Trace metal supplementation in wastewater sludge digesters

Farryad Ishaq

A thesis submitted to
The University of Birmingham
for the degree of
DOCTOR OF PHILOSOPHY

Department of Civil Engineering
School of Engineering
The University of Birmingham
November 2011

ABSTRACT

Multiple studies have shown the importance of transition metals for the microorganisms involved in anaerobic digestion. Deficiencies in metals can translate into suboptimal digester performances and therefore where present an opportunity to stimulate anaerobic digestion exists. Previous supplementation studies that have used EDTA complexed metals have shown conflicting results.

Assays supplemented with cobalt-EDTA on its own consistently responded to a level equal to or higher than that from assays supplemented with a combination of metals. The results seemingly indicate that cobalt was the most commonly deficient metal in the sludge digesters. However this study has raised questions about the bioavailability of other metals as EDTA chelates, particularly nickel-EDTA. Differing levels of bioavailability for different EDTA complex metals explain how conflicting results for EDTA complexed metals can exist. Metal deficiencies were present 69% of the time in the wastewater sludge digesters samples. Given the questions raised about the bioavailability of metal-EDTA chelates the prevalence of deficiencies was likely to have been higher. At a site specific level it was found that a lower than expected V_{max} for a given sites acetoclastic methanogen population corresponded with a positive response from supplementation and therefore it can act as an indicator of metal requirement.

AKNOWLEDGEMENTS

I would like to thank my supervisor Dr. C. Carliell-Marquet for all the valuable support and insight she provided throughout the period of the research. I wish to extend my thanks also to John Edgerton for all the help in the laboratory and to Peter Doughty and Peter Vale from Severn Trent Water for all their support for this research. Many thanks also to Tom Curtis's group from Newcastle University for their help with microbiological analysis.

Finally and most importantly I would like to thank God for guiding me through all the peaks and troughs to make this possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

ACN	Acetate capacity number
AD	Anaerobic Digestion
Al	Aluminium
ATP	Adenosine tri-phosphate
BA	Barium
BMP	Biochemical methane potential
BOD	Biochemical oxygen demand
Ca	Calcium
Ce	Cerium
CH_4	Methane
$\Delta G°$	Change in Gibbs's free energy
CH_3COOH	Acetate
CHP	Combined heat and power
CPR	Chemical phosphorus removal
COD	Chemical oxygen demand
Co	Cobalt
Cu	Copper
CO_2	Carbon dioxide
cm	Centimetre(s)
°C	Degree(s) Celsius – unit of temperature
d	Days(s)
Defra	Department for environmental food and rural affairs
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
Dy	Dysprosium
E^o	Reduction potential
EDTA	Ethylenediaminetetraacetic acid
EEC	European economic community
Er	Erbium

et al.	And others
Eu	Europium
EU	European union
Fe	Iron
FISH	Fluorescence in situ hybridisation
g	Grams(s)
GC	Gas chromatograph
Gd	Gadolinium
g/l	Grams per litre
h	Hour(s)
H_2	Hydrogen
HCl	Hydrochloric acid
HCO_3	Bicarbonate
Hg	Mercury
HNO_3	Nitric acid
Ho	Holmium
HRT	Hydraulic retention time
H_2O	Water
H_2SO_4	Sulphuric acid
IA	Intermediate alkalinity
ICP	Inductively coupled plasma (spectrophotometer)
IHT	Inter hydrogen transfer
K	Kelvin- unit of temperature in equations
K	Potassium
kg	Kilogram(s)
kJ	Kilojoules(s)
kW	Kilowatts(s)
kWh	Kilowatt hour(s)
l	litre(s)
La	Lanthanum
Lu	Lutetium

M / mol	Molar concentration
m	Meter(s)
mm	Millimetre(s)
µm	Micrometre(s)
m^3	Cubic metre(s)
m^2	Square metre(s)
MCR	Methyl-coenzyme M reductase
MO	Micro-organism(s)
mg	Milligram(s)
mg/l	Milligrams per litre
Mg	Magnesium
Mn	Manganese
min	Minute(s)
Msc	*Methanosarcinaceae*
Mst	*Methanosaetacea*
N	Normality
Na	Sodium
n	Sample size
Nd	Neodymium
Ni	Nickel
NH_3	Ammonia
OLR	Organic loading rate
P	Phosphorus
PA	Partial alkalinity
%	Percentage
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration ($\log_{10}$)
Pr	Praseodymium
qPCR	Quantification PCR
r^2	Regression coefficient
RNA	Ribonucleic acid

rpm	Revolutions per minute
RR	Ripley's ratio
RSD	Relative standard deviation (%)
s	Second(s)
SD	Standard deviation
Sm	Samarium
SMA	Specific methanogenic activity
SR	Strontium
SRB	Sulphate reducing bacteria
SRT	Solids retention time
STP	Standard temperature and pressure
TA	Total alkalinity
Tb	Terbium
Tm	Thulium
TM	Trace metal combination
TS	Total solids
UK	United Kingdom
UPW	Ultra pure water
V	Vanadium
VFA	Volatile fatty acids
$\mathbf{V_{max}/V_{max\ acetate}}$	Maximum acetoclastic activity (ml CH_4/h)
$\mathbf{V_{plant\ acetate}}$	Full-scale plant acetoclastic activity (l CH_4/l sludge. d)
VS	Volatile solids
WWTW	Wastewater treatment works
Yb	Ytterbium
Zn	Zinc

CHAPTER 1 INTRODUCTION

Suspended bacterial growth anaerobic digesters are commonly used to degrade (digest) municipal wastewater sludges. The primary goal of anaerobic digestion (AD) of wastewater sludges is to reduce the organic content of the sludge with concurrent reduction in odour and pathogens (Parkin and Owen, 1986). Methane (CH_4) is the end product of the microbial decomposition of organic matter in anaerobic environments.

Methane escaping into the atmosphere is of great concern as it is a more potent greenhouse gas than Carbon dioxide (Gerardi, 2003). Anaerobic digestion is an effective waste management and renewable energy strategy; it combines a reduction of greenhouse gas emission by capturing methane from the decomposition of organic materials and provides energy in the form of biogas which can be used to generate heat and power, or as a transport fuel, either locally or injected into the gas grid (defra, 2010). Also the treated material known as the digestate has great value as it can be used as a fertiliser or a soil conditioner.

According to the UK department of energy and climate change (DECC) the contribution of all renewables to UK energy consumption was 3.3% in 2010 (DECC, 2010). Britain has an EU mandated target to meet 15% of its energy requirements from renewable sources by 2020; this covers energy used for heating and transport as well as electricity. The government has also set a further target to cut carbon emissions by 60% by 2050 against a 1990 baseline. Given the UK renewable obligations and objectives anaerobic digestion is highlighted as a process which can make an important contribution in helping the government meet its targets and obligations (defra, 2010).

Anaerobic digestion represents a process which has significance at a worldwide level due to its relevance in helping tackle global climate change. It is of great interest therefore to further the understanding of the AD process in order to promote maximal output, specifically in respect to organic content reduction and the methane generation that is coupled to it.

Multiple studies have shown the importance of transition metals such as cobalt (Scherer *et al.*, 1983, Scherer and Sahm, 1981, Zandvoort *et al.*, 2006) for the microorganisms involved in AD. Whereas often sufficient metals appear to be retained in municipal wastewater sludge digesters to support optimal microorganism growth and activity, it's evident that the present elements are not always bioavailable (Speece, 1988). Consequently deficiencies in metals translate into suboptimal performances and therefore an opportunity to supplement metals exists in these digesters to maximise AD performance.

The advantages of supplementing metals in studies has been demonstrated previously in many ways including; increases in methane production (Fathepure, 1987, Gonzalez-Gil *et al.*, 2003, Gonzalez-Gil *et al.*, 1999, Jansen *et al.*, 2007), methanogenic growth (Mah *et al.*, 1978, Scherer and Sahm, 1981) substrate utilisation rate (Murray and Berg, 1981, Speece *et al.*, 1983, Zitomer *et al.*, 2008), reduction in COD (Takashima *et al.*, 2011, Takashima and Speece, 1989) and improvements in digester health and stability (Climenhaga and Banks, 2008, Murray and Berg, 1981).

Most supplementation studies add metals as chloride salts and studies that have used chelating compounds namely EDTA have shown conflicting results. Majority of studies find EDTA bound metals to be soluble but non-bioavailable (Aquino and Stuckey, 2007, Babich and Stotzky, 1983, Bartacek *et al.*, 2008, Pankhania and Robinson, 1984, Speece, 1988). However some studies have reported certain EDTA complexed metals to have had a much quicker stimulatory effect in comparison to the chloride salt, in terms of specific methane activity and substrate removal, suggesting higher bioavailability (Fermoso *et al.*, 2008) . Further understanding of the bioavailability of EDTA metal chelates is important in helping supplementation of metals in their most bioavailable forms and helping to avoid wasteful dosing.

An optimised metal dosing strategy to digesters aims to achieve maximum effect on the biological activity whilst avoiding wasteful dosing and limiting introduction of metals in to the environment (as part of effluent). In order to achieve this, better understandings of metal requirements alongside bioavailability in terms of metal speciation and microbiological uptake characteristics is required.

Principal Aim

The aim of this study is to investigate the potential of EDTA complexed metals to enhance methanogenesis of wastewater sludge and to further the understanding of the relationship between metal requirements, methanogenic populations and methane output in order to facilitate effective identification of when digesters would benefit from supplementation.

CHAPTER 2 LITERATURE REVIEW

2.1 Anaerobic Digestion- Process Overview

The process of anaerobic digestion involves the complex interaction of several groups of microorganisms. The process can be summarised as having three stages, the first stages involves the action of facultative and fermentative bacteria; these bacteria hydrolyse particulate compounds into monomeric soluble sugars, long chain fatty acids, amino acids and alcohols (Zeikus, 1979).

In the second stage the products of hydrolysis are further fermented to produce acetate, formate, methanol, H_2 and CO_2. This process is known as acidogenesis. The products of this process can then be further oxidised in a process known as acetogenesis producing acetate, H_2 and CO_2 (Schink, 1992). The microorganisms of AD have a complex and intricate relationship with one another. For example the oxidation of substrates such as butyrate, propionate or ethanol are endergonic reactions (energy requiring) i.e. the $\Delta G°$ for propionate oxidation is +76.1 kJ/reaction, however this reaction becomes exergonic (energy producing) when the hydrogen partial pressure is reduced i.e. the $\Delta G°$ for propionate oxidation becomes -25.6 kJ/reaction (Thauer *et al.*, 1993). Therefore hydrogen producing bacteria in acetogenesis stage grow in syntrophy with hydrogen utilising bacteria such as the methanogens or the sulphate-reducing bacteria (SRB). The process of hydrogen being produced by one group of bacteria i.e. the acetogens and used by another i.e. the methanogens is known as interspecies hydrogen transfer (IHT). In the final step of anaerobic digestion the products of fermentation are converted to CH_4 and the organisms mediating these reactions are known as the methanogens.

The Sulphate-reducing bacteria (SRB) are group of bacteria found in anaerobic digesters, the SRB reduce sulphate to hydrogen sulphide and can compete with the methanogens for their substrates such as acetate and hydrogen. At lower acetate concentrations SRB's are found to outcompete the methanogens for its substrate however the opposite result is found at higher acetate concentrations. Sulphate reducing bacteria produce hydrogen sulphide as an end product which is inhibitory to many trophic groups involved in AD including the methanogens (Widdel, 1988). This again emphasises the intricate nature of the microorganism relationships that exists within a digester.

2.2 The Methanogens

Methanogens are the only microorganisms known to produce CH_4 as a catabolic end product (Thauer, 1998). Despite sharing this unique ability, great diversity exists amongst the methanogens. Significant differences are found for example in cell wall composition and in growth conditions between different lineages (Sprott and Beveridge, 1993); optimum growth temperatures for some methanogens are at below 20°C whereas others are nearer 100°C (Boone *et al.*, 1993)

Comparative genetic analysis of 16S ribosomal RNA in the 1970's (Woese and Fox, 1977) revealed the methanogens to belong to an evolutionary unique kingdom; termed the archaea. Archaea are similar to bacteria in appearance and organisation of chromosomes (Londei, 2005) and have remarkable similarities with eukaryotic homologues especially in respect to DNA replication apparatus (Olsen and Woese, 1996). However the methanogens utilise unique cofactors and have unique cell wall compositions and metabolic pathways in comparison to members of other kingdoms (Gerardi, 2003).

Methanogens belong to the *Euryarchaeota* phylum. They are further classified into five orders: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales* and *Methanosarcinales*. Methanogenesis amongst all species involves conversion of a methyl group to methane. However the origin of the methyl group varies. Methanogenic species which generate CH_4 by the reduction of H_2/CO_2 are known as hydrogenotrophic methanogens and those which form CH_4 by acetate decarboxylation are known as acetoclastic methanogens. From the five orders only the *Methanosarcinales* can metabolise acetate to CO_2 and CH_4. Methanogens with high optimum growth temperatures (hyperthermophiles) are only found among the *Methanobacteriales*, *Methanococcales* and *Methanopyrales* orders (Boone *et al.*, 1993). Therefore more closely related species are found within the same taxonomic groups.

Acetoclastic methanogens are responsible for approximately 70% of the total CH_4 generated during anaerobic digestion (AD) of domestic wastewater sludge (Levett, 1990). Within the order Methanosarcinales, only two genera of methanoarchaea are known to utilise acetoclastic methanogensis. The first being the genus *Methanosarcina* which is a member of the family *Methanosarcinaceae* and the second is the genus *Methanosaeta* which belongs to the family *Methanosetaceae* (Schink, 1992). *Methanosaeta* is believed to only utilize acetate whereas *Methanosarcina* species utilize a range of compounds in preference to acetate e.g. methanol and other methylated compounds (Smith and Ingramsmith, 2007). *Methanosarcina* species form large pockets of coccoid cells whereas *Methanosaeta* are typically rods cells which often form long filaments and large aggregates (Jetten *et al.*, 1992). These morphological differences can be seen clearly using scanning electron microscopy as shown in Figure 2.1. Methanogens belonging to *Methanosarcina* genus tend to have a thick, rigid outer envelope whereas *Methanosaeta* species are characterised by a thin fibrillar outer sheath (Zeikus, 1977). Therefore despite belonging to the same taxonomic order and having the ability to utilise the same substrate, members of both species still maintain significant differences.

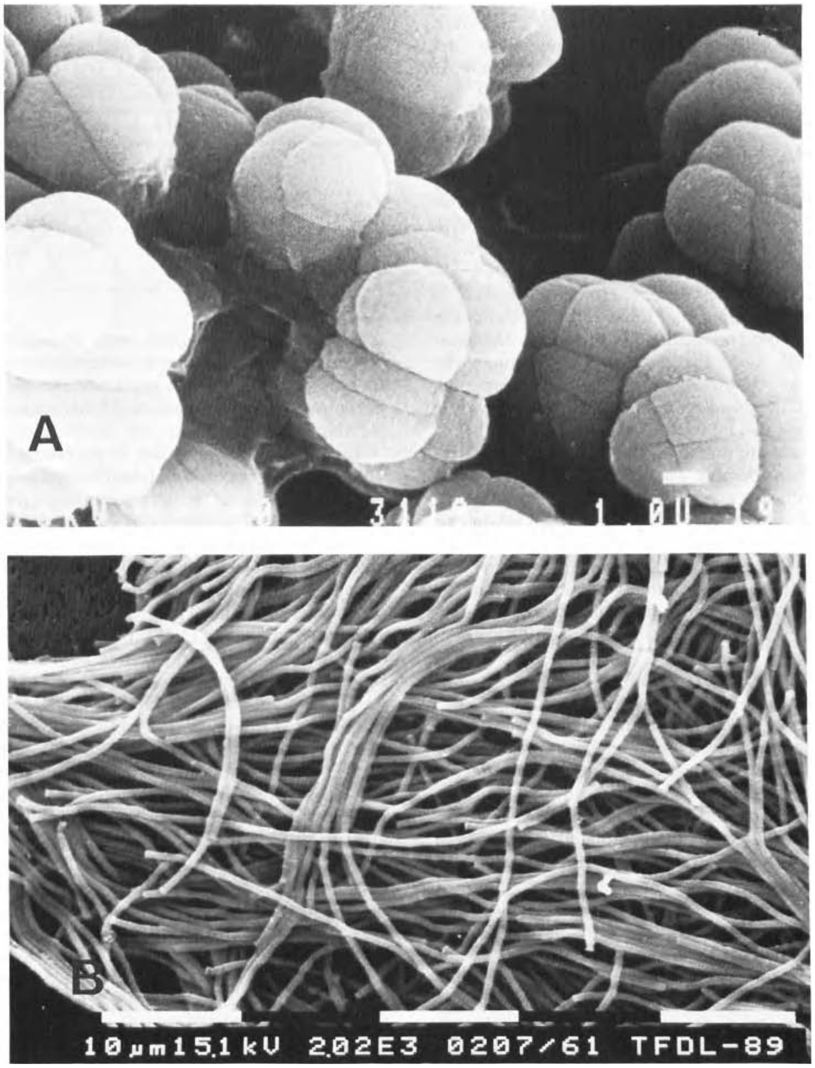


Figure 2.1 Scanning electron photograph of a *Methanosarcina barkeri* strain (A) and of a *Methanosaeta soehngenii* strain (B). From Jetten *et al.*, 1992

As well as morphological differences between the two genera there are also significant metabolic differences present even when breaking down the same substrate (acetate). The initial steps of acetate metabolism involve the activation of acetate to acetyl-CoA (Ferry, 2002). In *Methanosaeta* the reaction is catalysed by the enzyme acetyl-CoA synthetase (Kohler and Zehnder, 1984) whereas *Methanosarcina* uses acetate kinase and phosphotransacetylase for the activation (Kenealy and Zeikus, 1982). The enzyme acetyl-CoA synthetase system described for the *Methanosaeta* requires a higher energy input, which helps explain the different kinetic parameters reported for the 2 genera. (Jetten *et al.*, 1992). *Methanosarcina* requires a minimum threshold concentration of approximately 1mM acetate for growth compared to the much lower values of approximately 50 µM for the genus *Methanosaeta* (Jetten *et al.*, 1992). Table 2.1 highlights other differences between the two lineages; *Methanosarcina* has a higher yield and faster doubling time, however it has a higher K_s value which indicates lower affinity. Therefore *Methanosaeta* can be expected to prevail over *Methanosarcina* in the low acetate environments of anaerobic waste digesters (Griffin *et al.*, 1998).

Table 2.1 Comparison of selected physiological parameters of *Methanosaeta* and *Methanosarcina*. Adapted from (Jetten *et al.*, 1992)

	Methanosarcina	*Methanosaeta*
K_s (mM)	3.0	0.5
Yield (g/mol Acetate)	2.1	1.4
Doubling time (Days)	0.5-2	1-12

2.3 Species Identification

Multiple techniques are available to enable quantification of methanogens in environmental samples. These techniques include measurement of factor F_{420}; a cofactor unique to methanogens which is involved in electron transfer (Jacobson and Walsh, 1984). When oxidized factor F_{420} displays autofluorescence under ultra violet light (Mink and Dugan, 1977). This compound has an absorption maximum at 420nm wavelength and that's where it gets its name. Some of the other techniques available to identify species include those based on 16S ribosomal RNA gene sequencing. These include fluorescence *in situ* hybridization (FISH) which uses fluorescent probes that can bind specifically to 16S rRNA-targeted sequences. These sequences are chosen to so that they bind only in the target organism and once bound the level of fluorescence can be measured to quantify microbial population. Another quantification technique based upon 16S rRNA sequences is quantification PCR (qPCR), which works by amplifying the target sequence as in normal PCR whilst allowing quantification at the same time via the use of a flurophore. The autofluorescence techniques offer great value for generating fast and cost effective quantification data however the most powerful techniques are considered as those which are primarily based upon 16S ribosomal RNA gene sequencing (Sekiguchi *et al.*, 2001), as these allow identification and quantification of different lineages of methanogens whereas the measurement of factor F_{420} for example cannot differentiate between the different groups. Also the concentration of F_{420} has been found to vary among different methanogens, therefore the level of F_{420} in a sludge sample cannot accurately be used to quantify the level of methanogens present (Reynolds and Colleran, 1987).

Consequently the sequence based techniques are good for generating specific data, however due to them being relatively laborious and expensive, simple techniques such as measuring autofluorescence still have great value for generating fast and cost effective general quantification data.

2.4 Biochemistry of Methanogenesis

All methanogenic pathways converge into a common terminal step which consists of three key stages as can be seen illustrated in Figure 2.2. In the first stage the methyl (CH_3) group from factor III is transferred to coenzyme M (H-S-CoM, 2-thioethanesulfonate) via methyltransferase. In the second stage coenzyme M and coenzyme B (H-S-CoB, 7-thioheptanoylthreoninephosphate) react to form the heterodisulphide CoM-S-S-CoB, this is catalysed by methyl-coenzyme M reductase (MCR). The third stage involves the regeneration of coenzyme M and coenzyme B via reduction of the heterodisulphide CoM-S-S-CoB complex by the enzyme hetrodisulphide reductase (HDR) (Ferry 2002).

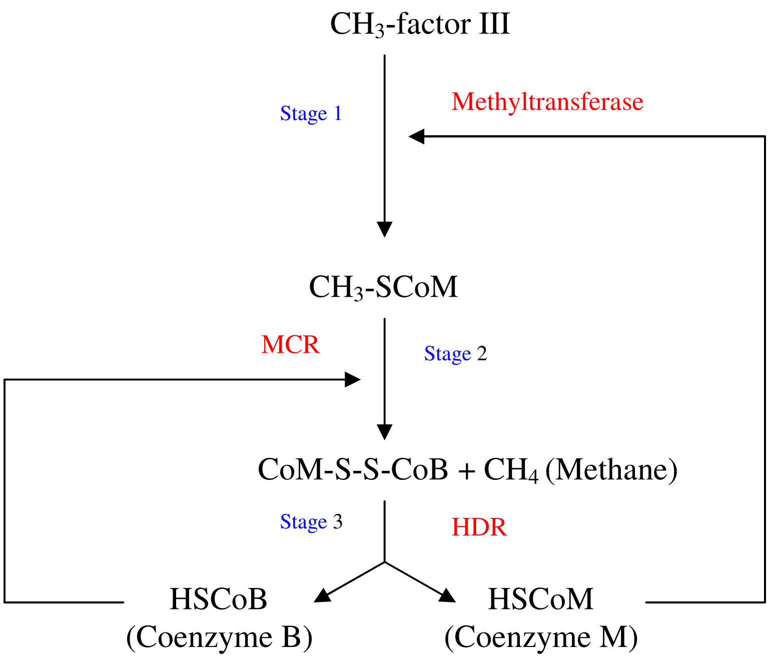


Figure 2.2 Reactions and enzymes common to all methanogenic pathways. Stage 1 is catalysed by Methyltransferase, stage 2: methyl–coenzyme M reductase (MCR), stage 3: heterodisulphide reductase (HDR).

The first step in methanogenesis from acetate and CO_2 are endergonic reactions and since the oxidation step appears not to generate any net ATP formation it is believed the methanogens obtain their energy needs from the reduction of heterodisulphide CoM-S-S-CoB catalysed by HDR (Thauer, 1998). The electrons required for this reduction are derived from the methanogenic growth substrate e.g. H_2, formate etc.

As can be seen from the terminal step illustrated in Figure 2.2, enzymes are very important in catalysing reactions essential for methanogenesis. Many enzyme active sites contain non-protein cofactors that allow specialised chemical functions (Petsko and Ringe, 2004). A cofactor is non-protein molecule or ion that assists in ligand binding or catalysis and cofactors that are organic compounds are often referred to as coenzymes (Petsko and Ringe, 2004). The MCR complex which was previously described is one example of a methanogenic enzyme which makes use of a cofactor. In fact every mol of the MCR enzyme contains 2 mol of non-covalently bound coenzyme F_{430}; which is a nickel tetrapyrole that is a uniquely methanogenic and characterised by its low molecular mass and detection at its absorption maximum of 430nm (Ellefson *et al.*, 1982). The use of such specific organic and inorganic cofactors within enzymes influences the nutritional requirements of the methanogenic archaea.

2.5 Metal Requirements

Transition metals have low ionisation energies and are able to exist in a wide range of oxidation states. This property allows the metals to catalyse electron transfer processes and consequently transition metal complexes are commonly found as cofactors within active sites of enzymes involved in a wide range of biochemical reactions. (Lewis and Evans, 1997). Therefore in addition to macronutrients such as carbons, nitrogen, sulphur and phosphorous, organisms also have a requirement for metals. This section explores the methanogens requirement for transition metals.

Table 2.2. Metal stimulation of pure cultures of methanogens.
(Adapted from Zandvoort *et al.*, 2006)

Pure culture	Substrate for methanogenic conversion	Stimulating concentration [μM]	Reference
Methanosarcina barkeri	Methanol	Fe(II) 35	(Lin *et al.*, 1990)
Methanosarcina barkeri	Methanol	Co 1, Ni 1, Se 1, Mo 1	(Scherer and Sahm, 1981)
Methanothrix soehngenni	Acetate	Fe 20-100, Co 2, Ni 2, Mo 2	(Fathepure, 1987)
Methanobacterium thermoautotrophicum	H_2/CO_2	Fe (>5), Co(>0.01), Ni (>0.01), Mo(>0.01)	(Schonheit *et al.*, 1979)
Methanospirillum hungatei	H_2/CO_2	(Mn) 50	(Pankhania and Robinson, 1984)

Table 2.2 shows the wide range of metal concentrations that have been reported to stimulate pure cultures of methanogens. Calculating exact requirements of MO's for metal requirements can be difficult even from studying pure cultures. Changes in speciation of the metal governed by differences in pH and presence of ligands and competing ions could result in changes in bioavailability (Hughes and Poole, 1991), this could result in overestimation of required quantities. Also it's possible certain metal requirements may have been met from the experimental apparatus, this would obscure the results. For example nickel dissolving from parts such as stainless steel syringes may lead to underestimation of required quantities. These reasons may account for some of the differences reported in the literature. However observed variations are to an extent expected given the diverse metabolic pathways employed by different methanogens especially between hydrogenotrophic methanogens and those of the acetoclastic and methyltophic methanogens.

A study on the cell metal content of 10 methanogens by Scherer *et al*. (1983) also showed great variation exists in metal content/requirements between different species. The study found in general the content of transition metals in cells to descend in order of Fe>Zn>Ni>Co>Mo>Cu>Mn. Iron content with some exception was quite uniform across the methanogens examined, with content representing average values of 0.11 to 0.15% of cell weight. Cobalt within the Methanosarcinae order was found at 25-80ppm which equates to 0.0025 to 0.008 % of the cell weight. Cell content studies help develop a greater understanding of metal requirements, the values from the study demonstrate that transition metals are required in trace amounts with Fe expected to be required at relatively higher amounts; approximately 30 fold higher than those of Co. This is consistent with the metal requirements demonstrated by Lin *et al.* (1990) and Scherer and Sahm (1981) for *Methanosarcina barkeri* as can be seen in Table 2.2. Interestingly Scherer *et al.* (1983) demonstrated that variation in metal content was present even if the methanogens were phylogenetically closely related and converting the same substrate. This observed difference indicates either the cells have the ability to tolerate lower or higher concentration than the optimum or that even closely related species have significant nutritional diversity.

2.6 Metals as Cofactors

The oxidation-reduction properties of metals are critical for their function in enzymes. The reduction potential E^o (V) value is a measure of the tendency for an oxidized species to accept electrons and a more positive E^o indicates a more favourable reaction. The reduction potential therefore allows evaluation of the favourability of a reaction by comparing the difference in E^o values of the oxidant and reductant. Amongst the metal reduction reactions shown in Table 2.3 we can see that $Co^{3+}$and Fe^{3+}, when accepting one electron represent the most favourable reduction reactions under standard conditions.

Table 2.3 Selected half-reaction standard reduction potentials E^o (V) (Housecroft and Constable, 1997a)

$Ti^{2+}(aq) + 2\,e^- \longrightarrow Ti(s)$	–1.63
$V^{3+}(aq) + e^- \longrightarrow V^{2+}(aq)$	–0.26
$Cr^{3+}(aq) + e^- \longrightarrow Cr^{2+}(aq)$	–0.41
$Mn^{2+}(aq) + 2\,e^- \longrightarrow Mn(s)$	–1.19
$Fe^{2+}(aq) + 2\,e^- \longrightarrow Fe(s)$	–0.44
$Fe^{3+}(aq) + e^- \longrightarrow Fe^{2+}(aq)$	0.77
$Co^{2+}(aq) + 2\,e^- \longrightarrow Co(s)$	–0.28
$Co^{3+}(aq) + e^- \longrightarrow Co^{2+}(aq)$	1.92
$Ni^{2+}(aq) + 2\,e^- \longrightarrow Ni(s)$	–0.25
$Cu^{2+}(aq) + e^- \longrightarrow Cu^{+}(aq)$	0.15
$Cu^{2+}(aq) + 2\,e^- \longrightarrow Cu(s)$	0.34

Within methanogens metals occupy diverse roles, for example Ni plays a role in structural stability of some methanogens and helps maintain integrity of membranes and sheath in bacteria (Patel and Can, 1984). Nickel is also an essential component of carbon monoxide dehydrogenase (CODH); CODH is a key enzyme complex in the methanogenesis from acetate. This enzyme complex makes use of both Ni and Fe, as it is composed of two enzyme components: a Ni/Fe-sulphur component and a corrinoid/Fe-sulphur component

(Ferry, 1999). The CODH complex is also involved in the formation of acetate by acetogens from H_2/CO_2 and methanol (Bainoitti and Nishio, 2000). All methanogenic pathways converge into the final step of co-enzyme M reduction with coenzyme B which is catalysed by methyl-coenzyme M reductase (MCR). This enzyme has already been described as having a tightly bound nickel porphinoid factor 430 (F_{430}), again highlighting the importance of Ni. As well as roles within CODH, iron has been widely documented as being a cofactor for a diverse range of enzymes; this is consistent with the relatively high levels found with methanogenic cells. Iron within methanogens is generally found to be associated with ferrodoxin and cytochrome proteins (Kamlage and Blaut, 1993, Steigerwald *et al.*, 1992) which are involved in electron transfer.

Cobalt is also considered an essential nutrient for both acetogens and methanogens (Patel and Can, 1984); The majority of cobalt content in the cells is believed to be most likely present as part of corrinoid compounds (Stupperich *et al.*, 1987). Corrinoid compounds have corrin rings as their basic component which when complexed with cobalt form the basic subunit for vitamin B_{12} and related cobamide enzymes (Bell, 1977b) As previously mentioned CODH has been shown to contain a corrinoid component (Ferry, 1999). The terminal steps of methanogenesis which are common to all pathways were described in the previous section as involving the transfer of the methyl (CH_3) group from factor III to coenzyme M via methyltransferase (Ferry, 2002). Factor III itself is a corrinoid derivative (Stupperich *et al.*, 1990) and the enzyme methyltransferase is a membrane associated complex, which harbours a corrinoid prosthetic group (Thauer, 1998). Prosthetic group is a term given to a cofactor when it is tightly bound to the enzyme (Bolsover *et al.*, 2011). This emphasises the importance of cobalt as a cofactor within methanogenesis, particularly for methyl transfer reactions.

Figure 2.3 illustrates these roles of cobalt within the terminal methanogenic step and demonstrates how a cobalt deficiency could inhibit the process. The figure highlights the importance of this metal as indicated by the inhibition (marked by a cross) caused at step 1 as transfer of the methyl group to co-enzyme m requires methyltransferase and factor III both of which have cobalt corrinoid components. Also it can be seen that limitation of cobalt would likely have a more profound effect on methanogenic pathways from acetate over hydrogenotrophic methanogenesis as it's found to be associated with CODH which is employed in acetoclastic methanogenesis. Eikmanns and Thauer (1985) further substantiated this point by showing that a corrinoid antagonist inhibited methanogenesis from acetate whilst not affecting methanogenesis from H_2 and CO_2 at the tested concentration.

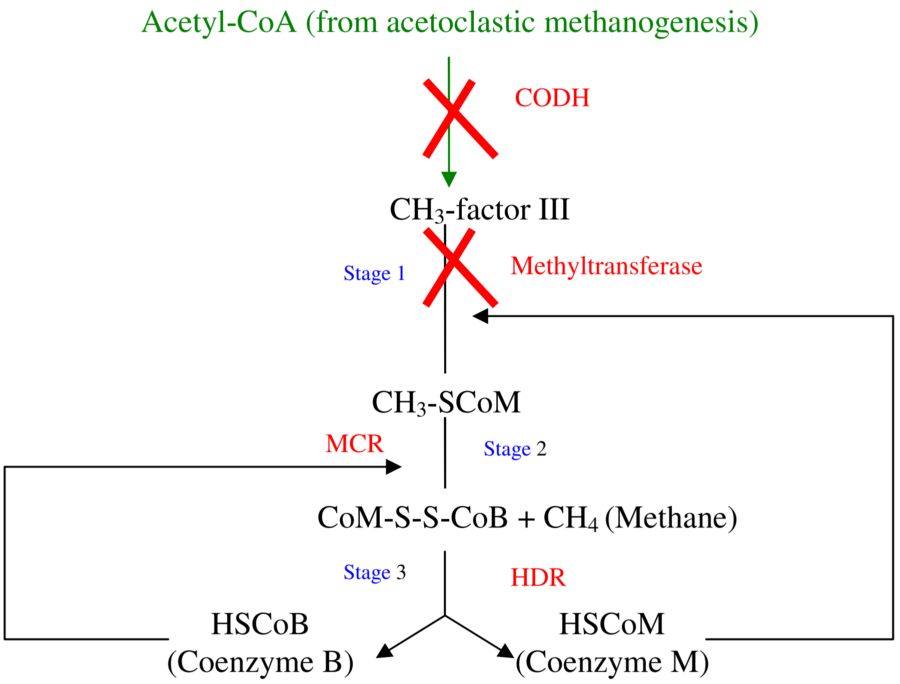


Figure 2.3 Key methanogenic pathways inhibited by cobalt deficiency. Breakdown of acetyl-CoA via CODH in acetoclastic methanogenesis and stage 1 catalysed by methyltransferase would be inhibited by cobalt deficiency. Stage 1-3 common to all methanogenic pathways and CODH employed in methanogenesis from acetate.

Methanogens only grow under anoxic conditions, however some have been shown to contain an iron superoxide dismutase which helps protect the cell from oxidative stress allowing the cells to survive if exposed to oxygen (Brioukhanov *et al.*, 2000, Kirby *et al.*, 1981, Rocha *et al.*, 1996). Superoxide dismutases can also contain manganese, copper, nickel and zinc. Zinc is another relatively abundant metal in methanogens relative to other transition metals; it has similar properties to calcium including charge neutralization, which serves to maintain integrity of the membrane sheath (Patel and Can, 1984).

Diverse roles for metals have been identified within methanogenic archaea and studies have shown methanogens to be stimulated by various different metals (Zandvoort *et al.*, 2006), Interestingly all methanogens have been found to require cobalt (Co), nickel (Ni) and iron (Fe) (Whitman, 1985) highlighting their common significance. Table 2.4 lists some of the key roles of Fe, Co and Ni within methanogens and methanogenesis which have already been discussed.

Table 2.4. Selected trace elements found in methanogenic enzymes/proteins

Enzyme	Metal(s)	Reference
Methyltransferase	Co	(Beveridge and Doyle, 1989)
CO-dehydrogenase	Co, Ni, Fe	(Ferry, 1999)
Methyl-CoM-reductase	Ni	(Hausinger, 1994)
Hydrogenase	Ni	(Hausinger, 1987)
Ferrodoxin	Fe	(Steigerwald *et al.*, 1992)
Cytochrome	Fe	(Kamlage and Blaut, 1993)

2.7 Metal Speciation

In anaerobic digesters metals are not only found as free ions but as different chemical species. The conditions in the reactors such as: pH, redox potential and levels of anions such as phosphorous and sulphur influences the speciation of the metals. Speciation of metals in a digester can be classified into those that exist in soluble forms and those that exist in the insoluble forms in the solid fraction. Gould and Genetelli (1975) summarized the speciation of metals in anaerobically digested sludge as shown in Table 2.5.

Table 2.5 Forms of metals in anaerobically digested sludge (Gould and Genetelli, 1975)

A. Soluble metals	Free (hydrated) metal ions Inorganic complexes Organic complexes
B. Metals in the solid fraction	
i. Precipitates	Carbonates Sulphides Phosphates Hydroxides
ii. Co-precipitates	Co-precipitation with iron sulphides Co-precipitation with iron oxides
iii. Adsorbates	Metals sorbed to clay minerals Metals sorbed to precipitates
iv. Organometallic complexes	Simple complexes Chelation
v. Metals present in dead and live biota	Metals bound to and accumulated within microbial cells

Soluble metals are generally defined as those not retained after centrifugation through a 0.45µM membrane; they consist of free (hydratated) metals ions and complexed forms with inorganic and organic ligands. Soluble inorganic complexes are formed between non-alkali metals (e.g. Ca^{2+} and Mg^{2+}, and transition metals) and anions such as HCO_3^-, CO_3^{2-}, OH-, SO_4^{2-}, S^{2-}, Se^{2-} and halides via ion pairing (Callander and Barford, 1983). A wide range of organic compounds are able to complex with metal ions, these compounds can be either non-specific or specific organic chelators. Non-specific organic chelators include synthetic organic substances such as ethylenediaminetetraacetic acid (EDTA) and natural organic substances such as humic acid. The metal specific organic chelators are those generally synthesized by some microorganism to facilitate metal uptake (Callander and Barford, 1983).

The solid fraction of anaerobically digested sludge consists of inorganic precipitates, mineral particles such as clay and sand, organics such as particulate humic matter and microbial cells and cell debris. Precipitation of the trace elements lowers the free metal concentration, which can have adverse effects by making essential nutrients no longer directly available to MO's, however it can also be advantageous if the concentrations of metals are at toxic levels. The main anionic compounds responsible for precipitating metals during anaerobic digestion are carbonate (CO_3^{2-}), sulphide (S^{2-}), and phosphate (PO_4^{3-}) (Callander and Barford, 1983). Precipitation occurs when the solubility product (K_{sp}) of the precipitate is exceeded.

The carbonate ion exists in equilibrium with other species and only forms a minute percentage of the total compound in solution. The ions are distributed in a typical digester (pH 7.3) as 0.089% CO_3^{2-}, 11% as solubilised CO_2, and 89% as HCO_3^- (Callander and Barford, 1983). At a constant partial pressure of CO_2 the relative fraction of carbonate species is a linear function of pH. A rise in pH of one unit results in a hundred-fold increase in carbonate ions (Mosey 1971). This highlights the importance of pH in influencing metal speciation profiles. Using equilibrium calculation it has been shown that under typical anaerobic digestion conditions the alkali earth metals (magnesium and calcium) are preferentially precipitated as carbonates (Callander and Barford, 1983)

Sulphide is formed during the process of anaerobic metabolism. It is derived from sulphide, sulphate and sulphur-containing compounds in the digester feed and is distributed between H_2S in the gas phase, H_2S, HS^- and S^{2-} in solution and metal sulphide precipitates in suspension (Callander and Barford, 1983). The form responsible for sulphide precipitation of metals is S^{2-} which forms only 0.00017% of the total sulphide in solution in an anaerobic digester (pH 7.3). The remainder is made up of approximately 33% H_2S and 67% HS^-

(Callander and Barford, 1983). Although S^{2-} is the species responsible for sulphide-precipitation, the important factor in this process is the total soluble sulphide rather than just the concentration of free sulphide ions initially present (Mosey, 1971). The K_{sp}'s of the transition metals indicates that they are preferentially precipitated as sulphides provided the concentration of the metal does not exceed that of the sulphide. If this is the case then the remainder metals are precipitated as carbonates with the exception of Ni which is precipitated as Ni-phosphate as it has a lower K_{sp} compared to that of Ni-carbonate.

Co-precipitation occurs when a chemical element is precipitated with other elements, this process involves mixed-solid formation and adsorption (Francis and Dodge, 1990). Ferrous sulphide precipitation is an example of a very common process in anaerobic digester and associations of trace elements with iron sulphides have been demonstrated (MacNichol and Beckett, 1989). Studies have shown that As, Cu, Co, Ni and Pb tend to accumulate within iron sulphides whereas Cr and Zn tend not to associate with it (Forstner, 1981). Therefore it is likely that some trace elements get co-precipitated alongside sulphide precipitates during anaerobic digestion.

Therefore it can be seen that a metal can exist in different states within the reactor medium and understanding metal speciation is important as it has the ability to influence metal uptake.

2.8 Uptake Mechanisms of the Methanogens

The Methanogens belong to the *Archaeal* Kingdom. Archaea are similar to bacteria in appearance and organisation of chromosomes (Londei, 2005), however they have remarkable similarities with eukaryotic homologues especially in respect to DNA replication apparatus (Olsen and Woese, 1996). Metal uptake mechanisms for methanogens are currently poorly understood and likely to have many variations between species given the wide range of metal uptake mechanisms characterised in closely related microorganisms. Analysis of processes within eukaryotic and bacterial homologues helps give insight into the mechanisms likely employed by the methanogens and consequently the factors which can affect them.

In order for metals to be incorporated as enzyme cofactors they first need to be internalized by the microorganisms (MO's) involved. According to a review by Nies (1999) MO's use two types of metal uptake mechanisms. One is fast, unspecific, constitutively expressed and driven by the chemiosmotic gradient across the cell membrane, whereas the other is energy dependent, slow and substrate specific. There are studies which support the fast unspecific mechanism as being responsible for most of the cations which are accumulated by the MO's (Nies, 1999). The substrate specific uptake of metals into biological systems has been described to involve two distinct steps; where firstly the metal is passively sorbed onto the biomass before an energy dependent mechanism internalizes it (Lawson *et al.*, 1984, Liu *et al.*, 2001, Oleskiewsicz and Sharma, 1990, Rudd *et al.*, 1984).

Acquiring of metals onto the biomass is likely to be the first step in metal uptake; this process is known as adsorption. Adsorption is believed to be passive as it is observed with living or dead organisms, it is shown to be reversible and dependent on dissolved organic matter (DOM) and pH (Butter *et al.*, 1998, Fowle and Fein, 2000, Guo *et al.*, 2001, Huang and Morehart, 1990, Wang *et al.*, 2003). The process is believed to occur as a result of electrostatic interactions and complexation between free metals and negatively charged groups. These groups primarily include carboxyl and phosphate groups found on extracellular polymers.

Gould and Genetelli (1978) demonstrated in their study that an increased pH correlated with an increased sorption of the metal to the digested sludge solids. This illustrates ion exchange as by increasing pH the concentration of positively charged hydrogen atoms decreases and this means less competition for binding sites on weakly acidic ligands, allowing greater metal binding. As well as competition from hydrogen atoms there is also competition between metals ions for binding sites. Gould and Genetelli (1984) examined this competition using Zn, Ni, Cd and Cu. The study found that by increasing the concentration of one metal the level of binding of the other metal decreased. Another factor likely to influence metal accumulation is cell-density, higher biomass concentration may lead to lower metal accumulation due to cells attaching to one another and lowering cell surface area for metal interactions (Al-Asheh and Duvnjak, 1995).

The pH dependence of microbial metal accumulation can vary greatly between different organisms and for different metals. Changes in pH can result in changes to the microbial surface, altering binding affinities and consequently metal uptake, (Ledin, 2000). It is a common occurrence for metal accumulation to increase with increasing pH although the opposite relationship is also reported (Ledin, 2000) . This finding is interesting as lowering pH in general increases metal solubility (Alloway and Jackson, 1991) which would be expected to improve availability as precipitated metals for example are considered not to be directly available (Gonzalez-Gil *et al.*, 2003). This highlights the importance of maintaining pH stability in digesters.

Other mechanisms proposed for uptake of metal ions involve facilitated diffusion aided by secreted organic compounds (Pirt 1975). In aerobic bacteria secretion of microbial products is seen in times of metal shortage e.g. siderophores are released to scavenge for iron (Nielands 1967). This mechanism does not rely on adsorption and is believed to be very specific. The siderophore chelates the iron and then is recognized by an outer-membrane receptor before being internalized. Interestingly Kim *et al.* (1995) demonstrated that *Methanobacterium bryantii* made use of at least 3 different forms of extracellular proteins when exposed to high levels of copper. Although released for detoxification, Kim *et al.* (1995) demonstrated that methanogens also made use of organic compounds to bind metals and it's therefore perceivable that methanogens may also secrete ligands for metal uptake similar to the siderophore uptake process. Bridge *et al.* (1999) showed in their study that certain sulphate reducing bacteria produce extracellular metal binding proteins which are able to bind copper and zinc, but noted this was unlikely to be the sole mode of acquiring metals due to the low binding affinities found. These findings combined with the lack of reports on extracellular ligands amongst the anaerobes make it unlikely that secreted organic compounds form a significant mode of metal uptake for methanogens.

A magnesium transport system which has preferential affinity for Mg^{2+} but also the ability to non-specifically transport divalent ions has been commonly described among prokaryotes (Nies and Silver, 1989, Webb, 1970). Functional homologues of the magnesium transporter system proteins have been characterised in the archaea; such as in the methanogenic organism *Methanococcus jannaschii* (Smith *et al.*, 1998). Consequently the magnesium transport system may be considered responsible for some uptake of metal ions in methanogens. The relatively non specificity of this system represents an economical solution for accumulating metals (Komeda *et al.*, 1997) however due to relatively low affinites found for cations such as cobalt (Nies and Silver, 1989) the system is probably not the major route of uptake for all trace metals. Jarrell and Sprott (1982) and Baudet *et al.* (1988) studied nickel uptake within *Methanobacterium bryantii* and *Methanosaeta concilii* respectively, both studies demonstrated cobalt affected nickel uptake, whereas other ions (NH_4^+ K^+ Ca^+ Fe^{2+} Mn^{2+} and Mg^{2+}) did not. This implied that nickel and cobalt were being taken up via a separate route to other ions and importantly not as an alternative substrate to the magnesium transport system. Cobalt and nickel are often present in very low levels in sludge digesters and therefore it would be expected that high affinity transporters capable of transporting across the concentration gradient would be required.

Two main families of nickel and cobalt transporter proteins with high affinities are known; ATP-binding cassette (ABC) and secondary permeases of the NiCoT family (Rodionov *et al.*, 2006). Recently genome studies of *Methanosarcina acteivorans*, *Methanosarcina berkeri* and *Methanosarcina mazei* have revealed the presence of transporters belonging to the NikABCDE group of permeases which are part of the ABC family of transporters (Rodionov *et al.*, 2006, Zhang *et al.*, 2009). This finding supports the hypothesis that substrate-specific high affinity transporters are employed for cobalt and nickel uptake in methanogens and also helps explain why studies found other cations to not affect their uptake.

Factors that influence a metals uptake are related to the speciation of the metal, which in turn is related to concentration of anions and their corresponding K_{sp}'s. The reactor pH plays a significant role in this as it influences levels of anion species that are responsible for metal precipitation and alters binding capacity primarily through reactions of ion exchange. As discussed methanogens employ diverse uptake mechanisms, consequently uptake of any one metal will be affected by other metals which share the same uptake pathway. If the metal is being taken up by a non-specific pathway such as the magnesium transport system then it is likely to be influenced by a larger range of metals compared to those which are taken up by a more specific mechanism such as nickel and cobalt.

2.9 Measuring Metal Speciation and Bioavailability

We have acknowledged that trace elements are not only present as free ions but exist inside the reactors as a range of different chemical species. The behaviour of metals inside the reactors is important in determining their bioavailability for uptake. Consequently there is a need to study the fate of trace elements in the anaerobic reactors. Single and sequential extraction techniques have been developed and applied to characterize the speciation of metals. All sequential extraction procedures aim to divide metal species into specific fractions by sequential removal of metal phases (from weakly to strongly bound). Generally the extracting agent is of decreasing pH and increasing reactivity

The sequential extractions method was initially applied to speciation of metals in soils and sediments. This procedure was first adapted to metal speciation studies in anaerobically digested sludge by Stover *et al.* (1976). Since the first adaptation other extraction techniques have been developed but the Stover method is the most widely used. The main factors which govern extraction efficiency are: concentration of extractant used, solid:reagent ratio, pH, temperature and the sample grain size (Pickering, 1986, Psenner and Puckso, 1988). In the heterogenous matrix typical of anaerobically digested sludge competition from other components against the ion of interest for the extractant can lead to saturation of the extractant and/or longer extraction times (Pickering, 1986). The method and length of sample storage also affects speciation profiles and therefore to obtain the most accurate results analysis is advised to be done immediately after sample collection

The main problem with using sequential extraction procedures is that the extractants are non-selective and trace element redistribution can occur between phases during extraction (Rapin *et al.*, 1986). As the extraction procedures can only give crude divisions between different chemical species it is recommended to refer to fractions as operationally-defined or extraction-method determined rather than being from a particular metal phase (e.g. metal sulphides). Other intrinsic disadvantages include the possible introduction of artefacts i.e. by altering the forms of metals or introducing new chemical species that were not initially present (Rapin *et al.*, 1986). Despite the inherent disadvantages sequential extraction procedures provide a useful tool to gain insight to metal behaviour and bioavailability in reactors.

2.10 Chelation

Transition metals are characterised by their ability to form complexes/coordination compounds, in which the d-block metals function as Lewis acids by accepting an electron pair and bind via coordinate bonding to molecules or ions functioning as Lewis bases (an electron pair donator). The surrounding molecules or ions are referred to as ligands; this term is derived from the Latin verb 'ligare' which means 'to bind' (Housecroft and Constable, 1997a). A single ligand can coordinate with a metal through more than one atom, the number of atoms through which a ligand coordinates with a metal is knows as the denticity of the ligand. The term denticity is derived from the Latin word for teeth, Table 2.6 shows the nomenclature used for ligands.

Table 2.6 Ligand denticity nomenclature

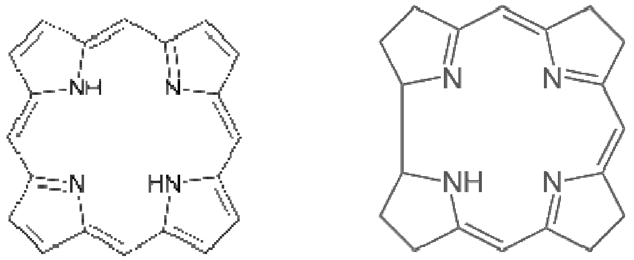
Number of donor atoms bound to central metal	**Denticity**
1	Monodentate
2	Bidentate
3	Tridentate
4	Tetradentate
5	Pentadentate
6	Hexadentate

Any ligand which binds with more than one atom to a metal ion is collectively known as polydentate. When a ligand employs this type of bonding it is referred to as a chelating ligand and the complex formed as a chelate. The formation of chelates is generally thermodynamically favoured compared to complexes with similar monodentate ligands and usually the higher the denticity of the ligand the more stable the chelate produced, this is known as the ‘chelate effect’ (Snoeyink and Jenkins, 1980). Chelation of metal modifies the metal species in solution in terms of the complexes solubility, toxicity and potentially biostimulatory properties (Snoeyink and Jenkins, 1980)

Chelation occurs when a ligand has at least two donor atoms with the ability to form a bond with a single metal. Donor atoms are characterised by their electronegative nature and therefore tend to be found on right hand side of the periodic table, commonly found in group 5 (nitrogen, phosphorus, arsenic and antimony) and in group 6 (oxygen, sulphur, selenium and tellurium) (Bell, 1977b). A metal’s oxidation state will dictate its preference for ligands. Hard metals, which have a high positive charge (often 3+ 4+) and/or high charge density, favour donor atoms which are highly electronegative such as fluoride and chloride. Whereas soft metals, which have lower positive charges (often 1+ to 2+) and/or low charge densities, favour less electronegative atoms such as sulphide (Housecroft and Constable, 1997a).

Donor atoms can be part of both acidic and basic functional groups. Basic groups contain an atom carrying a lone pair of electrons whereas acid groups can loose a proton and coordinate with a metal atom. Basic groups of particular relevance include amino ($-NH_2$), carbonyl (=O), alcohol (-OH), ester/ether (-O-) and thioether (-S-) groups whereas acidic groups include carboxylic ($–CO_2H$), sulphonic ($-SO_3H$) and phosphoric (-PO) groups. For chelation to occur the ligand must have a structure whereby the functional groups are within proximity of the single metal atom in order to allow multiple coordinate bonds to form. Ligands do often have some flexibility present in their carbon chains to allow it to accommodate the metal ion but the donor atoms within the ligand need to be appropriately situated. (Bell, 1977b).

Many important biological compounds are tetradentate chelates such as the porphin and corrin molecules. (Swaddle, 1997). Porphyrins contain 4 pyrrole subunits which are able to coordinate with 4 nitrogen atoms. Figure 2.4 shows both the porphine ring which is the basic unsubstituted unit of porphyrins and the corrin ring, which although appears very similar and also coordinates with 4 donor atoms is in fact chemically very different due to not being fully conjugated (Reddy, 2003). These tetradentate complexes are essential for life in many organisms, for example they are found in the O_2 carrier protein haemoglobin which contains an iron-porphyrin, whereas the corrin ring complexed with cobalt forms the basic subunit for vitamin B_{12} and in related cobamide enzymes (Bell, 1977b), which are discussed later in the chapter.



Porphin Corrin

Figure 2.4 Porphine and corrin molecules (Lindoy, 1989).

The rate of formation of complexes varies greatly; with some reactions reported to be virtually complete within seconds at typical natural water concentrations such as those of Co^{2+} with $EDTAH^{3-}$ whereas the coordination reaction of Fe^{3+} with the ligand Cl^{-} takes several hours (Snoeyink and Jenkins, 1980). These differences are due to wide range of factors and are related to the reduction potential of the central metal involved as well as the ligand properties in terms of presence and location of appropriate donor atoms. These reactions are described as ‘labile’ for when very they are very fast and ‘inert’ for when they are very slow. These describe the kinetics of interaction whereas the magnitude of the equilibrium constants (K) for a given reaction reveals the favourability and stability of the complex produced (Housecroft and Constable, 1997b). The larger the equilibrium constant, also known as the stability constant when stated for the complex formation, the more stable the complex. A ligand will preferentially associate with a metal that will produce a complex with a higher K and furthermore a ligand is able to scavenge complexed metal ions if they are present in a less stable complex providing that it is sufficiently labile (Snoeyink and Jenkins, 1980). Metal behaviour in a given system is affected by a wide range of factors and use of modelling systems based on stability constants of metal complexes is crucial for predicting the behaviour of metals in aqueous solutions.

2.11 EDTA

Ethylenediaminetetraacetic acid (EDTA) with the chemical formula $C_{10}H_{16}N_2O_8$ is a common polydentate chelating agent. At neutral pH values EDTA looses hydrogen atoms to form the $EDTA^{4-}$ anion (Sillen and Martell, 1971), and functions as a hexadentate ligand as it's able to coordinate at six sites using the four acetate groups and the two nitrogen atoms (Snoeyink and Jenkins, 1980). EDTA forms soluble and stable 1:1 stoichometric complexes with numerous metal ions, with stability generally increasing with the valence state of the ion (Nielsen, 2010). The ligand is utilised in a wide range of pharmaceutical and industrial applications including; treatment for metal poisoning and as part of food preservatives (Myers, 2007). EDTA works as a food preservative by sequestering metals, preventing their incorporation into bacterial enzymes responsible for the breakdown of food, thereby increasing food shelf life (Nielsen, 2010).

Table 2.7 Stability constants of selected EDTA complexes (Bell, 1977a)

Cation	Lg K	Cation	Lg K	Cation	Lg K
Mg^{2+}	8.69*	**Co^{2+}**	16.31	**Gd^{3+}**	17.37
Ca^{2+}	10.96	**Ni^{2+}**	18.62	**Tb^{3+}**	17.93
Sr^{2+}	8.63*	**Cu^{2+}**	18.80	**Dy^{3+}**	18.30
Ba^{2+}	7.76*	**Zn^{2+}**	16.50	**Ho^{3+}**	18.74
V^{2+}	12.7	**Hg^{2+}**	21.8	**Er^{3+}**	18.85
V^{3+}	25.9	**La^{3+}**	15.50	**Tm^{3+}**	19.32
Mn^{2+}	14.04	**Ce^{3+}**	15.98	**Yb^{3+}**	19.51
Fe^{2+}	14.33*	**Pr^{3+}**	16.40	**Lu^{3+}**	19.83
Fe^{3+}	25.1	**Nd^{3+}**	16.61		
Sm^{3+}	17.14	**Eu^{3+}**	17.35		

Values at 293 K and $\mu = 0.1$ in KNO_3* (or 0.1 in KCl).

Table 2.7 displays the stability constants of EDTA with different metals, from the data we see the wide range of ions which EDTA can complex with, as well as the complexes which are preferentially formed i.e. those with higher K values. From the shown stability constants in Table 2.7 we can derive that under standard conditions EDTA would preferentially complex to V^{3+}> Fe^{3+}> Hg^{2+}> Lu^{3+}> Yb^{3+}> Tm^{3+}> Er^{3+}> Cu^{2+}> Ho^{3+}> Ni^{2+}> Dy^{3+}> Tb^{3+}> Gd^{3+}> Eu^{3+}> Sm^{3+}> Nd^{3+}> Zn^{2+}> Pr^{3+} before binding to Co^{2+}. The stability constant values represent the relative affinities of the ligand for the metal in isolated conditions, however in real systems with presence of more than ligand or metal the stability constants vary considerably (Bell, 1977a).

Due to its hexadentate nature EDTA is relatively a very strong chelator and therefore very effective in reducing the free metal concentration for whole range of cations, keeping the metal in a soluble complexed form. Babich and Stotzky (1983) demonstrated reduced toxicity to microorganisms from high nickel concentrations with the addition of EDTA; this shows that by chelating with EDTA the metal species were unable to cause negative impact and it implies the free metal ion is the form most responsible for its biological effect.

Chelation by a ligand is able to affect the availability of a metal to an organism; it will make the metal more available if the target organism has a metal binding component (essentially an uptake mechanism) with higher stability constant than that of the ligand complex and less available if it doesn't (Callander and Barford, 1983).

Several studies have reported that metals complexed with EDTA become unavailable to the microorganisms (Aquino and Stuckey, 2007, Babich and Stotzky, 1983, Pankhania and Robinson, 1984, Speece, 1988). However recently Fermoso *et al.* (2008) reported Co-EDTA^{2-} had a stimulatory effect in terms of specific methane activity and substrate removal in methanol-fed UASB reactors, however the study did also highlight the effect was short lived in comparison to that from $CoCl_2$. The EDTA keeps more metal in solution which leads to washout in the effluent whereas dissolution from sulphide precipitates was proposed as giving rise to free cobalt ions over a longer period.

Interestingly studies on photosynthetic algae chlorella have found EDTA chelation to increase Fe^{3+} availability whereas reducing the availability of Mg^{2+} (Pirt and Pirt, 1977). This study concluded that the organism must have an iron-binding component with a higher stability constant than that of EDTA-Fe^{3+}. Nozoe and Yoshida (1992) showed a stimulatory effect with EDTA-Ni in decomposition of volatile fatty acids in paddy soil, their results were explained by possible EDTA alleviating nickel toxicity, EDTA-Ni being directly available or the nickel being made available by the methane producing bacteria. In the latter study it seems unlikely the effect was that of inhibition limitation as gas production in EDTA-Ni supplemented tests was enhanced above that of the control. The observed stimulation in these studies seems to be either due to the organisms having metal binding components with higher stability constants to those of the EDTA complexes or it can be alternatively explained if the complexed metal is being freed by displacement with another metal which is able to produce a complex with a higher stability constant. So for example in the case of Fe^{3+} it may be displaced by V^{3+} and in the case of the EDTA-Ni complex, possibly with metals such as V^{3+}, Fe^{3+} or Cu^{2+}. The bioavailability of EDTA metal complexes is discussed further in the next section of the literature review.

2.12 Specific Methanogenic Activity Test

For AD research purposes both continuous (and semi-continuous) and batch feed techniques have been used in laboratories to simulate full scale digesters The former simulates actual full-scale digester operating conditions more closely, however such test are generally more costly in terms of equipment, time, and personnel in comparison to batch bioassay techniques which do not have the same limitations (Owen *et al.*, 1979).

A biochemical methane potential (BMP) batch test was first described by Owen *et al.* (1979) as a simple inexpensive test to reliably measure the biodegradability of material subjected to anaerobic digestion. However the BMP type test has since been used as a flexible investigatory tool with more wider uses, for example its been used to evaluate the effect of potentially inhibitory/toxic compounds on sludge (Borja *et al.*, 1996, Colleran *et al.*, 1991, Dolfing and Bloeman, 1985) and in determining the microbial composition of sludges via the use of specific substrates (Soto *et al.*, 1993, Valcke and Verstraete, 1983). When the serum bottle batch test is used to determine the activity of the methanogens, the test is known as the specific methanogenic activity (SMA) test. When substrates are easily hydrolysed methanogenesis is the rate limiting step in AD (Noike *et al.*, 1985). Due to the importance and sensitivity of the methanogenic step the SMA test has been found to be very effective as a digester monitoring tool. Conklin *et al.* (2008) proposed the use of an acetate capacity number (ACN) to determine digester capacity and stability, the ACN value compares the methane production rate from acetate in a batch test to that of the actual plant acetoclastic production rate. The study concentrated on acetate as previous research has shown approximately 70% of methanogenesis in domestic sludge comes via the precursor acetate (Jeris and McCarty, 1965, Smith and Mah, 1966). Therefore the acetoclastic methanogens are seen as good representatives of the overall methanogenic activity and capacity of the digester. The study showed that when the ratio was above 1 there was excess capacity in the digester and when it was below 1 it indicated a condition approaching failure. Therefore the technique allows the determination of safe loading changes whilst also serving as an effective monitoring tool to indicate process upsets. Digester upsets can be very costly and effective digester monitoring requires an early detection to allow for a prompt corrective response. Research by Schoen *et al.* (2009)

verified that indicators which reflect the biokinetic state of the digester such as the ACN values were effective in predicting impending failure.

The Batch tests are a highly versatile and efficient means for digester monitoring and anaerobic digestion research. Although the use of the BMP/SMA test is well acknowledged in the literature there is no standardised protocol for it and different techniques have been developed by different researchers (Ince *et al.*, 1995, Owen *et al.*, 1979, Raposo *et al.*, 2006, Schoen *et al.*, 2009, Shelton and Tiedje, 1984, Valcke and Verstraete, 1983, Van Den Berg *et al.*, 1974).

2.13 Trace Metal Supplementation of Anaerobic Digesters – Review of Previous Research and Knowledge Gaps

Table 2.8 summarizes some key studies on digester metal supplementation with emphasis on cobalt supplementation given the importance of the metal highlighted in the previous section.

Table 2.8 Summary of key research papers

Reference	Type of Digester	Main Feed Substrate	Supplements	Bioavailability taken into account	Effect/Summary
Speece, 1988	BMP tests comparing sludge from 30 different conventional municipal wastewater treatment plants.	Acetate and propionate	Iron, cobalt and nickel. Each metal added as 10mg l^{-1}	Observed that even though soluble iron, cobalt and nickel appear to be ample in the sludges, their supplementation caused stimulation in 8/9 of the digesters. Hypothesised that naturally formed chelators bind metals so tightly that they are not bioavailable. Also noted EDTA bound metals in their studies was not available- results not shown.	Paper concentrating on differences in operational parameters. Showed 8/30 digesters showed stimulation in acetate utilisation rate (AUR) and 9/30 for propionate utilisation rate (PUR). However only considered 20% plus stimulation as significant and so didn't show lower effects. Therefore other digesters may have been stimulated as well albeit to a lesser extent. Also PUR stimulation was generally higher up to 200% whereas AUR up to 111%. In acetate tests iron gave highest effect when supplemented individually followed by nickel and then cobalt. In pyruvate tests it was Ni with highest effect and then Fe and Co having similar effects.

					Combinations of all three gave best results in both tests. Interestingly the site which had highest soluble iron also responded the highest to it.
Jansen *et al.*, 2007	*Methanosarcina* species enriched culture.	Methanol	Iron, nickel and cobalt	In absences of microorganisms Ni did not precipitate whereas Fe and Co precipitation was observed. Fe precipitated more slowly and stayed at a higher concentration.	Methanogenic activity increased with total Co and Ni concentrations and decreased with increasing sulphides concentrations. Increase in activity believed to be related to increased concentration of dissolved Co as Ni stayed same after raising added concentrations of both metals.
Fathpure, 1987	Pure culture of *Methanothrix (Methanosaeta) soehngenii.*	Acetate	Iron, nickel, cobalt and molybdenum as chlorides	Researcher explained inhibition as likely due to nonspecific binding of trace elements with the carrier proteins that are involved in uptake and incorporation. An excess of a particular element may saturate the carrier molecules and thereby restrict the uptake of other essential metal ions. A metal ion in excess	The results showed that the minimum amount of a Fe needed for the complete conversion of acetate to CH_4 was 0.1 mM. The results indicated that the addition of Co, Ni, or Mo at a concentration of 2 µM markedly stimulated CH_4 production. Tubes depleted of Co, Ni, or Mo produced 36, 59, or 83%, respectively, of the total CH4 produced at the optimal concentration (2 µM). The addition of higher concentrations (>5uM) of Co or Mo resulted in decreased CH_4 production, possibly because

				may also replace the essential metal of an enzyme.	of toxic effects. Interestingly no inhibition was noted for Ni even at concentrations of up to 10 uM.
Scherer and Sahm, 1981	Pure culture of *Methctnosarcina burkeri.*	Mainly methanol	Range of supplements including cobalt, nickel and molybdenum as chlorides		Growth was found to be dependent on cobalt and molybdenum. In the presence of 10^{-6} M Co and 5 x 10^{-7} M Mo optimal growth occurred. Furthermore it could be demonstrated that nickel and selenium each in a concentration of 10^{-7} M stimulated the growth of this methanogenic bacterium while the following elements tested had no influence: B, Cr, Cu, Mn, Pb.
Mah *et al.*, 1978	Methanosarcina species culture.	Acetate and methanol	Range of nutrients including cobalt and yeast extract		Yeast extract was highly stimulatory to growth from methanol but could not support significant growth or methane production on its own. The doubling time of the culture on defined medium was 73 h compared to doubling times of 11.6 and 11.1 h on low- and high yeast-extract media, respectively highlighting effect of limitations as 6-7 times slower. Vitamins or a combination of cobalt and manganese (but not manganese alone) stimulated growth above that in the basal medium and was

					approximately equivalent to growth in defined medium. Comparable level of stimulation from acetate was not shown.
Gonzalez-Gil *et al.*, 2003	Methanosarcina species culture from a full-scale anaerobic reactor treating wastewater from a chemical factory that produces formaldehyde from methanol. Biogas measured in serum bottles.	Methanol	Cobalt and nickel	Bioavailability of trace elements was shown to be greatly increased by the addition of yeast extract. This was explained as due to the formation of dissolved bioavailable complexes, which favor the dissolution of metals from sulphides. The total dissolved concentrations of both nickel and cobalt in the presence of intact yeast extract were indeed higher than in its absence.	Study concentrated on effect of yeast and bioavailability. Nickel and cobalt was added in the range of 0 to 400 µM, and sulphide was added at 1 mM. The biogas production rate increases with increasing metal concentration. In the presence of sulphides, metals seem to be limiting the rate of methanogenesis. However yeast extracts seem to overcome these limitations. The use of cysteine instead of sulphides as sulphur source also helped to avoid metal limitations. Therefore proposed trace doses of yeast extract may be effective in keeping additions of essential metals to anaerobic reactors at a minimum.
Gonzalez-Gil *et al.*, 1999b	Anaerobic granular sludge from a full scale expanded granular sludge bed was used. The sludge treated wastewater	Methanol	Cobalt and nickel as chlorides	Found continuous addition of metals better than spiking in one go. Precipitation-dissolution kinetics of metal sulphides believed to be	Three methane production rate phases recognized. 1) Exponential increase 2) temporary decrease 3) arithmetic increase. Ni and Co supplementation accelerated phase 1 and 3 and reduced 2. Phase 2 was eliminated when Ni and Co added

	from production of formaldehyde. Lab scale reactors used in study.			responsible for micronutrient limitations.	continuously rather than all in one go.
Murray and Berg, 1981	BMP tests seeded with sludge from fixed-film digester treating bean blanching waste and lab designed down flow fixed-film reactors tests as well.	Acetate and bean blanching waste	Nickel, cobalt as chloride salts and molybdenum as sodium molybdate		Conversion of acetic acid was stimulated by the addition of 100nM Ni and 50nM Co, especially when in combination. Molybdenum only had slight effect when in combination with Ni and Co. The addition of these traces metals to fixed-film digesters greatly enhanced reactor performance as allowed accumulation of a thicker methanogenic fixed film.
Speece *et al.*, 1983	Lab scale continuously fed digester. Inoculum from acetate-enriched methanogenic culture which had been taken from a municipal sludge digester.	Acetic acid	Study concentrated on nickel supplementation, but also studied effects of nickel in combination with iron, cobalt and yeast extract. Metals supplemented	Authors acknowledged metals may precipitate as sulphides but hypothesised the acidified feed pulses would make metals momentarily available for microorganisms.	Nickel and yeast extract shown to be key stimulants to obtain highest acetate utilisation rate. Daily phosphate addition appeared to be required to prevent digester crashing. In the absence of nickel, specific acetate-utilisation rates were in the ranges of 2 - 4 g acetate g^{-1} VSS day $^{-1}$. In the presence of nickel acetate-utilization rates of 10 g acetate g^{-1} VSS day $^{-1}$ were observed. When yeast extract was supplemented along with nickel,

			as chlorides.		specific acetate-utilisation rates as high as 12-15 g acetate g^{-1} VSS day $^{-1}$ were observed. Indicated nickel, iron and/or cobalt must be supplemented to achieve high VSS concentrations.
Zitomer *et al.*, 2008	Serum bottle tests on biomass from municipal wwtw which use thermophilic and mesophilic digesters.	Acetate and propionate	Iron nickel and cobalt as chlorides. The 3 Metals added to reach concentrations of 25mg/l each in the system.	Noted higher requirements for metals in thermophilic systems may be due to increase in requirements or possible decrease in bioavailability at higher temperatures.	The acetate and propionate utilisation rates were observed to statistically increase at thermophilic temps compared to mesophilic (irrespective of nutrients addition). Biomass was assayed for potential methane rate increases resulting from nutrient addition. The majority of samples (77%) benefited from nutrients. Propionate and acetate utilisation rates increased as much as 50% and 35% respectively. Propionate utilisation rates were more frequently stimulated, especially in thermophilic systems, demonstrating increased methane production rates of from 14 to 50%.
Kida *et al.*, 2001	Continuously stirred tank reactor (CSTR). Seeding sludge from sewage works.	Acetate	Cobalt and nickel as chlorides		Addition of Ni^{2+} and Co^{2+} to the synthetic wastewater drastically increased the maximum dilution rate of the cultivation. The concentrations of F_{430} and corrinoids, and the methanogenic

					activity decreased with decreasing amounts of Ni^{2+} and Co^{2+} added. These results suggest that Ni^{2+} and Co^{2+} were required for the methane-producing reactions via increases of coenzymes F_{430} and corrinoids.
Takashima *et al.*, 2011	Long term semi-continuous experiments. Suspended system in a 1 litre digester. Inoculum from municipal treatment plant.	Glucose	Iron, nickel, cobalt and zinc as chlorides		Study investigated minimum requirements for trace metals. Determined for milligrams per gram COD removed; iron 0.45 nickel 0.049 cobalt 0.054 and zinc 0.24. Values for thermophillic fermentation were 2.2-7 times greater. Study also highlights the amount of metals needed varies for different types of substrate. Values shown for acetate from Takashima and Speece (1989).
Aquino and Stuckey, 2003	Laboratory scale continuously stirred anaerobic reactors were used to investigate SMP production and BMP assays were used to determine the effects caused by the lack of specific nutrients.	Glucose	Range of nutrients used in the feed including ferrous chloride and cobalt chloride at 5mg/l each.	In subsequent study the research group showed an increase in methane production caused by addition of SMP so remains possible cells releasing specific chelators as found in aerobic bacteria however not conclusive.	It can be seen that the lack of vitamins and metal nutrients slightly affected the rate of methane production This study was carried out to investigate the effect of nutrient deficiency on SMP production. Study reported that DNA analysis suggested part of SMP might be biomass associated products (BMP) produced as a result of cell lysis, although the release of specific chelators (such

					as siderophores) to scavenge metal nutrients might also have contributed to SMP production. BMP analyses showed that the lack of macronutrients N and P caused the worst effect on the anaerobic digestion and resulted in the highest SMP production. These SMP may be organic compounds released as a mechanism of dumping of electrons that could not be invested in cell synthesis due to the lack of macronutrients.
Aquino and Stuckey, 2007	BMP tests	Glucose	Iron , copper, citric acid, EDTA and NTA	Paper investigates the effect of chelating agents on the bioavailability of Fe and Cu. It was expected that the stoichiometric addition of Fe^{2+}, which has a high complexation constant with EDTA, should be able to displace the complexed metal nutrients, thus reversing effects of EDTA but this was only seen when added at start of study. Paper	The results presented in this paper showed that the presence of free 1 mM EDTA reduced the methane production rate, NTA did not. The addition of soluble microbial products (SMP) did not change metal distribution in anaerobic systems, it caused an increase in the rate of methane production, paper explained this was believed to be due to direct uptake of Cu-SMP complexes.The third series of BMP bottles was carried out to investigate the effect of chelating agents in mitigating copper toxicity. As EDTA found inhibitory it was not tested and citric acid and SMP did not result in any

				suggested inhibitory effect not reversible. However inhibition perhaps related to metals with higher stability constants than EDTA-Fe^{2+} such as Ni^{2+} and Co^{2+}.	mitigation, NTA offered the best protection explained by efficient competition with binding sites on cell surface which reduced uptake.
Fermoso *et al.*, 2008	UASB	Methanol	$CoCl_2$ and $CoEDTA^2$	$CoCl_2$ creates a pool of cobalt into the granular sludge matrix due to the high cobalt retention (around 90%). Only 8% of the supplied Co $EDTA^{2-}$ is retained. EDTA could cause metal extraction from the metal active sites of the enzymes involved in methylotrophic methanogenesis. However, the metal content of the sludge (zinc, nickel and iron) was not affected. In contrast, the calcium content of the granule	The paper investigated the effect of chelation of cobalt to restore the performance of a cobalt limited reactor. When Co-$EDTA^{2-}$ was dosed in the first cobalt pulse dose, the cobalt limitation was clearly overcome immediately after dosage as shown by the high increase of SMA (233%), whereas the SMA of sludge did not increase at all after the first $CoCl_2$ pulse addition, suggesting higher bioavailability. Subsequent Co-$EDTA^{2-}$ doses did not have same effect. The study noted that due to the side-effects EDTA has on the granule matrix or microbial cells it is therefore an unsuitable ligand for cobalt dosing in full-scale applications. Cobalt added as chloride is more retained in the granular sludge compared to cobalt bound to EDTA. Study

				decreased. Calcium is a structural ion for the granular matrix and thus decreased calcium concentrations lead to a decreased granular strength.	noted dissolution of the cobalt sulfide precipitate has previously been proposed to provide enough free Co^{2+} to supply the cobalt requirements of *Methanosarcina bakeri* enrichment. Cobalt chloride hexahydrate at time of study was also noted as being 25% cheaper than cobalt bound to EDTA.
Climenhaga and Banks, 2008	Lab scale tests	Source-separated foodwaste	The trace element solution used followed the recipe of Gonzalez-Gil *et al*. (2001). Which notably included 2mg/l $CoCl_2 6H_2O$ FeCl2 4H2O and1mg/l EDTA.	Authors noted it's possible that either the influent feedstock lacks all of the trace elements specifically required for methanogenic metabolism, or that the elements are present but not bioavailable. With sulphides precipitation and metal- long chain fatty acids interactions highlighted as processes that are able to reduce bioavailability.	This paper showed the requirement of trace metals for stable digestion of mixed food waste; non-supplemented reactors demonstrated subsequent methanogenic failure. The reactor operating at a 25 day HRT with no supplementation failed after 40 days. Micronutrients suggested as having a possible role in supporting biomass resistance to inhibition or toxicity.
Bartacek *et al*., 2008	SMA tests with biomass from mesophilic UASB reactor treating	Methanol	$CoCl_2$ and $CoEDTA^2$	Complexation (and precipitation) with EDTA, phosphates and	Study noted the borders between cobalt's nutritional requirement and toxicity is very narrow. The optimal concentration was shown

	alcohol distillery Wastewater.			carbonates was shown to decrease the toxicity of cobalt and thus shown to reduce the bioavailability of cobalt.	to be approximately 7 µmol/l whereas a free cobalt concentration of approximately 18 µmol/l is already significantly toxic (50% inhibition). Difference in literature values for toxicity accounted for by varying magnesium and calcium levels which compete with cobalt for uptake.
Florencio *et al.*, 1993	UASB	Methanol and acetate	Cobalt		When trace elements were supplied individually, cobalt greatly stimulated methanogenesis which equalled the stimulation observed with the complete trace element mixture. No remarkable influence of any trace element was observed when acetate was used as the substrate.
Vlyssides *et al.* 2006	Laboratory-scale UASB	Synthetic milk wastewater	Ferrous iron (Fe^{2+})	Model developed takes into account all biological and physiochemical reactions.	The ferrous iron addition stimulated the biological activity of the sludge. The addition of ferrous iron induced a stable and excellent COD conversion rate. A mathematical model which simulates the dynamic behaviour in a UASB reactor was developed and validated. The model was able to simulate the accumulation of iron in the sludge bed and its effects on the biological activity of the anaerobic sludge. Using the model

					significant increases in the maximum growth rate of methanogens and acidogens was revealed.
Kim M *et al*. 2003	1 litre batch reactors seeded with homogenized UASB granules.	Dog food was used to simulate primary sludge	$CaCl_2$, $FeCl_2$, $CoCl_2$ and $NiCl_2$		Study looked at effect of pH and nutrients supplementation on hydrolysis and acidogenesis stages at both mesophilic and thermophilic temps. Greatest rates occurred when pH controlled at 6.5. The reactors supplemented with nutrients showed more acids production and higher rates of hydrolysis at both temps in terms of SCOD production, VSS removal and organic acids production implying importance of nutrient supplementation for hydrolysis and acidogenesis.
Cánovas-Díaz and Howell, 1986	Pilot scale downflow fixed-film reactor and Lab scale downflow fixed-film reactors.	Butyric acid-Treating cheese factory effluent	Nickel as chloride.		Adding of Ni 30mg/l caused a dramatic increase in butyric acid utilisation in pilot scale reactors. Utilisation and methane production were slightly higher in lab scale tests compared to control.
Hoban and Van Den Berg, 1979	Enriched mixed culture. Municipal anaerobic sewage digesters.	Acetic acid	$FeCl_2$	Large percentage of Fe was found to precipitate as carbonate. Ferrous carbonate strongly related to production	Initially batch reactors (serum bottles) were carried out. Addition of iron markedly increased the rate of acetic acid conversion to methane. Optimum concentrations (in respect to methane production)

	Anaerobic digesters fermenting food plant wastes.			of CO_2 during digestion. Explained often limiting factor is soluble Fe content.	of Fe were 5-10 mM. Even 20mM Fe increased activity however at such high levels Fe was often slightly inhibitory for the first 2 to 4 d. Continuous-flow reactors also showed stimulatory effect of Fe. The activity was increased by 100% in municipal sewage treating digesters. Results indicate that optimisation of the conversion of acetic acid to methane in methanogenic fermentations requires soluble iron levels many times higher than those often required for maximum growth and activity in microbial cultures.
(Schonheit *et al.*, 1979)	Pure culture of *Methanobacterium thermoautotrophicum.*	H_2 and CO_2	Ni, Co, Mo, Fe, Cu. Mn, Zn, Ca, Al and B		10 µmol Fe, 20nmol Co, 20nmol Mo and 150nmol Ni required for the synthesis of 1g cells (dry weight). A dependence of growth on Cu. Mn, Zn, Ca, Al and B could not be demonstrated. Higher amount of nickel required than cobalt.

Table 2.8 highlights multiple studies which have demonstrated the benefits of maintaining optimal cobalt concentrations. The advantages of supplementing cobalt in studies where the metal is limiting has been expressed in many ways including; increases in methane production (Fathepure, 1987, Gonzalez-Gil *et al.*, 2003, Gonzalez-Gil *et al.*, 1999a, Jansen *et al.*, 2007), methanogenic growth (Mah *et al.*, 1978, Scherer and Sahm, 1981) substrate utilisation rate (Murray and Berg, 1981, Speece *et al.*, 1983, Zitomer *et al.*, 2008), reduction in COD (Takashima *et al.*, 2011, Takashima and Speece, 1989) and improvements in digester health and stability (Climenhaga and Banks, 2008, Murray and Berg, 1981).

As highlighted earlier in the literature review transition metals have low ionisation energies and are able to exist in a wide range of oxidation states. This property allows the metals to catalyse electron transfer processes and consequently transition metal complexes are commonly found as cofactors within active sites of enzymes involved in a wide range of biochemical reactions (Lewis and Evans, 1997). The reduced methane production caused by deficiencies in metals is therefore commonly attributed to a reduction in functional cofactors available to catalyse metabolic reactions. Kida *et al*. (2001) demonstrated this in their study by showing that with decreasing amounts of Ni^{2+} and Co^{2+}, the methanogenic activity decreased alongside a reduction in concentrations of F_{430} and corrinoids. The opposite relationship was true for increasing amounts of Ni^{2+} and Co^{2+}. Gilles and Thauer (1983) in their study on the biosynthesis of factor F_{430} found uroporphyrinogen III to be an intermediate in the biosynthesis of the conenzyme in *Methanobacterium thermoautotrophicum.* Without nickel in the culture the intermediate uroporphyrinogen III was found to accumulate and once nickel is added the cofactor is subsequently formed from it. The addition of nickel to *Methanobacterium bryantii* culture has been shown to stimulate F_{430} synthesis over a 4 hour period (Jarell and Sprott, 1982). The synthesis of cofactors from close intermediates helps explain the quick response observed to metal stimulation.

Other explanations for reduced activity from metal deficiency include the possibility of other metals taking the place of the deficient metal and functioning in its role albeit at a lower efficiency. This would explain the reduction in activity observed however such relevant observations in the literature are limited mostly to tungsten and molybdenum enzymes (Bertram *et al.,* 1994) thus reduced activity is more likely associated with a reduced number of cofactors and therefore a reduced number of functional enzymes.

Research by Bartacek *et al.* (2008) on cobalt emphasises the importance of understanding nutritional requirements as the study showed the border between the optimal and inhibitory concentrations is very narrow; with 7 µmol/L found as optimal and 18 µmol/L already giving rise to 50% inhibition. At elevated concentration heavy metals are believed to cause disruption of enzyme function and structure by binding of the metals with thiol and other groups on protein molecules or by replacing naturally occurring metals in enzyme prosthetic groups (Vallee and Ulner, 1972). However within literature we find a range of optimal metal concentrations reported (Takashima *et al.*, 2011, Takashima and Speece, 1989). In studies where different feed substrates are used differences can be expected as different substrates would ultimately select for a different consortium of methanogens and hence different nutrient requirements would be observed. For similar reasons differences can also be expected when the inoculum sludge used in the study is sourced from digesters treating different wastes. However variability in data between studies can also be attributed to differences in the digester media conditions

The study by Bartacek *et al.* (2008) attributed the differences in the literature for cobalt toxicity concentrations as due to varying magnesium and calcium levels which compete with cobalt for uptake. As discussed previously the metal uptake mechanisms employed by methanogens can be subject to competition from other ions and can be affected by changes in reactor conditions, particularly by those that influence precipitation and the sorption of metals onto the biomass surface. This process of adsorption is important in the uptake process. Metal precipitation in a digester is important for retaining metal within sludges however it's believed to make the precipitated metal no longer directly available (Gonzalez-Gil *et al.*, 2003). Maintaining pH stability in digesters is important in respect to metal uptake as although lower pH values help keep metals in solution by preventing precipitation (Speece *et al.*, 1983), higher pH values are better for surface metal accumulation (Ledin, 2000). Therefore it is understandable how even studies with a similar experimental set up in respect to inoculum and feed source may still report different optimum metal concentrations.

Whereas often sufficient cobalt appears to be retained in digester to support optimal methanogenic growth and activity, it’s evident that the present elements are not always bioavailable even in the soluble form. Research by Speece (1988) surveyed 30 different conventional suspended system municipal wastewater digesters and amongst several other parameters the response to nutrient supplementation, using a SMA technique, was investigated. It was shown that even though soluble iron, cobalt and nickel appeared to be ample in the sludges, their supplementation caused stimulation in 9 of the digesters. The study explained this as due to presence of naturally formed chelators which are able to complex metals tightly so that they are unavailable to the microorganism consortia. Interestingly analysis of the data provided in the study reveals that the site which had the highest soluble iron also responded the highest to it; this is consistent with the researcher’s hypothesis as precipitation is unlikely to be the cause of the metal unavailability for that particular site.

It is also noteworthy that studies have highlighted that propionate utilisation rate (PUR) to be more responsive to metal supplementation relative to when acetate is used as the feed substrate (Speece, 1988, Zitomer *et al.*, 2008). This may possibly be explained by the fact approximately 70% of methanogenesis in domestic sludge comes via the precursor acetate (Jeris and McCarty, 1965, Smith and Mah, 1966) and therefore the sludge is more selected to acetate utilisation. Consequently when high concentrations of propionate is used as a test substrate for such sludges the methanogenic consortia are possibly more in need of adaptation, which may explain the higher nutrient requirement and thus the higher observed response to supplementation in the studies. As the studies with acetate as the feed substrate imitate a full scale digester treating municipal wastewater more closely, they provide a stronger indicator of actual metal requirement for such digesters.

As can been seen in Table 2.8, most supplementation studies add metals as chloride salts and studies which have used chelating compounds namely EDTA have shown conflicting results. Majority of studies find EDTA bound metals to be soluble but non-bioavailable (Aquino and Stuckey, 2007, Babich and Stotzky, 1983, Bartacek *et al.*, 2008, Pankhania and Robinson, 1984, Speece, 1988). This would imply that the methanogens do not have a metal binding component (essentially an uptake mechanism) with higher stability constant than that of the ligand complex. Furthermore the study by Aquino and Stuckey (2007) showed 1mM of free EDTA to be inhibitory, which is likely to be associated with the chelating agents ability to bind with a wide range of cations to produce stable complexes (shown in Table 2.7). Consequently the inhibition was attributed in the study to a loss in metal uptake capacity. However as previously shown Fermoso *et al.* (2008) compared cobalt supplementation as bound to EDTA with $CoCl_2$ and found Co-EDTA had a much quicker stimulatory effect in terms of specific methane activity and substrate removal in methanol-fed UASB reactors. The authors inferred that cobalt bound to EDTA has a higher bioavailability or is more efficiently transported within the granular sludge. It is important to note that the metal cobalt is only required in trace amounts and in the latter study was supplemented at 5 μM, which gave rise to SMA increasing by as much as 233% after the first dose of Co-EDTA. It is understood that free EDTA at the high concentration tested is inhibitory; however this would not be issue for metals such as cobalt as they required in trace amounts and would thus require corresponding (stoichiometric) EDTA concentrations at levels a few orders of magnitude less than that found to be inhibitory.

The variable response to EDTA metal chelates as found in the literature may also be explained by differences in the level of bioavailability for different metal EDTA chelates, as it has been shown (Table 2.7) that EDTA binds with different metals with different stability constants and we have also seen that microorganisms employ various uptake mechanisms. Therefore it is conceivable that for metals which bind with EDTA with a high stability constant and yet are taken up by a relatively non-specific pathway such as the magnesium transport system are in fact non-bioavailable when complexed with EDTA. Whereas other metals such as cobalt which, as discussed previously, is likely taken up by a higher affinity substrate specific pathway are bioavailable.

Previously it was suggested that displacement of EDTA complexed metal may be occurring within the digester medium and the effects reported may in actual fact be indirect. If this is the case it can provide sound reasoning as to why studies can report conflicting data for EDTA metals, because certain metals may actually be non-bioavailable but would still illicit a response in cases where they are freed from the complex via displacement with a metal that is able to produce a higher stability constant. However Aquino and Stuckey (2007) in their study, where 1mM free EDTA was shown to be inhibitory, also tested the effects of spiking with a stoichiometric (1mM) level of Fe^{2+} to see if the effects can be reversed, as Fe^{2+} produces a complex with a relatively high stability constant. To the contrary the study stated Fe^{2+} did not result in any improvements in methane production and concluded the effect to be irreversible. This would imply that displacement of the complexed metals is an unlikely factor however this study is not conclusive as it failed to acknowledge that essential trace metals such as cobalt, nickel and zinc all complex with higher stability constants in standard conditions and therefore are unlikely to be displaced. Also as highlighted earlier in the literature review ligands are able to scavenge complexed metal ions from a complex providing that it is sufficiently labile, (Snoeyink and Jenkins, 1980) so it may not have been possible to displace all of the metals responsible for the observed inhibition, or at least not in the time frame of the batch tests used. Therefore metal displacement may still be a significant factor for the variability of literature data on the bioavailability of the EDTA complexed metals, as well as the other factors discussed. Further understanding of the bioavailability of EDTA metal chelates is important in helping supplementation of metals in their most bioavailable forms and helping to avoid wasteful dosing of non-bioavailable chelates.

An optimised metal dosing strategy aims to achieve maximum effect on the biological activity whilst avoiding wasteful dosing and limiting introduction of metals into the environment (as part of effluent). In order to achieve this, better understandings of metal requirements alongside bioavailability in terms of metal speciation and microbiological uptake characteristics is required. This study concentrates on key practical aspects of trace metal supplementation in wastewater sludge digesters; recognising when the digester is deficient in metals and would therefore benefit from supplementation and also determining whether metal EDTA chelates are readily bioavailable to the methanogens.

CHAPTER 3 AIMS AND OBJECTIVES

3.1 Thesis Aims

1. To investigate the potential of metal EDTA complexes to enhance methanogenesis from anaerobic digestion of wastewater sludge.

2. To analyse the relationships between metal bioavailability, methanogenic populations and methane output, and to use this knowledge to identify when full-scale wastewater sludge digesters might benefit from metal supplementation

3.2 Thesis Specific Objectives:

1. Developing a standard diagnostic laboratory assay to understand digester response to trace metal supplementation.
2. Determining the potential of metal supplements to increase the maximum rate of methane production in sewage sludge digesters.
3. Investigating the relationship between total and soluble metals and methane production rates. Furthering the understanding of the importance of metal requirements.
4. Investigating the relationship between the methanogenic populations in digesters and methane production rates. Furthering the understanding of metal deficient digesters and their identification.
5. Investigating the relationship between metals in the soluble phase and response to trace metal supplementation. Furthering the understanding of the uptake and bioavailability of metal-EDTA complexes.
6. Investigating the relationship between differences in observed response to EDTA complexed metals and digester profiles (methanogenic population, methane production rate and metal content). Furthering understanding of bioavailability of metal-EDTA complexes.

3.3 Research Hypotheses

1. **That trace metal deficient anaerobic sludge digesters will produce more methane when supplemented by EDTA complexed metals**, measured as an increase in the acetoclastic methanogenic activity:
    a. EDTA metal complexes will generally not be directly bioavailable due to a combination of high stability constant chelation of EDTA and non-specific low affinity uptake pathways being employed by the methanogens.
    b. Except for EDTA complexed cobalt, which will be directly bioavailable as it is taken up by a high affinity system. The uptake of cobalt is likely to be unaffected significantly by other metals except nickel as highlighted in the literature review

2. **Metal deficient digesters will be characterised by a lower than expected acetoclastic methanogenic activity for the population of methanogens,** as a metal deficiency manifests as a lower activity due to lower levels of cofactors: A lower than expected activity in such a case will be an indicator for metal requirement. Whereas metal content of the sludge alone will not be a good indicator due to differences in bioavailability.

CHAPTER 4 METHODOLOGY

Within this chapter, the main methods and materials used throughout this research are presented: These are divided into three main sections:

1. An explanation of the experimental methodology and analyses associated with the specific methanogenic activity tests, conducted in serum bottles, which form the basis of the research presented in this thesis.
2. Details of the experimental methods and protocol used for identification and quantification of methanogenic populations in the full-scale digested sludge samples
3. General analytical methods used in this research for measurement of digested sludge characteristics, such as total and volatile solids and total and soluble metals.

4.1 Specific Methanogenic Activity Test

Within this section the experimental protocol is outlined for the specific methanogenic activity (SMP) test used, the method of biogas sampling is detailed and the method for the analysis of biogas composition described.

Table 4.1. Operational parameters of the digester sites studied (Details provided by Severn Trent Water)

	Digester site A	**Digester site B**	**Digester site C**	**Digester site D**
Digestion capacity (m^3)	6,780	80,000	18,180	10,800
Digester loading (m^3/d)	525	3618	1050	504
VS loading (kg/m^3.d)	2.6	1.5	1.9	1.5

Digester HRT/SRT (d)	13	22	17	21
Digestion type	Mesophilic	Mesophilic	Mesophilic	Mesophilic
Phosphate removal (iron-dosing)	No	No	Yes	Yes
% trade to domestic	7.22%	15.40%	7.61%	9.15%

Table 4.1 details the 4 digestion sites studied within this research. The sites were selected according to geographic proximity and their variation in operational parameters. The sites are themselves made up of several digesters and therefore for consistency samples were always taken from the same digester and same location. The information in Table 4.1 shows all the digestion sites chosen are mesophilic, with a wide range of digestion capacities. Two of the sites (C and D) employ chemical dosing (ferrous sulphate) to remove phosphorus from the wastewater. The resultant iron phosphate precipitates are settled out with the activated sludge and enter the digesters. Site A digesters are the most highly organically loaded with the shortest HRT, followed by Site C digesters and then B and D being almost identical in terms of organic loading and HRT.

4.1.1 Specific Methanogenic Activity Test

Specific methanogenic activity tests were conducted with digested sludge (taken from one of the target digesters for each test) used as the inoculum and acetate buffer used as the feed. As the feed is only acetate this test is designed to measure the acetoclastic methanogenic activity, although involvement of other methanogenic groups cannot be ruled out (discussed further in Section 6.2). Acetate buffer consisting of 99.75 % sodium acetate and 0.25% glacial acetic acid was used to maintain pH within optimal range pH 7 to 7.2 (Gerardi, 2003). In setting up the assay the first step was to determine the volatile solid (VS) content of the inoculum. The VS concentration of the inoculum will vary with time and was therefore always measured within a few hours of sludge collection. The substrate to inoculum ratio used in the SMA tests was chosen after developmental work (described fully in Section 5.1.2) to insure that CH_4 production would not be limited by substrate or inoculum availability. The objective was to calculate an accurate maximum methane production rate ($V_{max\ acetate}$) whilst avoiding excessive biogas production and digester souring. Thus, multiple substrate to inoculum ratios were tested using sludge from Digesters A to D, all based on 0.5 g of VS as inoculum with varying substrate concentrations; based on these tests, 60mM acetate (in 100ml sample volume) was chosen for all subsequent assays. Developing a standard diagnostic laboratory assay to understand digester response to trace metal supplementation was one of the objectives of this study and therefore its development will be discussed further in the next chapter.

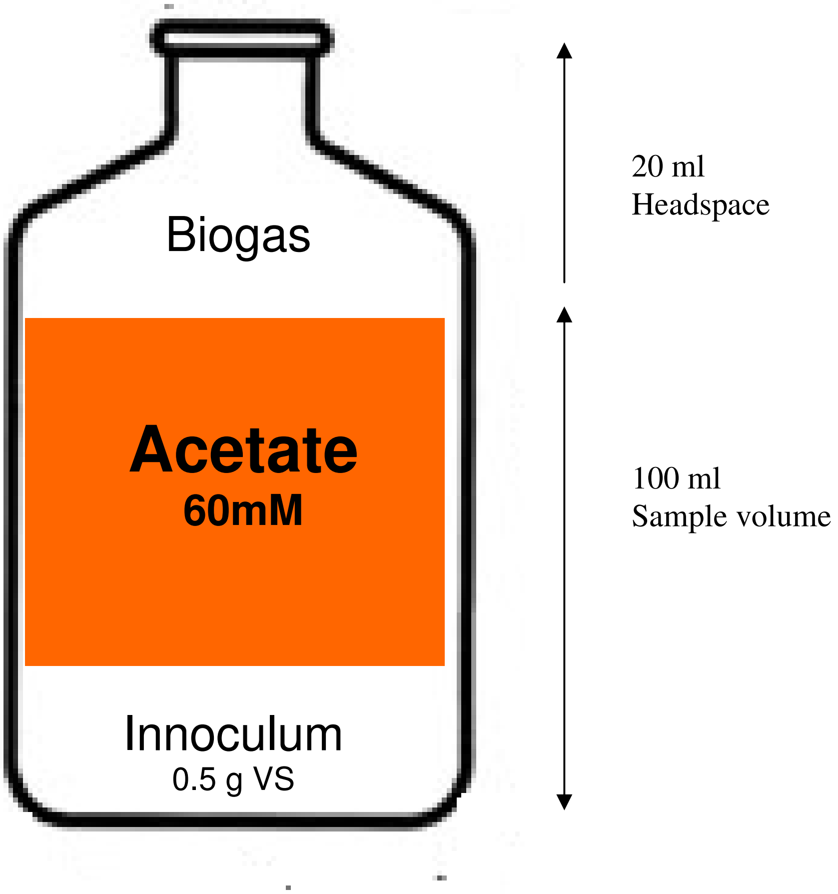


Figure 4.1 Illustration of specific methanogenic activity (SMA) test

The assays were performed in 120ml serum bottles as illustrated in Figure 4.1, with a 100ml sample volume and 20ml head gas space. The final volume was reached by adding de-ionized water to the bottle once the required mass of inoculum (equivalent of 0.5 g VS) and substrate are added. The bottles were sealed with a butyl rubber septum and aluminium crimp and kept at a constant temperature of 35°C. The tests ended when biogas production subsided to the base level of the non-feed control, which was approximately 13 days.

Supplementation assays were compared against a non-supplementation control to determine metal response and also to a non-feed control to allow correction for endogenous methane production. The non-feed control contained the quantity of inoculum added to the other assays plus de-ionized water to bring the volume to 100 ml. Each test was performed with 3-5 replicates.

4.1.2 Biogas Volume Calculation

Biogas accumulated in the headspace of the bottles and was measured and analysed to establish the percentage of CH_4 and CO_2. The pressure in the headspace was measured using a manometer and then converted into volume using the ideal gas equation.

$PV = nRT$

Where:

P	Pressure (N/m^2)
V	Volume (m^3)
n	Moles
R	Universal gas constant
T	Temperaure (K)

The converted biogas volumes were based on standard temperature and pressure 25°C and 1 atm (101.3 kPa).

4.1.3 Analysis of Biogas Composition

A 1 ml plastic syringe attached to a needle was used for sampling. The needles used were small and short to ensure that the needle tip did not touch the sludge and that the pierced holes in the septum were easily resealed. Needles were replaced when they became blunt. Around 1ml of biogas was extracted from the serum bottles for GC analysis. The sample in the syringe was expelled until it reached 0.5ml and at that point the sample was injected into the GC.

The analysis was carried out using a GC (Ai Cambridge, model GC94) with a Spherocarb column (mesh size 80-100). The carrier gas helium was set at a flow rate of 30ml per minute. The GC was calibrated using three standards: 1) 100% CH_4, 0% CO_2; 2) 50% CH_4, 50% CO_2; 3) 0% CH_4, 100% CO_2. The GC identified three gases, air, CH_4 and CO_2. The air fraction was considered to arise mainly from the sampling method and did not represent an actual portion of the biogas generated. For this reason only CH_4 and CO_2 figures are reported.

4.1.4 Methane Percentage Correction

As shown by Kim and Daniels (1991) unexpected errors in methane measurement occurred when analysing sealed bottles incubated at higher than room temperature. The study proposed the values generated by the GC should be corrected for the difference in temperature between the serum bottle and the syringe (at room temperature), this correction is summarised as:

$$y = \frac{x}{a} \times 100 - 100$$

Where:

y = change in the GC methane caused by temperature change (%)
x = incubation temperature of the serum bottles (K)
a = room temperature (K)

The GC standards used were at room temperature and the assays were kept at 35°C. Increases in temperature lead to increases in gas volume and therefore the temperature difference was identified as a source of error in the GC's methane quantification. Consequently room temperature was recorded every time GC analysis was performed and the methane percentage values were corrected according to value generated by the above equation.

4.1.5 Cumulative Methane

Biogas volume was integrated with methane percentage data to generate cumulative methane curves. Data was analysed and the maximum methane rates was extracted. These rates were then adjusted to represent the theoretical acetoclastic methanogenic activity ($V_{max\ acetate}$) by correcting endogenous methane produced from the non-feed controls. Approximately 70% of methanogenesis in domestic sludge is believed to occur via the precursor acetate (Jeris and McCarty, 1965, Smith and Mah, 1966) therefore upon this assumption methane produced by the non-feed controls was multiplied 0.7 to correct for the relative stoichiometry from the precursor acetate, as according to method described by Conklin *et al*. (2008).

4.1.6 Trace Metal Solution

All metals supplemented in this study were complexed as EDTA chelates. Trace metal supplementation studies were initially carried out using a defined mixture of trace elements. Table 4.2 details the composition of the mix used, which is referred to as the TM solution. After starting with the TM solution at a range of concentrations for each study, subsequent assays also incorporated supplementation of cobalt-only at equivalent concentrations. Appendix A details the experimental programme followed for each sample tested.

Table 4.2 Trace element composition of TM solution

Metal	Concentration (mg/l)
Fe	33.6
Co	11.2
Mn	11.2
Ni	11.2
Zn	1.12
Mo	1.12
B	0.112
Cu	0.112
Se	0.112

4.1.7 Soluble Metal Tracking

To track the evolution of metals in the soluble phase during the tests, selected studies were run with numerous replicates (15-20 for each unique assay). Samples were destroyed in duplicate at timed intervals during a test. The samples were centrifuged immediately and the supernatant was filtered through a 0.45 μm membrane filter, the samples were then acidified and analysed using an ICP-MS. Appendix A details the experimental programme followed for each sample tested.

4.1.8 Acid Washing

All glassware and plastic ware associated with the SMA assays were acid washed to remove inorganic contamination using the following procedure:

1. Wash in hot water with phosphorus-free soap.
2. Rinse thoroughly with tap water (5 times) and reverse osmosis water (ROW) (3 times).
3. Soak overnight in 10% HCl.
4. Rinse in ROW (3 times).
5. Soak overnight in ROW.
6. Dry in a hot cabinet.

4.2 Methanogenic Population Studies

Within this section the experimental methods and protocol are outlined for the techniques used for identification and quantification of methanogenic populations

4.2.1 Quantification PCR Methodology

Immediately after collection approximately 20ml of all sludge (digested) samples were frozen. Quantification PCR was later carried out on a selected number of these sludge samples. Quantification PCR (qPCR) is a technique that amplifies the target DNA/RNA sequence chosen as in standard PCR but also allows quantification at the same time via the use of a flurophore. The technique was used in this study to detect and quantify methanogens at the family-taxonomic level for *Methanosarcinaceae* (Msc) and *Methanosaetacea* (Mst) which both belong in the taxonomic order *Methanosarcinales*. For this study genomic DNA for qPCR studies was extracted from 250 µL inoculum sludge samples using FastDNA® SPIN KIT by MP Biomedicals LLC and a Hybaid® Ribolyser.

Table 4.3 Primers used for qPCR

Target group	Primer	Sequence (5' to 3')	Size (bp)	Annealing temperature	Ref.	Non Target matches
Methanosarcinaceae	Msc 380f	GAA ACC GYG ATA AGG GGA	448	62ºC	(Yu *et al.*, 2005)	0.7%
	Msc 828r	TAG CGA RCA TCG TTT ACG				
. *Methanosaetaceae*	Mst 702f	TAA TCC TYG ARG GAC CAC CA	126	62ºC	(Yu *et al.*, 2005)	0.2%
	Mst 826r	CCT ACG GCA CCR ACM AC				

4.2.2 QPCR conditions

Real-time quantitative PCR (qPCR) was performed using the iQ multicolor detection system (Bio-Rad Laboratories). Quantitative PCR reactions were performed in a total reaction volume of 20 µl, containing 3 µl DNA (both 1/10 and 1/100 dilutions used), 10 µl of buffer supermix (Bio-Rad), 0.5 µl of each primer (reverse and forward) for target group as detailed in Table 4.3, 0.2 µl SYBR green probe and ddH_2O. Thermal conditions were 95°C for 5 minutes, followed by 39 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 30 seconds. In each qPCR run, standards of 10^8 to 10^3 copy number/ml^{-1} were used and a non-template negative control was included to monitor possible contamination. Data were evaluated using the iQ 5 software (Bio-Rad Laboratories). The qPCR raw data reveals the number of copies of the targeted sequence; an organism can have a variation of the number of operons containing the target sequence per cell. However amongst closely related species the numbers appear conserved, for the archaea a single operon per cell appears to be most common (Acinas *et al.*, 2004), therefore for the purposes of this study it was assumed one copy translates to one cell. Sample runs in which the negative control responded were discounted and the test repeated. Data reported as averages of the repeats from the best amplifying dilution.

4.2.3 Errors from QPCR

Possible errors in quantification can arise from non-specific selectivity of primers, inappropriate sample selection coupled with heterogeneity, presence of humic acids and pipetting errors. The selectivity of the primers used in this study has been demonstrated before (Yu *et al.*, 2005), all samples used were well mixed, the presence of humic acid was countered by using dilutions of original DNA to prevent inhibition and the best amplifying dilution was used. The extracted nucleic acid must be free of contaminants that can inhibit enzymes used in the molecular technique, such as restriction endonucleases or *Taq* polymerase. Therefore a commercially available kit was used which gives high DNA recovery and elimination of inhibitory substances that can affect PCR.

The curves generated from the standard had a R^2 value of average 0.97 which indicates sound technique and amplification. The R^2 value of a standard curve represents how well the experimental data fit the regression line. which gives a measure of the variability across assay replicates of the standards, as well as whether the amplification efficiency is the same for different starting template copy numbers. In this study, the average coefficient of variation between 14 samples from 4 different digesters extracted in duplicate was relatively small at 10.03%. However due to all the variations associated with qPCR process only differences of at least 2 fold were considered significantly different.

4.2.4 FISH

Fluorescence in situ hybridisation (FISH) is technique that allows detection and location of a specific genetic sequence via the use of a fluorescent probe; it works on whole cells so does not require DNA extraction and can help reveal information about community structure. In order to verify data from qPCR, FISH was also performed on selected number of samples. For FISH analysis the samples had to be frozen in 50% ethanol immediately after collection. The FISH analysis was performed using the MST and MSC probes highlighted earlier alongside a total archaea probe, ARC 915. The FISH procedure involves 4 steps; fixation step, followed by a dehydration step, a hybridisation step where the probe is attached followed by a washing step before finally the slides are imaged using a confocal laser scanning microscope. The protocol was as follows:

Fixation step:

1. Take an appropriate amount of sample (typically 1ml) and centrifuge at 13,000 x g for 3 minutes in sterile (autoclaved) Eppendorf tubes. Remove supernatant.

2. Wash once with phosphate buffered saline (PBS) by adding 1 ml of 1 x PBS (i.e 1%) and mix using vortex. Centrifuge the solution at 13, 000 x g for 3 minutes and remove supernatant.

3. Resuspend the pellet in 0.25 ml of PBS (1%).

4. Add 0.75 ml of PFA fixative (see above) and vortex.

5. Store/incubate the suspension for 3hrs-overnight at 4°C.

6. After fixation, wash the cells by centrifuging the cells at 13,000 x g for 3 minutes and adding 1 ml of PBS (1%) and vortexing.

7. Repeat PBS wash step.

8. Remove the supernatant and add 1ml PBS (1%): absolute ethanol solution (1;1, v/v) and mix.

Dehydration of fixed samples:

1. Apply 5-30 µl of PFA or ethanol-fixed sample material onto a microscope slide (or onto one field of a teflon-coated slide). Ensuring you take into account negative control.

2. Dry for approx. 15 minutes at 46°C or longer at room temperature.

3. Dip slide for 3 min each into 50 %, 80 % and 96 % (v/v) ethanol. The dehydrating effect of the ethanol effect of the ethanol concentration series disintegrates cytoplasmic membranes which thus become permeable to oligonucleotide probes.

4. Dry the slides for a couple of minutes at 46°C.

5. After drying samples on slide apply 5 µl of 0.1% agarose.

Hybridisation:

1. Prepare 1ml of fresh hybridisation buffer per slide with correct hybridisation stringency, this ensures that a probes binding will be target specific. Stringency is achieved by adding formamide, formamide interferes with the hydrogen bonds

that stabilises nucleic acid duplexes. The stringencies of the probes used for our study were 50% and 35%.

2. The pH of the buffer is adjusted by addition of Tris/HCl. SDS in buffer reduces surface tension of the buffer, allowing it to spread more evenly over biomass on the microscopic slide and to penetrate thicker samples more easily.

3. Add 10 µl formamide hybridisation buffer to the well.

4. Add 1 µl (50ng/ µl) of probe to slides- with no probes in negative controls.

5. Put a piece of tissue paper into a 50 ml screw top plastic tube and pour remaining hybridisation buffer onto the tissue paper.

6. Immediately place the slide horizontally into the tube and close tube. Place the tube in horizontal position onto a rack and incubate it in an oven at 46°C for 1-5 hrs. Tightly sealed plastic tube functions as a moisture chamber preventing the evaporation of solution from slide. In particular, the evaporation of formamide can cause non-specific probe binding to non-target cells.

Washing:

1. Prepare 40ml of washing buffer in a 50 ml tube. The NaCl concentration in the washing buffer is prepared according to the formamide concentration in the hybridisation buffer. A highly stringent hybridisation (high formamide concentration) requires a highly stringent washing step (low Na^+ concentration in the washing buffer). Preheat buffer to 48°C.

2. Remove the screw-cap tube containing the slide from the hybridisation oven, immediately wash away the hybridisation buffer with 1ml of wash buffer.

Transfer the slide into the remaining wash buffer quickly (using tweezers and working in fume cupboard)

3. Transfer tube containing the slide and wash buffer back into 48°C water bath and incubate for 10-15 mins.

4. Take the slide out and dip into ice-cold ddH_20.

5. Air dry the slide as quickly as possible.

6. Repeat hybridisation steps with the lower stringency probe. Only hybridisations with probes requiring the same formamide concentration can be performed on the same slide at the same time. A sample can be sequentially hybridised with more stringent hybridisations being performed first.

Microscopy:

1. Apply two drops of antifadent close to the left and right of the slide. (Frozen slide should be warmed to room temperature first).

2. Put a microscope cover slip on top and wait until the antifadent (glycerol-pbs solution) has spread over the whole slide. Too much antifadent can blur the microscope image.

3. Finally observe the sample under an epifluorescence microscope or confocal laser scanning microscope equipped with suitable filters or lasers.

4.3 General Analysis

Table 4.3 lists the characterisation analysis carried out on all collected sludge samples to understand their physiochemical properties. This section outlines the methods used for these tests.

Table 4.4 Sludge characterisation analysis list
Acid digestion for total metals
Soluble fraction metal analysis
VS
TS
Alkalinity
Ripley's Ratio

4.3.1 Acid Digestion (Total Metal)

The method followed in this research was based on the one used by Smith (2006). The procedure was as follows:

1. Measure 20 ml sludge and transfer into a 250 ml glass beaker.
2. Add 2.7 ml analytic grade HCl, 30 ml UPW and 1.3 ml analytic grade HNO_3.
3. Place beaker on hotplate and boil until approximately 10ml remains.
4. Remove from hotplate and allow for it to cool before adding a further 2.7 ml analytic grade HCl, 30 ml UPW and 1.3 ml analytic grade HNO_3.
5. Return the solution to the hotplate and boil until approximately 10 ml remains.
6. Leave to cool and then filter through a Whatman 540 filter into a 100 ml volumetric flask.
7. Rinse the filter paper and beaker with UPW and then filter through again to capture residual metals.
8. Add UPW to flasks to bring volume to 100 ml.

4.3.2 Metal Analysis

The Atomic Absorption Spectrometry (AAS) instrument (Perkin Elmer AAnalyst 800) was used to measure the concentration of metals in samples after acid digestion for total metals and after filtering supernatant through membrane filter of 0.45 µm for the soluble metals in the liquid phase. Metals were quantified using inductively coupled mass spectrometry using an ICPMS Agilent 7500ce Octopole reaction and an ICP-MS Thermo Scientific ICAP 6000 series.

4.3.3 Total and Volatile Solid

TS and VS concentrations were measured in triplicate for all the sludge samples analysed. The basic procedure is described in Standard Method (2540 B and E, APHA AWWA WEF, 1992). Clean tins were introduced in a furnace at 500 ºC for 1 hour. Once the tins were cool they were weighed (weight noted as W1). Samples of 20ml digested sludge were transferred into the pre-weighed tins. The tins were then placed into an oven at 105ºC for 2 hours approximately (until all the liquid evaporated). After this step the tins were left to cool in the desiccators, weighted (weight noted as W2) and then transferred to the 500 ºC furnace for at least 1 hour. In the final step the tins were cooled and weighted (weight noted W3). Concentration of TS and VS can be calculated using the following equations:

TS (g/l) = (W2 - W1)/Volume
VS (g/l) = (W2 – W3)/Volume

This analysis was performed within a few hours of sludge collection and the VS values were used to determine the volume of sludge needed to reach 0.5 g VS for each serum bottle.

4.3.4 Alkalinity and Ripley's Ratio

The method used is based on the titration method described in Standards Methods (2320, APHA AWWA WEF, 1998). Sample size for the test was 20 ml. First the sample was pipetted into a beaker with a small magnet stirrer to keep the sample well mixed. The sample was titrated using 0.1 N sulphuric acid to the end points of pH: 5.75, 4.5 and 4.3. The end point of pH 4.5 is used for total alkalinity calculation, while the other two points are used to calculate the Ripley's Ratio.

Alkalinity is a measure of the ability of a solution to buffer acids. Total Alkalinity (TA) is calculated as mg $CaCO_3$ using the following equation:

$$TA = \frac{A \times N \times 5000}{S}$$

Where:

TA = Total alkalinity (mg $CaCO_3$/l)

A = Volume of standard acid used to reach end point of pH 4.5 (ml)

N = Normality of standard acid used (in this case 0.1N)

S = Volume of sample used (ml)

Parameters of alkalinity or volatile fatty acids (VFAs) give an idea about the digester's stability. Ripley *et al.* (1986) stated that ratios values below 0.3 are good and values as high as 0.8 are indicative of a stressed digester. The equation to calculate RR is:

$$RR = \frac{(TA - PA)}{PA}$$

RR = Ripley's ratio

TA (total alkalinity) = Volume of standard acid used to reach end point of pH 4.3 (ml)

PA (partial alkalinity) = Volume of standard acid used to reach end point of pH 5.75 (ml)

CHAPTER 5 RESULTS AND INTERPRETATION

This chapter is split into six main sections each relating to the six objectives outlined in Chapter 3 – Aims and Objectives, in corresponding order.

5.1 Standard Diagnostic Laboratory Assay

Developing a standard diagnostic laboratory assay to understand digester response to trace metal supplementation was the first objective of this study. A rapid laboratory test to determine digester response to trace metal supplementation was required that could be related back to the full-scale digester. The following section details the development of the test and its precision.

5.1.1 Standard Diagnostic Laboratory Assay

The SMA test developed by Conklin *et al.* (2008) was used as the basis for this trace metal supplementation study. Conklin *et al.* (2008) demonstrated a simple methodology which is designed to calculate the maximum methane production rate from acetoclastic methanogenesis, termed $V_{max\ acetate}$. The study concentrated on acetate as previous research has shown approximately 70% of methanogenesis in domestic sludge comes via the precursor acetate (Jeris and McCarty, 1965, Smith and Mah, 1966). The study used acetate as the feed and corrected the endogenous methane production from the no feed control as shown in section 4.1.1. The study related the calculated $V_{max\ acetate}$ to the full-scale digester by calculating the use of an acetate capacity number (ACN), the ACN value is the ratio between the $V_{max\ acetate}$ from a batch test to that of the actual plant acetoclastic production rate. The study demonstrated that when the ratio was above 1 there was excess capacity in the digester and when it was below 1 it indicated a condition approaching failure. The study used this to determine digester stability and safe loading changes. From a trace metal supplementation point of view this approach allows any increase in the $V_{max\ acetate}$, from trace metal supplementation, to be to be related back to the full scale digester in terms of a potential increase in digester capacity.

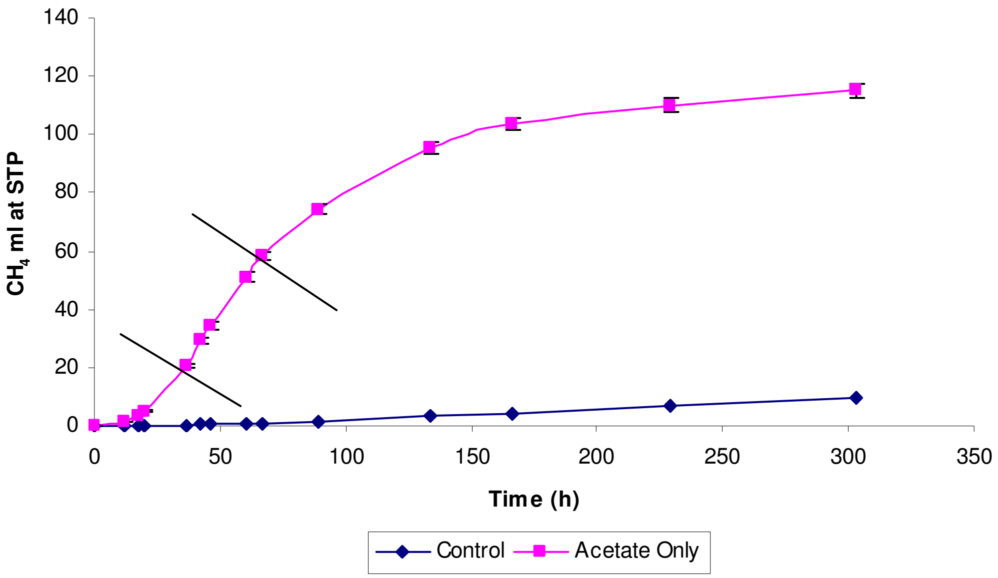


Figure 5.1 Cumulative methane curves

Figure 5.1 illustrates typical cumulative methane curves generated in this study using the serum bottle test methodology outlined in section 4.1. The $V_{max\ acetate}$, simplified as V_{max}, is calculated from the linear portion of the curve, as highlighted. Although our tests were run until the biogas production subsided, to obtain the V_{max} the tests would only need to be run typically for 3-4 days.

5.1.2 Method Development

This study adapted the described test by Conklin *et al.* (2008) in a number of ways. Different substrate to inoculum ratios were tested to ensure CH_4 production was not limited by substrate or inoculum availability. The objective was to calculate an accurate V_{max} whilst avoiding excessive biogas production. Thus multiple substrate to inoculum ratio were tested for different digesters, all based on 0.5 g of VS as inoculum with varying substrate concentrations. The volatile solid content was used as an indicator for the biomass content. Although this fraction will also include organics that were not fully digested the VS content was kept constant to reduce variability in the methanogen levels. The efficacy of this will be discussed later in the chapter from the viewpoint of methanogenic quantification which was later carried out on the collected sludge samples.

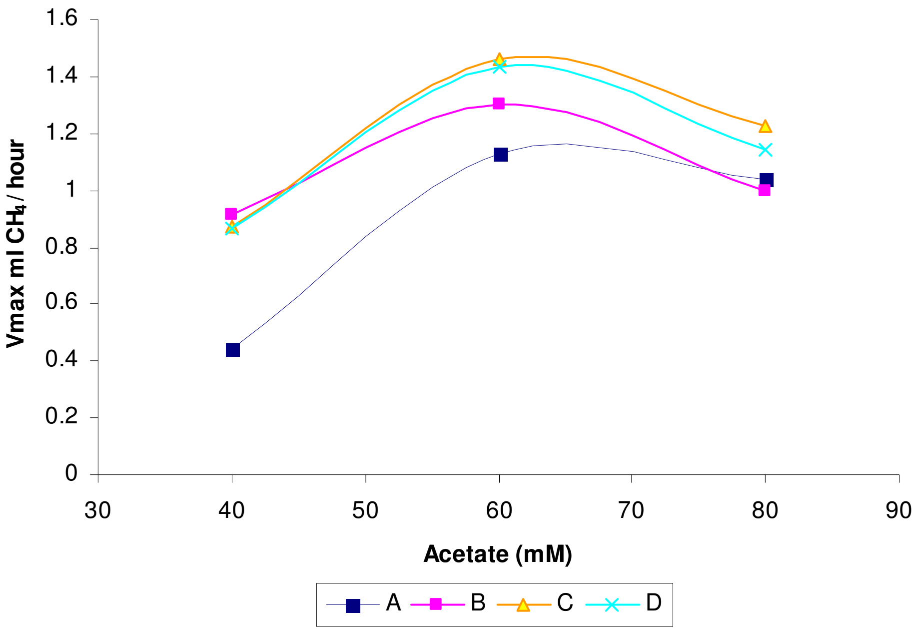


Figure 5.2 Digester V_{max} rates from different feed concentrations

Figure 5.2 demonstrates the results found with different acetate concentrations. All studies indicated 60mM gave the highest V_{max} based on 0.5 g VS. At the higher concentration of 80mM the V_{max} values generated across the digesters were noticeably lower; this may be attributed to sodium toxicity at the higher concentration. Consequently 60mM acetate was used for all subsequent tests.

As shown by Kim and Daniels (1991) unexpected errors in methane measurement occurred when analysing sealed bottles incubated at higher than room temperature. The GC standards used were at room temperature whilst the assays were kept at 35°C. Increases in temperature lead to increases in gas volume and therefore temperature differences are a source of error in the GC's methane quantification. Consequently the methane percentage values generated from the GC in this study were corrected as described in section 4.1.4.

5.1.3 Variation between Replicates

Table 5.1 highlights the V_{max} from the samples studies without any metal supplementation. The samples are labelled according to the digester they were sourced from (first letter) followed by the date (month and year). The table highlights the relative standard deviation of the replicates. Sources of error in the methodology include gas leaks from bottles, inaccuracy in pipetting sludge/feed and errors associated with gas sampling. It can be seen that the average relative standard deviation (RSD) from three replicates among the sixteen samples was 3.81%. An advantage of the SMA serum bottle test used in this research is that the simplicity of the experimental setup resulted in very few gas leaks and hence the relatively low recorded variation between replicate serum bottles. This demonstrates the precision of the methodology used. The variability in metal supplemented assays was found to be slightly higher, perhaps due to the nature of metal interaction with the biomass, thus 4-5 replicates were run for supplementation assays. The average RSD of replicates in supplementation studies was 4.23% over 56 unique assays.

Table 5.1 $V_{max\ acetate}$ rates without supplementation

Sample	V_{max} CH_4 ml/h	STDEV of Replicates	RSD (%)
A0909	1.36 (n=3)	0.02	1.43
A0210	1.25 (n=3)	0.04	3.26
A0310	1.11 (n=3)	0.03	3.10
A0510	1.56 (n=3)	0.02	1.51
A0810	1.44 (n=4)	0.04	2.70
A0111	1.46 (n=4)	0.05	3.40
B0310	1.88 (n=3)	0.13	6.99
B0710	1.30 (n=4)	0.05	3.73
B1109	1.22 (n=3)	0.06	5.05
C0310	1.46 (n=3)	0.08	5.76
C0810	1.45 (n=4)	0.06	3.90
C1109	1.20 (n=3)	0.05	4.23
C0211	1.22 (n=4)	0.08	6.34
D0610	0.92 (n=3)	0.04	4.55
D0710	1.43 (n=4)	0.04	2.97
D1109	0.89 (n=3)	0.02	2.05
AVERAGE	1.32	0.05	3.81
STDEV	0.24		1.62
RSD(%)	18.35		

Table 5.2 Digester breakdown for $V_{max\ acetate}$ without supplementation

Digester A Average V_{max} (CH_4 ml/h)	1.36 n = 6
RSD(%)	11.7
Digester B Average V_{max} (CH_4 ml/h)	1.47 n = 3
RSD(%)	24.6
Digester C Average V_{max} (CH_4ml/h)	1.33 n = 4
RSD(%)	10.7
Digester D Average V_{max} (CH_4 ml/h)	1.08 n = 3
RSD(%)	28.0

Table 5.2 details the average V_{max} from each site and their respective variations. It is evident that V_{max} was found to vary between digester sites as well as for within the same digester in different sampling periods. Differences amongst digesters are to a degree expected given that each digester is treating a different feed sludge composition. This could mean a selection towards different mix of methanogens i.e. hydrogenotrophic, methanotrophic and acetoclastic methanogens. Also each digester has different operational parameters as highlighted in section 4.1; specifically differences in the solid retention time (SRT) may influence methanogenic populations. A higher population of acetoclastic methanogens would be expected to exhibit a higher $V_{max\ acetate}$. Digester B and D had the highest variations between samples, whereas Digester A and C were a lot more uniform, although their figures are based on higher sample numbers. Variation within the same digester is likely to be caused by operational variability at that digestion site leading to differences in the methanogenic populations. Factors which can cause variations in the maximum acetoclastic activity in a digester include presence of compounds e.g. metals, sulphide, ammonia or deficiencies in essential macro and micro nutrients, or operational factors that could affect acetoclastic activity, such as falling digester temperatures, variable organic loading rates and feed composition, and unfavourable pH or alkalinity ranges in the

digester. The relationship between trace metal content and V_{max} will be explored in the following section.

5.2 Trace Metal Supplementation

This section presents the results from trace metal supplementation of the SMA serum bottle digesters. The results reveal the prevalence of metal deficiencies in sewage sludge digesters and the potential of trace metal supplements to increase the maximum rate of methane production.

5.2.1 Trace Metal Solution

All metals supplemented in this study were complexed as EDTA chelates. Trace metal supplementation studies were initially carried out using a mixture of trace elements and Table 5.3 details the composition of the mixed trace element solution which was used. This solution will hereafter be referred to as the TM solution. Figure 5.3 further illustrates the relative composition of the metals, consisting mainly of iron (Fe) at 48.15% and cobalt (Co), manganese (Mn) and nickel (Ni) each at 16.05% of the total elements as mg/l. As well as metals the TM solution used also contained relatively small concentrations of selenium (Se) and boron (B), each at only 0.16% of the total elements as mg/l. It should be noted that the TM solution is a commercially available solution marketed at full-scale anaerobic digesters and that the composition of this solution was not designed specifically for this research project. However the bespoke single trace metal solutions for subsequent tests were designed specifically for this research project.

Table 5.3 Trace element composition of TM solution

Metal	**Concentration (mg/l)**
Fe	33.6
Co	11.2
Mn	11.2
Ni	11.2
Zn	1.12
Mo	1.12
B	0.112
Cu	0.112
Se	0.112

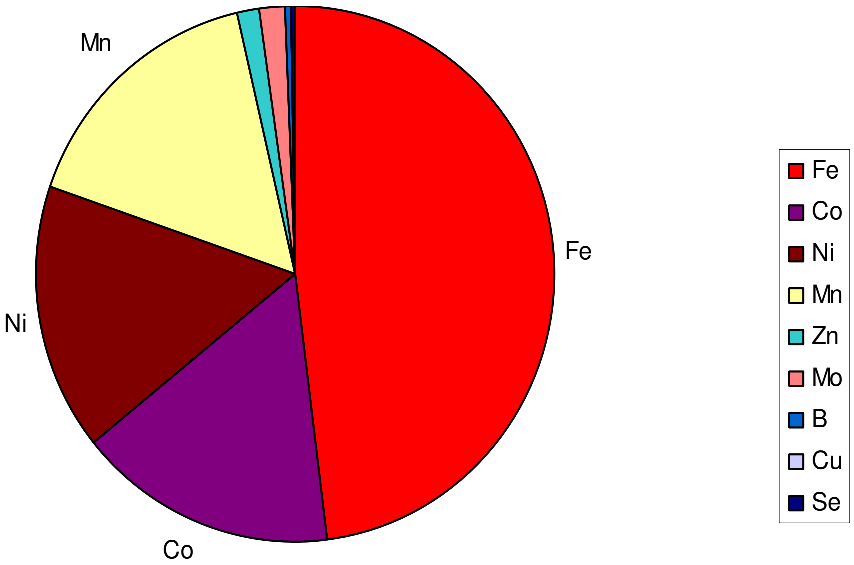


Figure 5.3 Trace element solution composition

5.2.2 Effect of TM Solution on V_{max}

In this study only assays which responded with a change in V_{max} that was beyond that of the standard deviation (SD) from the non-supplemented control plus the SD from supplemented assays were considered a positive result. Figure 5.4 is an example of cumulative methane curves produced in this study. The example in Figure 5.4 is from sample A0310; it shows the methane profile from assays supplemented with 20ml TM solution alongside the non-supplemented acetate-only control. As can be seen the methane profile for the supplemented assays indicates more methane was being produced even in the lag phase before the linear portion of the curve, however for the purposes of this only increases in the V_{max} are reported due to their applicability to the full scale digester via the ACN ratio. Sample A0310 responded with a 20% increase in the V_{max} after supplementation, which indicates the presence of a metal deficiency in digester A at the time of sampling. It is important to note the methodology used in this research is limited to investigating short term effects of metal supplementation. Therefore any increase in V_{max} is attributed to an increase in functional cofactors as explained in section 2.13, and methanogenic growth is considered only negligible given the timeframe of the recorded V_{max} and the slow growth rates reported for the prevalent methanogens.

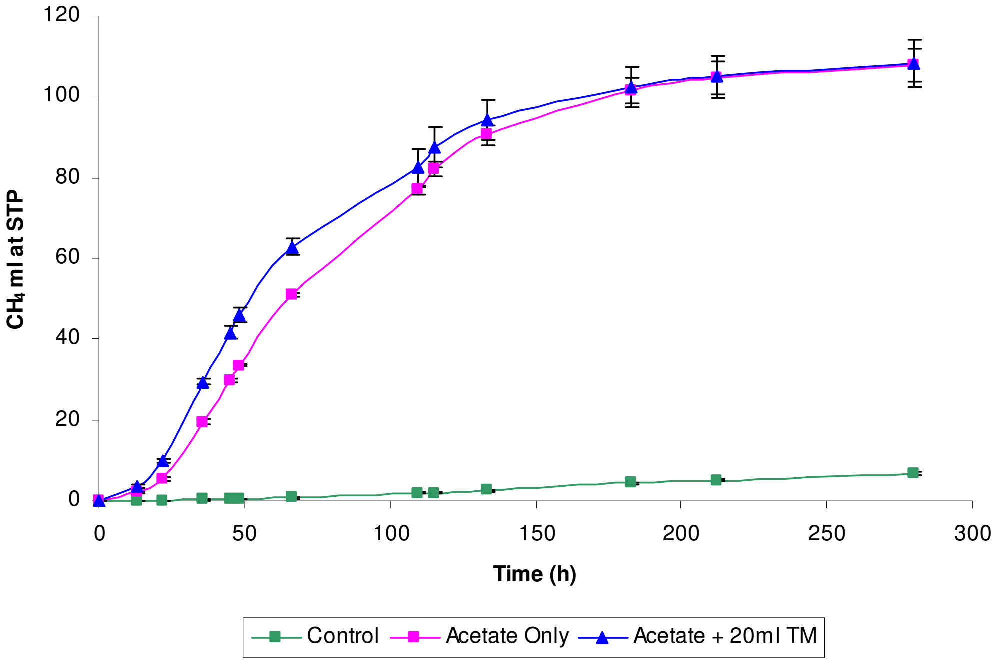


Figure 5.4 Cumulative methane curves- sample A0310

In metal supplementation assays within this study the TM solution was added at different concentrations and we see that the response found varied for different samples. Table 5.4 helps illustrate this point as it shows sample A0310 benefitted the most with 20ml of the TM solution whilst in sample C0310 it was 10ml which gave rise to the highest response. In C0310 it would appear the benefit with respect to an increased V_{max} is cancelled out with inhibitory effects at the higher concentrations. This highlights that understanding of metal requirements is important in order to maximise benefits from supplementation. The lower response at the higher concentration is likely to be due to one or more of the TM metals reaching the toxicity threshold; the point at which a metal goes beyond tolerance levels and becomes inhibitory. The fact Digester A in this case responded at a higher concentration may be due to it having a lower metal content relative to Digester C and thus a higher degree of deficiency. Metal content of all four digesters will be detailed and interpreted later in the chapter.

Table 5.4 Response to TM solution at different volumes

A0310	Acetate Only	Acetate + 10ml TM	Acetate + 15ml TM	Acetate + 20ml TM	Acetate + 25ml TM
V_{max} rate(ml/hr)	1.11	1.15	1.16	1.341	1.32
Stdev	0.03	0.07	0.08	0.12	0.09
RSD (%)	3.1	6.0	7.2	8.7	7.2
% change from acetate		2.98	3.85	**20.31**	**18.07**

C0310	Acetate Only	Acetate + 10ml TM	Acetate + 15ml TM	Acetate + 20ml TM	Acetate + 25ml TM
V_{max} rate(ml/hr)	1.46	1.67	1.48	1.547	1.47
Stdev	0.08	0.11	0.09	0.03	0.09
RSD (%)	5.8	6.9	6.2	2.2	5.9
% change from acetate		**14.46**	1.53	6.06	1.02

5.2.3 Effect of Cobalt only on V_{max}

Each sample tested responded differently to metal supplementation in terms of percentage change in the rate of methane production from acetate, and the TM supplement concentration it responded best to. After testing Digester A to D with the TM solution, individual metal supplementation studies were then carried out and it quickly became apparent that cobalt was very important in the response observed. It was found that when cobalt was supplemented individually, digesters would consistently respond with an increased V_{max} to a level equal or higher than that measured with the TM solution. Figure 5.5 illustrates this point well, with higher responses observed with cobalt only for Digester A, B and D; although the standard deviation between replicates suggests the change may only be marginally greater than that of the TM solution in A and B. Digester C has a slightly less positive response to cobalt supplementation, when compared to TM supplementation, although in both cases a marked increase in V_{max} occurs as a result of supplementation in comparison to the non-supplemented control SMA test. This would suggest that cobalt is either the most commonly deficient essential trace metal (essential for acetate conversion to methane) in sewage sludge digesters, or that cobalt is the only metal within the TM solution that is readily bioavailable within the timeframe of the SMA tests.

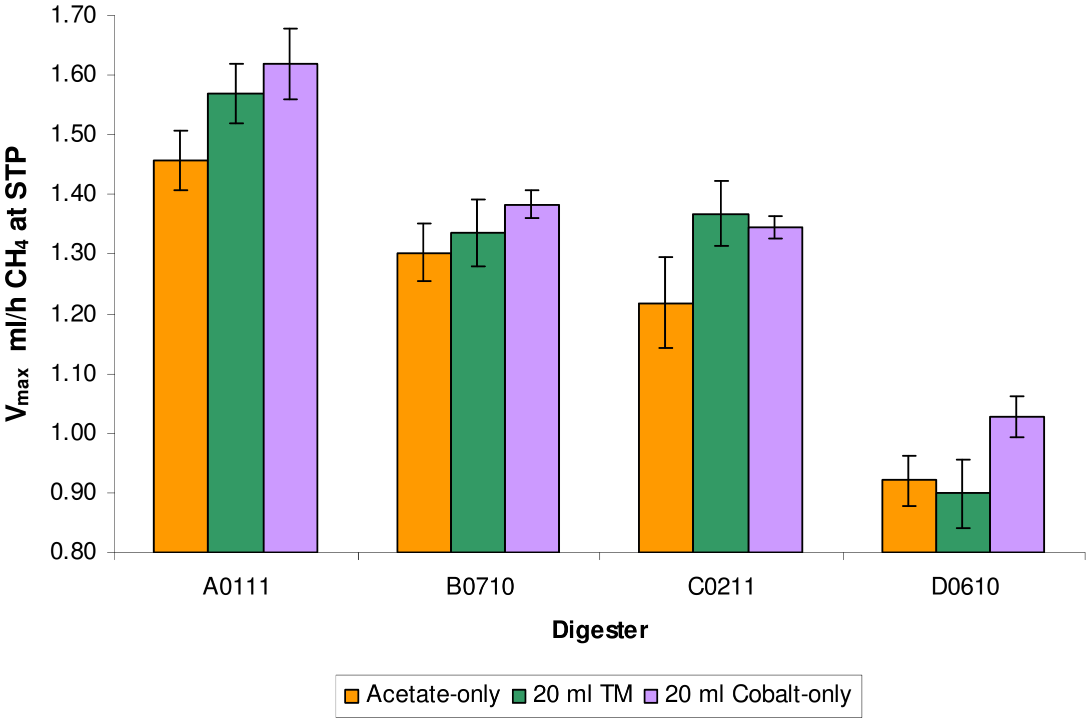


Figure 5.5 TM solution vs. cobalt-only

5.2.4 Potential of Metals to Increase V_{max}

In assays on sixteen samples collected from the four different digesters this study found trace metal supplementation to give a beneficial effect in terms of the V_{max} in eleven samples tested. This equates to 69% of occasions when the full scale digesters would have benefitted, at least with respect to an immediate increase in the rate of conversion of acetate to methane from trace metal supplementation. The response in each sample ranged from 5.7% to 20.3% increase in the V_{max}, with only increases above the two standard deviations for the replicates for that particular study being considered as positive results. The average response from metal supplementation in the 11 studies that benefitted from supplementation and thus were believed to be metal deficient was 10%.

As previously described the ACN ratio can be used to determine the maximum capacity of the digester. Table 5.5 details the digester loading conditions of the four digesters as detailed in section 4.1 and demonstrates how a 10% increase in the V_{max} would relate to each digesters. The table is drawn assuming the digester was at the ACN ratio of 1 before supplementation. Therefore the full scale digester acetoclastic methane production rate ($V_{plant\ acetate}$) shown is actually calculated using the average V_{max} found for the digesters in this study before supplementation as detailed in Table 5.2, converted to litres of CH_4 per litre of sludge per day. A 10% increase would cause the ratio to increase from 1 to 1.1, which accordingly translates into a 10% increase in the capacity of the digester that can support a 10% higher maximum organic loading (VS loading). The table shows how this proportionally equal change in the V_{max} has a different effect in each digester in terms of total additional CH_4 that can be potentially generated. The additional CH_4 is calculated by using the additional VS loading, assuming a COD to VS ratio of 1.5 and a conversion of COD to methane using the relationship of 0.35 m^3 CH_4 produced from a kg of COD (McCarty, 1964). In accordance with the different operational parameters its evident a 10% increase would result in the highest change in terms of extra methane potential from Digester B, with additional 285 m^3/d , and the lowest from Digester D, with additional 40 m^3/d of methane .

Table 5.5 ACN ratio and organic loading

	Dig A	**Dig B**	**Dig C**	**Dig D**
VS loading in feed (g/l/ d)	2.6	1.5	1.9	1.5
Digester loading (l/d)	525000	3618000	1050000	504000
Theoretical $V_{plant\ acetate}$ (l CH_4/l sludge. d)	0.91	0.98	0.89	0.72
$V_{max\ acetate}$ after a 10% increase (l CH_4/ l sludge. d)	1.00	1.08	0.98	0.79
ACN	1.1	1.1	1.1	1.1
Max Possible VS loading (g/l/d)	2.86	1.65	2.09	1.65
Additional VS loading (g/l/ d)	0.26	0.15	0.19	0.15
Total additional CH_4 (m^3/d)	**72**	**285**	**105**	**40**

Table 5.6 gives a breakdown of the maximum response found for each sample (including TM and cobalt-only assays) with the percentage change figures in bold for those studies with responses above the standard deviations. These details are further illustrated in Figure

5.5 where the V_{max} from the non-supplemented acetate only and those from the maximum observed after stimulation (including TM and Co-only) are presented with the corresponding standard deviations for each digester

Some trends can be seen from Table 5.6 and Figure 5.5. Digester A alone benefitted from supplementation for every sample collected, although the response seen did vary dramatically, with the lowest response being 5.3% and the highest being 20.3%. This indicates digester A was the most commonly metal deficient and that the level of deficiency tended to vary. Digester C and D responded on some occasions only; indicating the digesters didn't always have metal deficiencies. Interestingly, Digester B was the only digester not to respond to the TM solution but did express a stimulation of 6.3% with cobalt only. Digester B's response profile would suggest it is the least commonly deficient in metals. Metal content of all four digesters will be detailed later in the chapter. Digester D on the other hand was found to display an inhibitory effect to metal supplementation; interestingly the V_{max} was reduced by 19.3% in response to supplementation with cobalt-only. The inhibitory effect highlights the associated risk of heavy metal toxicity and the need to determine if a digester is metal deficient before dosing

Table 5.6 Response of SMA digestion tests to metal supplementation

Sample	V_{max} of Non-Supplemented Control	V_{max} Post-Supplementation	Change (%)
A0210	1.25	1.36	**8.49**
A0310	1.11	1.34	**20.31**
A0510	1.56	1.66	**6.55**
A0810	1.44	1.54	**7.17**
A0909	1.36	1.44	**5.73**
A0111	1.46	1.62	**11.12**
B0310	1.88	1.95	3.35
B0710	1.30	1.38	**6.26**
B1109	1.22	1.19	-2.07
C0310	1.46	1.67	**14.46**
C0810	1.45	1.47	1.33
C1109	1.20	1.26	5.56
C0211	1.22	1.37	**12.23**
D0610	0.92	1.03	**11.58**
D0710	1.43	1.30	-8.92
D1109	0.89	0.96	**7.57**

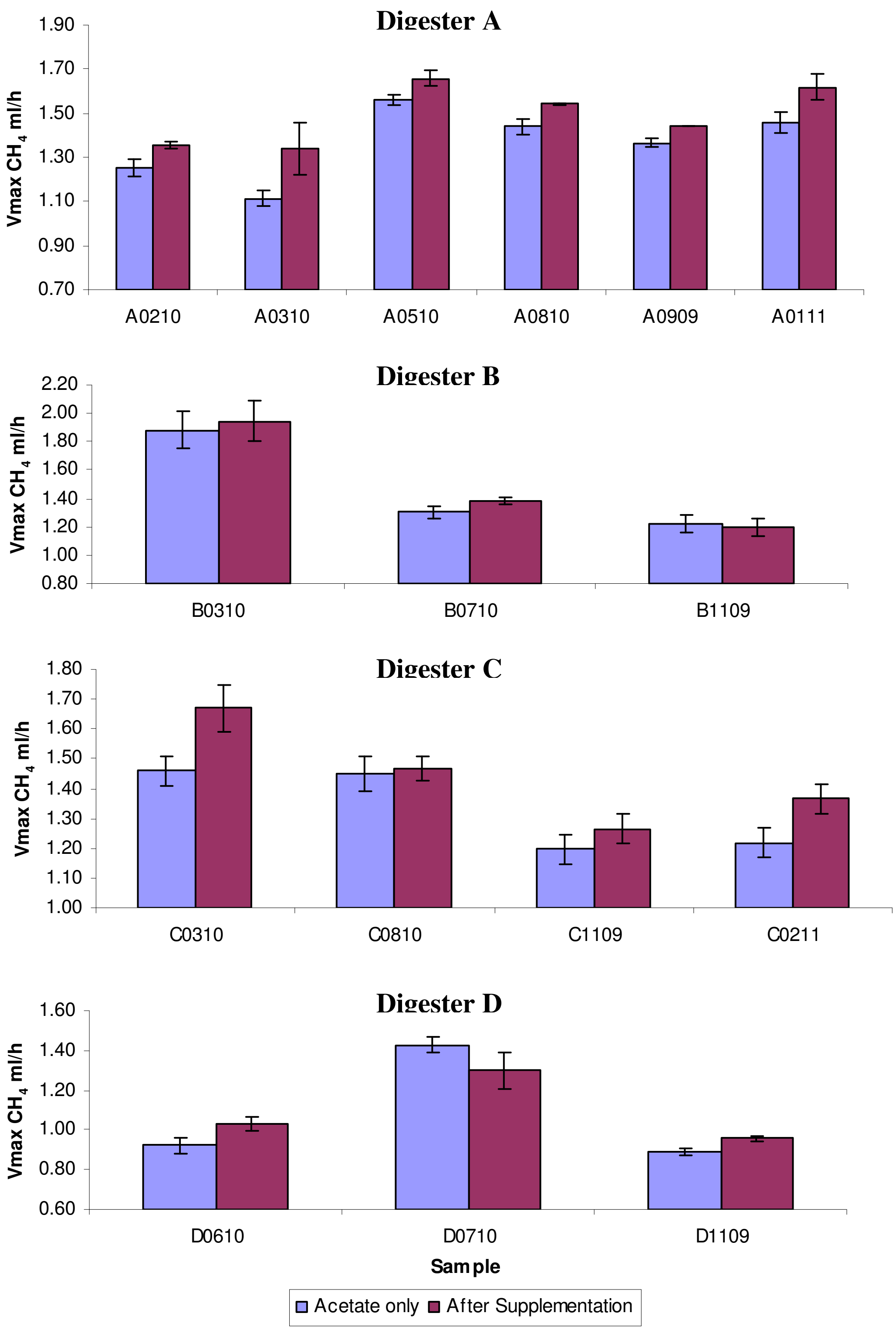


Figure 5.5 Response to metal supplementation

Out of sixteen studies highlighted in Table 5.6, seven samples were tested with cobalt only out of which five were found to respond positively. Also cobalt supplemented on its own induced a response that was equal to or higher than that observed from the combination TM solution, despite being at the same concentrations in both. The possibility of a significantly higher V_{max} from cobalt-only might be explained by metal uptake competition. If another metal is competing with the uptake of cobalt into the methanogenic cell then less cobalt would be taken up, meaning less of the requirement for cobalt is satisfied. Uptake competition of cobalt with nickel in anaerobic digesters has already been demonstrated to occur in Chapter 2 (literature review) and nickel is present in the TM solution. The observed effects of supplementing only cobalt, in comparison to the TM solution, suggest that cobalt is the most commonly deficient metal in sewage sludge digesters, and/or that other metals in the TM solution affect methanogenic cobalt uptake hence limiting the beneficial effect of cobalt, and/or that cobalt is the only metal within the TM solution that is bioavailable to the acetoclastic methanogens. Bioavailability of the EDTA-chelates will be discussed later in the chapter

5.3 Relationship between Metal Content and V_{max}

This section analyses the relationship between metals in the total and soluble fraction of the collected sludge samples and their relationship with the measured V_{max}, emphasising the importance of metal requirements.

5.3.1 Total Metal Content of Digesters

The digesters' total metal content alongside other key elements, as determined using acid digestion, is detailed in Tables 5.7 and 5.8 as an average for each element; sample by sample breakdown is found in appendix B. The data shown in Tables 5.7 and 5.8 includes standard deviations (SD) for each digester and is expressed as a fraction of the total solid content. Noticeably iron (Fe) levels are considerably higher in both Digester C and D, this is because they are both iron dosed for phosphate removal whereas Digester A and B are not. It can be seen that the standard deviation values for cobalt are greater than the average for all digesters; this is because in most samples cobalt was not found from ICP analysis. Undetected cobalt in samples means it was either not present or was present but below the detection limit of 1 mg/kg TS. Either way the data shows cobalt is very scarce in the four digesters, which is consistent with the strong response seen to cobalt supplementation. The averages in Table 5.8 for cobalt are as such due to one or two samples for each digester having detectable cobalt, which in some cases was erroneous (see appendix A), likely due to experimental error. Out of seven SMA digestion studies where cobalt was supplemented on its own, two did not respond, these were samples C0810 and D0710; interestingly neither of these digesters contained detectable quantities of cobalt. Therefore non-detected cobalt does not necessarily indicate cobalt requirement as cobalt requirements are likely to be relatively low.

In section 5.2.3 it was highlighted that Digester A alone responded to supplementation in all studies; this is consistent with the low concentrations of metals, such as Fe, Mn, Ni and Co, in this digester, all of which are supplemented in the mixed TM solution. Digester D on the other hand responded negatively at times, implying heavy metal toxicity as a result of TM solution supplementation, which is understandable given that it already has high iron levels and the highest nickel average. Iron in Digester D was present at an average of 52.2 g/kg TS in comparison to 10.7, 25.0 and 58.6 g/kg TS in Digester A, B and C respectively. Whereas the average nickel content in Digester D was 355 mg/kg TS, in comparison to 301, 286 and 310 mg/kg TS in Digester A, B and C respectively. In fact sample D0710 which responded negatively had a total nickel concentration of 522 mg/kg TS; the highest recorded from any sample, suggesting nickel toxicity as one possible cause of the inhibition in that particular study.

Table 5.7 Total fraction elements g/kg TS (1/2)

	Ca	Al	Na	Mg	P	Fe	K	S	Mn
Digester A	**29.69**	**7.83**	**7.69**	**4.55**	**22.08**	**10.69**	**4.00**	**9.20**	**0.25**
stdev	18.30	4.27	4.16	1.50	6.16	2.07	1.96	3.22	0.07
Digester B	**50.58**	**8.22**	**3.50**	**3.76**	**24.39**	**24.97**	**3.98**	**8.29**	**0.84**
stdev	25.93	2.48	1.09	0.54	7.38	3.26	0.39	2.48	0.21
Digester C	**49.26**	**3.95**	**13.76**	**3.58**	**28.92**	**58.59**	**4.86**	**10.97**	**0.34**
stdev	29.93	2.20	23.29	0.85	6.85	15.15	2.70	2.48	0.10
Digester D	**37.53**	**6.54**	**2.11**	**4.95**	**32.31**	**52.23**	**4.03**	**11.46**	**0.33**
stdev	5.98	7.80	2.51	2.97	6.10	1.21	1.10	0.71	0.18

Table 5.8 Total fraction elements mg/kg TS (2/2)

	Cu	Zn	Ni	Co	Mo	Se	Pb	B	Cd	Cr
Digester A	**561**	**624**	**301**	**2**	**2**	**2**	**124**	**16**	**22**	**213**
stdev	113	101	230	3	3	3	73	25	43	92
Digester B	**494**	**1898**	**286**	**5**	**4**	**5**	**163**	**104**	**2**	**288**
stdev	72	182	142	8	6	8	31	106	3	72
Digester C	**396**	**1202**	**310**	**41**	**1**	**3**	**138**	**351**	**2**	**88**
stdev	196	564	188	71	3	6	66	634	4	24
Digester D	**598**	**977**	**355**	**3**	**4**	**1**	**61**	**24**	**1**	**60**
stdev	208	450	257	5	6	1	80	42	2	52

5.3.2 Soluble Metal Content of Digesters

Tables 5.9 and 5.10 detail average concentrations of the elements found in the soluble fraction after filtering the supernatant through a 0.45 μm membrane; sample by sample breakdown of elements in the soluble fraction content is found in appendix C. Again cobalt was generally not found in the ICP analysis, meaning it's either not present or present but below the detection limit of 0.1mg/l. Digester A has the lowest average for nickel, at 1.75 mg/l, however the SD of 2.06 demonstrates the high relative variability in the digester over different sampling periods. This high relative SD values seen for all metals across digesters emphasises the variable nature of the sludge. Consistent with the total fraction metal data, Digester D had the highest average for nickel at 3.35 mg/l in the soluble fraction. However despite having high iron levels in the total fraction, relatively low amounts of iron are found in the soluble fraction. Digester D had 0.37mg/l average iron in the soluble fraction, which was the lowest amongst the four digesters.

Table 5.9 Elements in soluble fraction mg/l (1/2)

	Ca	K	Mg	Na	P	S	Fe	Ni	Zn
Digester A	**140.00**	**65.33**	**28.58**	**152.17**	**49.08**	**52.00**	**0.76**	**1.75**	**0.56**
stdev	181.11	26.96	6.32	32.99	19.75	59.44	0.86	2.06	0.48
Digester B	**162.50**	**47.17**	**12.67**	**89.83**	**45.83**	**9.50**	**1.10**	**2.20**	**16.03**
stdev	148.49	35.93	12.50	16.46	33.49	13.44	1.56	2.53	24.31
Digester C	**384.25**	**397.88**	**59.38**	**234.00**	**18.78**	**38.67**	**2.28**	**2.64**	**1.45**
stdev	476.58	666.69	71.23	341.06	20.61	56.27	2.67	2.03	0.84
Digester D	**55.50**	**65.83**	**25.33**	**41.50**	**1.60**	**17.00**	**0.37**	**3.35**	**0.57**
stdev	54.77	32.33	6.01	9.99	2.77	8.49	0.64	2.86	0.98

Table 5.10 Elements in soluble fraction mg/l (2/2)

	Co	**Cu**	**Cd**	**Al**	**Cr**	**B**	**Mn**	**Pb**	**Mo**	**Se**
Digester A	**0.00**	**0.35**	**0.01**	**0.33**	**0.02**	**0.16**	**0.04**	**0.24**	**0.01**	**0.00**
stdev	0.01	0.58	0.01	0.41	0.03	0.19	0.05	0.29	0.01	0.00
Digester B	**0.00**	**0.77**	**0.02**	**0.27**	**0.02**	**4.33**	**0.07**	**0.87**	**0.00**	**0.00**
stdev	0.00	1.08	0.03	0.47	0.04	1.04	0.12	0.78	0.00	0.00
Digester C	**0.00**	**1.06**	**0.01**	**0.09**	**0.02**	**19.50**	**0.05**	**0.35**	**0.00**	**0.01**
stdev	0.00	1.02	0.02	0.18	0.04	37.35	0.10	0.44	0.00	0.02
Digester D	**0.00**	**0.03**	**0.01**	**0.12**	**0.01**	**0.10**	**0.02**	**0.29**	**0.00**	**0.00**
stdev	0.00	0.05	0.02	0.20	0.02	0.17	0.03	0.51	0.00	0.00

5.3.3 Metals vs. V_{max}

In section 5.1.3 it was highlighted that the V_{max} is found to vary between digester sites as well as within the same digester over different sampling periods. Figure 5.6 plots metals from the total fraction, consisting of all the metals found in the TM solution (as outlined in section 5.2.1), against V_{max}. Interestingly Figure 5.6 reveals that overall there is no correlation with metal content in the total fraction and $V_{max,}$ however when looking at each digester, as denoted by the same icons, greater site specific relationships can be seen. It is understandable why such relationships across digesters may not exist, as many factors contribute to the acetoclastic methanogenic activity i.e. influent sludge profile, operational parameters such as SRT, presence of metals other than the ones measured and shown, presence of toxic compounds, and differences in the level of bioavailability of the metals. Notably from the figure Digester C represented by the green diamond icons appears to demonstrate a positive relationship with metal content and V_{max} whereas no clear relationship can be seen in the figure for Digester A and B, whilst Digester D (blue round icons) appears to have a negative relationship. A negative relationship for Digester D would be consistent with inhibited responses found to supplementation as it would indicate one or metals becoming inhibitory. Further data points would be required to establish the exact nature of the relationships suggested. Figure 5.7 shows the same metals but from the soluble fraction and again reveals no overall correlation with metal content and V_{max}. The absence of correlation amongst digesters indicates metal content cannot be used to predict the methanogenic activity; the activity found is likely to be related to a range of factors such as methanogenic populations and nutrient bioavailability.

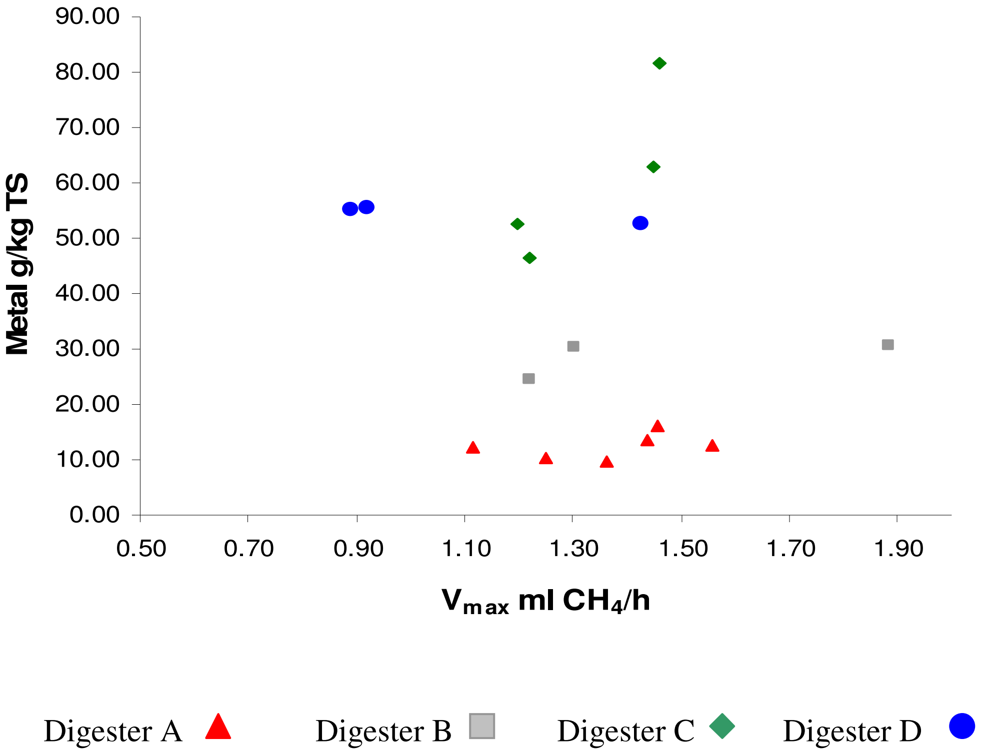


Figure 5.6 Total fraction metals vs. V_{max}

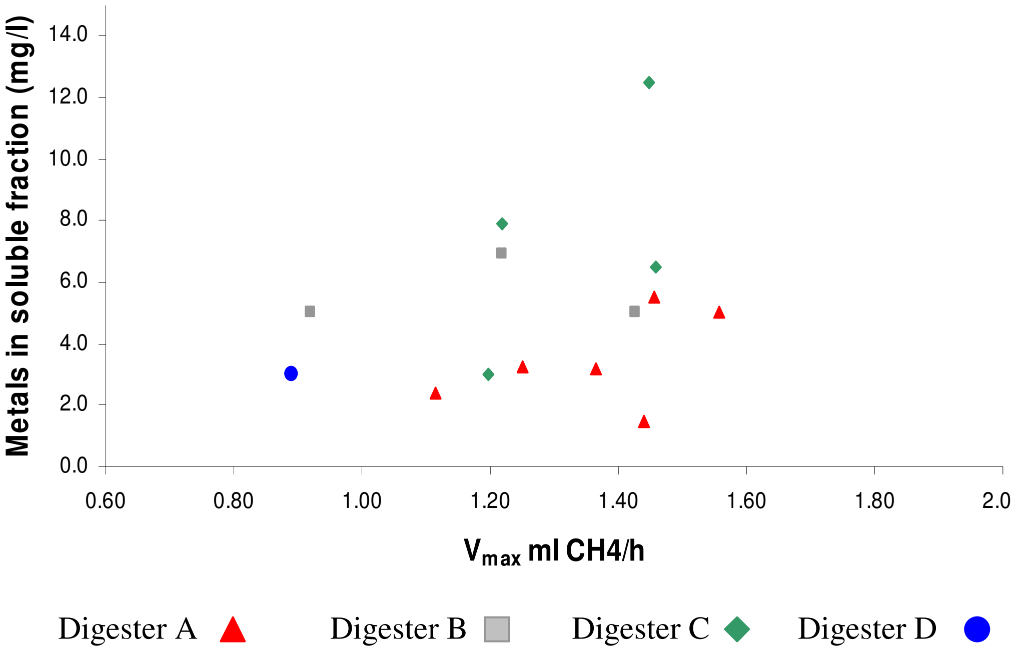


Figure 5.7 Soluble fraction metals vs. $V_{max.}$

5.3.4 Site Specific Metals vs. V_{max}

As seen in the previous section no correlation can be found amongst digesters for metal content with V_{max} however site specific relationships were suggested. Figures 5.8 to 5.16 further illustrate these relationships.

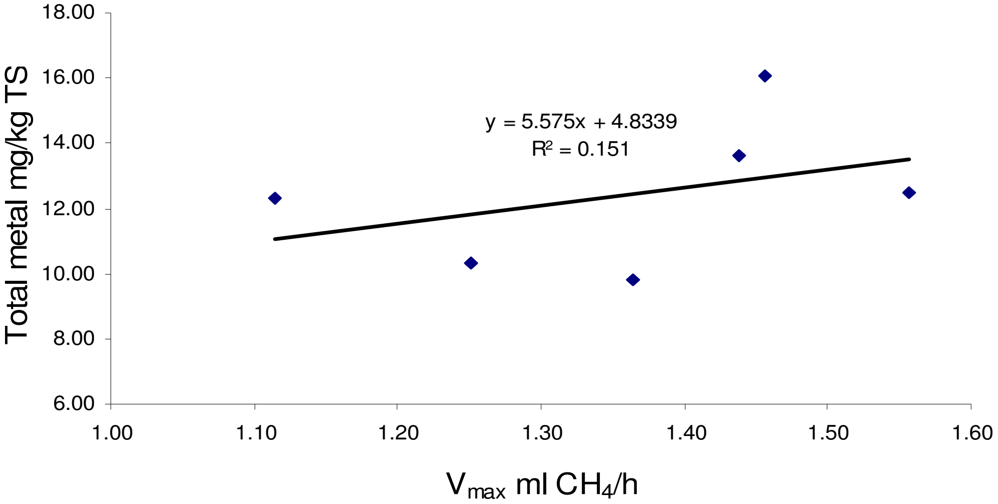


Figure 5.8 Digester A total fraction metals vs. V_{max}

Figure 5.8 plots metals from the total fraction, consisting of all 7 metals found in TM (as outlined in section 5.2.1), against V_{max} for Digester A. A weakly positive relationship of V_{max} with total metals in the digested sludge is suggested from this limited data set but this is not statistically significant.

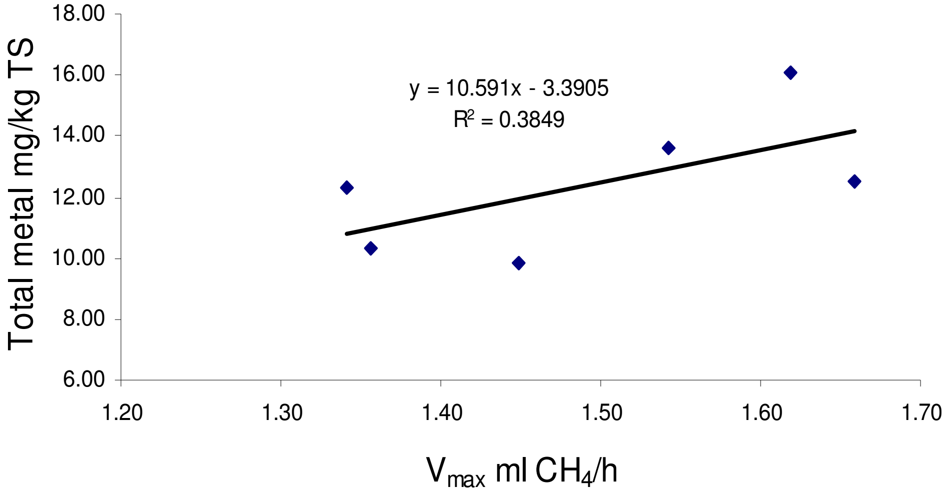


Figure 5.9 Digester A total fraction metals vs. post-supplementation V_{max}

Figure 5.9 plots metals from the total fraction, consisting of all 7 metals found in TM against the maximum V_{max} observed after supplementation for Digester A. Interestingly plotting the graph with the post-supplemented V_{max} rates reveals a better fit to the positive linear trend line. Student's t-test reveals the relationship is significant at the 80% level of confidence.

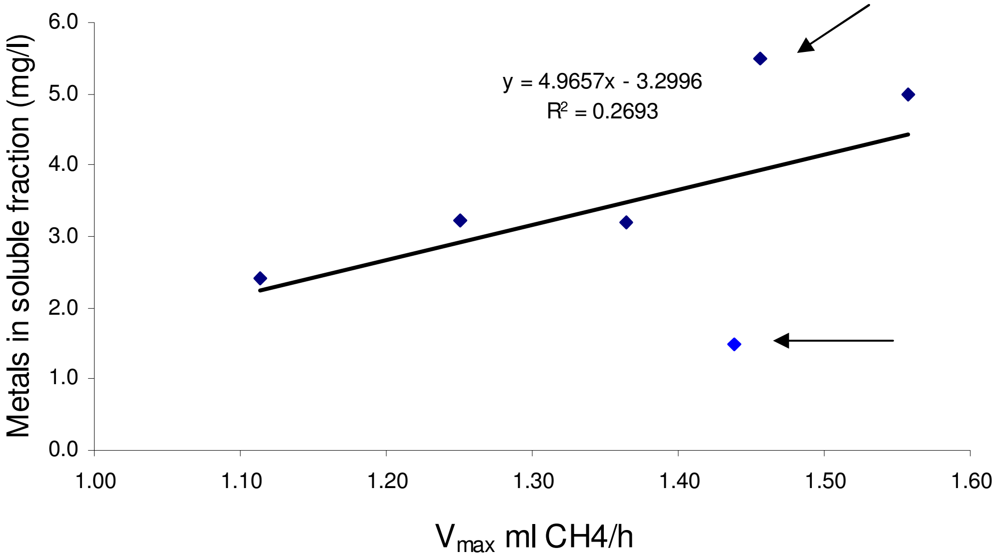


Figure 5.10 Digester A soluble fraction metals vs. V_{max}

Figure 5.10 plots metals from the soluble fraction, consisting of all 7 metals found in TM against V_{max} for Digester A. Again the linear trend line has a positive gradient indicating higher metals in soluble fraction corresponding with a higher observed V_{max}. Although The R^2 value however is low due to very distinct exceptions found as highlighted by the 2 arrows the positive relationship is arguably clearer compared to the total fraction data seen in Figure 5.8.

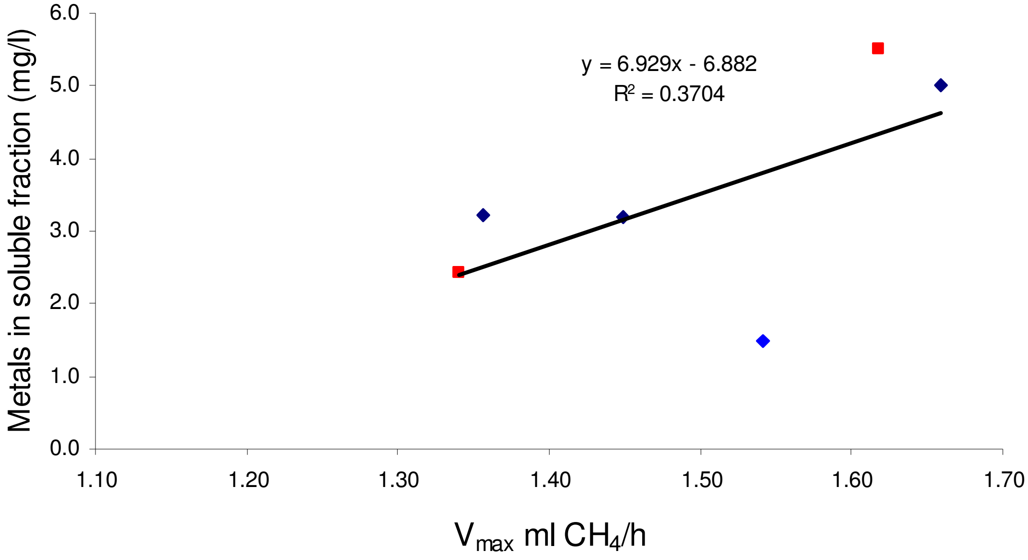


Figure 5.11 Digester A soluble fraction metals vs. Post Supplementation V_{max}

Figure 5.11 plots metals from the soluble fraction, consisting of all 7 metals found in TM against the maximum V_{max} observed after supplementation for Digester A. As with the total fraction sample, a better fit is seen with the post supplementation V_{max} data. The two points on the graph indicated by square markers signify the digester samples which gave the greatest response to trace metal supplementation in the study. The two points are on opposite ends of the trend line, indicating that a low metal content is not necessarily a good indicator of metal requirement or supplementation response.

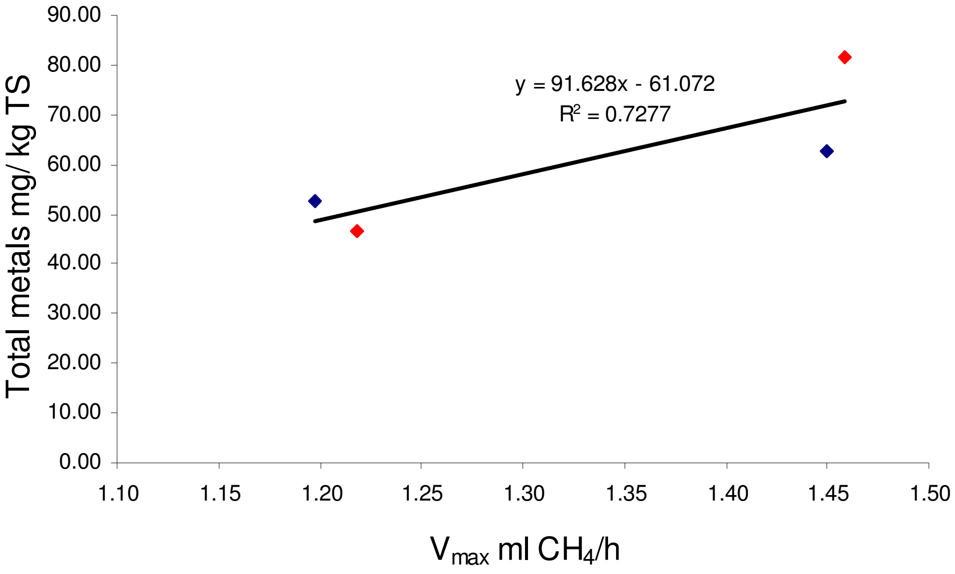


Figure 5.12 Digester C total fraction metals vs. V_{max}

Figure 5.12 plots metals from the total fraction, consisting of all 7 metals found in TM, against V_{max} for Digester C. For this digester, clustering of the data into two distinct areas of V_{max} makes is difficult to infer a relationship between total metals and V_{max}. There is a slight indication that higher V_{max} values were sometime associated with higher total metals values in the digester but a much wider spread of data is needed to confirm this. Overall, it should be noted that Digester C has a much higher total metal content than Digester A, 58.6 compared with 10.7 g/kg TS, which is mainly due to higher iron levels (iron-dosing for phosphate removal).

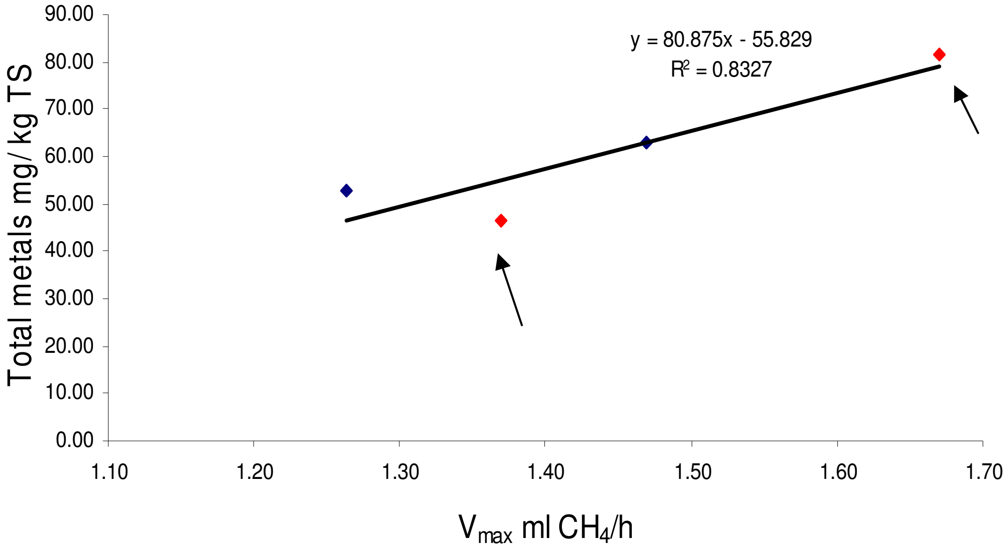


Figure 5.13 Digester C total fraction metals vs. Post Supplementation V_{max}

Figure 5.13 plots metals from the total fraction, consisting of all 7 metals found in TM, against the maximum V_{max} observed after supplementation for Digester C. As was seen in digester A a stronger relationship with post supplementation V_{max} data is seen again. The two arrows on the figure highlight the samples which responded significantly, one has high metal content and high V_{max} whilst the other had low metal content and a lower rate. Interestingly the sample which had the highest V_{max} and metal content still responded positively to supplementation, emphasising here again that the metal content alone is not a good indicator for metal requirement. The relationship is significant at the 90% confidence level. Higher number of data points would be required to show a relationship with a higher certainty.

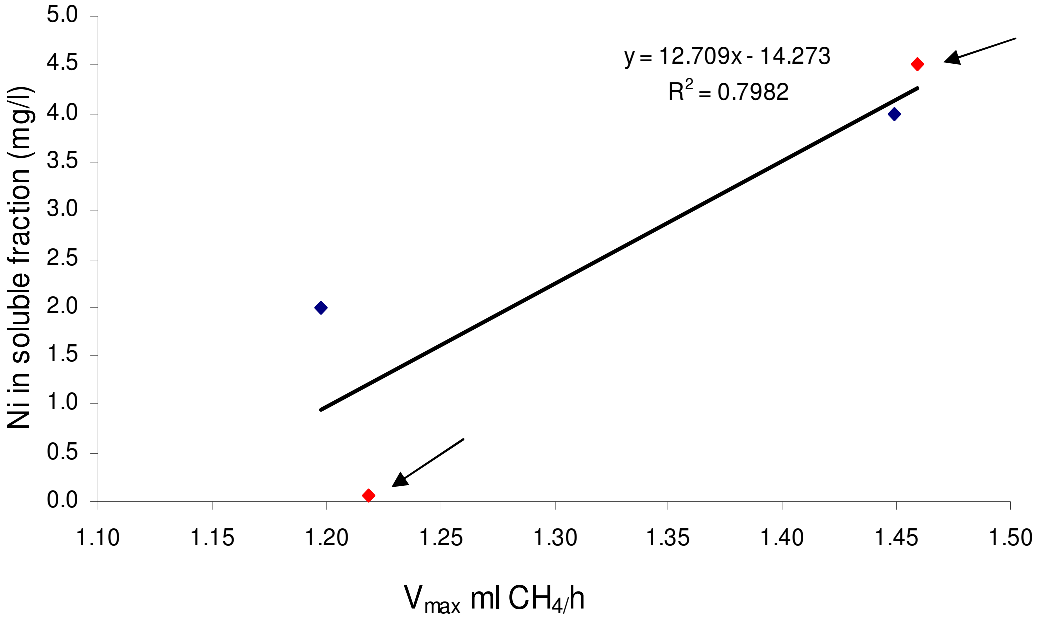


Figure 5.14 Digester C soluble fraction Ni vs. V_{max}

Figure 5.14 plots nickel from the soluble fraction against the V_{max} for Digester C. Again, clustering of the data points into two areas makes it difficult to infer a statistically significant relationship between soluble nickel in the digester and V_{max}, however, the data suggests an upward trend of soluble nickel and V_{max}, at concentrations of nickel below 4.5 mg/l. At higher concentrations, this is likely to become a negative relationship due to nickel toxicity. The two arrows on the figure again highlight the samples which responded significantly. The high nickel content sample still responded whereas a notably lower soluble nickel content sample did not. These results would either suggest that supplementation was either not related to nickel or that the relatively high soluble nickel sample was still deficient in nickel possibly due to lack of bioavailability of the already present metal. Bioavailability of the metal-EDTA chelates is discussed later in the chapter.

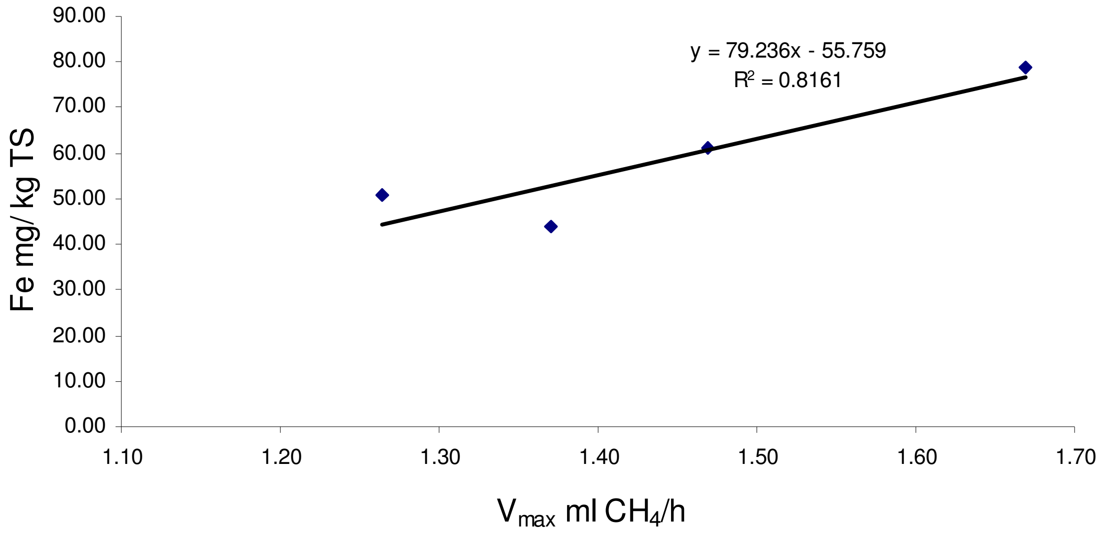


Figure 5.15 Digester C total fraction Fe vs. V_{max}

Figure 5.15 plots iron from the total fraction against the V_{max} for Digester C. Demonstrating a relatively strong relationship, which is interesting as Digester C is found to have the highest average iron content amongst the four digesters at 58.6 g/kg TS. This suggests that, despite relatively high levels of certain metals within the sludge, the metals may not be sufficiently bioavailable to the acetoclastic methanogens to allow acetoclastic methanogenesis to proceed at its maximum rate within the digester. The relationship is significant at the 90% confidence level. Digester C in Figure 5.5 was shown as the only digester where a slightly less positive response to cobalt supplementation was found, when compared to TM supplementation, although in both cases a marked increase in V_{max} occurs as a result of supplementation, in comparison to the non-supplemented control SMA test. Therefore the iron in TM solution may have been responsible for the apparent greater response.

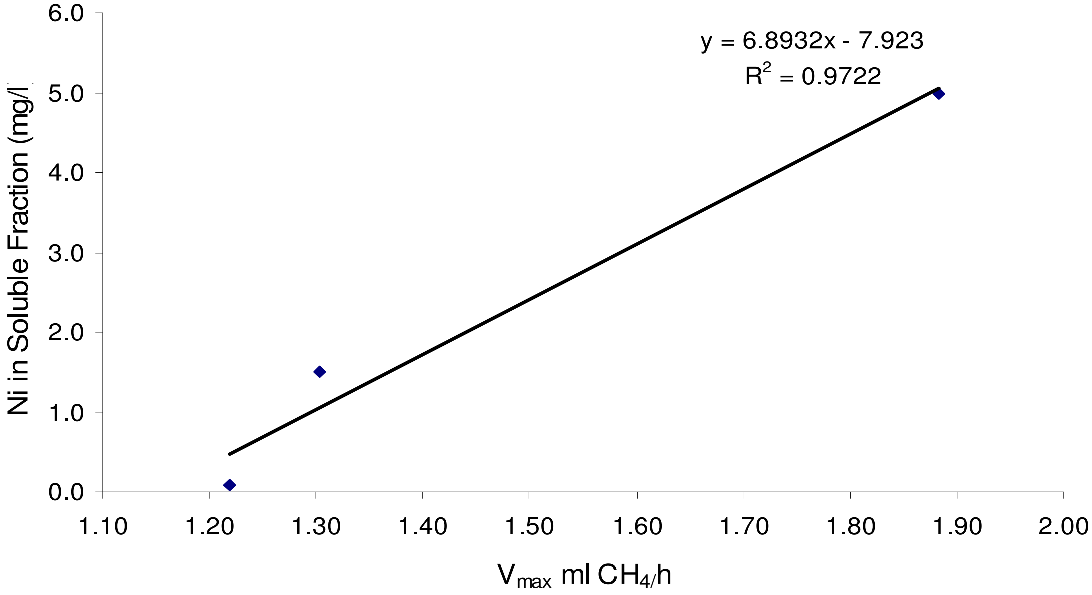


Figure 5.16 Digester B soluble fraction Ni vs. V_{max}

Figure 5.16 plots nickel from the soluble fraction against the V_{max} for Digester B. A lack of mid-range data points is problematic in inferring a relationship between soluble nickel and V_{max} for this digester, but it is strongly apparent that the highest V_{max} recorded for Digester B coincided with the highest soluble nickel concentrations in that digester. The relationship is only weakly significant (80% confidence level) due to the low number of data points, however, it is worth including because of the potential significance of soluble nickel to the V_{max} rate for a particular digester, which is worthy of further investigation.

Although further sample points are required to establish statistically significant correlations with a higher degree of confidence, Figures 5.8 to 5.16 help to demonstrate a positive relationship between the total and soluble fraction metals with V_{max} at a site specific level. A positive relationship emphasises the importance of metals for methanogenic activity and helps explain variations seen between different samples from the same digester. The reasons sample points within a digester may not fit exactly to a linear graph include; differences in levels of macronutrients, differences in levels of other metals (other than the ones measured and shown), presence of toxic compounds and differences in the bioavailability of the metals present. These factors are all able to influence the methanogenic activity. Interestingly the response of samples to supplementation is not always related to the metal content i.e. low metal content does not necessarily indicate a high response, as shown in Figure 5.11 where the highest responding samples lie on opposite ends of the trend line. Moreover, as shown in Figure 5.13, of the four data points illustrating the relationship between total metals and V_{max} for Digester C, the only two samples which responded positively to TM supplementation are seen at opposite ends of the total digester metal spectrum. These graphs emphasise the point that the metal content alone is not a good indicator for metal requirement.It was found that stronger relationships between metal content and activity are present when the maximum V_{max} post-supplementation is used to plot the data rather than the V_{max} from the non-supplemented acetate control. This is seen as supporting the mechanism proposed to be responsible for increases in V_{max} in this study: which in section 5.2.1 was stated as being attributed to an increase in functional cofactors whereas the effect of growth within the timescale was considered negligible. If the metals in these fractions are considered reflective of the longer term metal supply for the methanogens, then the observed results indicate the importance of these metals in increasing methanogenic populations and generally maintaining their health. The observed better fit from the post-supplementation V_{max} is seen as reflective of the methanogens susceptibility to the effects of acute (short-term) deficiencies of metals. An acute deficiency is seen as responsible for reduction in viable cofactors, without affecting population size, which would explain why subsequently after supplementation points are drawn closer to the positive trend lines. If growth was playing a valuable part in stimulation then points would be expected to be moving to the right without necessarily improving the trend line fit.

The importance of metal requirement is demonstrated by the positive relationships found between metals and methanogenic activity, at a site specific level. However the metal content alone is not a good indicator for metal requirement. Furthermore the inhibitory effect seen in assays from Digester D highlights the associated risk of heavy metal toxicity at high concentrations. Consequently there is a need to determine if a digester is metal deficient before dosing. In order to determine if a digester is metal deficient the study of the methanogenic population is considered important as it can help provide further insight into the digester condition and the requirement for metals.

5.4 Methanogenic Populations

This section analyses the relationship between the population of acetoclastic methanogens in the sludge sampled from Digesters A to D and their relationship with the observed V_{max} measured in the laboratory SMA tests. This section introduces the use of the population data alongside the V_{max} from the non-supplemented control as a tool to determine if a digester is metal deficient

5.4.1 Populations

Quantification PCR (qPCR) was carried out on a selected number of the collected sludge samples according to the methodology outlined in section 4.2.1. Table 5.11 reveals the average levels of the acetoclastic methanogens, *Methanosarcinaceae* (Msc) and *Methanosaetacea* (Mst) for each digester alongside the variation between the different sampling periods. Each digester was found to have members of Mst as the dominant acetoclastic methanogens, which is expected as it has a higher affinity than Msc for acetate, allowing it to prevail in the normal, low acetate conditions of a sewage sludge digester. Figures 5.17 and 5.18 illustrate this well where imaging from fluorescence in situ hybridisation (FISH) is shown for digester A. Both images show a sample hybridised with two different fluorophores, a red probe binding to all members of the archaea kingdom and a blue probe binding to the respective methanogen (Mst or Msc). The presence of the respective methanogen in each case would illicit the hybridisation of both probes revealing a magenta colour. It can be seen that the hybridisation of the Mst probe was more prevalent as illusrated by the higher levels of the magenta fluorescence in Figure 5.17, which is consistent with the qPCR results from the same samples.

Table 5.11 Average methanogenic populations

		Mst	Msc	Combined
Digester A n = 5	Average (cell no./ml)	2.13 x10^9	2.22 x10^8	2.35 x10^9
	RSD (%)	36.85	40.34	35.27
Digester B n = 3	Average (cell no./ml)	9.80 x10^8	1.49 x10^7	9.95 x10^8
	RSD (%)	11.85	109.05	13.18
Digester C n = 3	Average (cell no./ml)	6.86 x10^8	4.16 x10^7	7.28 x10^8
	RSD (%)	43.54	52.59	41.92
Digester D n = 3	Average (cell no./ml)	1.07 x10^9	3.30 x10^8	1.40 x10^9
	RSD (%)	34.81	143.27	32.91

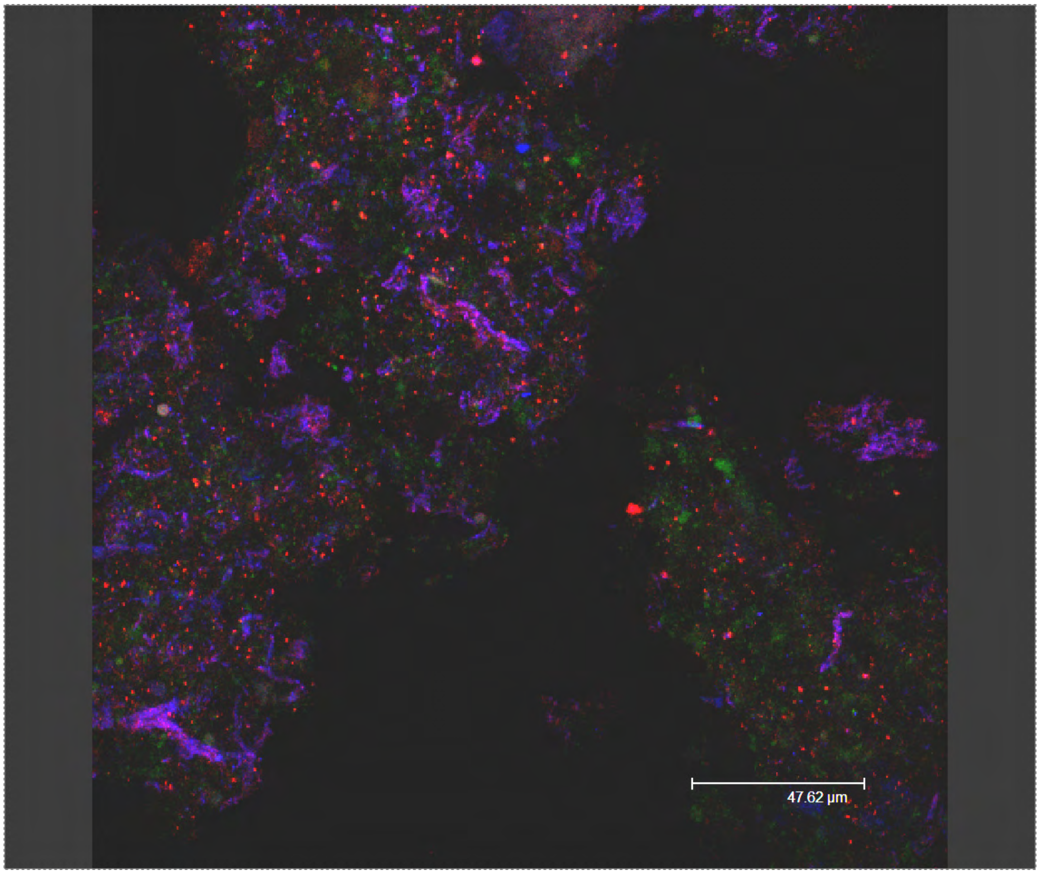


Figure 5.17 Prevalence of Mst Digester A

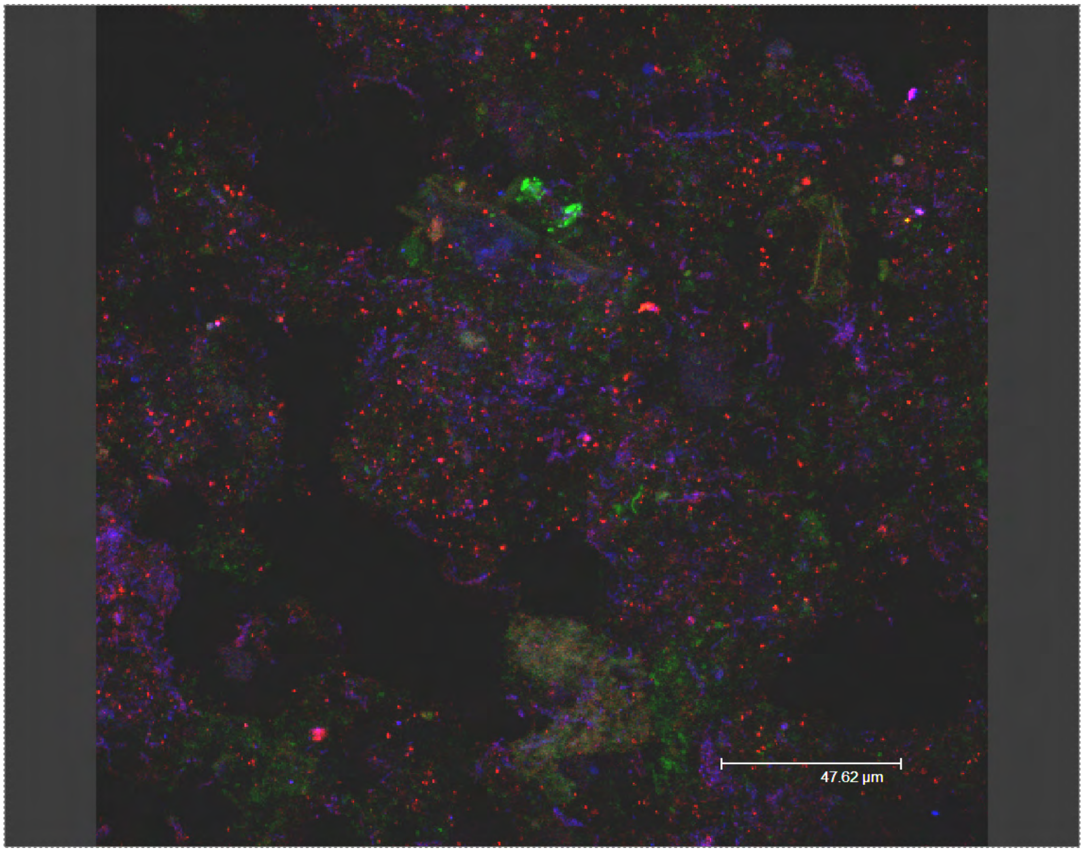


Figure 5.18 Prevalence of Msc Digester A

5.4.2 Population vs. V_{max}

Figure 5.19 plots the relationship between the combined acetoclastic methanogenic populations from the collected samples against their corresponding V_{max}. The relationship seen between the two variables reveals no correlation amongst the sites, as was the case for metals and V_{max}. Samples from the same digester, as displayed by the same icons, appear again to demonstrate a more site specific relationship. It can be seen from Table 5.11 the relative proportions found for Mst to Msc varied for different sites, however the samples from the same digester were found to follow a similar trend. The average Mst/Msc ratios were 10, 143, 18 and 61 for Digester A, B, C and D respectively. A higher ratio indicates a higher prevalence of Mst, interestingly both digesters B and D have the highest SRT, which corresponds with a higher prevalence of Mst; possibly due to longer periods of low acetate conditions at the higher SRT, which suit *Methanosaeta* species. As *Methanosaeta* and *Methanosarcina* species have different kinetic parameters as highlighted in section 2.2, different ratios found in different digesters helps explain why a different V_{max} can be observed even if the combined acetoclastic population values are very similar.

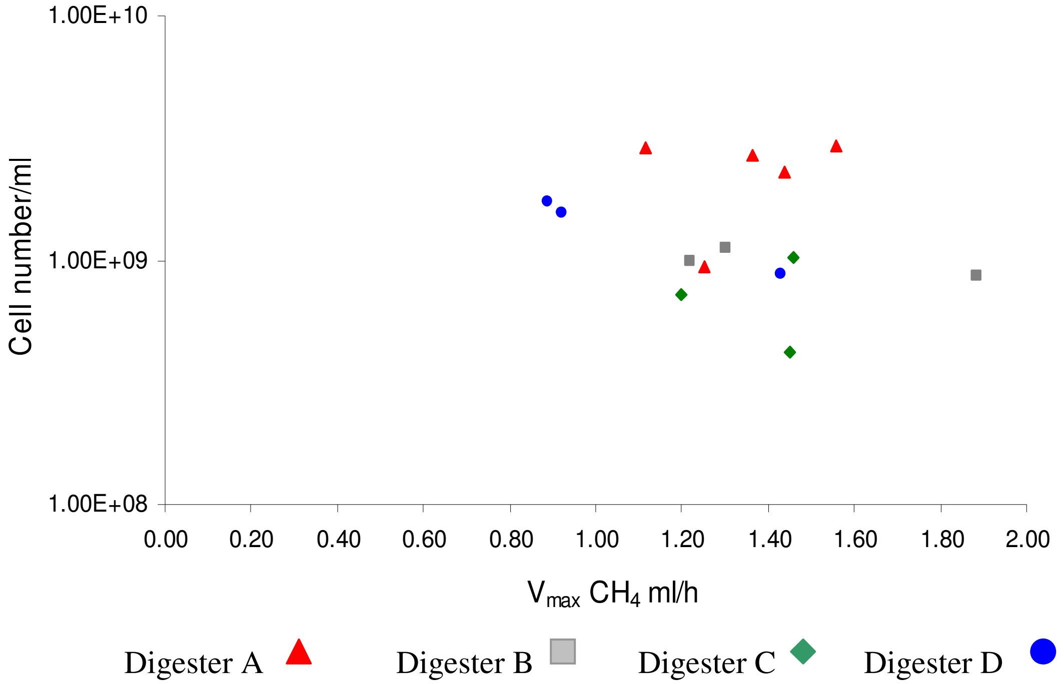


Figure 5.19 Methanogen populations vs. V_{max}

5.4.3 Digester A population vs. V_{max}

In order to understand site specific relationships, digester A was further analysed as it has the most data points. Figure 5.20 plots the acetoclastic methanogens against V_{max} for digester A and reveals an interesting relationship. Most points follow a positive relationship with an increase in activity related to an increase in the observed population, except for one quite notable exception, which is highlighted. The respective sample would have been expected to exhibit a significantly higher V_{max} on grounds of its high population and thus would have been expected to sit further right in the graph. Figure 5.21 plots the same data excluding the aforementioned sample, revealing a positive relationship which is significant at the 80% level of confidence using a student t-test statistical analysis. The sample point that didn't fit the curve was in fact sample A0310, which has previously been shown to have responded with a 20.3% increase in the V_{max} following supplementation with TM solution. Therefore the lower than expected V_{max} for this sample from Digester A corresponds to a strongly positive response of V_{max} to trace metal supplementation. This outcome seems to suggest that the A0310, while having a methanogenic population similar to samples that showed a more robust V_{max} in the SMA tests, was experiencing short-term limitation of bioavailable trace metals, which was limiting the rate of conversion of acetate to methane in the SMA test. Hence, when these were supplemented in the laboratory SMA test, the relatively numerous acetoclastic methanogens could respond strongly to the increased metal bioavailability, resulting in a large increase in V_{max}. Long-term trace metal limitation would most likely also be accompanied by a decrease in acetoclastic methanogenic numbers; the combination of a reasonable methanogenic population with a lower than expected V_{max} could be a good indicator of short term or intermittent trace metal limitation.

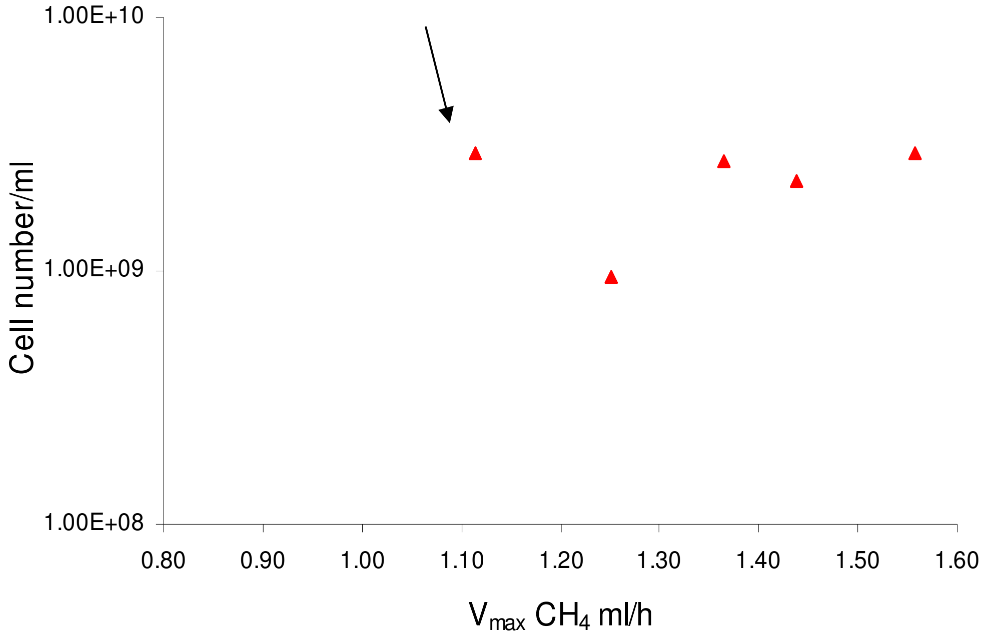


Figure 5.20 Digester A methanogen populations vs. V_{max}

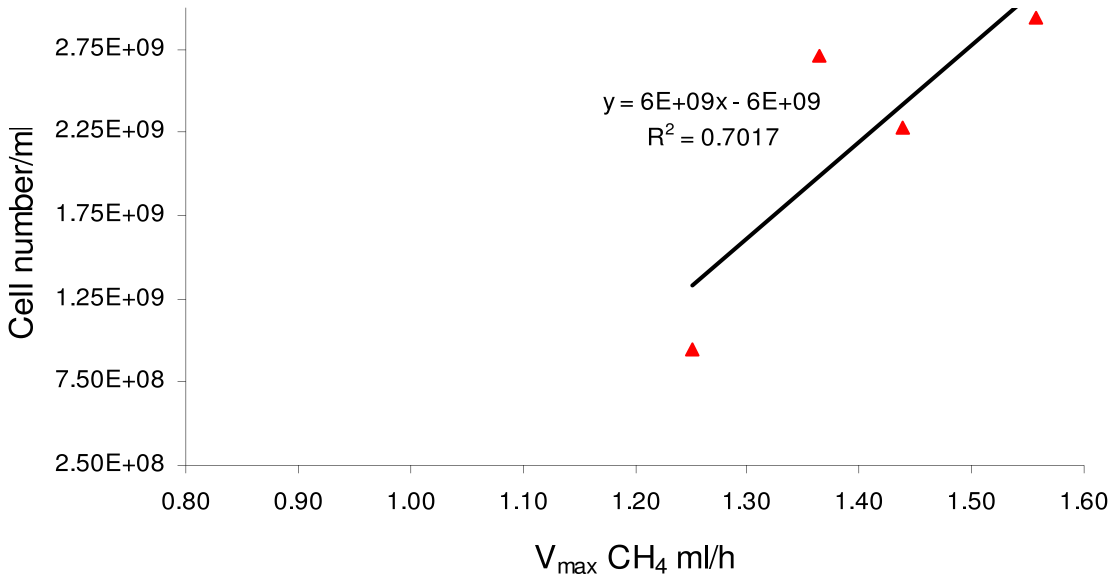
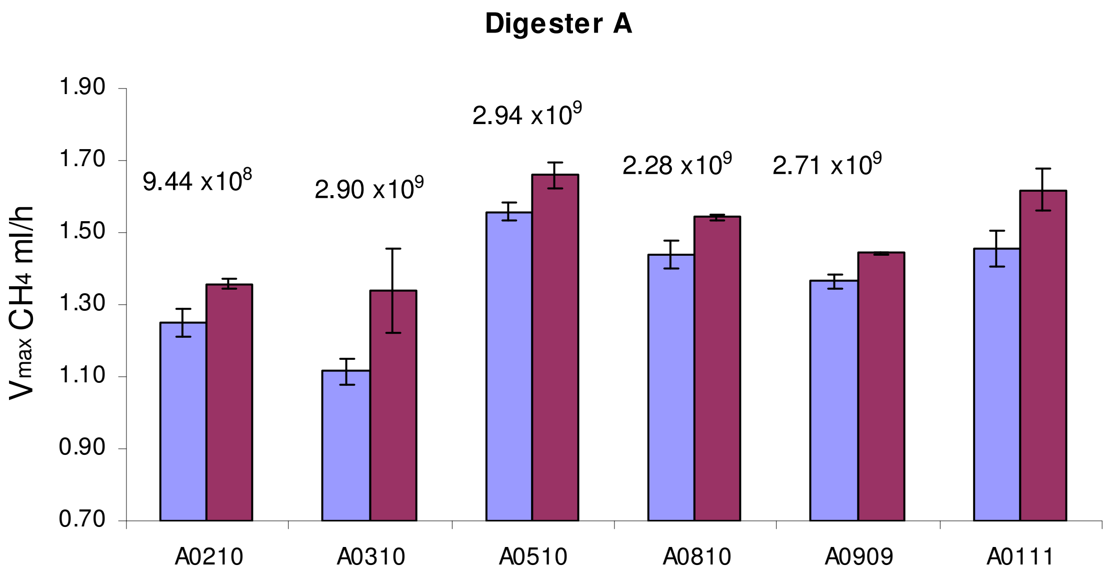
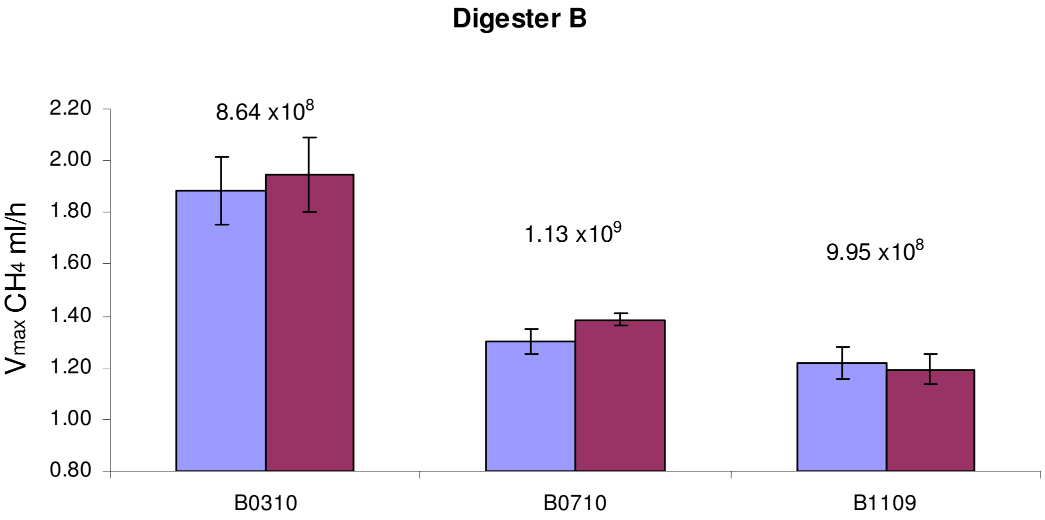


Figure 5.21 Digester A methanogen populations vs. V_{max} – excluding sample A0310

5.4.4 Response vs. Population

Figure 5.22 presents the different V_{max} values observed for each digester in the acetate only control and the maximum observed after stimulation as previously shown in Figure 5.5. Additionally Figure 5.22 also includes the combined population of acetoclastic methanogens for each of the samples qPCR was carried out for. Digester A gave a positive response in all samples and as illustrated in the previous section sample A0310 had a lower than expected V_{max} given its population levels and responded greatly, in this manner it is seen as possibly a good indicator for metal requirement. Both *Methanosaeta* and *Methanosarcina* species are responsible for the acetoclastic methanogenic activity found and as the ratios between Mst and Msc are similar in samples from the same digester, the combined acetoclastic population is shown for each sample.


Digester A
V_{max} CH_4 ml/h
1.90
1.70
1.50
1.30
1.10
0.90
0.70
9.44 x10^8
2.90 x10^9
2.94 x10^9
2.28 x10^9
2.71 x10^9
A0210
A0310
A0510
A0810
A0909
A0111


Digester B
V_{max} CH_4 ml/h
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
8.64 x10^8
1.13 x10^9
9.95 x10^8
B0310
B0710
B1109

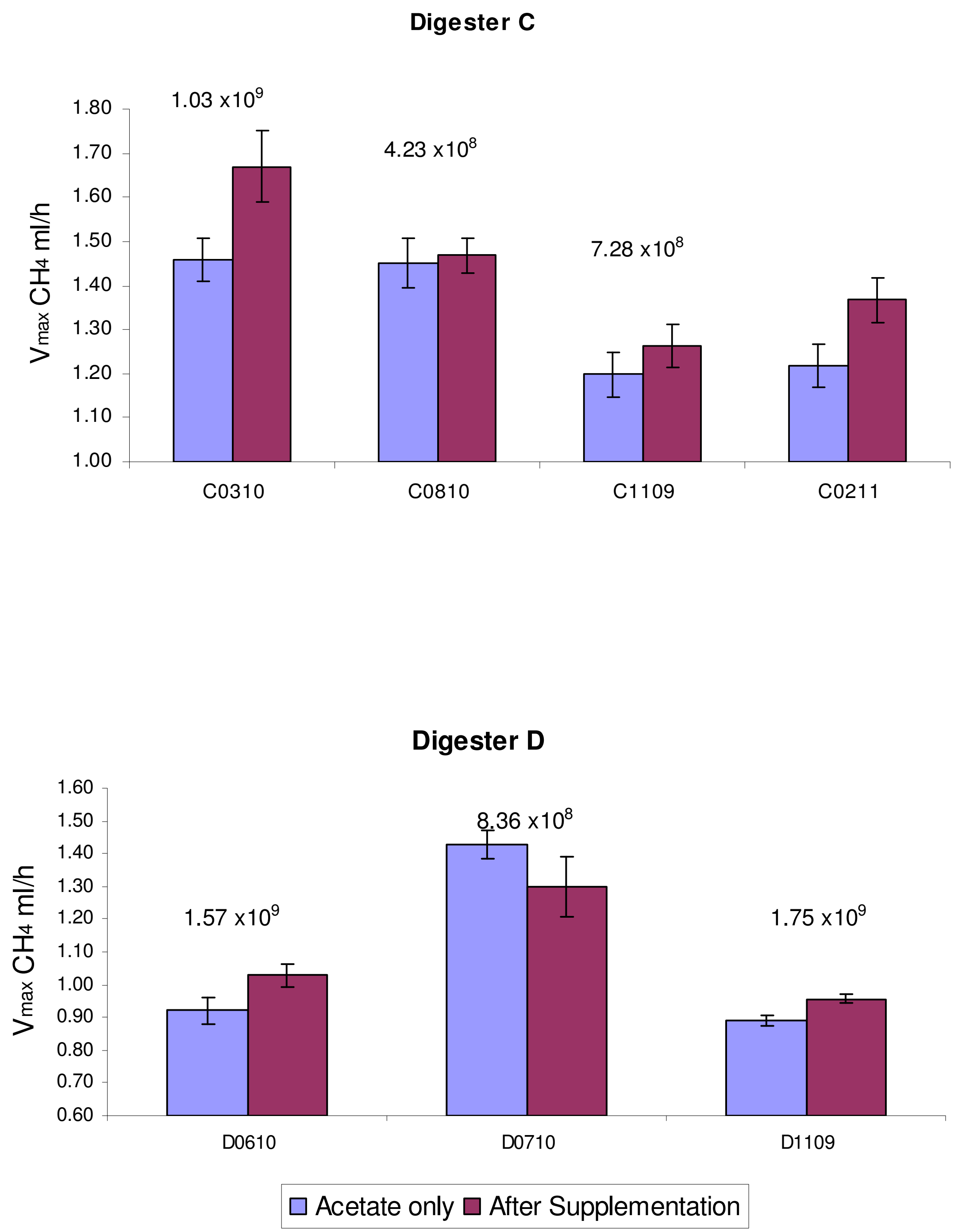


Figure 5.22 Digester responses and populations

Figure 5.22 reveals that digester C sample C0310 was found to have a substantially higher population than the others samples and yet its control V_{max} was no different from sample C0810 despite the 2.4 fold difference in population between the two samples. Consistent with the findings from digester A sample C0310 responded with a 14.5% increase, whereas C0810 did not respond. This relationship is also true in digester D where both samples D1109 and D0610 responded positively. Digester B only ever responded to cobalt-only stimulation in sample B0710, it can be seen that samples B1109 presented a control V_{max} of 1.22 ml CH_4/h in comparison to 1.88 CH_4/h from B0310, which is 65% higher. If using the same approach as in with digester A, it would be expected that sample B1109 would have responded positively as its population is not considered different, however it didn't. Sample C1109 had a population profile similar to that of C0810, in fact the combined acetoclastic population level was found to be higher, for the purposes of reporting with high degree of confidence, only differences of at least two fold were considered as actually different (explained in section 4.2.3). Therefore sample C1109 would have been expected to display a V_{max} similar to that of C0810 but yet it was 17% lower. Although a response of 5.6% was seen it was within the standard deviations of the two sets of replicates and therefore the result was not considered a positive response.

A lower than expected V_{max} for a given sites combined acetoclastic methanogen population is found to correspond with a strong response from supplementation in Digesters A and C. However in accordance with this both samples C1109 and B1109 would also have been expected to respond. This apparent lack of response can be explained by one or more of the following reasons; the samples were deficient in metals or nutrients other than those supplemented, the samples were not metal deficient but rather under toxic shock, or the metals supplied were not bioavailable to the methanogens. Bioavailability of the metal-EDTA chelates is discussed in the following sections.

5.4.5 Volatile Solid Content as an Indicator of Methanogenic Population

In section 5.1.2 it was stated that the volatile solid content was used as an indicator for the biomass. The VS content was kept constant to reduce variability in the methanogen levels and in accordance with the fixed VS content of 0.5g an acetate concentration was selected to ensure CH_4 production was not limited by the substrate. After quantifying the methanogenic populations using qPCR the benefit of fixing the VS was then analysed. Table 5.12 details the variation seen in the total acetoclastic methanogens for each site between different samples. The actual variation was determined after multiplying the population in the collected sludge samples (cell no./ml) by the corresponding ml of sludge used for that study to achieve 0.5 g VS. Whereas the hypothetical variations are those which would have been seen given the population (cell no./ml) if a fixed volume of 36ml digester sludge was used as the inoculum. The 36ml volume was chosen as it represents the equivalent volume used in the study by Conklin *et al*. (2008) upscaled for a 100ml working sample volume.

Table 5.12 Actual vs. hypothetical variations in acetoclastic population

Sample	Actual Variations	Hypothetical Variations
Digester A n = 5	46.33	35.27
Digester B n = 3	38.74	41.92
Digester C n = 3	9.98	13.18
Digester D n = 3	37.34	32.91
Average	**33.09**	**30.81**

Interestingly Table 5.12 reveals there was no overall benefit in fixing the VS content. According to the data the variation would have been 2.3 % less if a fixed volume had been used however the difference is not considered significant given the variation between qPCR replicates was found to be at 10.0% (as detailed in section 4.2.3), therefore no difference can be inferred from the data and therefore no benefit can be demonstrated. The volatile solid fraction would have included organics that were not fully digested, as well as all microorganisms in the digester; therefore the finding that fixing the VS content is not demonstrated to reduce the variability in acetoclastic methanogen population is understandable.

5.5 Soluble Fraction Tracking

A varied response of biological systems, including anaerobic digesters, to metal- EDTA chelates is reported in the literature (as detailed in section 2.13) with many studies reporting them to be soluble but not bioavailable. This section investigates the evolution of metals in the soluble phase of the assays with time, exploring the bioavailability of the metal-EDTA chelates.

5.5.1 Soluble Fraction Tracking

In order to explore the bioavailability of the metal-EDTA chelates being supplemented, SMA batch tests with a large number of replicates were carried out (as described in section 4.1.7). The samples were destroyed at timed intervals and changes in the soluble fraction were analysed. It is important to note that metals being removed from the soluble fraction are not necessarily being taken up by the methanogens or any other microorganism. Removal of the metal from the soluble fraction may also be due to changes in the metals speciation, which can transfer the metal into the solid fraction for examples as precipitates, co-precipitates, adsorbates or organometallic complexes (as highlighted in Table 2.5). Figure 5.23 shows the soluble fraction evolution of Fe, Co and Ni over time in sample A0810. The sample A0810 responded with a 7.1% increase in the V_{max} after supplementation with 20ml TM solution and similarly with 6.6% after supplementation with the equivalent concentration of cobalt on its own. Digester A, as previously highlighted in section 5.3, has relatively low iron in the total and soluble fractions and the lowest soluble nickel average of 1.75mg/l. Sample A0810 was also found not to have any cobalt detected in both fractions (total and soluble). Given digester A's consistent response to supplementation in this study and its relatively low metal concentrations it may be inferred that it has deficiencies in more than one metal. Figure 5.23 reveals that in assays from sample A0810 only cobalt levels decreased, nickel remained unchanged and interestingly iron levels were increasing as the assays continued. This would appear to suggest that among the three metals only cobalt was being taken up.

The soluble fraction tracking studies provide fascinating insight into the dynamics of metal-EDTA chelate bioavailability. As has been previously highlighted chelation by a ligand such as EDTA is able to affect the availability of a metal to an organism; it will make the metal more available if the target organism has a metal binding component (essentially an uptake mechanism) with a higher stability constant than that of the ligand complex and less available if it doesn't (Callander and Barford, 1983). If it's assumed the sample is deficient in all three of the metals then it would suggest that only cobalt is bioavailable. This would explain why cobalt supplemented on its own had an equal response (within error) to assays where it was supplied as part of the TM solution. This is interesting as nickel is expected to be taken up competitively with cobalt, meaning via the same pathway. If nickel is not bioavailable then it may be explained by the fact Ni-EDTA has a higher stability constant than Co-EDTA under standard conditions (detailed in Table 2.7), which may be sufficiently different to make it non-bioavailable to the methanogens.

The observation of iron increasing helps demonstrate that the displacement of EDTA complexed metal may be occurring within the digester medium. It was highlighted earlier in the literature review that a ligand is able to scavenge metal ions from a complex, providing that it is sufficiently labile, if it can produce a complex with a higher stability constant (Snoeyink and Jenkins, 1980). This opens the question as to whether certain EDTA complexed metals are directly bioavailable or not, because it would mean non-bioavailable complexed metals can still illicit a response in cases where they are freed from the complex via displacement with a metal, which is able to produce a higher stability constant. Moreover this can provide sound reasoning as to why studies can report conflicting data for EDTA metals, as for certain metals the positive effect seen may have been indirect.

Although iron levels are gradually increasing in Figure 5.23, the iron in the medium is not thought to be responsible for the displacement of cobalt. The cation Co^{2+} forms a complex with EDTA that has a higher stability constant than Fe^{2+}; therefore displacement between the two cations is unlikely. Figure 5.24 illustrates that iron levels were found not be increasing at comparative levels in the assays with cobalt-only, which supports the aforementioned point.

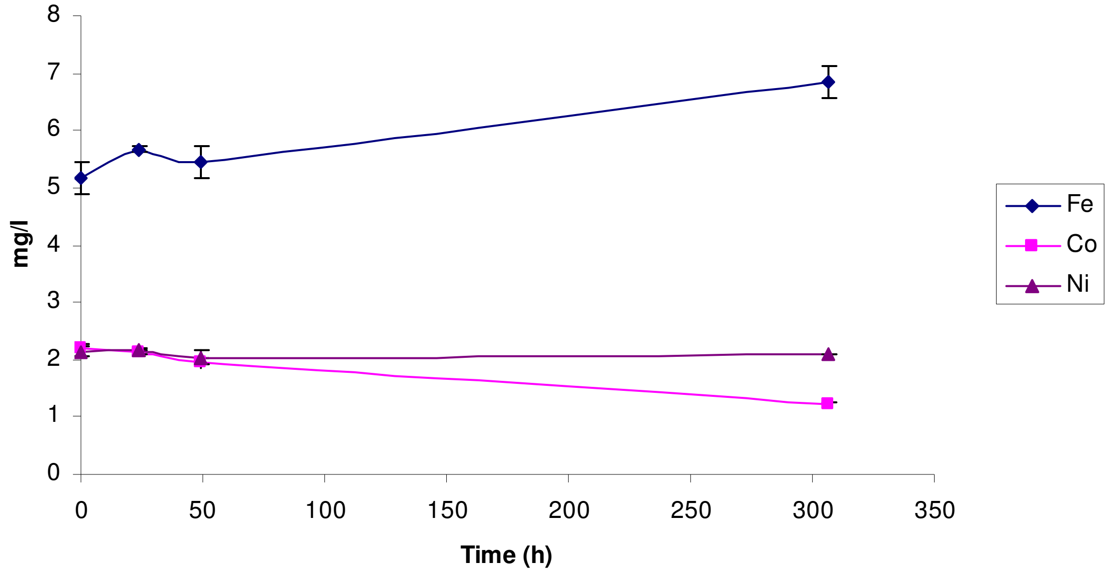


Figure 5.23 Soluble metals tracking after TM supplementation-A0810

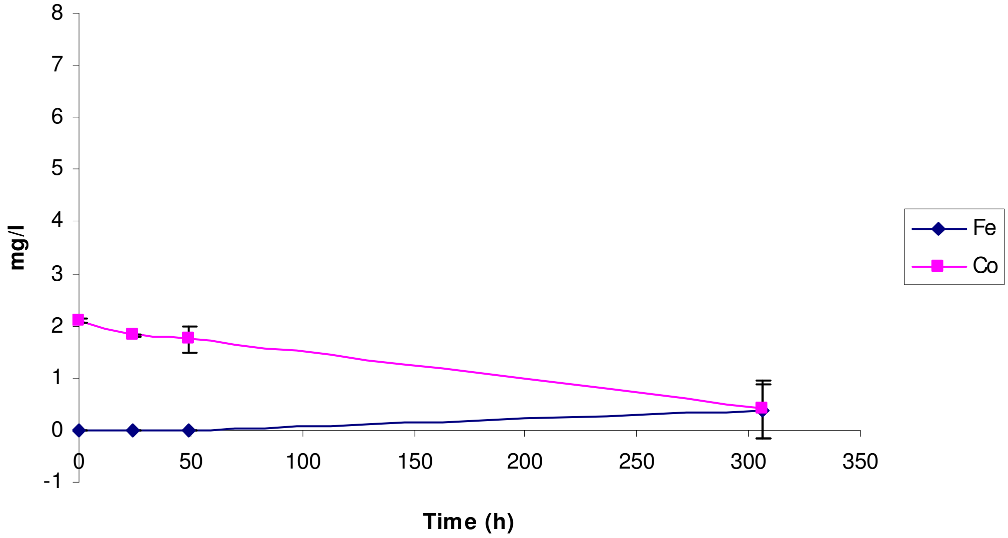


Figure 5.24 Soluble metals tracking after cobalt supplementation- A0810

Figure 5.25 shows the soluble phase evolution for sample B0710. Sample B0710 did not respond to TM solution supplementation but displayed a 6.3% increase after supplementation of cobalt on its own. Digester B has the lowest average nickel content in the total fraction at 286 mg/kg TS (as seen in Table 5.7), this coupled with data seen in Figure 5.16, where its strongly apparent that the highest V_{max} recorded for Digester B coincided with the highest soluble nickel concentrations, help to demonstrate the importance of nickel in this digester. Therefore the fact a response was only seen when the assays were supplemented cobalt-only raises questions about the bioavailability of EDTA-Ni. Interestingly the soluble phase tracking experiment reveals that the nickel concentration did come down by approximately 50% by end of study. This observation alongside the no response seen may be explained by just a change in nickels speciation, and/or the likelihood of uptake from other microorganisms found within the digester and/or slow uptake by the acetoclastic methanogens with the response requiring a longer time to be manifested, which would be consistent with the very low change at the start.

It is interesting to note that also unlike sample A0810 Figure 5.25 shows iron levels to be gradually decreasing in B0710 after TM solution supplementation. However Figure 5.26 shows the results from assays supplemented cobalt-only and demonstrates iron to be following a close to proportional increase with the decreasing cobalt, iron increased from 0.36 mg/l to 2.67mg/l, whereas cobalt decreased from 4.2mg/l to 0.42 mg/l. It was previously suggested that displacement between the two irons is unlikely. Although Figure 5.26 seems contrary to this, the increase in iron may represent an indirect displacement, in which cobalt is taken up by microorganisms before free EDTA complexes with another available cation; in this case possibly iron.

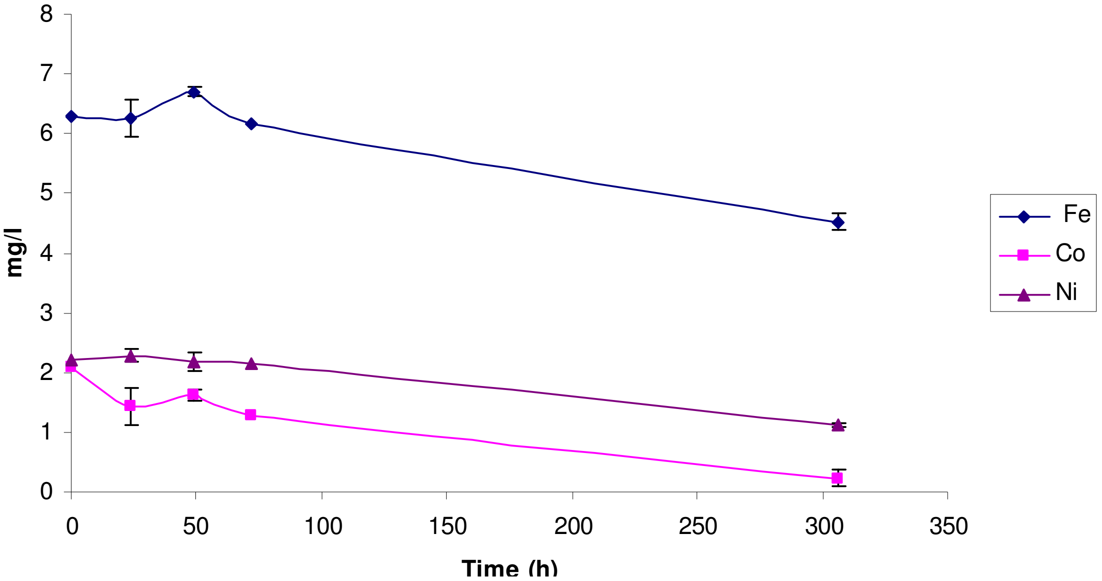


Figure 5.25 Soluble metals tracking after TM supplementation- B0710

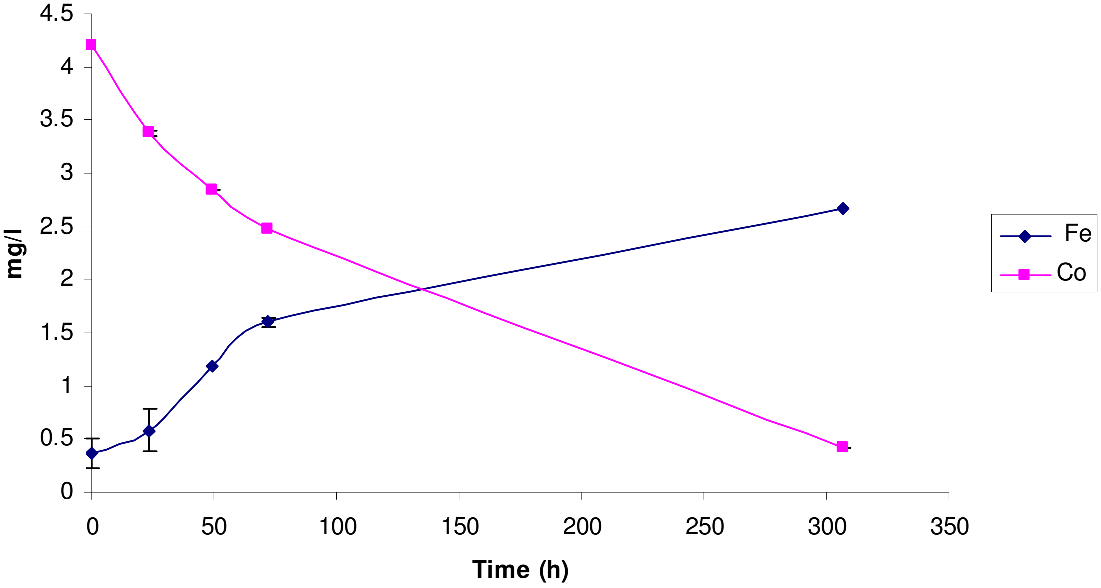


Figure 5.26 Soluble metals tracking after cobalt supplementation- B0710

Figures 5.27 and 5.28 show the soluble phase evolution for sample C0810. Sample C0810 did not respond to either the TM solution or cobalt on its own. In section 5.4.4 sample C0810 is used as a reference for other samples which did and didn't respond due to its acetoclastic methanogen population/high V_{max} and corresponding no-response from supplementation. Thus the sample is accordingly not seen as deficient in metals. Never the less it can be seen that cobalt was still stripped out of the soluble phase in the assays, which may just be due to the metal changing its speciation as previously described. However other reasons for the decrease without any accompanying response include the likelihood of uptake from other microorganism found within the digester or even still by the acetoclastic methanogens themselves but the response requiring a longer time to be manifested. The nickel content stayed the same in sample C but iron levels increased both after TM supplementation and when cobalt-only was supplemented as illustrated in Figure 5.28.

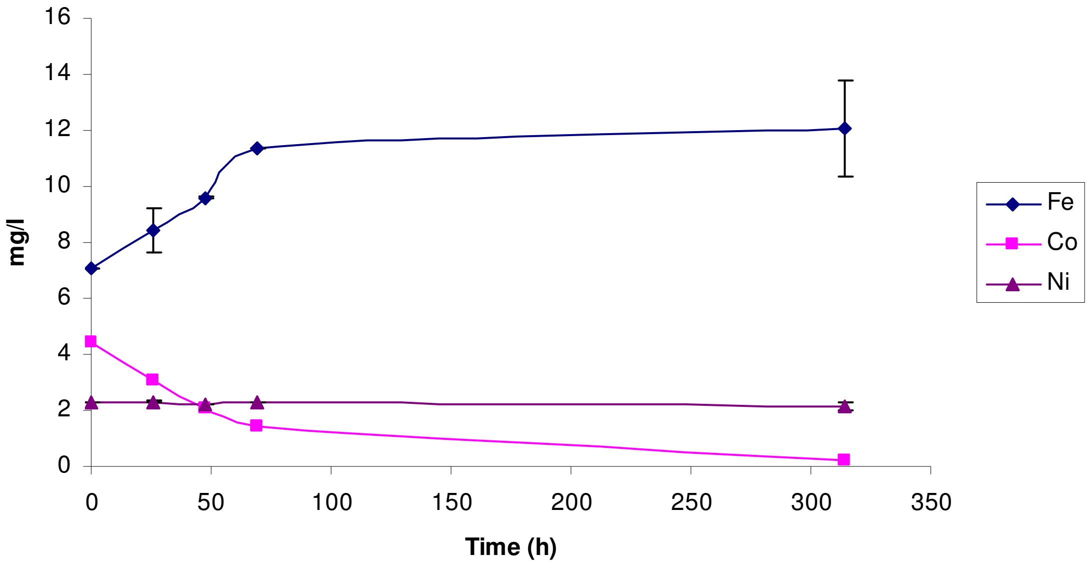


Figure 5.27 Soluble metals tracking after TM supplementation-C0810

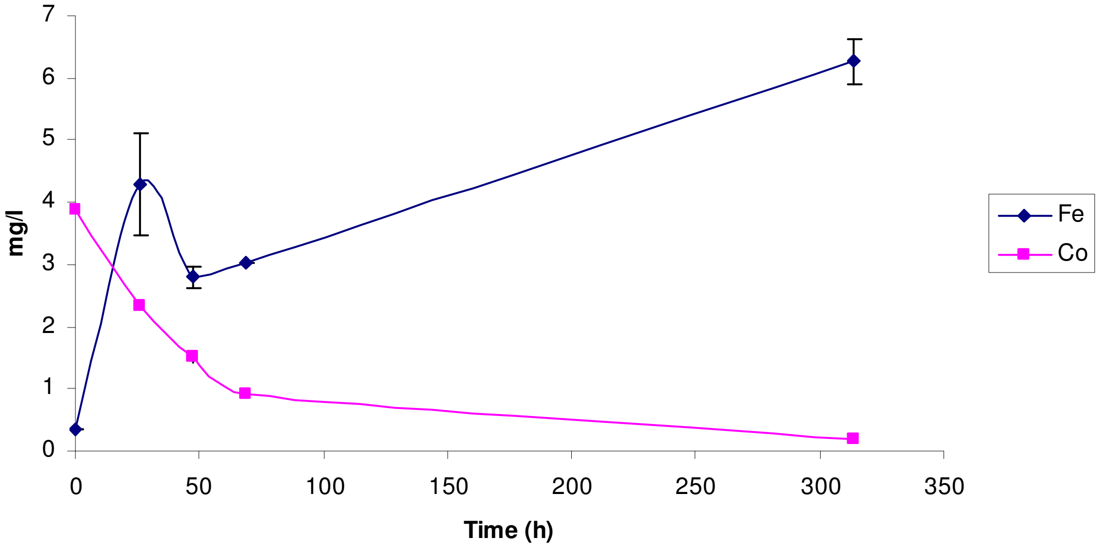


Figure 5.28 Soluble metals tracking after cobalt supplementation- C0810

5.5.2 Cobalt Uptake

As noted in section 5.2 in this study it was consistently found that when cobalt was supplemented on its own, it induced a response that was equal to or higher than that observed from the combination TM solution, despite being at the same concentrations in both. Figures 5.29 and 5.30 both display the decrease in cobalt, after being supplemented on its own or as part of the TM solution, over the first 50 hours of the study for samples A0810 and B0710 respectively. Only the first 50 hours are shown as that was the period in which the V_{max} (linear phase in the methane curve) was normally found to occur. For both samples it was found cobalt levels decreased more rapidly when supplemented on its own, as shown by the more negative gradient values, y = -0.0053 and y = -0.0175 for Co as part of TM solution in sample A0810 and B0710 respectively, in comparison to y = -0.0073 and y = -0.0275 from cobalt only in the respective samples. If the reducing cobalt is assumed to be all taken up by the methanogenic population then the graphs show cobalt is taken up faster when on its own. The slower uptake in the TM solution suggests competition is occurring for binding sites. This provides a reason as to how a higher V_{max} from cobalt-only supplementation may be found in comparison to TM solution supplementation despite cobalt being at the same concentration in both sets of assays. If another metal is competing with the uptake of cobalt then less would be taken up in a given time, meaning less of the requirement for the metal is satisfied and thus a lower increase in the V_{max} would be observed. Furthermore nickel is present in the TM solution at the same level as cobalt and uptake competition of cobalt with nickel has been shown by previous studies, as discussed in Chapter 2 (literature review). To demonstrate with certainty that nickel is the metal that is competing for uptake and not other metals in the TM solution, experiments with and without nickel would need to be carried out.

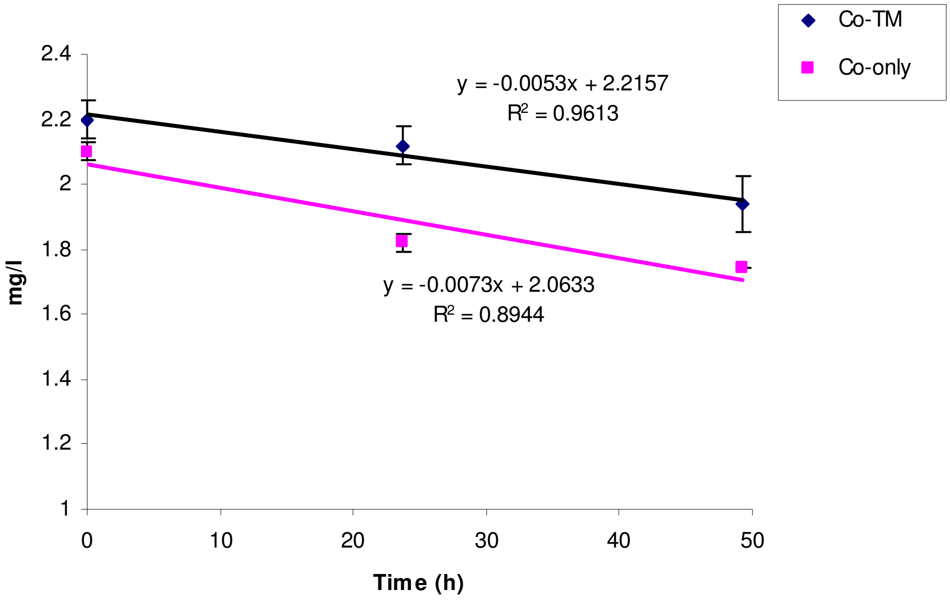


Figure 5.29 Sample A0810 cobalt vs. cobalt-TM

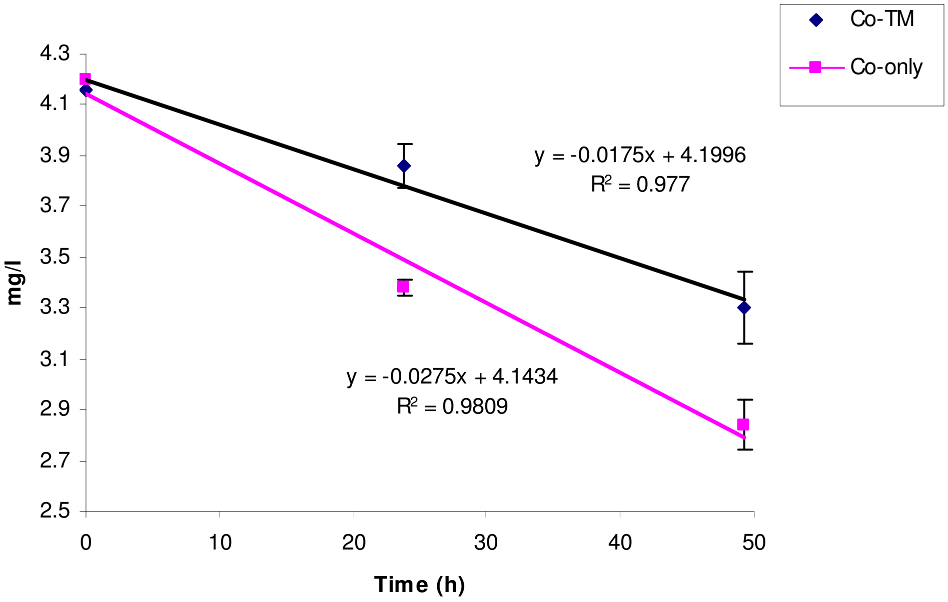


Figure 5.30 Sample B0710 cobalt vs. cobalt-TM

As highlighted previously metals being removed from the soluble fraction don't necessarily represent the fraction of metals being taken up by the microorganisms. However analysis of the evolution of metals in the soluble fraction helps to further the understanding of metal behaviour within the digester medium. From the soluble tracking experiments it was found that cobalt is consistently removed from the soluble fraction, nickel stayed at the same level in two studies but decreased somewhat in one, whereas iron levels generally increased from the starting levels. The effect of cobalt supplementation on V_{max} has been previously shown; cobalt supplemented on its own consistently induced a response that was equal to or higher than that observed from the combination TM solution. These findings further support cobalt as being the only metal among the three that is readily bioavailable as an EDTA complex. However further work in the form of longer term studies and with supplementation of metals individually would need to be carried out with digested sludge deficient in the respective metal to demonstrate the actual bioavailability of individual complexes. Additionally the observation of iron increasing demonstrates that the displacement of EDTA complexed metal may be occurring within the digester medium. This opens the question as to whether certain EDTA complexed metals are directly bioavailable or not, because it would mean non-bioavailable complexed metals can still illicit a response in cases where they are freed via displacement with a metal that is able to produce a higher stability constant. Consequently further work on bioavailability would also require examination of other metals present in the media as well as those being supplemented.

5.6 Irregular Samples

Section 5.4 introduced the use of the population data alongside the V_{max} from the non-supplemented control as a tool to determine if a digester is metal deficient. The approach highlighted that samples B1109 and C1109 would have been expected to respond given their normal acetoclastic methanogen populations and yet low V_{max}'s relative to other samples from the same digester at different times. This section explores the reasons for the no response seen by drawing together all data, helping to provide further insight into the bioavailability of the metal-EDTA chelates used in the study.

5.6.1 Sample B1109

Sample B1109 had a lower than expected V_{max} for its methanogenic population in relation to other samples from the same digester but yet did not respond to supplementation. Metal content can help give further insight into the reasons for the apparent lack of response. Tables 5.13 and 5.14 detail the metal content of the sample in the total and soluble fractions respectively, data is shown alongside the average for the digester. Data previously seen in Figure 5.16, where it's strongly apparent that the highest V_{max} recorded for Digester B coincided with the highest soluble nickel concentration, helps to demonstrate the importance of nickel in this digester. Furthermore from Tables 5.13 and 5.14 it can be seen that the concentration of nickel was substantially lower than the average for the site, the total fraction it was near 50% lower and in the soluble fraction it was 95% lower, suggesting a nickel deficiency was present. If this digester was indeed nickel deficient in sample B1109, this would provide support to the use of low V_{max}/normal methanogenic population data to identify trace metal deficiencies in digesters. The nickel deficiency diagnosis for sample B1109 is, however, not supported by the fact that the SMA test did not respond to TM supplementation, which contains EDTA-complexed nickel, unless the nickel-EDTA complex was not directly available to the acetoclastic methanogens within the timeframe of the SMA test.

Digester B was only found to have responded once in this study with cobalt only supplementation. The no response found in sample B1109 may therefore be related to cobalt, which incidentally was present in this sample at relatively high concentration of 13.6 mg/kg TS (appendix B provides metal content data in mg/kg TS), in comparison to below detection limits of 1mg/kg TS for most other samples in the study. Therefore sample B1109 was not likely to have been cobalt deficient, which provides further support to cobalt being the only readily bioavailable metal as an EDTA complex.

Table 5.13 Total metals for samples B1109 and C1109

All values in g/kg TS	B1109	Digester B Average	C1109	Digester C Average
Ca	21.23	50.58	17.85	49.26
Na	4.76	3.50	48.68	13.76
Mg	4.33	3.76	3.49	3.58
K	4.25	3.98	1.95	4.86
Fe	21.23	24.97	43.81	58.59
Zn	1.70	1.90	1.95	1.20
Mn	0.93	0.84	0.39	0.34
Cu	0.43	0.49	0.25	0.40
Ni	0.15	0.29	0.03	0.31
Co	0.01	0.00	0.02	0.04

Table 5.14 Soluble metals for samples B1109 and C1109

All values in mg/l	B1109	Digester B Average	C1109	Digester C Average
Ca	47	162.5	120.0	384.3
Na	97	89.8	-	234.0
Mg	25	12.7	33.0	59.4
K	85	47.2	41.0	397.9
Fe	2.2	1.1	5.1	2.3
Zn	4.1	16.0	2.3	1.5
Mn	0.2	0.1	0.19	0.0
Cu	0.3	0.8	0.22	1.1
Ni	0.09	2.2	0.1	2.6
Co	0.0	0.0	0	0.0

5.6.2 Sample C1109

Sample C1109 was also highlighted as an irregular sample in the sense it also had a low V_{max}/normal methanogenic population but yet it did not respond to supplementation. Tables 5.13 and 5.14 also show the metal content in the total and soluble fractions for sample C1109 alongside the digester averages. The tables reveal that like sample B1109 sample C1109 also had substantially low nickel in both fractions relative to the averages for the digester. Nickel was present at only 10% of the average in the total fraction and at only 3.8% of the average in the soluble fraction, again suggesting likelihood of a nickel deficiency for this sample, which if correct would provide further support to the use of low V_{max}/normal methanogenic population data to identify short-term trace metal deficiencies in digesters. It is important to note that low values do not necessarily indicate a deficiency as microorganisms have very low requirements for nickel and what is seen present in the average fraction is not all necessarily bioavailable anyway. Nevertheless such dramatic differences in the levels do present the possibility of a deficiency being present for nickel. Furthermore the iron content in sample C1109 as found in the total fraction was below average at 43.8 g/kg TS, which is 25.3% less than the average, which indicates an iron deficiency may also have been present. The importance of iron in Digester C was illustrated in section 5.3.4 where a relationship, which was significant at the 90% confidence level, was seen with Fe in the total fraction and V_{max}. However iron in the soluble fraction was at 5.1 mg/l, which is considerably above the average of 2.3mg/l, therefore a deficiency in iron was not as likely as a deficiency in nickel in sample C1109. Although it cannot be said with absolute certainty that the sample was nickel deficient, the lack of a positive response given the low level of nickel found alongside the low V_{max} measured creates doubt over the bioavailability of the nickel-EDTA complex. Furthermore consistent with sample B1109 sample C1109 also had relatively high cobalt at 16.2 mg/kg TS. Therefore sample B1109 was not expected to be cobalt deficient, which again provides further evidence to cobalt being the only readily bioavailable metal as an EDTA complex.

A nickel deficient sludge not responding to EDTA-nickel can be explained if the complex has a higher stability constant than that of the methanogenic uptake component, essentially making it non-bioavailable. Furthermore displacement of nickel by other metals, as previously described, will be quite minimal given the fact it forms a complex which already has a very high stability constants under standard conditions (as shown in Table 2.7). However as previously discussed aside from the limited bioavailability of EDTA-metal complexes, there are also other explanations for the lack of response from supplementation with the samples such as the possibility of deficiencies in metals or nutrients other than those supplemented and/or the samples being under toxic shock. Although no indicators were found for the digesters being under toxic shock the potential of another nutrient being deficient can still explain why a positive response was not recorded for the samples. Furthermore the methodology used in this research is only recording the short term effects of supplementation. Therefore further work in the form of longer term studies and with supplementation of metals individually would need to be carried out in sludge knowingly deficient in the respective metal to demonstrate the actual bioavailability of individual complexes.

CHAPTER 6 DISCUSSION

The principal aim of this study was to investigate the potential of EDTA complexed metals to enhance methanogenesis of wastewater sludge and to help identification of when full-scale wastewater sludge digesters might benefit from supplementation. Within the following chapter the following points are discussed;

- The efficacy of the method used and developed
- The prevalence of metal deficiencies
- Digester variability
- Site specific relationships
- Identification of metal requirement
- And the bioavailability of metals when complexed with EDTA

6.1 Specific Methane Activity Test

Although the SMA/BMP class of tests conducted in serum bottles recognisably simulate the full scale digester less closely than continuous or semi-continuous lab scale reactors, they have been shown to be effective in determining biodegradability of compounds and in digester monitoring (Conklin *et al.*, 2008, Ince *et al.*, 1995, Owen *et al.*, 1979, Raposo *et al.*, 2006, Schoen *et al.*, 2009, Shelton and Tiedje, 1984, Valcke and Verstraete, 1983, Van Den Berg *et al.*, 1974). The tests are simple and inexpensive, which serve as a flexible investigatory tool allowing multiple replicates to be easily run at the same time. Therefore the use of the SMA test is considered the best approach for the explorative research of this thesis.

The research presented in this study focuses on the effect of trace metal supplementation on the methanogenesis stage of anaerobic digestion only. The applicability of the SMA test to the full scale test via the ACN ratio relies on methanogenesis being the rate limiting step in the digester. If the rate limiting step is not methanogenesis in a particular digester then increases seen in methanogenic activity in the SMA test cannot be directly related to the full scale digester as increases in organic loading. If the methanogens are producing methane at a faster rate than the products of hydrolysis are being formed then increasing digester loading in such an instance has an associated risk of causing digester upsets and possible failures. The rate of methanogenesis has been shown previously to occur more slowly than the rate of hydrolysis of simple substrates such as starches, but more rapidly than hydrolysis of more complex substrates such as cellulose (Noike *et al.*, 1985). Therefore in AD of wastewater sludge the hydrolysis phase would commonly exist as the rate limiting step, however with the increasing use of pre-treatment technologies such as two-phase digestion (acid/gas phase digestion), thermal pre-treatment (CAMBI process), mechanical pre-treatment and co-digestion with rapidly biodegradable substrates, methanogenesis is increasingly present as the rate limiting step. Therefore stimulating the methanogenesis in such an instance equates to stimulating the rate of the overall process and accordingly the results can be applied to the full scale digester.

This study measures changes in the maximum activity rate, termed V_{max}, to understand the effect of metals. However as recommended by Speece (1996) serum bottle tests can be used to the same effect by simply measuring the differences in cumulative methane after a fixed term (Speece, 1996). Table 6.1 compares the use of the two methodologies in terms of the percentage change seen after supplementation using both methodologies. The cumulative methane data represents methane produced after 50 hours and was calculated using the methane production curves for the same assays as those represented for the V_{max} data (cumulative methane curves for all assays can be found in Appendix D). Only increases above the two standard deviations for the replicates for that particular study were considered as positive results and accordingly samples which met this condition are represented as bold in the percentage change field.

From Table 6.1 it can be seen that the majority of positively responding samples as determined by increases in the V_{max} can also be identified by an increase in the cumulative methane after 50 hours. Samples such as A0210 interestingly display very similar increases under both methodologies with 8.5% increase seen in V_{max} after supplementation and 8.9% increase seen in the cumulative methane. Other samples such as A0310 display a much higher increase in cumulative methane at 30.2% after supplementation compared to 20.3% increase in V_{max}, such differences between the two methodologies can be explained by the fact the V_{max} is only takes into consideration the linear phase of the curve whereas the cumulative methane value would also include any effects seen during the initial lag phase of the methane production profile.

Table 6.1 V_{max} vs. cumulative methane

Sample	V_{max} of Non-Supplemented Control	V_{max} Post-Supplementation	Change (%)
A0210	1.25	1.36	**8.5**
A0310	1.11	1.34	**20.3**
A0510	1.56	1.66	**6.6**
A0810	1.44	1.54	**7.2**
A0909	1.36	1.44	**5.7**
A0111	1.46	1.62	**11.1**
B0310	1.88	1.95	3.4
B0710	1.30	1.38	**6.3**
B1109	1.22	1.19	-2.1
C0310	1.46	1.67	**14.5**
C0810	1.45	1.47	1.3
C1109	1.20	1.26	5.6
C0211	1.22	1.37	**12.2**
D0610	0.92	1.03	**11.6**
D0710	1.43	1.30	-8.9
D1109	0.89	0.96	**7.6**

Sample	Cumalative Methane of Non-Supplemented Control (ml)	Cumalative Methane Post-Supplememtation (ml)	Change (%)
A0210	43.4	47.2	**8.9**
A0310	34.9	45.5	**30.2**
A0510	62.2	64.4	3.6
A0810	52.4	55.1	**5.2**
A0909	52.8	57.1	**8.3**
A0111	39.3	45.5	**15.8**
B0310	50.3	50.5	0.3
B0710	42.8	46.4	**8.2**
B1109	40.2	40.0	-0.4
C0310	51.3	53.3	3.9
C0810	51.9	49.3	-4.9
C1109	43.2	44.4	2.9
C0211	43.6	48.1	**10.3**
D0610	24.8	27.2	**9.8**
D0710	42.8	40.7	-5.0
D1109	27.4	26.8	-2.2

The advantages of using the cumulative methane method alone is that it removes the need to take as many biogas and methane readings as possible in order to form a rate. However it should be noted from Table 6.1 samples A0510, C0310 and D1109 were all found to respond positively under the V_{max} methodology with increases of 6.6 %, 14.5% and 7.6% respectively, however using cumulative methane production data no positive effect can be inferred for any of these samples. This difference may possibly be explained by the metal supplementation potentially causing an initial negative effect in these assays during the lag phase as the microorganisms adapt to the change in conditions. It is also noteworthy that in this study no sample displayed a positive response with an increase in the cumulative methane without also displaying an increase in V_{max}; these points together suggests that determining the V_{max} is a more sensitive method to ascertain the effect of nutrients in a serum bottle batch test in comparison to just comparing the cumulative methane after a fixed term alone. Also as explained in section 5.1.1 the V_{max} as described by Conklin *et al.* (2008) can be used to determine the maximum capacity of the digester, this means any increases in the V_{max} can consequently be used to determine safe increases to digester organic loading, as shown in section 5.2.4. Therefore despite calculating V_{max} being a more laborious approach it is considered a stronger method to investigate the effect of metal supplementation.

The use of acetate as a feed in this study was chosen as previous research has shown approximately 70% of methanogenesis in domestic sludge comes via the precursor acetate (Jeris and McCarty, 1965, Smith and Mah, 1966). The methodology used in this study is designed to target activity specifically related to acetoclastic methanogens, however it must be acknowledged that methanogenesis from acetate can also occur via syntrophic acetate oxidation to involve the hydrogenotrophic methanogens (Zinder and Koch, 1984). In syntrophic acetate oxidation, acetate-oxidising bacteria catalyse the production of CO_2 and H_2 from the oxidation of acetate, with the products subsequently utilised by the hydrogenotrophic methanogens to produce methane. The process occurs with syntrophic interaction of the two microbes, it should be noted however that energetically the first step is extremely unfavourable on its own (Hattori 2008). Given the experiments used in this study it is probable the acetoclastic route would have been predominant in the tests.

Furthermore any potential contribution of the syntrophic pathway to the methane production in individual assays does not affect the results of the study as all assays are compared to controls, which have the same feed and contain inoculum from the same source.

If the acetoclastic methanogenesis contribution to total methane production varies significantly amongst digesters then this can affect the application of the V_{max} to the full scale digester. A digester for example which has a higher proportion of methane produced via hydrogenotrophic methanogenesis will likely present with a relatively lower V_{max} with the acetate fed SMA test. The ACN ratio for such a digester would then present a lower value indicating less capacity than what is present, as the methodology calculates the plants actual acetoclastic activity with the assumption 70% of the methane is being produced via acetate pathway. This again could result in unnecessary decreases in digester loading. The difference in the relative prevalence of acetoclastic to hydrogenotrophic methanogens that make up the digesters methanogenic consortium could also explain the differences seen in V_{max} from different digesters. However differences between sites are more likely associated with differences in the number of acetoclastic methanogens, associated with differences in operational parameters and metal content, as well as differences in ratios of *Methanosaeta* and *Methanosarcina* species in different sites, variability between digesters is discussed further in section 6.3.

Hydrogenotrophic methanogenesis activity involves reduction of one mole of CO_2 by four moles of H_2 to generate one mole of methane and 2 Moles of H_2O (Thauer, 1998). The methodology used for the SMA test in this study converts increases in gas pressure to biogas production and then relates it to methane production using concomitant gas analysis. As the end reduction in hydrogenotrophic methanogenesis involves a reduction in gas pressure and volume, it results in 1 mole of gas (methane) being produced from 5 moles of gas (CO_2 and H_2), it means the hydrogenotrophic activity has associated decreases of gas pressure so it can not be measured accurately using the same experimental set up.

The correcting of the methane for temperature differences as described section 4.1.4 is important to produce an accurate V_{max}. Without the correction the methodology would present the V_{max} to be higher than what it actually was. An over calculation of the V_{max} would be mean the ACN ratio would wrongly indicate a higher amount of excess capacity. Furthermore if the ACN ratio is subsequently used to increase the digester loading, the associated risk of placing too much stress on a digester is that it can lead to costly digester failures.

In section 5.1.2 it was stated that the volatile solid content was used as an indicator for the biomass. The VS content was kept constant to reduce variability in the methanogen levels and in accordance with the fixed VS content of 0.5g the acetate concentration of 60mM was selected as it was shown to be sufficient to ensure CH_4 production was not limited by substrate. Incidentally the methanogenic population data from qPCR analysis revealed no benefit of using the measure. The volatile solid fraction includes organics that were not fully digested, as well as all microorganisms in the digester, which explains how such variation can still exist. Variability in the acetoclastic methanogens can affect the V_{max} as a higher than expected population may mean the feed concentration being used may be insufficient. If substrate is limiting during the linear phase an inaccurately low V_{max} will be measured. This could lead to the ACN ratio wrongly indicating a lower capacity, which if acted upon would result in unnecessary decreases in digester loading. The highest level of variation in methanogen population was seen in digester A at 46.3%. Incidentally the variation between the methanogenic population in samples used to determine the substrate concentration (as in section 5.1.2) was itself 39.3% therefore this provides confidence that acetate at 60mM was a sufficient feed concentration to use for accurate V_{max} determination in this study.

Other limitations of the methodology include the use of water to make up the reactor volume to 100ml, which could have a harmful effect on the microorganisms by means of changing the media conditions. The effect of this was minimised by ensuring pH of the acetate feed solution was set at 7.2. This ensured that the overall pH of the assays once the sludge had been added was within expected range (pH 7-7.8); this was verified by measuring pH at the start of all assays. This study compares the V_{max} values of assays that are typically generated within the first 50 hours of the assay. Therefore a key limitation of the approach used in this study is that it only reflects the short term effects of metal supplementation and therefore cannot represent longer term benefits, which previously have been highlighted in the literature such as increased growth rates (Mah *et al.*, 1978, Scherer and Sahm, 1981) and ability to cope with toxic shock (Climenhaga and Banks, 2008, Murray and Berg, 1981). General sources of error in the methodology can include gas leaks from bottles, inaccuracy in pipetting sludge/feed and errors associated from gas sampling. However the test being used is very simple and was not known to leak in this study. It can be seen that the average RSD among the sixteen samples is relatively low at 3.81%. This demonstrates the precision of the methodology used. Furthermore the assays were all treated exactly the same with the only variable being the metal supplements. Therefore they are perfectly comparable to other assays from the same sample, thus all responses to supplementation seen in this study are accurate for the short term effects, relative to the acetate-only fed assays

Although in this study the SMA tests were run until the biogas production subsided, they would actually only need to be run typically for 3-4 days to obtain the V_{max}, making the test more rapid. The gas volume measurement and composition analysis was carried out manually in this study, however online gas measurement devices are commercially available that can automate the measurement and generate methane production curves. Therefore the methodology used in this study can be very easily adapted to become a rapid and effective way for wastewater treatment works to monitor digesters on site and apply in accordance with the approach discussed in section 6.5.

6.2 Prevalence of Metal Deficiencies

This study demonstrated that deficiency was present 69% of the time, with a positive response to metal-EDTA chelates used as the indicator of metal deficiency. However the supplementation with the TM solution would only have been beneficial if the deficiency is in one or more of the metals being supplemented and providing that metal is readily bioavailable. Furthermore as discussed in the previous section the methodology is limited to recording only short term effects. Therefore it remains possible the prevalence of deficiencies may have actually been higher.

The response in each sample ranged from a 5.7% to 20.3% increase in the V_{max}, with only increases above the two standard deviations for the replicates of that particular study being considered as positive results. The use of two standard deviations means the difference in V_{max} is above the variability seen in the repeats and therefore the positive recorded results are unlikely to be due to experimental error. Different digesters responded differently; Digester A responding on all occasions demonstrating a very high prevalence of metal deficiency in the digester and Digester B only responding in one assay from three separate sampling periods suggesting a low prevalence of deficiencies in the digester. The average response from metal supplementation in the eleven studies that benefitted from supplementation, and thus were known to be metal deficient, was 10.1%. Increases in V_{max} in the serum bottle tests can be related back to the digester as previously shown via the ACN ratio, with an increase in the V_{max} related to an ability to cope with a higher rate of organic loading. In order for metal supplementation to be considered viable and to persuade wastewater treatment works to supplement their digesters, any economic benefits gained from supplementation would need to be greater than the costs incurred. In section 5.2.4 it was demonstrated that a 10% increase in the V_{max} across the digesters would result in the highest change in terms of extra methane potential from Digester B, with additional 285 m^3/d, and the lowest from Digester D, with additional 40 m^3/d of methane. To understand the economic viability of dosing further work is required in the form of costing supplementation relative to increases in potential methane production, as well as any additional revenue from treating imported sludge and its associated digestate. Such costing can then be calculated back and represented as a minimum increase in the V_{max} that is required for supplementation to be considered cost effective for each digester.

6.3 Digester V_{max} Variability

Amongst all digester samples no correlation was found with methanogen population with V_{max}, however as seen with Digester A, a site specific relationship was demonstrated. Only Digester A was detailed as there were too few data points for the other digesters. The V_{max}

value measured from the SMA test indicates the acetoclastic methanogenic activity of the sludge, which is a product of the population of the only two genera of methanogens known to be able to metabolise acetate to produce methane; *Methanosaeta* and *Methanosarcina*. The reason why no cross site correlation was seen is mainly due to differences in the relative populations of Mst and Msc (as described in Section 5.4.2). The Mst/Msc ratios were found to follow a site specific trend, which was shown to correspond with the SRT of the digester, with a higher prevalence of Mst associated with a higher SRT. A higher acetate concentration in a digester can cause selection pressure for a higher level of *Methanosarcina* over *Methanosaeta* species. *Methanosarcina* species have a lower affinity for the acetate hence why overall *Methanosaeta* species dominate in digesters. However *Methanosarcina* species have a higher V_{max} and therefore can outcompete at higher acetate conditions (Griffin *et al.* 1998). A higher proportion of *Methanosarcina* species would display a higher V_{max} in comparison to an equal population with a higher proportion of *Methanosaeta* species. Therefore differences in the relative populations can explain why a different V_{max} can be observed even if the combined values are very similar. Higher levels of both acetoclastic methanogens without any changes to the ratios would also be expected to give rise to a higher V_{max}.

As well as SRT and the Mst/Msc ratio the acetoclastic methanogenic activity would also be affected by factors such as differences in influent sludge profile, presence of toxic compounds, and differences in the level of bioavailability of the metals present. Presence of toxic compounds can especially have a negative effect on activity and lead to a lower than expected V_{max} for a sample. Determining presence of toxic compounds is difficult given the fact most compounds can generally exhibit a toxic effect, which is dependent on the concentration. This is understood even for metals, as they have an associated risk of causing toxicity at higher concentration. At elevated concentration heavy metals are believed to cause disruption of enzyme function and structure by binding of the metals with thiol and other groups on protein molecules or by replacing naturally occurring metals in enzyme prosthetic groups (Vallee and Ulner, 1972). However determining if a metal is at an inhibitory concentration can be difficult given a range of optimal metal concentrations reported in literature as previously discussed in section 2.13.

6.4 Site Specific Relationships

The differences in the V_{max} measured in the SMA test caused by differences in the combined and relative acetoclastic methanogen populations also help explain why no correlation was found with V_{max} and metal content across digesters. Although further sample points are required to establish statistically significant correlations with a higher degree of confidence, section 5.3.4 helped to demonstrate a positive relationship between the total and soluble fraction metals with V_{max} at a site specific level. A positive relationship emphasises the importance of metals for methanogenic activity and helps explain variations seen between different samples from the same digester site. Acetoclastic activity from samples from the same site will be expected to have a higher degree of similarity compared to samples from across digesters as no/or limited change to digester operational parameters would be expected and the composition of the influent would also be expected to be less different than between separate sites. Therefore fewer of the factors which may influence the acetoclastic methanogenic activity as highlighted in the previous section would be applicable.

The sometimes weak relationship between metals and V_{max} seen in section 5.3.4 demonstrates that changes in activity of the acetoclastic methanogenic activity cannot just be attributed to changes to metal content. The main reasons sample points within a digester may not fit exactly to a positive trend line for metal content with V_{max} include differences in levels of macronutrients such as phosphorus, presence of toxic compounds and also differences in the bioavailability of the metals that are present. Higher level of precipitation may make the metal no longer directly available (Gonzalez-Gil *et al.*, 2003), changes in level of the main anionic compounds responsible for precipitating metals during anaerobic digestion such as carbonate, sulphide, and phosphate may increase precipitation and therefore affect level of bioavailability (Callander and Barford, 1983). Sulphide is especially important given the K_{sp}'s of the transition metals indicate that they are preferentially precipitated as sulphides. Also presence of chelating organic compounds can make the metal less bioavailable if the target organism does not have a metal binding component which has a higher stability constant that that of the ligand-metal complex

(Callander and Barford, 1983). It must also be acknowledged that the total fraction metals measured and plotted against V_{max} in section 5.3 only include the 7 metals, which constitute the TM solution; therefore differences in levels of other metals may also be responsible for weaker relationships seen.

It was found that stronger relationships between metal content and activity are present when the maximum V_{max} post-supplementation is used to plot the data rather than the V_{max} from the non-supplemented acetate control. This is seen as supporting the mechanism proposed to be responsible for increases in V_{max} in this study: which in section 5.2.1 was stated as being attributed to an increase in functional cofactors whereas the effect of growth within the timescale was considered negligible. If the metals in these fractions are considered reflective of the longer term metal supply for the methanogens, then the observed results would be indicating the importance of the metals in increasing methanogenic populations and generally maintaining their health. Thereby emphasising the longer term benefits of supplementation, which are not recorded in this study. The observed better fit from the post-supplementation V_{max} is seen as reflective of the methanogens susceptibility to the effects of acute deficiencies of metals. An acute deficiency is seen as responsible for reduction in viable cofactors, without affecting population size, which would explain why subsequently after supplementation points are drawn closer to the trend line. If growth was playing a valuable part in stimulation then points would be expected to be moving to the right without necessarily improving the trend line fit.

Given the loosely positive relationship between V_{max} and metal content described in section 5.3.4 changes observed in the V_{max} values in samples from the same digester at different times can to an extent be attributed to changes in metal content. Figure 6.1 illustrates the effects of how metal deficiencies are believed to influence the V_{max}. The illustration splits the effect of a decrease in the level of metals available to the methanogens into a short term and long term effect. In the short term a decrease in the metal content is expected to reduce the V_{max} by decreasing the number of cofactors whereas in the longer term the adverse effect on stability and growth would be expected cause a change in the equilibrium leading to a decrease in the methanogenic population and thus a decrease in the V_{max}. Accordingly the beneficial effect from supplementation in this study is attributed to satisfying shorter term metal requirements.

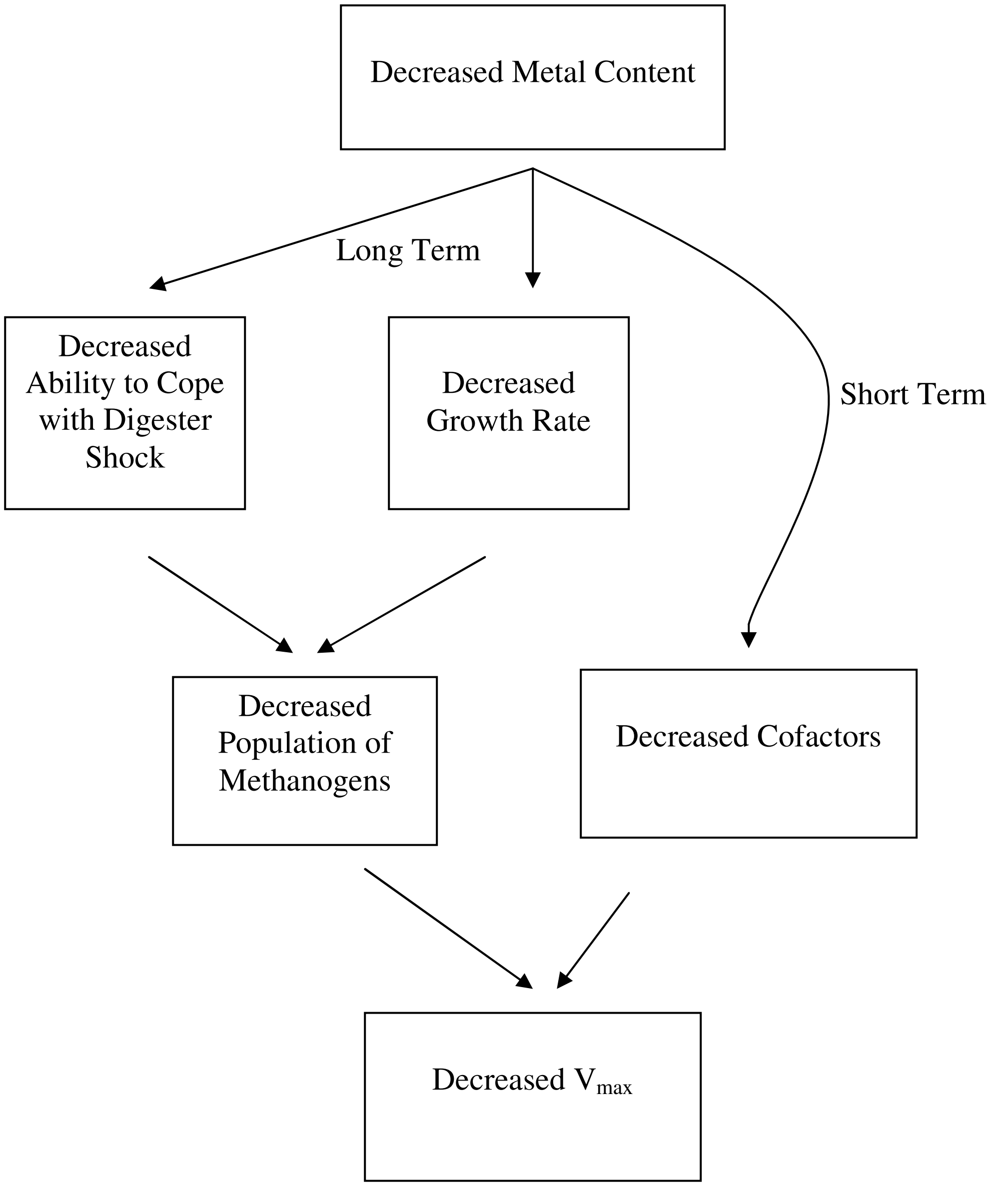


Figure 6.1 Short-term and long-term effects of metal deficiencies on V_{max}

It was found that metal content alone was not a good indicator of response to supplementation in this study. Low metal content did not necessarily correspond with a high response, as shown in section 5.3.4. This may be due to short term limitation of the methodology used or another reason for this maybe due to some of EDTA complexed metals supplemented in this study not being readily bioavailable. Bioavailability of the supplements is discussed further in section 6.6. The lower responses seen from higher TM solution concentrations coupled with the inhibitory effect seen in assays from digester D emphasise that there is an associated risk of heavy metal toxicity at high concentrations. Consequently there is a need to determine if a digester is metal deficient before dosing.

6.5 Metal Requirement Indicator

Although further sample points are required to establish statistically significant correlations with a higher degree of confidence, a site specific relationship was demonstrated in digester A for a higher acetoclastic methanogenic population with a higher V_{max}. Furthermore as hypothesised (section 3.3) a lower than expected V_{max} for a given sites combined acetoclastic methanogen population was found to correspond with a metal deficiency. The sample A0310 had the aforementioned profile and responded with a 20.3% increase in the V_{max} following supplementation with TM solution. Therefore the combination of a reasonable methanogenic population with a strong response to trace metal supplementation could be a good indicator of short term or intermittent trace metal limitation and related benefit from supplementation.

A lower than expected V_{max} for the combined acetoclastic methanogen population was also found to be characteristic of positively responding samples from digesters C and B. In accordance with Figure 6.1, the increase in the V_{max} is attributed to increases in the number of cofactors; therefore the aforementioned approach is seen as an indicator of an acute deficiency. A longer term deficiency would be expected to result in a shift in the equilibrium with a lower level of methanogens being present. It is expected that a deficiency related decrease in the population would be corrected by metal supplementation, with a higher population leading to a higher V_{max}. However this effect would be expected in the long term and cannot be demonstrated by the methodology used in this study. Therefore according to the results from this study the best indicator of metal requirement and benefit from supplementation is found when the V_{max} is lower than expected for the acetoclastic population of that particular site.

This approach can prove to be a useful indicator to trace metal deficiencies and an alternative to continuously supplementing metals in SMA tests, thereby decreasing costs. Theoretically observation of a lower than expected V_{max} can be used as a signal to initiate supplementation. Accordingly trace supplementation can then be carried out with SMA tests to determine metal response and the optimal dose to use. Finally the results of the SMA tests can then be applied in terms of the correct concentration of metals to be dosed in the full scale digester alongside the appropriate increase in digester loading as calculated via the ACN ratio. The major limitation of this approach is that quantification of methanogens using qPCR is a very laborious and expensive procedure, which requires a lot of specialist equipment and training. Therefore such an approach is not practical for use by wastewater treatment works.

The use of fluorescence based quantification may be a useful tool for wastewater treatment works. The autofluorescence techniques such as measurement of factor F_{420} can offer great value for generating fast and cost effective quantification data (as discussed in section 2.3). Factor F_{420} is a cofactor unique to methanogens which is involved in electron transfer (Jacobson and Walsh, 1984). The major limitation however in using such a technique is the inability to differentiate between the different methanogenic groups, which means the acetoclastic methanogens responsible for the V_{max} cannot be separately quantified. Also the concentration of F_{420} has been found to vary among different methanogens, therefore the level of F_{420} in a sludge sample cannot accurately be used to quantify the level of methanogens present (Reynolds and Colleran, 1987). However if site specific relationships exist as they appear to do for metal content, population and V_{max} then autofluorescence quantification techniques may be able to be used as illustrated in Figure 6.2 as an alternative to qPCR. Further work would be required to determine whether qPCR data can in fact be calibrated with autofluorescence quantification data at a site specific level and to determine its accuracy.

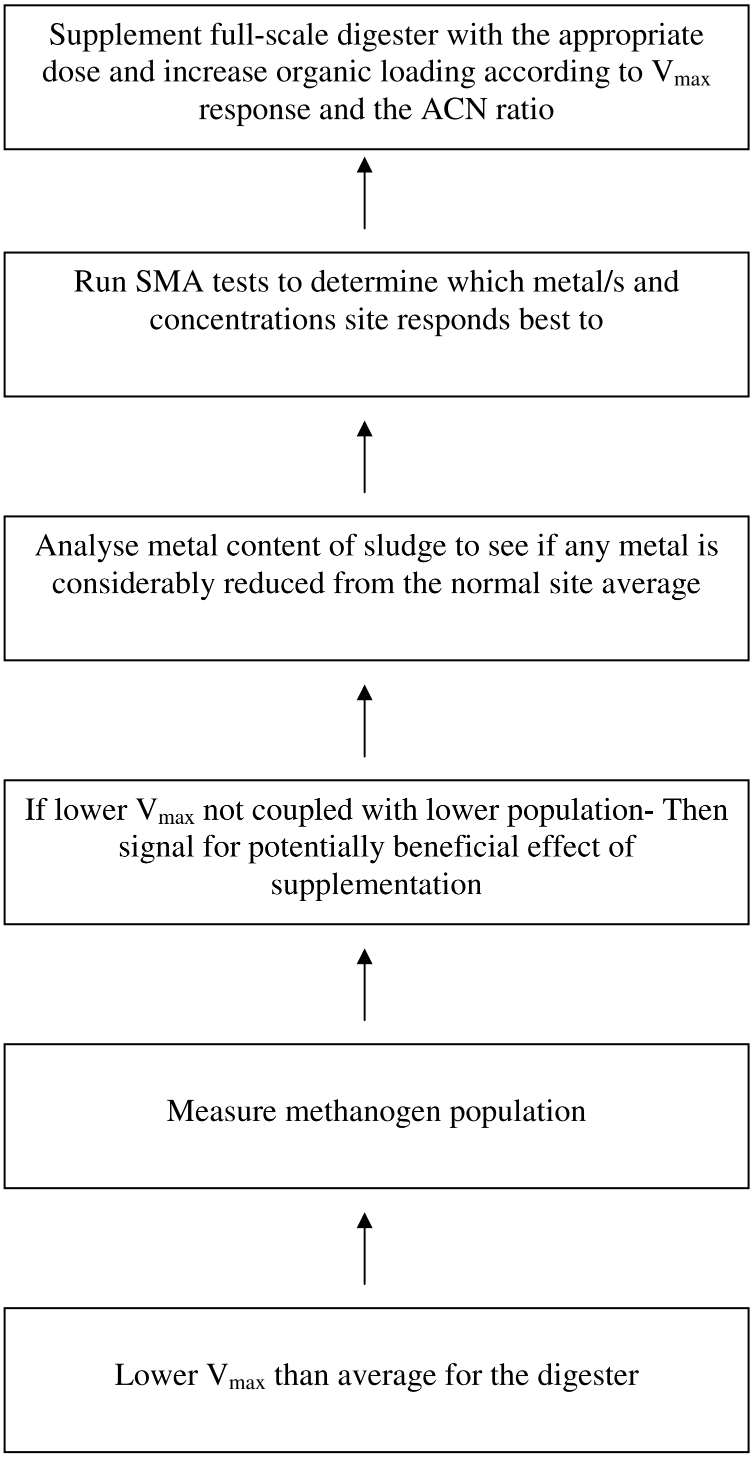


Figure 6.2 Application of SMA test with the methanogen population incorporated

The use of methanogen population as described can be a useful indicator for short term metal deficiencies. Figure 6.2 further illustrates how the SMA test can be applied by wastewater treatment works with methanogenic populations data incorporated. Notably the interpretation of metal content to determine deficiencies would need to be interpreted with caution as deficiencies in metals can still exist despite high levels found in sludge as its evident metals are not always bioavailable. In section 5.3.4 this point was illustrated with digester C having a positive relationship between increasing iron levels and increasing V_{max} that was significant at 90% level of confidence despite already having the highest average iron content amongst the four digesters at 58.6 g/kg TS. Therefore despite relatively high levels of presence, certain metals within the sludge may not be sufficiently bioavailable. However supplementation of metals in the SMA test would be able to confirm requirements via positive responses, thereby acting as another layer of control. The results of SMA test can then be applied as previously discussed in terms of the correct concentration of metals to be dosed in the full scale digester alongside the appropriate increase in digester loading as calculated via the ACN ratio.

The major limitation of using methanogen population in the described manner is that it does not differentiate between toxicity and metal requirements. In the event of toxicity and digester stress the population would also be expected to present such a reduced activity. However the low V_{max}/ normal population indicator would be expected to be used alongside other parameters such as VFA content and pH, which are already used by wastewater treatment works to determine if a digester is stressed.

The use of low V_{max}/normal methanogenic population data to identify short term trace metal deficiencies in digesters is not supported by the lack of response found in samples C1109 and B1109 as described in section 5.4.4. However both samples were found to have substantially lower nickel levels. Whereas lower nickel content does not necessarily indicate a deficiency, nevertheless such dramatic differences described in the levels do present the possibility of a deficiency being present for nickel. If both digesters were indeed nickel deficient as expected, this would provide support to the use of low V_{max}/normal methanogenic population data to identify trace metal deficiencies in digesters. However it raises question as to why the two samples did not respond.

This apparent lack of response can be explained by one or more of the following reasons; the samples were deficient in metals or nutrients other than those supplemented, the samples were not metal deficient but rather under toxic shock or the complexed metals supplied were not bioavailable to the methanogens. Interestingly no indicators were found for the digesters being stressed such as Ripley's ratios above 0.8, pH values outside the range of 7.7-8, prolonged lag phases in the methane curves. Given the variability associated with qPCR analysis it may be argued that the populations may have actually been lower than measured, which may explain why lower than expected V_{max} was observed and hence no response seen. However as demonstrated in section 4.2.3 the variability between qPCR replicates was relatively low at 10.0%. Furthermore it should be noted that the measured populations in both samples B1109 and C1109 were actually higher than the respective samples they were compared to, therefore an inaccuracy in qPCR data is an unlikely explanation for the observed lack of response from the two samples. Therefore due to the expected nickel deficiency and lack of response recorded doubts are raised about the bioavailability of the nickel-EDTA chelate being supplemented.

6.6 Bioavailability of Metals as EDTA Chelates

As has been previously highlighted chelation by a ligand such as EDTA is able to affect the availability of a metal to an organism; it will make the metal more available if the target organism has a metal binding component (essentially an uptake mechanism) with a higher stability constant than that of the ligand complex and less available if it doesn't (Callander and Barford, 1983). Furthermore studies which have used EDTA complexed metals have shown conflicting results. Majority of studies find EDTA bound metals to be soluble but non-bioavailable (Aquino and Stuckey, 2007, Babich and Stotzky, 1983, Bartacek *et al.*, 2008, Pankhania and Robinson, 1984, Speece, 1988).

Soluble fraction evolution experiments as shown in Section 6.5 provided further insight into the dynamics of metal-EDTA chelate bioavailability. Metals being removed from the soluble fraction as shown in section 5.5 are not necessarily representative of the metals being taken up by the methanogens or any other microorganism. Removal of the metal from the soluble fraction may also be due to changes in the metals speciation, which can transfer the metal into the solid fraction, for example as precipitates, co-precipitates, adsorbates or organometallic complexes (as highlighted in Table 2.5). However analysis of the evolution of metals in the soluble fraction helps to further the understanding of metal behaviour within the digester medium.

From the three soluble tracking experiments it was found that cobalt is consistently removed from the soluble fraction in all studies, nickel stayed at the same level in two studies but decreased somewhat in the other, whereas iron levels generally increased from the starting concentration. These findings would appear to suggest cobalt is the only metal among the three metals that is readily bioavailable to the acetoclastic methanogens. Different metals are known to form complexes with EDTA with a variety of stability constants (as detailed in Table 2.7) and the methanogens are known to employ a diverse range of uptake mechanisms (as discussed in section 2.8). Therefore as discussed in section 2.13 it is conceivable that metals that are able to bind with EDTA with a high stability constant and yet are taken up by a relatively non-specific pathway such as the magnesium transport system would not be readily bioavailable to the methanogens when complexed with EDTA. Whereas other metals such as cobalt, which are taken up by a higher affinity substrate specific pathway would be readily bioavailable.

In section 5.6 it was shown that that samples C1109 and B1109, which were expected to be deficient in metals other than cobalt, did not respond to supplementation. Furthermore in this study assays supplemented with cobalt only consistently responded to a level equal to or higher than that from the TM solution, despite being at the same concentrations in both. This alongside the results from the soluble metal tracking experiments would suggest that cobalt was the only metal readily bioavailable when complexed with EDTA. If accurate this supports the hypothesis of metals being non-bioavailable to methanogens when complexed to EDTA except for cobalt. Also differing levels of bioavailability for different EDTA complexed metals would help explain how conflicting results for EDTA complexed metals can exist in the literature. However further work in the form of longer term studies and with supplementation of metals individually would need to be carried out in studies deficient in the respective metal to demonstrate the actual bioavailability of individual complexes.

Fermosa *et al.* (2008) demonstrated EDTA complexed cobalt had a much quicker stimulatory effect in terms of specific methane activity and substrate removal in comparison to supplementation of cobalt chloride (Fermoso *et al.*, 2008). Therefore the results from the study suggested improved bioavailability of cobalt when complexed with EDTA in comparison to the chloride salt. However the study recommended against the use of Co-EDTA for two reasons. Firstly the Co-EDTA was found to have a much shorter lived effect given that in comparison to cobalt chloride supplementation less was retained within the solid fraction. The study proposed the dissolution of the cobalt sulphide precipitate from the solid fraction was sufficient to provide enough free Co^{2+} to supply the cobalt requirements. Secondly EDTA was noted to have a negative effect of decreasing calcium content; the study was carried out in UASB digesters and calcium is a structural ion for the granular matrix and thus decreased calcium concentrations lead to decreased granular strength. However when applying the findings to conventional suspended system digesters used for municipal wastewater sludge the same conclusion is not drawn. Fixed film digesters have very long solid retention times of over 100 days in comparison to typical 13-22 days found for the digesters used in this study. Furthermore the granulation of the methanogenic archaea does not occur in such systems. Therefore in sewage sludge digesters the use of Co-EDTA to treat cobalt deficiencies would be advantageous given its higher level of bioavailability. These findings are consistent with the strong stimulatory effect seen for Co-EDTA in this study.

The findings that nickel values stayed constant in the soluble phase tracking experiments casts doubt about the nickel-EDTA complex's bioavailability. Although uptake into the methanogens may still be taking place despite nickel values remaining constant; this can occur if the nickel taken up is replaced by more nickel from the solid fraction. However the samples C1109 and B1109, as discussed in the previous section, were both expected to be nickel deficient but yet did not respond to nickel containing TM solution supplementation.

The lack of response from the two samples alongside the findings from the soluble phase tracking experiments suggests that the nickel EDTA was not readily bioavailable. Further studies with supplementation of nickel-EDTA individually in nickel deprived reactors are required to conclusively determine its exact level of bioavailability. If nickel-EDTA isn't bioavailable then it may be explained by the fact Ni-EDTA has a higher stability constant than Co-EDTA under standard conditions (detailed in Table 2.7), which may be sufficiently different to make it non-bioavailable to the methanogens.

The observation of iron increasing helps demonstrate that the displacement of EDTA complexed metal may be occurring within the digester medium. It was highlighted earlier in the literature review that a ligand is able to scavenge metal ions from a complex, providing that it is sufficiently labile, if it can produce a complex with a higher stability constant (Snoeyink and Jenkins, 1980). This opens the question as to whether certain EDTA complexed metals are directly bioavailable or not, because it would mean non-bioavailable complexed metals can still illicit a response in cases where they are freed from the complex via displacement with a metal, which is able to produce a higher stability constant. Moreover this provides further reasoning as to why studies can report conflicting data for EDTA metals, as for certain metals the positive effect seen may have been indirect. Furthermore as nickel already forms a complex with EDTA that has a comparatively high stability constant it means displacement with nickel would be quite limited, which provides further possible reasoning to why no response was seen in samples C1109 and B1109.

Overall deficiency for cobalt in wastewater sludge digesters appears to be most common however this thesis raises doubts about the bioavailability of other metals when complexed with EDTA, particularly nickel-EDTA. To demonstrate the actual bioavailability of individual complexes further work in the form of longer term studies with supplementation of metals individually would need to be carried out. To elucidate a complexes bioavailability it would need to be supplemented to sludge knowingly deficient in the respective metal. Furthermore the observation of iron levels increasing demonstrates that the displacement of EDTA complexed metal may be occurring within the digester medium. This means further work for determining individual metal bioavailability must take into consideration other metals present as displacement effects may make a directly non-bioavailable complex appear bioavailable.

CHAPTER 7 CONLUSIONS AND RECOMMENDATINS

Within this chapter, conclusions which relate to the aims of this research are presented. These are followed by recommendations for application and further work in this area.

7.1 Conclusions

1. In this study assays supplemented with cobalt on its own consistently responded to a level equal to or higher than that from assays supplemented with a combination of metals. The results suggest that cobalt was the most commonly deficient metal in wastewater sludge digesters. However this study has raised questions about the bioavailability of other metals as EDTA chelates, particularly nickel-EDTA. Differing levels of bioavailability for different EDTA complexed metals explain how conflicting results for EDTA complexed metals can exist in the literature.

2. This study demonstrated that metal deficiencies were present 69% of the time in the wastewater sludge digesters samples, with a positive response to metal-EDTA chelates used as the indicator of metal deficiency. Given the questions raised about the bioavailability of metal-EDTA chelates the prevalence of deficiencies was likely to have been higher.

3. This study demonstrated a reoccurring theme of site specific relationships. At a site specific level it was found that a lower than expected V_{max} for a given sites combined acetoclastic methanogen population corresponded with a positive response from supplementation and therefore it can act as an indicator of metal requirement.

7.2 Recommendations and Further Work

The SMA test methodology described in this study can be used as an indicator for metal deficiencies. Figure 7.1 illustrates the recommended use of the test for wastewater treatment works.

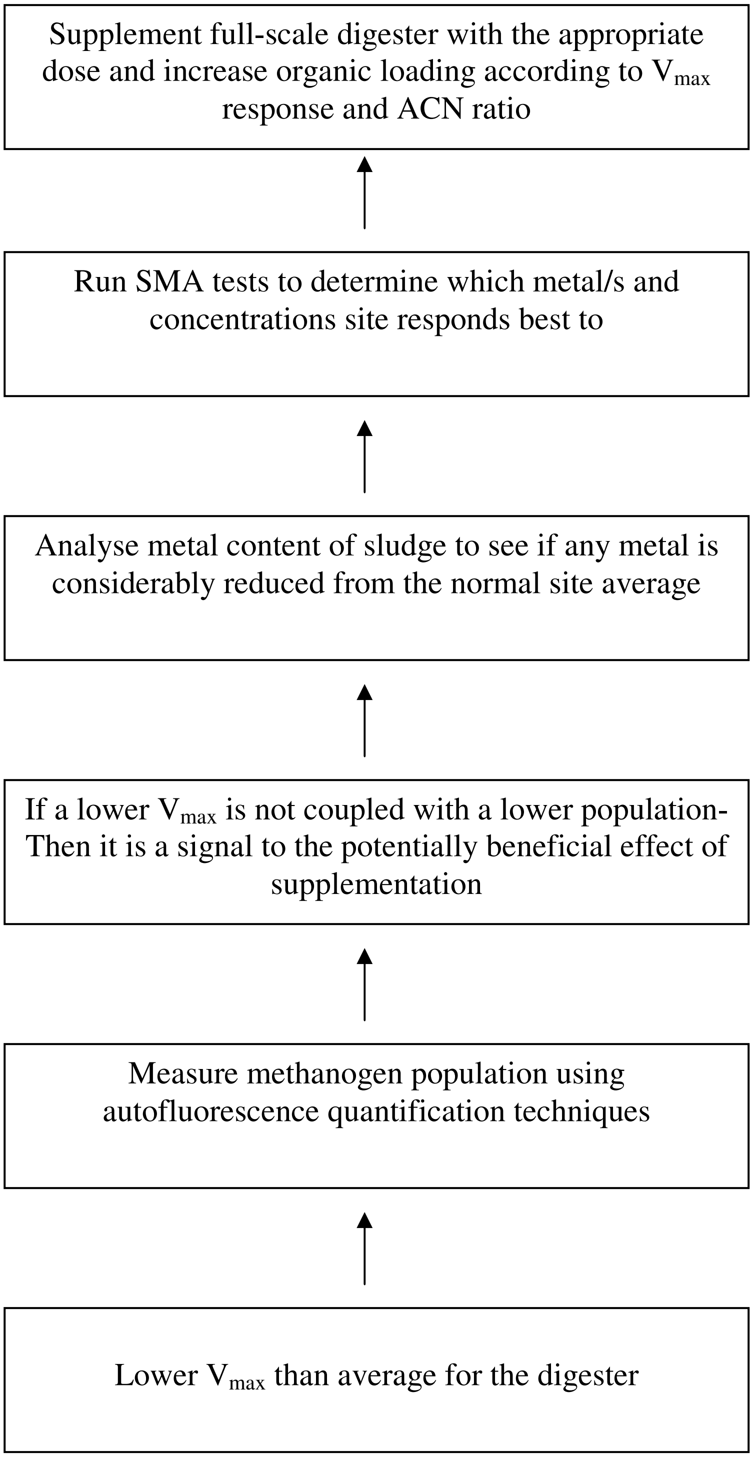


Figure 7.1 Application of the SMA test

A lower than average V_{max} observation should be followed by quantification of the methanogen population using fluorescence based techniques. If a lower V_{max} is not coupled with a lower methanogenic population then it indicates supplementation maybe beneficial. Metal content data should be used as an indicator to determine which metals are limiting based on the site specific profile. Supplementation studies using the SMA methodology should be carried out to determine metal response and the optimal dose to use. Finally the results of SMA test can then be applied in terms of correct concentration of metals to be dosed in the full scale digester alongside the appropriate increase in digester loading as calculated via the ACN ratio. The recommended use of the methodology as described relies on site specific relationships and therefore the digesters standard profile needs be known first.

Further work with metal deficient sludges is required to verify the use of a lower than expected V_{max} for a given sites combined acetoclastic methanogen population as an indicator for metal requirement. In addition further work is required to determine if qPCR results can in fact be calibrated with autofluorescence quantification data and to ascertain its accuracy. It is expected autofluorescence techniques such as those based on measuring factor F_{420} will correlate with qPCR data at a site specific level. The existence of the aforementioned correlation would mean the technique can be used by wastewater treatment works to determine changes in the methanogenic population. Autofluorescence quantification would serve as a more practical alternative to qPCR, which is a very laborious and expensive procedure.

Supplementation strategies aim to achieve the maximum effect on the biological activity whilst avoiding wasteful dosing and limiting introduction of metals in to the environment (as part of effluent). Therefore the determination of bioavailability of individual metal-EDTA complexes is important, as it will help to determine the most readily bioavailable forms of metals to supplement at times of metal deficiencies. This study raises doubts about the level of bioavailability of metal-EDTA chelates other than cobalt-EDTA. To fully elucidate a complexes bioavailability it would need to be supplemented to sludge knowingly deficient in the respective metal. It is expected that metals which form a complex with EDTA that has a high stability constant and are taken up by non-specific low affinity uptake system will not be readily bioavailable. Only metals that are taken up by high affinity specific uptake system are expected to be readily bioavailable to the methanogens when complexed with EDTA. Although in methanogens nickel has been shown to be taken up via a related pathway to cobalt, it is expected the difference in the stability constants of Ni-EDTA and Co-EDTA is sufficient to make Ni-EDTA non-bioavailable. Any response observed from Ni-EDTA supplementation in nickel deficient samples is expected to be related to the presence of other metals that can form a higher stability constant with EDTA. The possible displacement effect described for Ni-EDTA in wastewater sludge digesters is expected to be limited and where present it is expected to be primarily related to metals such as copper.

In order for metal supplementation to be considered viable and to persuade wastewater treatment works to start dosing their digesters, any economic benefits gained from supplementation would need to be greater than the costs incurred. The promotion of metal dosing would require further work in the form of costing supplementation relative to the increase in energy recovery and any additional revenue from treating imported sludge and its associated digestate. Such costing can then be calculated back and represented as a minimum increase in the V_{max} that is required for supplementation to be considered cost effective for a particular digester. Furthermore long-term studies would be required to determine full benefits from supplementation such as those on growth and stability. These benefits should also be taken into consideration for an accurate cost-benefit analysis of metal supplementation.

REFERENCES

Acinas, S.G., Marcelino, L.A., Klepac-Ceraj, V. and Polz, M.F. (2004) Divergence and Redundancy of 16S rRNA Sequences in Genomes with Multiple rrn Operons. **Journal of Bacteriology**, 186: (9): 2629-2635.

Al-Asheh, S. and Duvnjak, Z. (1995) Adsorption of Copper and Chromium by *Aspergillus carbonarius*. **Biotechnology Progress**, 11: (6): 638-642.

Alloway, B.J. and Jackson, A.P. (1991) The Behaviour of Heavy Metals in Sewage Sludge-Amended Soils. **The Science of the Total Environment**, 100: 151-176.

Aquino, S.F. and Stuckey, D.C. (2007) Bioavailability and Toxicity of Metal Nutrients during Anaerobic Digestion. **Journal of Environmental Engineering**, 133: (1): 28-35.

Aquino, S.F. and Stuckey, D.C. (2003) Production of Soluble Microbial Products SMP in Anaerobic Chemostats under Nutrient Deficiency. **Journal of Environmental Engineering**, 129: (11): 1007–1014.

Babich, H. and Stotzky, G. (1983) "Toxicity of Nickel to Microbes: Environmental Aspects". In Allen, I.L. (Ed.) **Advances in Applied Microbiology**. Academic Press, New York, USA.

Bainoitti, A.E. and Nishio, N. (2000) Growth Kinetics of *Acetobacterium sp*. On Methanol-Formate in Continous Culture. **Journal of Applied Microbiology**, 88: (2): 191-201.

Bartacek, J., Fermoso, F.G., Baldó-Urrutia, A.M., Hullebusch, E.D van. and Lens, P.N.L. (2008) Cobalt Toxicity in Anaerobic Granular Sludge: Influence of Chemical Speciation. **Journal of Industrial Microbiology and Biotechnology**, 35: (11): 1465-1474.

Bell, C.F. (1977a) "Aminopolycarboxylic Acids". **Principles and Applications of Metal Chelation**. Clarendon Press, Oxford UK.

Bell, C.F. (1977b) "Properties of Ligands and Chelate Rings". **Principles and Applications of Metal Chelation**. Clarendon Press, Oxford, UK.

Bertram, P.A., Schmitz, R.A., Linder, D. and Thauer, R.K. (1994) Tungstate can Substitute for Molybdate in Sustaining Growth of *Methanobacterium thermoautotrophicum*. **Archives of Microbiology**, 161: (3): 220-228.

Beveridge, T.J. and Doyle, R.J. (1989) **Metal Ions and Bacteria**. Wiley Interscience, New York, USA.

Bolsover, S.R., Shephard, E.A., White, H.A. and Hyams, J.S (2011) **Cell Biology: A Short Course.** John Wiley and Sons, New Jersey, USA.

Boone, D., Chynoweth, D., Mah, R., Smith, P. and Wilkie, A. (1993) Ecology and Microbiology of Biogasification. **Biomass and Bioenergy**, 5: (3-4): 191-202.

Borja, R., Sánchez, E. and Weiland, P. (1996) Influence of Ammonia Concentration on Thermophilic Anaerobic Digestion of Cattle Manure in Upflow Anaerobic Sludge Blanket (UASB) Reactors. **Process Biochemistry**, 31: (5): 477-483.

Brioukhanov, A., Netrusov, A., Sordel, M., Thauer, R.K. and Shima, S. (2000) Protection of *Methanosarcina barkeri* against Oxidative Stress: Identification and Characterization of an Iron Superoxide Dismutase. **Archives of Microbiology**, 174: (3): 213-216.

Butter, T.J., Evison, L.M., Hancock, I.C. and Holland, F.S. (1998) The Kinetics of Metal Uptake by Microbial Biomass: Implications for the Design of a Biosorption Reactor. **Water Science and Technology**, 38: (6): 279-286.

Callander, I.J. and Barford, J.P. (1983) Precipitation, Chelation, and the Availability of Metals as Nutrients in Anaerobic Digestion. I. Methodology. **Biotechnology and Bioengineering**, 25: (8): 1947-1957.

Cánovas-Díaz, M. and Howell, J.A. (1986) Effect of Nickel on Methane Production and Butyric Acid Utilization in a Downflow Fixed-Film Reactor. **Biotechnology Letters**, 8: (4): 287-292.

Climenhaga, M.A. and Banks, C.J. (2008) Anaerobic Digestion of Catering Wastes: Effect of Micronutrients and Retention Time. **Water Science and Technology**, 57: (5): 687-692.

Colleran, E., Concannon, F., Golden, T., Geoghegan, F., Crumlish, B., Killilea, E., Henry, M. and Coates, J. (1991) Use of Methanogenic Activity Tests to Characterize Anaerobic Sludges, Screen for Anaerobic Biodegradability and Determine Toxicity Thresholds against Individual Anaerobic Trophic Groups and Species. **Water Science and Technology**, 25: (7): 31-40.

Conklin, A., Chapman, T., Zahller, J., Stensel, H. and Ferguson, J. (2008) Monitoring the Role of Aceticlasts in Anaerobic Digestion: Activity and Capacity. **Water Research**, 42: (20): 4895-4904.

DECC (2010) "Digest of United Kingdom energy statistics ". Department of Energy and Climate Change, UK

DEFRA (2010) "Accelerating the Uptake of Anaerobic Digestion in England: an Implementation Plan". Department for Environment, Food and Rural Affairs, UK.

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

Eikmanns, B. and Thauer, R.K. (1985) Evidence for the Involvement and Role of a Corrinoid Enzyme in Methane Formation from Acetate in *Methanosarcina barkeri*. **Archives of Microbiology**, 142: (2): 175-179.

Ellefson, W.L., Whitman, W.B. and Wolfe, R.S. (1982) Nickel Containing Factor F430 :Chromophore of the Methylreductase of Methanobacterium. **Proceedings of the National Academy of Sciences of the USA**, 79: (12): 3707-3710.

Fathepure, B.Z. (1987) Factors Affecting the Methanogenic Activity of *Methanothrix soehngenni VNBF*. **Applied Environmental Microbiology**, 53: (12): 2978-2982.

Fermoso, F.G., Bartacek, J., Chung, L.C. and Lens, P.N.L. (2008) Supplementation of Cobalt to UASB Reactors by Pulse Dosing: CoCl2 versus CoEDTA2- Pulses. **Biochemical Engineering Journal**, 42: (2): 111-119.

Ferry, J.G. (1999) Enzymology of One-Carbon Metabolism in Methanogenic Pathways. **FEMS Microbiology Reviews**, 23: (1): 13-38.

Ferry, J.G. (2002) Methanogenesis Biochemistry. **Encyclopedia of Life Sciences.** John Wiley & Sons Ltd, New Jersey, USA.

Florencio, L., Jenicek, P., Field, J.A. and Lettinga, G. (1993) Effect of Cobalt on the Anaerobic Degradation of Methanol. **Journal of Fermentation and Bioengineering**, 75: (5): 368-374.

Forstner, U. (1981) "Metal Transfer Between Solid and Aqueous Phases". In Forstner, U. & Whitman, G.T.W. (Eds.) **Metal Pollution in the Aquatic Environment**. Springer Verlag, Berlin, Heidelberg, New York.

Fowle, D.A. and Fein, J.B. (2000) Experimental Measurements of Heavy Metals by EPS of Activated Sludge. **Water Science and Technology**, 43: (6): 59-66.

Francis, A.J. and Dodge, C.J. (1990) Anaerobic Microbial Remobilisation of Toxic Metals Coprecipitates with Iron Oxide. **Environmental Science and Technology**, 24: 373-378.

Gerardi, M.H. (2003) **The Microbiology of Anaerobic Digesters**. John Wiley & Sons, Inc, New Jersey, USA.

Gilles, H. and Thauer, R.K. (1983) Uroporphyrinogen III, an Intermediate in the Biosynthesis of the Nickel-Containing Factor F430 in *Methanobacterium thermoautotrophicum*. **European Journal of Biochemistry**, 135: (1): 109-112.

Gonzalez-Gil, G., Jansen, S., Zandvoort, M.H., Leeuwen, H.P. van. (2003) Effect of Yeast Extract on Speciation and Bioavailability of Nickel and Cobalt in Anaerobic Bioreactors. **Biotechnology and Bioengineering**, 82: (2): 134-142.

Gonzalez-Gil, G., Kleerebezem, R. and Lettinga, G. (1999) Effects of Nickel and Cobalt on Kinetics of Methanol Conversion by Methanogenic Sludge as Assessed by On-Line CH4 Monitoring. **Applied and Environmental Microbiology,** 65: (4): 1789–1793.

Gould, M.S. and Genetelli, E.J. (1975) Heavy Metal Distribution in Anaerobically Digested Sludges. **In Proceedings of the 30th Industrial Waste Conference**, Purdue University, Indiana, USA, 689-699.

Guo, L., Hunt, B.J., Santschi, P.H. and Ray, S.M. (2001) Effect of Dissolved Organic Matter on the Uptake of Trace Metals by American Oysters. **Environmental Science and Technology**, 35: (5): 885-893.

Hattori, S. (2008) Syntrophic Acetate-Oxidizing Microbes in Methanogenic Environments. **Microbes and Environments,** 23: (2): 118-127.

Hausinger, R.P. (1987) Nickel Utilization by Microorganisms. **Micobiological Reviews**, 51: (1): 22-42.

Hausinger, R.P. (1994) Nickel Enzymes in Microbes. Sceince of the Total Environment **Sceince of the Total Environment**, 148: (2-3): 157-166.

Hoban, D.J. and Van Den Berg, L. (1979) Effect of Iron on Conversion of Acetic Acid to Methane During Methanogenic Fermentations. **Journal of Applied Microbiology**, 47: (1): 153-159.

Housecroft, C.E. and Constable, E.C. (1997a) "Coordination Complexes of the D-Block Metals". **Chemistry: An Integrated Approach**. Addison Wesley Longman Ltd, Massachusetts, USA.

Housecroft, C.E. and Constable, E.C. (1997b) "Thermodynamics and Electrochemistry". **Chemistry: An Integrated Approach**. Addison Wesley Longman Ltd, Massachusetts, USA.

Huang, C.P. and Morehart, A.L. (1990) The Removal of Cu(II) from Diluted Aqueous Solutions by *Saccharomyces cerevisae*. **Water Resources**, 24: (4): 433-439.

Hughes, M.N. and Poole, R.K. (1991) Metal Speciation and Microbial Growth—The Hard (and Soft) Facts. **Journal of General Microbiology**, 137: (4): 725-734.

Ince, O., Anderson, G.K. and Kasapgil, B. (1995) Control of Organic Loading Rate Using the Specific Methanogenic Activity Test During Start-Up of an Anaerobic Digestion System. **Water Research**, 29: (1): 349-355.

Jacobson, F. and Walsh, C. (1984) Properties of 7,8-Didemethyl-8-Hydroxy-5-Deazaflavins Relevant to Redox Coenzyme Function in Methanogen Metabolism. **Biochemistry**, 23: (5): 979-988.

Jansen, S., Gonzalez-Gil, G. and van Leeuwen, H.P. (2007) The Impact of Co and Ni Speciation on Methanogenesis in Sulfidic Media—Biouptake versus Metal Dissolution. **Enzyme and Microbial Technology**, 40: (4): 823-830.

Jarell, K.F. and Sprott, G.D. (1982) Nickel Transport in Methanobacterium bryantii. **Jounal of Bacteriology**, 151: (3): 1195-1203.

Jeris, J.S., McCarty, P.L. (1965) The Biochemistry of Methane Fermentation Using C^{14} Tracers. **Journal of the Water Pollution Control Federation**, 37: (2): 178–192.

Jetten, M.S.M., Stams, A.J.M. and Zehnder, A.J.B. (1992) Methanogenesis from Acetate: a Comparison of the Acetate Metabolism in *Methanothrix soehngenii* and *Methanosarcina spp*. **FEMS Microbiology Letters**, 88: (3-4): 181-197.

Kamlage, B. and Blaut, M. (1993) Isolation of a Cytochrome-Deficient Mutant Strain of *Sporomusa sphaeroides* not Capable of Oxidizing Methyl Groups. **Journal of Bacteriology**, 175: (10): 3043-3050.

Kenealy, W.R. and Zeikus, J.G. (1982) One-Carbon Metabolism in Methanogens: Evidence for Synthesis of a Two-Carbon Cellular Intermediate and Unification of Catabolism and Anabolism in Methanosarcina barkeri. **Journal of Bacteriology**, 151: (2): 932-941.

Kida, K., Shigematsu, T., Kijima, J., Numaguchi, M., Mochinaga, Y., Abe, N. and Morimura, S. (2001) Influence of Ni2+ and Co2+ on Methanogenic Activity and the Amounts of Coenzymes Involved in Methanogenesis. **Journal of Bioscience and Bioengineering**, 91: (6): 590-595.

Kim, B.K. and Daniels, L. (1991) Unexpected Errors in Gas Chromatographic Analysis of Methane Production by Thermophilic Bacteria. **Applied and Environmental Microbiology**, 57: (6): 1866-1869.

Kirby, T.W., Lancaster, J.R. and Fridovich, I. (1981) Isolation and Characterization of the Iron-Containing Superoxide Dismutase of *Methanobacterium bryantii*. **Archives of Biochemistry and Biophysics**, 210: (1): 140-148.

Kohler, H.P.E. and Zehnder, A.J.B. (1984) Carbon Monoxide Dehydrogenase and Acetate Thiokinase in *Methanothrix soehngenii*. **FEMS Microbiology Letters**, 21: (3): 287-292.

Komeda, H., Kobayashi, M. and Shimizu, S. (1997) A Novel Transporter Involved in Cobalt Uptake. **Proceedings of the National Academy of Sciences**, 94: (1): 36-41

Lawson, P.S., Steritt, R.M. and Lester, J.N. (1984) Adsorption and Complexation Mechanisms of Heavy Metal Uptake in Activated Sludge. **Journal of Chemical Technology and Biotechnology**, 34: (B): 253-262.

Ledin, M. (2000) Accumulation of Metals by Microorganisms - Processes and Importance for Soil Systems. **Earth-Science Reviews**, 51: (1-4): 1-31.

Levett, P.N. (1990) **Anaerobic Bacteria**. Open University Press, Buckingham, UK

Lewis, R. and Evans, W. (1997) "Chemical Families". **Chemistry**. Macmillan Press Ltd, Basingstoke, UK.

Lin, D., Kakinozo, T., Nishio, N. and Nagai, S. (1990) Enhanced Cytochrome Formation and Stimulated Methanogenesis Rate by the Increased Ferrous Concentration in *Methanosarcina barkeri* culture. **FEMS Microbiology Letters**, 68: (1-2): 89-92.

Lindoy, L.F. (1989) **The Chemistry of Macrocyclic Ligand Complexes**. Cambridge University Press, Cambridge. UK.

Liu, Y., Lam, M.C. and Fang, H.H.P. (2001) Adsorption of Heavy Metals by EPS of Activated Sludge. **Water Science and Technology**, 43: (6): 59-66.

Londei, P. (2005) Evolution of Translational Initiation: New Insights from the Archaea. **FEMS Microbiology Reviews**, 29: (2): 185-200.

MacNichol, R.D. and Beckett, P.H.T. (1989) The Distribution of Heavy Metals between the Principal Components of Digested Sewage Sludge. **Water Research**, 23: (2): 199-206.

Mah, R.A., Smith, M.R. and Baresi, L. (1978) Studies on an Acetate-Fermenting Strain of *Methanosarcina*. **Applied and Environmental Microbiology**, 35: (6): 1174-1184.

McCarty, P.L. (1964) Anaerobic Waste Treatment Fundamentals. **Public Works**, 95: (9): 107-112.

Mink, R.W. and Dugan, P.R. (1977) Tentative Identification of Methanogenic Bacteria by Fluorescence Microscopy. **Applied and Environmental Microbiology,** 33: (3): 713-717.

Mosey, F.E. (1971) Toxicity of Cadmium to Anaerobic Digestion: its Modification by Inorganic Anions. **Water Pollution Control**, 70: (5): 584-598.

Murray, W.D. and Berg, L.v.d. (1981) Effects of Nickel, Cobalt, and Molybdenum on Performance of Methanogenic Fixed-Film Reactors. **Applied and Environmental Microbiology**, 42: (3): 502-505.

Myers, R.L. (2007) **The 100 Most Important Chemical Compounds**. Greenwood Publishing Group, Connecticut, USA.

Nielsen, S.S. (2010) "Traditional Methods for Mineral Analysis". **Food Analysis**. Springer, New York, USA.

Nies, D.H. (1999) Microbial Heavy-Metal Resistance. **Applied Microbiology and Biotechnology**, 51: (6): 730-750.

Nies, D.H. and Silver, S. (1989) Metal Ion Uptake by a Plasmid-Free Metal-Sensitive Alcaligenes eutrophus Strain. **Jounal of Bacteriology**, 171: (7): 4073-4075.

Noike, T., Endo, G., Chang, J-E., Yaguchi, J-I. and Matsumoto, J-I. (1985) Characteristics of Carbohydrate Degradation and the Rate-Limiting Step in Anaerobic Digestion. **Biotechnology and Bioengineering**, 27: (10): 1482-1489.

Nozoe, T. and Yoshida, K. (1992) Effect of Ni-EDTA on Production of Methane and Decomposition of Volatile Fatty Acids in Paddy Soil. **Soil Science and Plant Nutrition**, 38: (4): 763-766.

Oleskiewsicz, J.A. and Sharma, V.K. (1990) Stimulation and Inhibition of Anaerobic Processes by Heavy Metals-a Review. **Biological Wastes**, 31: (1): 45-67.

Olsen, G.J. and Woese, C.R. (1996) Lessons from an Archaeal Genome: What are we Learning from *Methanococcus jannaschii*? **Trends in Genetics**, 12: (10): 377-379.

Owen, W.F., Stuckey, D.C., Healy Jr, J.B., Young, L.Y. and McCarty, P.L. (1979) Bioassay for Monitoring Biochemical Methane Potential and Anaerobic Toxicity. **Water Research**, 13: (6): 485-492.

Pankhania, I.P. and Robinson, J.P. (1984) Heavy Metal Inhibition of Methanogenesis by *Methanospirillum hungatei GP1*. **FEMS Microbiology Letters**, 22: (3): 277-281.

Parkin, G.F. and Owen, W.F. (1986) Fundamentals of Anaerobic Digestion of Wastewater Sludges. **Journal of Environmental Engineering**, 112: (5): 867-920.

Patel, G.B. and Can, J. (1984) Characterization and Nutritional Properties of *Methanothrix concilii sp. nov*., a Mesophilic, Aceticlastic Methanogen. **Microbiology**, 30: (11): 1383-1396.

Petsko, G.A. and Ringe, D. (2004) **Protein Structure and Function**. New Science Press, Ltd, London, UK.

Pickering, W.F. (1986) Metal Ion Speciation-Soils and Sediments (A Review). **Ore Geology Reviews**, 1: 83-146.

Pirt, M.W. and Pirt, S.J. (1977) Photosynthetic Production of Biomass and Starch by Chlorella in Chemostat Culture. **Journal of Applied Chemistry and Biotechnology**, 27: (6): 643-650.

Psenner, R. and Puckso, R. (1988) Phosphorous Fractionation: Advantages and Limits of the Method for the Study of Sediment P Origins and Interactions. **Ergebnisse der Limnologie**, 30: 43-59.

Rapin, F., Tessier, A., Campbell, P.G.C. and Carignan, R. (1986) Potential Artefacts in the Determination of Metal Partitioning in Sediments by Sequential Extraction Procedure. **Environmental Science and Technology**, 20: (8): 836-840.

Raposo, F., Banks, C.J., Siegert, I., Heaven, S. and Borja, R. (2006) Influence of Inoculum to Substrate Ratio on the Biochemical Methane Potential of Maize in Batch Tests. **Process Biochemistry**, 41: (6): 1444-1450.

Reddy, K.H. (2003) "The Chemistry of Vitamin B12 and Related Compounds". **Bioinorganic Chemistry**. New Age International Publishers Ltd, New Delhi, India .

Reynolds, P.J. and Colleran, E. (1987) Evaluation and Improvement of Methods for Coenzyme F420 Analysis in Anaerobic Sludges. **Journal of Microbiological Methods**, 7: (2-3): 115-130.

Ripley, L., Boyle, W. and Converse, J. (1986) Improved Alkimetric Monitoring for Anaerobic Digestion of High Strength Wastes. **Journal of Water Pollution Control Federation**, 58: (5): 406-411.

Rocha, E., Selby, T., Coleman, J. and Smith, C.J. (1996) Oxidative Stress Response in an Anaerobe, *Bacteroides fragilis*: a Role for Catalase in Protection Against Hydrogen Peroxide. **Journal of Bacteriology**, 178: (23): 6895-6903.

Rodionov, D.A., Hebbeln, P., Gelfand, M.S. and Eitinger, T. (2006) Comparative and Functional Genomic Analysis of Prokaryotic Nickel and Cobalt Uptake Transporters: Evidence for a Novel Group of ATP-Binding Cassette Transporters. **Journal of Bacteriology**, 188: (1): 317-327.

Rudd, T., Sterrit, R.M. and Lester, J.N. (1984) Formation and Conditional Stability Constants of Complexes Formed Between Heavy Metals and Bacterial Extracellular Polymers. **Water Research**, 18: (3): 389-384.

Scherer, P., Lippert, H. and Wolff, G. (1983) Composition of the Major Elements of 10 Methanogenic Bacteria Determined by Inductive Coupled Plasma Emission Spectroscopy. **Biological Trace Elements Research**, 5: (3): 149-163.

Scherer, P. and Sahm, H. (1981) Effect of Trace Elements and Vitamins on the Growth of *Methanosarcina barkeri*. **Acta Biotechnologica**, 1: (1): 57-65.

Schink, B. (1992) **The Prokaryotes**. Springer Verlag, New York, USA.

Schoen, M.A., Sperl, D., Gadermaier, M., Goberna, M., Franke-Whittle, I., Insam, H., Ablinger, J. and Wett, B. (2009) Population Dynamics at Digester Overload Conditions. **Bioresource Technology**, 100: (23): 5648-5655.

Schonheit, P., Moll, J. and Thauer, R.K. (1979) Nickel, Cobalt and Molybdenum Requirement for Growth of *Methanbacterium thermoautotrophicum*. **Archives of Microbiology**, 123: (1): 105-107.

Sekiguchi, Y., Kamagata, Y. and Harada, H. (2001) Recent Advances in Methane Fermentation Technology. **Current Opinion in Biotechnology**, 12: (3): 277-282.

Shelton, D.R. and Tiedje, J.M. (1984) General Method for Determining Anaerobic Biodegradation Potential. **Applied and Environmental Microbiology**, 47: (4): 850-857.

Sillen, L.G. and Martell, A.E. (1971) **Stability Constants**. Chemical Society, London, UK.

Smith, J. (2006) **An Investigation into the Anaerobic Digestibility of Iron-dosed Activated Sludge.** PhD, University of Birmingham, Birmingham, UK.

Smith, K. and Ingramsmith, C. (2007) *Methanosaeta*, the Forgotten Methanogen? **Trends in Microbiology**, 15: (4): 150-155.

Smith, P.H. and Mah, R.A. (1966) Kinetics of Acetate Metabolism during Sludge Digestion. **Applied and Environmental Microbiology**, 14: (3): 368-371.

Smith, R.L., Gottlieb, E., Kucharski, L.M. and Maguire, M.E. (1998) Functional Similarity between Archaeal and Bacterial CorA Magnesium Transporters. **Journal of Bacteriology**, 180: (10): 2788-2791.

Snoeyink, V.l. and Jenkins, D. (1980) "Coordination Chemistry". **Water Chemistry**. John Wiley & Sons, New Jersey, USA.

Soto, M., Méndez, R. and Lema, J.M. (1993) Methanogenic and Non-Methanogenic Activity Tests- Theoretical basis and experimental set up. **Water Research**, 27: (8): 1361-1376.

Speece, R.E. (1988) A Survey of Municipal Anaerobic Sludge Digesters and Diagnostic Activity Assays. **Water Research**, 22: (3): 365-372.

Speece, R.E. (1996) **Anaerobic Biotechnology for Industrial Wastewaters**. Archae Press, Tennessee, U.S.A.

Speece, R.E., Parkin, G.F. and Gallagher, D. (1983) Nickel Stimulation of Anaerobic Digestion. **Water Research**, 17: (6): 677-683.

Sprott, G.D. and Beveridge, T.J. (1993) "**Microscopy"** In Ferry, J.G (Ed.) **Methanogenesis**. Chapman and Hall, New York, USA..

Steigerwald, V.J., Pihl, T.D. and Reeve, J.N. (1992) Identification and Isolation of the Polyferredoxin from *Methanobacterium thermoautotrophicum* strain delta H. **Proceedings of the National Academy of Sciences***,* 89: (15): 6929-6933.

Stupperich, E., Hans-Jürgen Eisinger and Schurr, S. (1990) Corrinoids in Anaerobic Bacteria. **FEMS Microbiology Letters***,* 87: (3-4): 355-359.

Stupperich, E., Steiner, I. and Eisinger, H.J. (1987) Substitution of Co Alpha-(5-Hydroxybenzimidazolyl)Cobamide (Factor III) by Vitamin B12 in *Methanobacterium thermoautotrophicum*. **Journal of Bacteriology***,* 169: (7): 3076-3081.

Swaddle, T.W. (1997) "Chelation". **Inorganic Chemsitry: An Industrial and Environmental perspective**. Academic Press Ltd, New York, USA.

Takashima, M., Shimada, K. and Speece, R.E. (2011) Minimum Requirements for Trace Metals (Iron, Nickel, Cobalt, and Zinc) in Thermophilic and Mesophilic Methane Fermentation from Glucose. **Water Environment Research**, 83: (4): 339-346.

Takashima, M. and Speece, R.E. (1989) Mineral nutrient requirements for high-rate methane fermentation of acetate at low SRT. **Water Pollution Control Federation**, 61: (11-12): 1645-1650.

Thauer, R.K. (1998) Biochemistry of Methanogenesis: a Tribute to Marjory Stephenson. **Microbiology***,* 144: (9) 2377-2406.

Thauer, R.K., Hedderich, R. and Fischer., R. (1993) **Methanogenesis**. Chapman & Hall, New York, USA.

Valcke, D. and Verstraete, W. (1983) A Practical Method to Estimate the Acetoclastic Methanogenic Biomass in Anaerobic Sludges **Water Pollution Control Federation***,* 55: (9): 1191-1195.

Vallee, B.L. and Ulner, D.D. (1972) Biochemical Effects of Mercury, Cadmium, and Lead. **Annual Review of Biochemistry***,* 41: 91-128.

Van Den Berg, L., Lentz, C.P., Athey, R.J. and Rooke, E.A. (1974) Assessment of Methanogenie Activity in Anaerobic Digestion: Apparatus and Method. **Biotechnology and Bioengineering***,* 16: (11): 1459-1469.

Wang, J., Huang, C.P. and Allen, H.E. (2003) Modeling Heavy Metal Uptake by Sludge Particulates in the Presence of Dissolved Organic Matter. **Water Resources***,* 37: (20): 4835-4842.

Webb, M. (1970) Interrelationships Between the Utilization of Magnesium and the Uptake of Other Bivalent Cations by Bacteria. **Biochimica et Biophysica Acta**, 222: (2): 428-439.

Whitman, W.B. (1985) "Methanogenic Bacteria". **The Bacteria**. Academic Press, New York USA.

Widdel, F. (1988) "Microbiology and Ecology of Sulphate and Sulfur-Reducing Bacteria". In Zehnder, A.J.B. (Ed.) **Biology of Anaerobic Microrganisms** John Wiley & Sons, New York, USA.

Woese, C. and Fox, G. (1977) Phylogenetic Structure of the Prokaryotic Domain: The Primary Kingdoms. **Proceedings of the National Academy of Sciences of the USA**, 74: (11): 5088-5090.

Yu, Y., Lee, C., Kim, J. and Hwang, S. (2005) Group-Specific Primer and Probe Sets to Detect Methanogenic Communities Using Quantitative Real-Time Polymerase Chain Reaction. **Biotechnology and Bioengineering**, 89: (6): 670–679.

Zandvoort, M.H., van Hullebusch, E.D., Fermoso, F.G. and Lens, P.N.L. (2006) Trace Metals in Anaerobic Granular Sludge Reactors: Bioavailability and Dosing Strategies. **Engineering in Life Sciences**, 6: (3): 293-301.

Zeikus, J.G. (1977) The Biology of Methanogenic Bacteria. **Bacteriological Reviews**, 41: (2): 514-541.

Zeikus, J.G. (1979) Microbial Populations in Digesters. In Stafford, D.A, Wheatley, B.I. and Hughes, D.E. (Ed.) **Proceedings of the First International Symposium on Anaerobic Digestion**, Applied Science Publishers, London, UK.

Zhang, Y., Rodionov, D., Gelfand, M. and Gladyshev, Vadim. (2009) Comparative Genomic Analyses of Nickel, Cobalt and Vitamin B12 Utilization. **BMC Genomics**, 10: (1): 78.

Zinder, S.H. and Koch, M. (1984) Non-Aceticlastic Methanogenesis from Acetate: Acetate Oxidation by a Thermophillic Syntrophic Coculture. **Archives of Microbiology**, 138: (3): 263-272.

Zitomer, D.H., Johnson, C.C. and Speece, R.E. (2008) Metal Stimulation and Municipal Digester Thermophilic/Mesophilic Activity **Journal of Environmental Engineering**, 134: (1): 42-47.

APPENDICES

Appendix A Experimental Programme

Samples and Experiments				
Sample	TM Solution Supplementation	qPCR	Cobalt-only Supplementation	Solubility Tracking
A0909	✓	✓		
A0210	✓	✓		
A0310	✓	✓		
A0510	✓	✓		
A0810	✓	✓	✓	✓
A0111	✓		✓	
B0310	✓	✓		
B0710	✓	✓	✓	✓
B1109	✓	✓		
C0310	✓	✓		
C0810	✓	✓	✓	✓
C1109	✓	✓		
C0211	✓		✓	
D0610	✓	✓	✓	
D0710	✓	✓	✓	
D1109	✓	✓		

Appendix B Total Fraction Element Content for Each Sample

As determined using acid digestion

Ca, Al, Na, Mg, P, Fe, K and S shown in g/kg TS all others shown in mg/kg TS

Sample	**Ca**	**Al**	**Na**	**Mg**	**P**	**Fe**	**K**	**S**
A0210	15.54	7.25	8.81	5.70	16.58	8.81	3.63	-
A0310	16.87	4.62	-	4.34	21.37	10.77	6.92	4.78
A0510	20.10	1.70	4.31	2.11	18.15	10.63	3.56	8.92
A0810	53.09	11.82	5.47	3.59	26.43	11.77	0.96	11.99
A0909	19.19	8.62	14.49	5.87	17.62	8.22	3.92	-
A0111	53.37	12.98	5.39	5.68	32.32	13.94	5.01	11.12
C0810	83.42	5.31	2.54	4.23	35.94	78.67	3.90	13.24
C0211	64.18	4.10	1.70	4.18	33.20	61.05	5.23	8.32
C0310	31.60	0.79	2.11	2.41	25.46	50.81	8.36	11.34
C1109	17.85	5.60	48.68	3.49	21.10	43.81	1.95	-
B0310	60.12	5.82	2.85	3.68	22.97	27.17	4.16	6.53
B0710	70.38	10.78	2.90	3.27	32.38	26.52	3.54	10.04
B1109	21.23	8.07	4.76	4.33	17.83	21.23	4.25	-
D0610	43.43	4.44	1.45	3.78	30.33	53.36	5.19	11.96
D0710	31.48	0.00	0.00	2.74	27.43	50.96	3.00	10.96
D1109	37.68	15.17	4.89	8.32	39.15	52.37	3.92	-

	Mn	**Cu**	**Zn**	**Ni**	**Co**	**Mo**	**Se**	**Pb**	**B**	**Cd**	**Cr**
A0210	300.5	476.7	621.8	88.1	6.2	6.2	4.7	129.5	40.9	13.0	196.9
A0310	164.8	494.5	604.4	274.7	0.0	0.0	0.0	164.8	0.0	109*	164.8
A0510	200.5	551.4	451.1	651.6	0.0	0.0	0.0	0.0	0.0	0.0	250.6
A0810	218.8	612.7	744.0	262.6	0.0	0.0	0.0	218.8	0.0	0.0	262.6
A0909	348.6	470.0	704.9	50.9	7.0	6.7	5.1	133.2	54.8	11.0	66.6
A0111	286.4	763.7	620.5	477.3	0.0	0.0	0.0	95.5	0.0	0.0	334.1
C0810	442.7	684.1	1328.0	362.2	0.0	0.0	0.0	201.2	0.0	0.0	120.7
C0211	305.0	348.6	740.7	392.2	0.0	0.0	0.0	87.1	0.0	0.0	87.1
C0310	226.0	301.3	791.0	452.0	146*	0.0	0.0	75.3	1298	0.0	75.3
C1109	389.5	251.5	1947.3	34.1	16.2	5.7	13.0	186.6	105.5	8.1	67.3
B0310	594.1	475.2	1940.6	435.6	0.0	0.0	0.0	158.4	0.0	0.0	237.6
B0710	977.1	572.8	2055.3	269.5	0.0	0.0	0.0	134.8	101.1	0.0	370.6
B1109	934.2	433.1	1698.5	152.9	13.6	11	13.6	195.3	212.3	5.9	254.8
D0610	219.8	527.5	879.1	483.5	0.0	0.0	0.0	0.0	0.0	0.0	87.9
D0710	217.4	434.8	583.5	521.7	0.0	0.0	0.0	30.9	0.0	0.0	0.0
D1109	538.3	832.0	1468.2	58.7	8.8	11	2.4	151.7	73.4	3.9	93.0

* Erroneous result

Appendix C Soluble Fraction Element Content for Each Sample

All data in mg/l

Sample	Ca	K	Mg	Na	P	S	Fe	Ni	Zn
A0210	55.0	65.0	30.0	170.0	47.5	-	1.6	0.2	1.1
A0310	60.0	65.0	36.0	200.0	55.0	-	1.2	0.3	0.8
A0510	148.0	101.0	24.5	163.5	44.0	29.0	0.0	5.0	0.0
A0810	500.0	39.5	35.5	148.0	40.5	119.5	0.0	1.5	0.0
A0909	46.0	32.0	25.0	120.0	24.0	-	1.8	0.1	1.0
A0111	31.0	89.5	20.5	111.5	83.5	7.5	0.0	3.5	0.5
C0810	96.0	25.0	13.0	23.5	1.0	2.5	0.0	4.5	1.0
C0211	227.0	130.0	26.0	51.0	28.0	10.0	4.0	4.0	2.0
C0310	1094.0	1395.5	165.5	627.5	43.5	103.5	0.0	2.0	0.5
C1109	120.0	41.0	33.0	-	2.6	-	5.1	0.1	2.3
B0310	330.0	43.0	13.0	71.0	27.0	19.0	0.0	5.0	44.0*
B0710	110.5	13.5	0.0	101.5	84.5	0.0	104.8	1.5	0.0
B1109	47.0	85.0	25.0	97.0	26.0	-	2.2	0.1	4.1
D0610	0.0	79.0	19.5	50.0	0.0	11.0	0.0	5.0	0.0
D0710	109.5	89.5	31.5	30.5	0.0	23.0	0.0	5.0	0.0
D1109	57.0	29.0	25.0	44.0	4.8	-	1.1	0.0	1.7

Sample	Co	Cu	Cd	Al	Cr	B	Mn	Pb	Mo	Se
A0210	0.0	0.2	0.0	0.5	0.0	0.4	0.1	0.4	0.0	0.0
A0310	0.0	0.1	0.0	0.5	0.0	0.2	0.1	0.3	0.0	0.0
A0510	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A0810	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A0909	0.0	0.3	0.0	1.0	0.1	0.3	0.1	0.7	0.0	0.0
A0111	0.0	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C0810	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C0211	0.0	2.5	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0
C0310	0.0	0.5	0.0	0.0	0.0	75.5	0.0	0.0	0.0	0.0
C1109	0.0	0.2	0.0	0.4	0.1	2.5	0.2	0.9	0.0	0.0
B0310	0.0	2.0	0.0	0.0	0.0	3.5	0.0	1.5	0.0	0.0
B0710	0.0	0.0	0.0	0.0	0.0	5.5	0.0	0.0	0.0	0.0
B1109	0.0	0.3	0.1	0.8	0.1	4.0	0.2	1.1	0.0	0.0
D0610	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D0710	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D1109	0.0	0.1	0.0	0.4	0.0	0.3	0.1	0.9	0.0	0.0

* Erroneous result

Appendix D Methane Production Data and Curves for Each Sample

The following section provides the average cumulative methane data for each sample series alongside standard deviations between replicates. The methane production curves are shown for each unique assay (plotted with error bars illustrating standard deviation) alongside separate figures illustrating the curve profiles, in the first 100 hours, for the most relevant assays from the series.

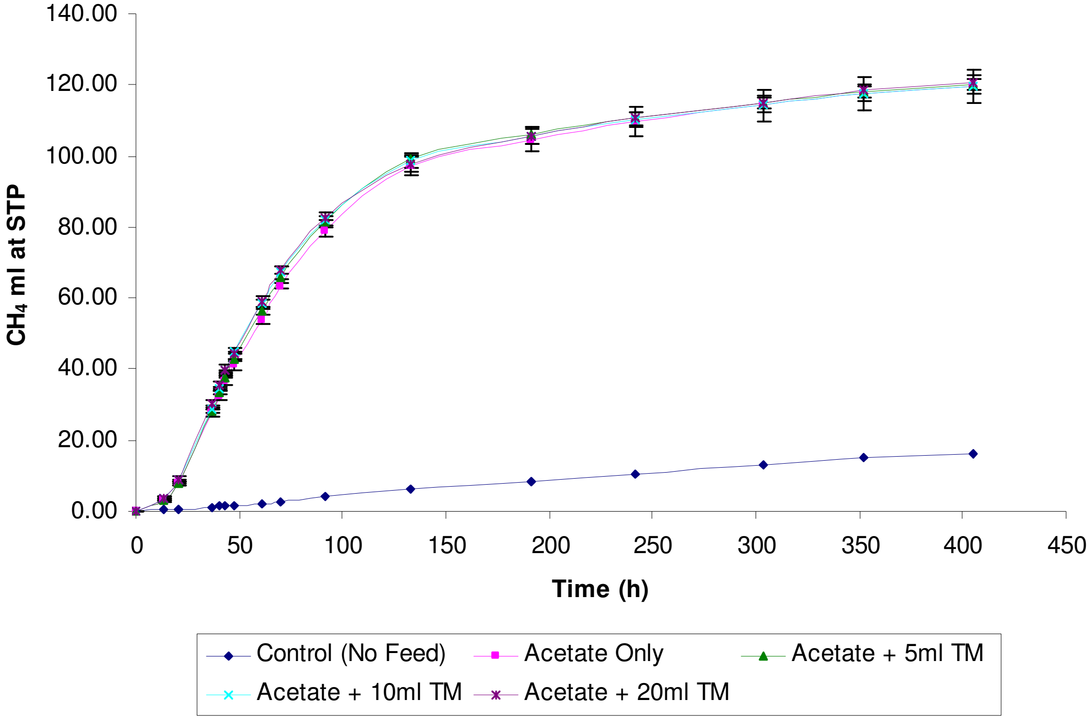
The table below details the periods in each sample from which the V_{max} was calculated.

Sample V_{max} period	
Sample	V_{max} Time (h)
A0909	41.25 – 68.25
A0210	36 – 48.5
A0310	36 – 48.5
A0510	21 – 44.25
A0810	27.75 – 48.5
A0111	36.25 – 45.75
B0310	40.75 – 48
B0710	28.5 – 52.75
B1109	36 – 48.5
C0310	38.25 – 45.75
C0810	27.75 – 48.5
C1109	25.75 - 68
C0211	37 – 44.75
D0610	41.25 – 68.25
D0710	39.75 – 46.25
D1109	40 - 50

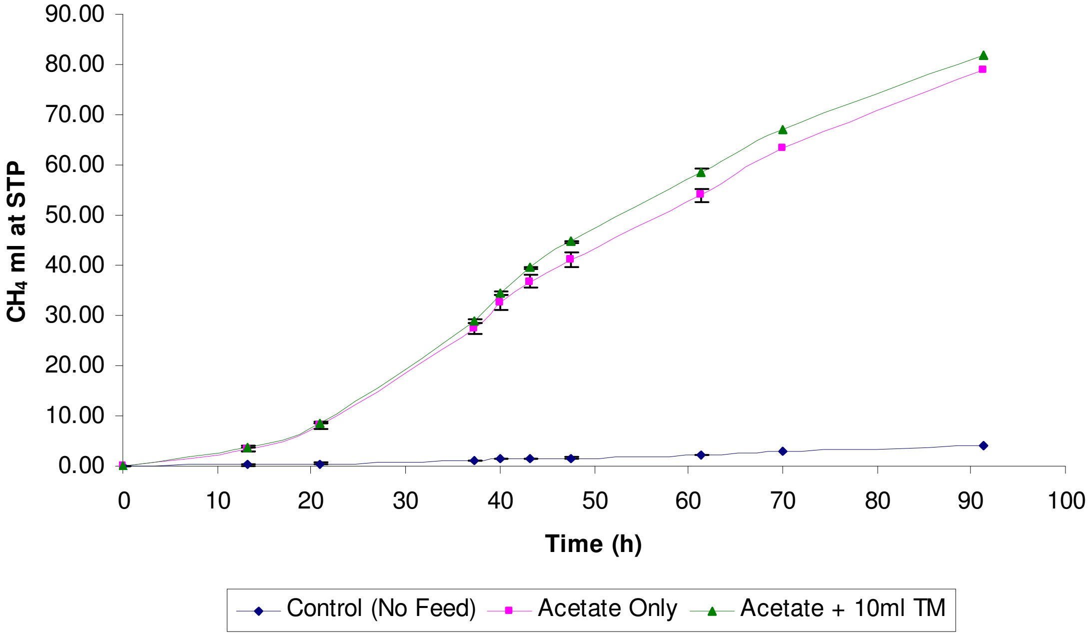
Sample A0210

Average Cumulative Methane (CH_4 ml at STP) from Replicates					
Time (h)	Control (No Feed)	Acetate Only	Acetate + 5 ml TM	Acetate + 10 ml TM	Acetate + 20 ml TM
0	0.00	0.00	0.00	0.00	0.00
13.25	0.31	3.33	3.34	3.56	3.61
21	0.46	8.05	7.73	8.64	9.04
37.25	1.22	27.53	28.09	28.96	30.36
40	1.38	32.63	33.50	34.44	35.55
43.25	1.51	36.79	37.85	39.45	39.87
47.5	1.62	41.01	42.82	44.71	44.38
61.25	2.29	53.97	56.47	58.47	58.95
70	2.87	63.42	65.79	67.13	67.89
91.25	3.97	78.84	81.37	82.02	82.72
132.75	6.28	97.32	99.16	98.64	97.67
191.5	8.31	104.62	106.27	105.62	105.45
241.25	10.53	109.65	110.58	110.12	110.57
303.75	12.99	114.20	114.87	114.35	115.16
351.75	14.94	117.60	118.02	117.54	118.44
405.5	16.09	119.70	120.09	119.70	120.60

Standard Deviation from Replicates					
Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 5 ml TM n = 5	Acetate + 10 ml TM n = 5	Acetate + 20 ml TM n = 5
0	0.000	0.000	0.000	0.000	0.000
13.25	0.134	0.194	0.303	0.598	0.279
21	0.136	0.532	0.399	0.304	0.878
37.25	0.055	1.137	0.666	0.439	0.820
40	0.061	1.338	1.014	0.447	1.009
43.25	0.075	1.385	1.258	0.288	1.249
47.5	0.083	1.418	1.862	0.132	1.459
61.25	0.059	1.377	2.256	0.963	1.865
70	0.074	0.913	2.268	1.920	1.147
91.25	0.230	1.437	2.088	2.240	0.542
132.75	0.403	2.878	2.020	2.224	1.930
191.5	0.595	3.271	2.032	2.281	1.934
241.25	0.672	4.026	2.082	2.002	1.829
303.75	0.729	4.462	2.164	1.984	1.678
351.75	0.715	4.639	2.111	2.029	1.762
405.5	0.706	4.838	2.130	1.936	1.910



CH4 ml at STP
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0
50
100
150
200
250
300
350
400
450
Time (h)
Control (No Feed)
Acetate Only
Acetate + 5ml TM
Acetate + 10ml TM
Acetate + 20ml TM

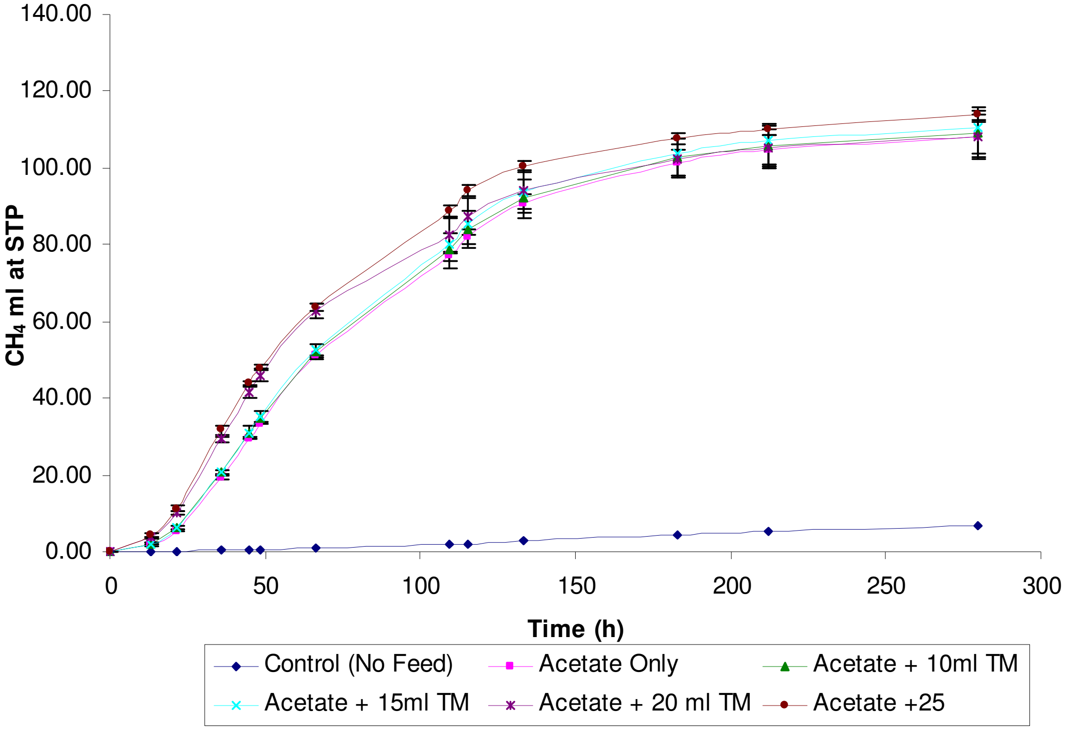


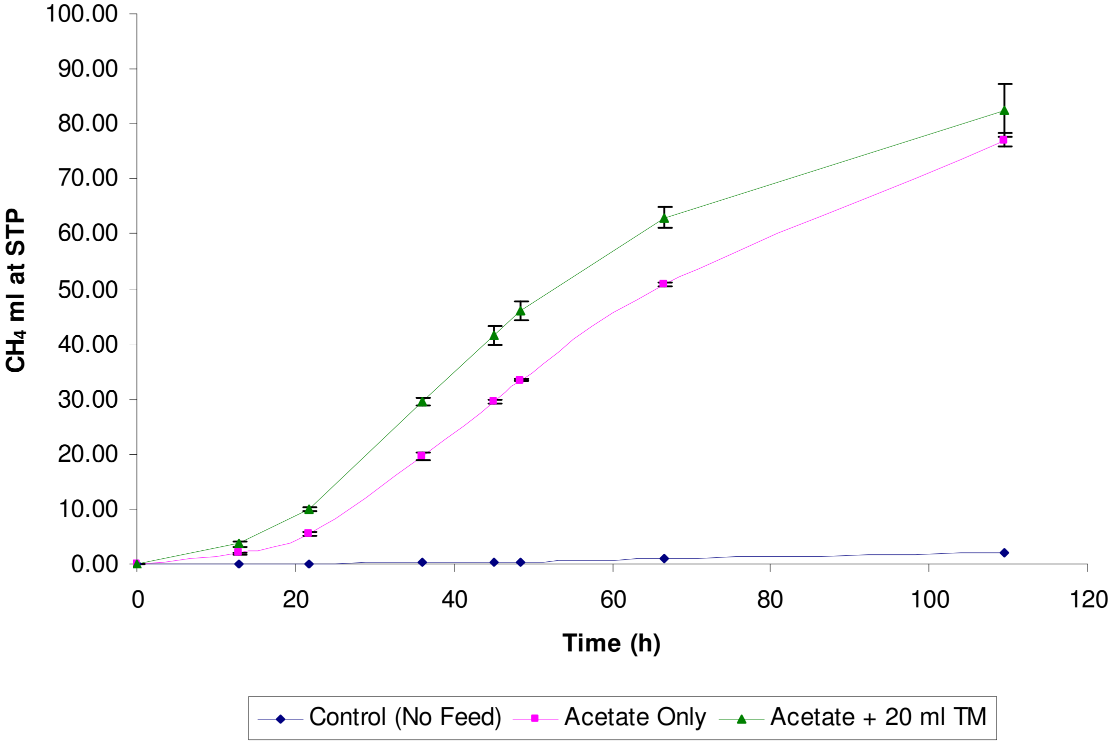
CH4 ml at STP
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
10
20
30
40
50
60
70
80
90
100
Time (h)
Control (No Feed)
Acetate Only
Acetate + 10ml TM

Sample A0310

Average Cumulative Methane (CH_4 ml at STP) from Replicates						
Time (h)	Control (No Feed)	Acetate Only	Acetate + 10 ml TM	Acetate + 15 ml TM	Acetate + 20 ml TM	Acetate + 25 ml TM
0	0.00	0.00	0.00	0.00	0.00	0.00
13	0.02	2.04	2.06	2.13	3.66	4.20
21.75	0.05	5.53	6.07	6.23	9.99	11.32
36	0.29	19.55	20.60	20.70	29.40	31.62
45	0.36	29.67	31.03	31.09	41.72	43.77
48.5	0.36	33.47	34.93	35.20	46.10	47.99
66.5	0.89	50.96	52.14	52.60	62.93	63.73
109.5	1.95	77.06	78.49	80.03	82.46	88.79
115.25	2.02	82.04	83.98	85.58	87.42	94.12
133.5	2.69	90.58	92.07	93.67	94.23	100.47
183	4.33	101.53	102.84	104.02	102.48	107.59
212.25	5.18	104.68	105.74	107.11	105.05	110.21
279.75	6.68	107.95	108.96	110.61	108.23	114.09

Standard Deviation from Replicates						
Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 10 ml TM n = 5	Acetate + 15 ml TM n = 5	Acetate + 20 ml TM n = 5	Acetate + 25 ml TM n = 5
0	0.000	0.000	0.000	0.000	0.000	0.000
13	0.018	0.177	0.330	0.268	0.428	0.470
21.75	0.026	0.354	0.420	0.403	0.459	0.817
36	0.073	0.658	0.954	0.517	0.686	1.125
45	0.087	0.357	1.419	0.912	1.728	0.582
48.5	0.087	0.242	1.565	1.218	1.707	0.560
66.5	0.235	0.370	1.767	1.525	1.929	1.082
109.5	0.177	1.150	1.365	1.744	4.663	1.579
115.25	0.203	1.756	1.639	1.520	5.009	1.494
133.5	0.209	2.394	1.974	1.449	5.026	1.523
183	0.281	3.351	2.355	1.687	5.055	1.392
212.25	0.347	3.925	2.339	1.714	5.207	1.444
279.75	0.498	4.055	2.449	1.793	5.906	1.564

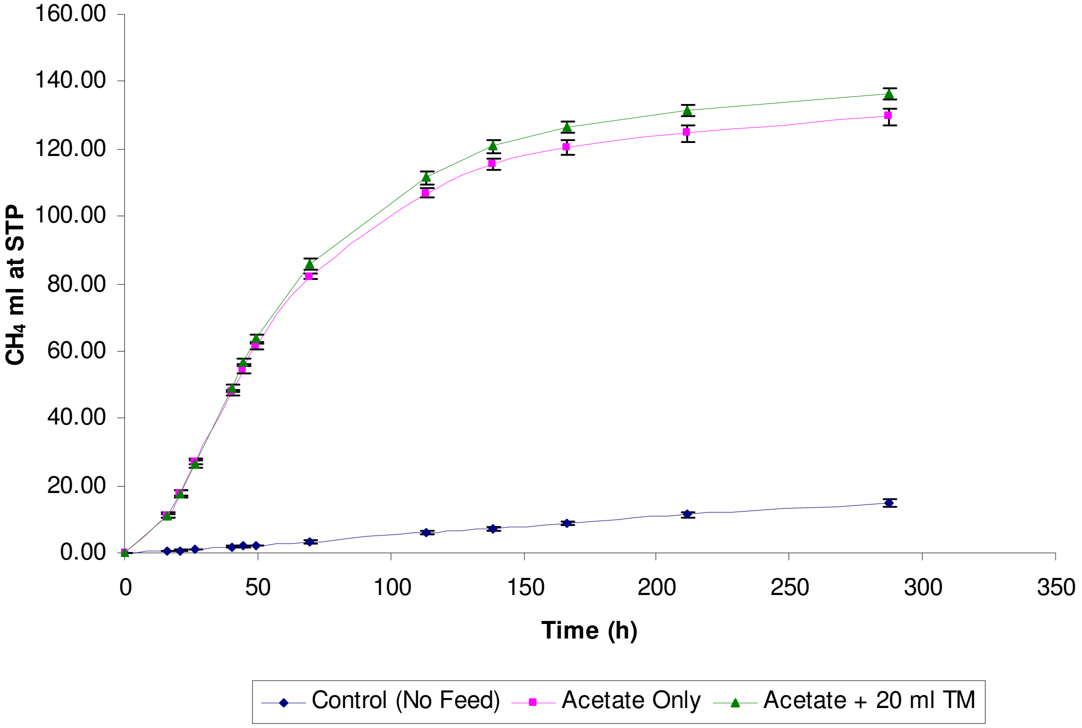

140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH_4 ml at STP
0
50
100
150
200
250
300
Time (h)
Control (No Feed)
Acetate Only
Acetate + 10ml TM
Acetate + 15ml TM
Acetate + 20 ml TM
Acetate +25


100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH_4 ml at STP
0
20
40
60
80
100
120
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM

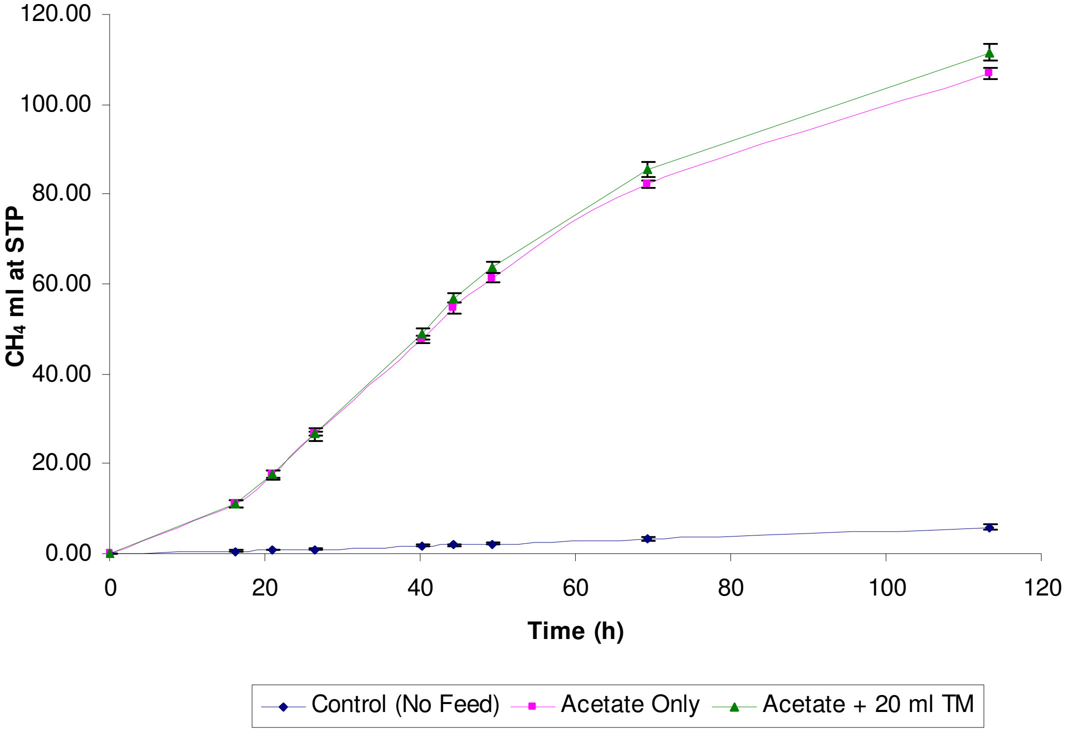
Sample A0510

Average Cumulative Methane (CH_4 ml at STP) from Replicates			
Time (h)	Control (No Feed)	Acetate Only	Acetate + 20 ml TM
0	0.00	0.00	0.00
16.25	0.62	11.02	11.17
21	0.81	17.77	17.51
26.5	0.99	26.78	26.57
40.25	1.78	47.66	48.90
44.25	2.02	54.68	56.79
49.25	2.18	61.41	63.62
69.25	3.44	82.14	85.59
113.25	5.91	106.92	111.49
138.75	7.31	115.42	120.72
166.5	8.97	120.25	126.64
211.25	11.38	124.55	131.17
287.5	14.73	129.54	136.25

Standard Deviation from Replicates			
Time (h)	Control (No Feed) n = 3	Acetate Only n = 4	Acetate + 20 ml TM n = 4
0	0.000	0.000	0.000
16.25	0.049	0.708	0.801
21	0.078	0.777	1.146
26.5	0.114	0.528	1.377
40.25	0.168	0.966	1.305
44.25	0.215	1.146	1.033
49.25	0.251	1.157	1.289
69.25	0.428	0.878	1.670
113.25	0.530	1.313	1.825
138.75	0.511	1.790	1.799
166.5	0.603	2.097	1.609
211.25	0.836	2.219	1.656
287.5	0.969	2.431	1.543



160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH₄ ml at STP
0
50
100
150
200
250
300
350
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM



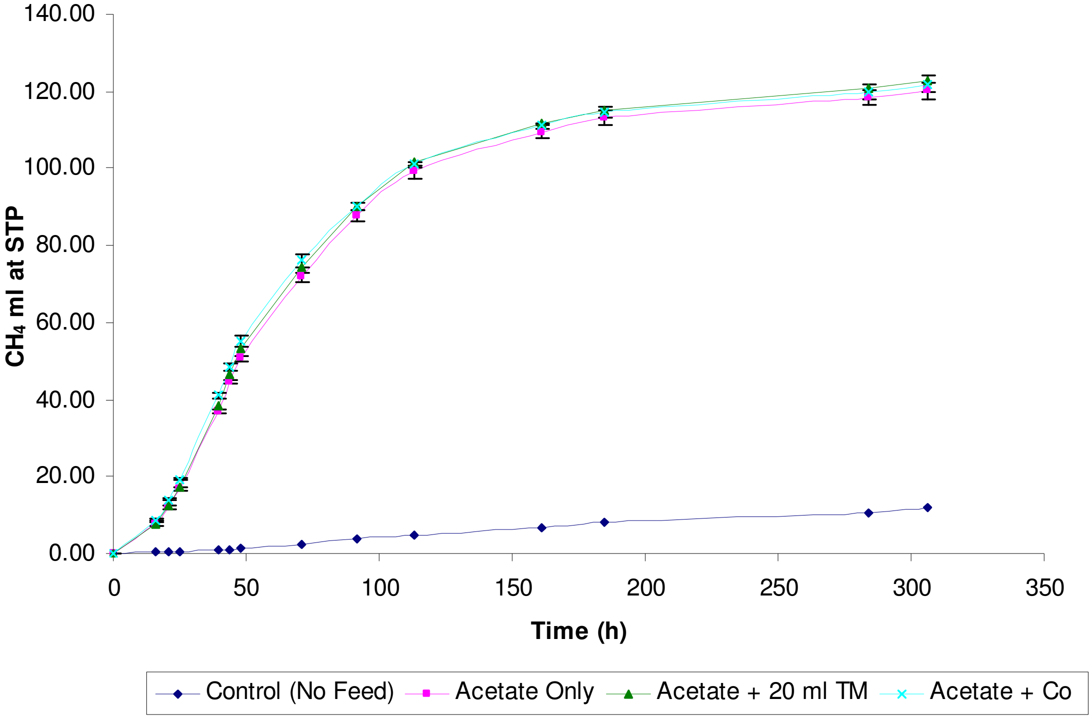
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH₄ ml at STP
0
20
40
60
80
100
120
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM

Sample A0810

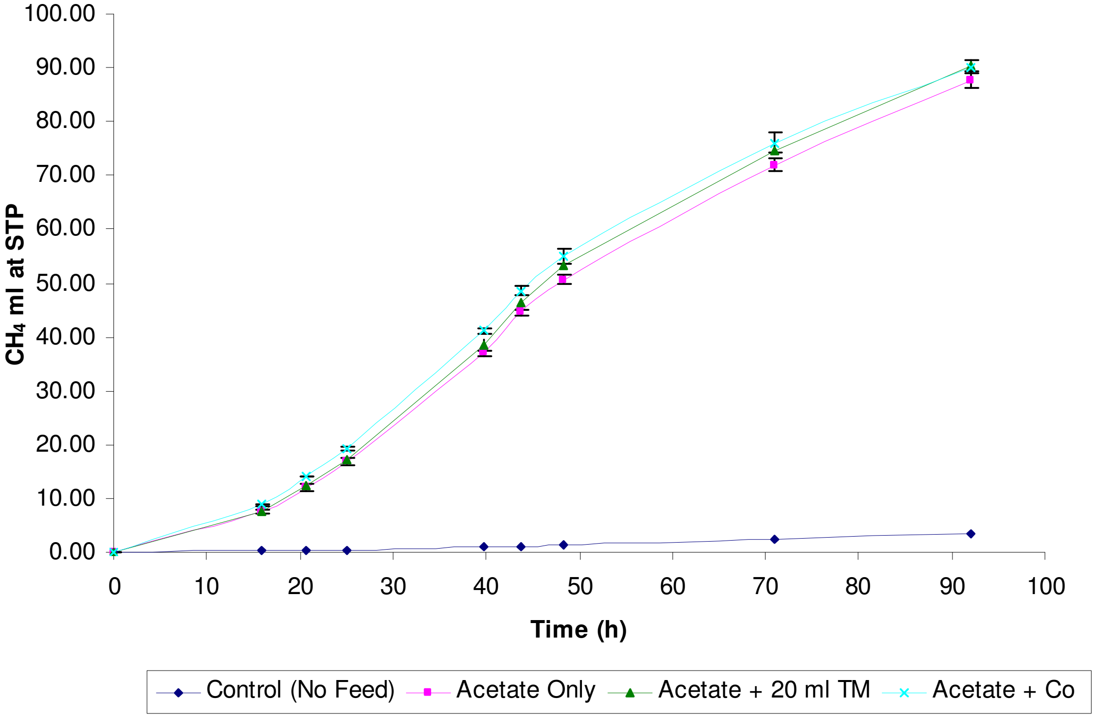
Average Cumulative Methane (CH_4 ml at STP) from Replicates				
Time (h)	Control (No Feed)	Acetate Only	Acetate + 20 ml TM	Acetate + Co*
0	0.00	0.00	0.00	0.00
16	0.36	7.51	7.47	8.80
20.75	0.46	12.06	12.30	14.09
25	0.51	16.77	17.16	19.29
39.75	1.02	37.07	38.52	41.09
43.75	1.15	44.54	46.49	48.57
48.25	1.36	50.66	53.25	55.08
71	2.47	71.85	74.43	76.06
92	3.61	87.75	90.31	90.20
113	4.72	99.04	101.63	100.95
161	6.72	109.52	111.52	111.08
185	7.93	113.20	115.19	114.48
284.25	10.74	118.37	120.70	119.92
306	11.93	120.16	122.77	121.90

Standard Deviation from Replicates				
Time (h)	Control (No Feed) n = 3	Acetate Only n = 4	Acetate + 20 ml TM n = 3	Acetate + Co* n = 3
0	0.000	0.000	0.000	0.000
16	0.110	0.409	0.053	0.226
20.75	0.093	0.575	0.075	0.100
25	0.077	0.585	0.305	0.263
39.75	0.142	0.528	0.839	0.610
43.75	0.169	0.511	0.513	0.924
48.25	0.183	0.719	0.440	1.453
71	0.197	1.199	0.177	1.785
92	0.180	1.648	0.365	1.116
113	0.173	1.800	0.463	0.567
161	0.150	1.764	0.617	0.818
185	0.149	1.803	0.696	1.530
284.25	0.157	1.935	0.703	2.021
306	0.196	2.124	0.678	2.188

* Cobalt was supplemented at the equivalent concentrations to that present within 20 ml of the TM solution - 224 µg.



140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH$_4$ ml at STP
0
50
100
150
200
250
300
350
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + Co

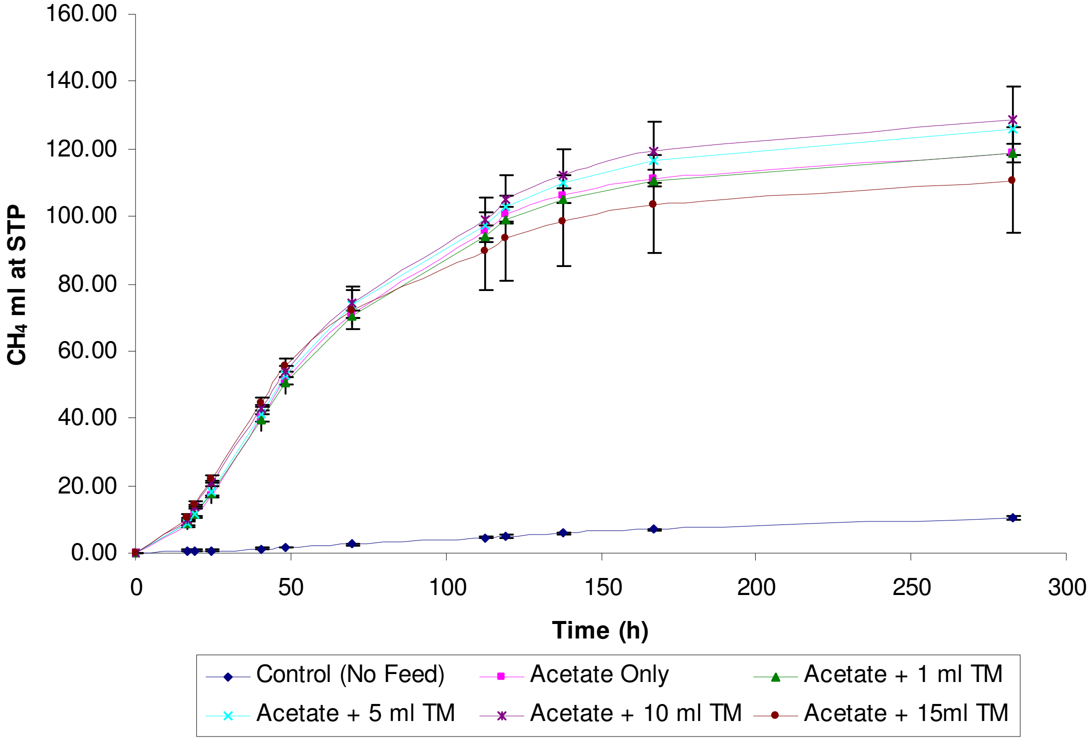


100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH$_4$ ml at STP
0
10
20
30
40
50
60
70
80
90
100
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + Co

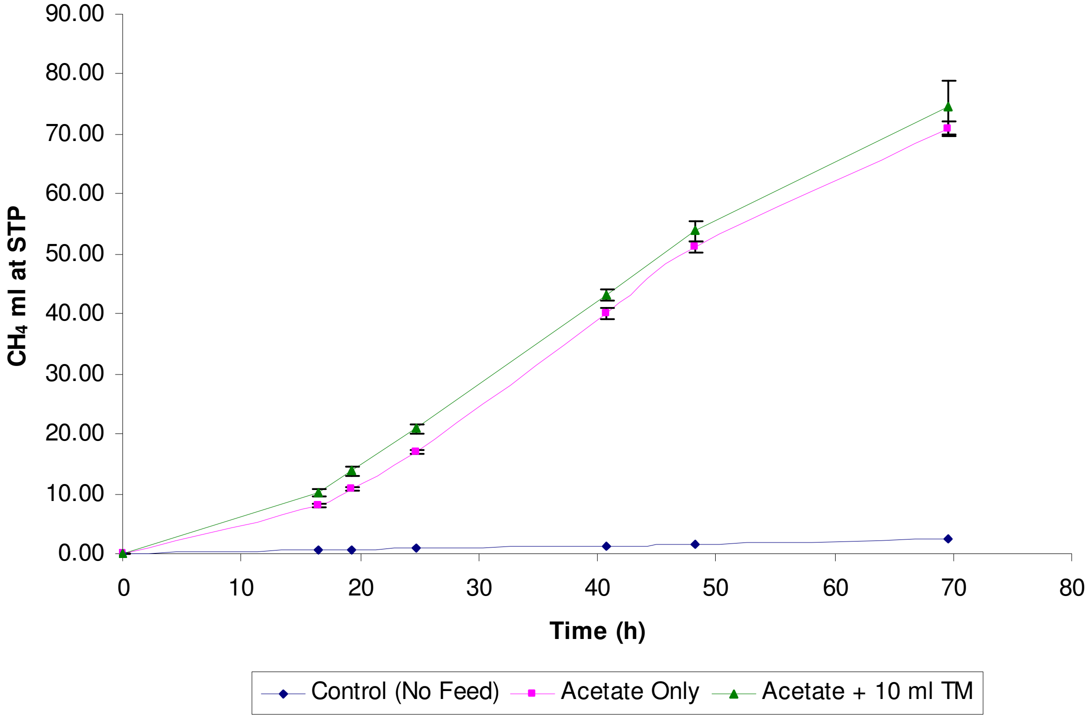
Sample A0909

Average Cumulative Methane (CH_4 ml at STP) from Replicates						
Time (h)	Control (No Feed)	Acetate Only	Acetate + 1 ml TM	Acetate + 5 ml TM	Acetate + 10 ml TM	Acetate + 15 ml TM
0	0.00	0.00	0.00	0.00	0.00	0.00
16.5	0.59	8.09	8.54	8.67	10.06	10.66
19.25	0.65	10.78	11.44	11.72	13.73	14.46
24.75	0.81	16.97	17.42	17.96	20.82	22.22
40.75	1.36	40.14	39.86	41.02	43.10	44.77
48.25	1.62	51.15	50.57	52.71	53.79	55.80
69.5	2.54	70.92	70.21	73.83	74.49	72.17
113	4.62	95.50	93.84	97.52	98.89	89.45
119	4.99	100.76	99.22	103.06	104.91	93.56
137.75	5.80	106.18	104.98	109.83	112.06	98.67
167	6.94	111.16	110.74	116.49	119.06	103.54
283	10.44	118.80	118.90	125.98	128.43	110.67

Standard Deviation from Replicates						
Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 1 ml TM n = 3	Acetate + 5 ml TM n = 3	Acetate + 10 ml TM n = 3	Acetate + 15 ml TM n = 3
0	0.000	0.000	0.000	0.000	0.000	0.000
16.5	0.251	0.381	1.375	0.570	0.571	0.632
19.25	0.274	0.449	1.900	0.751	0.689	0.747
24.75	0.309	0.344	2.661	1.614	0.851	1.110
40.75	0.209	0.869	3.327	1.600	0.952	1.241
48.25	0.192	0.945	3.407	0.251	1.704	1.730
69.5	0.259	1.278	2.686	1.690	4.429	5.713
113	0.319	1.983	1.966	2.466	6.422	11.531
119	0.331	2.151	1.931	2.764	7.283	12.658
137.75	0.368	2.346	2.406	3.229	8.070	13.678
167	0.419	2.545	2.605	3.742	9.004	14.627
283	0.633	2.817	2.955	4.377	10.155	15.589



160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH$_4$ ml at STP
0
50
100
150
200
250
300
Time (h)
Control (No Feed)
Acetate Only
Acetate + 1 ml TM
Acetate + 5 ml TM
Acetate + 10 ml TM
Acetate + 15ml TM



90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH$_4$ ml at STP
0
10
20
30
40
50
60
70
80
Time (h)
Control (No Feed)
Acetate Only
Acetate + 10 ml TM

Sample A0111

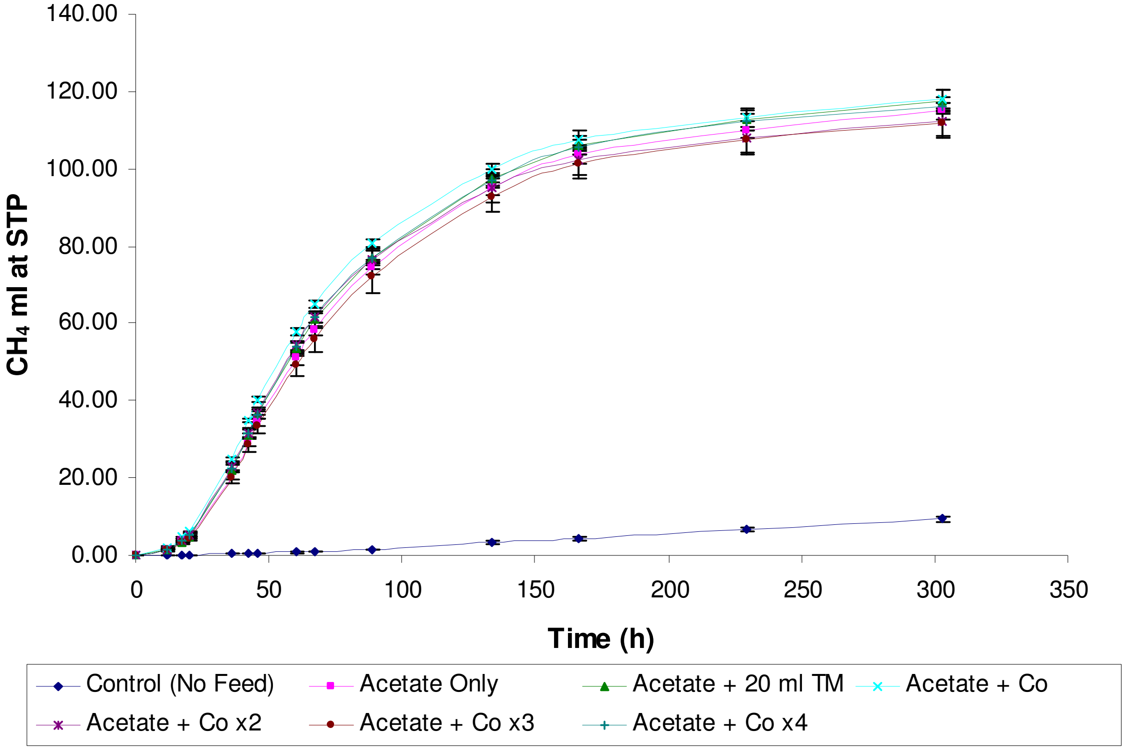
Average Cumulative Methane (CH_4 ml at STP) from Replicates

Time (h)	Control (No Feed)	Acetate Only	Acetate + 20 ml TM	Acetate + Co*	Acetate + Co x2	Acetate + Co x3	Acetate + Co x4
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	0.07	1.50	1.40	1.99	1.64	1.32	1.55
17.5	0.09	3.61	3.50	4.63	3.90	3.23	3.75
20	0.09	4.89	4.71	6.13	5.23	4.40	5.05
36.25	0.30	20.55	21.86	24.87	22.86	20.10	22.50
42	0.38	29.27	31.08	34.70	31.70	28.53	31.37
45.75	0.39	34.38	36.69	40.36	36.80	33.41	36.35
60.25	0.76	51.05	53.33	57.98	54.36	49.15	53.87
67	0.87	58.31	61.10	65.03	61.44	55.89	61.55
88.75	1.46	74.44	77.17	80.72	76.30	71.97	77.15
133.5	3.21	95.25	97.68	99.67	94.88	92.66	96.99
166	4.29	103.46	106.16	107.35	102.24	101.15	105.52
229.25	6.68	110.02	112.62	113.25	108.03	107.68	112.08
303	9.46	114.99	117.73	117.94	112.49	111.63	116.19

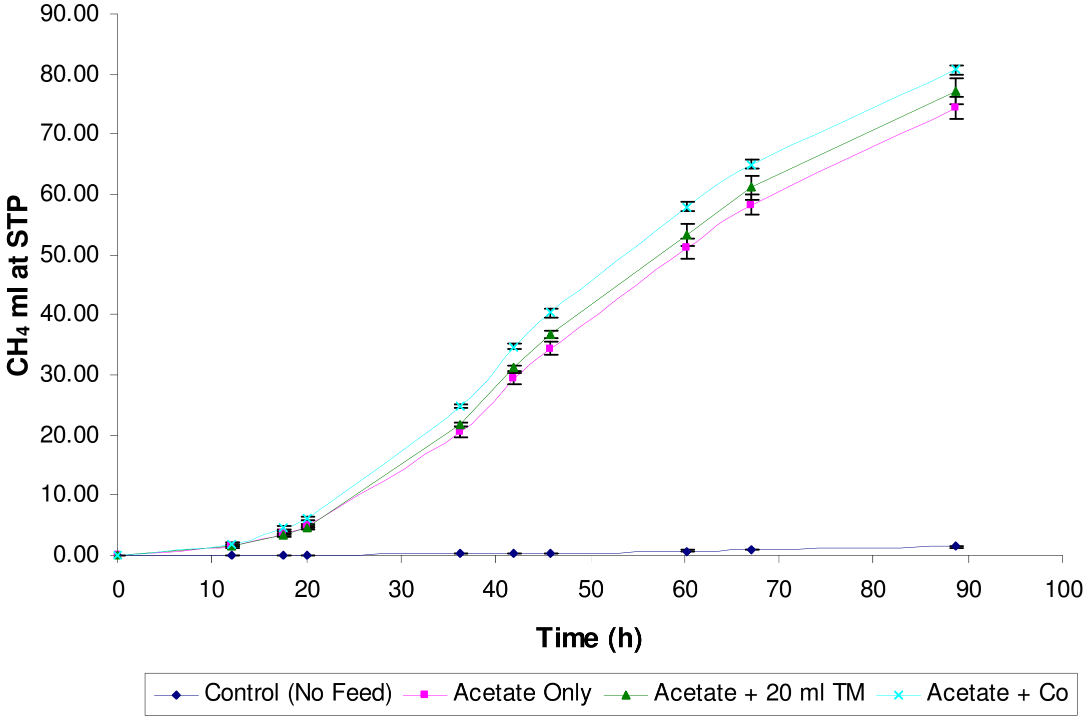
Standard Deviation from Replicates

Time (h)	Control (No Feed) n = 3	Acetate Only n = 4	Acetate + 20 ml TM n = 4	Acetate + Co* n = 4	Acetate + Co x2 n = 4	Acetate + Co x3 n = 4	Acetate + Co x4 n = 4
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
12	0.045	0.047	0.099	0.161	0.216	0.180	0.242
17.5	0.044	0.272	0.290	0.285	0.340	0.466	0.464
20	0.044	0.346	0.305	0.296	0.355	0.591	0.579
36.25	0.021	0.926	0.320	0.374	1.095	1.528	0.946
42	0.033	0.903	0.446	0.453	1.220	1.816	1.102
45.75	0.042	1.159	0.522	0.782	1.296	1.893	1.223
60.25	0.070	1.644	1.792	0.886	1.154	2.760	1.340
67	0.085	1.680	1.960	0.785	1.253	3.112	1.467
88.75	0.177	1.875	2.057	0.813	2.390	3.878	1.578
133.5	0.372	2.219	2.305	1.717	3.573	3.664	1.679
166	0.476	2.060	2.471	2.337	3.939	3.670	1.926
229.25	0.581	2.210	2.558	2.443	4.495	3.325	2.231
303	0.640	2.269	2.657	2.263	4.738	3.077	2.167

* Cobalt was supplemented at the equivalent concentrations to that present within 20 ml of the TM solution - 224 µg.
Co x2 = 448 µg
Co x3 = 672 µg
Co x4 = 896 µg



CH₄ ml at STP
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0
50
100
150
200
250
300
350
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + Co
Acetate + Co x2
Acetate + Co x3
Acetate + Co x4

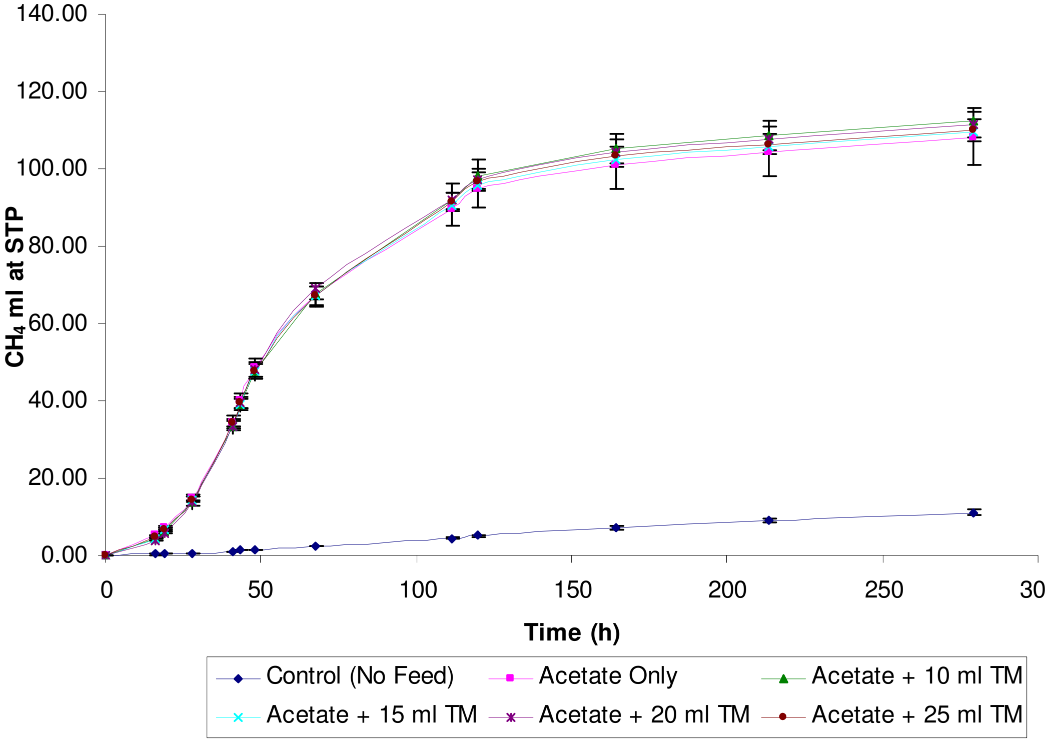


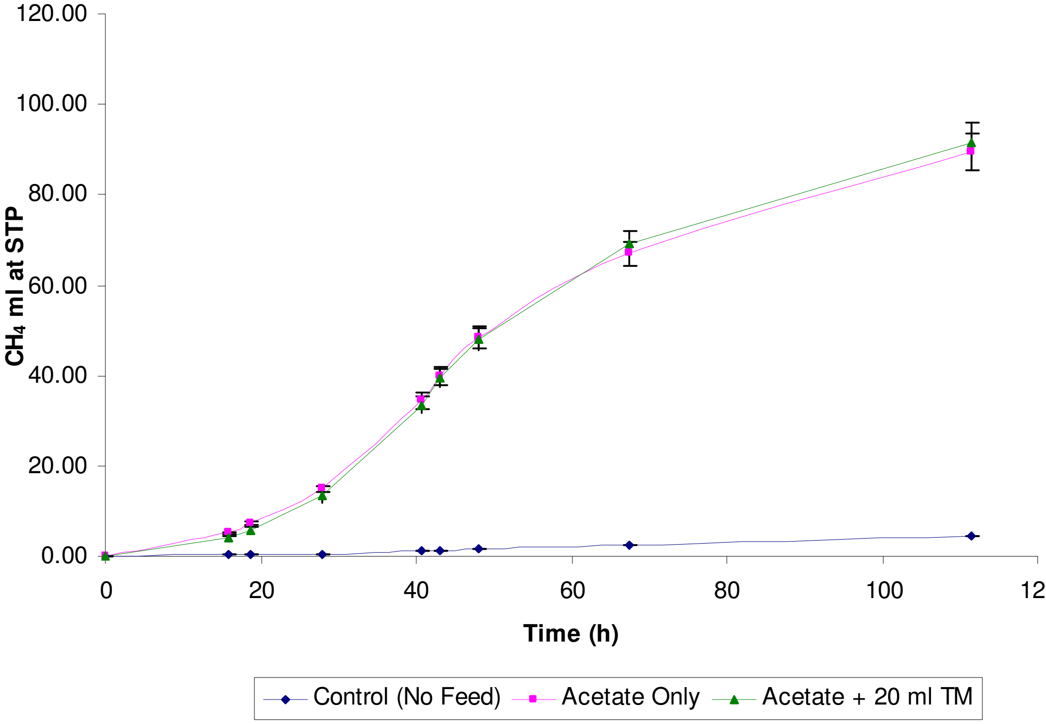
CH₄ ml at STP
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
10
20
30
40
50
60
70
80
90
100
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + Co

Sample B0310

Average Cumulative Methane (CH_4 ml at STP) from Replicates						
Time (h)	Control (No Feed)	Acetate Only	Acetate + 10 ml TM	Acetate + 15 ml TM	Acetate + 20 ml TM	Acetate + 25 ml TM
0	0.00	0.00	0.00	0.00	0.00	0.00
15.75	0.27	5.13	4.29	4.50	3.99	4.56
18.75	0.37	7.28	6.20	6.49	5.79	6.73
28	0.47	14.93	13.62	14.32	13.61	14.46
40.75	1.17	34.37	33.14	33.48	33.50	34.07
43	1.28	40.02	39.07	39.03	39.37	39.34
48	1.49	48.43	47.62	47.53	48.03	47.72
67.5	2.41	67.00	67.48	66.98	69.17	67.23
111.5	4.46	89.53	92.14	90.50	91.71	91.47
120	5.11	94.90	98.19	95.70	97.37	96.68
164.5	7.08	101.15	105.21	102.51	104.31	103.17
213.5	8.91	104.50	108.72	105.95	107.78	106.38
279	11.17	107.91	112.40	109.62	111.37	110.04

Standard Deviation from Replicates						
Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 10 ml TM n = 5	Acetate + 15 ml TM n = 5	Acetate + 20 ml TM n = 5	Acetate + 25 ml TM n = 5
0	0.000	0.000	0.000	0.000	0.000	0.000
15.75	0.070	0.282	0.337	0.580	0.429	0.478
18.75	0.092	0.266	0.563	0.874	0.576	0.653
28	0.130	0.591	1.709	1.514	0.692	0.594
40.75	0.273	1.654	1.728	1.160	2.281	0.934
43	0.358	2.065	2.012	1.413	2.546	1.035
48	0.408	2.594	2.468	1.645	3.133	1.652
67.5	0.566	2.659	2.834	1.005	2.849	2.366
111.5	0.990	4.155	4.141	0.789	5.062	2.238
120	1.109	5.132	4.417	0.866	4.981	2.178
164.5	1.413	6.297	3.884	1.180	4.743	2.540
213.5	1.682	6.446	3.719	1.397	4.827	2.698
279	1.962	6.742	3.512	1.620	4.736	2.935


140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH$_4$ ml at STP
0
50
100
150
200
250
300
Time (h)
Control (No Feed)
Acetate Only
Acetate + 10 ml TM
Acetate + 15 ml TM
Acetate + 20 ml TM
Acetate + 25 ml TM

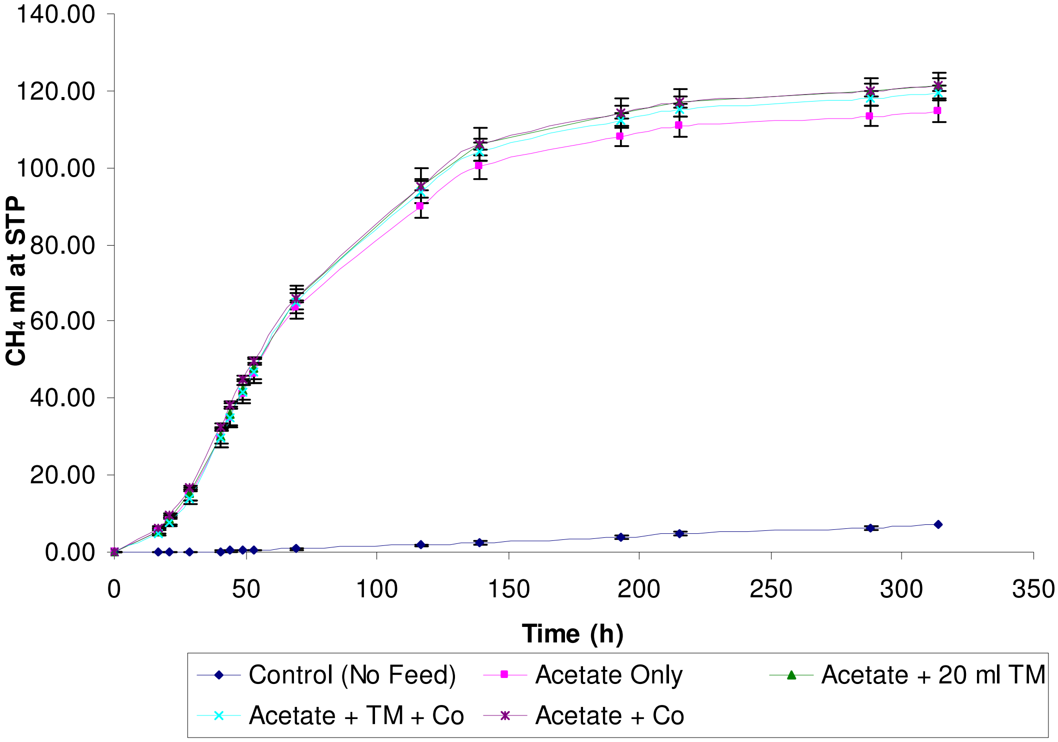

120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH$_4$ ml at STP
0
20
40
60
80
100
120
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM

Sample B0710

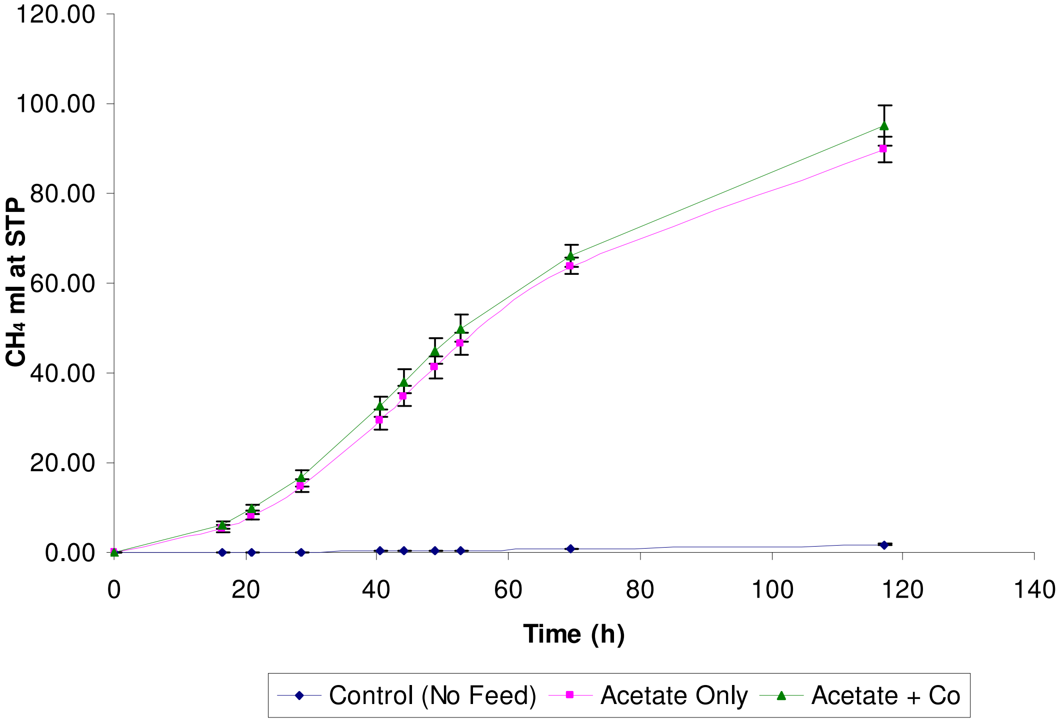
Average Cumulative Methane (CH_4 ml at STP) from Replicates					
Time (h)	Control (No Feed)	Acetate Only	Acetate + 20 ml TM	Acetate + 20 ml TM + Co	Acetate + Co*
0	0.00	0.00	0.00	0.00	0.00
16.5	0.06	5.29	5.33	4.83	6.27
21	0.09	8.34	8.44	7.75	9.65
28.5	0.14	14.90	15.24	14.01	16.56
40.5	0.23	29.59	30.30	29.50	32.48
44	0.26	34.88	35.83	35.11	38.13
48.75	0.34	41.19	42.28	41.59	44.82
52.75	0.41	46.43	47.74	47.05	49.91
69.5	0.72	63.78	65.78	65.13	66.14
117	1.76	89.62	95.24	93.87	95.29
138.75	2.35	100.12	105.98	104.08	105.93
192.5	3.77	108.17	114.28	112.42	114.36
215.5	4.68	110.69	116.93	115.03	117.07
287.5	6.27	113.43	119.75	117.92	120.07
314	7.31	114.84	121.26	119.42	121.59

Standard Deviation from Replicates					
Time (h)	Control (No Feed) n = 3	Acetate Only n = 4	Acetate + 20 ml TM n = 4	Acetate + 20 ml TM + Co n = 4	Acetate + Co* n = 4
0	0.000	0.000	0.000	0.000	0.000
16.5	0.037	0.669	0.802	0.724	0.240
21	0.037	0.940	1.100	1.065	0.427
28.5	0.014	1.533	1.683	1.764	0.534
40.5	0.067	2.160	2.226	2.367	0.913
44	0.016	2.409	2.738	2.778	1.020
48.75	0.013	2.507	2.851	3.049	1.110
52.75	0.010	2.531	3.038	3.029	0.732
69.5	0.072	1.898	2.514	4.259	1.220
117	0.144	2.836	4.496	3.004	1.232
138.75	0.125	3.075	4.429	2.355	1.390
192.5	0.206	2.665	3.743	1.778	1.583
215.5	0.231	2.636	3.619	1.734	1.639
287.5	0.354	2.731	3.534	1.871	1.667
314	0.412	2.951	3.541	1.957	1.694

* Cobalt is added at the equivalent concentrations to that present within 20 ml TM solution - 224 μg.
Samples labelled ‘Acetate + 20 ml TM + Co’ were supplemented with 20 ml TM with an additional 224 μg of cobalt.



140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH₄ ml at STP
0
50
100
150
200
250
300
350
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + TM + Co
Acetate + Co

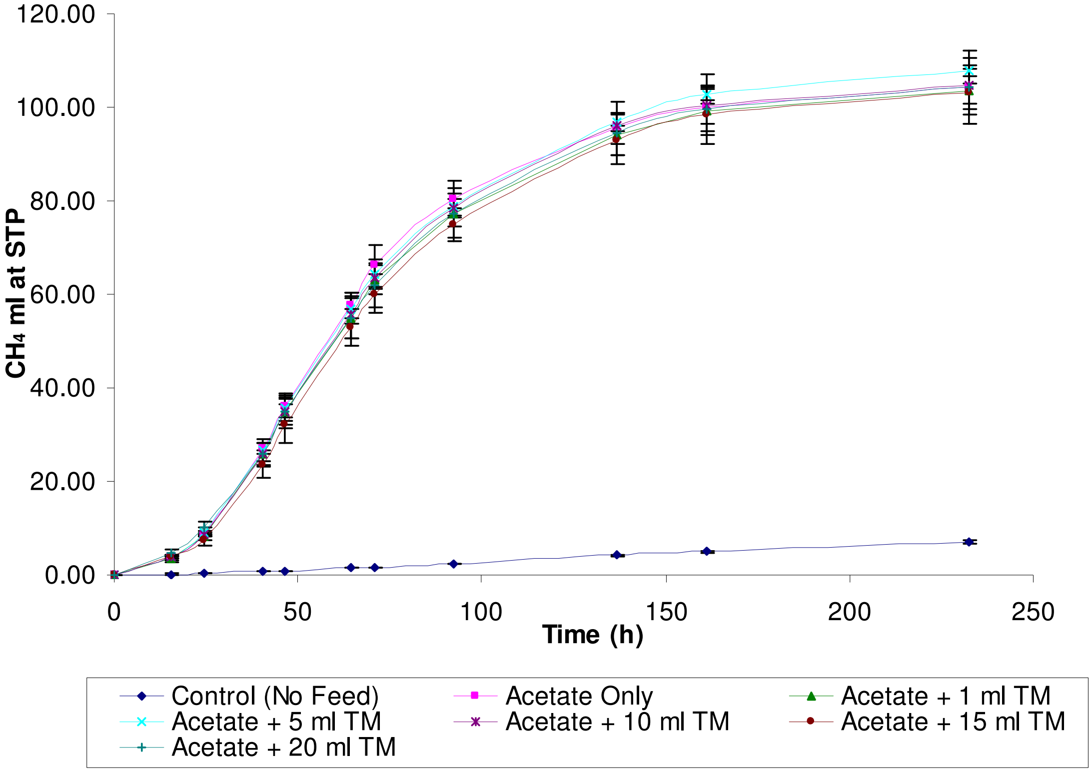


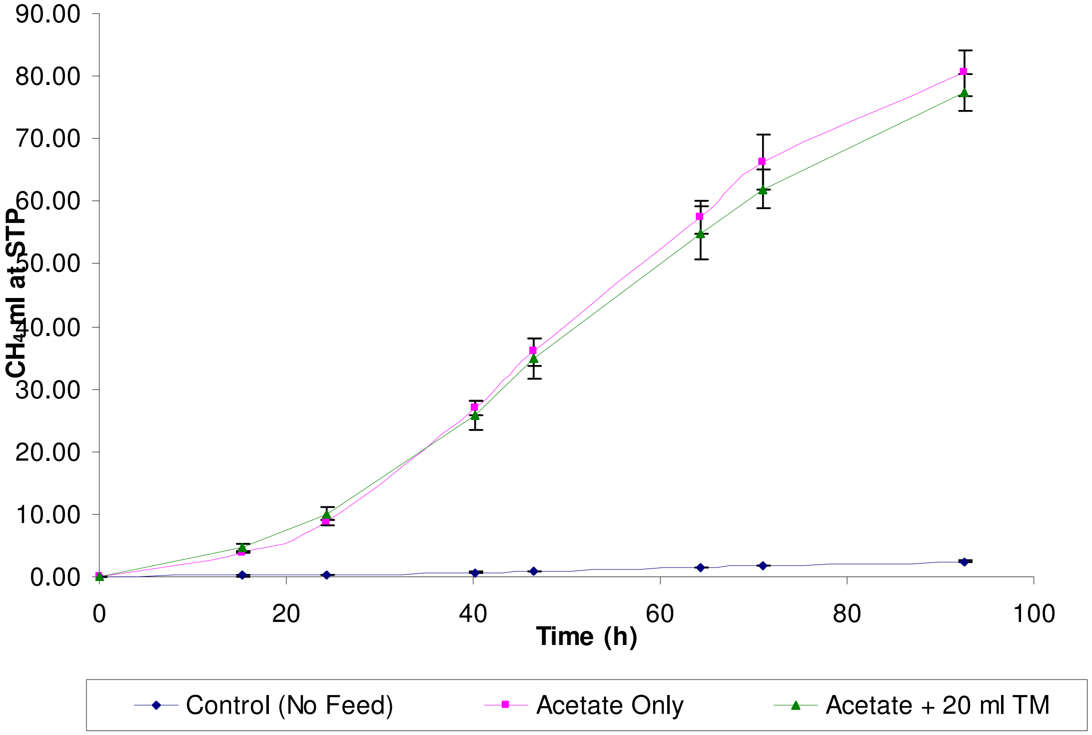
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH₄ ml at STP
0
20
40
60
80
100
120
140
Time (h)
Control (No Feed)
Acetate Only
Acetate + Co

Sample B1109

Average Cumulative Methane (CH_4 ml at STP) from Replicates							
Time (h)	Control (No Feed)	Acetate Only	Acetate + 1 ml TM	Acetate + 5 ml TM	Acetate + 10 ml TM	Acetate + 15 ml TM	Acetate + 20 ml TM
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15.25	0.19	3.89	3.56	3.91	4.09	3.76	4.75
24.25	0.27	8.68	8.39	9.19	8.54	7.54	10.07
40.25	0.73	26.91	25.69	26.72	26.00	23.70	25.79
46.5	0.90	35.97	34.75	35.88	34.95	32.34	34.88
64.25	1.44	57.55	54.82	57.00	55.85	52.90	54.94
71	1.66	66.16	63.20	64.43	63.39	60.05	62.00
92.5	2.46	80.54	77.42	79.01	78.46	74.98	77.45
136.5	4.15	95.50	94.08	96.93	95.94	92.86	94.56
161.25	5.05	100.11	99.17	102.75	100.44	98.41	99.71
232.75	7.13	104.49	103.68	107.86	104.88	102.99	104.33

Average Cumulative Methane (CH_4 ml at STP) from Replicates							
Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 1 ml TM n = 3	Acetate + 5 ml TM n = 3	Acetate + 10 ml TM n = 3	Acetate + 15 ml TM n = 3	Acetate + 20 ml TM n = 3
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
15.25	0.049	0.208	0.655	0.427	0.774	0.568	0.743
24.25	0.049	0.463	1.030	0.938	0.948	1.196	1.391
40.25	0.157	1.133	2.435	2.339	1.447	2.935	2.249
46.5	0.187	2.278	3.318	3.080	1.937	4.167	2.804
64.25	0.270	2.686	4.352	3.290	1.665	3.938	4.490
71	0.305	4.418	3.157	3.190	1.265	4.069	4.748
92.5	0.419	3.627	2.868	2.724	1.238	3.510	5.311
136.5	0.526	3.323	4.434	1.901	0.184	3.207	6.720
161.25	0.691	3.621	4.994	2.141	0.628	3.324	7.436
232.75	0.959	3.842	5.298	2.569	1.322	3.519	7.760

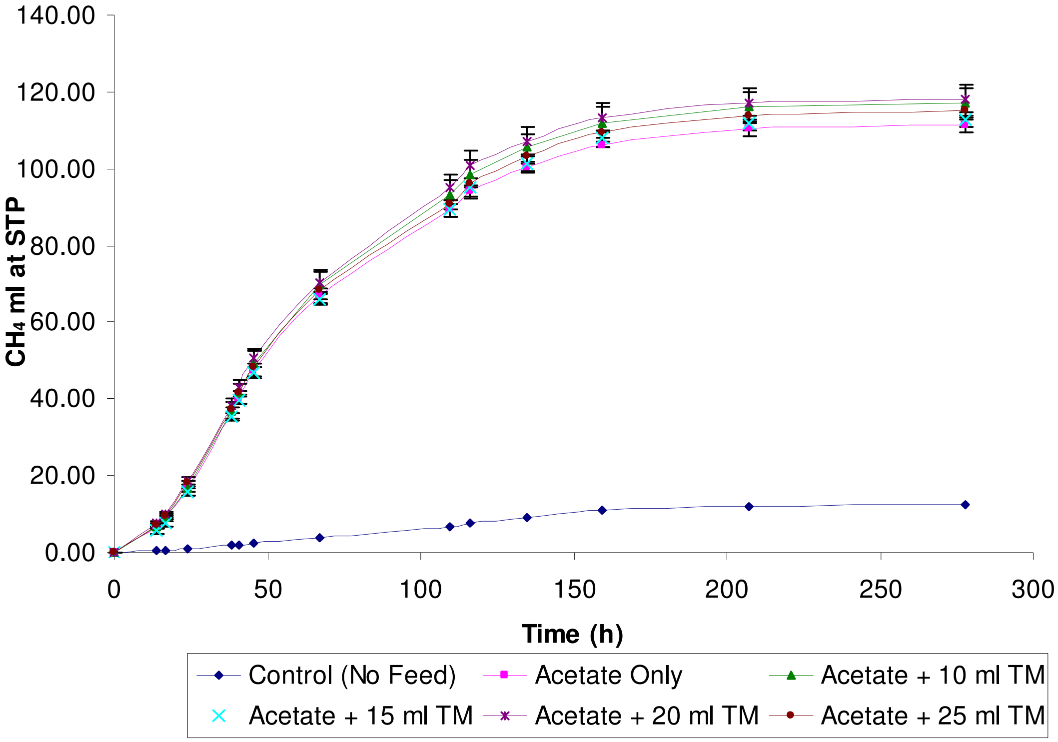

120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH4 ml at STP
0
50
100
150
200
250
Time (h)
Control (No Feed)
Acetate Only
Acetate + 1 ml TM
Acetate + 5 ml TM
Acetate + 10 ml TM
Acetate + 15 ml TM
Acetate + 20 ml TM


90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH4 ml at STP
0
20
40
60
80
100
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM

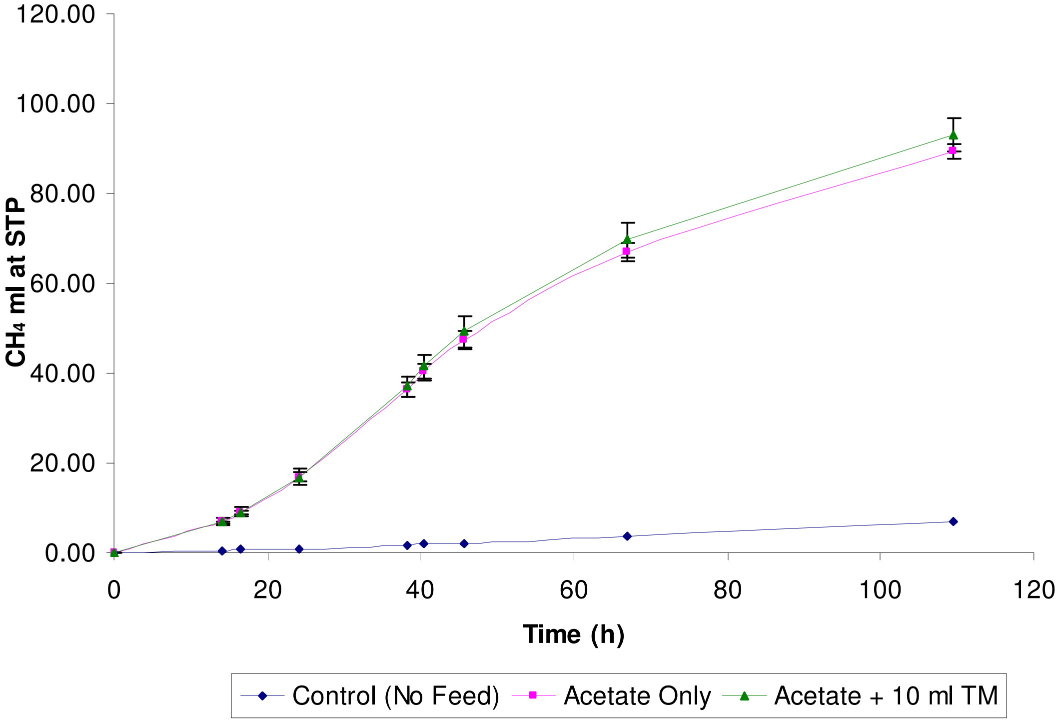
Sample C0310

Average Cumulative Methane (CH_4 ml at STP) from Replicates						
Time (h)	Control (No Feed)	Acetate Only	Acetate + 10 ml TM	Acetate + 15 ml TM	Acetate + 20 ml TM	Acetate + 25 ml TM
0	0.00	0.00	0.00	0.00	0.00	0.00
14	0.55	6.79	6.85	5.67	7.52	7.35
16.5	0.64	9.00	9.06	7.75	9.87	9.64
24.25	0.96	16.83	16.93	15.73	18.46	18.19
38.25	1.82	36.18	36.97	35.32	38.25	37.09
40.5	1.92	40.29	41.51	39.88	42.77	41.43
45.75	2.20	47.42	49.22	46.83	50.55	48.49
67	3.77	66.86	69.70	66.16	70.29	68.14
109.5	6.77	89.30	93.11	89.55	95.27	90.80
116.25	7.45	93.95	98.54	95.02	100.87	96.25
134.5	8.85	100.25	105.38	101.52	107.15	102.99
159.25	10.91	106.27	112.04	107.87	113.19	109.61
207.25	12.09	110.36	115.97	111.66	116.93	113.91
277.75	12.53	111.13	116.88	112.63	117.87	114.93

Standard Deviation from Replicates						
Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 10 ml TM n = 5	Acetate + 15 ml TM n = 5	Acetate + 20 ml TM n = 5	Acetate + 25 ml TM n = 4
0	0.000	0.000	0.000	0.000	0.000	0.000
14	0.056	0.338	0.799	0.695	0.664	0.334
16.5	0.066	0.418	1.030	0.853	0.799	0.444
24.25	0.075	1.047	1.909	1.022	1.266	0.306
38.25	0.015	1.633	2.131	1.004	1.898	0.517
40.5	0.033	1.812	2.607	1.087	2.077	0.495
45.75	0.073	2.027	3.368	1.265	2.356	0.748
67	0.186	1.954	3.908	1.823	2.936	1.073
109.5	0.611	1.707	3.681	2.029	3.388	2.374
116.25	0.701	1.672	3.593	2.327	3.633	2.284
134.5	0.847	1.221	3.714	2.226	3.896	2.075
159.25	1.001	0.876	3.914	2.138	3.914	1.968
207.25	1.186	2.079	4.061	1.988	4.093	1.759
277.75	1.276	1.937	3.959	1.944	4.101	1.711



140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH₄ ml at STP
0
50
100
150
200
250
300
Time (h)
Control (No Feed)
Acetate Only
Acetate + 10 ml TM
Acetate + 15 ml TM
Acetate + 20 ml TM
Acetate + 25 ml TM



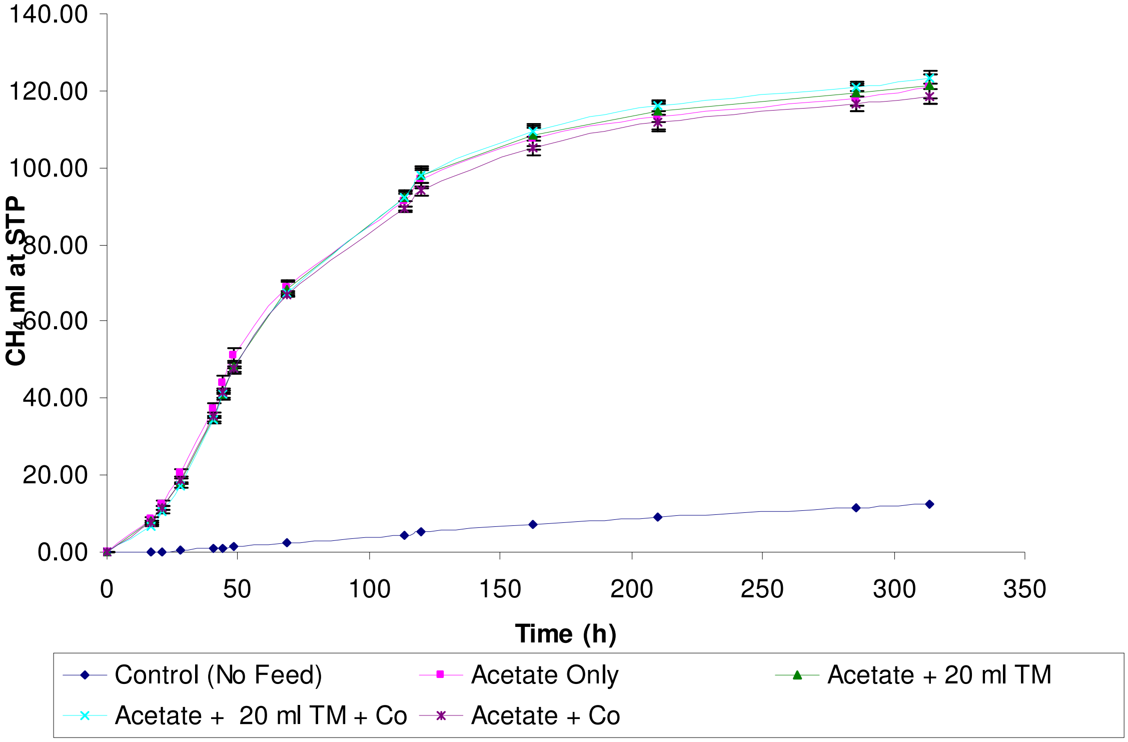
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH₄ ml at STP
0
20
40
60
80
100
120
Time (h)
Control (No Feed)
Acetate Only
Acetate + 10 ml TM

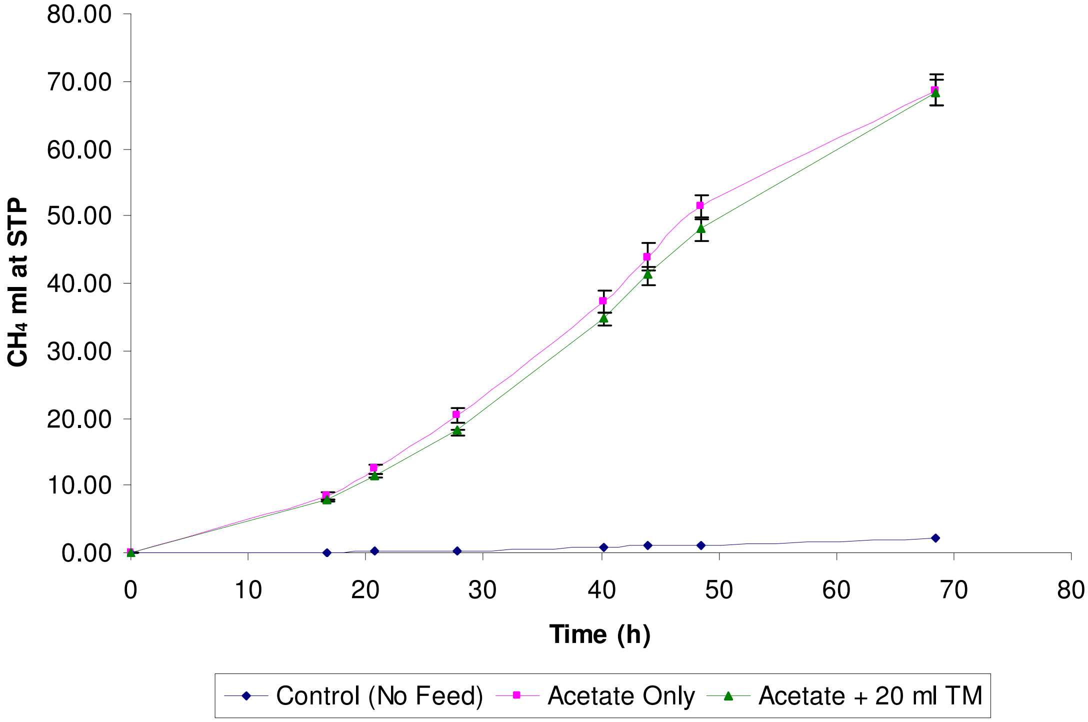
Sample C0810

Average Cumulative Methane (CH_4 ml at STP) from Replicates					
Time (h)	Control (No Feed)	Acetate Only	Acetate + 20 ml TM	Acetate + 20 ml TM + Co	Acetate + Co*
0	0.00	0.00	0.00	0.00	0.00
16.75	0.04	8.42	7.93	6.92	7.65
20.75	0.15	12.45	11.32	10.38	11.41
27.75	0.30	20.50	18.15	17.02	18.42
40.25	0.83	37.25	34.93	34.33	35.49
44	0.96	43.91	41.25	40.90	41.68
48.5	1.20	51.36	48.12	47.86	47.91
68.5	2.27	68.61	68.31	67.34	66.90
113.25	4.54	91.48	92.22	92.15	89.11
119.75	5.06	97.13	98.09	97.87	94.29
162.25	6.94	107.75	108.70	109.41	105.10
210.25	8.89	113.28	114.71	116.02	111.68
285.5	11.28	118.04	119.24	120.89	116.44
313.5	12.64	120.74	121.35	123.07	118.64

Standard Deviation from Replicates					
Time (h)	Control (No Feed) n = 3	Acetate Only n = 4	Acetate + 20 ml TM n = 3	Acetate + 20 ml TM + Co n = 4	Acetate + Co* n = 4
0	0.000	0.000	0.000	0.000	0.000
16.75	0.014	0.579	0.393	0.305	0.399
20.75	0.029	0.711	0.199	0.540	0.615
27.75	0.050	1.080	0.668	0.463	0.876
40.25	0.136	1.658	1.300	0.629	0.594
44	0.146	1.958	1.507	0.857	0.430
48.5	0.155	1.597	1.911	1.119	0.312
68.5	0.187	2.277	1.927	0.621	0.267
113.25	0.261	2.379	2.357	1.005	0.807
119.75	0.299	2.549	2.811	1.619	1.621
162.25	0.295	2.868	2.897	1.507	2.072
210.25	0.295	3.264	2.922	1.252	2.078
285.5	0.323	3.440	3.044	1.136	1.872
313.5	0.326	4.230	3.119	1.139	1.825

* Cobalt was supplemented at the equivalent concentrations to that present within 20 ml TM solution - 224 µg.
Samples labelled ‘Acetate + 20 ml TM + Co’ were supplemented with 20 ml TM with an additional 224 µg of cobalt.

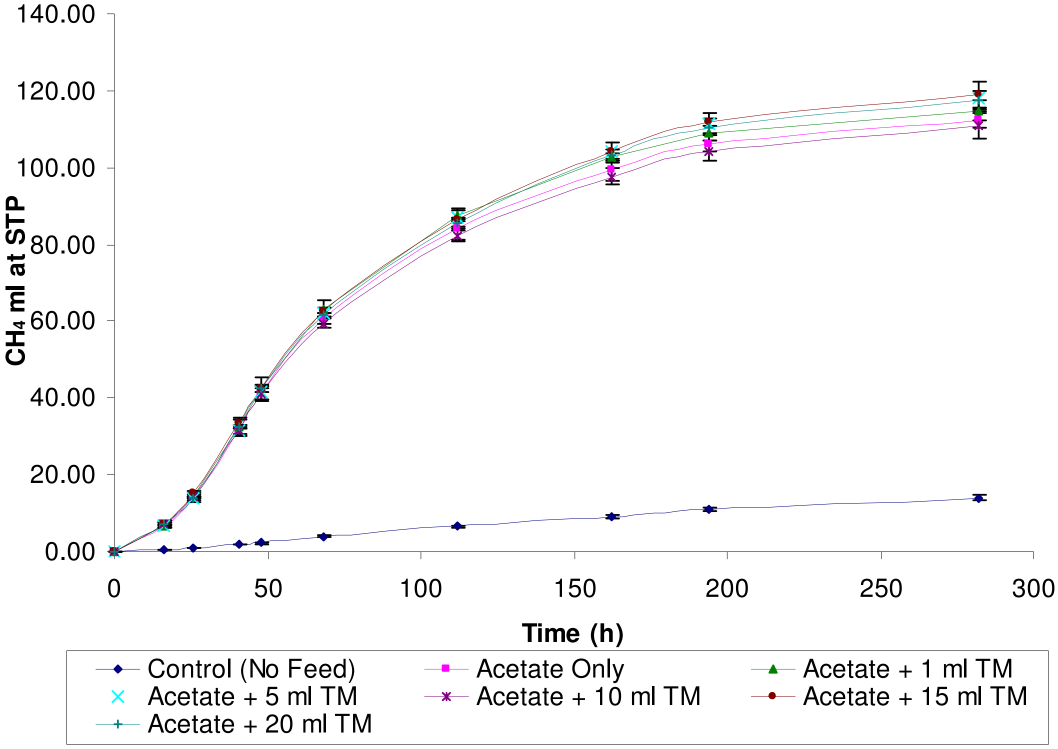

140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH$_4$ ml at STP
0
50
100
150
200
250
300
350
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + 20 ml TM + Co
Acetate + Co


80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH$_4$ ml at STP
0
10
20
30
40
50
60
70
80
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM

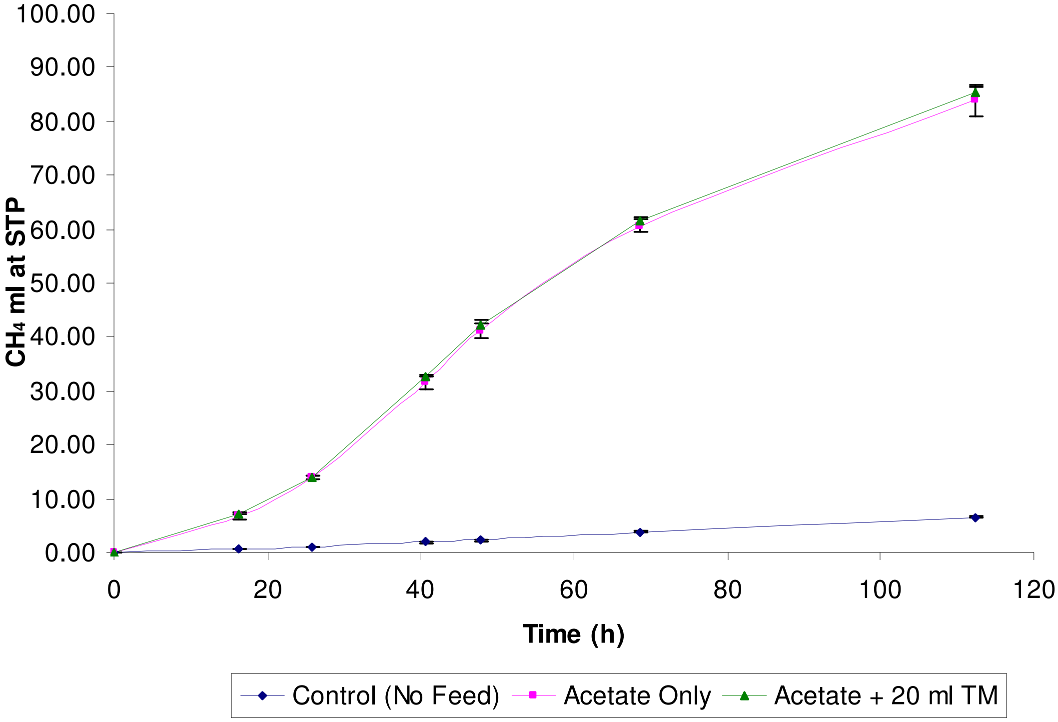
Sample C1109

Average Cumulative Methane (CH_4 ml at STP) from Replicates							
Time (h)	Control (No Feed)	Acetate Only	Acetate + 1 ml TM	Acetate + 5 ml TM	Acetate + 10 ml TM	Acetate + 15 ml TM	Acetate + 20 ml TM
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
16.25	0.56	6.68	6.71	6.59	7.08	7.36	6.99
25.75	0.93	14.06	14.15	13.64	14.37	15.07	13.92
40.75	1.91	31.53	32.35	31.50	31.56	33.35	32.50
47.75	2.28	41.03	42.51	41.45	40.95	42.73	42.32
68.5	3.88	60.66	63.12	61.96	59.45	62.83	61.63
112.25	6.61	83.91	87.52	87.04	82.40	86.32	85.46
162.25	9.17	99.55	102.63	104.35	97.61	103.93	103.21
194	10.87	106.25	108.93	111.31	104.37	111.57	110.59
282.25	13.93	112.47	114.88	117.96	110.88	118.78	117.49

Average Cumulative Methane (CH_4 ml at STP) from Replicates							
Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 1 ml TM n = 3	Acetate + 5 ml TM n = 3	Acetate + 10 ml TM n = 3	Acetate + 15 ml TM n = 3	Acetate + 20 ml TM n = 3
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
16.25	0.040	0.553	0.671	0.766	0.366	0.784	0.624
25.75	0.060	0.377	1.070	0.805	0.486	0.890	0.475
40.75	0.194	1.126	2.037	2.090	1.480	1.539	0.520
47.75	0.226	1.378	2.652	2.794	1.972	0.944	0.768
68.5	0.186	1.241	2.121	1.882	1.295	0.649	0.575
112.25	0.291	2.904	1.714	3.501	1.858	2.597	1.059
162.25	0.375	2.887	0.989	3.970	2.170	2.732	1.607
194	0.523	2.313	1.852	3.857	2.650	2.818	2.002
282.25	0.786	2.099	2.642	3.829	3.185	3.309	2.243



CH$_4$ ml at STP
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
0
50
100
150
200
250
300
Time (h)
Control (No Feed)
Acetate Only
Acetate + 1 ml TM
Acetate + 5 ml TM
Acetate + 10 ml TM
Acetate + 15 ml TM
Acetate + 20 ml TM



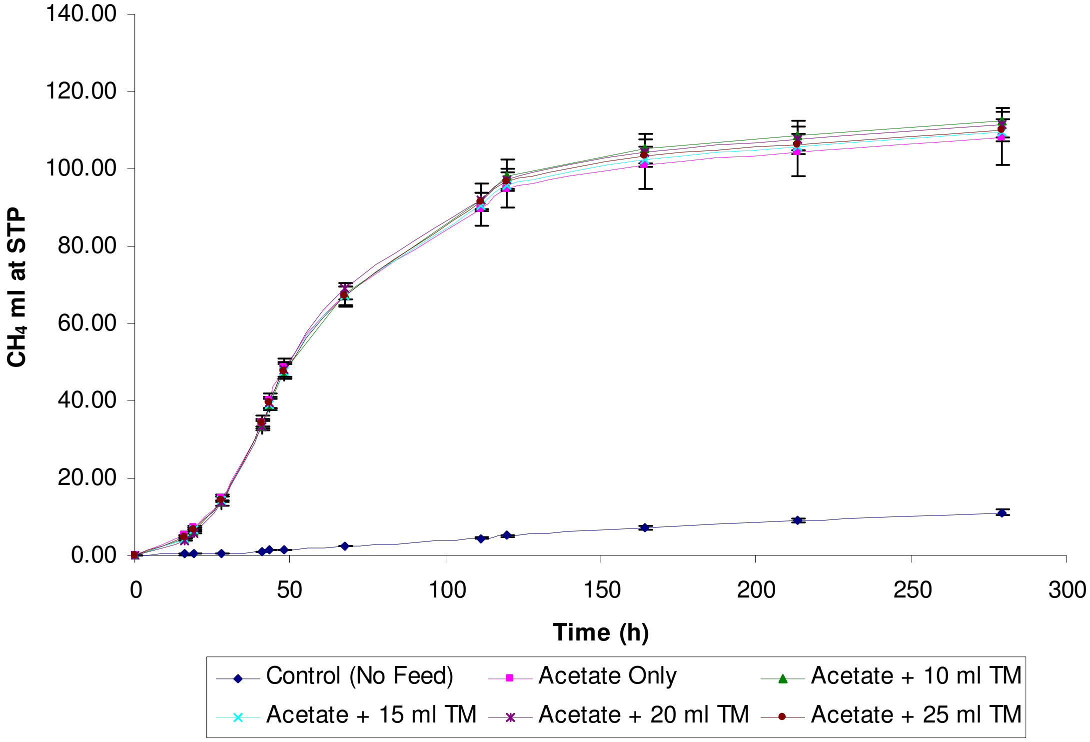
CH$_4$ ml at STP
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
20
40
60
80
100
120
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM

Sample C0211

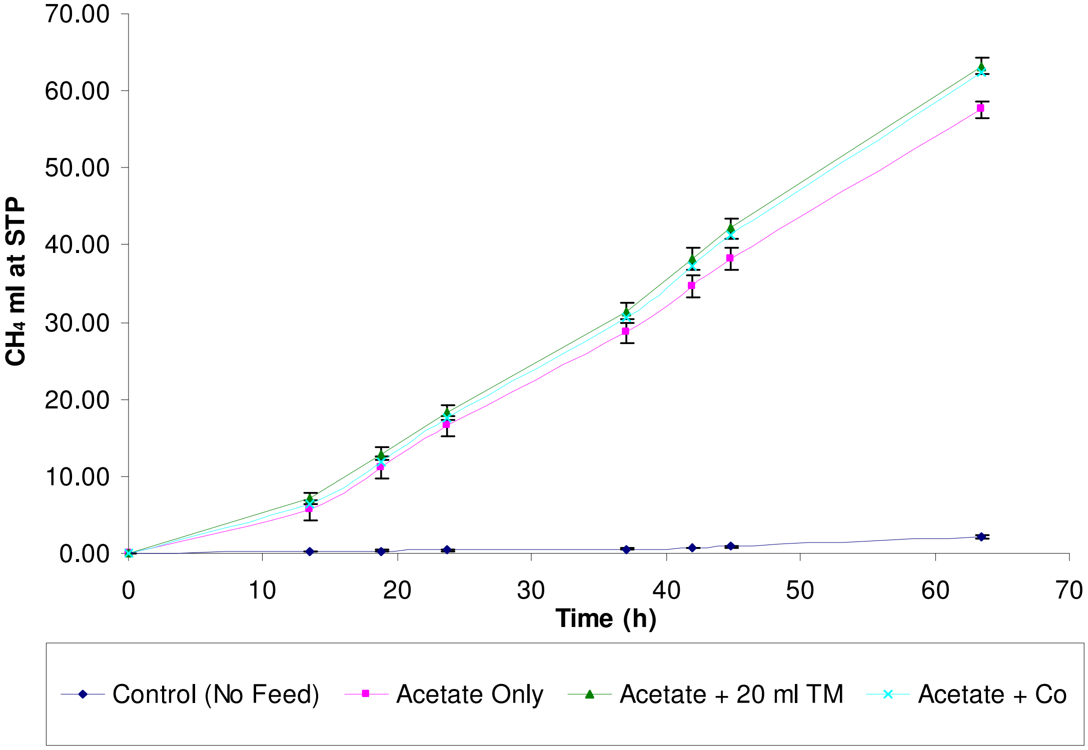
Average Cumulative Methane (CH_4 ml at STP) from Replicates							
Time (h)	Control (No Feed)	Acetate Only	Acetate + 20 ml TM	Acetate + Co x 0.5	Acetate + Co*	Acetate + Co x2	Acetate + Co x3
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
13.5	0.18	5.65	7.05	7.79	6.41	6.36	3.40
18.75	0.35	11.15	12.88	13.81	11.82	11.94	6.62
23.75	0.40	16.58	18.36	19.46	17.47	17.42	8.49
37	0.55	28.60	31.38	32.41	30.57	29.90	27.55
42	0.76	34.63	38.20	39.11	37.32	36.46	36.78
44.75	0.85	38.15	42.17	43.11	41.36	40.38	41.86
63.5	2.13	57.56	63.22	62.69	62.42	60.71	59.05
110.75	5.50	86.93	91.48	88.87	91.01	89.36	66.42
136	6.75	97.06	101.93	99.28	101.94	100.44	81.55
184	9.91	107.05	112.73	111.03	114.30	112.79	100.65
207.5	11.88	111.25	117.25	115.57	119.16	117.69	108.83
278.75	15.07	116.39	122.80	121.22	124.94	123.35	115.03
301.75	17.14	118.88	125.37	123.74	127.44	125.71	118.92

Standard Deviation from Replicates							
Time (h)	Control (No Feed) n = 3	Acetate Only n = 4	Acetate + 20 ml TM n = 4	Acetate + Co x0.5 n = 4	Acetate + Co* n = 4	Acetate + Co x2 n = 4	Acetate + Co x3 n = 4
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13.5	0.039	1.290	0.755	0.376	0.042	0.551	0.631
18.75	0.045	1.362	0.872	0.640	0.435	0.828	0.664
23.75	0.046	1.308	0.945	0.910	0.659	0.920	0.710
37	0.048	1.322	1.006	1.077	0.934	0.915	1.613
42	0.045	1.331	1.329	1.172	1.195	1.088	1.668
44.75	0.048	1.375	1.352	1.408	1.346	1.057	1.696
63.5	0.243	1.048	0.993	1.669	1.417	1.610	1.397
110.75	0.515	0.820	0.656	2.973	1.013	1.440	1.445
136	0.717	1.172	0.908	2.709	1.028	1.235	1.259
184	0.948	1.774	2.015	2.911	1.651	1.493	1.228
207.5	1.140	2.134	2.451	3.093	1.783	1.702	1.501
278.75	1.388	2.350	2.655	3.274	1.859	1.820	1.845
301.75	1.254	2.436	2.722	3.350	1.958	1.781	1.858

* Cobalt was supplemented at the equivalent concentrations to that present within 20 ml of the TM solution - 224 µg.
Co x0.5 = 112 µg
Co x2 = 448 µg
Co x3 = 672 µg



140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH₄ ml at STP
0
50
100
150
200
250
300
Time (h)
Control (No Feed)
Acetate Only
Acetate + 10 ml TM
Acetate + 15 ml TM
Acetate + 20 ml TM
Acetate + 25 ml TM



70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH₄ ml at STP
0
10
20
30
40
50
60
70
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + Co

Sample D0610

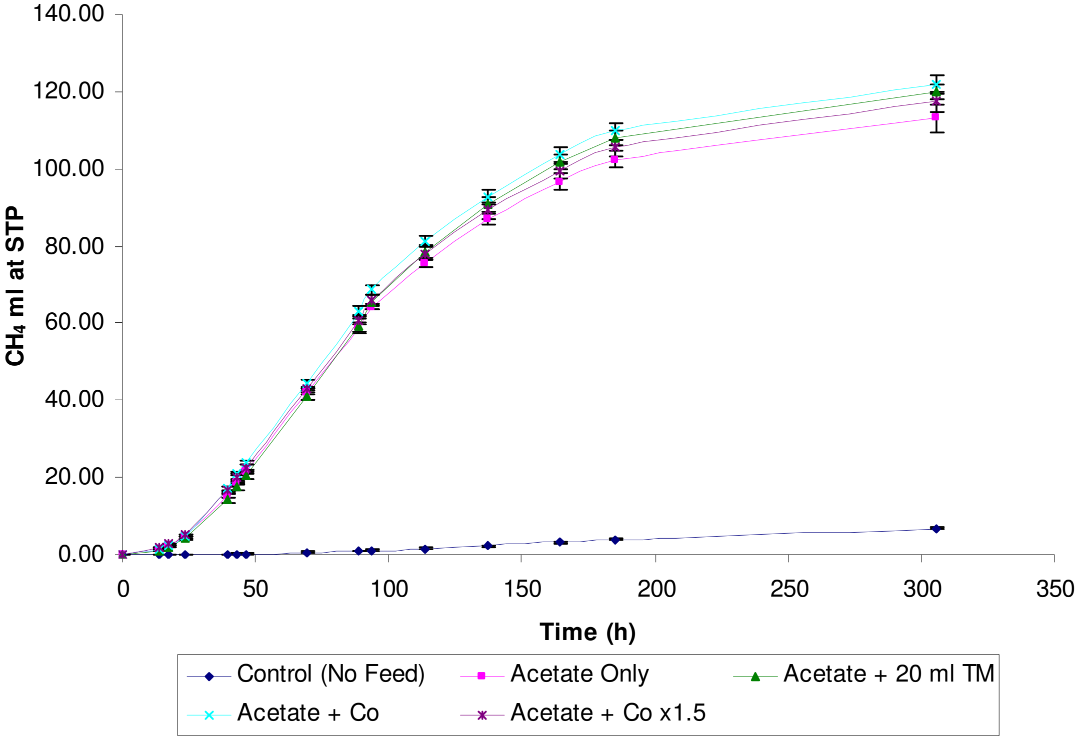
Average Cumulative Methane (CH_4 ml at STP) from Replicates

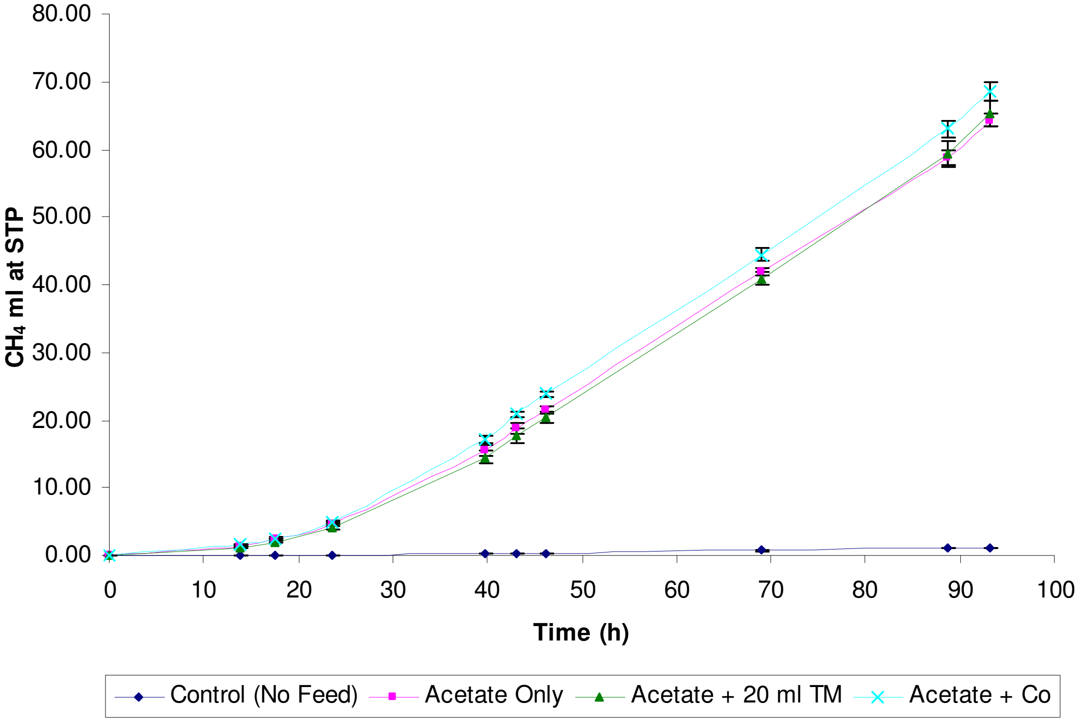
Time (h)	Control (No Feed)	Acetate Only	Acetate + 20 ml TM	Acetate + Co*	Acetate + Co x1.5
0	0.00	0.00	0.00	0.00	0.00
13.75	0.03	1.47	1.15	1.53	1.80
17.5	0.04	2.43	1.95	2.54	2.80
23.5	0.05	4.74	4.11	5.01	5.05
39.75	0.22	15.41	14.55	17.14	16.69
43	0.24	18.77	17.75	20.88	19.99
46.25	0.24	21.41	20.39	23.83	22.62
69	0.70	41.85	40.90	44.41	42.98
88.75	1.03	58.77	59.42	63.04	60.77
93.25	1.17	64.25	65.36	68.60	66.02
113.75	1.65	75.71	78.46	81.16	78.08
137.25	2.21	86.86	90.59	92.86	89.24
164.5	3.12	96.68	101.70	103.50	99.48
185	3.84	102.45	107.98	109.68	105.39
305.75	6.84	113.02	119.90	121.89	117.34

Standard Deviation from Replicates

Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 20 ml TM n = 4	Acetate + Co* n = 3	Acetate + Co x1.5 n = 4
0	0.000	0.000	0.000	0.000	0.000
13.75	0.001	0.059	0.112	0.101	0.092
17.5	0.003	0.054	0.151	0.151	0.154
23.5	0.009	0.018	0.264	0.228	0.282
39.75	0.009	0.828	1.012	0.577	0.910
43	0.013	0.787	1.049	0.432	0.922
46.25	0.013	0.556	0.920	0.378	0.785
69	0.034	0.466	0.966	0.940	0.091
88.75	0.044	1.223	1.841	1.283	1.115
93.25	0.054	0.930	1.861	1.317	1.381
113.75	0.094	1.280	1.826	1.596	1.733
137.25	0.182	1.541	1.941	1.848	2.080
164.5	0.229	2.017	1.996	2.028	2.194
185	0.255	2.191	2.027	2.137	2.293
305.75	0.297	3.618	1.929	2.497	2.535

* Cobalt was supplemented at the equivalent concentrations to that present within 20 ml of the TM solution - 224 μg.
Co x1.5 equates to addition of 336 μg of cobalt.


140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH4 ml at STP
0
50
100
150
200
250
300
350
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + Co
Acetate + Co x1.5

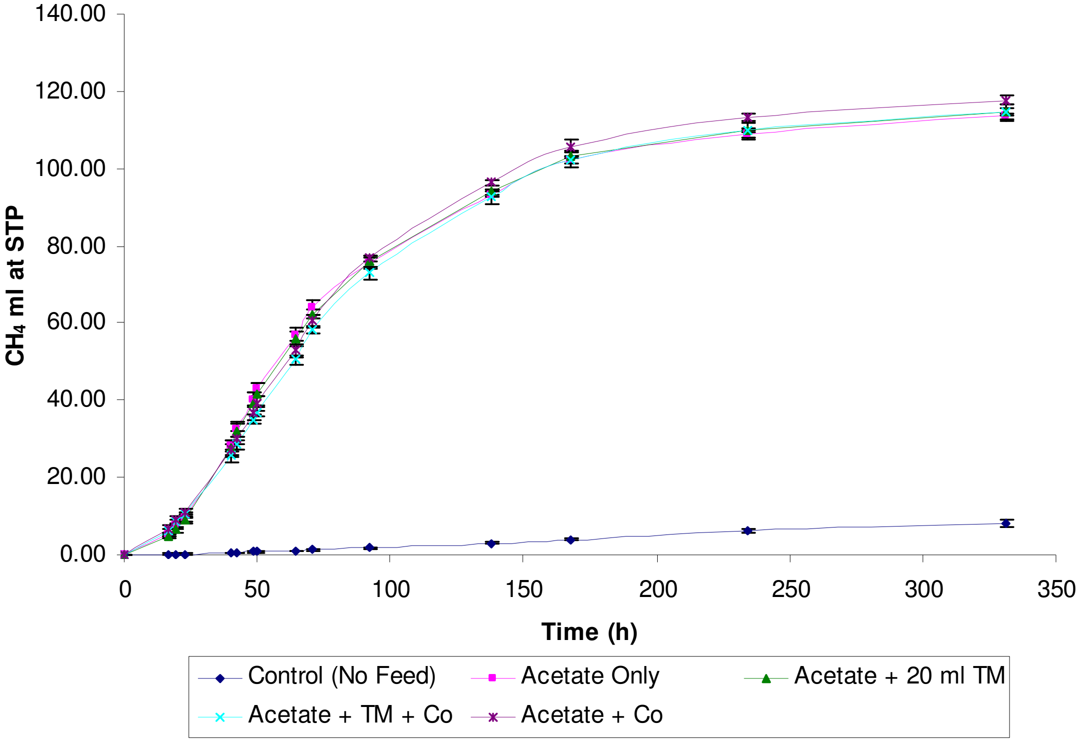

80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH4 ml at STP
0
10
20
30
40
50
60
70
80
90
100
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + Co

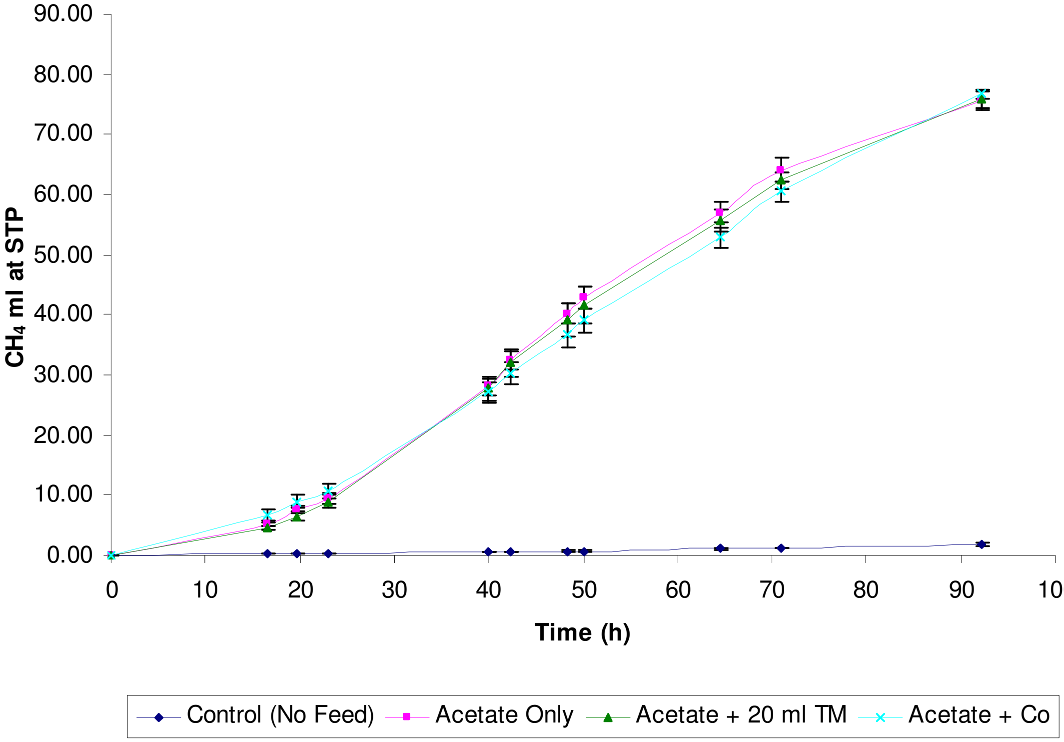
Sample D0710

Average Cumulative Methane (CH_4 ml at STP) from Replicates					
Time (h)	Control (No Feed)	Acetate Only	Acetate + 20 ml TM	Acetate + 20 ml TM + Co	Acetate + Co*
0	0.00	0.00	0.00	0.00	0.00
16.5	0.20	5.20	4.55	6.43	6.79
19.75	0.24	7.52	6.57	8.56	8.98
23	0.24	9.55	8.99	10.34	10.77
40	0.57	28.05	27.72	25.60	27.12
42.25	0.59	32.36	31.99	28.90	30.33
48.25	0.73	40.17	39.19	35.08	36.68
50	0.73	42.84	41.64	36.99	39.05
64.5	1.11	57.09	55.82	50.51	52.86
71	1.24	64.12	62.33	58.27	60.50
92.25	1.83	75.61	76.02	72.93	76.77
137.75	3.02	93.40	94.04	92.58	96.36
167.75	4.05	102.13	102.99	102.34	105.73
234	6.14	108.89	109.89	109.70	113.13
331.25	8.16	113.52	114.61	114.50	117.74

Standard Deviation from Replicates					
Time (h)	Control (No Feed) n = 3	Acetate Only n = 4	Acetate + 20 ml TM n = 3	Acetate + 20 ml TM + Co n = 3	Acetate + Co* n = 3
0	0.000	0.000	0.000	0.000	0.000
16.5	0.037	0.262	0.192	0.464	0.981
19.75	0.034	0.609	0.624	0.459	1.086
23	0.034	0.885	0.967	0.612	1.154
40	0.051	1.367	1.918	1.553	1.620
42.25	0.059	1.572	2.398	1.449	1.762
48.25	0.054	1.738	2.798	1.248	2.034
50	0.054	1.760	2.921	1.036	1.915
64.5	0.081	1.831	1.837	1.308	1.644
71	0.109	1.908	1.368	1.071	1.606
92.25	0.161	1.442	1.518	1.537	0.791
137.75	0.274	0.756	0.703	1.791	0.871
167.75	0.400	0.845	0.383	2.137	1.633
234	0.525	0.935	0.704	2.363	1.274
331.25	0.804	0.856	0.935	2.263	1.283

* Cobalt was supplemented at the equivalent concentrations to that present within 20 ml of TM solution - 224 μg.
Samples labelled ‘Acetate + 20 ml TM + Co’ were supplemented with 20 ml TM with an additional 224 μg of cobalt.


140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH4 ml at STP
0
50
100
150
200
250
300
350
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + TM + Co
Acetate + Co

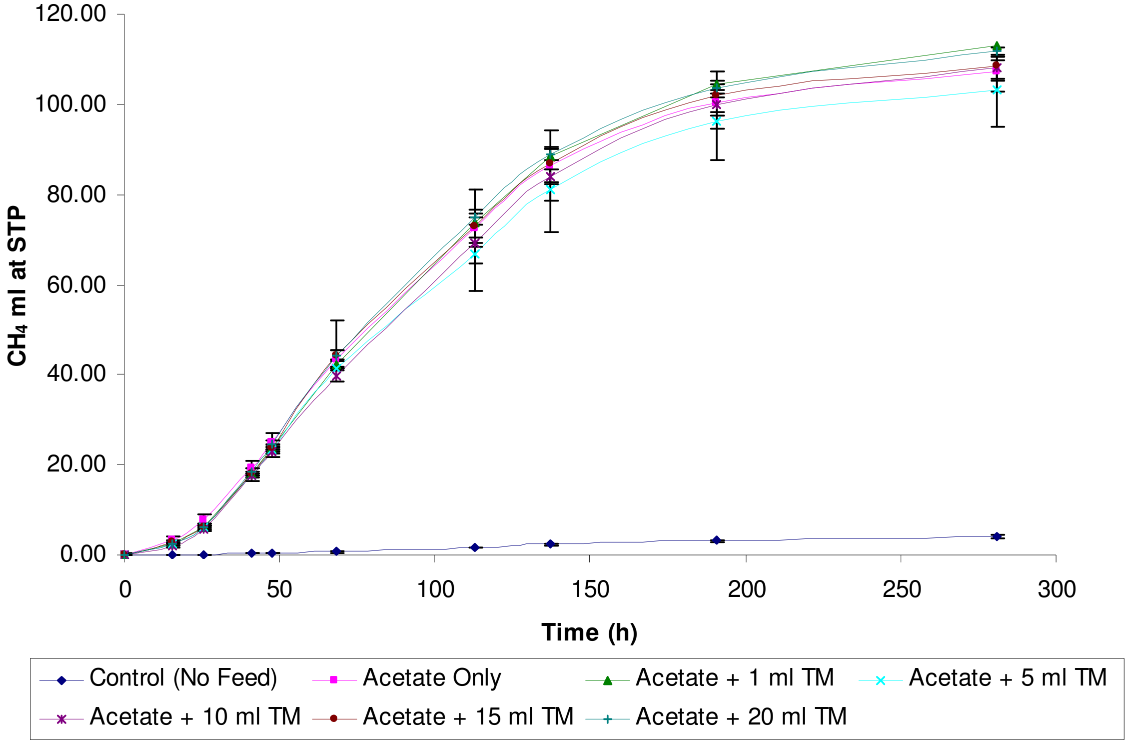

90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH4 ml at STP
0
10
20
30
40
50
60
70
80
90
100
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM
Acetate + Co

Sample D1109

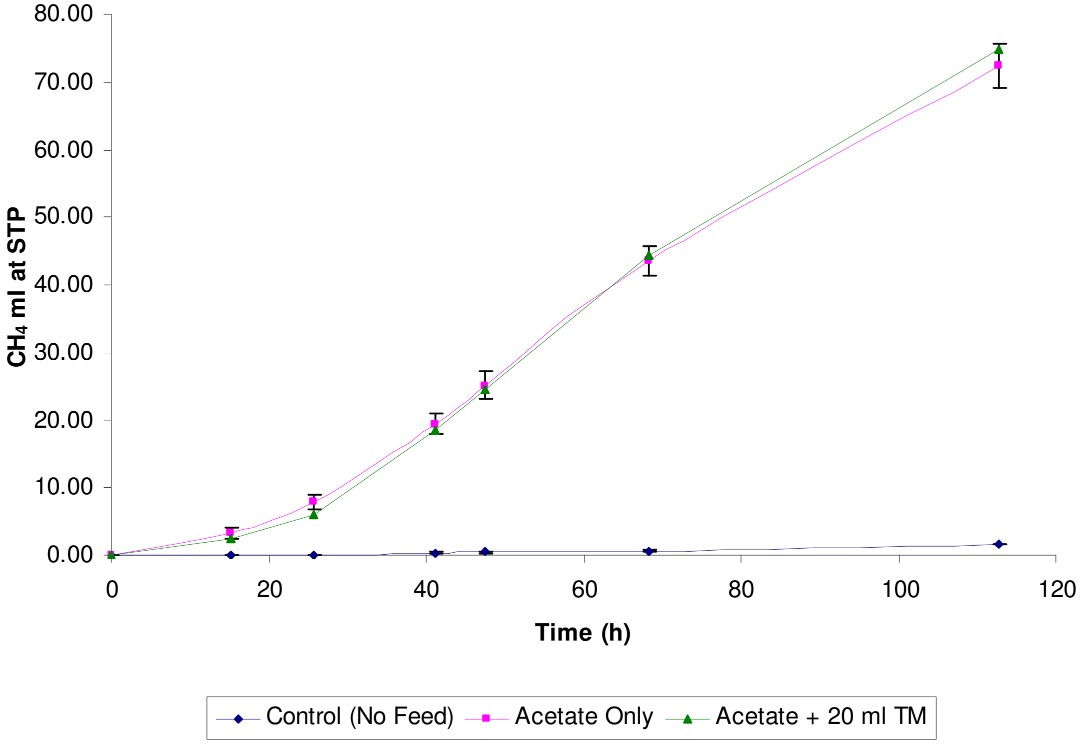
Average Cumulative Methane (CH_4 ml at STP) from Replicates							
Time (h)	Control (No Feed)	Acetate Only	Acetate + 1 ml TM	Acetate + 5 ml TM	Acetate + 10 ml TM	Acetate + 15 ml TM	Acetate + 20 ml TM
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
15.25	0.08	3.34	2.60	2.31	2.15	2.73	2.56
25.75	0.11	7.92	6.01	5.99	5.88	6.11	6.05
41.25	0.36	19.39	17.87	17.76	17.41	17.99	18.39
47.5	0.41	25.16	23.57	23.37	22.96	23.83	24.38
68.25	0.66	43.57	42.16	41.29	39.93	44.06	44.33
112.75	1.64	72.49	73.92	66.80	69.38	72.75	74.96
137.5	2.30	86.26	88.53	80.98	84.05	87.00	89.03
190.5	3.15	100.29	104.63	96.15	99.93	101.80	103.70
281	4.19	107.47	113.18	103.03	108.05	108.63	111.72

Average Cumulative Methane (CH_4 ml at STP) from Replicates							
Time (h)	Control (No Feed) n = 3	Acetate Only n = 3	Acetate + 1 ml TM n = 3	Acetate + 5 ml TM n = 3	Acetate + 10 ml TM n = 3	Acetate + 15 ml TM n = 3	Acetate + 20 ml TM n = 3
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
15.25	0.003	0.791	0.157	0.291	0.605	0.513	0.177
25.75	0.015	1.025	0.718	0.282	0.736	0.615	0.327
41.25	0.127	1.485	1.137	0.224	0.887	0.960	0.734
47.5	0.123	1.941	1.270	0.307	1.171	1.124	0.911
68.25	0.109	2.079	1.237	0.488	1.308	0.855	1.032
112.75	0.121	3.263	1.682	8.269	1.059	8.050	1.638
137.5	0.205	3.723	3.002	9.245	1.581	8.247	1.510
190.5	0.260	2.832	2.718	8.418	1.485	7.193	1.488
281	0.394	2.313	2.328	8.003	2.480	5.647	1.096

.



120.00
100.00
80.00
60.00
40.00
20.00
0.00
CH_4 ml at STP
0
50
100
150
200
250
300
Time (h)
Control (No Feed)
Acetate Only
Acetate + 1 ml TM
Acetate + 5 ml TM
Acetate + 10 ml TM
Acetate + 15 ml TM
Acetate + 20 ml TM



80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CH_4 ml at STP
0
20
40
60
80
100
120
Time (h)
Control (No Feed)
Acetate Only
Acetate + 20 ml TM

DISSEMINATION AND IMPACT

Omex Environmental Ltd Annual Conference
14- September 2011
Lincoln, UK

Omex Environmental Ltd is a manufacturer and supplier of nutrients for all types of industrial wastewater.

- Platform presentation

International Conference on Biogas Microbiology
14-Septemeber- 16-September
Leipzig, Germany

- Platform presentation (delivered by Cynthia Carliell-Marquet)

12th IWA Specialist Conference on Anaerobic Digestion- AD12
31-October - 4-November 2010
Guadalajara, Mexico

- Two full conference papers
- Platform presentation

Resource Efficiency KTN and WARMNET Annual Conference
30-June – 1-July 2008
Nottingham, UK

- Poster presentation

University of Birmingham Annual Doctoral Conference
14-May – 15-May
Birmingham, UK

- Platform presentation

Other Conferences Attended:

International Workshop on Anaerobic Digestion
10-December – 11-December 2009
Narbonne, France

Anaerobic Digestion Conference
5-May 2009
Loughborough, UK

IMPACT:

Full scale trace metal supplementation trial has been initiated at one of Severn Trent's wastewater treatment works after an increase of 34.4% in the V_{max} was demonstrated using the SMA methodology. The site was highlighted by Severn Trent as exhibiting a lower than normal methane output and was suspected of suffering from metal deficiencies. Interestingly the greatest response was found with cobalt-only supplementation, consistent with findings of this research. The supplementation trial is in its early phase however communication from Severn Trent has already revealed that after 25 days of the trial an increase has been found in the average daily electricity generation to **28% above** target in comparison to the **23% below** generation target (an average generation of 28751 kWh/day for the site) the site was found to be operating at for two months prior to supplementation. The above target electricity generation is a product of metal supplementation combined with an increase in loading of imported sludge. Therefore in accordance with this study the increase in methanogenic activity was able to be related to the digester as an increase in processing capacity.