3.2). These digesters were identified as D1, D2, D3 and D4; and were dosed incremental amount of either: Ni, Ni&Co, Co and no Co or Ni, respectively. i.e. D1 (Ni), D2 (Ni &Co), D3 (Co) and D4 (no Co or Ni), as shown in Table 3.1. The overall goal of this experimental regime was to develop data to populate regions A to D of a dose response curve for Co alone, Ni alone and Co and Ni in combination, Figure 1.2.

- This chapter is divided into two parts:

  PART ONE

  - Performance of digesters before their individual and combined dosage with Co and Ni (*Region A*).
  - Performance of digesters at stimulatory concentration of individual Co and Ni as well as that of their combination (*Region B*).

  PART TWO

  - Specific activity (assed in serum bottles) and performance of digesters at toxic concentrations of individual Co and Ni as well as that of their combination (*Region D*).

- Deduction of excess concentrations of individual Co and Ni, as well as that of their combination (*Region C).*
- Analytical measurements (presentation of physical and microbial parameters that affected digesters performance).

4.1 PART ONE

This part discusses performance of digesters at limiting concentration or less accessible concentration (*Region A*) of individual and a combination of Ni and Co, as well as performance of digesters at stimulatory concentration (*Region B*). At all dosages, biogas produced by digesters was recorded cumulatively in 10 minutes intervals using a data logger, this produced 144 data points per day, and these were summed up to produce daily biogas volume. Performance of digesters is discussed mainly in terms of daily cumulative volume of biogas. This gave the opportunity to compare overall biogas (hence methane) production across different digesters in a period in which they were dosed different micronutrients, e.g. (D1 compared to D4). This is also in line with the practical operation of anaerobic digesters as methane is cumulatively stored before it is used in a CHP plant so as to allow for consistent supply. Because the percentage of methane in biogas was found to be constant and also digesters were daily fed the same amount of acetate for most of the experimental period, a change in biogas production would also reflect a change in methane volume. Data of methane produced per gram of acetate fed per day is mainly used to compare to theoretical volumes of methane production from acetate, thus giving an indication of the efficiency of the digestion system employed. The percentage of methane in biogas was found to be constant at 64±1% throughout the experimentation period and across all digesters.

4.1.1 *Performance of digesters before dosage (Region A)*

This part examines performance of digesters before they were differentiated through their dosage of various micronutrients, this represents a region of limiting or non-bioavailable Ni and Co to microorganisms. Figure 4.1 shows that prior to differentiation of the digesters through their dosage with respective micronutrients, their biogas output was similar with a maximum range of 10%, being D2 above D4. Twelve days, which is 1.2 times the hydraulic retention time of the system was viewed as enough to examine similarity in performance of digesters prior to their differentiation, in addition to their previously viewed similarity in biogas production during the start up period (section 3.2.1). The reduction of Ni and Co with digestion operation time, up to almost zero expected after 3xHRT in D4, can also be used to examine biogas production at low Ni and Co concentrations, this can provide data for examination of region 'A' of the dose response curve when compared to the dosed digesters. The average measured Ni concentration across all digesters on day 12 was 2.427 µM ± (0.371) with a variance of (2%) on the mean. On the other hand the average measured Co concentration across all digesters at the same time was 1.145µM (±0.183) with a variance of 3% on the mean. In view of this low variability of Ni and Co concentration across digesters, the observed slight variation in performance of these digesters before their dosage cannot be attributed to the difference in their Ni and Co concentration by day 12; more so that each digester was seeded with the same seed sludge from the same container. Generally the concentration of both Ni and Co measured progressively with digester operation time in D4 are much higher than those calculated based on their background concentration in the seed sludge. The GFAAS used had a narrow linear range of 1 ppb-25 ppb this necessitated several dilutions of the samples before measurement in order to bring the analyte into the instrument's linear range of detection. These dilutions could have introduced uncertainties in the measurement of both Ni and Co. The difference in sensitivities of Ni and Co measurement

by GFAAS observed by Nollet and De Gelder (2000) could have led to the perceived higher reduction rate of measured Co concentration as compared to Ni. After 30 days of operation of digesters, the concentration of Ni and Co is anticipated to asymptote towards zero in those digesters that were omitted of either or both Ni and Co, e.g. D4.

Visual MIINTEQ simulation results, in appendix B, show that 10 % more Co in solution is anticipated to be in ion form (Co^{2+}) as compared to Ni at a pH of 7.5. Gustavsson (2012) observed a higher bioavailability of Co as compared to Ni in grain stillage digesters despite these digesters having contained high amount of SO_4^{2-} (0.8-1.1 g/L) and the inherent high interfering matrix of grain stillage. In view of this, the bioavailability of micronutrients can influence the amount of dosage that have to be administered before increase in biogas production is observed.

In contrast to grain stillage digesters, the average concentration of SO_4^{2-} across all acetate digesters used in this research was 0.576 mg/L or 0.006 mM (Table 4.2) which is more than a thousand times less than the concentration in Gustavsson (2012)'s grain stillage digesters. This implies that it is more plausible that there will be more ions in solution in an acetate digester compared to a grain stillage one; hence a lower dosage could be effective in an acetate digester.

4.2 Performance of Digesters at a daily dosage of 2.50 micro moles of their respective micronutrients

Biogas production from digesters following dosage of their respective micronutrients is shown in Figure 4.1(a). On day 12, 2.50 μ moles of Ni, Co and Ni and Co were daily dosed to D1, D2 and D3 respectively while D4 was not dosed. This is shown in chapter 3. This dosage

was chosen as a concentration of 0.5 µM of Ni had been shown to be catalytic by Fermoso (2008) during anaerobic digestion of methanol in UASB digesters.

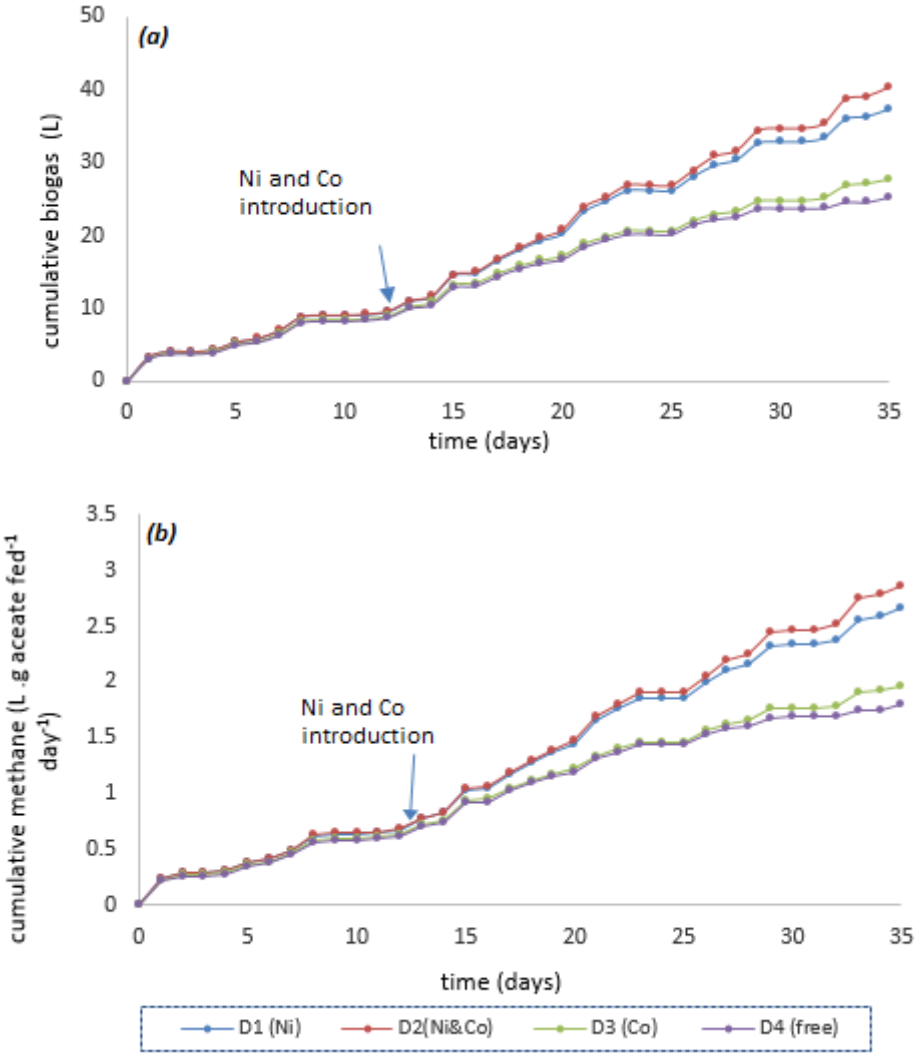


Figure 4.1 Digesters' performance before and after daily supplementation with 2.50 micro moles of their respective micronutrients. (*a*=cumulative biogas volume, *b*= cumulative methane L .g acetate fed^{-1} day^{-1}).

From Figure 4.1(a), three days after initial supplementation with 2.50 µ moles of micronutrients, D1 and D2 produced the same amount of biogas, but outperformed D3 and D4 by 1.0 L which also produced the same amount of biogas between themselves, i.e. (D1=D2) > (D3=D4). From day 15 to day 22, D1 and D2 produced the same volume of biogas (25.0 L each) during the same period; D3 and D4 also produced the same amount of biogas volume between themselves, 17 L each. Thirteen days (day 25) after initial dosage of micronutrients,

D2 exceeded D1 and D3 exceeded D4 by the same biogas volume, 3.0 L, i.e. D2 > D1 and D3>D4, Figure 4.1(a). At the end of the 2.5 μ moles daily supplementation period, D1, D2 and D3 had produced 68%, 85% and 13 % more biogas than D4. The sum of percentages by which D1 and D3 exceeded D4 is 81 %, which is close to D2's percentage exceedance of D4, (86%).

It is worth a reminder at this point that biogas production from digesters should not be compared retrospectively, but should be compared with reference to that of D4 (Control Digester). This is because it is not easy to maintain a steady state (zero rate of change) of methanogens with time under the used operation condition.

Figure 4.2 shows the average five day methane production from digesters. After dosage of micronutrients, D1 and D2 showed increase in methane or biogas production for the first 3 five day averages, then a decline was observed. No increase in methane or biogas production was observed from D2 and D4, a decline in methane production was observed on day 25 in all digesters. The observed average five day methane production per gram of acetate fed per day is significantly less than the theoretically expected one (more than 21 times less). This is possibly because not all of the fed acetate was converted to methane daily, as well as some being used for microbial growth and cell maintenance.

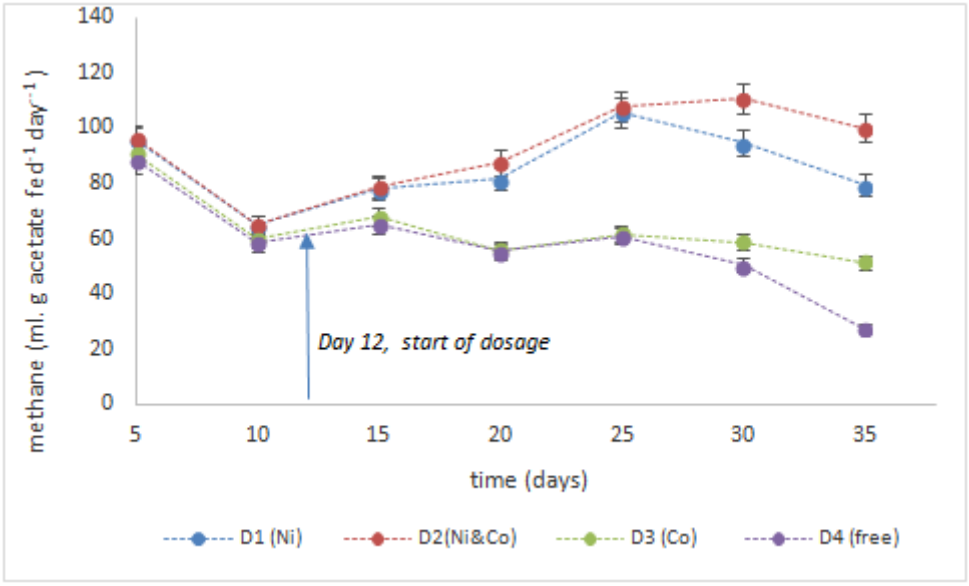


Figure 4.2. Average five day methane production from digesters when daily dosed 2.50 micro moles of Ni and Co

4.2.1 *Deduction from performance of digesters when daily supplemented with 2.5 micro moles of Ni and Co*

Following the dosage of micronutrients as per Table 3.1, those digesters that were dosed Ni (D1 and D2) almost immediately and simultaneously showed an almost equal increase in biogas volume output of 1.0 L above D4 (Figure 4.1a). As D3 and D4 produced almost the same amount of biogas between themselves despite D3's dosage, the increase in biogas production by D2 and D1 during the same period is credited to nickel's catalytic activity. The equality of biogas output of D1 and D2 lasted for 10 days (from day 12-22) Figure 4.1(a). From day 22-35, 13 days, D2 exceeded D1 in biogas production as simultaneously and by the same magnitude, D3 was exceeding D4. This increase in D2's performance above D1, which occurred at the time when D3 was exceeding D4 is credited to cobalt's catalytic activity.

The immediate increase in biogas production by the sludge following addition of nickel is in concurrence with the findings of Fermoso (2008) who observed an almost immediate increase in biogas production and a decrease in VFA (majority being acetate) accumulation from a

methanol fed UASB digester following Ni supplementation to 0.5 µM. An improved clustering and growth of Methanosarcina was observed almost instantaneously on supplementation of 0.5 µM of nickel by Fermoso et al. (2008). Since a large part of the VFAs in Fermoso et al. (2008)'s digester was acetate, this shows the ability of Ni to catalyse acetate degradation as observed through increased biogas production after its supplementation.

On the other hand, on supplementation to 0.5 µM of Co Fermoso (2008) observed an increased VFA accumulation in methanol fed UASB digester, as well as a slight increase in Methanosarcina population. In the work of Gustavsson (2012) in which two previously Ni and Co dosed grain stillage digesters were each omitted one of these elements; increased VFA and subsequent failure of a Ni omitted digester was observed following accumulation of up to 100 mM of VFA (a large part being acetate) 90 days after their respective omission, while only 10 mM of VFA accumulated in a Co deprived digester and it did not fail. This signifies the immediate need for Ni in improving removal of VFAs. A concentration of 0.1-0.3 mg/L of both nickel and Co was found to be necessary in order to successfully digest grain stillage anaerobically (Gustavsson, 2012). The larger concentration of 0.1-0.3 mg/L of Ni and Co needed in Gustavsson (2012)'s work as compared to 0.5 µM (0.029 mg/L) of Ni proved catalytic by Fermoso et al. (2008) can be explained by the simplicity (low matrix) and low sulphur content in a methanol feed digester as opposed to high matrix and high sulphur in grain stillage. High sulphur and interfering matrix can increase precipitation of micronutrients; thus rendering them less accessible to microbes. Low matrix and low sulphur content of the acetate digesters used in this experimentation possibly resulted in increased amount of soluble ions, hence their ability to exert the observed increase in biogas production around 0.5 µM (on day 15) , which is a similar concentration that was proved catalytic by Fermoso (2008).

A possible reason or explanation for the quicker (3 days) positive response following nickel as opposed to cobalt (13 days) supplementation could be due to the relative point (position) in methane generation catalytic chain of nickel action as compared to cobalt action. Ni is required for catalyses of the last step of methane production by methyl coenzyme M reductase, see Figure 2.9. Given that digesters were already in operation or running at the time of supplementation, the already present CH_3-S-CoM could have been quickly reduced to methane by H-S-CoB on Ni supplementation. The breakage of C-S bond during the reaction of CH_3-S-CoM and H-S-CoB has been identified by Scheller et al. (2013) to be both rate limiting and Ni induced, this suggest that Ni supplementation would increase methane production where CH_3-S-CoM and H-S-CoB are not limiting and are already present.

On the other hand, supplementation of Co is anticipated to increase production of methyl-coenzyme M as Co is vital for methyl transferases, Table 2.3 (Thauer, 1998). When supplemented to already running/ operating digesters the effect of this increased CH_3-S-CoM may not be readily realised or observed, especially if increase in biogas (CH_4) production is the main judgement or examination criteria. It is also worth noting that there are more Co dependent reactions (or steps) involved in the production of CH_3-S-CoM than are in its Ni dependent reduction with H-S-CoB leading to methane production. This could lead to the positive effect of Co on biogas production being more lagged compared to that of Ni. The many Co dependent methyl transferases leading to CH_3-S-CoM generation could also lead to Co being required in larger amounts than Ni before its effects on biogas production can be realised.

In an experiment conducted by Wolfe and McBride (1971) involving incubation of $^{14}CH_3$-B_{12} and CoM in the presence of cell free extracts of *Methanobacterium* M.O.H and ATP, $^{14}CH_3$-S-CoM was produced. The radioactive methylated coenzyme M ($^{14}CH_3$-S-CoM) was separated or purified using a Dowex 50 H^+ column (5 X 40 cm) and was eluted with water

(Wolfe and McBride, 1971). The purified $^{14}CH_3$-S-CoM was used as a substrate in methane assay in the presence of *Methanobacterium* M.O.H cell extracts (5.6 mg of protein) (Wolfe and McBride, 1971). Methane formation was found to be more rapid, 0.78 μ moles $^{14}CH_4$/hr per mg of protein than was observed from incubation of $^{14}CH_3$-B_{12}, 0.24 μ moles $^{14}CH_4$/hr per mg of protein; i.e. 3.25 faster. This could have been caused by the need to first produce $^{14}CH_3$-S-CoM from $^{14}CH_3$-$B_{12,}$ as methyl coenzyme M has been shown to be a unique intermediate for methanogenesis, (Thauer, 1998). It is worth recognition that those digesters that were dosed Ni (D1 and D2) showed higher biogas production quicker than D3 (Co). Three days after start of dosage, D1 and D2 produced the same biogas volume above D3 and D4, but 13 days after initial dosage, D3 was exceeding D4, as simultaneously D2 was exceeding D1.

Due to its simplicity, the observation point during anaerobic digestion experiments is normally the production of biogas or methane. In this case supplementation of Ni could appear to produce quicker results compared to that of Co especially during long term studies as CH_3-S-CoM might already be available in the system at the time of dosage. Possibly monitoring the accumulation of CH3-S-CoM would be a good criterion for judging the Co effect, but this is unlikely to be readily applicable due to the complexities envisaged.

Because Ni and Co are anticipated to alter biochemical reactions, hence biogas production through their interaction with microorganisms, which can be represented by VS; their interaction (numerical) with VS is discussed in section 4.3.

4.3 Relationship between volatile solids and Co and Ni when digesters were daily supplemented with 2.50 micro moles of Ni and Co

Ni and Co are anticipated to increase metabolic activity of methanogens, thus resulting in their increased utilisation of acetate, hence increased biogas production. For this to occur, Ni and Co have to be assimilated by methanogens, as such the relative abundance of these elements to methanogens should be considered. Volatile solids can be used to reflect the concentration of bacteria (Metcalf and Eddy, 2003). The ratio between volatile solids and metal ions of interest is important as it shows the theoretical interaction between microorganisms (VS) and ions of interest being, Ni and Co. It is anticipated that those digesters that had a high association of Ni and Co with volatile solids at stimulatory concentration would produce more biogas compared to those that did not. Data showing the measured concentration of metal ions and volatile solids on various days is presented in section 4.19.1.5. Each micronutrient is discussed in sections. 4.3.1 and 4.3.2

4.3.1 *Nickel*

The effluent obtained on day 12 before dosage of any micronutrient shows that there was an average measured Ni mass per VS of 18.55µg/gVS ±4.36 across all digesters, corresponding to 2.427 ±0.371 µM of Ni concentration (Table 4.6 and Table 4.7). This represents a Ni/VS mass ratio percentage reduction of 61% from the concentration of day 0, background concentration.

The average background nickel concentration in digesters D1 and D2 on day 12 was found to be 2.305 µM or (0.135 mg/L) corresponding to a Ni/VS mass ratio of 17.215µg/gVS. This concentration was found to be limiting the performance of digesters or not bioavailable to microorganisms. Following the dosage of 0.146 mg of Ni to D1 and D2, thereby increasing its

concentration in these digesters to 0.151 mg/L from 0.135 mg/L, an increase of 1.0 L of biogas production above D4 and D3 was observed 3 days later, Figure 4.1(a).

The minimum stimulatory Ni and Co concentration of 0.1-0.3 mg/L was reported by Gustavsson (2012) during anaerobic digestion of grain stillage, and a stimulatory concentration of 0.5 µM (0.029 mg/L) was reported by Fermoso (2008) during digestion of methanol. The observed stimulatory concentration of 0.151 mg/L in this research is contained within the range reported to having been stimulatory by Gustavsson (2012) and Fermoso (2008).

Given that 0.016 mg/L (0.151-0.135 mg/L) of Ni increase was achieved following its dosage, which is quiet small, it is likely or possible that the increase in methanogenic activity hence biogas volume production was caused by an increase in the bioavailability of Ni following its dosage rather than the increase in the total nickel in a digester. An effluent from a Ni devoid digester was found to have no decrease in Ni concentration, while that from a Co devoid digester showed Co concentration decrease at a rate of 0.1 µg/gTSS.d^{-1}, but a 50% increase in SMA with methanol as substrate of sludge from this Ni devoid digester following 5 µmol.L^{-1} dosage of Ni was reported by Zandvoort et al. (2006). This shows the need to dose Ni to a digester despite it's already presence, a possible reason for this is the need to increase its bioavailable concentration.

Immediately following the dosage of Ni on day 12, D1 and D2 had an average of 0.151 mg/L of Ni, while D3 and D4 had 0.112 mg/L, which represented a concentration of 0.038 mg/L of Ni more in D1 and D2 than in D3 and D4. The concentration in D4 is used as D3's is deemed an outlier as it was the highest amongst the three on day 12. This difference in total Ni concentration between the Ni dosed and non-dosed digesters is unlikely to solely account for the observed increase in biogas in D1 and D2 following Ni dosage. Possibly the background Ni of the seed sludge was not as available to methanogens as the newly dosed one. Increase in

bioavailable Ni could be exerted through its increase in the supernatant due to its dosage. Zandvoort et al. (2004) found that only 6% of the in-situ loaded Co to a methanol fed UASB reactor accumulated in granules as majority was lost in effluent. Fermoso et al. (2009) reported that this loss of Co is a major drawback of in situ loading of a UASB. The functionality of UASB relies on granulation of biomass, hence the need to maintain Co in granules as opposed to a CSTR where complete mixing of reactants is necessary. Since supplementation of micronutrients was done daily, this possibly maintained a constant pool of Ni ions in the supernatant, making them more accessible to microorganisms in line with Zandvoort et al. (2004)'s observation.

By the end of the daily 2.50 μ moles (≈0.147 mg) of Ni and Co supplementation period, day 35, the concentration of Ni in D1 and D2 is expected to have been 4.873 μ molar (0.286 mg/L) having increased from 0.745 μ molar (0.0437 mg/L) anticipated on day 12 immediately before dosage. This calculated concentration is within the stimulatory range of 0.1-0.3 mg/L reported by Gustavsson (2012). The computation was done using the combined effect of washout and dosing of Ni taking into account the already present calculated Ni on day 12 (before dosage). The evolution of micronutrients with time in digesters is presented in section 4.19.1.9.

4.3.2 *Cobalt*

After 3 days of 0.147 mg of Co dosage to D3 and D2 thereby increasing their calculated Co concentration to 0.109 mg/L from 0.040 mg/L, no difference in biogas production was observed between D3 and D4 contrary to the increase observed above D4 from both D1 and D2 after their dosage; this signify that the increase in D2's biogas production 3 days following the dosage of Co and Ni was solely due to nickel (Figure 4.1a). The long latency period (13 days) following cobalt supplementation can mostly be associated with the role cobalt play in acetate anaerobic digestion catalysis. On day 28, D2 and D3 had almost the same measured

cobalt of 0.052 mg/L as on day 12, 0.060 mg/L but lager Co/VS of 7.81µg/gVS was observed on day 12 compared to on day 28, 6.680 µg/gVS. The increase in biogas production observed 13 days after initial dosage; being D3 above D4 and D2 above D1 can be associated with the effect of the dosed Co, just that its effect was significantly lagged compared to that of Ni, especially in already running digesters. Fermoso (2008) did not observe an increase in methane production from methanol anaerobic digesters following Co dosage to a Co limited digester, this is a similar observation as in this research; more so that both methanol used by Fermoso (2008) and acetate used in this study were of similar purity, which discount the possibility of different bio availabilities having an effect.

During anaerobic digestion of grain stillage, Gustavsson (2012) found 10-16% more of Co to be in the sludge liquid phase as compared to Ni which was mostly associated with sulphides/organic matter. This Co in liquid phase was perceived as being bioavailable to microbes than Ni. This suggests that the late response of Co dosed digesters is not likely to be due to its unavailability to microbes, but likely to be due to its catalytic role in biogas production or its requirement in larger amount (higher thresh hold) compared to Ni, possibly due to the many intermediary steps involved in generation of methyl coenzyme M. On day 25 when the Co effect was observed, D2 and D3 had a calculated Co concentration of 0.230 mg/L, which is approximately 51% more than the 0.111 mg/L of Ni observed to have caused an increase in biogas production or methanogenic activity by day 15. Although the Co concentration of 0.230 mg/L observed on day 25 is within the range of 0.1-0.3 mg/L of Co observed to have been catalytic by Gustavsson (2012) during anaerobic digestion of grain stillage, it is higher than the Ni concentration of 0.5 µM that was reported having been catalytic by Fermoso (2008) during the anaerobic digestion of methanol; possibly owing to the high amount of sulphate in Gustavsson (2012)'s digesters which could have led to high precipitation of Ni and Co, hence the need to dose a large amount before stimulation occurs.

On day 35 the concentration of Co in D2 and D3 was calculated to have been 0.287 mg/L, which is within the stimulatory range reported by Gustavsson (2012).

Generally low stimulatory concentrations of micronutrients have been observed in digesters where a simple substrate such as methanol or acetate with low or no sulphates is digested. A concentration of 5 µM of Co was found to be stimulatory to a methanol digester (Zandvoort et al., 2006), 1 mM was also found to be stimulatory to methanol digester (Zandvoort et al., 2004) was well as 0.62 µmol-Ni/g VSS and 0.67µmol-Co/g VSS being found to be stimulatory to an acetate digester (Kida et al., 2001). On the other hand a concentration of 0.16 mg kg fresh matter $^{-1}$ of Se and 0.22 mg kg fresh matter^{-1} of Co (approximately 0.1-0.2 mg/L respectively) were found to be critical for the successful digestion of food waste at a loading rate of 5 g $VS^{-1}day^{-1}$ (Banks et al., 2012). The critical concentration of Co reported by Banks et al. (2012) is essentially the same as the 0.230 mg/L that was observed to having been catalytic on day 25 in this study, despite the expected high interfering metric from food digesters compared to the simplicity of acetate digesters.

Because of the inconsistency and variability of anaerobic digestion, especially where pure seed cultures were not used, it was found necessary to statistically test the significance of the difference in biogas (methane) production between the dosed digesters (D1, D2 and D3) and the control digester (D4) during the period when digesters where daily dosed 2.50 µ moles. The following section 4.4 discusses results of such statistical test.

4.4 Statistical test on differences in biogas production between digesters and the control when daily dosed 2.50 micro moles of Ni, Co and Ni and Co.

To test whether the differences between D1(Ni), D2(Ni &Co) , D3(Co) and the control (D4) is statistically significant, a paired two sample for means t-test at 5% confidence level was performed. That is, the mean differences of methane output, (ml) per gram of acetate fed per day, for each of the digesters daily supplemented with 2.50 μ moles of Ni (D1), Co (D3) and Ni and Co (D2) against that of the control digester (D4), were calculated and tested at 5% significance level.

The nominal micronutrients concentration in those digesters that were dosed increased from 0.745 μM (0.044 mg/L) on day 12 to 4.873 μM (0.286 mg/L) on day 35 for Ni and 0.679 μM (0.040 mg/L) to 4.867 μM (0.287 mg/L) for Co in the same period.

From the statistical test there was a significant difference in methane volume output in ml per gram of acetate fed per day between D1 (Mean=79.65) and D4 (Mean = 49.93) with $t(35) = 4.93$, $P = 4.81 \times 10^{-5}$. There was also a significant difference in methane volume output in ml per gram of acetate fed per day between D3 (Mean=54.68) and D4 (Mean = 49.93) with $t(35) = 2.48$, $P = 0.02$. Also, a significant difference in methane volume output in ml per gram of acetate fed per day between D2 (Mean=79.65) and D4 (Mean = 49.93) with $t(35)= 4.93$, $P = 4.81 \times 10^{-5}$ was realised. The significance of Co dosage was observed to be less than that of individual Ni dosage in accordance with the observation made in Figure 4.1(b). Daily dosage of 2.50 μ moles of Ni, Co and Ni and Co led to production of significantly more biogas or methane from the dosed digesters compared to the non-dosed digester during the dosage period.

Having established the significance/need to dose Ni and Co individually as well as in combination; the contribution to biogas production by individual Ni and Co when co-dosed was examined and is presented and discussed in section 4.5.

4.5 Delineation of individual effect of Ni and Co when daily co-dosed at 2.50 micro moles

The individual contribution of Ni and Co to biogas production when co-dosed was not delineated and separately examined in the work of Gustavsson (2012). Drawing inspiration from Gustavsson (2012)'s work, the individual contribution of Ni and Co when daily co-supplemented at 2.5 µ moles is delineated and presented in Figure 4.3 and Figure 4.5.

To the best of the author's knowledge this is the first presentation of delineation of Co and Ni's effect on biogas production when co-dosed in an acetate digester. Their individual effects were also found to be in agreement with the catalytic interconnectivity or co-dependence suggested in Figure 2.9, section 2.4.2.1. This delineation of the effects of Ni and Co also tests the idea that the effects of Ni and Co when co-dosed are synergistic or additive.

4.5.1 *The effect of Ni in a Ni and Co dosed digester*

Figure 4.3 was obtained by comparing the biogas production profile (time and amount) of D2 and D3, i.e. (D2-D3) and that of D4 and D1, i.e. (D4-D1); if the difference in biogas volume output from each of these two pairs is due to the presence and absence of Ni respectively; then the sum of their difference is anticipated to be zero or close to zero. This computation was done using biogas volumes presented in Figure 4.1(a). Indeed the magnitude of the difference at each point in time was almost the same, and the sum of the differences between these pairs

of digesters was zero or almost zero at each point in time as presented in Figure 4.3. This observation held true daily for 23 days of dosage (day 12-35).

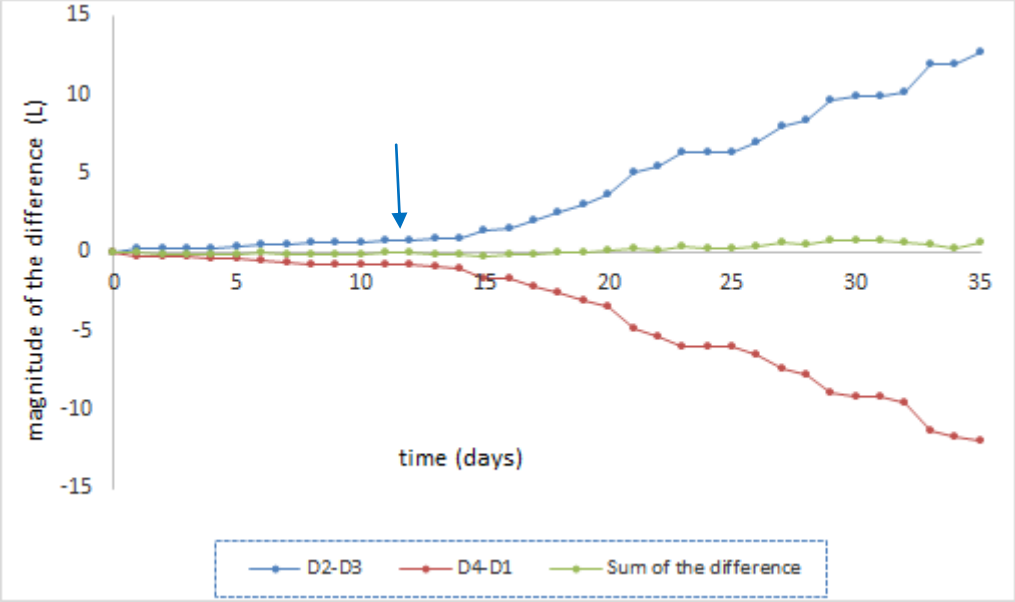


Figure 4.3 The magnitude of the difference between D2 and D3 and D4 and D1 as well as the sum of the differences, showing the Ni effect. This was obtained when digesters were daily dosed 2.50 µ moles of Ni and Co.

Recall that dosage of digesters is as follows; D1= [Ni only], D2= [Ni and Co], D3= [Co only] and D4 = [no Ni and Co]. Conceptualisation of these digesters as sets (as in mathematics) and micronutrients dosed as elements of each set make the above computation more understandable.

To further check the significance of the observation made in Figure 4.3, a simple linear regression was conducted using Microsoft Office Excel 2013 to predict the amount of biogas that a Ni and Co dosed digester (D2) exceed a Co only dosed digester (D3) by, based on the amount of biogas by which a Ni only dosed digester, D1, exceed a non--dosed digester (D4) by. The line fit plot of this analysis is presented in Figure 4.4. The amount by which D2 exceeded D3 based on the amount by which D1 exceed D4 could be predicted from the following equation: Y= 1.051X, with an R^2 of 0.998 ≈1. This shows that the effect of Ni dosage in both D2 and D1 is almost the same; hence the Ni effect can be isolated from that of

Co when Ni and Co are co-dosed. This also shows/ supports the repeatability of exertion of the dosed Ni effect, as it had almost the same effect in both D1 and D2.

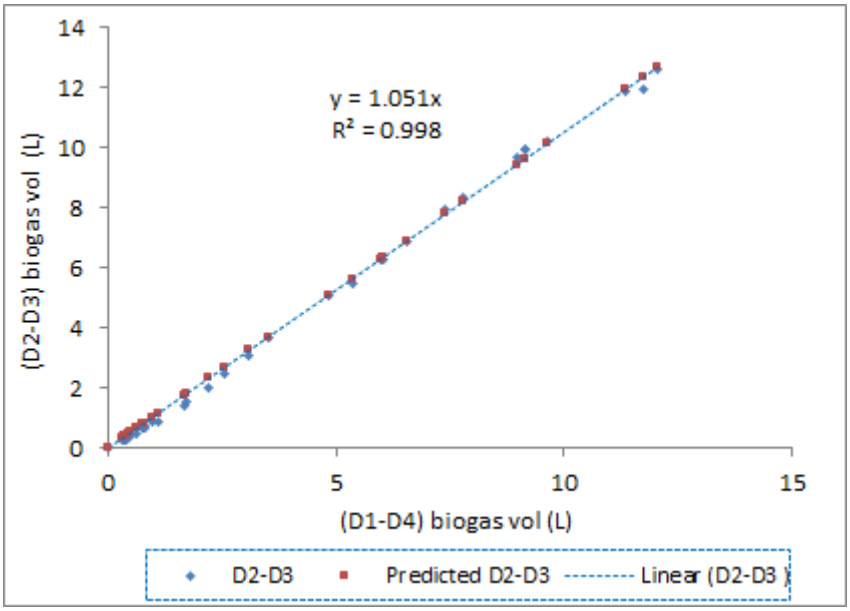


Figure 4.4. A line fit plot of the linear regression that correlates the effect of Ni dosage in D2 and in D1.

4.5.2 *The effect of Co in a Ni and Co dosed digester*

A similar computation as was done to separately examine the Ni effect when co-dosed with Co was done to separately examine the effect of Co when co-dosed with Ni. This was based on the same expectation as was during the examination of the effect of sole Ni.

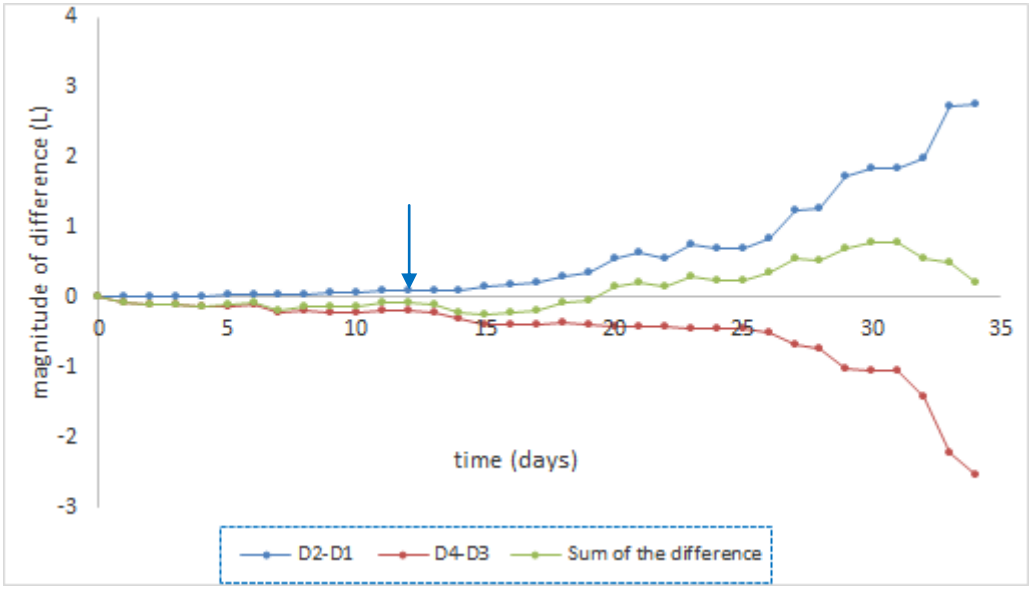


Figure 4.5 The magnitude of the difference between D2 and D1 and D4 and D3 as well as the sum of the differences, showing the Co effect. This was obtained when digesters were daily dosed 2.5 0 µ moles of Ni and Co.

The computation was done by comparing the difference in biogas output between D2 and D1, i.e. (D2-D1) and D4 and D3, i.e. (D4-D3). The findings are presented in Figure 4.5. The magnitude of the difference at each point in time between these pairs of digesters is almost the same, and the sum of the differences between these pairs of digesters is less than one litre, (1.0 L) at each point in time. This observation also held true daily for 23 days of dosage.

The effect of Co is not as highly correlated to its dosage as that of Ni as shown by its lower R^2 value of 0.923 as compared to the higher R^2 value of 0.998 of Ni effect. This is possibly because Co catalysis a series of reactions involved in methyl transfer up to the point of generation of methyl-coenzyme M, as opposed to Ni which only catalysis one last step being the reduction of methyl-coenzyme M with coenzyme B to produce methane (Thauer, 1998), see Table 2.3. A similar observation was made by Zandvoort et al. (2006) who observed a linear increase in SMA of Ni limited sludge after its dosage, while Co dosage produced an increase in SMA in a non-linear manner.

To further check the significance of the observation made in Figure 4.5, a simple linear regression was conducted using Microsoft Office Excel 2013, to predict the amount of biogas that a Ni and Co dosed digester (D2) exceed a Ni only dosed digester (D1) by, based on the amount of biogas by which a Co only dosed digester, D3, exceed a non-dosed digester (D4) by. The line fit plot of this analysis is presented in Figure 4.6. The amount of biogas by which D2 exceeded D1 based on the amount by which D3 exceed D4 could be predicted from the following equation: $Y = 1.275X$ with an R^2 value of 0.923. This also shows/supports the repeatability of exertion of the dosed Co effect in two different digesters, D3 and D2, as these effects are almost the same.

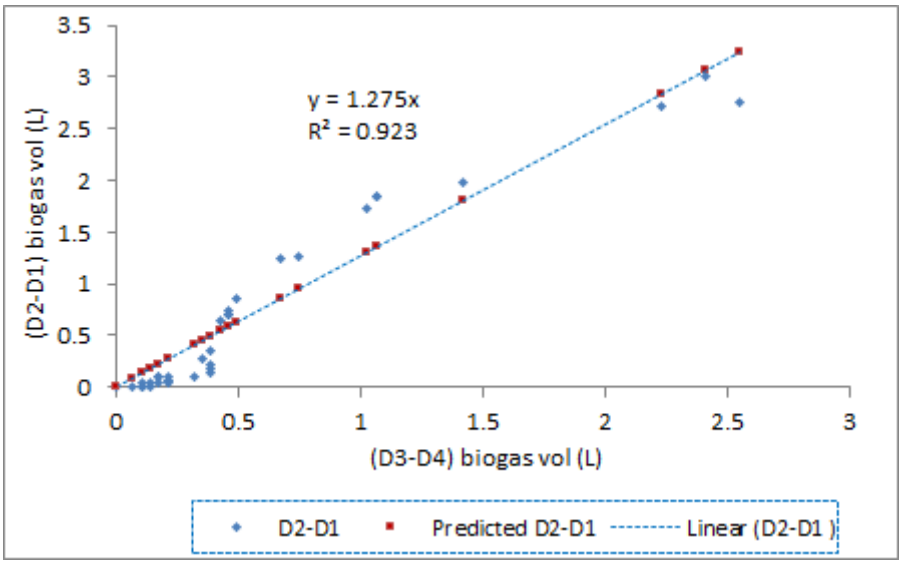


Figure 4.6 A line fit plot of the linear regression that correlates the effect of Co dosage in D2 and in D3.

As Co and Ni catalyse different steps during methanogenesis (from acetate and other substrates) their individual contribution to biogas production can be delineated. The schematic view in Figure 2.9 suggests that co-dosage of these two elements would be synergistic or additive in catalysing biogas production from acetate. Several researches concurs that Cot plays a pivotal role in the catalyzation of activities of methyltransferases (Ludwig and Matthews, 1997, Blaylock and Stadtman, 1964, Barker et al., 1958). For methane production from acetate to occur, the methyl group of acetate has to be transferred through a series of enzyme mediated reactions as presented in Table 2.3 up to the point of generation of CH_3-S-CoM. Enzymes involved in this methyl transfer are believed to be activated by the presence of cobalt (Thauer, 1998). These enzymes are produced by methanogens (Ludwig and Matthews, 1997, Gärtner et al., 1993). In the presence of cobalt, increased growth of methanogens was observed (Ishaq, 2012, Fermoso, 2008). Cobalt catalysis methanogenesis through increasing the rate of production of methyl-coenzyme M (Thauer, 1998) which is a key intermediate in methanogenesis.

It is believed that Ni catalyses the terminal step during methanogenesis. Being either the reduction of methyl coenzyme M with proton from Coenzyme B (H-S-CoB) leading to the production of methane and a heterodisulphide product of CoB-S-S-CoM (Scheller et al., 2013, Scheller et al., 2010, Thauer, 1998) or the generation of a methyl radical and its (methyl radical) subsequent quenching of hydrogen from coenzyme B (Blomberg et al., 2014, Pelmenschikov and Siegbahn, 2003, Pelmenschikov et al., 2002) and the later formation of a heterodisulphide product of CoB and CoM. As such the concomitant presence of Co and Ni during acetate anaerobic digestion is anticipated to lead to independent catalyses of two separate steps; being CH_3-S-CoM generation and its (CH_3-S-CoM) subsequent demethylation and reduction by hydrogen from H-S-CoB; thereby producing methane.

In section 4.11, Figure 4.15 it is shown that the sum of percentage biogas volume in excess of control of D1 and D3 (D1+D3) is almost equal to the individual percentage in excess of control by D2, i.e., [D1+D3≈D2] following daily dosage of 2.50 μ moles of Ni and Co, D1+D3 = (82±3) % in excess of control while D2 = (86±1) % in excess of control. Despite having not conducted experiments to quantify the intermediate CH_3-S-CoM and its subsequent disappearance, the above additive effect of Ni and Co in biogas production is most likely due to the postulated reaction interconnection suggested in Figure 2.9.

The observed direct proportionality between the amount of CH_4 produced from CH_3-S-CoM and the amount of CH_3-S-CoM added, coupled with the direct proportionality between the amount of CH_3-S-CoM produced from CH_3-B_{12} and the amount of CH_3-B_{12} added (Wolfe and McBride, 1971) combined with knowledge that reduction of CH_3-S-CoM to CH_4 is Ni induced (Scheller et al., 2010) and that Co is involved in CH_3-S-CoM production; make a case for co-requisite of Ni and Co in anaerobic digestion of acetate, as well as explaining the observed additive effect of their co-dosage.

4.6 Overall performance of digesters when daily dosed 3.75 and 5.00 micro moles of Ni and Co; days 35-90

This section discusses biogas production from digesters in two regions; when daily dosed 3.75 µ moles and 5.00 µ moles in that order. During these periods, magnesium acetate was omitted from the feed as it was not available in the laboratory; instead the proportion of sodium acetate and calcium acetate in 30 mM acetate feed was increased from 25:35 percent respectively to 1:1. The possible effects of this new feed on the 'health' of methanogens are considered with relevance to biogas production.

4.6.1 *Performance of digesters when daily dosed 3.75 micro moles of their respective micronutrients; days 35-60*

On day 40 the composition of 30 mM acetate fed to digesters was changed from: sodium acetate, calcium acetate and magnesium acetate in percentage molar ratio of 25:35:40 respectively to sodium acetate: calcium acetate in molar ratio of 1:1. This was because magnesium acetate stock got depleted; so while awaiting its arrival the feed had to be changed to keep digesters alive. Through this change, the concentration of sodium in the feed was doubled from 7.5 mM to 15.0 mM and the feed stock pH increased to 8.7 from 7.0-7.5.

On day 35, daily micronutrients supplementation was increased to 3.75 µ moles. For the first five days following dosage increase D1, D2 and D3 continued to exceed D4 in production of biogas by 1 L/d for D1 and 2 L/d for both D2 and D3 after which no considerable biogas production was observed from all four digesters (Figure 4.7a) for five consecutive days.

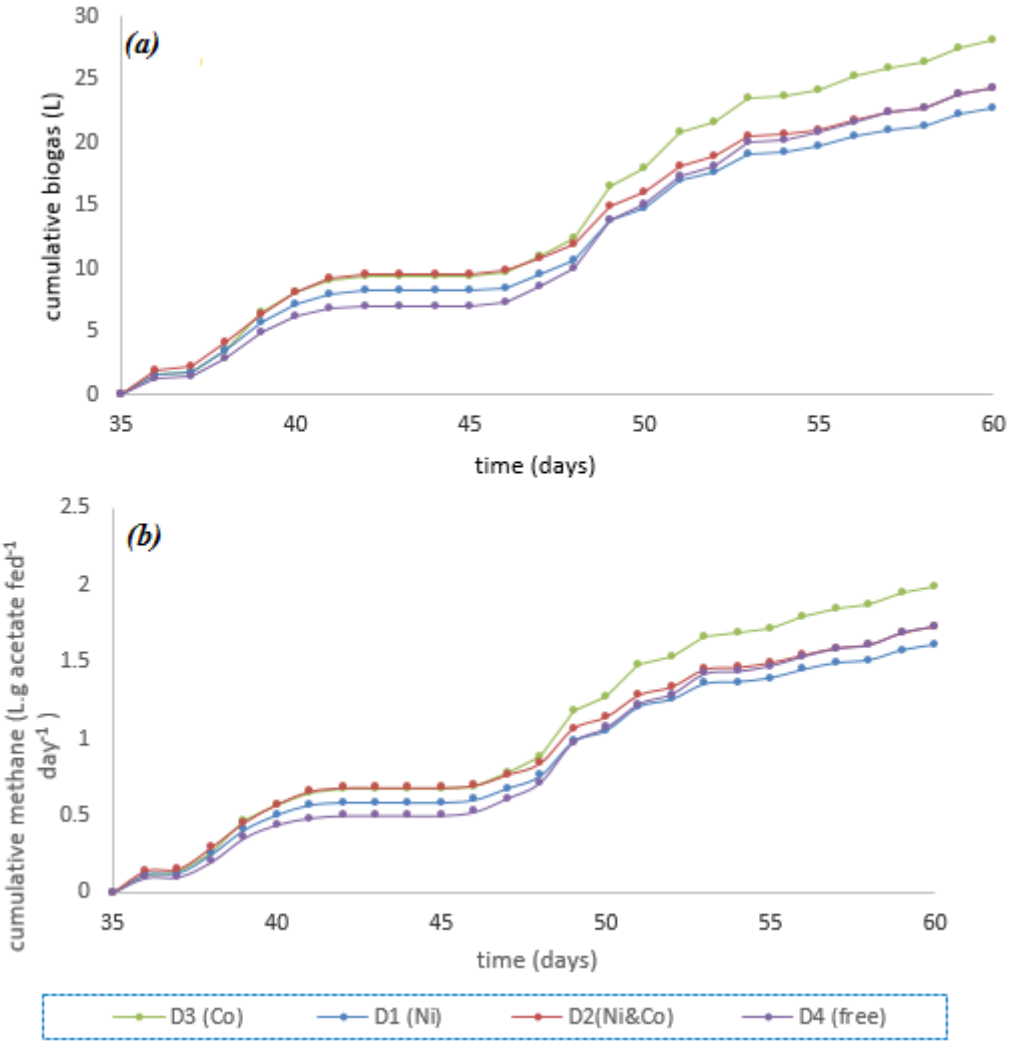


Figure 4.7. Digesters' performance when daily dosed 3.75 micro moles of their respective micronutrients. (a=cumulative biogas volume, b= cumulative methane L .g acetate fed^{-1} day^{-1}).

This lack of biogas production can be associated with the increase in digesters pH due to a change in feed composition as well as sodium toxicity to microbes. A sodium concentration of 6 g/L was reported to having been toxic to methanogens by Bashir and Matin (2004), a higher lethal sodium concentration of 16 g/L was reported by Chen et al. (2003). Both authors (Bashir and Matin, 2004, Chen et al., 2003) observed that pre-exposure of methanogens to sodium increase their tolerance to it through acclimatization. The effluent pH increased from 7.5 on day 42 to around 8.5 on day 43. Despite having only increased the sodium concentration from 0.17 g/L to 0.34 g/L due to feed change, which is considerably lower than

the toxicity concentration reported by Bashir and Matin (2004) and Chen et al. (2003) the five day (day 40-45) lack of biogas production is associated with acclimatization of microbes to a new feed stock composed of sodium acetate and calcium acetate at a molar ratio of (1:1) and a pH of 8.7 as compared to 7.0-7.5, Figure 4.7(a). Bashir and Matin (2004) observed that magnesium concentration of 0.55 g/L was optimal for averting toxicity due to 6 g/L of sodium in a whey anaerobic digester, so omission of magnesium (0.30 g/L) which is close to 0.55 g/L) from the feed possibly compounded the problem of sodium toxicity to digesters. The percentage of methane in biogas (64%) remained the same during the period of lack or magnesium and high pH, Figure 4.7(b), this could potentially mean that the pathway that was involved in methanogenesis prior to feed and pH change was hindered or interfered with.

On day 47 all digesters showed a slight increase in biogas production with D2 and D3 producing 2 L above D4 while D1 produced 1 L above D4. The difference in Performance of digesters during the period of lack of magnesium acetate in feed stock, day 40-73, cannot be attributed solely to differences in micronutrients fed to them.

4.6.2 *Performance of digesters when daily dosed 5.00 µ moles of their respective micronutrients; days 60-90.*

Following the increase of daily micronutrients supplementation from 3.75 µ moles to 5.00 µ moles, the status quo of digesters performance remained the same with D3 leading in biogas production as shown in Figure 4.8(a).

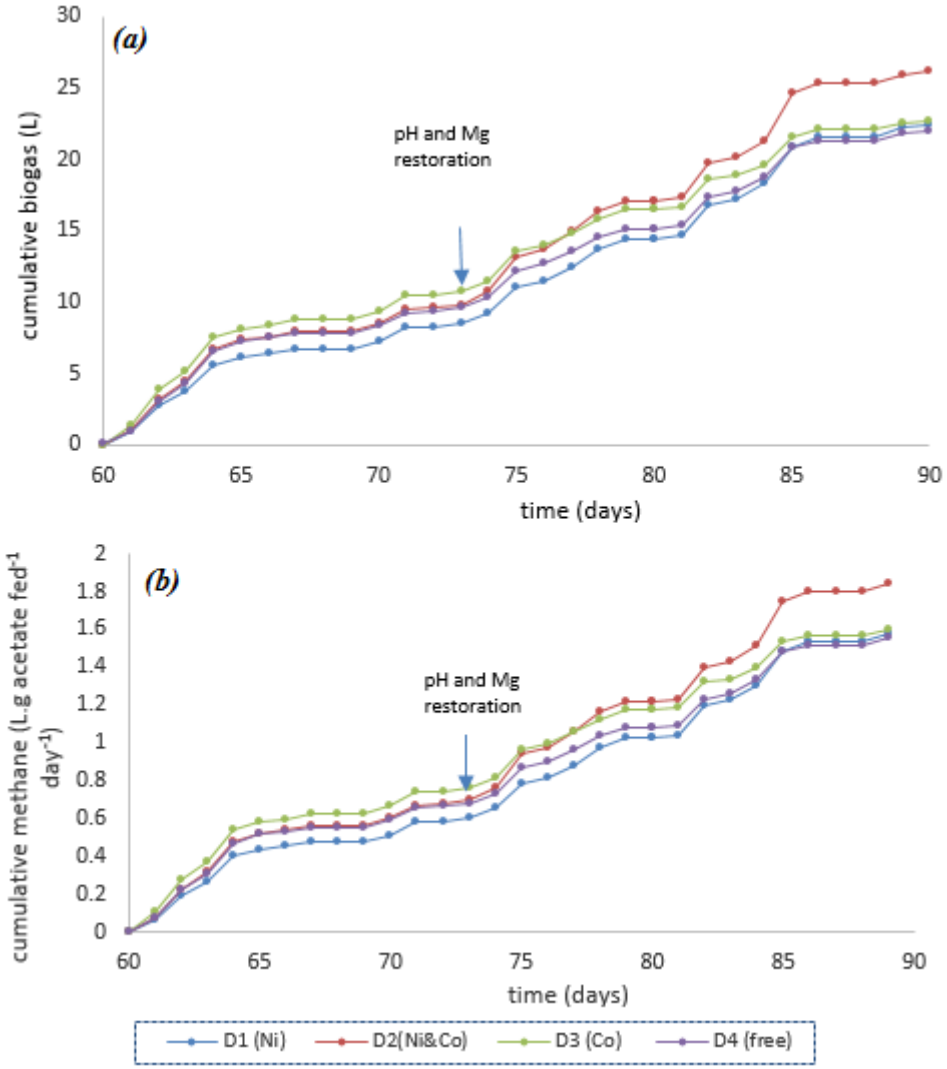


Figure 4.8. Digesters' performance when daily dosed 5.00 micro moles of their respective micronutrients. (a=cumulative biogas volume, b= cumulative methane L .g acetate fed^{-1} day^{-1}).

On day 73 following availability of magnesium acetate the feed pH was restored, and on day 74 a marked increase of 2.0 L/d in biogas production was observed across all four digesters. By the end of the 5.00 µ moles dosage period, D2 was leading in biogas production having produced on average 0.8 L/d and D1 had caught up with both D3 and D4 having produced 0.6 L/d. The percentage of methane in biogas was constantly 64±1%, Figure 4.8(b).

The difference in performance of digesters during the period of lack of magnesium acetate in feed stock (day 40-73) cannot be attributed to different micronutrients fed to them. A change of pH (outside methanogens' optimum) and an increase in sodium concentration as well as lack of magnesium in the feed stock are anticipated to have had a negative effect on biochemical activity of methanogens. Mortimer et al. (1981) reported the optimum pH for Methanosarcinales to be near neutral to slight acidic. qPCR data in section 4.19.2 shows that Methanosaetaceae were the dominant family across all digesters during the period that covered omission of magnesium acetate, hence these conditions were out of their optimal growth pH

During cultivation of nine strains of methanogens belonging to the order Methanosarcinales,Yu et al. (2006) observed that the concentrations of the extracted DNA of each strain were not identical because of the different concentration of each strain by the end of the incubation cycle. In line with this view, the change in DNA/gVS equivalence of biomass, as well as in 16s rRNA/µg DNA observed in this study, indicate the change in abundance of total and specific methanogens respectively with digester operation time. It is shown in section 4.19.2, Figure 4.44 that the DNA yield from biomass/sludge reduced from an average of 5 µg DNA/g VS equivalent biomass for D1, D3 and D4 (on day 22) to an average of less than 1 µg/g VS equivalent biomass (on day 77). This indicated low abundance of methanogens in digesters during period of high feed pH and high sodium concentration due to omission of magnesium acetate, hence during this period low biogas production was observed. The DNA yield from D2 on day 22 was about 1 µg DNA/g VS equivalent biomass, the reason for this low yield has not been identified.

After restoration of pH as well as sodium and magnesium concentration to desired level (similar to the ones observed in day 0-35) the dosage of micronutrients was increased to 7.50 µ moles per day. The performance of digesters under this dosage is presented in section 4.7.

4.7 Performance of Digesters when daily dosed 7.50 micro moles of their respective micronutrients; days 90-129

On day 90, daily dosage of micronutrients was increased to 7.50 µ moles from 5.00 µ moles. As with 2.50 µ moles of Ni and Co supplementation on day 12, Ni dosed digesters (D1 and D2) showed the highest increase in biogas production almost immediately being: 0.4 L for D1 and 0.5 L for D2 while D4 and D3 produced 0.3 L, Figure 4.9(a). All digesters then ceased producing biogas for almost 3 days, then picked on day 95. Up until day 112, D3 produced slightly less biogas compared to D4, 0.2-0.3L less.

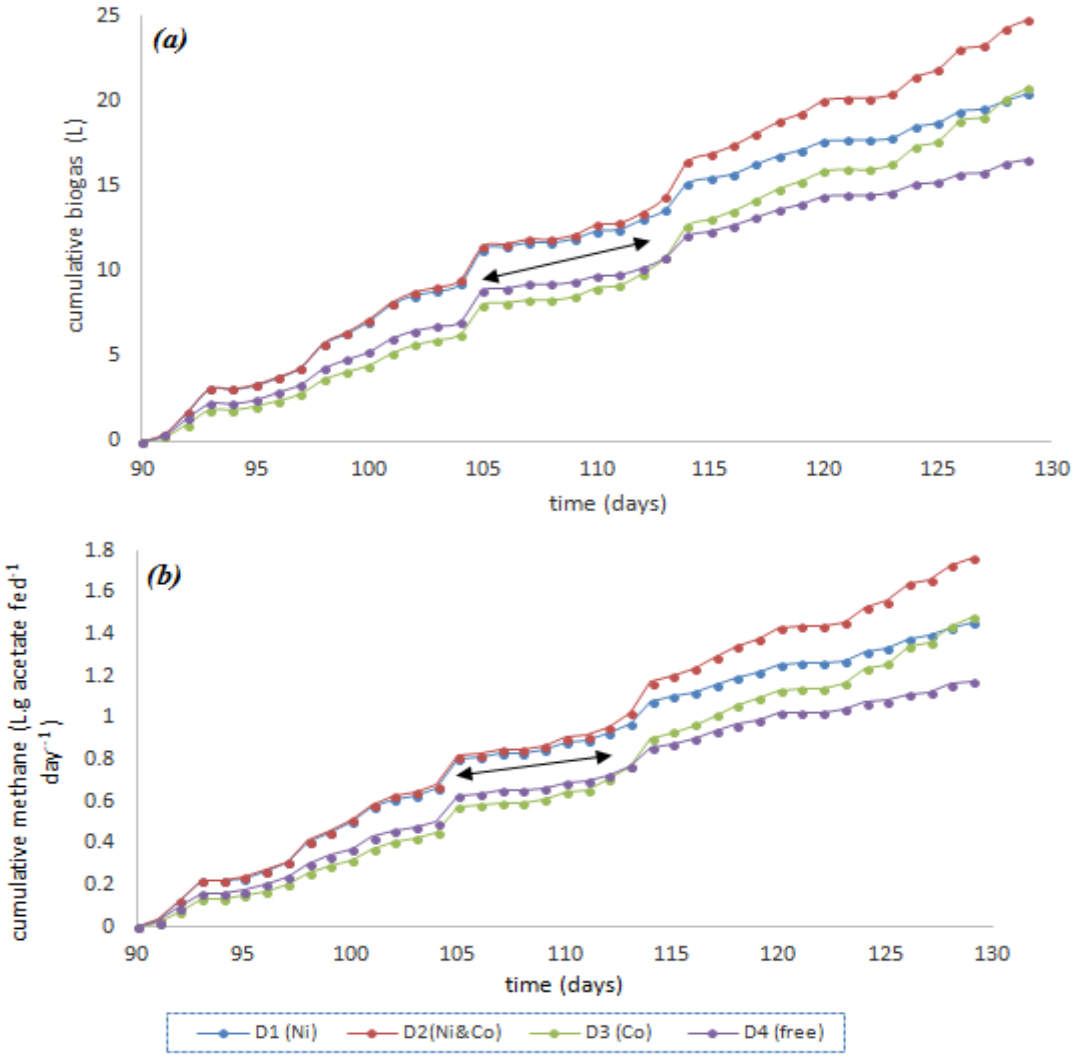


Figure 4.9 Digesters' performance when daily dosed 7.50 micro moles of their respective micronutrients. (a=cumulative biogas volume, b= cumulative methane L .g acetate fed^{-1} day^{-1}). Note low biogas production at arrow point.

On day 114, D3 exceeded D4, as D2 was exceeding D1 in biogas production: again simultaneously and with the same magnitude (1.0 L).This happened in a similar manner as was observed in Figure 4.1(a) following the daily dosage of 2.50 µ moles of Ni and Co. By day 127 both D1 and D3 had produced similar amount of biogas, approximately 19.5 L and 19.1 L respectively, this was mainly due to low biogas production by D1 while D3 continued to increase its biogas production (by approximately 1.0 L) Figure 4.9(a).

On day 103 which is 3xHRT from the pH restoration point (day 73) it is anticipated that the concentration of magnesium in digesters was restored to 0.3 mg/L and that sodium concentration was also restored to 0.17 g/L from that of 0.34 g/L due to its decrease in the

feed. On day 103, pH was found to be around neutral. By the end of daily 7.5 µ moles supplementation period, the condition of digesters had been restored to as before omission of magnesium acetate, hence the pattern of biogas production by digesters is observed to be getting similar to that seen at the time of daily supplementation of 2.50 µ moles.

On day 122 DNA yield of D1 and D4 were about 4.5 and 6.0 µg/g VS equivalent biomass respectively, both having increased from less than 0.5 µg/g VS equivalent biomass on day 77, section 4.19.2, Figure 4.44. On day 122, D2 and D3 had the lowest DNA yield of less than 0.5 µg/g VS equivalent biomass. With the exception of D1 which was dominated by Methanomicrobiales, all other digesters were dominated by Methanosarcinales on day 122 (Figure 4.45) as conditions which favour their growth were prevalent. Because of these reasons, the pattern of biogas production during period of daily dosage of 7.50 µ moles, Figure 4.9(a) can be compared to that observed during daily dosage of 2.50 µ moles, Figure 4.1(a).

At the end of the 7.50 µ moles daily supplementation period, day 90-129, D1, D2 and D3 had exceeded D4 in biogas output by the following percentages: 24, 50 and 26 respectively. It also held true that the sum of percentage exceedance of D4 by D1 and D3 equalled that by which D2 exceeded D4; written mathematically as D1+D3=D2, i.e. 50. This is presented in Figure 4.15. This again is in line with the view that the effect of Co and Ni when co-dosed at stimulatory concentrations are additive with respect to biogas production.

When compared with day 0-35, a further reduction of the five day average methane production from around 80-120 ml. g acetate fed^{-1} day^{-1} (Figure 4.2) to around 60-80 ml. g acetate fed^{-1} day^{-1} (Figure 4.10) for D2 was observed from day 95-115. On average all digesters showed reduction in biogas/methane production from day 95 to day 129 as compared to from day 0-35. This is in line with the observed reduction in Methanosaetaceae

across all digesters with digestion time. The highest reduction in five day average methane production from day 115-125, Figure 4.10, correspond to the time of marked reduction in Methanosarcinales population, which D1 was leading in, as well as in reduction of biogas production compared to all dosed digesters. With the temporal reduction in Methanosaetaceae, the amount of methane produced from digesters further deviated from the theoretical value.

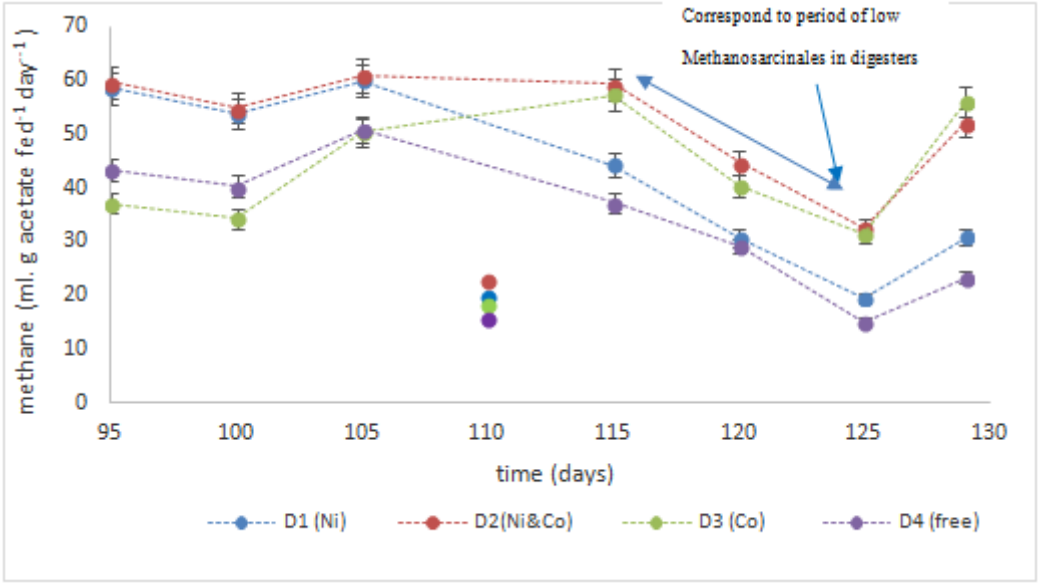


Figure 4.10. Average five day methane production from digesters when daily dosed 7.50 micro moles of Ni and Co.

As was done when digesters were daily dosed 2.50 µ moles, a statistical test was conducted in cognisance of the inherent variability in anaerobic digestion systems, to test the significance of dosage of Ni and Co to digesters on methane/biogas production. Section 4.8 presents and discuses results of such test.

4.8 Statistical test on differences in biogas production between digesters and the control when daily dosed 7.50 micro moles of Ni, Co and Ni and Co.

A paired two sample for means t-test at 5% significance level was conducted to test the statistical significance of the methane output per gram of acetate fed per day (ml CH_4.g acetate fed^{-1} day^{-1}) from those digesters that were daily dosed 7.50 μ moles of Ni (D1), Co (D3) and Ni and Co (D2) against that of the control digester (D4). The nominal micronutrients concentration in those digesters that were dosed increased from 10.397 μM (0.610 mg/L) on day 90 to 14.924 μM (0.876 mg/L) on day 129 for Ni and 10.379 μM (0.611 mg/L) to 14.924 μM (0.879 mg/L) for Co in the same period.

There was a statistically significant difference in methane volume output (ml) per gram of acetate fed per day between D1 (Mean=36.44) and D4 (Mean = 29.45) with t(39) = 4.67, P = 3.53×10^{-5}.

There was also a significant difference in methane volume output (ml) per gram of acetate fed per day between D3 (Mean=37.07) and D4 (Mean =29.45) with t(39) = 3.15, P = 3.15×10^{-3} . Also a significant difference in methane volume output (ml) per gram of acetate fed per day between D2 (Mean= 44.24) and D4 (Mean = 29.45) with t(39)=6.72, P = 5.21×10^{-8} was observed. The significance of co-dosage of Ni and Co was observed to be larger than that of individual Ni and Co dosage in accordance with the observation made in Figure 4.9(b). Like during the daily dosage of 2.50 μ moles, daily dosage of 7.50 μ moles of Ni, Co and Ni and Co led to production of significantly more biogas or methane from the dosed digesters compared to the non-dosed digester.

The individual effect of Ni and Co when daily co-dosed at 7.50 μ moles (day 90-129) was examined in the same manner as was done when they were daily co-dosed at 2.50 μ moles

(day 12.35). This examined the repeatability of the previously made observation, whereby the effect of Ni and Co were delineated when daily dosed at 2.50 µ moles; this is important as digesters were interrupted by a period of high pH, high sodium concentration as well a lack of magnesium in the feed. Section 4.9 presents and discusses results of such delineation.

4.9 Delineation of individual effect of Ni and Co when daily co-dosed at 7.5 micro moles

As with 2.50 µ moles daily dosage, the individual effects of Ni and Co when daily co-dosed at 7.5 µ moles were delineated and are presented in Figure 4.11 and Figure 4.12 respectively. These were computed in the same manner as was done during the 2.5 µ moles daily dosage period.

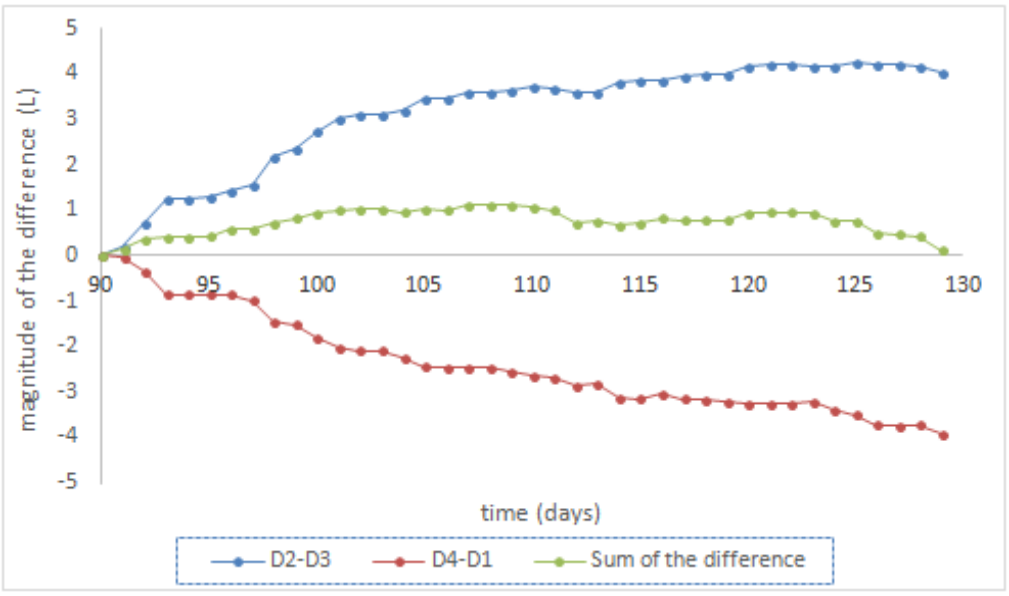


Figure 4.11. The magnitude of the difference between D2 and D3 and D4 and D1 as well as the sum of the differences, showing Ni effect. This was obtained when digesters were daily dosed 7.50 µ moles of Ni and Co.

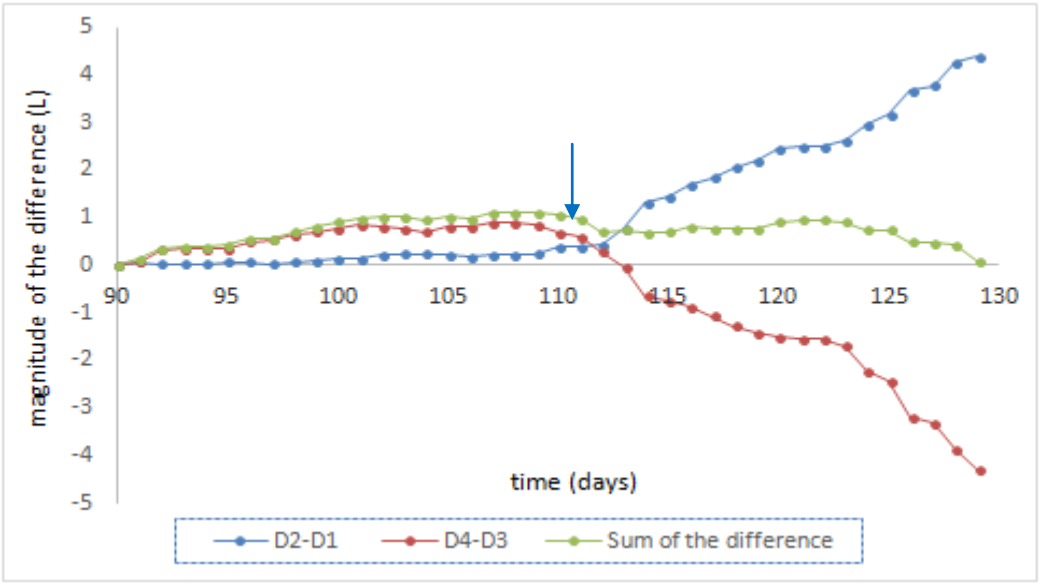


Figure 4.12. The magnitude of the difference between D2 and D1 and D4 and D3 as well as the sum of the differences, showing Co effect. This was obtained when digesters were daily dosed 7.50 µ moles of Ni and Co.

On day 114, D3's biogas production was larger than that of D4 (by 1.0 L) while D2 was exceeding D1; again this happened simultaneously and with the same magnitude of exceedance, Figure 4.9(a). The period from day 73 to day 113 is 4 times the HRT of the digestion system. This mean that by day 113, more than 90% of the reactor contents that were present in day 73 had been turned over. Lowering of digesters effluent pH to almost 7.0 on or around day 113 was observed. By day 113, the effect of lack of magnesium acetate or increased concentration of sodium in digesters is expected to have been diminished, so the pattern of digesters' biogas production was getting similar to that which occurred during the period of daily dosage of 2.50 µ moles; this could explain why D3 was exceeding D4.

The sum of percentage exceedance of D4 by D1 and D3 (50±2) % is found to be equal to that by which D2 exceed D4 (50±1) %; .i.e. [D1+D3=D2]. This is shown in Figure 4.15. This observation supports the prior observed positive effect of Co and Ni following their daily co-dosage at 2.5 µ moles. Figure 4.15 shows that the catalytic effect of Ni and Co when co-dosed

are additive during anaerobic digestion of acetate. Crucially, the co-dependence or co-requisite of Ni and Co in anaerobic digestion of acetate is also highly supported. The ability to delineate the Ni and Co effect when co dosed, Figure 4.11 and Figure 4.12 respectively as well as their additive effects provide support of the interconnectivity of Ni and Co's catalytic activity suggested in Figure 2.9.

A simple linear regression was conducted using Microsoft Office Excel 2013 to predict the amount of biogas that D2 exceeds D3 by, based on the amount of biogas by which D1 exceed D4; thus correlating the Ni effect in both D2 and D1. The same regression was done to predict the amount of biogas that D2 exceeds D1 by, based on the amount by which D3 exceed D4; thus correlating the Co effect in both D2 and D3.

The line fit plots of this analysis are presented in Figure 4.13. The amount of biogas by which D2 exceeded D3 based on the amount by which D1 exceed D4 could be predicted from the following equation: $Y = 1.273X$, with an R^2 of 0.923, while the amount by which D2 exceed D1 based on the amount by which D3 exceed D4 could be predicted from $Y=1.079X$, with an R^2 of 0.673. As was the case during the daily dosage of 2.5 µ moles, the coefficient of determination for Co effect is lower than that of Ni effect when determined using biogas data. It would be interesting to see what the correlation would be if CH_3-S-CoM is used instead of biogas data for Co effect determination as Co is plays a vital part in enzymes that lead to production of CH_3-S-CoM.

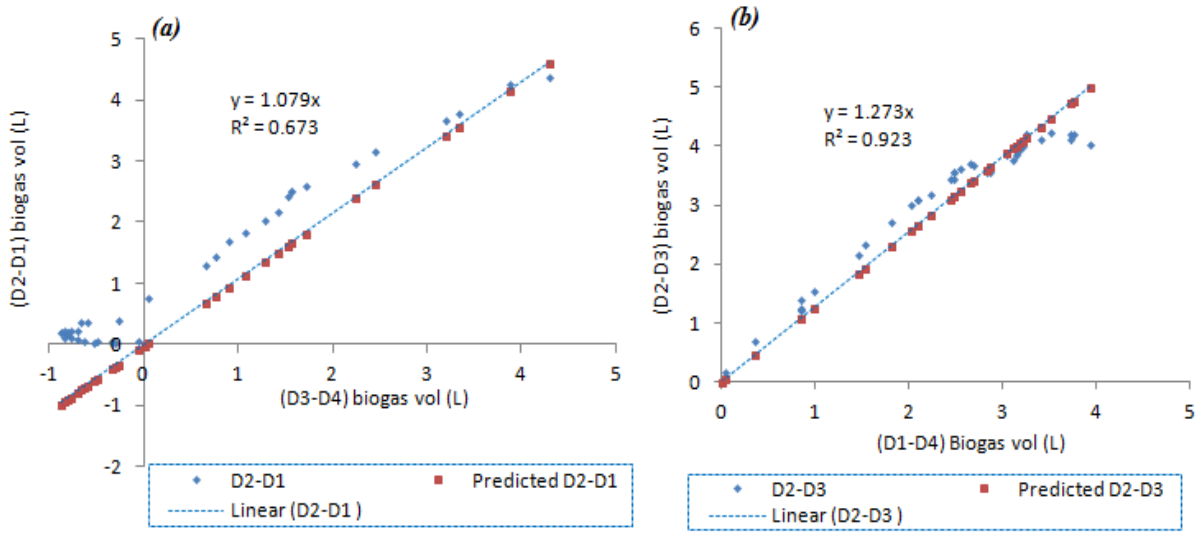


Figure 4.13. Line fit plot of the linear regression that correlates the effect of: (a) Co dosage in D2 and in D3, (b) Ni dosage in D2 and in D1.

4.10 Relationship between volatile solids and Ni and Co when digesters were daily dosed 7.50 micro moles of Ni and Co

This section shows possible degree of association of Ni and Co with biomass in the same manner as was done during the 2.50 µ moles daily dosage period. This is important as Ni and Co are anticipated to exert their effect through interaction with microorganisms, which can be represented by the use of volatile solids (VS). It was anticipated that at stimulatory concentrations those digesters that had a high association of Ni and Co with volatile solids will produce more biogas compared to those which do not. Digesters that were not dosed neither Ni or Co were expected to show a decrease in these elements' concentration with time while those that were dosed were expected to show an increase in their concentration. Data showing the measured concentration of metal ions and volatile solids on various days is presented in section 4.19.1.5.

The measured cobalt in D1 and D4 was 0.028 mg/L (0.473 µM) and 0.015 mg/L (0.249 µM) respectively on day 120, while its calculated concentration is anticipated to be almost zero.

The measured Co mass per VS in these reactors were 2.43 µg/g VS for D1 while D4 had 1.27 µg/g VS on day 120, Table 4.5. The cobalt concentrations present were found to be limiting biogas production of these digesters as D3 and D2 with an average measured Co concentration of 0.736 µM or 0.043 mg/L, corresponding to 3.31µg/g VS produced more biogas than D4. For data on measured cobalt concentrations see Table 4.4.

The average measured Co/VS of D1 and D4 on day 120 was 1.85 µg/g VS. This mean that there was more association of Co with VS in D3 and D2 than in D1 and D4, ≈ 44%more, hence this Co was able to exert its catalytic effect in D3 and D2 as shown by their higher production of biogas compared to D1 and D4, Figure 4.9(a).

The measured cobalt in D2 and D3 despite being lower than the dosed was found to be stimulatory to biogas production as D2 produced more biogas that D1, as well as D3 producing more biogas than D4. The calculated concentration of Co on day 120 in those digesters that were dosed Co was found to be 0.872 mg/L while in those digesters that were not dosed Co it is expected to be undetectable or almost zero. This was calculated taking into account the washoutand daily dosage of Co in a CSTR type digester. By the end of the period of 7.50 µ moles daily dosage, Co is expected to have increased from 0.612 mg/L on day 90 to 0.879 mg/L on day 129, while Ni is anticipated to have increased from 0.610 mg/L to 0.876 mg/L in the same period.

Ni measured in D4 on day 120 was 0.035 mg/L or 0.604 µM corresponding to a Ni/VS concentration of 3.07 µg/g VS, Table 4.6 and Table 4.7 respectively. This represents a percentage molarity reduction of 72% from that of day 12. This nickel concentration was found to be limiting the performance of D4, as it produced the least amount of biogas compared to those digesters dosed Ni by the end of the 7.5 µ moles daily supplementation period, Figure 4.9(a). The amount of nickel measured in D2 on day 120 was 0.293 mg/L (5.0 µM) corresponding to Ni/VS ratio of 17.78 µg/g VS. This represents approximately six times

more association of Ni with VS in D2 compared to in D4. The concentration of Ni present in D1 and D2 was found to be stimulatory to biogas production as these digesters exceeded D4 in biogas production in addition to D2 exceeding D3. Theoretical or calculated Ni concentration in those digesters that were dosed Ni was on day 120 found to be 0.869 mg/L, while in those digesters that were not dosed Ni it is expected to be almost zero. The calculation took into account both washout and daily dosage in a CSTR type digester.

The overall overview of performance of digesters in comparism to their control digester (D4) at all dosages is presented in section 4.11

4.11 Performance of digesters in comparism to a control digester at all dosages used: 2.50 micro moles to 7.50 micro moles

The average methane production per gram of acetate fed per day under each dosage condition is shown in Figure 4.14. A general decrease with time in the amount of methane, hence biogas produced is observed. This is correlated to the observed degrease in Methanosaetaceae population across all digesters with time. The decrease in the family Methanosaetaceae could be as a result of the used hydraulic retention time of 10 days; a digester operated at a 10 day hydraulic retention time was found to have a decline in Methanosaetaceae population accompanied by an increase in Methanosarcinaceae population byYu et al. (2006). Also in line with the observation made by Yu et al. (2006) the seed sludge used in this research was found to contain a higher proportion of Methanosaetaceae than Methanosarcinaceae possibly owing to the large retention time used in sewage digestion plant as well as the low acetate concentration in the effluent (digested seed sludge). The change in pH due to omission of magnesium acetate in the feed could also have negatively affected Methanosaetaceae more

than Methanosarcinaceae as these have different resilience level to change in environmental conditions as alluded to by Mortimer et al. (1981).

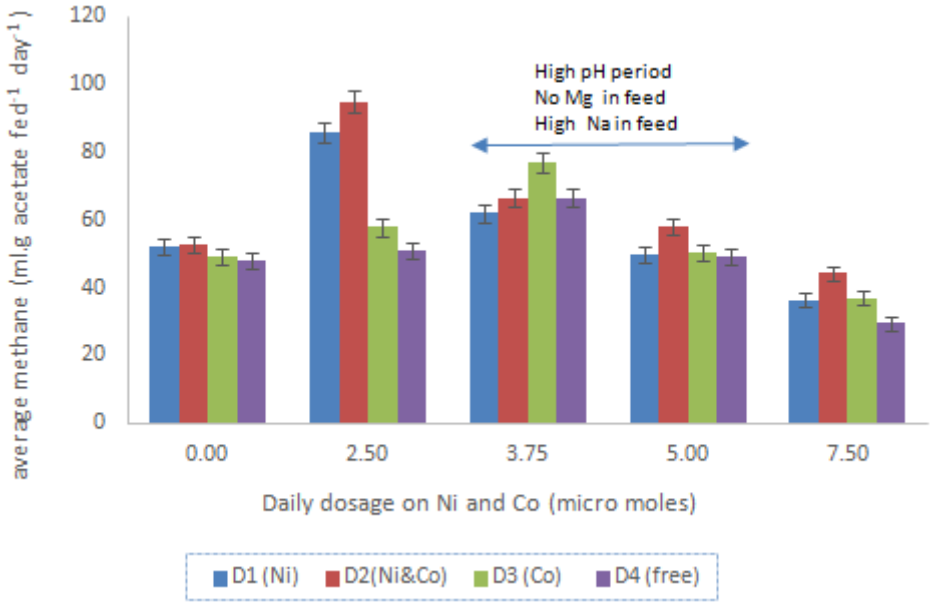


Figure 4.14. Average methane production (ml) from all digesters per gram of acetate fed per day under each dosage condition.

Before dosage of micronutrients, digesters produced similar amount of biogas, whereas after their dosage they produced different amount of biogas with the dosed digesters producing more biogas than the control digester at suitable pH and feed composition, Figure 4.14. The percentage by which a dosed digester exceeded the control digester (D4) in biogas or methane production at the end of a particular dosage period is presented in Figure 4.15. This was calculated via:

$$\text{Percentage Exceedance} = (\text{DX} - \text{D4})/\text{D4} * 100 \qquad Eq(12)$$

Where: DX=D1, D2 and D3's total biogas production under particular dosage conditions.

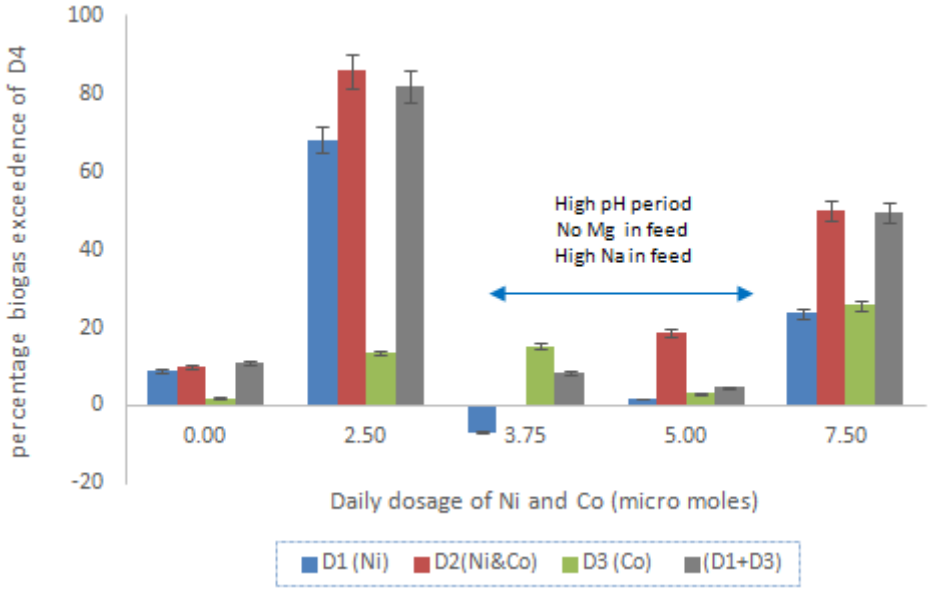


Figure 4.15. Percentage exceedance of the control by experimental digesters at various daily dosages of their respective micronutrients.

Figure 4.15 shows that D1, D2 and D3's biogas output increased by 59, 76 and 12 percent respectively as compared to D4's following their daily supplementation with 2.5 µ moles of their respective micronutrients over 23 days. This shows the individual and combined importance of Ni and Co to acetate degrading methanogens. The percentage performance increase of D2 (76) was observed to be close to the sum of that of D1 and D3, being 59+12=71%. This is in line with the view that Co and Ni are co-required as micronutrients for methanogens. The co-presence of these elements increased biogas production in an additive manner.

At the time of daily dosage of 3.75 µ moles and 5.00 µ moles of Ni and Co, all digesters showed reduced performance due to a change in pH as a result of lack of magnesium acetate or sodium toxicity or both. But following pH restoration and sodium concentration reduction, the performance of digesters was restored after a lapsed period equivalent to four times the hydraulic retention time of the system. At the end of the period through which digesters were daily dosed 7.50 µ moles of Ni and Co, the sum of percentage biogas volume output in excess of D4 by D1 and D3 (24+26 =50%) was equal to the percentage exceedance of D4 by D2

which is 50%. This again shows the additive effect of Ni and Co dosage in biogas production from acetate at stimulatory concentrations. This addictiveness is postulated to be due to Ni and Co's requisite in the interdependent enzyme system that catalyses methanogenesis.

The consistence/persistence of the observed equality of the sum of the individual effects of dosage of Ni and Co, to that of their combined dosage was tested or examined over their entire dosage period under suitable feed pH, day 0-12 and day 90-129. This was done to test or confirm that their additive effects were not caused by abnormal outlier values, as Figure 4.15 was obtained based on the total biogas produced over the a particular dosage period, not daily cumulative biogas volumes.

4.11.1 *Correlation of the sum of the individual effects of Co and Ni dosage to that of their combined dosage.*

A regression analysis was conducted using Microsoft Excel 2013 to test the equality of the sum of biogas volume by which D1 and D3 exceeded D4 to that by which D2 exceeded D4. This test if the additive nature of the effect of Ni and Co dosage held true throughout their dosage period at suitable pH and feed composition. Daily cumulative biogas was used in this analysis.

From the analysis, during the period of daily dosage of 2.5 μ moles and 7.5 μ moles the sum of biogas volume by which D1 and D3 exceeded D4 was almost equal to that by which D2 exceeded D4, Figure 4.16(a). A higher coefficient of determination (R^2) of 0.998 Figure 4.16(a) was realised during the period of daily dosage of 2.50 μ moles as compared to that of 0.942 observed during 7.50 μ moles daily dosage period, Figure 4.16(b). This is possibly due to the overall change in methanogens abundance with time as well as the changed feed effect.

The equal nature of the sum of biogas volume by which D1 and D3 exceeded D4 to that by which D2 exceeded D4 over two lengthy periods; day 12-35 and day 90-129 at different daily dosages; 2.5 μ moles and 7.5 μ moles respectively, shows to a great extent the repeatability

of this additive phenomenon. The ability to reproduce the biogas production pattern after 55 days of disruption due to feed composition change (day 35-90) also adds to the reproducibility of this observation. The observation that each of these elements produce similar amount of increase in biogas production in different digesters strongly suggests that it is the same order or families of methanogens that respond to the dosage of these elements; tentatively the order Methanosarcinales through the family Methanosaetaceae as per qPCR data.

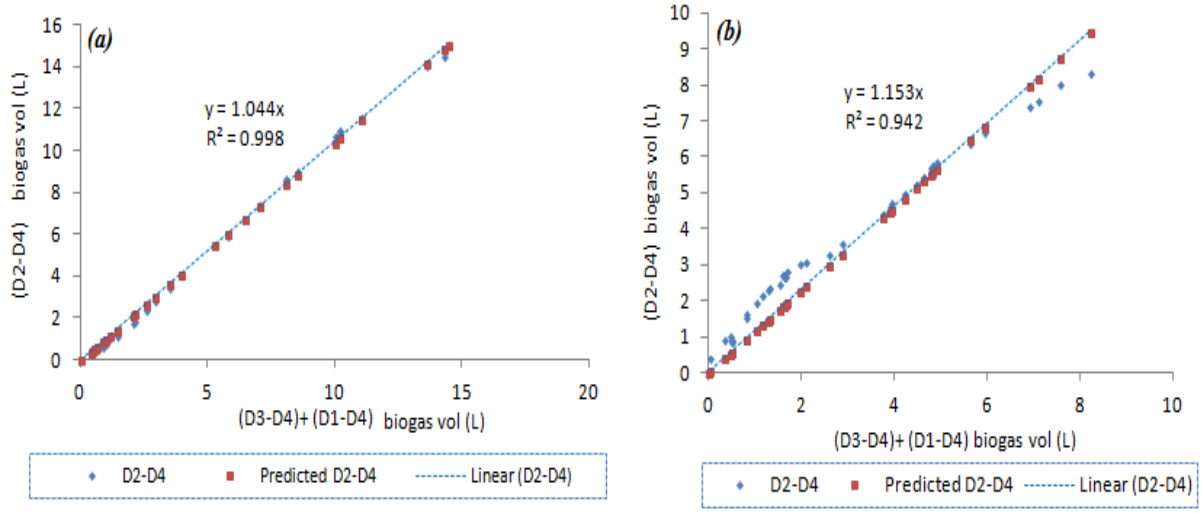


Figure 4.16. Comparism of the sum of the individual effect of Ni and Co dosage to that of their combined dosage at dosages of: a=2.50 µ moles, b= 7.50 µ moles.

D2 and D4 were dominated by Methanosaetaceae from day 7 until day 129 (see section 4.19.2) when differentiation of digesters through their dosage of micronutrients was ceased as per Table 3.1. As such the performance of D2 as compared to D4 from day 12 until day 129 on average reflect the response of Methanosaetaceae to the dosage of Ni and Co. This could explain the constant percentage of methane of 64 ±1 % produced by these digesters in this digestion period as Methanosaetaceae are only able to obtain energy from acetate splitting into carbon dioxide and methane (Kendall and Boone, 2006).

On day 129, D3 (Co) had 43% of its methanogens as Methanosarcinaceae while on day 122 D1 (Ni) was dominated by Methanomicrobiales by more than 98%. qPCR analysis for

methanogens on samples obtained on day 122 and 129 shows that all digesters experienced a marked reduction (>10 times) in Methanosarcinales (Figure 4.46). qPCR analyses was not conducted for D1 on day 129 as SMA assay was conducted with its effluent to check if this digester was still alive in view of its lack of biogas production, Figure 4.9(a), hence no sample was kept. This reduction in Methanosarcinales population was accompanied by cessation of biogas production from day 120-124 across all digesters, Figure 4.9(a). D1 had the highest reduction in Methanosarcinales and an increase in Methanomicrobiales as well as the highest reduction in biogas production. The increase in Methanomicrobiales in D1 was not further investigated. The increase in Methanomicrobiales observed in digesters D1 suggest that in addition to acetate splitting, acetate oxidation and possibly subsequent reduction of CO_2 by H_2 to CH_4 may have occurred in D1 as Methanomicrobiales are capable of CO_2 reduction by H_2 to CH_4 (Garcia et al., 2006). It is not clear why Methanomicrobiales increased in a Ni only dosed digester as Co is essential for syntrophic acetate oxidation and hydrogenotrophic methanogenesis, in addition to Ni being essential for the latter stage (Banks et al., 2012, Thauer, 1998). The increase in Methanomicrobiales seems to have not led to a significant or measurable biogas production as during the time of their dominance no measureable biogas was detected as shown in Figure 4.9(a). Gustavsson (2012) reported an increase in Methanomicrobiales in a Co as well as in a Ni limited mesophilic grain stillage digester, with a more evident increase observed in a Co as compared to a Ni limited digester. The increase in Methanomicrobiales reported by Gustavsson (2012) was also accompanied by digester instability as well as decrease in Methanosarcinales.

The observed increase in Methanomicrobiales at the time of reduction in Methanosarcinales and onset of reactor instability calls for the need for a comprehensive micronutrient dosage to digesters so as to improve their resilience. In food waste digesters containing high amount of ammonia that potentially led to inhibition and subsequent loss of acetoclastic methanogens,

the need for dosage of selenium (Se) and Co in maintaining digester stability and high organic loading rates was demonstrated by Banks et al. (2012). Their experiment showed the loss of acetoclastic methanogens and an increase in Methanomicrobiales; thus, dosage of Co and Se could potentially lead to recovery of D1 with Methanomicrobiales taking over biogas production, hence improving digester resilience to loss of acetoclastic methanogens.

4.12 Potential of recovery of D4 with Ni dosage; day 129-143

In view of continued lack of biogas production by D4, Figure 4.9(a), it was found necessary to examine the potential or possibility for its recovery through sole Ni dosage. Ni was chosen over Co because it had been shown to cause an increase in biogas production quicker than cobalt during the 2.50 μ moles daily supplementation period, day 12-35, Figure 4.1(a) as well as in the study conducted by Fermoso (2008). The effect of dosage of Co to D1 and Ni to D3 were also examined. It was anticipated that these digesters will have an increased biogas production after inclusion of Co and Ni respectively in their feed i.e. D1+Co and D3+Ni. D2 was not dosed Ni or Co in order to examine if its biogas production will reduce as Ni and Co washes out with effluent. Ni and Co were supplemented at 2.50 μ moles per day. By this day the calculated concentration of Ni and Co in those digesters that had been previously dosed these elements was 14.924 μM (≈0.880 mg/L) while in those digesters that had not been dosed was expected to be almost zero on day 129.

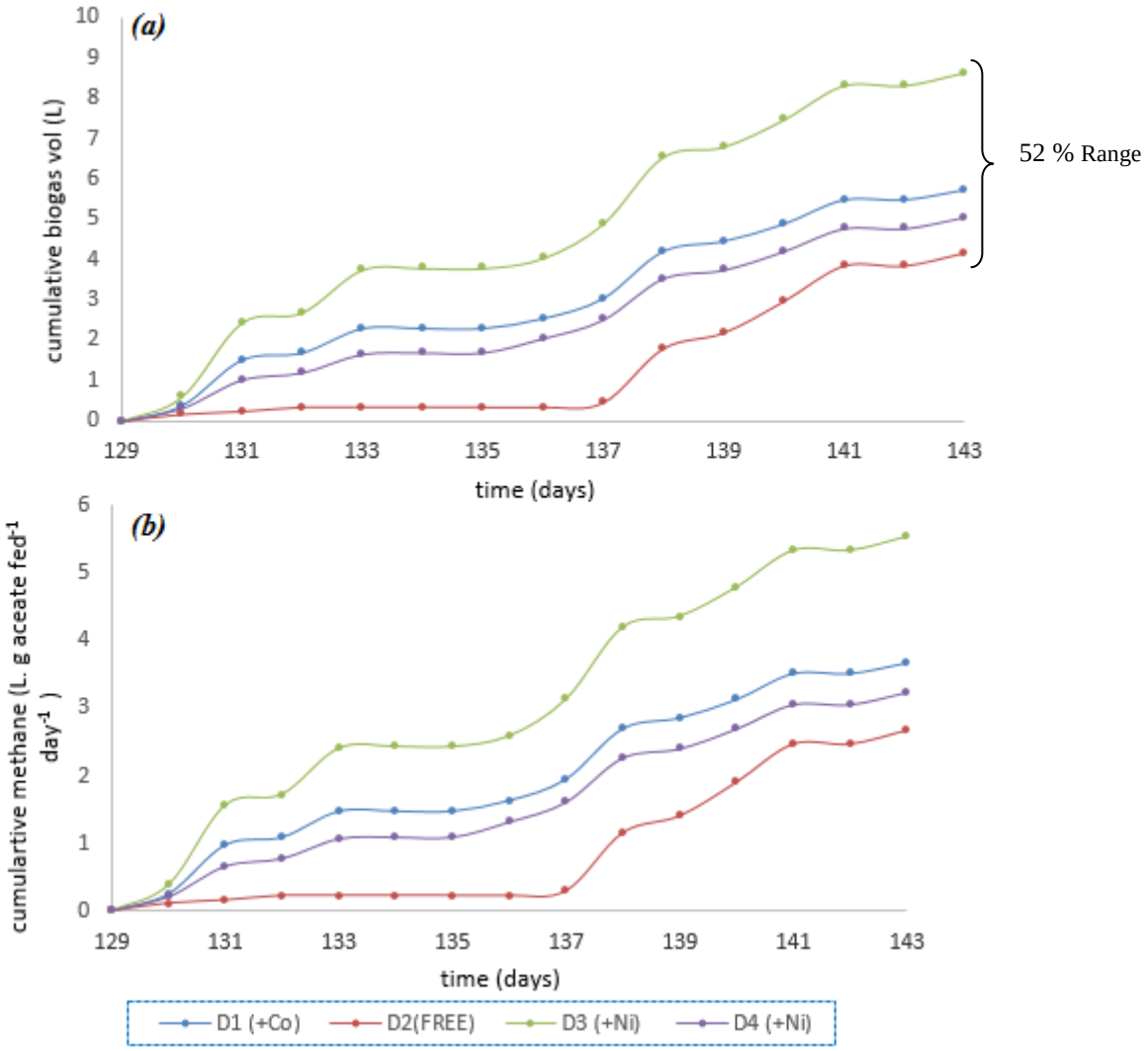


Figure 4.17. Digesters' performance under changed micronutrients dosage as per the legend. (a=cumulative biogas volume, b= cumulative methane L .g acetate fed^{-1} day^{-1}).

Digesters were fed 0.6 g acetate L^{-1} day $^{-1}$ (≈ 60 mM) instead of 1.8 g acetate L^{-1} day^{-1} (≈ 30 mM); this was done because no effluent was withdrawn from digesters until day 135 in order to allow for recovery of D4 as its effluent was visibly thin. Following this change, digesters produced biogas as follows: D3, 1.5 L/d, D1, 0.75 L/d, D4, 0.5 L/d while D2 produced less than 0.5 L/d, Figure 4.17(a). D2 did not produce considerable amount of biogas from day 129-137, this cannot be associated with its non supplementation at this point, as no effluent was withdrawn until day 135. qPCR analysis presented in section 4.19.2, Figure 4.46, shows that on day 122 and 129 there was a marked reduction in methanogens population, especially

Methanosarcinales. Given that these had been the major proportion of total methanogens population especially Methanosaetaceae, their reduction in population is postulated to have caused the lack of or great reduction in biogas production in all digesters around this period (day 122-129).

On day 135, 600 ml of effluent was withdrawn and all digesters were fed 9.0 g acetate as normal. This was followed by a slight biogas production from D3, D1 and D4, but this production cannot be credited to the new feed as no biogas was produced prior to feeding, as such acetate is deemed to have been available at all times prior to feeding. On day 137 all digesters showed an increase in biogas production with D3 having the highest production of 2 L/d while all digesters produced approximately1 L/d.

From day 123-129, before Ni addition to D4, it produced approximately less than 1.0 L of biogas (0.2 L/d) while it produced 1.0 L from day 129-131 (0.5 L/d), Figure 4.17(a). This signalled a slight recovery of D4 due to nickel dosage. qPCR data (Section 4.19.2) shows that 86% of methanogens in a sample from D4 obtained on day 129 were Methanosaetaceae, this suggest slight activation of this family through Ni dosage.

From day 123-129, D1 produced less than 1.0 L of biogas ($\leq$ 0.2 L/d) while from day 129-131 following its daily supplementation with 2.5 µ moles of Co, it produced 1.5 L (0.8 L/d). Microbial data show that on day 122, D1 was dominated entirely by Methanomicrobiales, except for 1% of Methanosaetaceae. Since Methanomicrobiales cannot utilise acetate for energy production (Garcia et al., 2006) if they were the dominant methane producers, they would have grown in syntrophy with an acetate oxidiser, but not a considerable/measurable amount of biogas was produced by D1 at the time of their dominance (day 122) Figure 4.17(a). The composition of methane in biogas did not change with the new dosage, Figure 4.17(b).

By the end of this period, day 129-143, biogas production had been recovered in D4 through sole nickel dosage and also within a short period of time (2 days). A similar observation was made on day 12 as biogas production increased following daily dosage of 2.5 µ moles of nickel to digesters, Figure 4.1(a). Biogas production was also recovered in D1 (Figure 4.17a) after six days of no biogas production, this signified the need for both Co and Ni in a digester, especially a Methanosarcinales dominated one.

On day 129 the measured Ni and Co concentrations were 0.402 µM and 0.248 µM (0.024 and 0.015) mg/L respectively in D4; Table 4.4 and Table 4.6. These corresponds to 1.27 Co µg/g VS and 2.19 Ni µg/g VS, these concentrations are in the range previously found to be limiting. Note that Day 120 Co concentration is more applicable than the one obtained on day 129. The concentration of Ni in D3 on day 129 was not detected, most likely it was below detectable level (1.0 ppb).

4.13 Performance of digesters following their daily co-dosage with 2.50 micro moles of Ni and Co

In order to examine if the prior observed variability in performance of digesters at suitable feed composition and pH was due to their dosage with different micronutrients, on day 143 all digesters were daily dosed 2.50 µ moles of Ni and Co. Digesters' performance following this change in dosage is presented in Figure 4.18. After this change, digester's performance was observed to be getting similar or closer to each other.

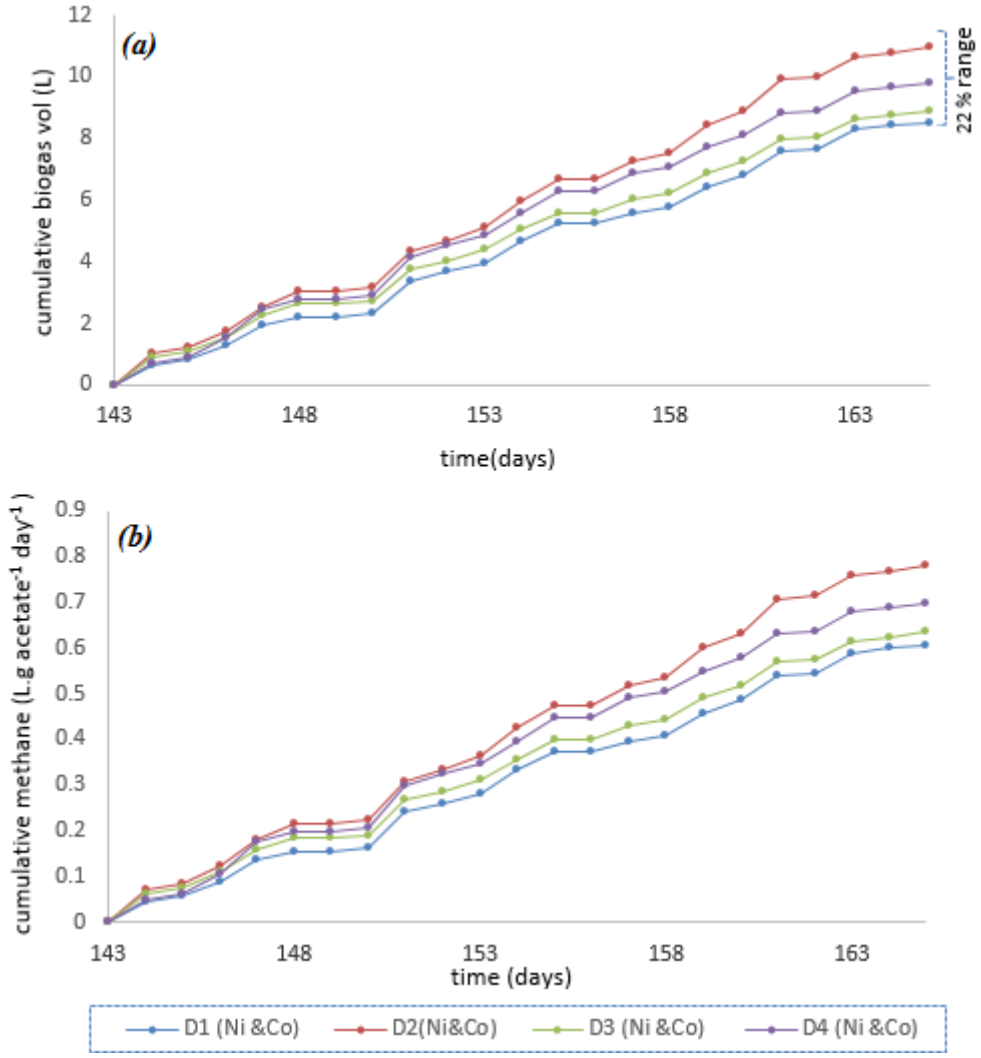


Figure 4.18. Performance of digesters when they were all daily dosed 2.50 µ moles of Ni and Co. (a=cumulative biogas volume, b= cumulative methane L .g acetate fed^{-1} day^{-1})

From day 129 to 165 (36 days) the percentage range of total biogas produced was 52% while that of from day 143-165, when all digesters were dosed the same micronutrients was reduced to 22%**.** This is the lowest percentage range across digesters performance while running under a suitable feed composition and pH following micronutrients dosage. This lowering of percentage range or variability of digesters performance is attributed mainly to their supplementation with the same micronutrients, hence removing the cause of prior observed variability in their biogas production. This again exhibits the need to co-dose Ni and Co for

increased biogas production from acetate digesters, especially those which are deemed to be at risk of acidity.

4.14 Summary of part one

Biogas production from 5.0 L digesters operated at a loading rate of 1.8 g acetate L^{-1} d^{-1} was investigated before and after their daily supplementation with Ni, Co and a combination of Ni and Co while a control digester was not dosed. Results show that prior to their differentiation through dosage of their respective micronutrients, digesters biogas production was similar, with a maximum range of 10%; being D2 above D4. At the time of dosage of Ni and Co to digesters, their measured average concentrations of these elements were respectively, 2.427 µM ± (0.371) and 1.145 µM (±0.183). At the same time the calculated concentration of Ni and Co was found to be 0.745 µM and 0.679 µM. These concentrations were found to be either limiting or not available to methanogens for utilisation as after dosage of Ni and Co biogas production increased. From day 12-35 digesters were daily dosed 2.5 µ moles as follows: D1 (Ni), D2 (Ni and Co), D3 (Co) while D4 was the control. Following dosage, those digesters that were dosed Ni (D1 and D2) showed an immediate and almost equal increase in biogas production while that which was dosed Co only (D3) showed an increase in biogas production above D4 13 days later.

The proportion of methane in biogas did not vary with dosage of micronutrients nor temporally, it was constantly 64±1 percent. Before and after dosage of micronutrients, qPCR data show that the dominate methanogens across all digesters were of the family Methanosaetaceae, section 4.19.2; owing to their dominance in the seed sludge. As such biogas production from digesters from day 12-35 mainly reflects the response of Methanosaetaceae to dosage of Ni and or Co. By the end of the period during which digesters

were daily supplemented with 2.5 µ moles of Ni and Co; D1, D2 and D3 had produced 68, 86 and 14 percent more biogas that D4 respectively. The sum of percentage biogas volume by which D1 and D3 exceeded D4 was found to be close to that by which D2 exceeded D4 (68+14=82). This supports the additive nature of catalytic effect of Ni and Co in biogas production. The contribution to biogas production by Ni and Co when co-dosed was delineated and each presented separately. These were found to be in line with the interconnectivity and co-dependence in terms of biogas production, possibly through the interconnectivity mechanism suggested in section 2.4.2.1, Figure 2.9. The delineated contribution of each element as well as their additive effect in biogas production were examined through regression analysis and a strong correlation was found for each.

On day 35 the daily dosage of Ni and Co was increased to 3.75 µ moles, i.e 0.22 mg of Ni and 0.221 mg of Co. The acetate loading was kept constant at 1.8 g acetate L^{-1} d^{-1} (≈30 mM). On day 40, the composition of the feed was changed due to depletion of magnesium acetate in the laboratory. The new feed was composed of calcium acetate and sodium acetate in the molar ratio of 1:1, leading to doubling of sodium in feed from 7.5 mM to 15.0 mM and the feed stock pH increasing to 8.7 from 7.0-7.5. This possibly resulted in sodium toxicity to methanogens (Bashir and Matin, 2004) and or a pH shock (Mortimer et al., 1981). DNA yield data show that on day 77 (4 days after pH restoration) the yield across all digesters was reduced to below 1µgDNA/g VS equivalent biomass section 4.19.2. This is further seen as a reflection of the sensitive nature of methanogens that limit their growth and metabolism when environmental conditions are out of those physiological optimum for their growth (Mortimer et al., 1981).

On day 60-90, the daily dosage of Ni and Co was increased to 5.00 µ moles of Ni and Co, i.e. 0.293 mg of Ni and 0.295 mg of Co. Biogas production from digesters during periods which

coincided with high pH and increased sodium concentration are not credited to their dosage with different micronutrients.

On day 73, on arrival of magnesium acetate, the pH was restored and 4*HRT (day 113) from the day of pH restoration biogas production increased from all digesters. On day 77 Methanosarcinaceae accounted for: 29%, 20%, 5% of methanogens in D1, D2 and D4 respectively, while a negligible amount was observed in D3 which was mainly dominated by Methanosaetaceae (4.19.2).

From day 90-129, the daily dosage of Ni and Co to digesters was increased to 7.50 µ moles i.e. 0.440 mg of Ni and 0.442 mg of Co. By the end of this dosage period, biogas production from digesters was observed to be getting similar to that observed during the period of daily dosage of 2.50 µ moles (day 12-35). This is anticipated to be due to turning around of digester contents at more than 3*HRT of the digestion system, thus restoring pH to near neutrality as well as lowering Na concentration hence its toxicity chances. At the end of the period of 7.50 µ moles daily dosage, day 90-129; D1, D2 and D3 had cumulatively produced 24, 50 and 26 percent more biogas than D4 respectively. The sum of the percentage by which D1 and D3 exceeded D4 was found to be equal to that by which D2 exceeded D4; this again is credited to the additive nature in terms of biogas production of the effects of co-dosage of Ni and Co to acetate digesters.

The contribution to biogas production by Ni and Co when co-dosed was delineated at the end of the period of daily 7.50 µ moles supplementation. The results were found to be similar to those obtained during delineation of the Ni and Co effect during the 2.50 µ moles dosage period. This provide compelling support for the additive nature of the catalytic effect of co-dosage of Ni and Co in biogas production, as well as their interdependence or co-requisite as suggested in 2.4.2.1, Figure 2.9.

The measured Ni and Co in D4 on day 120 was 0.604 and 0.248 µM respectively, while the calculated ones were almost zero (Figure 4.36, section 4.19.1.6). These concentrations were found to be limiting biogas production of D4 as those digester that were dosed Ni and or Co produced more biogas than D4. qPCR data (4.19.2) show that D2 and D4 were dominated by the family Methanosaetaceae on day 77, 122 and 129. This suggests that the dominant digestion pathway in these digesters during these periods was acetoclastic cleavage by *Methanosaeta sp*. Methanosaetaceae were also the dominant family in D3, but on day 77 and day 129 there was 31 % and 40% of Methanosarcinaceae respectively in this digester. D1 was dominated by Methanomicrobiales on day 122 at about 98%, this is the highest recorded Methanomicrobiales abundance in this study, possibly this family grew in syntrophy with an acetate oxidiser. The contribution to biogas production of this family was not further investigated in this study; but it is not expected to be significant since on day 122 no biogas production was observed in D1 as well as on day 129, leading to conduction of SMA test to check if this digester was still alive, that is why there is no microbial data for D1 on day 129.

Dosage of 2.50 µ moles of Ni to D4 from day 129-143 led to recovery of its biogas production within two days. This showed the importance of Ni to Methanosaetaceae as they were the dominant family in D4 on day 129 as per qPCR data, section 4.19.2 , Figure 4.45(d).

From day 143 to 165, all digesters were supplemented with 2.5 µ moles of a combination of Ni and Co. After this change, digesters produced similar amount of biogas with a total percentage range of 22%, being D2 above D1 by day 165. This lowering of percentage range in digesters biogas production is attributed mainly to their supplementation with the same micronutrients, hence removing the cause of prior observed variability in their biogas production. Before differentiation of digesters through their dosage with different micronutrients, day 0-12, their biogas production was similar or close to each other with a maximum range of 10% being D2 above D4; this shows that variation in biogas production

between digesters after their differentiation thorough dosage was mainly due to the dosed micronutrients. On day 158, Methanosarcinaceae were the most abundant family of methanogens in D1, D2 and D3 with 70%, 70% and 51% abundance respectively, while D4 had only 20%, but it had Methanosaetaceae accounting for 67%. Dosage of Ni and Co led to an increase in the population of Methanosarcinaceae hence producing a shift towards their dominance. This could possibly have been exacerbated by the effect of the used 10 day hydrolytic retention time on Methanosaetaceae (Yu et al., 2006) as well as the increase in the real time acetate concentration that would favour growth of Methanosarcinaceae as not all of the fed acetate was daily converted to biogas (Conklin et al., 2006).

Daily co-dosage of 2.50 and 7.50 μ moles of Ni and Co was found to have an additive effect on biogas production. This is most likely due to the co-requisite of Ni and Co in methanogenesis. When co-dosed, the effect of Ni dosage and that of Co dosage on biogas production were delineated, thus further supporting their co-requisite. During the period of daily dosage of 3.75 and 5.00 μ moles of Ni, Co and Ni and Co there was no increase in biogas production; this was associated with sodium toxicity and a high pH as magnesium acetate was omitted from the feed and the proportion of sodium acetate in the feed was increased. These conditions are out of those physiological optimum for growth of Methanosarcinales (Kendall and Boone, 2006); thus providing compelling support that biogas production prior to this change in conditions was due to Methanosarcinales. Daily dosage of 2.50 μ moles of Ni was found to cause an increase in biogas production quicker (3.3 times) than that of cobalt. During the period where digesters were differentiated through their dosage with different micronutrients, the dominate methanogen family across all digesters was Methanosaetaceae followed by Methanosarcinaceae, with the exception of a Ni only dosed digester, D1, which on day 122 was dominated by Methanomicrobiales. Towards the end of

the experimental period there was a general observed increased in the presence of Methanosarcinaceae.

To test the significance of the effect of individual dosage of Ni, Co and that of their combined dosage, a t-test was conducted at 95% confidence interval on each of the experimental digesters against a control digester (D4). All digesters were found to have produced significantly more biogas (methane) than a control digester. The significance of the individual dosage of Co on biogas production was found to be lower than that of the individual Ni dosage as well as that of the combination of Ni and Co dosage.

Through regression analysis, the effect on biogas production of the dosage of Ni to D1, i.e. D1-D4 and to D2 i.e. D2-D3 were found to be highly correlated; as well as the effect of dosage of Co to D3, i.e. D3-D4 and to D2 i.e. D2-D1. During both the 2.50 μ moles daily dosage period and the 7.50 μ moles daily dosage period a stronger correlation was observed for the Ni effect in D1 and D2 than for the Co effect in D3 and D2. This is possibly due to these element's points of catalytic action during methanogenesis from acetate. With a regression analysis summation of the effect of Ni and Co when separately dosed i.e. [(D1-D4) + (D3-D4)] was found to be strongly/ highly correlated to that of their combined dosage (D2-D4) during both the 2.50 μ moles dosage period and the 7.50 μ moles dosage period. This support the co-requisite for Ni and Co during anaerobic digestion of acetate, which is possibly expressed through the interconnectivity presented in Figure 2.9-page 38.

More biogas hence methane was produced during the period of daily dosage of 2.5 μ moles (day 12-35) than during the period of daily dosage of 7.5 μ moles (day 90-129). The average daily methane production (ml) per gram of acetate fed by all digesters from day 12-35 was: 85.50, 94.78, 57.89 and 51.00 for D1, D2, D3 and D4 respectively. The same measure from day 90-129 was: 36.43, 44.24 36.06 and 29.46 for D1, D2, D3 and D4. These represent a percentage reduction from each digester of 57%, 53%, 35% and 42% for D1, D2, D3 and D4

respectively. This reduction in methane production hence biogas is possibly due to reduction in Methanosarcinales population with digestion time, more especially the family Methanosaetaceae with digestion time.

As such the positions of daily dosage of 2.50 and 7.50 μ moles of Ni and Co relative to each other on the dose response curve were not able to be determined from this study; only that these daily dosages were simulative to methanogenesis can be determined.

Standardisation of biogas data with methanogens data obtained through qPCR is mathematically possible, but the low accuracy of qPCR in quantifying the abundance of microorganisms hinders the practicality of this. Also qPCR data was obtained at a much lower frequency compared to biogas data which was measured in 10 minutes intervals.

Individual and combined daily dosage of 2.50 μ moles of Ni and Co from day 12 to day 35, thereby increasing their nominal concentrations from 0.272 μM (0.016 mg/L) and 0.199 (0.012 mg/L) to 4.873 μM (0.286 mg/L) and 4.867 μM (0.286 mg/L) respectively, was found to be stimulatory to biogas production from the anaerobic digestion of acetate. At 2.50 μ moles daily dosage the dosed digesters produced significantly more biogas, hence methane than a non-dosed digester (control). The stimulatory or catalytic effect of Ni (increased biogas production) was observed at its nominal concentration of 1.898 μM (0.111 mg/L) on day 15, while that of Co was observed at its nominal concentration of 3.901 μ M (0.230 mg/L) on day 25. It is inclusive whether this disparity was due to the lagged Co effect or the higher threshold for Co in this digestion system.

Also individual and combined daily dosage of 7.50 μ moles of Ni and Co from day 90 to day 129 thereby increasing their nominal concentrations from 10.397 μM (0.610 mg/L) and 10.379 μM (0.612 mg/L) to a concentration of (14.924 μM), approximately 0.876 mg/L for Ni and 0.879 mg/L for Co, was found to be stimulatory to biogas production from anaerobic

digestion of acetate. At 7.50 μ moles daily dosage, dosed digesters also produced significantly more biogas than a non- dosed digester (control).

The catalytic effects, in terms of biogas production, of Ni and Co when co-dosed were found to be additive. This to a large extent supported the need for their co-dosage in acetate anaerobic digesters. With and without dosage of Ni or Co, the percentage of methane in biogas was found to be constant at 64±1%.

4.15 PART TWO

This part examines the onset of the reduction of the production of biogas from anaerobic acetate digestion due to a high concentration of individual and a combination of Ni and Co. This section in essence examines the onset of region D of the hypothetical dose response curve. A similar dose response curve was proposed by Fermoso (2008) for a methanol UASB reactor. The reduction or cessation of biogas production at high Ni and/Co dosage is anticipated to be caused by the toxicity of these elements to methanogens. Knowledge of the toxic concentration of micronutrients of interest to methanogens is necessary as it informs the digester operator which concentration not to cross or dose beyond.

It is important to consider the total concentration of metal ions (micronutrients) of interest as their toxicity is not expected to be exerted in a selective manner as does their catalyzation of biogas production in cognisance of the mechanism through which toxicity is exerted such as: disruption of DNA replication, disruption of enzyme production as well as damage to the cell structure (Babich and Stotzky, 1983). This is in contrast to activation concentration which is expected to be specific to each enzyme. i.e. the Ni catalysed or activated enzyme or enzymes will not affect the Co catalysed enzymes, but toxicity of Co, Ni or their combination is expected to affect the viability of the whole microbial cell. The effects of Ni and Co toxicities were found to be different from each other by Wu et al. (1994) thus suggesting that each of this ion exert toxicity on its own, but their combined toxicity affect the viability of the microbial cell as a whole. As such when co-dosed, the total Ni and Co ions concentration is taken into account rather than their individual concentrations in assessing their toxicity.

To avoid a sudden collapse of the digesters due to a high concentration of Ni and Co, most of this part of the research was done off-line using effluent from digesters. 100.0 ml of a well mixed effluent from each digester was pre-concentrated to 20.0 ml through decantation in

120.0 ml serum bottle. After 80.0 ml of supernatant was discarded, 20.0 ml of 300 mM acetate solution was added together with 60.0 ml of RO water, creating an acetate solution of at least 60 mM. A desired amount and combination of Ni and Co was then dosed to this serum bottle to a final desired concentration. This acetate solution was then digested in a 37±2°C temperature controlled room. The biogas produced was measured daily using a manometer. These experiments were carried out in triplicates, and as the control serum bottles were not dosed any micronutrients, they gave an indication of the specific biogas production potential of their parent digesters on the day of the sample obtainment.

It is worth noting that the percentage of methane in the serum bottles when there was no difference in the biogas volume between the dosed and non-dosed serum bottles did not vary with or without dosage of micronutrients, as such the percentage difference in the biogas production between the dosed and non-dosed serum bottle was the same as the percentage difference in methane production between the dosed and non-dosed serum bottles. This is shown in appendix A. The concentration of the methane in the serum bottles when the biogas production was not impaired by high concentration of Ni and Co was 75±1%. When toxicity was exerted by a high concentration of Ni and Co, the percentage of the methane was reduced to about 45% (at 504.00 µM concentration). The biogas production of non-dosed serum bottles gave an indication of the specific methanogenic activity and specific biogas production (SBP) of each digester's sludge, this is because the difference in volatile solids concentration across the digesters was quite small (Figure 4.32) and can be approximated to 10.0 g/L. The performance of serum bottles dosed a high concentration of Ni and Co were standardised by that of the control serum bottle from the same parent digester.

4.16 Performance of digesters and serum bottles at high Ni and Co ion concentration

On days shown in Table 4.1, the effluent from each digester was used to conduct a biogas production assay with acetate as the substrate following dosage of various amounts of Ni and Co, also shown in Table 4.1. This was done to define the toxic range of micronutrients of interest (Ni and Co). This toxic range is represented by region 'D' of the hypothetical dose response curve.

Table 4.1 Regime used in conduction of specific biogas production assay at high metal ion concentration.

Day of SMA start and effluent obtainment	Number of moles added ($X\ 10^{-6}$))	Total metal ion target conc. µM of Ni, Co, Ni and Co in bottle (excluding the background conc).
75	50.400	504.00
95	12.600	126.00
109	6.300	63.00
113	3.150	31.50
122	1.575	15.75

On days shown in Table 4.1, 600.0 ml of a well-mixed effluent was obtained from each digester and biogas production assay at various metal ion concentration was conducted in triplicates using 100.0 ml of effluent per serum bottle. The other half of the effluent, 300.0 ml was used for control studies, also in triplicates. Biogas production from a serum bottle that was dosed to a large concentration of micronutrients (Ni and Co) was compared to that which was at the same concentration as the parent digester. The parent digester concentration level was used as a control as opposed to the use of the non-dosed digester (D4) as those microorganisms that had been exposed to particular metal ions tend to develop more tolerance due to their acclimatization. This has been shown during the study of Na toxicity by Bashir and Matin (2004) who observed that prior exposed of cell to Na improve their tolerance to it. With the exception of D4's effluent which was dosed both Ni and Co, while the parent

digester was not dosed, all other biogas production study samples were dosed the same metal ion combination as their parent digester i.e. (D1(Ni), D2(Ni and Co), D3(Co) and D4 (Ni and Co). Where both Ni and Co were added (D2 and D4 serum bottles) the total targeted ions concentration was composed of the sum of individual Ni and Co ion's concentration. The following graphs show biogas production from serum bottles when dosed to various Ni and Co concentrations. A general trend of increase in biogas volume production deficit between the dosed and non- dosed serum bottles was observed with increase in dosage of Ni and Co.

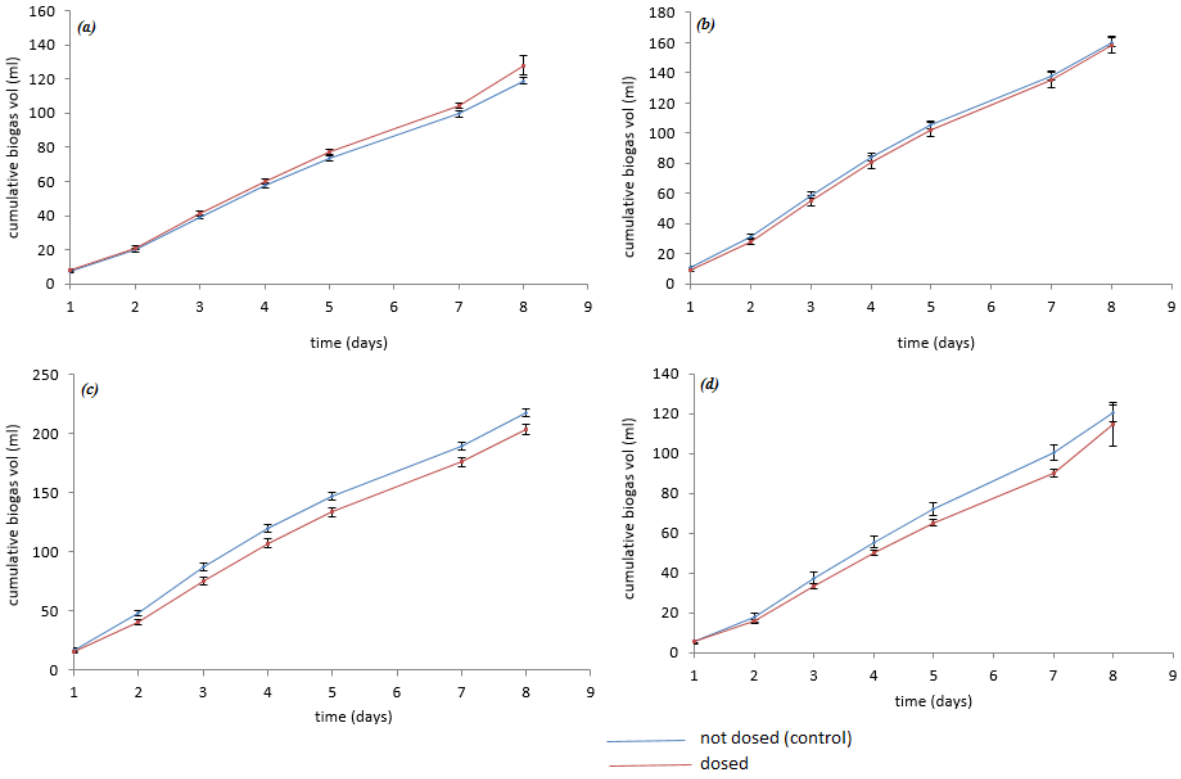


Figure 4.19. Cumulative biogas production from serum bottles when dosed to 15.75 µ molar. (a= D1, b=D2, c=D3 and d=D4).

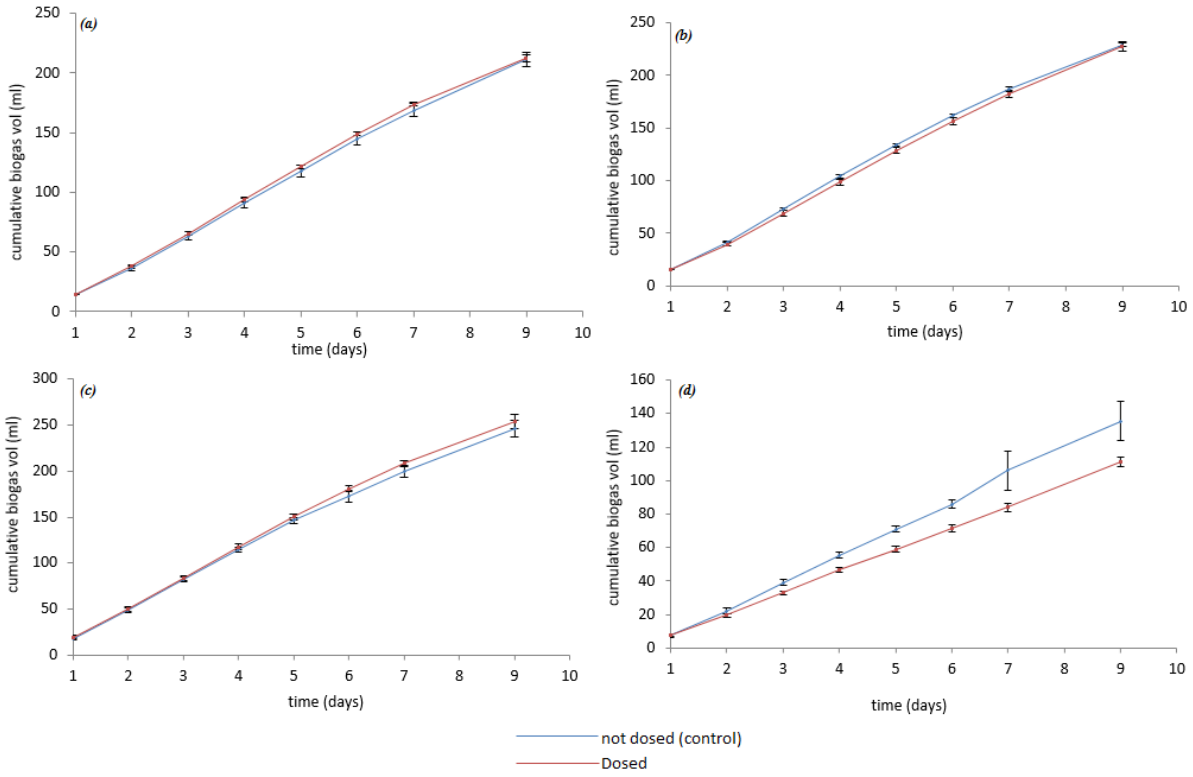


Figure 4.20. Cumulative biogas production from serum bottles when dosed to 31.50 µ molar. (a= D1, b=D2, c=D3 and d=D4).

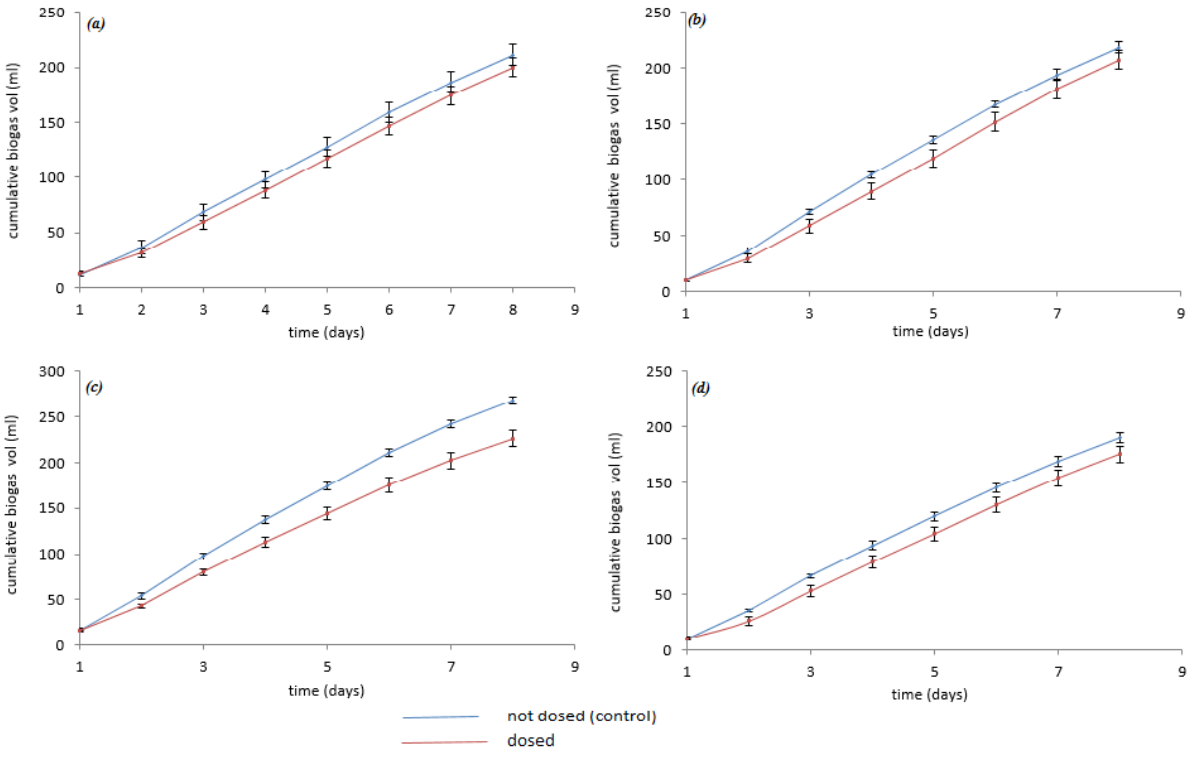


Figure 4.21. Cumulative biogas production from serum bottles when dosed to 63.00 µ molar. (a= D1, b=D2, c=D3 and d=D4).

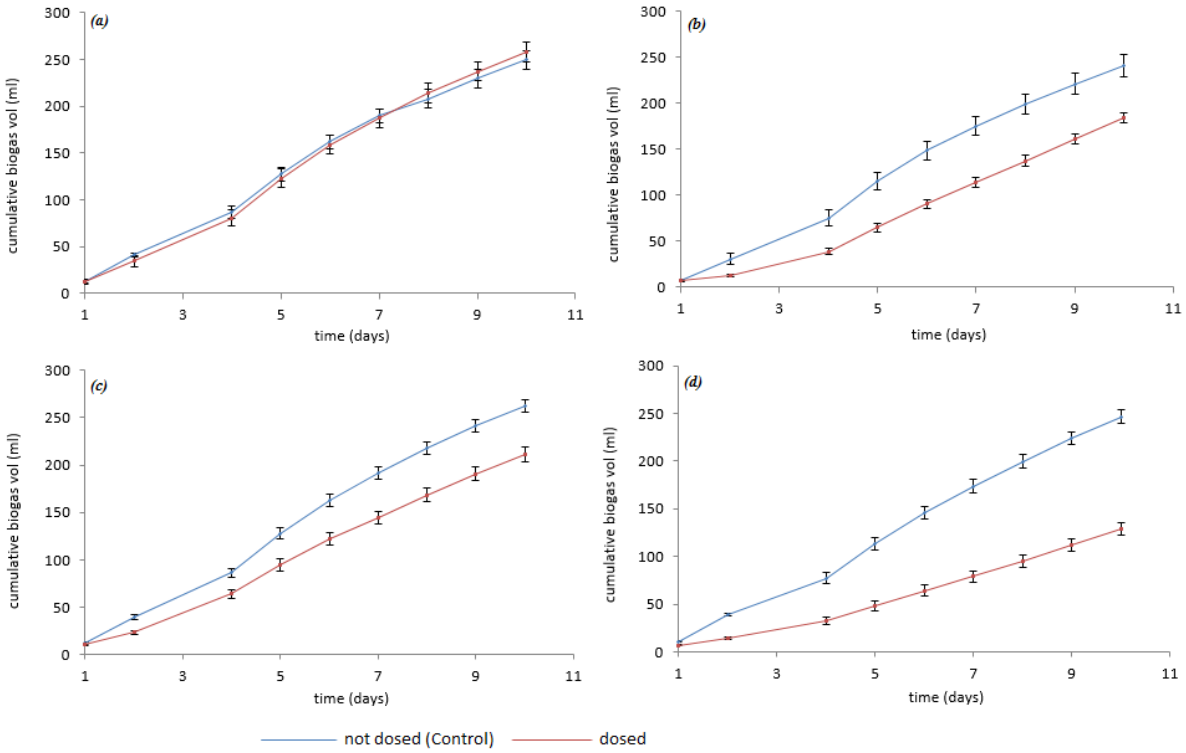


Figure 4.22. Cumulative biogas production from serum bottles when dosed to 126.00 µ molar. (a= D1, b=D2, c=D3 and d=D4).

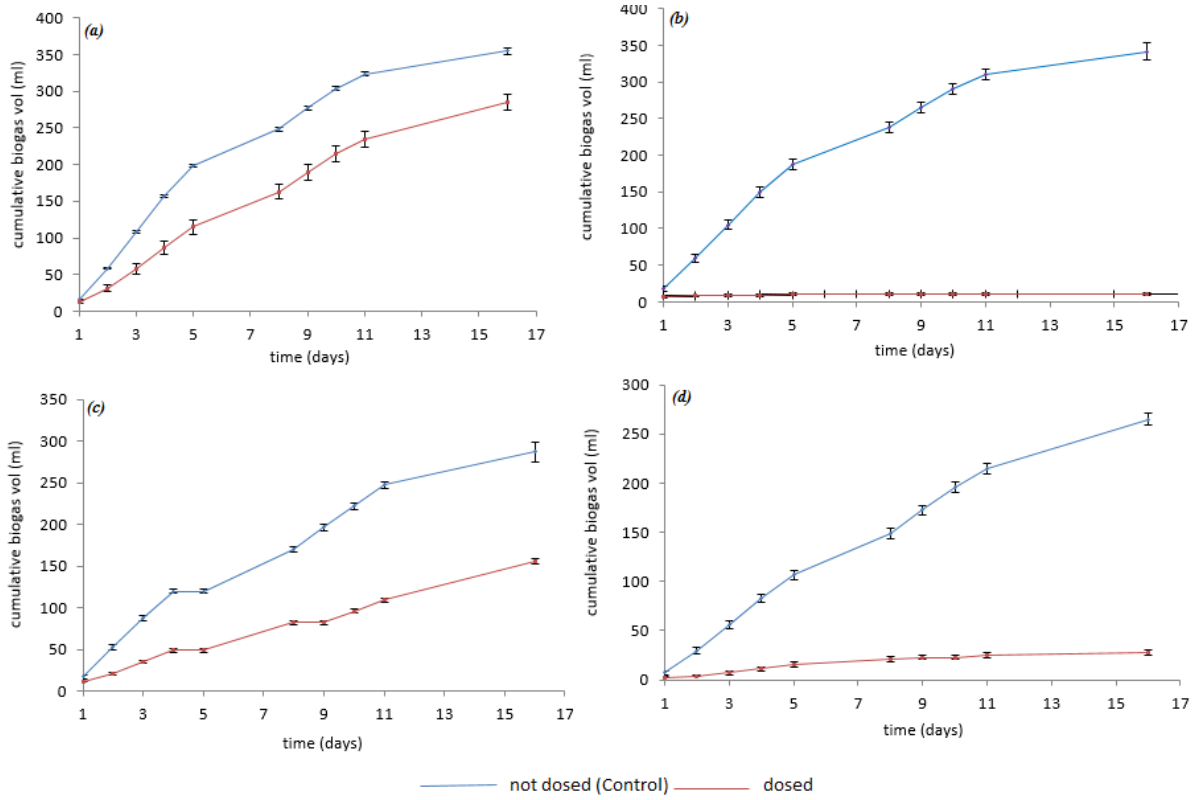


Figure 4.23. Cumulative biogas production from serum bottles when dosed to 504.00 µ molar. (a= D1, b=D2, c=D3 and d=D4).

The percentage difference or deficit of biogas production between the dosed and non-dosed serum bottles are shown in Figure 4.24. These were obtained by using cumulative biogas volume produced during the first four days of incubation. The first four days cumulative biogas production period was selected because it covered the period during which maximum biogas production occurred in serum bottles (V_{max}), which possibly coincides with the exponential phase of microbial growth. The V_{max} of the SMA test conducted in a similar manner was found by Ishaq (2012) to be within 3-4 days of incubation. Biogas production from serum bottles is presented in presented in Figure 4.19 to Figure 4.23. The percentage biogas volume deficit between the dosed and non-dosed serum bottles was calculated from:

$$\%\ \text{volume Deficit} = (\text{Non Dosed} - \text{Dosed})\ /\text{Dosed} * 100\% \qquad Eq(13)$$

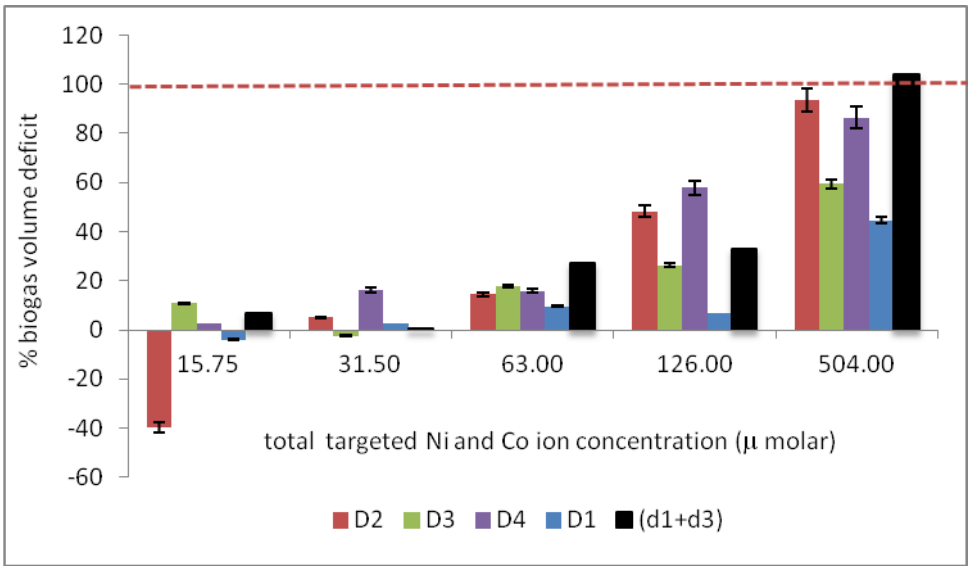


Figure 4.24. Percentage deficit in biogas volume compared to control as dosage of individual and combined Ni and Co to serum bottles is increased (summary of Figure 4.19 to Figure 4.23).

Individual and a combined dosage of Ni and Co were observed to exert inhibition or impairment of biogas production when dosed to a total ion strength or concentration of 63.00 µM (Figure 4.21 and Figure 4.24). For Co only dosage an increasing degree or level of inhibition of biogas production was observed from a concentration of 63.00 µ M to 504.00 µM, while no clear trend of increase was observable from the Ni dosage at the same concentration, but less biogas was produced from the dosed serum bottle compared to the control serum bottle (Figure 4.24). D2's seed sludge produced 40% more biogas when dosed to a combined Ni and Co ion concentration of 15.75 µM compared to its non-dosed controls, while D4'seed sludge produced approximately 5% less biogas compared to its controls at the same dosage, Figure 4.24. This can possibly be due to D2's slightly higher concentration of volatile solids that made it withstand toxicity or due to the tolerance of methanogens in D2 to Ni and Co as this digester was already dosed Ni and Co before, thus allowing acclimatization of its methanogenic population. When dosed to 31.50 µM, serum bottles seeded with D2 and D4' sludge showed reduced biogas production of about 2 % and 18% respectively compared to their control serum bottles (Figure 4.24). This is the first observed point or concentration in

this study where both the prior exposed and non-prior exposed seed sludge showed inhibition to biogas production. This concentration possibly represent the turning point of combined dosage of Ni and Co to toxicity, hence inhibition or reduction of biogas production. The turning point to toxicity or inhibition for combined dosage of Ni and Co is important for consideration in operating digesters since combined dosage of Ni and Co have been shown to increase biogas production more than their individual dosage. The individual effect of dosage of Ni and Co was not clearly defined at 31.50 µ M. 95% and 85% less biogas was produced at a combined Ni and Co concentration of 504.00 µM by D2 and D4 serum bottles compared to their controls respectively. This represents almost a lethal concentration of these ions.

Lin and Chen (1999) observed a 50% inhibition of biodegradation of a mixture of VFAs composed of acetic, propionic and butyric acid by a Ni concentration of 81.0 mg/L at 1 day HRT. The observed inhibitory concentration of 81.0 mg/L in Lin and Chen (1999) is significantly larger than 3.70 mg/L (63.00 µM) that have shown clear inhibition in all serum, bottles in this research. It was also observed that bio granules for acidogenesis had a higher tolerance for Ni than methanogens (Lin and Chen, 1999). Given that qPCR data (section 4.19.2) shows that the dominant methanogen order was Methanosarcinales, this can explain the disparity observed in toxic concentration of Ni between the current research and the one by Lin and Chen (1999). Generally the point of onset of toxicity is dependent on the substrate being digested and the environment of the digester in operation, e.g. factors such has pH and redox potential affect exertion of toxicity to digesters, hence there is the need to examine the point of onset of toxicity for each Trace Elements (TE) supplemented digester.

When dosed to a total Ni and Co ion concentration of 126.00 and 504.00 µ molar, the percentage reduction in biogas production is greater than when individual Ni and Co are dosed to the same concentrations. In comparison to D1(Ni), D3(Co)'s seed sludge showed a greater reduction in biogas when dosed to a concentration of 63.00-504.00 µM. The reduction

of biogas production by serum bottles at high ion concentration is believed to be caused by toxicity of these elements to methanogens. No biogas volume production reduction was observed for D1 and D2 at 15.75 µM and for D3 at 31.50 µM.

In contrast to activation concentration dosage, the sum of percentage reduction of D1 and D3's seed sludge considerably exceed that of D2 or D4.i.e those digesters that were dosed both Ni &Co, Figure 4.24. This implies lack of selectivity in exertion of toxicity as opposed to prior observed selectivity in exertion of activation (Figure 4.15); as such toxicity due to Ni or Co cannot be delineated when they are co-dosed. This is possibly because as reported by Babich and Stotzky (1983) exertion of toxicity involves direct microbe-metal ion interaction as opposed to catalyzation of biogas production which mainly involves the activation of specific enzymes involved in catalyses of specific methanogenic route.

4.16.1 *Implication of Ni and Co toxicity in relation to their supplementation to digesters*

When supplementing Ni and Co individually or in combination to a digester which has volatile solids concentration of approximately (5-25) g/L, the total ion concentration of individual or sum of Ni and Co should not exceed 63.00 µM, as a reduction in the digester's performance at this concentration is anticipated based on biogas production from the serum bottles at this concentrations. When Ni and Co were dosed in combination, their threshold concentration for toxicity was reduced to the sum ion concentration of 31.50 µM, Figure 4.24. Gikas (2007) reported a toxicity concentration range of Co and Ni of 2.73-5.45 mM when investigating the effect of Ni and Co on activated sludge, which is significantly larger than 63.00 µM observed in the current research. The reason for this disparity is possibly due to the difference in matrix content of sewage sludge, especially raw sludge in an aeration tank as compared to an acetate digester as well as the different in response and tolerance of methanogens to Ni and Co compared to other microbes. Sewage sludge in an aeration tank is anticipated to contain more matrix than an acetate digester, as such most of the dosed metal

ions may not be readily available to exert toxicity in an aeration tank, hence the need to dose more before exertion of toxicity.

In view of the different performance of the parent digesters, it was deemed necessary to conduct a biogas production assay with acetate as the substrate at various volatile solids concentration (VS) and total metal ion concentrations (M^{z+}) in order to examine the relation between biogas production and the ratio of VS/M^{2+}; as the interaction of these variables affect biogas production. Bhattacharya et al. (1995) observed that the toxicity of Co to acetate digesting methanogens varied with Volatile Suspended Solids Concentration (VSS). The experiment they conducted involved incubation of acetate in serum bottles at various VSS and Co concentrations. Free Co/VSS and total soluble Co/VSS mass ratios of and exceeding 0.05 and 0.16 respectively caused complete failure of methanogenesis (Bhattacharya et al., 1995). The study by Bhattacharya et al. (1995) showed the importance of variation of VSS on the amount of Co that exert toxicity to methanogens. Because different digesters will have different VSS or VS, different amounts of Co or Ni will on-set exertion of toxicity in each digester.

Fresh biomass (sludge) was used as seed sludge to ensure that microbes are in the same condition of viability, as opposed to the use of already running digester's effluent. The seed sludge was obtained from the same WWTP in Birmingham, UK as before. A three day cumulative biogas volume was used in computation of percentage deficit from control of the dosed serum bottles. Three day biogas volume was selected in view of the high V_{max} of new biomass as well as the anticipated high biogas production rate due to large VS concentration used. SMA assay experiments performed by Ishaq (2012) seeded with similar amounts of biomass had their V_{max} within a period of 3-4 days.

Comparism of Ni and Co's toxicities depend on the number of moles (specifically equivalents) or reacting species as they have different molar masses. Because of this, their

toxicity is examined in this part of the research in terms of their number of moles interacting with VS, as microbes are part of the VS. The four major elements in bacterial/microbial structures usually occurs in the ratio $C_5H_7O_2N$ as was first presented by Porges et al. (1956) during casein oxidation, cited in Rittmann and McCarty (2001). The frequency of occurrence of $C_5H_7O_2N$ bears some indication into the amount of microorganisms or bacteria per sample of VSS or VS. i.e. VS mass (g) divided by $C_5H_7O_2N$ mass (113 g) has some indication of the quantity of bacteria/microorganisms in that VS sample. Bacterial structures are highly complex containing various amounts of carbohydrates, proteins, fats and other molecules (Rittmann and McCarty, 2001). The use of $C_5H_7O_2N$ to draw inference into the amount of bacteria or microorganism in water and wastewater sample has been widely employed (Mogens et al., 2008, Metcalf and Eddy, 2003, Rittmann and McCarty, 2001) as well as having been reported to be satisfactory for most practical purposes (Rittmann and McCarty, 2001).

The logarithm of the ratio of $nC_5H_7O_2N$: number of moles of metal ion (nM^{2+}) i.e. ($nC_5H_7O_2N$ /nM^{2+}) was used as an independent variable as it had been shown that toxicity depend on the variation or interaction of these two variables. Biogas production results from dosed and non-dosed serum bottles at various VS concentrations are presented in Figure 4.25 to Figure 4.27.

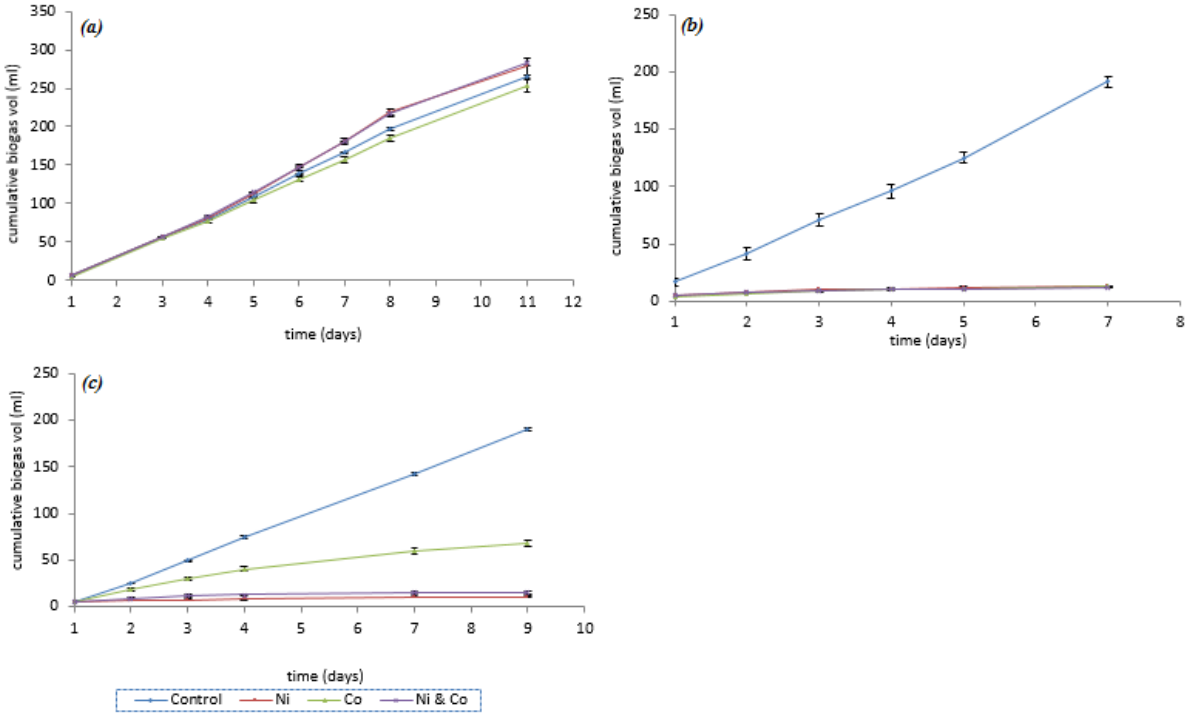


Figure 4.25. Cumulative biogas production from serum bottles at a VS concentration of 0.25 mg/L dosed to a final concentration of :a= 15.75 µM, b=504.00 µM and c= 1008.00 µM.

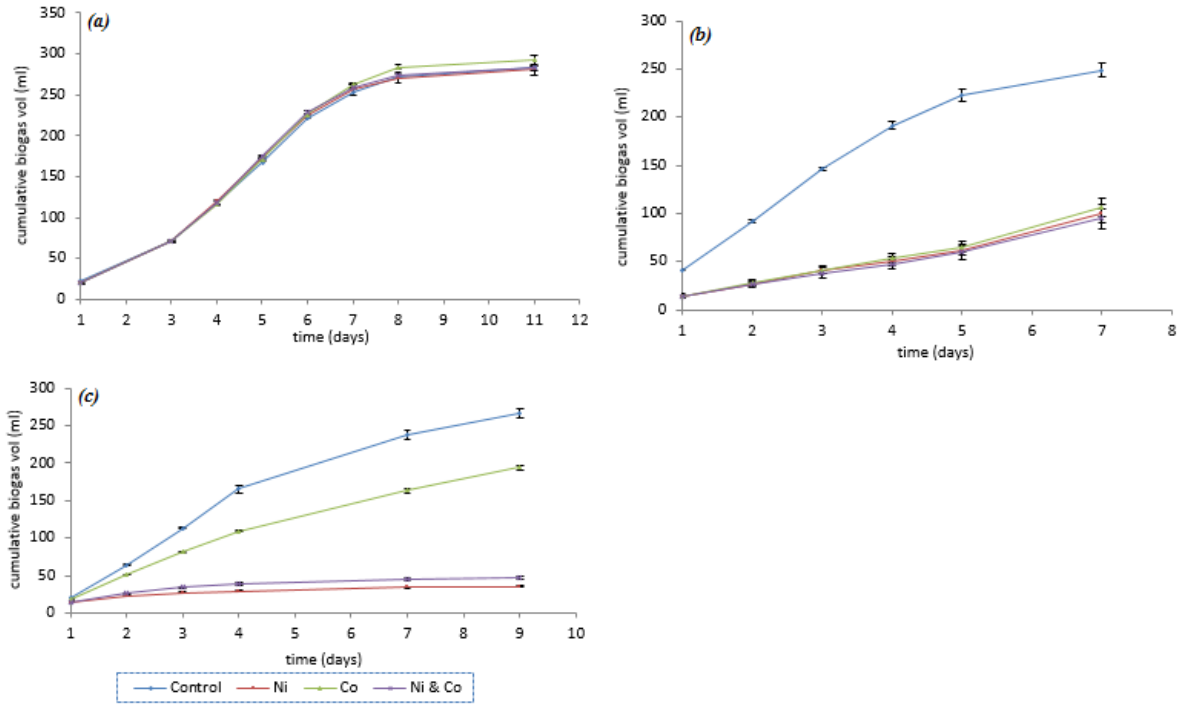


Figure 4.26. Cumulative biogas production from serum bottles at a VS concentration of 0.5 mg/L dosed to a final concentration of :a= 15.75 µM, b=504.00 µM and c= 1008.00 µM.

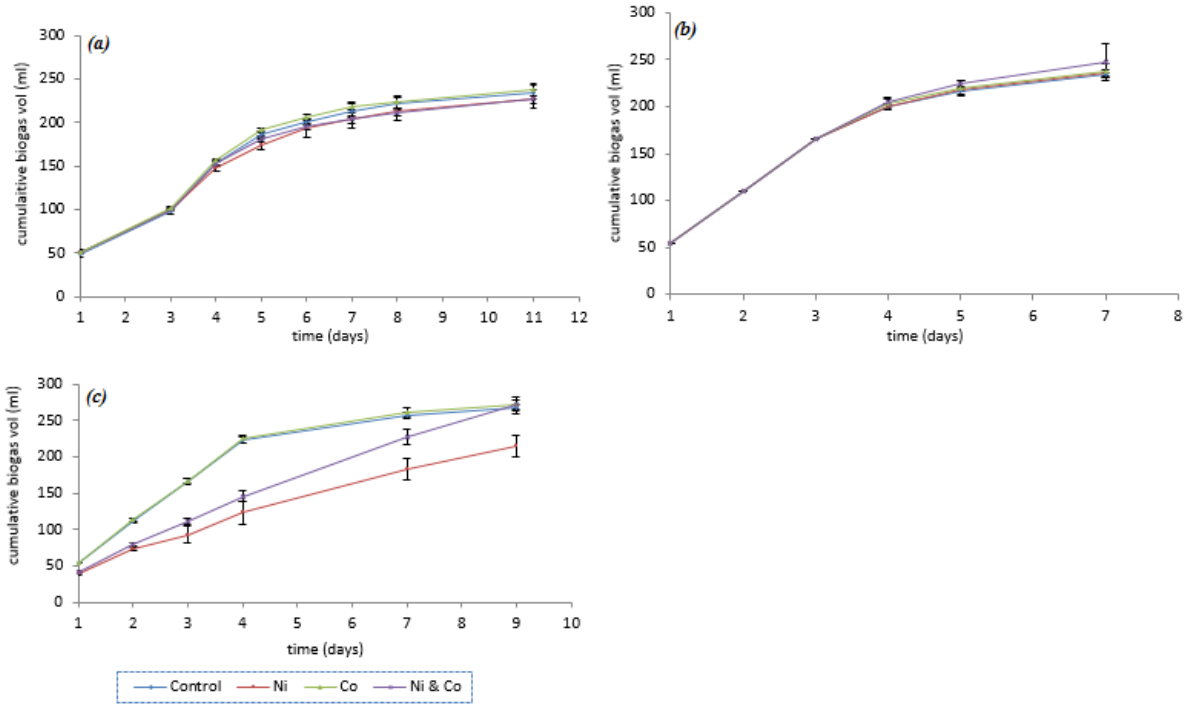


Figure 4.27. Cumulative biogas production from serum bottles at a VS concentration of 1.0 mg/L dosed to a final concentration of: a= 15.75 µM, b=504.00 µM and c= 1008.00 µM.

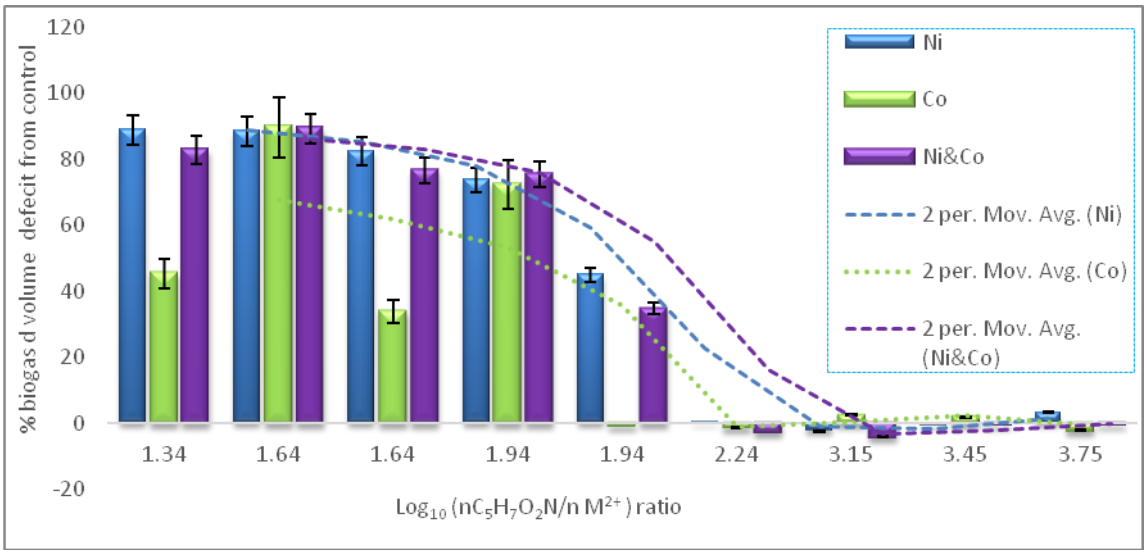


Figure 4.28. Histogram plot of the percentage deficit of the biogas production by the dosed serum bottles compared to the non- dosed ones expressed in Log_{10} ($nC_5H_7O_2N/n\ M^{2+}$) ratio. Summary of Figure 4.25 to Figure 4.27.

Figure 4.28 shows an increase in reduction of biogas production as the logarithm of the ratio VS/M^{2+} is reduced, i.e. reduction in biogas production is inversely proportional or related to the ratio of VS/M^{2+}. Figure 4.25 to Figure 4.27 shows that an increase in VS leads to reduction of biogas volume deficit for the same metal ion concentration. This observation is similar to the one made by Bhattacharya et al. (1995) where low VSS concentration resulted in increased toxicity of Co. The increase in Co toxicity at low VSS concentration was postulated to occur through the increase in free Co and soluble Co with reduction in VSS (Bhattacharya et al., 1995). The highest reduction in biogas production, hence toxicity, as VS concentration was increased was observed in Co dosed serum bottles, Figure 4.25(c) compared to Figure 4.27(c). The reduction of biogas production deficit as VS concentration is increased has implication on the toxicokinetics of the metal ions involved, tentatively; it implies that toxicity is exerted through direct microbe-metal ion interaction.

4.16.2 *Implication of biogas production from serum bottles at various metal ion and volatile solids concentration*

It is shown that an increase in sludge VS concentration led to a reduction of biogas volume deficit for the same metal ion concentration. This reduction in biogas production is assigned to toxicity of metal ions at high concentration, this further support the implication that toxicity is most likely exerted through direct metal-microbe interaction, other than an unfavourable change of the total digester environment. For example, if toxicity was caused by a change in environmental factors such as a change in redox potential, the same metal ion concentration would cause the same redox potential irrespective of VS concentration, as such would exert the same level of toxicity. At $\log(VS/M^{2+})$ ratio of 1.34, there was a high (more than 80%) deficit of biogas production from control for Ni dosed and Ni & Co dosed serum bottles, while sole dosage of cobalt produced about 40% deficit from control at the same $\log(VS/M^{2+})$ Figure 4.28. Given that the total ion concentration is the same in each of these

serum bottles, there is an observed high toxicity of Ni as compared to Co at log (VS/M^{2+}) ratio of 1.34. Toxicity of Ni and Co was observed to be exerted synergistically on activated sludge by Gikas (2007) at 2.73 mM and 5.45 mM respectively. These concentrations are higher that the observed toxicity concentration of 504.00 µM observed in this study, possible due to matrix interference in activated sludge as opposed to the simplicity of acetate fed digester; but nevertheless Gikas (2007)'s study and the current study both exhibits the inherent toxicity of Ni and Co to microorganisms.

At a log (VS/M^{2+}) ratio of below 2.24 (Figure 4.28) the onset of reduction in percentage biogas production was observed from both Ni, Co and Ni and Co dosed serum bottles. Above this figure (log mole ratio of 2.24) no reduction in biogas volume was observed. This can tentatively be the upper end of the saturation concentration, above which the onset of toxicity is observable. Figure 4.28 shows that on average (2 period moving average) toxicity was exerted in the following decreasing order: Ni and Co, Ni and then Co i.e. Ni and Co > Ni > Co. When using seed sludge from already operating digesters (Figure 4.24) co-dosage of Ni and Co was found to exert more toxicity that their individual dosage, especially when dosed to 126.00-504.00 µM of total Ni and Co ions. A mixture of chromium and cadmium was reported to have exerted inhibition of anaerobic degradation of acetate synergistically when dosed to 5 mg L^{-1} by Lin (1992); a similar observation of synergistic exertion of toxicity is made in the current study using Ni and Co.

Both biotic and abiotic factors have been reported by Babich and Stotzky (1983) to have an effect on the toxicity of Ni to microbes, so conditions of each digester will affect the degree to which an element is toxic. Mechanisms of metal ion uptake have been reported by Gadd and Griffiths (1978); these are non-specific binding of the metal to surfaces and metabolism mediated cellular uptake, with the latter being more important that the former. This support

the view that toxicity is exerted through direct-metal microbe interaction, so variation of VS concentration for the same ion concentration will affect the toxic metal ion concentration.

4.17 Confirmation of toxicity concentration to 5.0 L Digesters

To confirm the onset and applicability to continuously fed digesters of the prior observed inhibition of biogas production during SBP assay (in serum bottles) with acetate as the substrate, digesters were dosed 157.50 µ moles (≈ 9.24 mg) of Ni and Co individually and in combination on day 165; see Table 3.1. Dosage was done as follows: D1, D2 and D3 were dosed Ni, Ni & Co and Co respectively. No alteration was done to D4; as such it acted as a control digester again for toxicity confirmation study.

This meant that the dosed digesters had a concentration of 31.50 µM more of the ions of interest than the non- dosed digesters as was the case during toxicity study in serum bottle experiment. The dosage used was informed by SBP toxicity assay results summarised in Figure 4.24, in which toxicity was observed when Ni and Co were co-dosed to 31.50 µM in serum bottles seeded with D2 and D4's sludge.

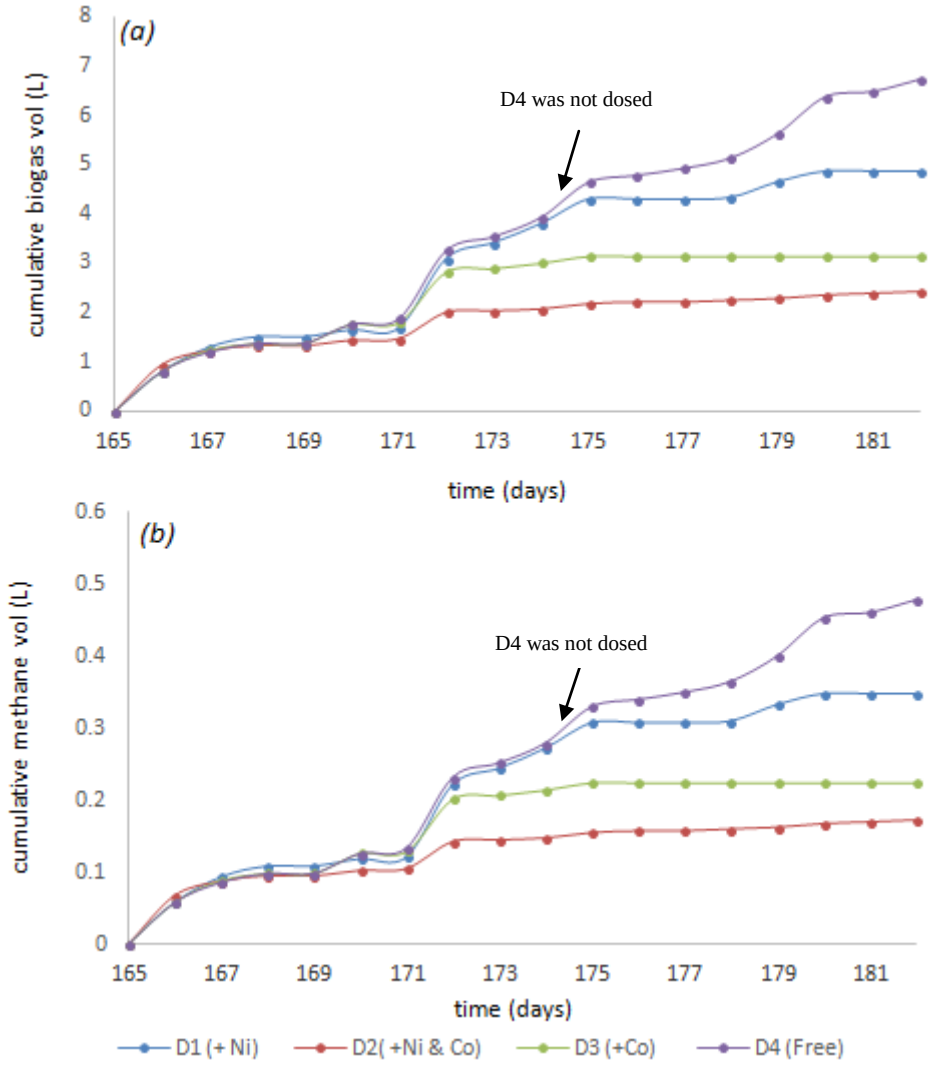


Figure 4.29 Digesters performance at sum ion (Ni & Co) concentration of (31.50-252.00 µM)

After a change in dosage, digesters produced about the same amount of biogas volume, Figure 4.29(a), until on day 169 when D2 produced the least amount of biogas. The percentage of methane in biogas did not change with or without dosage; it was constant at 64±1%, Figure 4.29(b). On day 171, individual and combined Ni and Co dosed to digesters was increased to 630.00 µ moles, thus increasing the total concentration of ions of interest to at least 157.50 µ M (≈ 9.24 mg/L). The number of moles added to digesters at this point is so large that the amount of Ni and Co removed with effluent is negligible.

An immediate reduction in biogas production was not observed at this point, but from day 172-173, the volume of biogas produced was considerably reduced in all digesters as

compared to the volume produced on day 171-172 (from approximately 1.5 L to 0.5 L). This is believed to be due to toxicity of the respective dosed metal ions at approximately 157.50 μ molar. Digesters D3 and D2 reduced biogas production the most, D1 showed more resilience to addition of high nickel concentration. This was an unexpected observation as prior alteration of pH and omission of magnesium due to change in feed stock composition produced a higher reduction in biogas production from D2 and D1. On day 175 a further 315.00 μ moles of total ions was supplemented to digesters in the same manner as before, making their concentration to be at least 220.50 μ M (≈.12.94 mg/L). After this alteration no further biogas production was observed from D1, D2 and D3 until three days later (day 178) at which some biogas production was observed in D1 (< l L). As D4 was not altered, it acted as a control experiment; hence this supports the view that reduction in biogas production from dosed digesters during the period of high micronutrient supplementation was caused by the supplemented metal ions.

4.18 Summary of part two

Region D of the hypothetical dose response curve was determined for the digestion system used through digestion of 100.0 ml of 60 mM of acetate off line in 120.0 ml serum bottles seeded with 20.0 ml of sludge from pre-concentration of 100.0 ml of effluent from each digester. The seed sludge contained approximately 10.0 g/L of VS. When digesters are supplemented with micronutrients, it is important to determine the point of onset of their toxicity so as to avoid their sudden failure due to toxicity of micronutrients at high concentration. When individual or a combination of Ni and Co were dosed to at least 15.75 μM (≈ 1.49 μ moles dosed) a slight reduction in biogas production was observed in D3 and D4's sludge while no reduction was observed in D2 and D1 sludge, in fact D2 sludge

produced 40% more biogas, Figure 4.24. This possibly represents a zone of reduced stimulation or a transition zone from stimulatory concentration to toxicity, given that more stimulation of biogas production was observed in D2 at a Ni and Co concentration of about 0.1-0.3 mg/L or (2.0-3.9 μM), Figure 4.1(a). At a concentration of at least 31.50 μ molar (dosed 3.15 μ moles) reduction in biogas production was observed for a combined dosage of Ni and Co, while at an individual and combined concentration of 63.00 μ molar (dosed 6.30 μ moles), a clear inhibition of methanogenesis was observed for all dosage combinations, i.e. Ni, Ni and Co and Co. This possibly represents a cross over concentration to toxicity for all dosage combinations, in a similar manner as was proposed by McCarty (1964). Reduction of biogas production increased when serum bottles were dosed a combination of Ni and Co to a total ion concentration of 126.00 μM (dosed 12.6 μ moles) up to almost a complete lethal concentration of 504.00 μM (dosed 50.4 μ moles).

Due to the difference in biogas production between digesters, it was found necessary to examine toxicity using fresh biomass at various VS concentrations. Biogas production from the dosed serum bottles was examined as a function of volatile solids and amount of metal ion dosed, (sum of Ni and Co or individually). At log (VS/M^{2+}) of 3.15, onset of toxicity or reduction in biogas production was observed in Ni and Ni &Co dosed serum bottles, while Co started to exert toxicity at log (VS/M^{2+}) of 2.24, Figure 4.28. The order of exertion of toxicity was found to be: (Ni & Co) > Ni > Co. This is in line with the observation made by Gikas (2008). As different microbes other than methanogens are functional or active in activated sludge as compared to in acetate digester, the consistency in the order of exertion of toxicity by Ni and Co possibly reflect their inherent toxicity.

4.18.1.1.1 Deduction of part C

A Ni and Co concentration range of 1.89-14.9 μM (≈ 0.11-0.88 mg/L) have been found to be stimulatory to biogas production from digesters; while a combined (in serum bottles) Ni and

Co concentration range of 31.50 μM -504.00 μM (≈1.86-29.70 mg/L) in D4's seed sludge has been found to be inhibitory to biogas production. Dosage of D4'seed sludge in serum bottles with a combined Ni and Co to a concentration of 15.75 μM (≈0.93 mg/L) did not cause an increase or significant inhibition (only ≤2%) of their biogas production; while their dosage to a combined Ni and Co concentration of 31.50 μM caused about 18% inhibition of their biogas production, Figure 4.24.In view of this, a concentration range of 15.75 μM -31.50 μM (≈0.93-1.86 mg/L) is postulated with great tentativeness to be the excess as well as the transitionary concentration to toxicity for the anaerobic digestion system used.

The resultant Ni and Co concentration in serum bottles seeded with sludge from dosed parent digesters is higher than the targeted concentration; the actual existing/nominal concentration cannot be estimated as the amount of analyte that was removed with 80.0 ml of the supernatant was not quantified. The concentration of Ni and Co in serum bottles seeded with D4's seed sludge is more representative or realistic (as this digester was not pre-dosed); as such it is the serum bottles that were seeded with this digester's seed sludge that are more reflective of the combined Ni and Co toxicity.

4.19 Analytical measurements

This section presents and discusses different physical and microbial parameters that affect microbial (methanogens) metabolism and consequently digesters' biogas output. These are discussed with relevance to the prior observed biogas output from digesters. Although these are presented in separation as physical and microbial parameter, their interaction in a digester influence each other, consequently influencing biological activity and ultimately biogas output from digesters.

4.19.1 ***Physical parameters measurement***

The physical parameters or condition of a digester largely affect its microbial or biochemical activities during anaerobic digestion. It is suggested from the work of Mathir (2013) that micronutrient dosage strategies should be toiler made for each digestion process as physical conditions across digesters vary at industrial level. The following is a brief discussion of measured physical parameters relevant to laboratory scale digesters used in this research.

4.19.1.1 Percentage of methane in biogas produced by digesters.

As the most crucial component of biogas, the percentage of methane in biogas was measured. The results are shown in Figure 4.30. The percentage of methane in the produced biogas from all digesters was constantly 64±1% during the entire experimental period irrespective of the actual biogas volume they produced, while incubated control serum bottles had 75% methane. Biogas produced in serum bottles was not free to escape, as such pressure build up leading to increased dissolution of carbon dioxide, hence the high methane percentages in the head space of serum bottles as compared to digesters. On the other hand, biogas was free to escape from digesters, hence no increased dissolution of CO_2 gas due to increased pressure occurred. This

result in lower methane percentage in the head space of digesters compared to in that of control serum bottles.

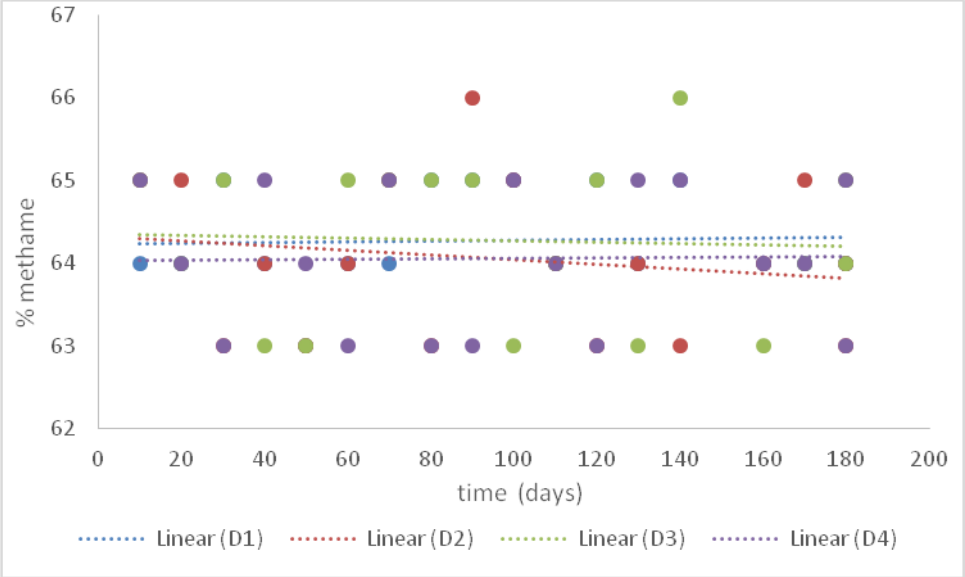


Figure 4.30. The percentage of methane in the biogas produced by the digesters.

4.19.1.2 pH

Given the known sensitive nature of methanogens to change in environmental conditions (Mortimer et al., 1981) and the potential effect of these changes in the stability of an operating digester, it was necessary to monitor the pH of a digester. The effluent pH was measured when it was leaving the digester to reduce the impact of aeration of the measurement.

Figure 4.31 shows the evolution of effluent pH measured over time for the four digesters.

The rise in effluent pH observed from day 40-75 was due to the increase in influent pH from 7.0 to 7.8 due to omission of magnesium acetate from the feed. Under a desired feed composition, the effluent pH was normally around 7.75 having increased from the feed pH of 7.0-7.50. The optimal pH for methanogens is reported by Mortimer et al. (1981) to be near neutral (6.5-7.5). The observed reduction in the peformance (biogas production) of the digesters around day 40 was caused by this increase in pH and possibly sodium toxicity as well.

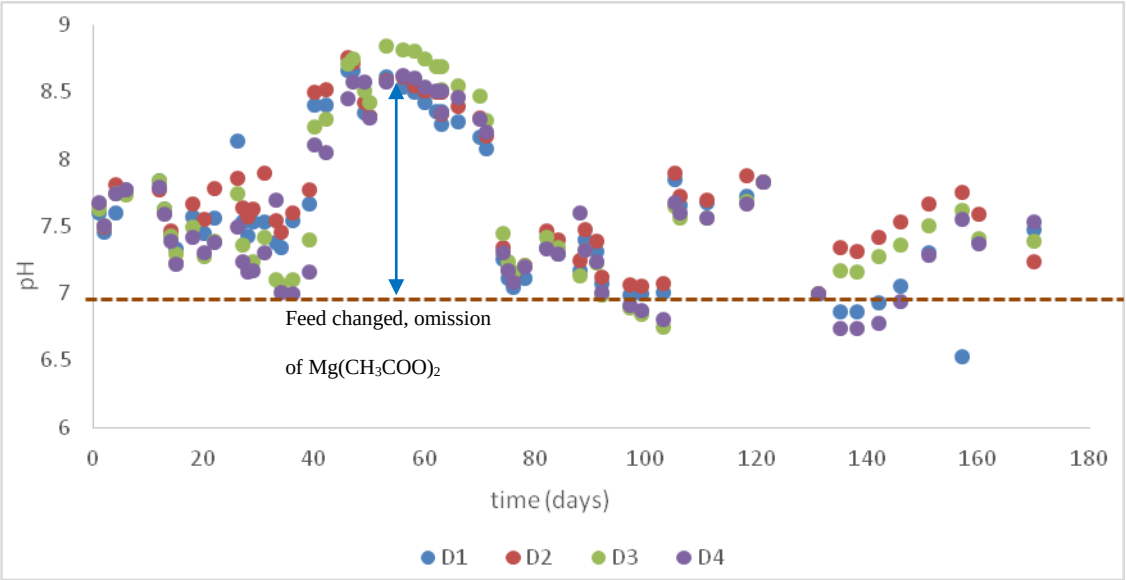


Figure 4.31. Evolution of effluent pH of the four digesters.

4.19.1.3 Solids concentration

Variation or change in total and volatile solids in a digester can be used to estinate, although with low accuracy, the change in microbial abaundance in a digester, especially volatile solids (VS) and volatile suspended solids (VSS). VS and VSS are at times used to determine the expected quantity of methane from an ananerobic digestor (Bond et al., 2011), as these can inform the biodegradable propotion of waste/subtrate being degested.

Figure 4.32 shows that volatile solids concentration was increasing with time across all four digesters, to almost having doubled (from the starting 5.0 mg/ml) by day 60. The change in volatile solids data is contrary to the reduction in abaundace of Methanosaetaceae with digestor's operation time; possibly due to the low accuracy of VS data as comapred to molecular (qPCR) data in reflecting the abaundance of microbial population. The use of molecular techniques in estimating the abundance (absolute quantification) of specific microbial groups (qualitative) and the use of the resulting data in bioengineering process control is highly advocated for by Yu et al. (2006). In cognisance of this, qPCR data, specifically 16S rRNA gene copy number/ µg DNA; was deemed to be more sensetive as well

as specific to changes in methanogenic population; hence it offered a better and real time reflection of evolutionary trend of methanogens in this research as compared to VS data.

The increase in VS obsserved in Figure 4.32 could be caused by the decomposition of ammonium acetate (CH_3COONH_4) into its gaseous products such as NH_3 and H_2O (Erdey et al., 1964). CH_3COONH_4 could have been formed in the acetate digesters, as they were fed NH_4Cl as part of macronutrients; as such ammonium ion (NH_4^+) could have reacted with the acetate forming CH_3COONH_4.. The decomposition of ammonium acetate is reported to satrt at a temperature of 100°C, with melting and quencequent loss of NH_3 occuring at 115°C by Erdey et al. (1964). On raising the temperature of combustion of NH_3CH_3COO to above 115°C, acetamide (H_2NOCCH_3) was reportedly formed, further increasing the temperarture to above 150°C, acetamide vapourised off, and the thermal gravimetry graph (TG) showed a 100% weight loss of the sample; (Erdey et al., 1964). Since samples were heated at 505°C in this research for VS measurements, this decomposition of ammonim acetate could have contributed to the increase in VS, which is contratry to microbial quantification data through qPCR .

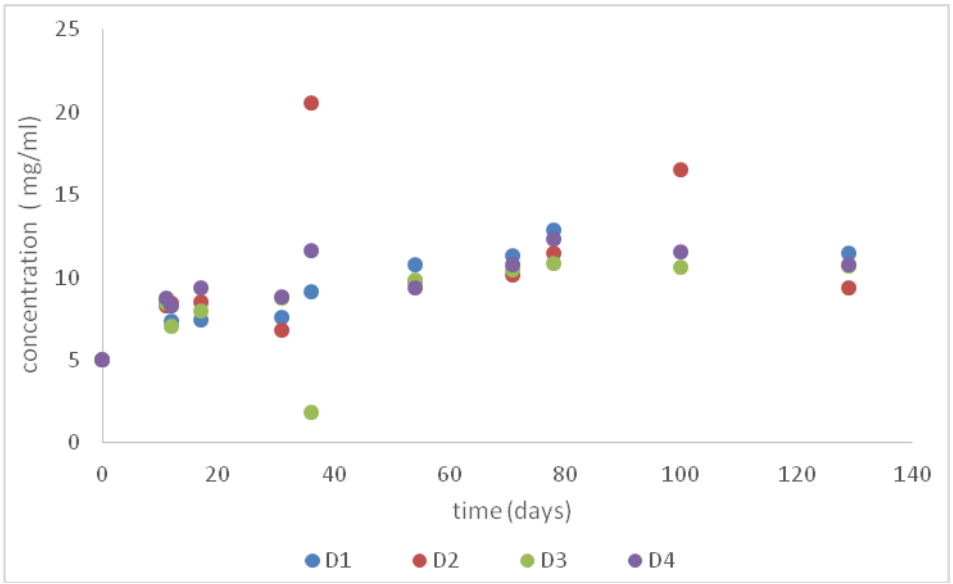


Figure 4.32 Change in VS concentration across all four digesters with time.

As with VS concentration, the TS concentration increased with time (Figure 4.33). This increase was mainly due to the formation of salts (from the feed). Formation of solids was observed during TS measurement as white residue with sharply pointed crystals which remained after evaporation of the sample at 105 °C.

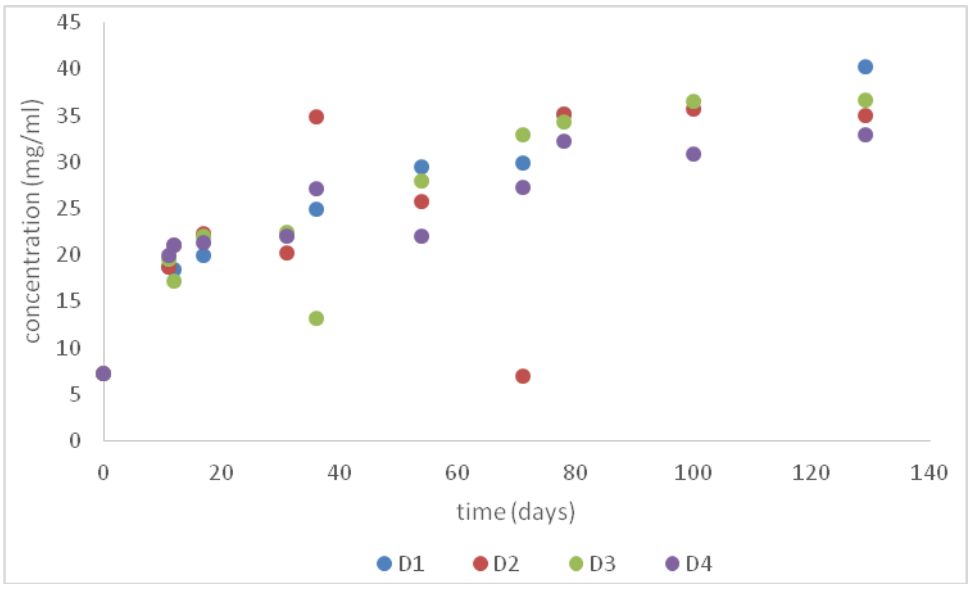


Figure 4.33 Change in TS concentration across digesters with time.

4.19.1.4 Effluent acetate concentration

Figure 4.34 highlights that the amount of acetate in the effluent increased with time. This was due to the fact that not all of the daily fed acetate was converted to biogas in one day. Again the increase in acetate concentration from day 45-75 was anticipated to be due to a change in feed pH. D1, D2, D3 and D4 had an 89, 90, 87 and 90% average acetate conversion or removal respectively by the end of 120 days in which acetate concentration in the effluent was measured. The 90% (highest) acetate removal rate of D4 was not consistent with its observed biogas production, having produced the lowest amount of biogas in this period, it was expected that this digester would have the highest amount of acetate in the effluent. No other VFA other than acetate was detected during measurement, which was the main reason for VFA measurement.

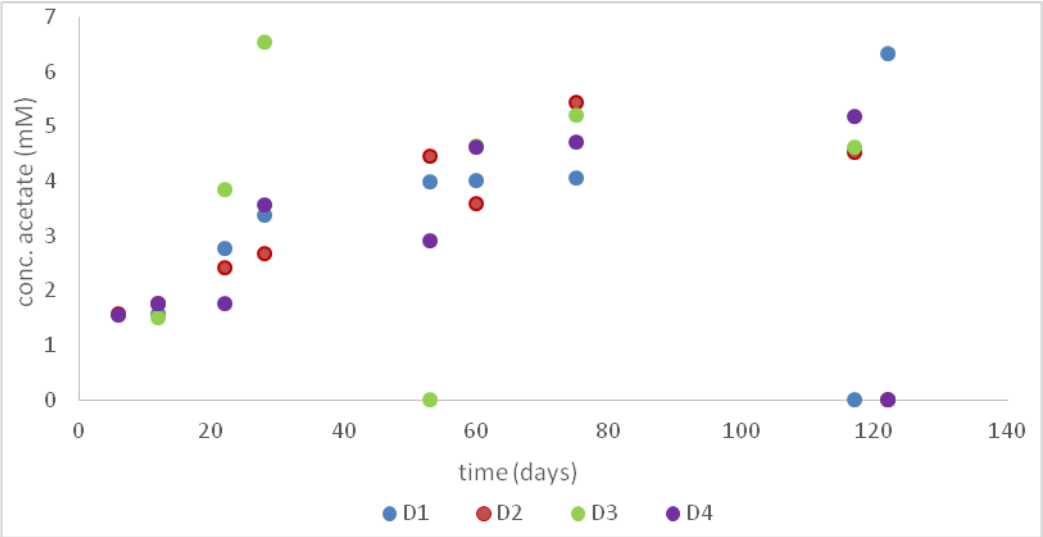


Figure 4.34 Evolution of effluent acetate concentration with time.

Table 4.2 shows the measured concentration of other ions in the effluent. Of most interest is the concentration of the sulphate ion in relation to the divalent cations added to the digesters, and the implication of the possibility of sulphate ion precipitating those cations. This was less likely to affect the soluble concentration of the dosed Ni and Co as there was a large pool of other divalent cations in solution such as iron (II) which was dosed to 5.0 µM.

Table 4.2 Concentration (mM) of other ions in effluent.

Ion	D1	D2	D3	D4
SO_4^{2-}	0.0028±0.0024	0.0155±0.0351	0.0042±0.0042	0.0034±0.0026
Cl^{2-}	0.5722±0.2569	0.5898±0.2745	0.5993±0.4434	0.5783±0.2706
PO_4^{3-}	0.0103±0.0087	0.0063±0.0097	0.0066±0.0093	0.0010±0.0105

Dissolved oxygen at the time of effluent obtainment was found to be 0.1% across all digesters. This is considered not significant for causing toxicity to methanogens (Mortimer et al., 1981).

4.19.1.5 Metal concentration in sludge

This section presents the back ground concentration of Ni and Co in sludge. This is important as the amount of metal ion in a digester will affect its biogas production. Prior to dosage of metal ions in a digester, it is important to know their background concentration so as to be able to correlate the performance of a digester with the dosed metal ion. The interaction or association of metal ion with VS is important as it shows the potential interaction of metal ions with methanogens or microorganisms, hence the likelihood of the dosed ions to exert their catalytic effect, which will be reflected as increased biogas production. The reduction in concentration of Ni and Co from those digesters that were not dosed Ni and Co will be shown in this section, as well as the increase in their concentration in those digesters that were dosed. The recovery, background and evolution of metal ions of interest before and after dosage are discussed below. This was to enable examination of whether the observed behaviour of each digester was due to experimental variables of interest, i.e. Ni & or Co dosage.

4.19.1.6 Percentage of Ni and Co recovered during extraction.

The amount of analyte that could be recovered by the extraction method employed was calculated based on the lowest amount of metal ion concentration resulting from supplementation of Ni and Co to digesters, minimum concentration of 0.5 µM for both Ni and Co. It was expected that if recovery is high at low metal ion concentration (0.5 µM) in higher concentration the same or higher recovery will be achieved based on the extraction method used.

To measure the percentage recovery, 1.0 L portion of seed sludge was dosed 0.50 µ moles of both Ni and Co to their final concentration of 0.50 µM, and then incubated in a temperature controlled room at 37±2 °C for 5 days. Biogas produced by this sludge was daily vented off by opening the lid of the jerry can. At the end of five days, the metal content of the sludge was measured according to Eaton et al. (2005). The sludge which was not dosed Ni and Co

was used to obtain the background concentration of the metal ions of interest. The findings are presented in Table 4.3. Note that only Ni measurement and computational data (mainly percentage recovery) are presented as the same extraction procedure was done for Co as was for Ni.

Table 4.3. Background concentration of Ni (µM) in the sludge and its (Ni) percentage recovery.

Dosage	Recovered (µM)	Background concentration (µM)	% Recovery
0.029 mg	4.576 ±0.312	4.077 ± 0.084	98± 0.3

At a concentration of 0.5 µM, 98% of the dosed Ni was recoverable in addition to the background concentration. The seed sludge was found to contain a background Co concentration of 3.00 µM and a 97% recovery was achieved. Percentage recovery was calculated through:

$$\text{Ni \% recovery} = \frac{\text{spiked mass}}{\text{spiked sample} - \text{original sample}} \qquad Eq(14)$$

Where: spiked mass = mass of Ni spiked,

spiked sample = mass of Ni measured in a spiked sample,

original sample = mass on Ni in the un spiked sample

4.19.1.7 Measured Cobalt

Table 4.4 shows reduction in the concentration of Co from those digesters that were not dosed Co (D4 and D1) while D3 exhibit an increase in its Co concentration. Samples obtained on day 12 and beyond had a slightly higher Co concentration that the one expected on day zero, possibly due to Co contamination from equipment. The lack of increase in Co concentration in D2 is mainly due to experimental error during metal extraction and quantification, as digester D2 was dosed Co in the same manner and amount as D3, as such it was expected to exhibit an increase in cobalt concentration.

Table 4.4. Presentation of evolution of Co concentration (µM) across all digesters with time.

	Digesters			
Days	D1	D2	D3	D4
0	0.60	0.600	0.600	0.600
12	1.327	1.175	0.891	1.187
28	0.787	0.706	1.075	1.267
52	0.738	0.692	1.155	0.575
120	0.473	0.777	*0.695*	0.248
129	0.429	1.150	*0.823*	2.917

Note; dosage was done after obtaining samples for metal analysis in all cases.

The distribution of cobalt mass per mass of volatile solids is presented in Table 4.5 and Figure 4.35. This is important for visualisation of its (Co) potential interaction with microorganisms (VS).

Table 4.5. Evolution of cobalt mass/Vs mass (µg/gVS.) across all digesters with time

	Digesters			
Days	D1	D2	D3	D4
0	35.36	35.36	35.36	35.36
12	10.67	8.18	7.44	9.00
28	6.11	6.09	7.26	7.90
52	4.05	4.16	6.91	3.63
120	2.43	2.78	3.85	1.27
129	2.20	7.20	4.52	15.96

4.19.1.8 Measured Nickel

Table 4.6 shows an increase in Ni concentration in D2, while in D4 a reduction was observed beyond day 12. Given that D2 was dosed Ni over time while D4 was not, this is a normal observation. The concentration of Ni in day 28-129 in D3 and day 52-129 in D1 could not be detected; possibly not enough analyte was extracted. Where Ni concentration readings in D1 and D3 were lacking, D2 and D4's readings respectively, were used to inform the readings in D1 and D3. The concentration of Ni measured beyond day zero (diluted background

concentration) was higher than that expected on day zero in non-Ni dosed digesters (D3 and D4); this could be as a result of Ni contamination from equipments used.

Table 4.6. Presentation of evolution of Ni concentration (µM) across all digesters with time.

	Digesters			
Day	D1	D2	D3	D4
0	0.185	0.185	0.185	0.185
12	2.309	2.300	2.969	2.129
28	1.835	1.552		1.874
52		2.388		1.363
120		5.000		0.604
129		4.250		0.402

Note; day 0 denotes background concentration.

The distribution of nickel mass per mass of volatile solids is presented in Table 4.7 and Figure 4.35. This have implications on the possible interaction of Ni and microorganisms (VS) as the more likely the contact between Ni and microorganisms, the more likely is the Ni to exert its effect. Those digesters which were dosed Ni had a higher ratio or association of Ni/VS; this Ni was believed to have caused the dosed digesters to produce more biogas than the non-dosed ones.

Table 4.7. Evolution of Nickel mass /mass of Vs (µg/g VS across all digesters with time.

	Digesters			
Day	D1	D2	D3	D4
0	47.83	47.83	47.83	47.83
12	18.49	15.94	24.71	15.06
28	14.18	13.33		12.41
52		14.3		8.55
120		17.78		3.07
129		26.51		2.19

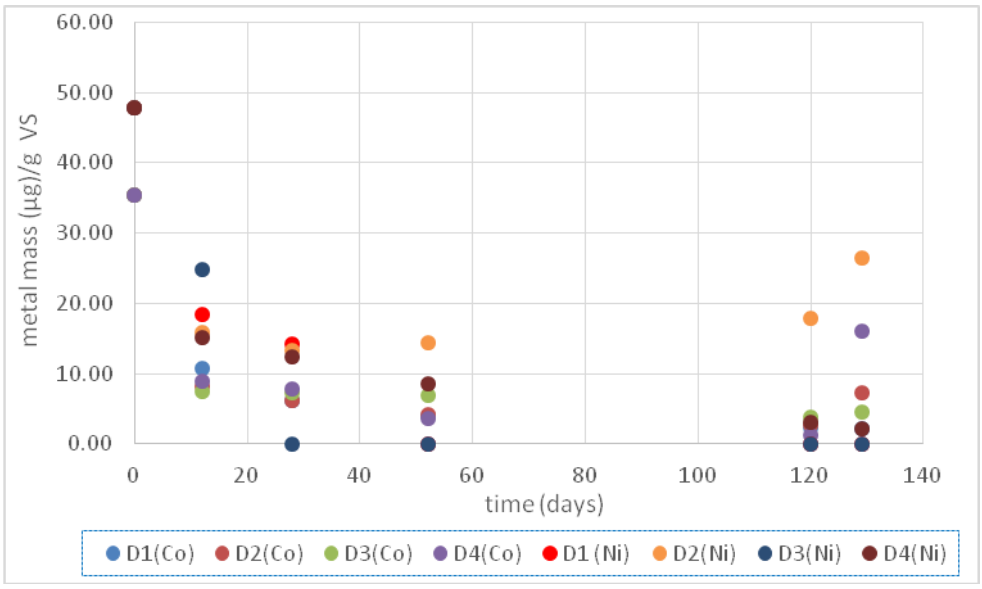


Figure 4.35. Variation of Co and Ni mass per Volatile solids mass across all digesters with time.

4.19.1.9 Evolution of Ni and Co in a Control Digester (D4)

This section presents the measured and calculated (theoretical) evolution of Ni and Co concentration with time. These concentrations when contrasted with those of dosed digesters will give an indication of the limiting concentration of Ni and Co in those digesters that were not dosed either one of these elements. The calculation of the concentration of Ni and Co in D4 with time was based on the ideal evolution of a conservative (non- reactive) tracer when dosed in a pulse manner (once) to an ideal CSTR with a continuous inflow of influent as described in Metcalf and Eddy (2003). The effluent concentration of Ni and Co in D4 could be estimated using:

$$C_t = C_0 e^{-\Phi} \qquad Eq(15)$$

Where:

C_t = Concentration of Ni or Co in the reactor at time t, ML^{-3}

C_o= initial concentration of Ni or Co in the reactor at time t= 0, ML^{-3}

t= time, T

Φ = normalised detention time, t/θ, dimensionless

θ= hydraulic retention time, T

Figure 4.36 shows the calculated (using equation 5) and measured evolution of Co and Ni concentration in D4 with time; a general trend of reduction in Ni and Co concentration was observed with measured Co showing the largest reduction by day 120 as compared to measured Ni, This was possibly due to the different sensitivities of the GFAAS to Ni and Co measurement. Supplementation of D4 with Ni to 0.5 µM on day 129 led to increased biogas production, Figure 4.17.

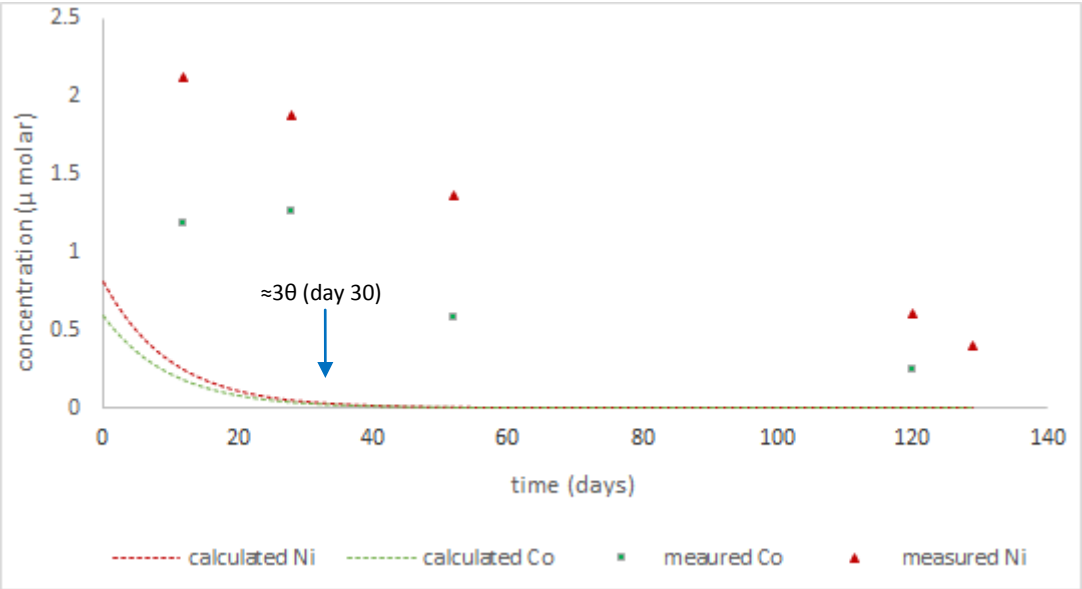


Figure 4.36 Variation of Ni and Co in D4 with time, showing both measured and calculated concentrations.

4.19.2 *Theoretical evolution of nickel and Cobalt concentration in dosed digesters*

In view of the small amount of Ni and Co which digesters were daily dosed, 2.50-7.50 µ moles, and the resulting challenges associated with both the recovery and the quantification of these elements, it was found necessary to make a theoretical calculation of the anticipated concentration of Ni and Co in the dosed digesters. The calculation was based on the principle of a CSTR, whereby the effluent contains the same contents as the digester. This calculation was conducted from day 0-129, as this covers the period through which digesters were

differentiated. The amount of Ni and Co remaining in a digester after each feeding and dosage was estimated via:

$$y_n = y_{n-1} - \left(\frac{1}{10} y_{n-1}\right) + \alpha \qquad Eq(16)$$

Where:

y_n= resultant number of Ni and Co moles in a digester on the n^{th} day

α= number of moles of Ni and Co added during feeding.

y_{n-1}= *(n^{th}—1) day*

Equation (16) is based on how digesters were feed acetate solution and dosed micronutrients. On daily bases, one tenth (500.0 ml) of the digester content was removed as effluent; because digester contents were completely mixed the same proportion of Ni and Co is expected to also be removed. Using equation (16) Figure 4.37 was obtained as the theoretical evolution of Ni and Co in dosed digesters with time, this basically represents the concentration of Ni and Co that microbes were daily exposed to, i.e. the daily exposure concentration.

Based on Figure 4.37, a Ni and Co concentration of 14.924 μ molar (≈ 0.88 mg/L) was expected in dosed digesters on day 129, which is significantly larger than the 0.1-0.3 mg/L of these elements observed to have been catalytic to biogas production by Gustavsson (2012); nevertheless exposure of methanogens to this concentration was observed to increase biogas production from dosed digesters as they produced more biogas than D4 by the end of the 7.50 μ moles daily dosage period.

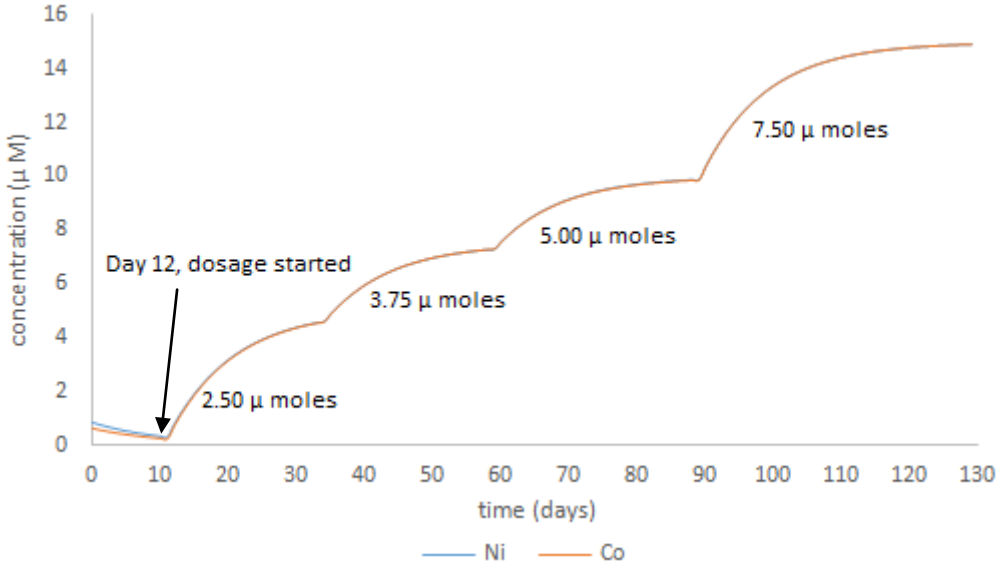


Figure 4.37. Theoretical evolution of Ni and Co concentration in dosed digesters from day 0-129, with daily dosages presented on the graph.

4.19.2.1 Estimation of the possible extent of precipitation of nickel and cobalt in digesters

Using Visual MINTEQ program, a simulation was run to assess the theoretical extent of precipitation of Ni and Co in dosed digesters. This was done at experimental pH, Figure 4.31, and the hypothetical concentration of Ni and Co, Figure 4.37. As per Visual MINTEQ, at a concentration of 14.924 µ molar and a pH of 7.5, 67% and 77% of soluble Ni and Co respectively are anticipated to exist as ions. This represents a 10% more potential bioavailability of Co than Ni. At a pH of 8.5 and a concentration of 7.567 µ molar (≈0.44 mg/L) the anticipated Ni and Co ions in aqueous phase reduces to 47% and 70 % respectively, presented in Appendix B. This shows that high pH in addition to being out of the optimum for methanogens growth can potentially lead to reduction of amount of ions in aqueous phase, which are mostly deemed bioavailable.

4.19.3 ***Microbial parameters***

With the intention of narrowing the search for identification of methanogens through qPCR, the effluent obtained on various days was examined with SEM to identify methanogens based on their morphological features as outlined by Mogens et al. (2008) and Mortimer et al. (1981). The majority of microorganisms were found to be closely attached to solids or clumps of sludge. This attachment to sludge particles hindered a clear viewing of the morphological features of these microorganisms. Figure 4.38(F) shows possible mitotic division of two cells, most likely daughter cells. This is typical of how *Methanosarcina sp* grows into grape like clamps over time. Figure 4.38(B) is a large spiral unidentified object, possibly a microbial cell component. High magnification viewing in Figure 4.38 A, C and G. show rod-like objects possibly Methanosaeta having clinched to solid materials, In Figure 4.389(E), sharp edges of salts can be seen; these possibly result from a reaction of acetate with other ions present in the feed.

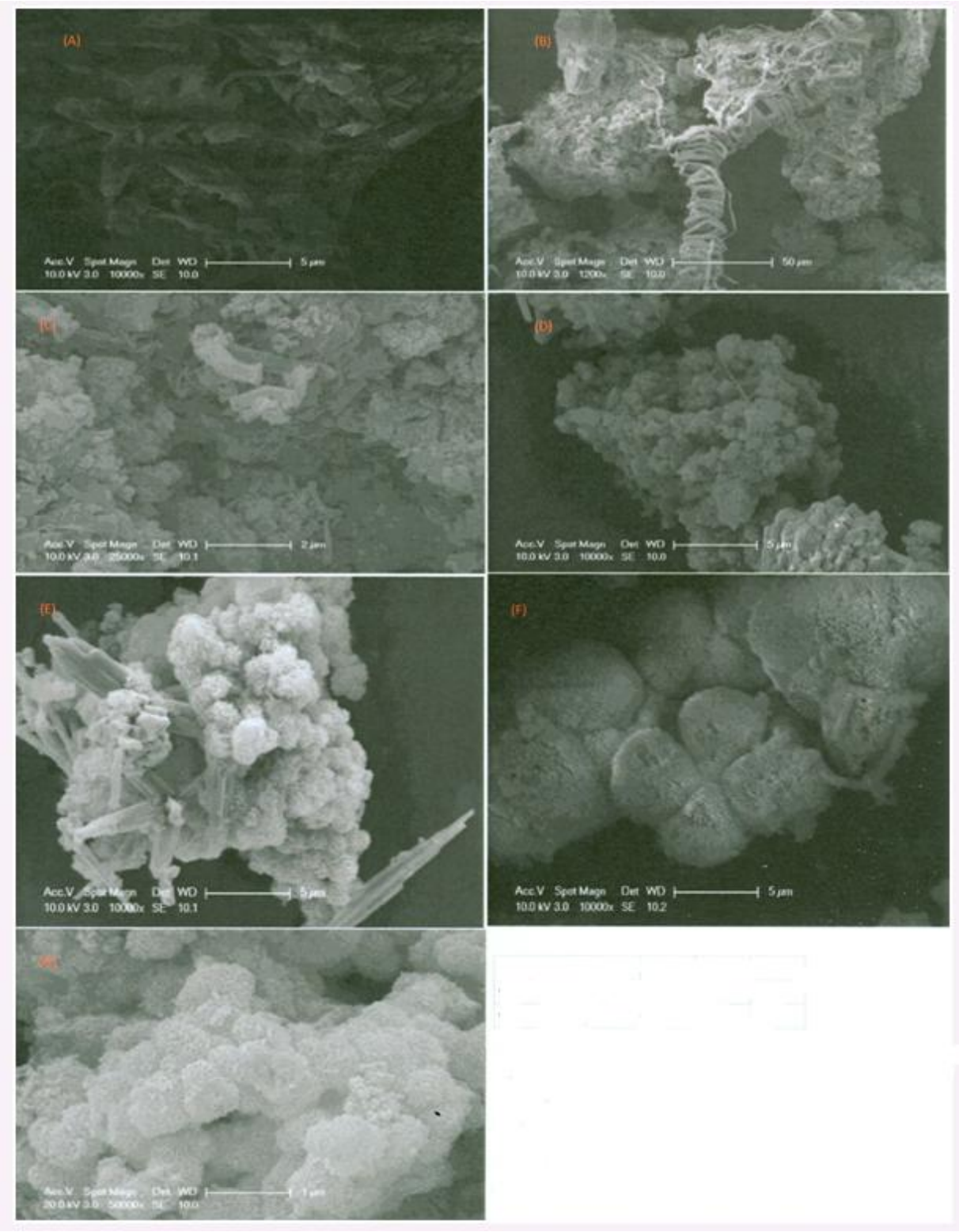


Figure 4.38. SEM images from samples from all digesters taken at different periods; L-R (a=D1 day 9,B=D2 day 9, C=D3 day9, D= D2 day 9, E and G=D3 day 99, F=D4 day 9 and G=D4, day 99)

The formation of a greyish to brownish ‘hard water scum’ or foam like product was observed in effluent from those digesters that were not dosed nickel, Figure 4.39

Figure 4.39 (A, B and E) read with Table 3.1. 100.0 ml of this hard water scum like product was analysed for TS and VS according to Eaton et al. (2005). The TS concentration of the scum from digesters D3 and D4 was 0.02 g/ml, the volatile component of the scum was respectively 43% and 49 % for these digesters. The volatility of this product suggests that it is an organic product, most likely a microbial product. The composition of this product was not investigated further, nor do the reasons as to why it seemed the presence of Ni avert its formation or accumulation. The addition of Ni to D3 and D4 led to the disappearance of this product as seen in Figure 4.39s (C, D and F*)* read with Table 3.1.From the reviews literature, no comparable work was found that reported the formation of a scum like product as a function of micronutrients dosage.

Figure 4.39. Presence of a scum or foam like product in the absence of Ni, L-R (a=all digesters day 100, b=D4 day 100, c=all digesters day 150, d= D2 and D4 day 150, e=D3 day 100 and F= D1 and D3 day 150).

4.19.1 *Biogas production from control serum bottles (activities)*

The biogas production potential of each digester's sludge was investigated. This gave an indication of the degree of microbial activity in each of the digesters on the day of sample obtainment. This microbial activity was indirectly measured as the biogas production potential of each digester using serum bottles. The biogas production potential relative to that of a control serum bottle was obtained by computing the percentage exceedance of the controls (those serum bottles that were not dosed metal ions during investigation of the effect of high metal concentration) among each other, i.e. controls used to obtain Figure 4.24. The biogas production of these serum bottles (controls) is presented in Figure 4.40(a-e).

Biogas production potential of each digester's sludge with acetate as the substrate in excess of that of control digester (D4) is presented in Figure 4.41.

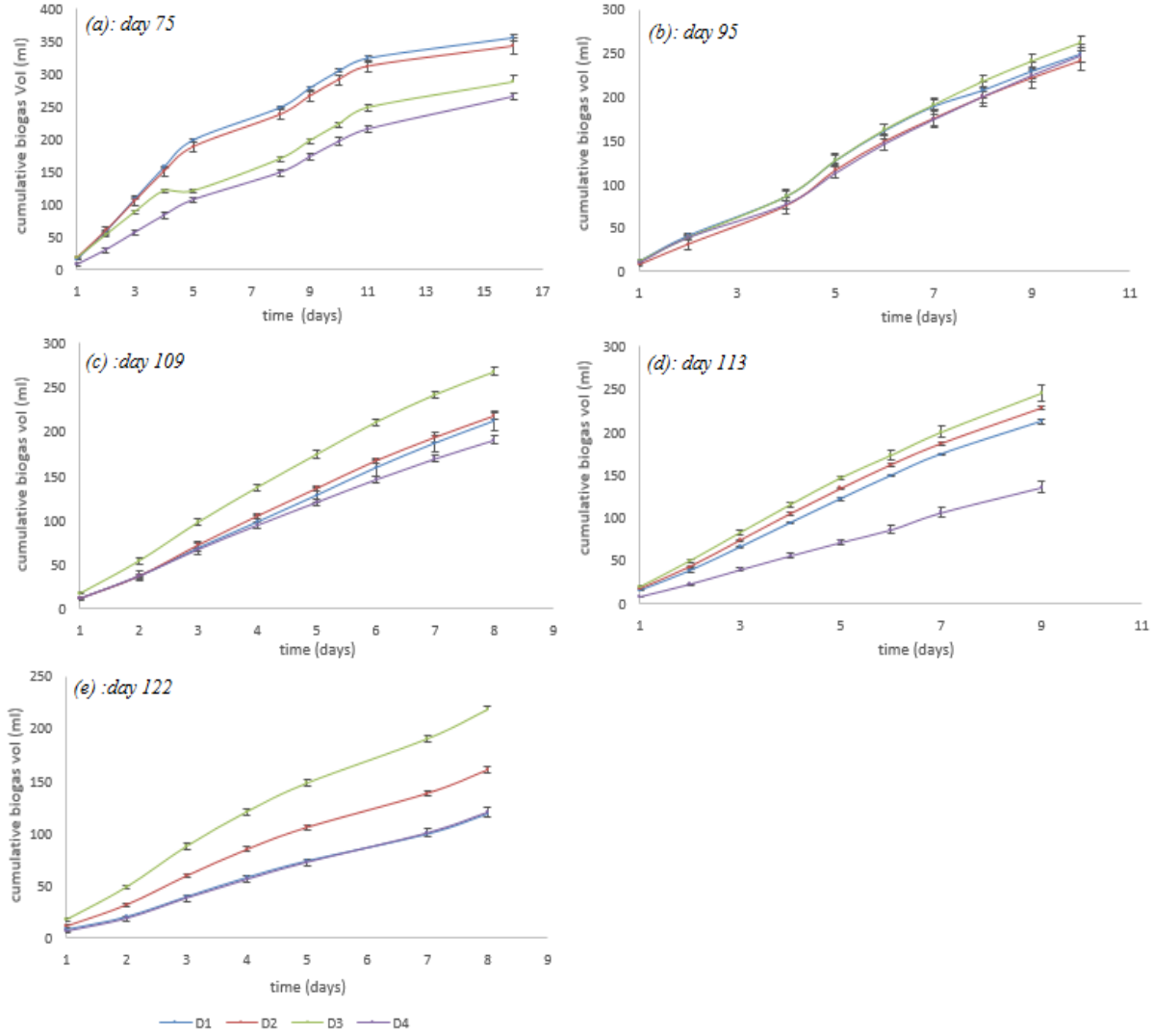


Figure 4.40. Biogas production potential of digesters investigated using 120.0 ml serum bottles on various days.

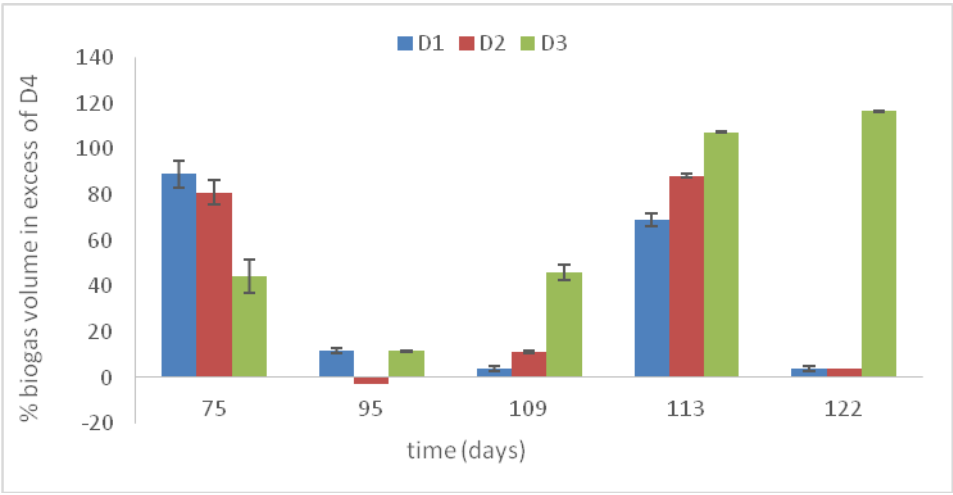


Figure 4.41. Percentage exceedance of D4's seed sludge in biogas production by each digester's seed sludge (D1-D3) investigated in 120.0 ml serum bottles.

A high exceedance (by 10%) of D4 by D3's biogas production from day 95 up to day 122 (by 120%) was observed, Figure 4.41. A possible explanation of why Co dosed digester's seed sludge (D3) had a high biogas production potential compared to D1 and D2's seed sludge could be that the seed sludge (20.0 ml residue) from D3 (Co) contained more residual acetate than that of other digesters (D1 and D2) as these produced more biogas hence utilized more acetate than D3.

Figure 4.9(a) shows that on day 114 and 125, D3 exceeded D4 and D1 respectively, thus indicating a healthier digester. The high exceedance of D4 by D3's biogas production potential on day 109 and 112 can also be associated with D3's performance on days 114 and 125, thus indicating the health of this digester relative to that of D4.

4.19.1 *Investigation of possible acetate oxidation to CO_2 and H_2 and subsequent CO_2 reduction by H_2 to CH_4.*

Due to expected diversity of microbial community in the seed sewage sludge, it was found necessary to consider the potential effect of acetate oxidation to CO_2 and H_2 with subsequent reduction of CO_2 by H_2 to CH_4. It is crucial to consider acetate oxidation in this research so as to insure that the effects of Ni and Co dosage observed are on methanogenesis from acetate rather than on products of acetate oxidation. This also help inform the dominate digestion pathways in digesters used, D1-D4.

On day 100, effluent obtained from D1-D4 was used to conduct biogas production potential assay in the same manner as before (20.0 ml residue from decantation of 100 ml of effluent was used for seeding) with the exception of dosing one group of serum bottles (3 per reactor) 100.0 mg/L of vancomycin chloride. This was adopted from a study done by Florencio et al. (1994) in which vancomycin was used to reduce/prevent oxidation of acetate to hydrogen and carbon dioxide, as well as to prevent the reverse reaction. Worth noting in that the mechanism of action of vancomycin is expected to not affect methanogens as it is based on prevention of

synthesis of the peptidoglycan layer of the cell wall (Hammes and Neuhaus, 1974). The individual performance of vancomycin dosed and non- dosed serum bottles are presented in Figure 4.42 (a-d). The triplicate average biogas volume by which those serum bottles that were not dosed vancomycin exceeded those that were dosed vancomycin is presented in Figure 4.43. From Figure 4.43, those serum bottles that were not dosed vancomycin exceed those which were dosed by around 20±5% biogas volume on day 1. This percentage of exceedance reduced with time to up to almost 10% or below after day 6. D1 and D2 had the most reduction in percentage exceedance of control with time followed by D3 then D4 for the first six days.

The acetate utilization route was not fully elucidated; nevertheless both qPCR data (abundance of Methanosaetaceae) and data from the vancomycin study suggest or provide circumstantial evidence to the view that the dominating pathway of acetate digestion in this study was through acetoclastic cleavage into CH_4 and CO_2. The acetate utilization route could best be determined through tracer experiments using radioactively labelled carbon of the methyl group of acetate, then quantifying the ratio of the radioactively labelled CH_4 to that of the radioactively labelled CO_2 as was done by Mah et al. (1978).

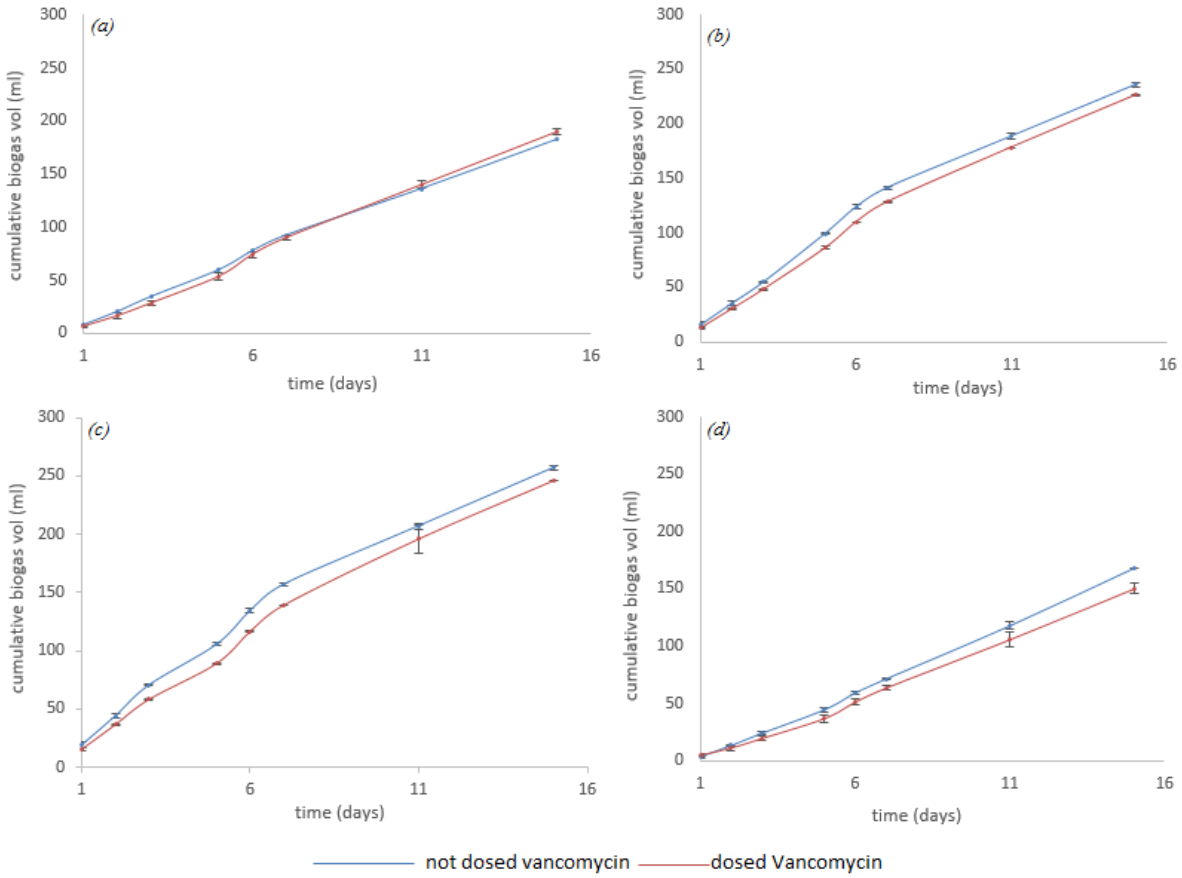


Figure 4.42. Biogas production potential of digester's seed sludge with and without dosage of Vancomycin chloride: a = D1, b = D2, C=D3, d=D4.

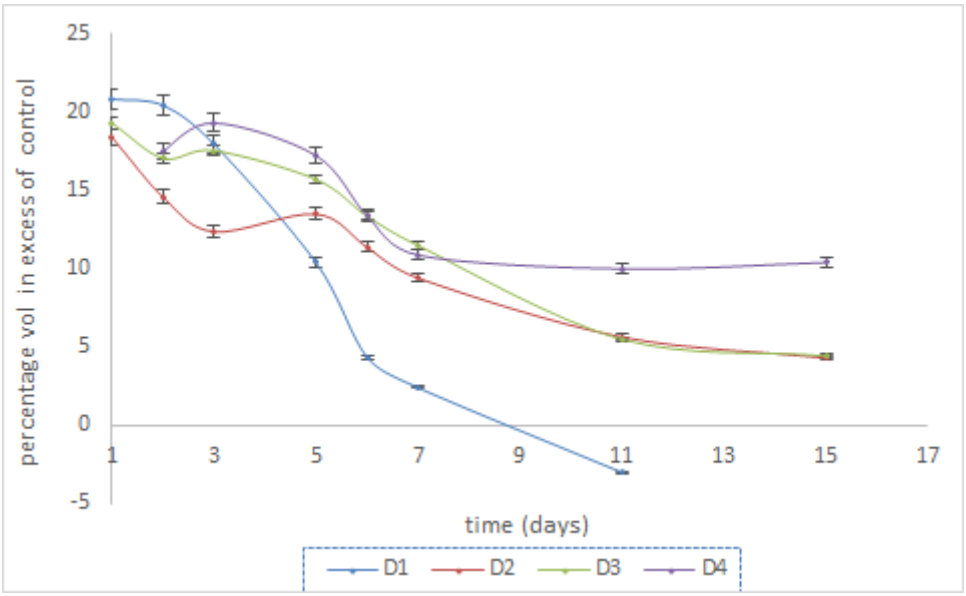


Figure 4.43. Comparism of biogas production by non-vancomycin dosed and vancomycin dosed seed sludge from each digester. Vancomycin chloride was dosed to 100.0 mg/L.

4.19.2 *Evolution of Key Microbial populations*

The relative abundance of methanogens was investigated. This is important as it can inform which pathway of biogas production was employed, as well as revealing which family of methanogens responded to which element, Ni or Co, and at what concentrations. Methanogenic genera were identified and quantified using genomics. Absolute quantification was done so it is possible to compare different time points and also different reactors.

The first two samples (bottles) taken on day 7 and day 22 have a similar appearance, a very dark colour due to the influence of the seed sludge. The other samples taken at later time points were white in colour with a limestone like texture. This change in appearance of samples was due to turning around of digester contents over time as well as formation of salts such as ammonium acetate.

Figure 4.44 shows the concentration (μg of DNA per gram of VS equivalent biomass) of DNA obtained after the DNA extraction i.e. the DNA yield. Form Figure 4.44, there was a reduction in yield across all four digesters on day 77, this period coincided with that of high pH due to omission of magnesium acetate from feed or it is due to sodium toxicity as the proportion of sodium was doubled in the feed. D4 was observed to have the highest yield from day 77 to day 158, a large proportion of which was Methanosaetaceae, Figure 4.45(d).

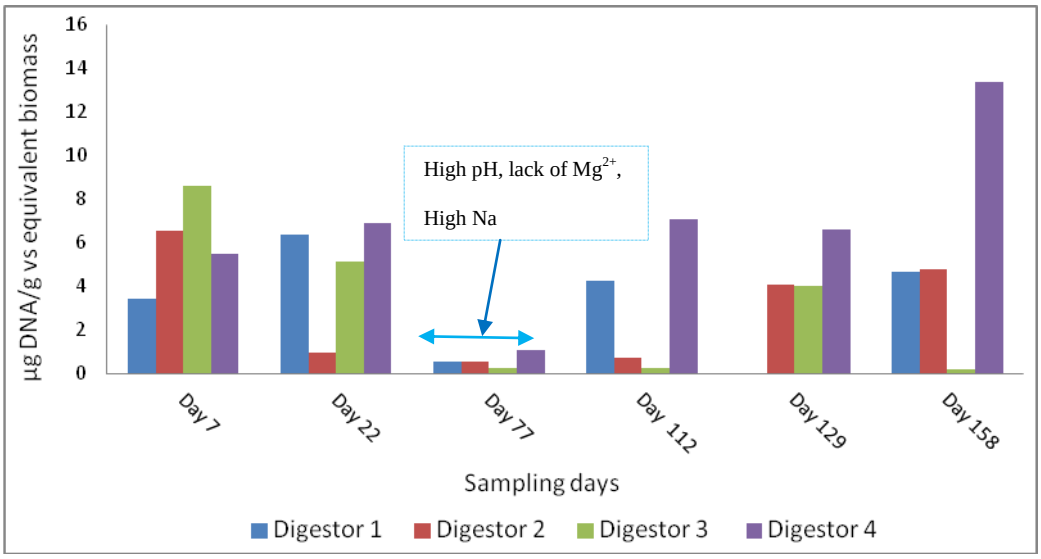


Figure 4.44. DNA yield from all digester with digestion time

Figure 4.45 shows the log 16S rRNA gene copy number/µg of DNA as a percentage of the total methanogens, based on µg of DNA. This identifies families of methanogens that were dominant in digesters on the day of sampling. D2 and D4 were mostly dominated by Methanosaetaceae, while a gradual increase in Methanosarcinaceae was observed in D3. Dosage of Ni and Co seemed to have maintained the dominance of Methanosaetaceae in D2.

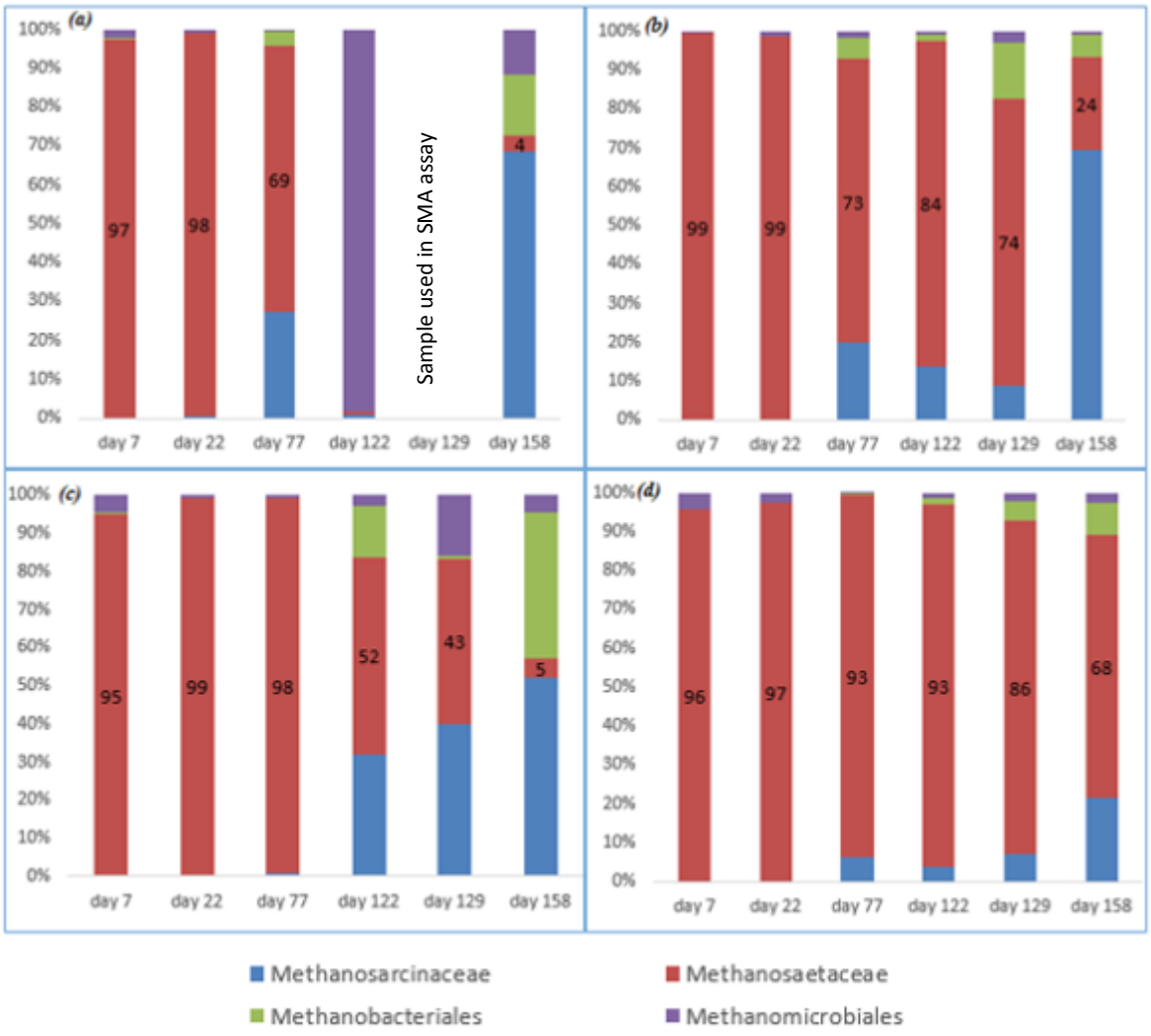


Figure 4.45. Profile of methanogens from all digesters based on log 16S rRNA gene copy number/µg of DNA; (a= D1, b=D2, c= D3 and d=D4.)

The order *Methanococcales* was not detected in any of the samples. Figure 4.45(*a-d*) shows which families of methanogens were abundant as a fraction of the extracted DNA; this indicates which methanogen family was mainly responsible for biogas production on the day of sampling. When this information (informing the abundant family) is linked to the dosed metal ion(s), it gave an indication of which methanogen family was responding to the dosage of Ni, Co and their combination.

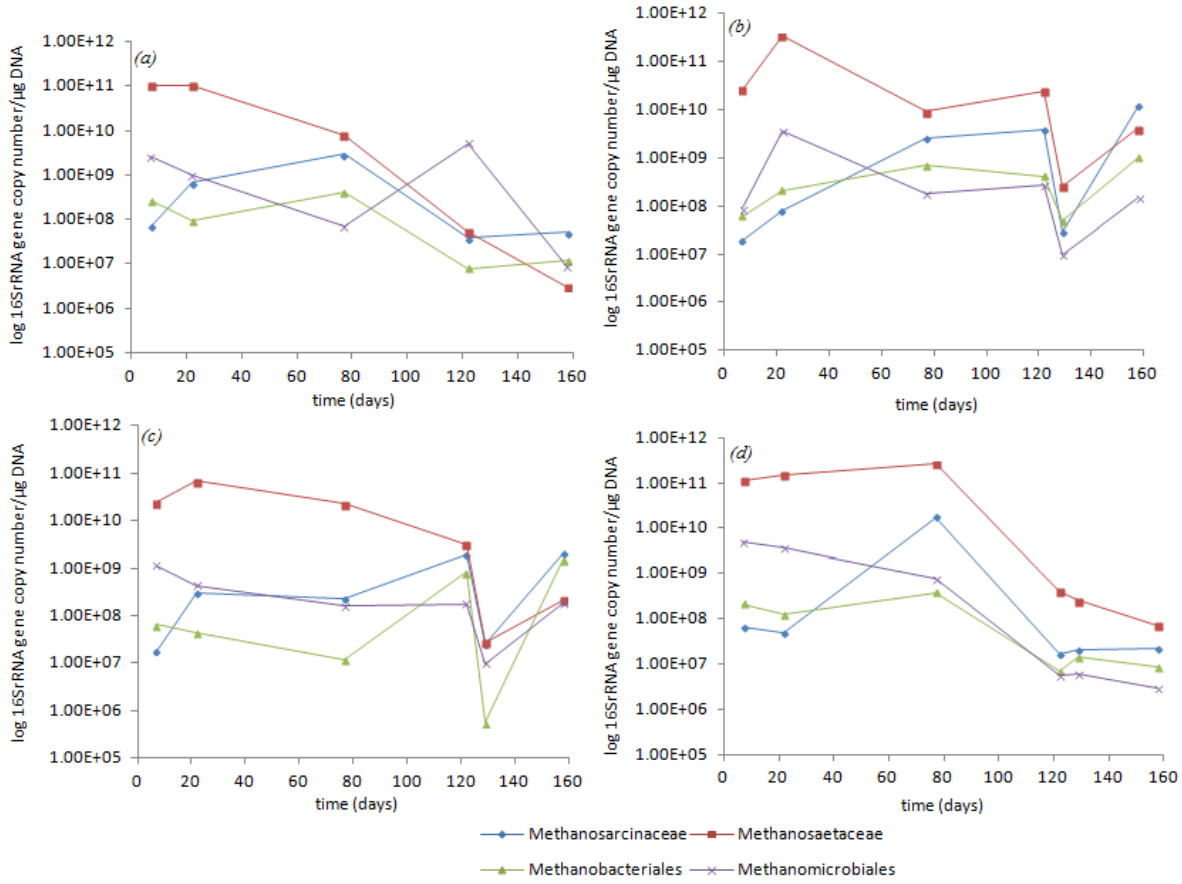


Figure 4.46. Absolute quantification profile of methanogens in log 16S rRNA gene copy number/ µg DNA;(a= D1, b= D2, C= D3 and d= D4).

Figure 4.46 shows that on day 7 and day 22, the dominant methanogen was of the family Methanosaetaceae as identified by the abundance of its 16s rRNA/µg DNA. It was shown that Methanosaetaceae were dominant in all digesters when they were operated identically (day 7). Overall there was more abundance of methanogens in D2 as compared to in other three digesters; this was in line with the observed high biogas production by this digester (D2) compared to digesters, Figure 4.15. When compared to D1 and D3, by day 158, D2 had the highest proportion of Methanosaetaceae, Figure 4.46(d). At the same time a large increase in Methanosarcinaceae and Methanobacteriales was seen in both D2 and D1. On day 122, there was dominance of Methanomicrobiales in D1, Figure 4.46(a) with little or no biogas production, Figure 4.1(a). Overall, there was a general dominance of Methanosaetaceae, with a later increase of Methanosarcinaceae in all digesters.

The general trend of change in Methanosarcinales showed an increase in Methanosarcinaceae, with time while Methanosaetaceae decreased, Figure 4.47. This trend of a shift towards Methanosarcinaceae from Methanosaetaceae could be caused by operation of digesters at 10 day hydraulic retention time, owing to the different growth rate of the two families,; Given that they both require Ni and Co for methanogenesis. A similar shift was observed by Yu et al. (2006) in reactors operated at a 10 day hydraulic retention time, and it was credited to the different growth rate of these families. The use of kinetic parameters given by Conklin et al. (2006)and Mogens et al. (2008)as well as equations (5) and (6) support the view that the use of the hydraulic retention time of 10 days could have been more favourable to accumulation of Methanosarcinaceae than Methanosaetaceae. The shift in Methanosarcinales population in all digesters with digestion time is presented in Figure 4.47.

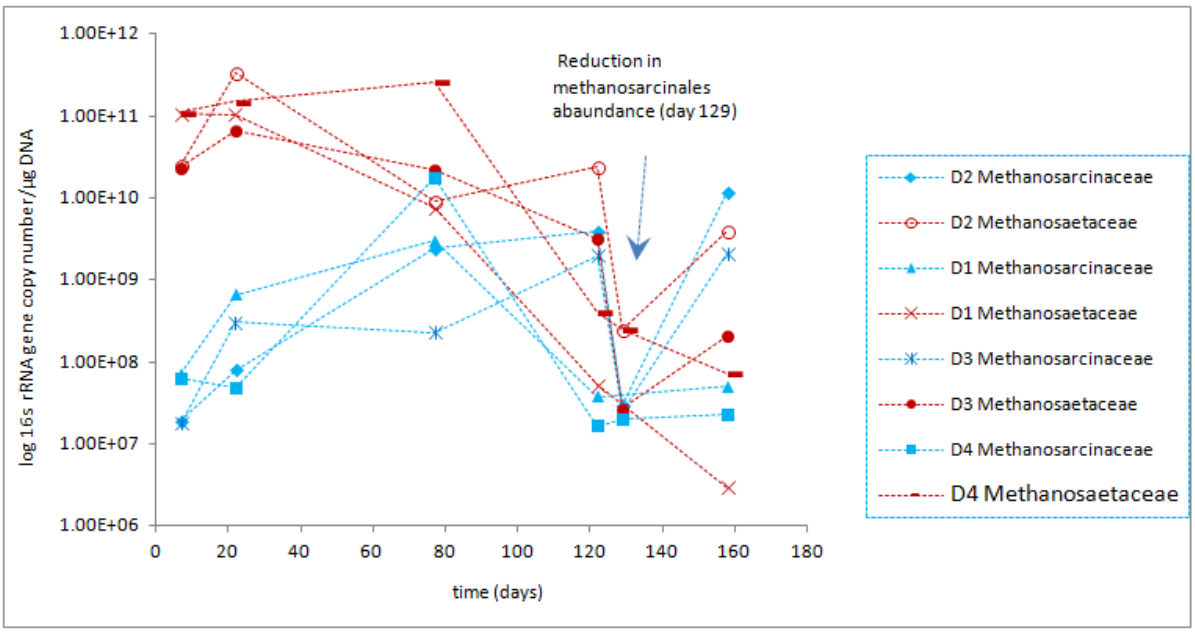


Figure 4.47. Evolution of Methanosarcinales in the digesters with time.

4.19.3 *Implication of evolution of key microbial populations*

The early dominance of Methanosaetaceae on day 7-22 was reflective of this family's dominance in the seed sludge. From day 7 up to day 22 is only 2.2 times the HRT, as such a

complete turnaround of the digester contents had not occurred. This was also observable as thick and dark qPCR samples in Figure 3.7 and Figure 3.8, as compared to the samples obtained at later days, day 129 and 158. As Methanosaeta are only able to obtain energy through acetate splitting, i.e., acetoclastic cleavage (Kendall and Boone, 2006) this is believed to be the dominant pathway for biogas production in this time period, day 7-22. Methanosaeta as compared to Methanosarcina have a low growth rate (*k)* and low half saturation coefficient (K_s) (Conklin et al., 2006).So in regions of low acetate concentrations (<25mg/L COD) as observed in Figure 4.34, Methanosaeta were anticipated to dominate the digester. As the overall performance of digesters was anticipated to go down with time, hence the concentration of acetate in effluent was also expected to increase, an increase of Methanosarcinaceae was observed in D2, D3 and D4 from day 77 to day158, Figure 4.47. In conjunction with this, there was a higher acetate (>25 g COD/L) in effluent by day 120 (Figure 4.34). At this acetate concentrations Methanosarcina are reported to have a kinetic advantage over Methanosaeta (Conklin et al., 2006). The later increase of Methanosarcinaceae is associated with their growth kinetics with respect to substrate (acetate) concentration as well as their generation time.

The contribution to biogas production by acetate oxidation to CO_2 and H_2 with the subsequent reduction of CO_2 by H_2 to CH_4 was not established in this study; but from the qPCR data as well as data from the study using vancomycin, if it occurred, it is believed to have contributed to a small percentage of methane in line with the normally observed 30%. The percentage of methane in biogas was the same (75±1%) between those serum bottles dosed vancomycin and non-dosed ones (data not shown).

Given that the seed sludge was dominated by Methanosaetaceae, Figure 4.45 and a general decrease with time of biogas production in all digesters occurred; it is strongly suggested that this decrease in biogas production with time was caused by the observed decrease in

Methanosaetaceae population with digestion time in all digesters, Figure 4.47. This further support that view that the dominant digestion pathway in this study was acetoclastic cleavage as Methanosaetaceae can only obtain energy form acetate splitting (Kendall and Boone, 2006).This decline in Methanosaetaceae population with digestion time hampered retrospective comparism of biogas production data between different time points. Standardization of biogas or methane data with VS data to reflect changing methanogenic populations is not advisable as VS measurements or data have low accuracy and specificity in reflecting methanogens population abundance. This is more applicable in this research as VS data could have been influenced by ammonium acetate decomposition during VS combustion at 505°C. This low accuracy of process oriented techniques such as change in VS in quantifying or evaluating methanogenic community is one of the reasons why Yu et al. (2006) highly advocated for the use of molecular techniques data such as genomics for engineering process control.

From day 0-129, when digesters were daily dosed 0.00-7.50 μ moles of Ni, Co and Ni and Co the dominate pathway of acetate digestion is believed to have been acetoclastic cleavage mostly by Methanosaeta, most likely of the species *M. concilii*, as opposed to *M. themophilia* based on their physiological growth requirements as the former is mesophilic while the latter is thermophilic (Kendall and Boone, 2006). In view of this, it was proposed that individual digesters' biogas output from day 0-129, during period of suitable pH mostly reflect degree of Methanosarcinales' metabolism, with the dominant family being Methanosaetaceae in the presence and absence of Ni, Co or their combination.

4.20 Project overview

This section presents an overview or summary of the interaction between various aspects of the anaerobic digestion system employed in this study, in essence an over view of the digester ecosystem with relevance to methanogens' activity is presented. This is viewed as the interaction between chemical and microbial systems of a digester.

Digesters were fed acetate daily at a loading rate of 1.8 g L^{-1} d^{-1}, targeting a daily acetate concentration of at least 30 mM. 500.0 ml of digester contents were removed daily as effluent. A general trend of reducing Methanosaetaceae while Methanosarcinaceae increased with digestion time was observed in all digesters. As Methanosaetaceae were the dominant family in early days of digesters operation, their reduction with digestion time is believed to have led to the observed reduction in biogas production from all digesetrs with digestion time, thus preventing positioning of the nominal Ni and Co concentration on the proposed dose response curve. Nevertheless the presence of these elements (Ni and Co) in a digester led to improved biogas production as the dosed digesters produced significantly more biogas than the control digester. The used 10 day hydraulic retention time possibly favoured the accumulation of Methanosarcinaceae than Methanosaetaceae, a similar observation was made by Yu et al. (2006) in a digester operated hydraulic retention time of 10 days. Also the combination of micronutrients and macronutrients that were fed to acetate digesters could not possibly provide all the essential nutrients needed by methanogenic population, as such their aggregate viability was possibly reducing with time. In line with the consequences of these factors it was observed that the overall concentration of Methanosaetaceae was reducing with digestion time. As not all of the daily fed acetate was concerted to biogas daily, this produced a situation where the digesters nominal acetate concentration were always increasing, so over time this possibly resulted in a situation where Methanosarcina were physiologically more

favoured by the digester environment as they outgrow Methanosaeta at high acetate concentration (Conklin et al., 2006, Kendall and Boone, 2006).

It is generally assumed that the metal ions in the aqueous phase are the most bioavailable while those associated with the solids and sulphates are the least bioavailable, and that those in aqueous phase are able to exert their effects more readily than those associated with sulphides or solids (Gustavsson, 2012). In this research the digester had low solids and sulphates concentration, as such the majority of the dosed metal ions were deemed to have existed in the aqueous phase. The difference in the availability of Ni and Co in aqueous phase, if any, would have been caused by their inherent chemistry. Visual MINTEQ simulation, 10% more Co than Ni was anticipated to be in the liquid phase. As dosing of Ni and Co was done daily in this study, the difference in bioavailabilities of these metal ions was not expected to cause process disturbance, in view if their co-dependence in biogas production.

qPCR data (section 4.19.2) show that prior to differentiation of the digesters through their dosage with different micronutrients, they were dominated by the family Methanosaetaceae. Methanosarcinaceae increased later (by day 77) across all digesters, their increase was observed in the following order; D1>D2>D4>D3. The observation that Methanosarcinaceae did not increase in D4 as much as in the other digesters during the entire experimentation time implies that the increase of this family in D1, D2 and D3 was as a response to supplementation of these digesters with Ni, Ni and Co and Co, respectively. At the time when D1 was dominated by the family Methanomicrobiales, no detectable amount of biogas was measured from this digester. This period also coincided with that of marked reduction in Methanosarcinales population across all digesters, day 122-129, but it was evident that D1 was the most affected digester. No further investigations were done as to why D1 had a higher increase in Methanomicrobiales.

In all the digesters the percentage of methane in the produced biogas was 64±1%. A study to examine the effect of non-methanogenic microorganisms on acetate oxidation was done through dosing of vancomycin to the serum bottles digesting acetate. At most, 20% reduction in biogas volume was observed from those serum bottles that were dosed Vancomycin on day 1, while no difference between the dosed and non-dosed serum bottles was observed in percentage of methane in the biogas produced. These observations together with the observed dominance of Methanosaetaceae, which can only obtain energy through acetate splitting to CO_2 and CH_4 provide circumstantial support to the view that the dominant pathway of methane production in this study was acetoclastic cleavage.

It is possible that the concentration of acetate in the seed sludge from the WWTP digester was already low, leading to the observed dominance of Methanosaetaceae on day 7. This is conceivable as a WWTP digester operator aims for as low as possible the concentration of VFA in the effluent, and Methanosaeta have a kinetic advantage over Methanosarcina at low acetate concentrations (Kendall and Boone, 2006). In view of this, the seed sludge used in this study might have had an inherent bias towards Methanosaetaceae.

It was found that co-supplementation of Ni and Co produced the largest increase in biogas production from acetate digesters; the second largest increase was caused by the dosage of Ni while Co dosage was observed to cause the least increase in biogas production. This can be represented as (Ni & Co) > Ni > Co. Using fresh/new biomass at higher Ni and Co concentrations, the order of exertion of toxicity (inhibition of biogas production)was also found to be: (Ni & Co) > Ni > Co.

Continuous reduction of Methanosaetaceae population with digestion time hampered comparism of biogas production during periods when digesters were daily dosed 7.50 µ moles (day 90-129) to periods when they were daily dosed 2.50 µ moles (day 12-35) i.e. the time dependence of methanogenic population abundance, especially Methanosaetaceae, hampered

the retrospective comparism of biogas production from digesters. Nevertheless overall the nominal concentrations of Ni and Co of approximately 0.1-0.3 mg/L present from day 15-35 and that of approximately 0.6-0.9 mg/L, present from day 90-129, were found to be stimulatory to biogas production from mesophilic anaerobic digestion of acetate. Thus the concentration range of approximately 0.1-0.9 mg/L of Ni and Co was found to be stimulatory to biogas production from anaerobic digestion of acetate.

CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS FOR FUTHER WORK

In this chapter conclusions relevant to the aims and objectives of this research are presented. Recommendations for further research in this topic of study area also presented.

The need to co-dose Ni and Co to anaerobic acetate digesters was established. Their co-dosage was found to lead to the production of more biogas as well as more abundance of methanogens (Methanosarcinales) than their individual dosage. Despite the need to dose Ni and Co, at large enough concentration, their exertion of toxicity was observed, which led to a reduced biogas production, this established the boundary concentration for the anaerobic digestion system used. Methanosarcinales were established as the dominant methane producers in this study, these were dominated by Methanosaetaceae family. Despite this, a shift from Methanosaetaceae to Methanosarcinaceae was observed with digester operation time; this is believed to have led to the observed reduction in biogas across all digesters with operation time.

The stimulatory and toxic concentrations of Ni and Co observed in this study are specific to the anaerobic digestions system employed; but the principles behind the biochemical catalyses/functionality of Ni and Co in methanogenesis are believed to be universally true, hence can be applied to different digesters.

The specific conclusion relevant to the aim and objectives of this research are presented as follows:

- Form reviewing the literature, it was found that when Ni and Co are dosed to digesters they lead to increased production and activation of enzymes that catalyse methanogenesis, resulting in increased biogas production. These biochemical roles of Ni and Co concurred with empirical observations made from review of the literature.

- A test regime for examining the effects of individual and combined dosage of Ni and Co to mesophilic anaerobic acetate digesters was developed. This test regime combined digester's biogas production data with their methanogenic population evolution data to evaluate the effects of their dosage.
- Individual and combined daily dosage of 2.50 µ moles of Ni and Co from day 12 to day 35, thereby increasing their nominal concentrations from 0.272 µM (0.016 mg/L) and 0.199 µM (0.012 mg/L) to 4.873 µM (0.286 mg/L) and 4.867 µM (0.286 mg/L) respectively, led to dosed digesters producing significantly more biogas than the non-dosed digester (control) in the anaerobic digestion system used.
- Individual and combined daily dosage of 7.50 µ moles of Ni and Co from day 90 to day 129 thereby increasing their nominal concentrations from 10.397 µM (0.610 mg/L) and 10.379 µM (0.612 mg/L) respectively, to a concentration of 14.924 µM, approximately 0.876 mg/L for Ni and 0.879 mg/L for Co, was found to be stimulatory to biogas production from the anaerobic digestion of acetate, as the dosed digesters produced significantly more biogas/methane compared to the control digester in this period.
- The combined daily dosage of 2.50 µ moles of Ni and Co from day 12 to day 35, thereby increasing their nominal concentrations from 0.272 µM (0.016 mg/L) and 0.199 µM (0.012 mg/L) to 4.873 µM (0.286 mg/L) and 4.867 µM (0.286 mg/L) respectively, as well the combined daily dosage of 7.50 µ moles of Ni and Co from day 90 to day 129 thereby increasing their nominal concentrations from 10.397 µM (0.610 mg/L) and 10.379 µM (0.612 mg/L) respectively, to a concentration of 14.924 µM, approximately 0.876 mg/L for Ni and 0.879 mg/L for Co led to production of more biogas than from their individual dosage in the anaerobic digestion system used.

- When co-dosed, the individual contribution to biogas production by dosed Ni and Co were delineated, and were found to be additive. This addictiveness was postulated to be caused by the interdependence of the Ni and Co catalysed enzymes which are crucial for methanogenesis.
- The increase of the nominal concentrations of Ni and Co from 0.275 μM (0.0162 mg/L) and 0.199 μM (0.012 mg/L) respectively, to their common concentration of 14.924 μM (Ni =0.867 mg/L; Co=879 mg/L) due to their daily dosage was found to be stimulatory/catalytic to biogas production at suitable pH and feed composition from the anaerobic digestions system used. Nevertheless the relative positions of these concentrations on the dose-response curve could not be determined due to the observed time dependent reduction in biogas production across all digesters. This decline in biogas production with time was postulated to be caused by the observed decline in Methanosaetaceae population with digestion time as determined by the 16s rRNA analysis.
- Methanosarcinales was the dominant order of methanogens in all digesters in this study, with the family Methanosaetaceae more abundant in early days of digestion, possibly owing to their dominance in the seed sludge used. Methanosaetaceae reduced with digestion time while Methanosarcinaceae increased; this was credited to the used hydraulic retention time (10 days) and acetate concentration; these factors favoured growth of Methanosarcinaceae in view of their maximum growth rate (μ_{max}) and half saturation constant (K_s) relative to those of Methanosaetaceae. Methanomicrobiales dominated in D1(Ni) at the time of marked reduction in Methanosarcinales population, this was accompanied by low or no biogas production. The cause of increase of this order was not identified.

- There was more abundance of methanogens (Methanosarcinales) in a digester co-dosed with Ni and Co (D2); this was believed to have caused this digester to produce more biogas than those digesters which were individually dosed Ni and Co.
- As the co-dosage of Ni and Co was found to be more important for biogas production than their individual dosage, the combined toxicity of Ni and Co was of more important consideration than that of their individual dosage. A combined (in serum bottles) Ni and Co concentration range of 31.50 μM -504.00 μM (≈1.86-29.70 mg/L) in D4's seed sludge was found to be inhibitory to biogas production for the anaerobic digestion system used.
- The excess concentration of Ni and Co for the anaerobic digestion system used was not conclusively determined. But considering that, dosage of D4's seed sludge in the serum bottles with a combined Ni and Co to a concentration of 15.75 μM (≈0.93 mg/L) did not cause an increase or significant inhibition (only ≤2%) of their biogas production; while their dosage to a combined Ni and Co concentration of 31.50 μM caused about 18% inhibition of their biogas production, a concentration range of 15.75 μM -31.50 μM (≈0.93-1.86 mg/L) is postulated with great tentativeness to be the excess as well as the transitionary concentration to toxicity for the anaerobic digestion system used.
- Using fresh biomass/sludge, it was found that when co-dosed to the same total ion concentration as their individual dosage; Ni and Co were a more potent inhibitor of biogas production, followed by individual Ni then Co, i.e. inhibition was exerted in the order: (Ni & Co) > Ni > Co.
- Region A (NOEL) of the proposed dose response curve for the anaerobic digestion system used was not determined as the concentration of Ni and Co due to the first applied daily dosage of 2.50 μ moles led to an increased biogas production.

Nevertheless, this higher biogas production of dosed digesters as compared to the control digester, whose background Ni and Co were continuously washing out with time, implies that its (control digester) nominal Ni and Co concentration were limiting its biogas production.

- The acetate utilization route was not fully elucidated in this study; nevertheless both qPCR data (abundance of Methanosaetaceae) and data from the vancomycin study provide circumstantial evidence to the view that the dominating pathway of acetate digestion was through acetoclastic cleavage.

5.1 Recommendations for further work

In relation to micronutrients dosage to anaerobic digesters further research is recommended along the following areas for improvement of both scientific knowledge and applicability of the findings to large scale digesters.

- The main challenge in this study has been the time dependent decline in biogas production with digestion time, due to a time dependent decline in Methanosaeta population, while the dosage of Ni and Co, hence resulting nominal concentration, was increased with time. This hampered correlation of dosage/nominal concentration with biogas production. This can be overcome by running several digesters and dosing them with different initial dosages on Ni and/Co, e.g. operating three digesters and daily dosing them with 2.50 μ moles, 5.00 μ moles and 7.50 μ moles and then comparing their biogas production profiles. In this way, the nominal concentrations of Ni in each digester will be compared in real time as opposed to retrospective comparism. A pull out digester system can also be used, where by one set of experiments e.g. investigation of Co effects is done and finalised, then a new set of experiments seeded with sludge from the same digester that was used to seed the first set of experiment is done, e.g. investigation of effects of Ni dosage.
- Testing the effect of Co dosage on the rate of accumulation/production of CH_3-S-CoM from acetate, as Co is anticipated to increase biogas production rate through increasing the rate of CH_3-S-CoM production in necessary. As such testing the effect of Co on what it is anticipated to do is important.
- Testing the effect of Ni dosage on the rate of disappearance of CH_3-S-CoM is recommended as Ni is believed to increase the rate of biogas production through

increasing the rate of reduction of CH_3-S-CoM to CH_4. Again testing the effect of Ni on what it is anticipated to do is important.

- Examination of the effect of dosing different number of moles of Ni and Co to a digester is also important, as possibly these elements are required in different amounts by methanogens. This may be because the production of CH_3-S-CoM requires several steps as opposed to its reduction (Thauer, 1998) and the increase in biogas production due to Co was shown to have been delayed in this research, possibly more Co was needed before increased biogas production due to its dosage could be observed, thus implying that more Co could have been required than Ni in the digestion system used.
- Determination of the acetate digestion route through isotope labelling of the methyl group of acetate ($^{14}CH_3COOH$), then quantifying the ratio of $^{14}CH_4/^{12}CO_2$ is recommended, as this will allow the digester operator to focus on a specific known route of methanogenesis.
- In light of Gikas (2007)'s research which showed improved settling and other important characterises of sewage sludge following the dosage of Ni and Co to an aeration tank, it will be interesting to examine the biogas production potential (Methane) of the sludge obtained from a Ni and Co dosed aeration tank. If improved biogas production is observed from this sludge, possibly dosing Ni and Co only once at the aeration stage could be more economical.

Appendix A: Percentage of Methane from serum bottles experiment digesting acetate as substrate

The percentage of methane measured from serum bottles from day 75-122 following their supplementation to 15.75-504.00 µM (Table 3.3) of Ni and/ Co are presented in Figure A- 1. Overall the control serum bottles, those that were not dosed high metal ion concentration, had a 75% prevalence of methane in terms of volume. Starting from the dosage to 63.00-504.00 µM of Ni, Co and Ni and Co, there was an observed reduction in the percentage prevalence of methane; this was also accompanied by a reduction in biogas volume from these serum bottles. When dosed to a concentration of 15.75-31.5 µM there was no observed disparity in percentage methane between control and experimental serum bottles.

The higher prevalence of methane in control serum bottles is believed to be due to the dissolution of CO_2 at high pressure caused by biogas in serum bottles. Serum bottles which were dosed high metal ions produced less biogas, as such had low pressure, there was observed low percentage of methane in these serum bottles; Figure A- 1. During measurement of biogas volume from serum bottles there was an observed effervescence across the length and breadth of the liquid phase, in the manner similar to that of a *fizzy drink*. It is also worth noting that the parent digesters, D1-D4, had on average 64% methane gas during the experimental period irrespective of the actual biogas volume they produced. This was because there was no pressure buildup in digesters as biogas was free to escape; as such no increased CO_2 dissolution, hence no excess methane in the headspace.

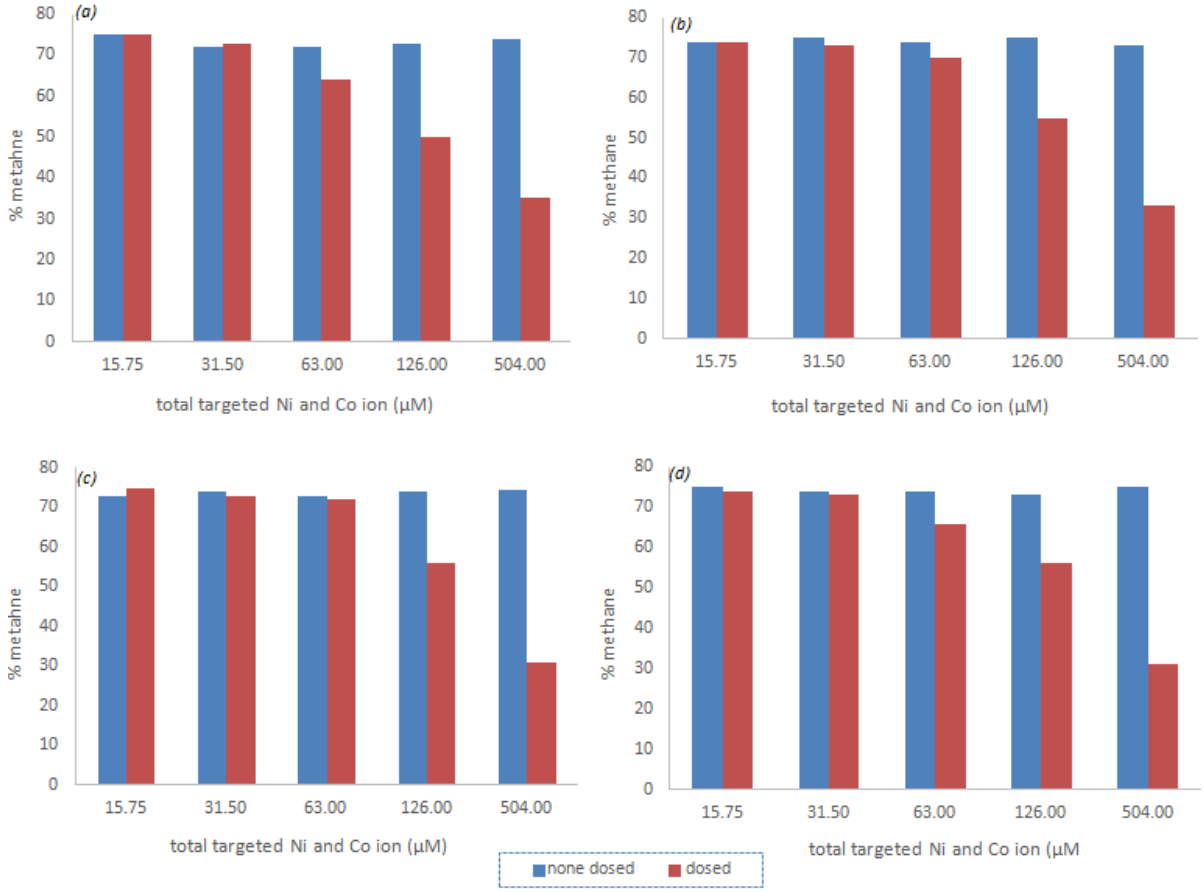


Figure A- 1. The percentage of methane in the biogas measured from serum bottles during toxicity assay seeded with sludge from all digesters; (a)= D1, (b)=D2, (c)= D3, (d)= D4.

Appendix B: Visual MINTEQ simulation and total solids change over time

The chemical compounds fed to the digesters were expressed in terms of their individual ions at their respective concentrations and ran on Visual MINTEQ to assess the likelihood of precipitation of Ni and Co. The outcomes with regard to Ni and Co are presented in Figure B-1 and Figure B-2. The pH values used in MINTEQ simulation were reflective of those of digesters during the period of omission of magnesium acetate from the feed as well as when magnesium acetate was available. At a pH of 8.5 as was the case when magnesium acetate was omitted from the feed, 47% and 70% of Ni and Co were anticipated to exist as ions in solution, while at a pH of 7.50 these percentages increased to 66% and 77% respectively. As

such the change of pH not only could have affected methanogens directly but also may have interfered with the bioavailability of Ni and Co.

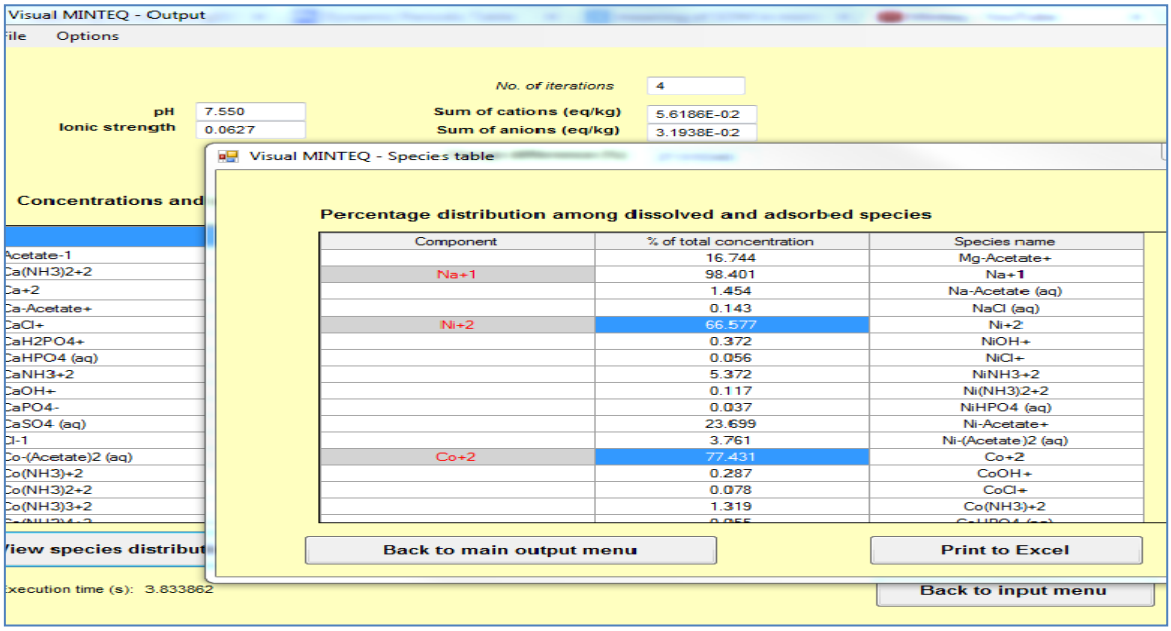


Figure B-1. Visual MINTEQ simulation showing percentage distribution of Ni^{2+} and Co^{2+} at a pH of 7.5, and a concentration of 0.731 mg/L.

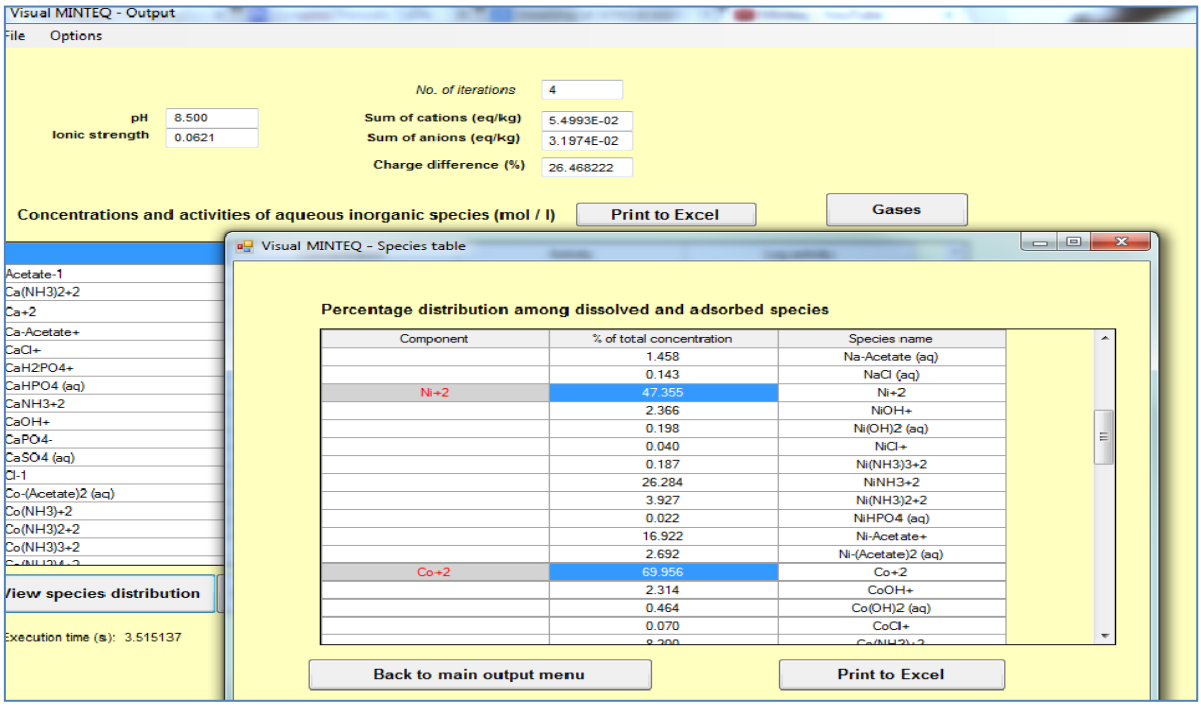


Figure B-2. Visual MINTEQ simulation showing percentage distribution of Ni^{2+} and Co^{2+} at a pH of 8.5 and a concentration of 0.428 mg/L.

Figure B- 3. Change in total solids content of the sludge over time, A = D1, B = D2, C =D3 and D =D4.

Note the visibly thin sludge 'B' of D4 on day 130, as compared to on day 10.

Appendix C: Treatment of uncertainties in measurements

Equations C-1 to C-6 show how uncertainties in measurements, especially of biogas volume, were treated during addition, division and multiplication as well as when a constant was involved in the computation. The gas counters used for biogas measurement were found to have an uncertainty margin of 2 ml after a triplicate calibration. During measurements of biogas production from serum bottles in triplicates, the standard deviation was taken as the uncertainty margin. These equations were adapted from Andraos (1996).The following situations applied.

An algebraic sum or difference: *if*

$$f = x + y + z \qquad \text{Eq (C-1)}$$

then $$\Delta f = \pm\sqrt{(\Delta x)^2 + (\Delta y^2) + (\Delta z^2)} \qquad \text{Eq (C-2)}$$

A product or a quotient: *if:*

$$f = \frac{x}{y} \qquad \text{Eq (C-3)}$$

then $$\Delta f = \pm\sqrt{\left(\frac{\Delta x}{x}\right)^2 + \left(\frac{\Delta y}{y}\right)^2} \qquad \text{Eq (C-4)}$$

A product involving constants: *if*

$$f = \alpha(x) \qquad \text{Eq (C-5)}$$

then $$\Delta f = \pm|\alpha|\Delta x \qquad \text{Eq (C-6)}$$

Dissemination

Conferences and workshops attended

April 2011: Presented in School of Civil Engineering Conference, The University of Birmingham. "Metal ions (Ni and Co) in anaerobic digestion of Acetate".

December 2012: Poster Presentation at 4th Asia-Pacific Young Water Professionals Conference, Tokyo, Japan. "Micronutrients in anaerobic digestion of Acetate: focusing on Cobalt ($Co^{2+)}$ and Nickel ($Ni^{2+)}$"

June 2013: Poster presentation. 13th World Congress on Anaerobic Digestion. Santiago de Compostela. Spain. "Micronutrients in anaerobic digestion of Acetate: focusing on Cobalt (Co2+) and Nickel (Ni 2+)".

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