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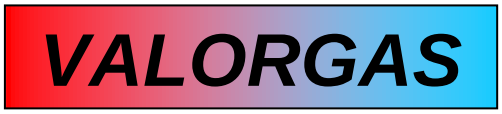
Biowaste as feedstock for 2nd generation



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D4.1: Description of problems associated with ammonia toxicity and trace metal deficiency in mesophilic and thermophilic digestion of high nitrogen wastes

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Revision [1]

D4.1: Description of problems associated with ammonia toxicity and trace metal deficiency in mesophilic and thermophilic digestion of high nitrogen wastes

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Revisions

Changes from version [0] consist of the addition of a Table of Contents and list of names of those mainly involved in preparing the report

Contents

D4.1 Description of problems associated with ammonia toxicity and trace metal deficiency in mesophilic and thermophilic digestion of high nitrogen wastes

Previous studies (Defra, 2010) have shown that food wastes have a high recoverable energy content (0.425 STP m^3 kg^{-1} VS_{added}), equivalent to ~100 m^3 of methane for each tonne of wet weight added to the digester. This methane yield can be produced consistently, but during extended run times the digester is likely to accumulate high concentrations of volatile fatty acids, in particular of propionic acid. This build-up may be related to the increasing concentration of ammonia as a result of the breakdown of organic nitrogenous materials: concentrations typically found in digestate can exceed 5000 mg l^{-1}. Similar conditions have previously been reported in food waste digestion trials at both mesophilic and thermophilic temperatures (Banks *et al.*, 2008). Although digesters can operate with high levels of VFA and ammonia for long periods there is a risk these conditions could result in sudden failure. This is most likely to occur when the concentration of propionic acid exerts sufficient acidity to overcome the pH buffering provided by the ammonia. If this happens, the pH in the digester can fall to a critical point where the methanogenic population fails, the digester becomes 'sour' and biogas production ceases.

To understand these unusual conditions in food waste digesters, it is necessary first to understand the biochemical routes by which methane is formed in the digestion process and the conditions needed by the different groups of microorganisms that carry out each stage in the conversion. The anaerobic digestion (AD) process is based on a close microbial association between acid-producing bacteria, acid-degrading bacteria and methanogens. These groups have to work together, as the energy yields from the biochemical reactions are low and would not proceed without syntrophy between the methanogens and the acid-degrading bacteria: this second group is therefore sometimes referred to as syntrophic acid oxidisers (also known as acetoclastic bacteria). These bacteria are able to oxidise the higher chain-length volatile fatty acids to acetic and formic acid, and in the process release hydrogen and carbon dioxide into solution. The methanogens convert these products into biogas, and in doing so ensure that the syntrophic acid-oxidising bacteria are able to function. If the end products of the acid oxidising bacteria (either formic acid or hydrogen) accumulate in the system, however, these organisms are unable to gain energy from the biochemical reactions, and therefore stop working. The removal of these intermediate products and their conversion into methane is thus essential to the proper functioning of the system, and forms the contribution of the methanogens to the syntrophy. By these reactions carbon in the waste is transformed into a mixture of carbon dioxide and methane. This escapes from the liquid phase as a gaseous product, in which the methane still retains oxidisable carbon that can be reclaimed as energy by combustion. The syntrophic relationship has one further important aspect, in that the biochemical energy released is very small. This limits the growth yield of the microbial population, and most of the original potential energy in the food waste ends up in the biogas, with very little excess microbial biomass yield.

The methanogens convert acetic acid, formic acid, hydrogen and carbon dioxide into methane. One group of methanogens (the acetoclastic methanogens) exclusively uses acetic acid to form biogas effectively by cleaving the molecule into two gaseous elements.

$$CH_3COOH \rightarrow CH_4 + CO_2$$

This group of methanogens is the one most commonly encountered in anaerobic digesters and provides the principal route through which methane is formed (in sewage sludge digestion, 70% of methane is typically formed by this group).

The second group of methanogens (hydrogenotrophic methanogens) form methane by chemical reduction of carbon dioxide with hydrogen, and this group is therefore most important for ensuring the syntrophy with the acid degraders

$4\,H_2 + CO_2 \rightarrow CH_4 + 2H_2O$

There is one other important product of the degradation of the higher chain-length volatile fatty acids: this is formic acid, which can result from the degradation of VFA with an odd number of carbon atoms, e.g propionic acid (C_2H_5COOH). Formic acid itself can also be converted to methane by the hydrogen and carbon dioxide route through a formate dehydrogenase enzyme system.

$4HCOOH \rightarrow 4CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O + 3CO_2$

The accumulation of formic acid will result in propionic acid not being degraded in the system, in the same way as hydrogen accumulation will stop higher all chain-length VFA being degraded and could thus account for the increased VFA concentrations seen in food waste digesters.

A potential explanation for this was put forward at the begining of the VALORGAS project based on results from earlier research and an outline understanding of the syntrophic reactions necessary to maintain stable and well-functioning digesters. This explanation is based on two major assumptions, namely:

- Ammonia is toxic to acetoclastic methanogens
- Food waste is deficient in the essential trace elements required for successful hydrogenotrophic methane production which, if present, could make up for the lost acetoclastic methanogenic activity.

The current deliverable was therefore designed firstly to review the state of knowledge on ammonia toxicity in anaerobic digesters, and secondly to establish the metabolic pathways in methane production and the dependence of these on enzymes or co-enzymes containing trace elements.

Although the above assumptions are interrelated, the deliverable report deals with them in two parts. Part A looks at the literature concerning ammonia toxicity in digesters that receive high nitrogen content substrate material. Part B examines the effects of trace elements in digesters as determined through experimental work, as well as reviewing the state of the art on the metabolic pathways leading to methane formation, the interdependencies within these pathways, and the role of trace elements in the enzyme systems that control them.

Part A Ammonia toxicity

A1 Introduction

In anaerobic digestion processes, ammonia is produced through the degradation of the nitrogenous matter present in the feedstock, mostly in the form of proteins (Kayhanian et al, 1999; Kotsyurbenko et al., 2004). In general, ammonia nitrogen is essential for bacterial growth: according to McCarty (1964) ammonia concentrations between 50 and 200 mg l^{-1} have a beneficial effect on anaerobic processes. At higher concentrations, however, ammonia is inhibitory to methanogenesis (McCarty, 1964; Gallert et al., 1998).

The quantity of ammonia generated from anaerobic biodegradation of an organic substrate can be estimated using the following stoichiometric relationship (Nielsen and Angelidaki, 2008).

$C_aH_bO_cN_d + (4a - b - 2c + 3d)/4\ H_2O = (4a + b - 2c - 3d)/8\ CH_4 + (4a - b + 2c + 3d)/8\ CO_2 + d\ NH_3$

In aqueous conditions ammonia exists mainly in two forms, as ammonium ion NH_4^+ and free ammonia $(NH_3)_{aq}$. The fraction of free ammonia relative to total ammonia is dependent on pH and temperature, as shown in the following formula (Hansen et al., 1998).

$$NH_3\ (\text{Free}) = \text{TAN} \times \left(1 + \frac{10^{-\text{pH}}}{10^{-(0.09018 + \frac{2729.92}{\text{T(K)}})}}\right)^{-1}$$

Where: NH_3 (Free) = free ammonia nitrogen concentration (mg l^{-1})
TAN = total ammonia nitrogen concentration (mg l^{-1})
T(K)= temperature in Kelvin

The proportion of free ammonia (FA) is of importance, as FA has been suggested as the main cause of inhibition (Angelidaki and Ahring, 1993; de Baere et al., 1984; Hansen et al., 1998; Kayhanian, 1994 and 1999; Kroeker et al., 1979). As the free ammonia fraction increases with pH and temperature, the concentration at thermophilic temperatures is expected to be higher than under mesophilic conditions at the same pH. Findings from several studies (Angelidaki and Ahring, 1994; Hansen et al., 1998) indicated that methane fermentation of wastes with a high ammonia concentration is more easily inhibited at thermophilic temperatures than at mesophilic temperatures, supporting the view that it is the free ammonia which causes toxicity. The digestion process becomes more sensitive towards ammonia when the pH value increases, which is again consistent with theory.

Under normal operating conditions, digesters treating waste rich in proteins tend to have high pH values, often above pH 8 (Borja et al., 1996). An increase in pH from 7 to 8 will lead to an eight-fold increase of the free ammonia concentration in mesophilic conditions (Hansen et al., 1998), and even more at thermophilic temperatures.

With reference to the two parameters controlling the process, pH and temperature, the following considerations should be taken into account:

1) pH affects the growth of microorganisms as well as the partitioning of TAN (Hansen et al., 1999; Hashimoto, 1983 and 1984; Kroeker et al., 1979). The equilibrium concentration between ammonium and FA follows the equation $NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O$ and depends on pH. The FA form of ammonia has been suggested as the toxic agent, therefore an increase in pH would result in increased toxicity (Borja et al., 1996). At high pH values the unionised form, free ammonia, dominates and this form is more inhibitory than the ammonium ion (NH_4^+).
2) Temperature affects both microbial growth rates and FA concentration. A higher process temperature may increase the metabolic rate of the microorganisms but also results in a higher FA concentration. Some authors have found that anaerobic fermentation of wastes with a high concentration of ammonia was more easily inhibited and less stable at thermophilic temperatures than at mesophilic temperatures (Braun et al., 1981; Parkin and Miller, 1983). Other studies, however, indicated that thermophilic flora tolerated at least twice as much FA as compared to mesophilic flora (Gallert and Winter, 1997)

Preliminary studies on the effect of ammonia on anaerobic digestion were carried out in 1964 by McCarty (cited by de Baere, 1984 and Borja et al., 1996) where it was postulated that the

mesophilic digestion process was inhibited at any pH when the total ammonia nitrogen exceed 3 g l^{-1}, while from 1.5 to 3 g l^{-1} was inhibited at pH from 7.4 to 7.6.

Inhibitory thresholds for ammonia have been reported in a number of studies, but the concentrations found vary significantly. Koster and Lettinga (1984) indicated that under mesophilic conditions, the maximum methanogenic activity was unaffected at TAN concentration of 680 mg l^{-1} (Free ammonia = 26.5 mg l^{-1}). However as the TAN concentration was increased to 1600 mg l^{-1} (Free ammonia = 60.3 mg l^{-1}), methanogenesis decreased to about 75% and declined further as the TAN increased. Kayhanian (1994) found that under thermophilic conditions, methane production decreased at total ammonia nitrogen concentration of 1000 mg l^{-1} (Free ammonia = 60 mg l^{-1}) when the digester was operated at pH of 7.5 or above. Other researchers have reported higher inhibitory thresholds at TAN concentrations of 1500-2500 mg l^{-1} (Hashimoto, 1986; van Velsen, 1979). This discrepancy illustrates the difficulties associated with reporting ammonia inhibition based on TAN rather than free ammonia, as the total ammonia inhibitory concentrations reported from different studies are not comparable unless the pH and temperature conditions are also cited. In the above studies by Koster and Lettinga (1984) and Kayhanian (1994), although reported inhibitory concentrations of ammonia are different, if converted to free ammonia they are more consistent.

Other factors such as microbial acclimation to high ammonia concentrations and cation antagonism effects (Chen et al., 2008; Lapp et al., 1975) could also contribute to the broad range of ammonia inhibitory thresholds reported in the literature. Higher tolerances can be achieved by acclimation of the anaerobic process to ammonia, as reported by van Velsen (1979). When digested sewage sludge and digested piggery manure acclimated to 815 mg l^{-1} and 2420 mg l^{-1} ammonia nitrogen respectively were used as inoculum in a batch experiment, the digested sewage sludge showed a longer lag phase of methane production as the ammonia nitrogen concentration increased in the range 730-4990 mg l^{-1}. Using digested piggery manure methane formation started immediately without a lag phase, although in both cases the maximum methane production rates decreased with increasing ammonia concentration.

Hensen et al. (1998) conducted a batch experiment to determine ammonia toxicity using inoculums acclimated to high ammonia. The study demonstrated that a free ammonia concentration of 1.1 g N l^{-1} (TAN=3.4 g l^{-1}) was needed to induce inhibition of the process. Below this value the specific apparent growth rates were found to be constant.

A number of other continuous studies have also reported adaptation of methanogenesis to ammonia concentrations far above the level believed to be inhibitory. Hashimoto (1986) observed that ammonia inhibition began at about 2.5 g N $l^{-1}$and 4 g N l^{-1} for unacclimated and acclimated thermophilic methanogens, respectively. Parkin and Miller (1983) reported that levels as high as 8–9 g l^{-1} of TAN could be tolerated with no significant decrease in methane production after acclimation. The experiments clearly demonstrated the possibility of obtaining stable digestion of manure with ammonia concentrations exceeding 5 g N l^{-1}after an initial adaptation period.

Since methanogens are the most sensitive amongst the complex microbial population involved in anaerobic digestion (Koster and Koomen, 1988) and the resistance to ammonia toxicity within methanogen species varies significantly (Borja et al., 1996; Koster and Koomen, 1988; Zeeman et al., 1985), the acclimation of the anaerobic process to a high concentration of ammonia may be the result of internal changes in the predominant species of methanogens, or of a shift in the methanogenic pathway (Zeeman et al., 1985).

In general, the literature on anaerobic digestion shows considerable variations in the inhibition/toxicity levels reported for TAN and FA, as shown in Table A1 and A2.

Table A1. Inhibition limit of FA and TAN in CSTR reactors.

Authors	Temperature	Substrate	Reactor	Inoculum	Inhibition limit FA mgN l^{-1}	Inhibition limit TAN gN l^{-1}	% reduction in CH4 production	pH
Sung and Liu, 2003	55	soluble non fat dry milk + NH4Cl	CSTR	acclim		5.77	64	6.40
Angelidaki et al., 1994	55	cattle manure	CSTR	acclim	600-800			7.4-7.9
Borja et al., 1996	55	cattle manure	UASB	acclim	500	7.00	72	
Zeeman et al., 1985	50	cattle manure	CSTR			1.70	initial inhibition	
Angelidaki and Ahring, 1993	55	cattle manure	Continuously fed reactor		900	4.00	25	
Hansen et al., 1998	55	swine manure	CSTR		1600		70	7.97
Hansen et al., 1998	60	swine manure	CSTR		2600		96	8.15
Kayhanian et al., 1999	55	OFMSW	Complete-mix reactor		45	1.2		7.20
Climenhaga and Banks, 2008	37	foodwaste	CSTR		> 1000	5.7		> 7.5
Gallert and Winter, 1997	55	OFMSW	CSTR	acclim	680-690	3.4 -3.5	50	7.60
Gallert and Winter, 1997	37	OFMSW	CSTR	acclim	220-280	3.0 -3.7	50	7.60
Gallert et al., 1998	55	OFMSW	CSTR with waste recirculation	acclim	251	1.83		

Acclim = acclimatised

Table A2. Inhibition limit for FA and TAN in BATCH assays

Authors	Temperature	Substrate	Reactor	Inoculum	Inhibition limit FA mgN l^{-1}	Inhibition limit TAN gN l^{-1}	% reduction in CH4 production	pH
Siles et al., 2010	52	glucose+Na2SO4+ammonia	Batch	acclim	620		21	
Liu and Sung, 2002	55	soluble non fat dry milk + NH_4Cl	Batch	acclim		10.00	100	
Hashimoto et al., 1986	55	cattle manure + NH_4Cl	Batch	acclim		4.00		7.2
Hansen et al., 1998	55	swine manure	Batch		1100			
Krylova et al., 1997	55	poultry manure+NH4Cl	Batch			2.6-8	80-90	
Benabdallah El Hadi T et al., 2009	55	synthetic OFMSW	Batch		468	6.07	50	7.5
Benabdallah El Hadi T et al,, 2009	37	synthetic OFMSW	Batch		215	4.08	50	7.5

Animal manures

A number of relevant studies on this topic were carried out on thermophilic digestion of manure. Zeeman et al. (1985) studied the influence of total ammonia concentration on thermophilic (50°C) digestion of cow manure, which in the Netherlands often contains total ammonia concentration up to 4 g l^{-1}. Results in a CSTR configuration showed inhibition at 1.7 g l^{-1} of TAN, but after acclimation a constant methane production was achieved even at higher ammonia levels (up to 3.3 g l^{-1} as TAN). Zeeman et al. (1985) carried out batch digestion trials, and observed a fourfold increase in methane production if the pH was reduced from 7.5 to 7.0.

Angelidaki and Ahring (1993) investigated the effect of ammonia on cattle manure digestion and found that an ammonia concentration of 4 g l^{-1} inhibited the process, but stable digestion could be maintained at up to 6 g l^{-1} after 6 months of operation, with a consequent reduction in methane production.

The combined effect of temperature and ammonia were studied by Angelidaki and Ahring (1994) in CSTR treating cattle manure. They observed a decrease in biogas yields both for low (2.5 g l^{-1}) and high (6 g l^{-1}) ammonia load, when the temperature was increased from 55 to 64°C. A negative effect at high ammonia loadings was also evident at a temperature of 45°C. These conditions corresponded to a FA concentration of 0.6-0.8 g l^{-1} which is higher than inhibition levels reported elsewhere for mesophilic digestion (80-150 mg l^{-1} of FA at pH 7.5) (Angelidaki and Ahring, 1994; Braun et al., 1981). Acclimation of the inoculum was suggested as the reason for this higher tolerance, as shown by Hashimoto (1986). Hashimoto (1986) found that if the inoculum was acclimatised at TAN between 1.4 and 3.3 g l^{-1}, inhibition began at 4 g l^{-1} compared to 2.5 g l^{-1} without acclimation.

Borja et al. (1996) tested the influence of ammonia on a thermophilic UASB process treating cattle manure, and observed initial inhibition at 5 g l^{-1} of TAN; but stable digestion could be maintained at 7 g l^{-1} after 6 months of operation although with lower gas production. It was also shown that strong inhibition occurred with rapid temperature increases but this could be reduced if the temperature was only increased gradually.

Krylova et al. (1997) tested different ammonia concentrations (as NH_4Cl) in the AD of poultry manure (batch tests) and observed no inhibition up to 2.6 g l^{-1} of TAN but a reduction of 80-90% in biogas production for concentrations ranging from 2.6 to 8 g l^{-1} of TAN. Hansen et al. (1997) observed a thermophilic AD inhibition treating swine manure in batch condition and found a limit value of 1.1 gN l^{-1} as free ammonia.

Municipal, commercial and industrial organic wastes

Liu and Sung (Liu and Sung, 2002; Sung and Liu, 2003) used acclimatized AD sludge at different ammonia concentrations with a substrate of non-fat dried milk, and observed in both batch and continuous tests that a strongly inhibitory TAN concentration for methanogens was between 8 and 13 g l^{-1} depending on acclimation conditions and the pH of the system.

Gallert and Winter (1997) studied the AD of OFMSW in CSTR configuration at thermophilic and mesophilic temperatures. During the thermophilic process 1.4 g l^{-1} of ammonia was released, whereas in the mesophilic process only 1 g l^{-1} ammonia was generated, presumably from protein degradation. The results reported a 50% inhibition in

methane production at 0.69 and 0.68 g l^{-1} of FA at thermophilic temperature, and a 50% inhibition at 0.22 and 0.28 g l^{-1} of FA at mesophilic temperature.

Kayhanian (1999) investigated ammonia inhibition in a pilot-scale high solids anaerobic reactor treating simulate OFMSW and showed an initial inhibition at a TAN concentration of about 1.2 g l^{-1} (FA 45 mg l^{-1} and pH of 7.2). Two strategies to mitigate ammonia inhibition were identified: the dilution of the digester contents with fresh water with the aim to correct ammonia overloads; and the adjustment of the feedstock C/N ratio from 27 to 32 (C/N ratio calculated using biodegradable carbon and total nitrogen values).

Climenhaga and Banks (2008) tested ammonia inhibition in bench-scale single-stage digesters treating food waste (a varied mix of fruits, vegetables, meats and fried foods). A constant organic loading rate (OLR) was maintained with different hydraulic retention times (25, 50, 100 and 180 days). The 100-day HRT reactors sustained total ammonia nitrogen (TAN) levels beyond 3 g l^{-1}, while in the 180-day HRT reactors anaerobic digestion continued at TAN concentrations exceeding 5.7 g l^{-1} at pH above 7.5. Free ammonia therefore exceeded 1000 mg l^{-1}, well beyond the threshold of 300 mg l^{-1} observed to have a severe inhibitory effect by Angelidaki and Ahring (1993). In this investigation, TAN appears to be more beneficial than detrimental, as it provides buffering capacity in the long HRT reactors, as opposed to the 25-day HRT reactor in which TAN was washed out and declined through the trial.

Benabdallah El Hadi et al. (2009) performed some batch tests at both mesophilic and thermophilic temperatures using a synthetic substrate simulating OFMSW. It appeared that not only free ammonia affected the methanogenic fermentation, but the ammonium ion also had similar effects. A 50% inhibition of biomethane production was observed at level of 215 and 468 mg FA l^{-1} under mesophilic and thermophilic conditions. However, methane generation under mesophilic and thermophilic conditions was reduced by 50% when the ammonium ion reached concentrations of 3860 and 5600 mg l^{-1} under mesophilic and thermophilic temperature conditions, respectively.

From the data in Tables A1 and A2, it could be concluded that in a CSTR configuration the inhibiting range for anaerobic digestion of cattle manure is 600-900 mg/l as free ammonia, while for swine manure it is from 1600 to 2600 mg l^{-1} of FA. Inhibition levels reported for batch assays of manure ranged between 2.6 and 8 g TAN l^{-1} or 1100 mg N l^{-1} as free ammonia. Anaerobic digestion of OFMSW in CSTR configuration appear to show the following inhibitory concentration range: 45-1000 mg FA l^{-1} and 1.83 – 5.7 g TAN l^{-1}, while anaerobic digestion of the same substrate in batch assays showed inhibitory concentrations in the range: 215-468 mg FA l^{-1} and 4.08-6.07 g TAN l^{-1}.

As expected, the reported inhibitory concentration of ammonia is different in different works. The significant difference in ammonia concentration can be attributed to the differences in substrates and inocula, environmental conditions (temperature, pH) and acclimation periods.

Considering microbiological aspects, at 1.1 g l^{-1} of FA ammonia affects organisms such as the acetate utilizing methanogens that are normally responsible for approximately 70% of methane production in the digestion of sewage sludge, while inhibition of the H_2-utilizing methanogens occurred at a higher FA concentration (>1.2 g l^{-1}) (Hansen et al., 1997).

In conclusion, the literature on anaerobic digestion shows considerable variations in the inhibition/toxicity levels reported for FA and TAN. The major reason for these variations is the complexity of the anaerobic digestion process where mechanisms such as antagonism, synergism, acclimation, and complexing could significantly affect the phenomenon of inhibition. Considerable further work is required to improve our understanding of ammonia toxicity which is clearly an important consideration in the digestion of food waste where TAN concentrations are likely to be in the range of 5-7 g l^{-1}.

A2 Mechanisms of Ammonia inhibition to Methanogenesis

Knowledge of the mechanisms of ammonia toxicity in anaerobic microorganisms is limited. Studies indicate, however, that it is sequential, involving firstly the diffusion of ammonia through cell membranes into methanogens and intra-cellular accumulation, which subsequently leads to toxic cytosolic enzyme and/or proton imbalance (Gallert et al., 1998, Sprott and Patel, 1986).

A2.1 Diffusion and accumulation of ammonia through cell membrane into cytosol

To understand the possible mechanisms of ammonia inhibition, it is important to consider the chemical interaction of ammonia and cells. In order to clarify these a physical model was proposed by Kayhanian et al (1999): in this model (**Figure 1**) the free ammonia molecule may diffuse passively into the cells of methanogens (De Baere et al 1984), causing proton imbalance and/or potassium deficiency (Sprott and Patel, 1986; Gallert et al., 1998). NH_3 enters into the cells and the different intracellular pH causes the conversion of part of this into ammonium (NH_4^+), absorbing protons in the process. The cells must then consume energy in proton balancing, using a potassium (K^+) pump to maintain the intracellular pH, thus increasing maintenance energy requirements, and potentially causing inhibition of specific enzyme reactions (Whittmann et al., 1995).

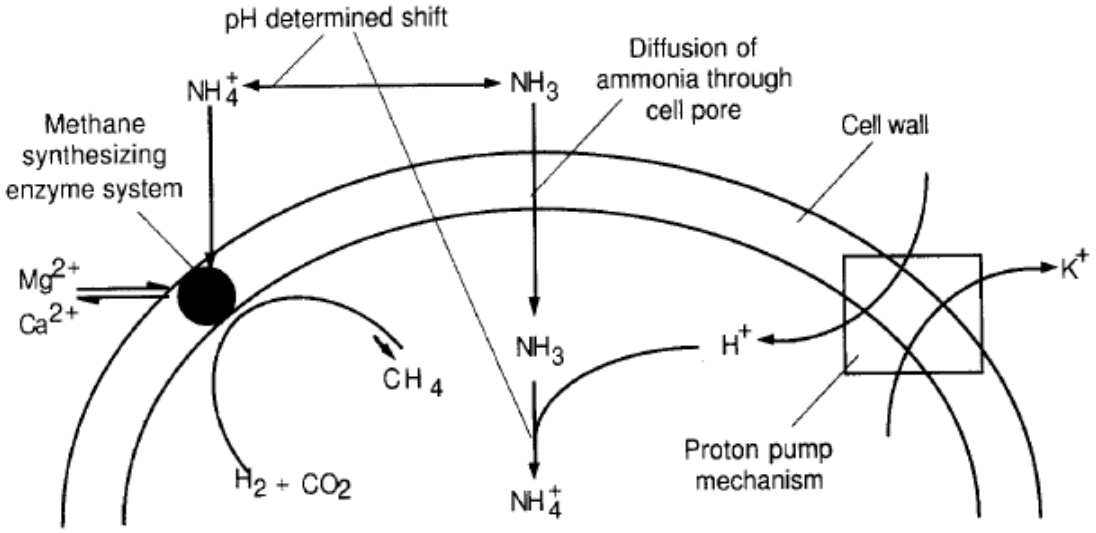

Figure A1. Proposed mechanism of ammonia inhibition in methanogenic bacteria (Kayhanian et al, 1999)

For most microorganisms, the energy equilibration in ATP is achieved by proton-translocating ATPases applying a proton motive force ($\Delta_{\mu H}^{+}$) across the cell membrane. The energy in the $\Delta_{\mu H}^{+}$ is the sum of the energies in the transmembrane pH gradient (ΔpH) and the transmembrane electrical gradient (Δψ)(Ni et al., 1994). For methanogens living in a slightly alkaline environment, studies have indicated that the transmembrane pH gradient (ΔpH) is small or even negative, therefore those methanogens can grow with a near-neutral cytosol even when the external pH is above 7 (Ni et al., 1994). However, the reversed ΔpH will lead to the accumulation of ammonium in the cytosol when a methanogen cell is exposed to an increased extracellular ammonia concentration (Kleiner, 1993). It is hypothesised that unionised free ammonia diffuses readily across the cell membrane, equilibrating the intracellular and extracellular concentrations of NH_3 and thereby leading to a rapid increase in cytosolic concentration of unionised ammonia. On the other hand, ammonium (NH_4^+) does not readily diffuse across cell membranes. The intracellular and extracellular concentrations of $NH_4^+$are dependent on NH_3 concentration and the local pH (pK_a = 9.24). Thus, cells whose intracellular pH is lower than the extracellular pH (i.e. negative ΔpH) have an intracellular NH_4^+ concentration greater than that of their environment. In cells with a very negative ΔpH, cytosolic NH_4^+ may constitute a considerable fraction of the intracellular cations. The procedures for measuring intracellular pH and cations concentrations are complex, however, and currently only very limited studies have been carried out on these membrane processes .

A study carried out using methanogen strain *Methanolobus taylorii* showed that most of the energy in the $\Delta_{\mu H}^{+}$ of the methanogen cell is accounted for by its large Δψ (i.e., the outside of the cell is more positively charged); its ΔpH is therefore small or even negative (Kleiner, 1993). The study discovered that for *M. taylorii*, the negative ΔpH allows it to grow with a near-neutral cytosol even when the external pH is above 7. The active extrusion of potassium by this methanogen was suggested as a mechanism by which it increases its Δψ, thereby allowing it to grow with a low or negative ΔpH (Ni et al., 1994, Hansen et al., 1998).

A2.2 Intracellular ammonia toxicity

Following the diffusion of ammonia into cell, at least two possible mechanisms of ammonia toxicity have been postulated (Kadam and Boone, 1996):

First is the direct inhibition of the activity of cytosolic enzymes by un-ionized ammonia. Kadam and Boone (1996) studied the active level of 3 ammonia-assimilating enzymes (glutamate dehydrogenase, glutamine synthetase, and alanine dehydrogenase) in 3 species of the family *Methanosarcinaceae*. The results showed diverse enzymic responses towards high levels of ammonia in growth media, which implied variable tolerances to ammonia between the three methanogen species.

Second, the NH_4^+ accumulated inside cells might be toxic due to its effect on intracellular pH (Sprott and Patel, 1986) or the concentration of other cations such as K^+ (Sprott et al., 1984). In a study conducted by Sprott et al. (1984) using pure culture *Methanospirillum hungatei* exposed to ammonia in a K^+ free buffer, it was observed that the methanogen lost up to 98% of the cytosolic K^+ through an ammonia/K^+ exchange reaction. The experiment also suggested that additions of NH_4OH or various NH_4^+ salts (or methylamine) were most effective in causing K^+ depletion in media of alkaline pH (i.e. higher unionised form free ammonia), suggesting that NH_3 was the active chemical species crossing the cell membrane and causing toxicity. It has also been reported that other essential cytosolic cations such as Mg^{2+} and Na^+ can be affected in the same way by ammonia (Kadam and Boone, 1996).

Based on the findings of these studies, it is reasonable to speculate that high ammonia could also affect the uptake of essential trace elements required for cell function and thereby cause micro-nutrient deficiency.

In either case, it is understandable that high pH and high total ammonia concentrations could exert their toxicities synergistically. At higher pH values, a larger fraction of total ammonia is unprotonated (about 0.5% at pH 7, but almost 65% at pH 9.5). Also, if methanogens growing at a higher pH establish a more negative ΔpH to maintain a near-neutral cytosol, then the potential toxicity due to NH_4^+accumulation would also be greater.

A3 Impact of ammonia toxicity on the diversity of methanogens and on the methanogenesis pathway in anaerobic digestion

It is generally acknowledged that of all the microorganisms involved in anaerobic digestion, the methanogens are the least tolerant to environment inhibitors and the most likely to cease growth due to ammonia inhibition (Kayhanian, 1994, McMahon et al., 2001, Kotsyurbenko et al., 2004).

Koster and Lettinga (2010) studied the changes in the microbial activity of the acidogenic and methanogenic population in granular sludge as ammonia nitrogen concentrations were increased in the range of 4051-5734 mg N l^{-1}. The experiment showed that the methanogenic population had lost 56.5% of its activity, while the acidogenic populations were hardly affected. The particular sensitivity of methanogens to ammonia toxicity will lead to the cessation of the methanogenesis stage of the anaerobic digestion, whilst the acid-producing stage continues. The consequent build-up of organic acids from inhibited methanogenesis will cause a rapid fall in pH and complete failure of the whole anaerobic processes (McMahon et al., 2001).

Among the methanogenic strains, the tolerance towards ammonia toxicity varies significantly. One early study indicated hydrogen-utilizing methanogens were more susceptible to ammonia than acetoclastic methanogens, and hydrogen accumulation subsequently causes build-up of propionate which in turn, acts as an inhibitor of the acetoclastic methanogens (Wiegant and Zeeman, 1986). There is now increasing evidence in the literature, however, that acetoclastic methanogens are more sensitive to ammonia toxicity than hydrogenotrophic ones at both mesophilic and thermophilic temperatures (Robbins et al., 1989, Schnürer and Nordberg, 2008, Angelidaki and Ahring, 1993). Koster and Koomen (1988) studied ammonia inhibition specifically of hydrogenotrophic methanogens using sludge that had never experienced ammonia inhibition before. The hydrogenotrophic population was found to grow well at ammonia concentrations as high as 6.3 g N l^{-1}. Unlike previous studies (Van Velsen, 1979, Koster and Lettinga, 1984, Koster, 1986) which used unadapted mixed populations of acetoclastic and hydrogenotrophic methanogens that were temporarily inhibited after exposure to ammonia and resumed methanogenesis only after an adaptation period, in the case of Koster and Koomen (1988) methanogenesis started without the requirement for acclimation. This indicated that the adaptation period was required only for acetoclastic methanogens, and this again supports other findings that hydrogenotrophic methanogens are less susceptible to ammonia toxicity.

A small number of toxicity studies have been carried out examining the different inhibition concentrations of ammonia towards hydrogenotrophic and acetoclastic methanogens. Jarrell et al. (1991) used pure culture to study the ammonia tolerance in four methanogen strains

commonly isolated from sludge digesters which can grow on H_2 and CO_2 (i.e. *Methanospirillum hungatei, Methanosarcina barkeri, Methanobacterium thermoautotrophicum,* and *Methanobacterium formicicum*). It was observed that *Methanospirillum hungatei,* the most sensitive to ammonia of the four strains investigated, can tolerate ammonia concentrations up to 4.2 g N l^{-1}; whilst the other three strains tested were resistant to concentrations above 10 g N l^{-1}. In similar pure culture studies conducted on four strains of thermophilic hydrogenotrophic methanogens, population growth could still be observed for some strains even at an ammonia concentration of 9 g N l^{-1} (Hendriksen and Ahring, 1991). The ammonia concentrations that can be tolerated by methanogens in those studies were significantly higher than in other studies using mixed cultures (Poggi-Varaldo et al., 1997), further indicating that hydrogenotrophic methanogens have higher tolerance towards ammonia toxicity.

As far as is known no literature to date has proposed a possible mechanism at a molecular microbiology level to account for the different sensitivities of the two groups of methanogens, or more specifically why ammonia should show greater toxicity to methanogens using the acetoclastic pathway. Some investigations have been conducted on the microbial response towards ammonia toxicity, as in Borja et al. (1996) who observed that inhibition of the acetoclastic populations showed a sigmoidal pattern. This finding coincided with results of the study conducted by Poggi-Varaldo et al. (1991) who found that the bacterial growth rate and the specific acetate-uptake rate were affected by the free ammonia concentration in a three-stage pattern: initial inhibition, plateau and final inhibition. This inhibition pattern could indicate that two inhibition mechanisms are involved, acting at different concentration levels. The hydrogenotrophic populations, however, exhibited a more linear pattern of inhibition (Borja et al., 1996).

Some other studies have focused on microbial diversity and morphology at genus and species levels. Sprott and Patel (1986) found that methane formation from obligate acetotrophs *Methanosaeta concilii* was completely inhibited at a total ammonia-N concentration of 560 mg l^{-1}, while methane formation from *Methanosarcina barkeri* was not inhibited at a total ammonia-N concentration of 2800 mg l^{-1}.

The 83 species of methanogens so far discovered (including six synonymous species) are divided into three main nutritional categories; 61 species (including five synonymous) of hydrogenotrophs oxidise H_2 and reduce CO_2 to form methane, while nine species (including 1 synonymous) of acetoclastic (or acetotrophic) methanogens utilize acetate to produce methane, of which only two species *Methanosaeta concilii* and *Methanosaeta thermophila* are obligate acetotrophs (i.e. the other 7 can also feed on H_2/CO_2 or formate depending on the availability of the carbon source) (Garcia et al., 2000).

Some researchers concluded that cell morphology plays a part in resisting ammonia toxicity. Demirel and Scherer (2008) attributed the particular susceptibility of acetate-utilizing methanogens *Methanosaetaceae* to the thin filaments cell morphology. Due to their cell shape, *Methanosaetaceae* offer a larger surface area to volume ratio than hydrogenotrophic methanogens growing as rods or *Methanosarcinaceae* consisting of thick clumps, and therefore the diffusion of free ammonia into the cells will be more effective. A similar speculation was also made by Wiegant and Zeeman (1986). A study by Hendriksen and Ahring (1991) even suggested that for some thermophilic hydrogenotrophic methanogens, high ammonia concentrations in the growth medium can induce formation of large cell

aggregates, which implied a possible defence mechanism for those strains against ammonia toxicity.

The selective inhibition of ammonia towards methanogens will have a profound impact on the diversity of the methanogenic population in an anaerobic digester treating ammonia nitrogen rich substrates: under the influence of ammonia, hydrogenotrophic methanogens will gradually become predominant and as the result the major methanogenesis pathway will shift to hydrogenotrophic.

Recent advances in molecular microbiology techniques such as Fluorescent in situ Hybridisation (FISH) and Quantitative Polymerase Chain Reaction (q-PCR) have made it possible to monitor changes in the methanogenic population in anaerobic digesters exposed to high concentrations of ammonia. Results from these studies have confirmed the change of dominance to hydrogenotrophic methanogens after exposure to ammonia.

Changes in methanogenic population and major methanogenic pathway were examined during start-up of a full-scale anaerobic sequencing batch reactor (ASBR) treating swine waste (Angenent et al., 2002). It was observed that after the increase in total ammonia concentration during the start-up process acetate degradation increased, however the acetoclastic methanogen population decreased based on the 16S ribosomal RNA (rRNA) levels. The findings are consistent with other studies suggesting that in some anaerobic systems a syntrophic relationship between an acetate-oxidizing organism (possibly a homoacetogen) and a hydrogen utilizing methanogen serves as the major route of methane production from acetate (Zinder and Koch, 1984).

Karakashev et al. (2005) studied the influence of environmental parameters on the diversity of methanogenic communities in 15 full scale-biogas plants treating either manure or sewage sludge as substrates under different conditions. The findings of this study indicated in plants operating at mesophilic ranges, where free ammonia concentrations were lower than in the thermophilic plants, the diversity of methanogenic population was broader. The dominance of the acetoclastic phylogenetic group *Methanosaetaceae* was observed in digesters fed with sewage sludge (ammonia concentration at 0.03-0.3 g N l^{-1}). However, *Methanosaetaceae* was never found to be dominant in digesters treating manure (ammonia concentration at 2.1-6.1 g N l^{-1}). According to the authors, inoculum and loading rates did not affect the diversity of methanogens in biogas reactors, but the concentrations of ammonia and volatile fatty acids (VFA) did have an effect. At high levels of NH_3 and VFA in manure digesters the dominance of *Methanosarcinaceae* was observed, while in sewage sludge digesters with low levels of NH_3 and VFA *Methanosaetaceae* dominated. Karakashev et al. (2006) also claimed that in the absence of *Methanosaetaceae*, the acetate oxidation to H_2/CO_2 with the subsequent generation of methane by hydrogenotrophic methanogens should be the dominant pathway. These results seem in agreement with the other studies (Angelidaki and Ahring 1993; Shigematsu et al. 2004).

The consequences of the change in methanogenic pathway for the apparent behaviour and maintenance strategies of the anaerobic digestion process are significant. Under normal, uninhibited conditions, 70% of the methanogenesis is attributed to the acetoclastic pathway and only 30% methane is formed via the hydrogenotrophic pathway (Jeris and McCarty, 1965, Gujer and Zehnder., 1983). Once the acetoclastic pathway is blocked, the hydrogenotrophic pathway starts to play a crucial role in keeping the hydrogen partial pressure low enough to make it thermodynamically possible for propionate and butyrate to be

converted into the methanogenic substrates acetate and hydrogen (Wolin and Miller, 1982). According to Gujer and Zehnder (1983), the hydrogen partial pressure should be maintained under 10^{-4} bar in order to sustain a healthy digestion process; furthermore hydrogen partial pressure is the parameter that most promptly indicates instability in the digestion process. Therefore if the digestion has been inhibited by ammonia and methanogenesis has become almost completely reliant on the hydrogenotrophic route, any further disturbance could cause an accumulation of hydrogen which subsequently results in a thermodynamic blockage of propionate degradation in a timescale of a fraction of a second (whereas acetoclastic blockage affects anaerobic digestion by reducing pH, which normally takes days). This may explain why anaerobic digesters treating nitrogen rich substrates such as food waste can experience stability problems making them prone to rapid and often irreversible operational failure (Neiva Correia et al., 2008; Banks and Zhang, 2010).

A4 Conclusions on ammonia toxicity

The above review has established that acetoclastic methanogens are inhibited at high ammonia concentrations and that this inhibition threshold is lower than that for hydrogenotrophic methanogens, in both thermophilic and mesophilic digesters. It is therefore likely that at the high ammonia concentrations reached in food waste digestion the acetoclastic methanogenic population will be lost or severely inhibited, and the direct formation of methane and carbon dioxide by cleaving of acetic acid would then be no longer possible. If under these conditions the autotrophic methanogens can continue to operate the acetic acid must first be converted to hydrogen and carbon dioxide. If this happens the process of methane production does not stop, only the route through which it forms changes; but current literature on ammonia toxcity does not offer any good explanation as to why these systems are likely to run at elevated VFA concentrations.

Part B The role of trace elements in anaerobic digestion

B1 Introduction

A hypothesis to explain previous observations concerning the accumulation of volatile fatty acids (VFA), and in particular of propionic acid, during food waste digestion was put forward at the start of the VALORGAS project. This was based on the knowledge that hydrogenotrophic methanogens can convert formic acid to methane, but making the assumption that if this population partly fails formic acid will accumulate. Limited evidence suggested that if this happens the syntrophic propionate-oxidising bacteria would be inhibited with the subsequent accumulation of propionic acid as an intermediate. Formic acid conversion to H_2 and CO_2 and subsequently to methane is thought to be dependent on a specific enzyme system (formate dehydrogenase) and it is not uncommon for this to include a selenium-containing amino acid which forms the complex molecule seleno-cysteine, or other metal enzyme complexes that might catalyse this reaction. The hypothesis proposed to explain the slow accumulation of VFA is that as time progresses a digester fed on food waste runs out of the trace elements necessary to form key enzyme complexes. If this hypothesis is correct then the enzyme system would fail, formic acid cannot be converted and propionic acid would accumulate as observed in food waste digesters.

A review of the literature on the role and importance of trace elements in anaerobic digestion was carried out to see if this added weight to the hypothesis proposed, and to compare and contrast the findings of others with the experimental work being carried out as part of the VALORGAS project.

Anaerobic digestion of biomass is a multi-stage process involving different types of microorganisms. In addition to macronutrients such as carbon, nitrogen, phosphorus and sulphur, trace elements play a crucial role in the growth and metabolism of anaerobic microorganisms where they are essential for many physiological and biochemical processes (Goodwin et al., 1990; Takashima and Speece, 1989). Many of these trace elements are metals (Zandvoort et al., 2006) and can be fundamental constituents of the anaerobic biomass or involved in enzyme activity. For example, Methanogenic Archaea are very diverse and vary greatly in their nutritional requirements and metabolic pathways. Different species have been shown to be stimulated by different trace metals (Ishaq et al., 2005). The methanogenic requirement for trace elements is not fully understood and this is a serious problem in commercial application of anaerobic biotechnology (Speece, 1983 and 2008).

Specific trace metals such as cobalt, nickel, tungsten or molybdenum serve as cofactors in enzymes which are involved in the biochemistry of methane formation (Zandvoort et al., 2006). If any required enzyme is limited, the whole process may be disturbed. Recent studies have highlighted potential stability problems in the anaerobic digestion of food waste (Climenhaga and Banks, 2008), and it is therefore necessary to define the trace element requirement for this material. The first part of this report therefore deals in general with role of trace elements in anaerobic digestion and the second part deals specifically with special factors that may need to be considered in relation to food waste digestion.

B1.1 The effect of trace elements addition on the anaerobic digestion of different substrates

A number of studies report trace element supplementation during either batch or continuous anaerobic digestion of different organic wastes. Evidence for the requirement comes from anaerobic digestion tests on wastewater sludges, energy crops (maize silage) and a variety of solid and liquid wastes.

B1.1.1 Trace elements requirements in anaerobic digestion of wastewater treatment biosolids

Ishaq and co-workers **(**Ishaq et al., 2010) looked at trace elements supplementation in municipal wastewater biosolids digesters and studied the effect of additions using biochemical methane potential (BMP) tests. Analysis of digested sludge samples taken from four different wastewater treatment works indicated sufficient trace metals were present and supplementation might not be necessary. The results of the BMP tests, however, showed that in one sample with low Fe (9000 mg kg^{-1} TS) and Co (8 mg kg^{-1} TS) contents, supplementation was of benefit. In this case the addition of 672 µg Fe, 224 µg Ni, 224 µg Co to each serum bottle used in the test resulted in a 20% increase in maximum methane production. In the three samples with higher concentrations of Fe (20000-80000 mg kg^{-1} TS) and Co (10-20 mg kg^{-1} TS) supplementation of Fe, Co, Ni did not improve biogas production.

Zitomer et al. (2008) looked at Ni, Co and Fe supplementation to full-scale thermophilic and mesophilic digesters treating either a mixture of primary sludge and waste activated sludge or activated sludge alone. The majority (77%) of digestate samples analysed in batch tests using acetate or propionate as the substrate benefited from the addition of 25 mg Ni l^{-1}, 25 mg Co l^{-1} and 25 mg Fe l^{-1} of the digestion mix. In some cases, supplementation by a single element increased the methane production and in other tests a mix of all three elements had no effect. From a biochemical viewpoint it was more often the rate of propionate utilisation which was increased compared to acetate (86% versus 33%). The average potential acetate and propionate utilisation rates were also found to be higher at a thermophilic temperature. After trace element addition thermophilic conditions also increased the methane production, with increases in the range 4% - 51%, compared to an increase from 7% to 36% under mesophilic conditions.

Hinken et al. (2008) carried out batch tests on anaerobic digester sludge and found that trace element supplementation did not give any particular improvement. He concluded that the concentration of trace elements already available in the system was sufficient.

B1.1.2 Trace elements requirements for the anaerobic digestion of maize silage and other crops

Phobeim and co-workers (Phobeim et al., 2010) studied Mo, Ni, and Co additions to batch digestion using a model substrate synthesised to simulate maize silage. The results showed an increase in methane yield of up to 30% using the three element mixture but this was also achievable using only nickel in the concentration range 0.4-10.6 μM Ni. Methane production was also increased by approximately 10% using Co in a concentration range of 0.4-2.0 μM whereas the addition of Mo did not significantly affect methane production. Hinken et al. (2008) quantified the trace metals in anaerobic digestates from different substrates: maize silage, rye crops, and wheat crops. They then tested different loading rates for maize, using acetate as a control, in batch tests with Fe, Co, Ni supplementation based on recommended dosages (Lettinga et al., 1996) which were: 11 mg Ni kg^{-1} COD_{in}, 9 mg Co kg^{-1} $COD_{in,}$ 7 mg Mo kg^{-1} COD_{in} . The metal additions were calculated by comparing metal concentrations in the substrate with these optimal concentrations. For maize silage, concentrations of Co, Fe, Ni were below detection limit and therefore received the full recommended dose. This resulted in 35% more biogas production than the control reactor where maize alone was fed.

Jarvis et al. (1997) used batch assays to examine if the addition of a single trace element (Fe, Ni, Co, Mo) or a mixture of trace elements could improve digestion of maize in a silage-fed mesophilic biogas process. Neither Mo at concentrations of 0.5 mg l^{-1} and 5mg l^{-1} nor Ni at concentrations up to 2.3 mg l^{-1} improved methane yields compared to the controls. Co at concentrations of 0.2 and 2.2 mg l^{-1} or a mixture of trace elements to give concentrations (mg l^{-1}) of 0.2 Co, 109 Fe, 0.7 Ni, and 0.5 Mo or Fe addition at 720 mg l^{-1} and 3600 mg l^{-1} all increased the biogas production. Addition of Ni at a final concentration of 23 mg l^{-1} inhibited methane production.

Gustavsson et al. (2010) investigated trace element supplementation in the digestion of a wheat stillage residue from bio-ethanol production. Supplementation of Ni (0.2 mg l^{-1}), Co (0.5 mg l^{-1}) and Fe (0.5 g l^{-1}) overcame the problems of poor process stability, low methane production, and accumulation of process intermediates.

Lebuhn et al. (2008) monitored biogas production for over a year in six laboratory-scale continuously stirred digesters fed on maize silage. The results showed process instability can be due to a deficiency of trace elements, and efficient long-term methanation is only possible if a cocktail of essential trace elements is provided. Co was reported to be the most limiting element and the recommended dose for stimulation was 0.02 - 0.4 mg l^{-1}; Mo and Se were also recommended but no dose was suggested. Alger et al. (2008) found that Co supplementation was required as a growth factor for cultures in corn stillage-fed thermophilic anaerobic sequencing batch reactors.

Clemens (2007) also commented that biogas plants operating with maize silage as single substrate consistently show a lack of trace elements and consequently a decrease in biogas production. This limitation leads to reduced methane yields and to considerable problems due to increasing process instability.

B1.1.3 Trace elements requirements for anaerobic co-digestion of mixed substrates

In many cases energy crops are mixed with manure or other co-substrates to enhance biogas production, and experience with these plants has showed these to be much less sensitive than mono-digestion (Hinken et al., 2008).

Hinken et al. (2008) used batch digesters to compare mono substrate digestion with co-digestion of energy crops and manure in which the proportion of manure was 10% and 50% on a VS basis. Comparison of anaerobic batch tests from a mono digestion plant and co-digestion plant showed great differences in biogas production: the co-digestion option showed a 70% increase in acetate utilization rate.

B1.2 Optimum concentration of trace elements

Other results found in the literature reported that the optimum or stimulatory concentrations of Co for batch cultures of methanogens were in a range between 5.9 mg m^{-3} and 120 mg m^{-3}. The optimum or stimulatory concentrations of Se were reported to range between 79 mg m^{-3} and 790 mg m^{-3}, while the optimum concentration for Mo was reported to be 48 mg m^{-3} (Demirel and Scherer, 2011; Takashima and Speece, 1990). Results are therefore still limited.

It is clear from the literature that deficiencies of trace metals in the AD process can arise when using mono-substrates like maize silage, but that problems are infrequent when using substrates like wastewater sludge which is rich in metals.

B2 Role of trace elements

B2.1 Trace elements present in anaerobic bacteria

Anaerobic bacteria are made up mainly of carbon, oxygen, hydrogen, nitrogen and phosphorus, typically in the proportions 45-55%, 20%, 10%, 6-14% and 1-3% respectively on a dry weight basis. Sulphur, sodium, potassium, calcium, and magnesium can each reach 1% depending on the specific bacterial strain. The remaining components make up less than 1% and are generally reported as 'trace' (Todar, web reference). With specific reference to methanogens and methane formation, the microbial regeneration time is a function of the concentration of macronutrients and the micronutrients present (Zhang et al., 2003). In order estimate the trace metals requirement of methanogens, accurate determination of intra-

cellular trace metals is useful. Scherer (1983) reported the major elements present in methanogenic bacteria as shown in Table B1.

Table B1. Elemental composition of methanogenic bacteria (percentage of wet weight)

Element	Min	Max
C	37.0	44.0
O	25.0	40.0
H	5.5	6.5
N	9.5	12.8
Na	0.3	4.0
K	0.1	5.0
S	0.6	1.2
P	5.0	2.8
Mg	0.1	0.5
Fe	0.1	0.3
Other	-	0.5

The literature shows, however, that the abundance of trace elements in anaerobic biomass is very variable and strongly dependent on the history of the anaerobic system. Many research papers have dealt with the metal composition of methanogenic biomass, and the following references are useful in this respect:

1. Nutrient requirement for anaerobic biomass (Hinken L et al., 2008)
2. Contents of micronutrients in acclimated-methanogens (Zhang et al., 2003)
3. Metal contents of inocula (Gustavsson et al., 2010)
4. Chemical composition (initial composition) of sludge granules - four full-scale anaerobic UASB- (Zandvoort et al., 2006)
5. Composition of full-scale digesters which treat wastewater (Ishaq et al., 2005)
6. Composition of liquid of full-scale plant (Feng et al., 2010)
7. Contents of trace elements in one Austrian agricultural biogas plant (digester sludge) (Pobeheim et al., 2010)
8. Composition of anaerobic sludge in a plants which treat municipal solid waste (office paper 30%, newspaper 30%, yard waste 35%, food waste 5%) (Lo et al., 2010)
9. Contents of trace metals in granular sludge of plant treating alcohol distillery wastewater and of plant treating paper mill (Hullebusch et al., 2005)
10. Contents of metals in granular sludge obtained from a full scale UASB reactor treating alcohol distillery wastewater (Zandvoort, 2003)
11. Digested sewage sludge in Japan, mesophilic and thermophilic condition (Uemura, 2010)
12. Composition of the major elements and trace elements of 10 Methanogenic bacteria determined by inductively coupled plasma emission spectrometry (Schreer, 1982)
13. Composition of digestate of biogas plants across Europe (Austria, Italy, Denmark, Germany, Poland, UK, Czech Republic) (Schattauer et al., 2011).

Table B2 summarises the metal contents found in anaerobic digester biomass and shows great variability in terms of trace elemental composition. The concentration range of Nickel, for example, varies from 0.7 to 230 mg kg^{-1} dry matter; the concentration range of Cobalt varies from 0.7 to 51 mg kg^{-1} dry matter; that for Manganese from 2 to 2569 mg kg^{-1} dry matter, for Copper 1 to 690 mg kg^{-1} dry matter, and for Selenium from 1 to 124 mg kg^{-1} dry matter.

The differences are probably due to the different origins of inocula and to factors such as the time for which the digester had been operational, the composition of material processed and the amount processed. Hinken et al. (2008) concluded after the analysis of different agricultural digesters that the concentration of certain micro and macro nutrients depended on substrate composition, operation time, and proportion of manure mixed with the substrate (for co-digestion plants).

Table B2. Presence of trace metals in anaerobic biomass

Metal	Nutrient requirement (Hinken et al., 2008)	Contents of micronutrients in acclimated-methanogens (Zhang et al., 2003)	Contents of inocula (Gustavsson et al., 2010)	Chemical composition of sludge granules - (Zandvoort et al., 2006)	Composition of full-scale digesters of wastewater (Ishaq et al., 2010l)	Composition of liquid of full-scale plant (Feng et al., 2010)	Composition of sludge from agricultural biogas plant (Pobeheim et al., 2010)	Composition of seeded sludge (Lo et al., 2010)
Unit	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**
Mn	334.29			4.55, 21.42, 25.62, 24.57		7.9	3.18	77.07
Cu	20.06	52.52		57.27, 10.50, 29.82, 63.00		2.6	0.48	6.12
Ni	2.01	16.12	1.13	10.79, 6.30, 22.05, 48.30	4.80, 7.11, 1.41, 2.76		0.06	1.89
Co		3.12	1.23	2.24, 6.51, 6.93, 10.71	0.33, 0,52, 0.52, 0,41		ND	0.33
Mo	1.67			ND, 10.29, 10.92, 7.56		0.25	0.02	0.12
Zn	53.49	52.56		63.08, 21.84, 476.07, 320.04		19	3.00	40.41
Fe	2674.29	382.72	1038.86	1.73, 5.44, 15.90, 7.56	383.74, 947.64, 1879.84, 2755.10		32.80	590.40
Se				ND, 0.40, 10.29, 26.04		ND	0.03	
W						ND		ND

ND = not detectable

Metal	Contents of trace metals in granular sludge of plant treating alcohol distillery wastewater (Hullebusch et al., 2005)	Contents of trace metals in granular sludge of plant treating paper mills (Hullebusch et al., 2005)	Contents of metals in granular sludge (Zandvoort, 2003)	Digested sewage sludge in Japan, mesophilic condition (Uemura, 2010)	Digested sewage sludge in Japan, thermophilic condition (Uemura, 2010)	Composition of methanogenic bacteria (Scherer, 1983)	Composition of digestate of biogas plants across Europe (Schattauer et al., 2011)
Unit	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1} dm**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**
Mn	5.20	53.88	2.4			< 5 - 25	6 - 74
Cu	57.00	29.49				< 10 - 160	0.45 -15
Ni	12.27	9.42	11.60	0.133	0.220	65 - 180	0.2 - 3.6
Co	2.21	13.67	1.70	3.52	3.49	10 - 120	0.03 -1.64
Mo				817	896	10 - 70	0.13 - 0.46
Zn	115.18	47.21	58.10			50 - 630	10-68
Fe	2249.30	10599.40	249.60			720 - 2150	48 - 1421
Se			1.80				0.01-0.4
W							

ND = no detectable

B2.2 The role of trace elements in biochemical reactions

Trace elements are important not only as part of the cell itself but also because they are involved in enzyme activity, and several important metal-based enzymes or co-enzymes have been identified in methanogens.

Co

Cobalt is present in specific enzymes and corrinoids. It is required for synthesis of vitamin B12 (cyanocbalamin) and it activates carboxylpeptidase. A corrinoid, such as vitamin B12, containing cobalt ion is known to bind to coenzyme M (CoM) methylase which catalyses methane formation in both acetoclastic methanogens and hydrogenotrophic bacteria. Co is essential for enzyme methyltransferase which catalyse the transfer of one methyl group. The common enzyme carbon monoxide dehydrogenase (CODH) uses cobalt as well. CODH plays an essential role in the acetogenic process (Müller 2003; Murakami and Ragsdale 2000; Thauer et al., 2008). Some lab tests have confirmed the positive effect of cobalt addition on methanogenesis. Feng et al. (2010) and Lo et al. (2010) employed a laboratory-scale test using industrial food waste and municipal solid waste to demonstrate the positive effect of Co on biogas production.

Cu

The role of copper in methanogenesis is subject to conflicting observations. Copper has been found in many methanogenic bacteria strains, but copper addition has not been found to have any noticeable stimulatory effects on biogas production. As the effect of this metal in methanogenesis has only been studied through being part of a trace metal mix supplementation, it is currently impossible to understand with any certainty the role of Cu in biogas production.

Mg and Mn

Mn stabilises methyltransferase in methane-producing bacteria and is often interchangeable with Mg in kinase reactions. It is not clear if these metals are effectively related to biogas production. As with copper, the roles of Mg and Mn in methanogenesis have only been studied by supplementation in trace metal mixes.

Fe

Iron plays numerous roles in anaerobic processes, primarily due to its extremely large reduction capacity. Zandvoort et al. (2003) studied the effect of Fe, Ni and Co on the conversion of methanol in an upflow anaerobic sludge bed reactor (UASB) with methanol as substrate. The results showed that only Fe had a significant effect on the methanol degradation rate and on methanogenic activity. It is clear that iron and nickel are present in the form of a Ni-Fe-S cluster and Fe-S cluster, and these are mainly subunits of enzymes such as hydrogenase and acetyl-CoA synthase (Lindahl and Chang 2001; Thauer et al., 2010).

Mo

Molybdenum is present in the common enzyme formate dehydrogenase (FDH). However, molybdenum may also inhibit sulphate reducing bacteria, limiting the formation of necessary sulphides. Mo seems to stimulate methane production from maize silage substrate (Jarvis et al., 1997; Pobeheim et al., 2010) and from municipal solid waste (Lo et al., 2010).

Ni

Many anaerobic bacteria are dependent on nickel when carbon dioxide and hydrogen are the sole source of energy. The nickel tetrapyrrole, coenzyme F_{430}, is known to bind to methyl-S-CoM reductase which catalyses methane formation from methyl-S-CoM in both acetoclastic and hydrogenotrophic methanogens. In addition, CODH is a nickel protein and may aid sulphur-

reducing bacteria. The role of Ni in methanogenesis is related to the following enzymes: CODH, methylreductase, hydrogenases and synthesis of F_{430}.

Uemura (2010) tested the role of Ni, Co, Fe in anaerobic digestion in completely stirred tank reactors fed with organic solid waste from kitchens and demonstrated that Ni was the most important metal for anaerobic digestion of the organic solid waste.

Se and W

Selenium is a component of several anaerobic bacterial enzymes and certain bacterial nucleic acids. A common selenium enzyme in anaerobic bacteria is formate dehydrogenase (FDH). Tungsten is also a component of the FDH enzyme.

Few studies have dealt with the influence of Se and W in methanogenesis. Jones and Stadtman (1977) demonstrated that the growth of *Methanococcus vannielii* (an anaerobic coccus able to produce methane) is markedly stimulated by selenium and tungsten. Feng et al. (2010) employed a laboratory-scale reactor treating food industry waste with the aim of investigating the effects of Co, Ni, Mo, B, Se, and W on biogas production. The results showed the highest methane production was linked to addition of Se and W in combination with Co.

These studies are not sufficient to give a clear idea about the role of Se and W in methanogenesis and deeper investigation of the scientific literature beyond that on anaerobic digestion shows that Se is an essential trace element for many organisms. The most important and best characterised biological form of Se is that of the amino acid selenocysteine (Sec), the 21st genetically encoded amino acid. It is structurally identical to cysteine (Cys), only with the thiol group replaced by a selenol group. The use of Sec can be partly explained by its high nucleophilicity and the fact that the selenol group is mostly deprotonated at physiological pH due to its lower pK_a value (5.2 for Sec, 8.3 for Cys) making it more reactive than Cys. Due to this trait, Cys is almost exclusively found in the catalytic site of numerous redox-active enzymes (Rother and Krzycki, 2010).

The only Archaea for which selenoproteins have been demonstrated, either by experimentation or prediction from genome sequence data, are the hydrogenotrophic methanogens although not all hydrogenotrophic methangens employ Sec (Kryukov and Gladyshev, 2004; Stock and Rother, 2009).

All Gram-positive bacteria for which selenoproteins have been experimentally demonstrated belong to the clostridial clade and are therefore strictly anaerobic. Members of this clade are able to grow by means of various mixed acid and alcohol fermentations (e.g. butyrate-, acetone/butanol-, Stickland-fermentation) and/or anaerobic respirations (e.g. sulfate- or acetogenic carbonate respiration). Metabolic pathways where selenoproteins are involved include autotrophic acetogenesis via the reductive acetylcoenzyme A (acetyl-CoA) pathway, glycine-dependent acetogenesis, acetogenesis via Stickland-fermentation, and purine/pyrimidinedependent acetogenesis. Selenoproteins are also found in these organisms involved in other processes, such as antioxidant defense, redox cycling and redox homeostasis, and even Sec biosynthesis itself (Stock and Rother, 2009).

Zn

Although zinc is part of enzymes such as formate dehydrogenase (FDH), super dismutase (SODM), and hydrogenase, it has not yet proven to be an essential metal for methanogenesis.

Table B3 summarises the work carried out on metal supplementation with the aim of showing the effect on biogas production. It is clear from the reported data that there is no definitive agreement on which metals are important and which are not: very often the same element is reported as essential for biogas production by one group of researchers and useless by a different group.

Table B3. Trace element function

Nutrient	General function	References
Cobalt ***Co***	Cobalt is present in specific enzymes and corrinoids	Kayhanian and Rich, 1995; Oleszkiewicz et al., 1990; Kida et al., 2001.
	It is required for synthesis of vitamin B_{12} (cyanocbalamin) and it activates carboxylpeptidase	Schattauer et al., 2011; Burgess et al., 1999.
	The common enzyme carbon monoxide dehydrogenase (CODH) uses cobalt. CODH and plays an essential role in acetogenic (acetate-forming) activity	Oleszkiewicz et al., 1990; Kayhanian and Rich, 1995; Schonheit and Thauer, 1979.
	The role of Co in methanogenesis is related to the enzyme methyltransferase	Somitsch, 2007.
Copper ***Cu***	Copper has been found in the analysis of many methanogenic bacteria strains.	Kayhanian and Rich, 1995.
	Copper may be a component in super dismutase (SODM) and hydrogenase.	Oleszkiewicz et al., 1990; Kirby et al., 1981.
	It is a metallic enzyme activator and it chelates other substances and reduces their toxicity.	Schattauer et al., 2011; Burgess et al., 1999.
	Copper addition has not been found to have any noticeable stimulatory effects in biogas production.	Kayhanian and Rich, 1995.
Magnesium ***Mg***	It is an enzyme activator for number of kinases and phosphotransferase.	Burgess et al., 1999.
	Magnesium is not related to biogas production.	Schattauer et al., 2011.
Manganese ***Mn***	Mn is a cofactor of various enzymes and activates isocitric dehydrogenase and malic enzyme systems of bacteria·	Schattauer et al., 2011; Burgess et al., 1999.
	It stabilises methyltransferase in methane producing bacteria and Mn is often interchangeable with Mg in kinase reactions. Mn is also involved in redox reactions	Oleszkiewicz et al., 1990; Burgess et al., 1999.
	Manganese has not yet been proven to improve methane production	Schattauer et al., 2011.

Nutrient	General function	References
Iron ***Fe***	Iron has been found to be present in methanogenic tissue in concentrations higher than that of any other trace metal. Iron plays numerous roles in anaerobic processes, primarily due to its extremely large reduction capacity. Iron is found in, and helps activate, numerous enzymes. In addition, iron may form sulphide precipitates and may promote excretion of extra cellular polymers. **The role of Fe in methanogenesis is related to the following enzymes:** - Formyl MF dehydrogenase - CODH, Acetyl-CoA synthesis - Hydrogenases	Schattauer et al., 2011; Kayhanian and Rich, 1995. Kayhanian and Rich, 1995; Brock et al., 1984. Somitsch, 2007.
Molybdenum ***Mo***	Molybdenum is present in the common enzyme formate dehydrogenase (FDH). However, molybdenum may also inhibit sulphate reducing bacteria, limiting the formation of necessary sulphides. Mo is a cofactor of various flavinous enzymes **The role of Mo in methanogenesis is related to the following enzymes:** - Formyl MF dehydrogenase - FDH	Oleszkiewicz et al., 1990. Schattauer et al., 2011. Somitsch, 2007.
Nickel ***Ni***	Many anaerobic bacteria are dependent on nickel when carbon dioxide (CO_2) and hydrogen (H_2) are the sole sources of energy. Most nickel is taken up by cells in a compound named F factor 430 (F_{430}). F_{430} has been found in every methanogenic bacterium ever examined. In addition, CODH is a nickel protein and may aid sulphur-reducing bacteria. Ni stabilises DNA and RNA, and it is a cofactor of urease. **The role of Ni in methanogenesis is related to the following enzymes:** - CODH - Methylreductase - Hydrogenases - Synthesis of F_{430}	Kayhanian and Rich, 1995. Diekert et al., 1981; Kayhanian and Rich, 1995; Hausinger, 1987; Thauer et al., 1980; Kida et al., 2001. Oleszkiewicz et al., 1990; Schattauer et al., 2011. Somitsch, 2007; Diekert et al., 1981.

Nutrient	General function	References
Selenium ***Se***	Selenium is a component of several anaerobic bacterial enzymes and certain bacterial nucleic acids. A common selenium enzyme in anaerobic bacteria is formate dehydrogenase (FDH). Selenium-dependent enzymes tend to be very reactive at neutral pH, have a low redox potential, and may help metabolize fatty acids. The catalysts which contain selenium are synthesised when selenium is present at extremely low concentrations.	Kayhanian and Rich, 1995; Schattauer et al., 2011; Stadtmann, 1980.
	The role of Se in methanogenesis is related to the following enzymes: - FDH - Formyl MF dehydrogenase - CODH, Acetyl-CoA synthesis	Stadtmann, 1980; Somitsch, 2007.
Tungsten ***W***	Tungsten is a component of the FDH enzyme. It is possible that tungsten may aid the metabolism of CO_2 and H_2 , in a manner similar to nickel.	Zellner et al., 1987; Kayhanian and Rich, 1995.
	Limited studies have been conducted on the effect of tungsten supplementation	Kayhanian and Rich, 1995.
Zinc ***Zn***	Zinc, like copper, is present in relatively large concentrations in many methanogens.	Kayhanian and Rich, 1995.
	It may be part of FDH, SODM, and hydrogenase.	Kayhanian and Rich, 1995; Kirby et al., 1981; Oleszkiewicz et al., 1990.
	It can stimulate cell growth and it is a cofactor of RNA- and DNA-polymerase	Schattauer et al., 2011.
	Zinc has not yet proven to be an essential metal	Kayhanian and Rich, 1995.

*CODH = the enzyme carbon monoxide dehydrogenase
**SODM = the enzyme super dismutase.
***FDH = the enzyme formate dehydrogenase

B3 Trace elements requirements for the anaerobic digestion of food waste or the Organic Fraction of Municipal Solid Waste (OFMSW)

This part of the review focuses specifically on the trace element requirement for anaerobic digestion of food waste and OFMSW both in mesophilic and thermophilic conditions.

The literature suggests that there are different nutritional requirement under thermophilic and mesophilic conditions. For example, Takashima and Shimada (2004) reported minimum requirements for iron, nickel, cobalt, and zinc in thermophilic methane fermentation from glucose to be 3.5, 0.40, 0.45, and 2.0 mg l^{-1}, respectively. These concentrations are ten times greater than those determined for mesophilic methane fermentation from acetate (Takashima and Speece, 1989), indicating a possible decrease in bioavailability or increase in nutrient requirements at thermophilic temperatures. Macleod and Forster (1988) showed that thermophilic biomass was more sensitive to the effect of toxic metals than mesophilic biomass, with the effects exhibited at much lower concentrations. In general, different substrates have different trace element requirements and too high a concentration of trace elements can have a negative effect and cause inhibition (Hinken et al., 2008; Isha. et al., 2010; Jarvis et al., 1997).

The literature suggests that food waste is heterogeneous in composition and there are several articles that give information on the presence of trace metals in this type of material:

1. Composition of food waste (Banks and Zhang, 2010)
2. Composition of food waste (Zhang et al., 2007)
3. Presence of trace metals into biodegradable organic fraction of municipal solid waste of United States (composition: 54% office paper, 18% newsprint, 17% yard waste - dry weight basis) (Kayhanian and Rich, 1995)
4. Composition of municipal solid waste (office paper 30%, newspaper 30%, yard waste 35%, food waste 5%) (Lo et al., 2010)
5. Presence of trace elements in a model substrate (Organic Solid Waste in Japan, % w/w, apple (10%), grapefruit (rind, 5%), orange (rind, 5%), banana (rind, 10%), cabbage (12%), potato (12%), carrot (12%), ground meat (5%), fish (5%), egg (4%), polished rice (10%), bread (5%), Japanese noodle (2.5%), and Chinese noodle (2.5%)) (Uemura, 2010)
6. Contents of Ni e Co in distillery wastewater (Kida and Sonoda, 1993)
7. Characteristics of food industrial wastewater (Oleszkiewicz and Romanek , 1989)
8. Composition of food waste (El-Mashad et al., 2010)

Table B4 summarises the typical trace element content of food waste.

Table B4. Typical trace metals presence in food waste

Metal	Food waste composition (Banks and Zhang, 2010)	Composition of food waste, (Zhang et al., 2007)	Biodegradable organic fraction of municipal solid waste of USA. (Kayhanian and Rich, 1995)	Composition of municipal solid waste (Lo et al., 2010)	Composition of trace elements in a model substrate (Uemura, 2010)	Contents of Ni e Co in distillery wastewater (Kida and Sonoda, 1993)	Characteristics of food industrial wastewater (Oleszkiewiez and Romanek, 1989)	Composition of food waste (El-Mashad et al., 2010)
Unit	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**	**mg kg^{-1}**
Mn	23.15 24.32 20.18	15.84		9.03				14.00
Cu		8.18	4 - 8 Av = 5.84	1.80				2.80
Ni		0.53	0.08 - 4 0.208	ND		1.13	0.08	ND
Co	0.02 0.09 <0.06		0.08 - 2.40 Av = 0.104	0.01	ND	0.36		
Mo	0.26 0.26 0.11		0.08 - 4.80 Av = 0.80	0.03				ND
Zn		20.06	25.60 - 71.20 Av = 45.60	1.87				
Fe	35.08 45.05 54.36	202.22	460 - 960 Av = 592	44.21	38.90		1.80	201.60
Se	0.28 0.10 <0.07		ND - 0.80 Av = 0.08					
W	0.26 0.26 <0.24		ND - 0.80 Av = 0.096	ND	ND			

ND = not detectable
Av = average

B3.1 Anaerobic digestion of food waste in mesophilic conditions

Source segregated food wastes are very high in potential energy and therefore make excellent substrates for biogas production through anaerobic digestion (Liu et al. 2009; Zhang et al. 2007). There are, however, many examples of food waste digestion where the AD process has suffered from high volatile fatty acids (VFA) concentration after extended periods of operation. These include a technical-scale trial using kitchen waste collected in the UK (Banks et al., 2008), and the digester failure in a 30,000 tonne per year food waste digester operated by Valorsul SA in Lisbon, Portugal (Neiva Correia et al., 2008). Similar problems were also observed repeatedly in smaller scale laboratory trials (Wang and Banks, 2003; Climenhaga and Banks 2007 and 2008; Zhang and Banks 2008; Banks and Zhang, 2010) when using source-segregated catering waste or domestic food waste was used as a sole substrate in the digestion process. In the study by Climenhaga and Banks (2008) source separated food residues (a varied mix of fruits, vegetables, meats and fried foods) were digested in bench scale single-stage digesters at a constant organic loading rate (OLR) and different HRTs (25, 50, 100 and 180 days). Initial problems with the digestion were overcome when the authors used a trace metal solution containing Fe, Cu, Zn, Mn, Mo, Co, Al and Se (Gonzalez-Gil et al., 2001). This work showed the importance of micronutrients in digestion of a mixed foodwaste feedstock: reactors supplemented with trace elements showed a stable digestion, while non-supplemented reactors showed methanogenic failure.

In a recent study the effect of trace metal addition, namely Co, Ni, Mo, B, Se and W, was investigated in a lab-scale work on biogas production from food industry residues (Feng et al., 2010). The laboratory-scale batch reactors worked at mesophilic temperature and trace elements were added with the daily feed. High addition of Se/W resulted in the highest production of methane in combination with a low level of Co. The ranges of Se and W concentrations in the reactors after addition varied from 8 to 800 mg m^{-3}, and 18 mg m^{-3} to 1.80 g m^{-3}, respectively, while Co concentration ranged between 60 mg m^{-3} and 6 g m^{-3}. Furthermore, this study suggested that the *Archaea* spp. in biogas reactors were more sensitive to trace metals levels when compared with the other members of the bacterial community.

In another recent batch study, using OFMSW as substrate, the effects of trace metals on mesophilic biogas production were investigated (Lo et al., 2010). Cr, Ni, Zn, Co, Mo and W all had the potential to enhance biogas production, with the exception in these tests being Zn. The useful concentrations of Cr, Ni, Co, Mo and W were reported to vary between 2.2 mg m^{-3} and 21.2 mg m^{-3}, 801 mg m^{-3} and 5,362 g m^{-3}, 148 mg m^{-3} and 580 mg m^{-3}, 44 mg m^{-3} and 52.94 g m^{-3}, and 658 mg m^{-3} and 40.39 g m^{-3}, respectively. Metal levels higher than threshold values would result in adverse effect, leading to inhibition of biogas production.

Banks and Zhang (2010) carried out a batch test in mesophilic conditions, using food waste as substrate. The aim of this study was to explore the possibility of regulating the metabolic pathway leading to methane production by trace element addition. The results showed that Se and Co are the key trace elements needed for the long-term stability of food waste digestion, but likely to be lacking in food waste. The minimum concentrations recommended for selenium and cobalt in digesters being fed food waste at moderate organic loading rates (~2-3 kg VS m^{-3} day^{-1}) was around 0.16 and 0.22 mg l^{-1} respectively. A total selenium concentration greater than 1.5 mg l^{-1} is likely to be toxic to the microbial consortium in the digester. Mo, W, and Ni are present in food waste in sufficient quantities

for moderate loadings, but may have to be supplemented in digestion at a high organic loading rate (5 kg VS m^{-3} day^{-1}).

The stimulatory effects of Ni, Co and Mo on biogas and methane production rates were previously studied in a methanogenic fixed-film reactor treating food residues (Murray and Berg, 1981). When Ni and Co were tested in combination, they stimulated methanogenesis to a much greater extent than could have been expected when they were added individually; and molybdenum increased methanogenic activity only when added in combination with both nickel and cobalt. Total biogas and methane production increased approximately 42% with addition of Ni (100 nM), Co (50 nM), Mo (50 nM).

B3.1.1 Problems encountered with food waste digestion

The work of Banks and Zhang (2010) showed clearly that VFA accumulation in mesophilic food waste digesters is a common problem and starts with acetate accumulation and may indicate inhibition of acetoclastic methanogenesis. This increase is followed by a decline in acetic acid concentrations and a subsequent increase in longer chain length VFA species, particularly propionic acid, probably due to product-induced feedback inhibition. Anaerobic digestion is a complex process and its metabolic routes are moderated and directed through syntrophic reactions between different groups of microorganisms (Pind et al., 2003; Speece 1983) and this feedback control is governed by the buildup of methane precursors, namely, hydrogen, formate, and acetate. It is now thought that this pattern is the result of ammonia inhibition of the acetoclastic methanogenic population followed by a shift in the population structure to give a predominantly hydrogenotrophic methanogenic population. Other work indicating such a shift in population under conditions of high ammonia concentrations and the importance of the hydrogenotrophic methanogenic population under these conditions has been shown on a number of occasions (de Bok, 2003; Plugge et al. 2009; Schnurer et al., 1994; Schnurer and Nordberg, 2008).

B3.2 Anaerobic digestion of food waste in thermophilic conditions

In addition to macro-nutrients, Kayhanian and Rich (1995) used micro-nutrients, namely Co, Cu, Fe, Mo, Ni, Se, W and Zn, during thermophilic pilot-scale digestion of OFMSW. The authors concluded that the nutrients supplementation helped to elevate the gas production by 30% and increase the stability of the reactor. However, it should be taken into account that this effect was observed due to the supplementation of both macro (N, K, P, and S) and micro-nutrients (trace metals) together. Furthermore, the authors also presented a literature survey on the stimulatory ranges of trace metals for anaerobic digestion of biomass. The stimulatory ranges for Co, Fe, Mo, Ni and Se were reported to be 0.05-0.19, 0-0.39, 0.16-0.3, 0.11-0.25, and 0.062 mg kg^{-1}, respectively.

Many studies have focused on the metals requirement when using other substrates like starch-based waste or maize silage. For example, Leighton and Forster (1997a) examined the toxic effect of heavy metal ions (Cu, Zn, Ni, Pb) on the performance of a two-phase thermophilic anaerobic reactor operated with a starch-based feed. In each case, four concentrations (0.5, 1, 2 and 3mM) were used and the addition was made continuously over a period of 30 hours. The individual addition of metals showed that all metals concentrations led directly to a fall in methane production and COD reduction, although this was not proportional to the metal concentration. The results also showed that Ni and Cu caused less impact than Zn and Pb. Another study from the same authors (Leighton and Forster, 1997b),

which utilised the same apparatus (two-phase thermophilic anaerobic reactors operated with a starch-based feed), suggested that the use of two phase systems did not offer any real protection to the methanogenic bacteria against metal toxicity.

A more recent study (Zhang et al., 2007) analysed food waste composition in California and the anaerobic digestibility and biogas methane yields were evaluated using batch tests performed at 50°C. Analysis showed that the food waste was well balanced in nutrients for anaerobic microorganisms: the methane yield was determined as 348 and 435 ml g^{-1} VS after 10 and 28 days of digestion respectively, and the average methane content of the biogas was 73%.

A recent study (Uemura, 2010) explored the mineral requirements for both mesophilic and thermophilic anaerobic digestion of organic solid waste. The tests were conducted in completely stirred tank reactors (CSTRs) under both thermophilic and mesophilic conditions. After failure of the reactor due to the acidification of the process, methane yield was restored by the addition of Ni, Co, Fe in mesophilic digesters but not in the thermophilic ones. The authors suggested that the metal requirement is higher in thermophilic anaerobic digestion than in mesophilic digestion. When additional minerals were added at the beginning of a test no acidification occurred in the thermophilic reactor.

B4 Metabolic pathways to methane production and the importance of trace elements

B4.1 Methanogenesis

All methanogenic archaea investigated to date rely on methanogenesis for energy conservation and growth. The number of substrates utilised by methanogens is quite limited reflecting the narrow ecological niche this group occupies: most methanogens are only able to grow with H_2 and CO_2/formate, some can utilise methylated compounds, and some can grow with acetate. These different substrate classes are metabolised via distinct, but overlapping, pathways (Deppenmeier and Müller, 2008; Ferry, 1993; Stock and Rother, 2009; Thauer, 1998; Thauer et al., 2008). Central to these pathways is methyl-S-CoM (2-methylthioethanesulfonate) which is formed by three different pathways depending on the primary substrate: these are shown in Figure 1 (Deppenmeier, 2002).

B4.1.1 Acetoclastic methanogenesis

This is carried out by two genera of the methanogenic archaea: *Methanosaeta* and *Methanosarcina.* These organisms cleave acetate to a methane and carbon dioxide as shown in Figure 2. The first step is activation of acetate to acetyl-CoA which is catalysed by AMP-forming acetyl-CoA synthetase (ACS) or the combined actions of acetate kinase (AK) and phosphotransacetylase (PTA). The next steps are similar in both genera and involve the cleavage of the carbon–carbon and carbon–sulfur bonds of acetyl-CoA which is facilitated by the five-subunit carbon monoxide dehydrogenase/acetyl-CoA decarbonylase enzyme complex (CODH/ACDS) and at this point the carbonyl group formed is oxidized to CO_2. The enzyme complex contains Co, Ni and Fe in its α and β subunits, and these are the active sites (Ferry, 1992; Murakami and Ragsdale, 2000). The methyl group formed is then transferred to the co-factor tetrahydrosarcinapterin (H_4SPT)which in turn is transferred to HSCoM by Methyltetrahydrosarcinapterin:CoM methyltransferase (MTR), a corrinoid-dependent integral membrane protein complex (Tallant et al., 2001). There is then a reductive demethylation of CH_3-S-CoM catalysed by the methyl-CoM methylreductase (MCR) which contains Ni

(Ermler, 1997; Kida et al., 2001). This final step to methane production is common to all methanogenic pathways and for all substrates. The trace elements which has been identified to be important in the acetoclastic methanogenesis pathway are shown in Table B5.

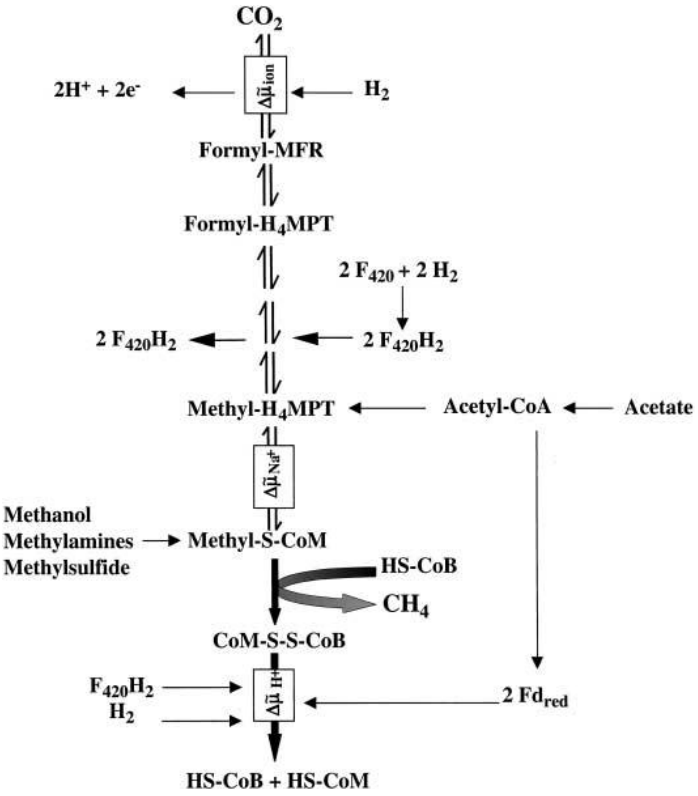

Figure B1. Schematic overview of the methanogenic pathways (Deppenmeier, 2002).

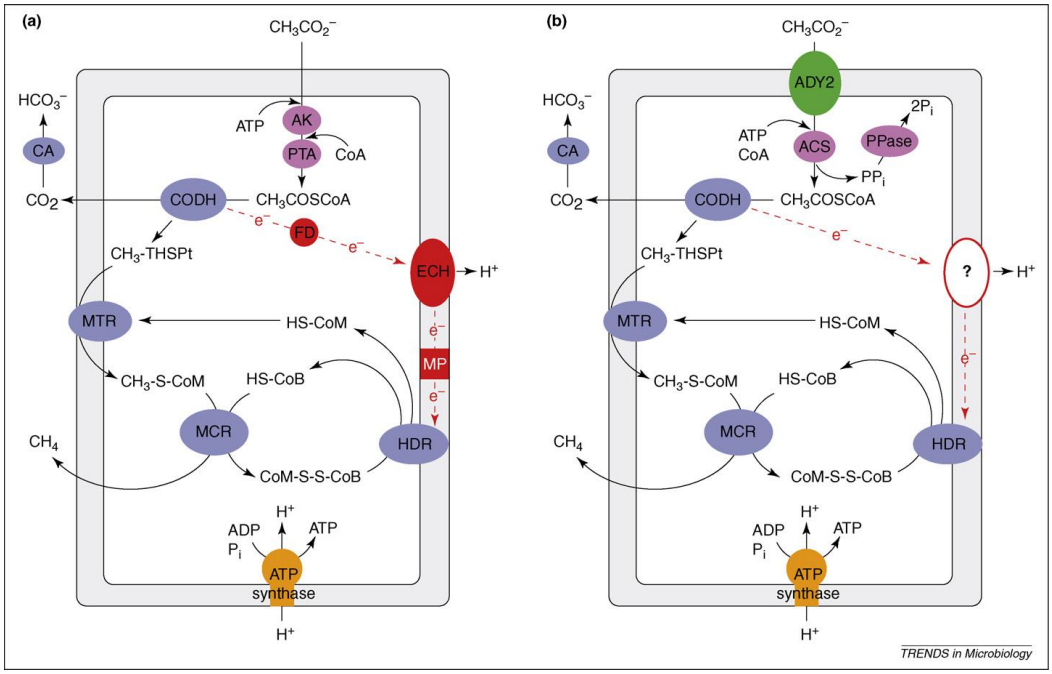

Figure B2. The proposed pathways for acetoclastic methanogenesis in *Methanosarcina mazei* and *Methanosaeta thermophila* (Smith and Ingram-Smith, 2007).

Table B5. Major metalloenzymes identified in acetoclastic methanogenesis.

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

B4.1.2 Hydrogenotrophic methanogenesis

In this pathway CO_2 is sequentially reduced to methane in seven steps as shown in Figure 3. This involves coenzyme-bound intermediates which use H_2 as the electron donor. Where formate is the source of both the H_2 and CO_2 this is first reduced via formate dehydrogenase (FDH), which may contain selenocysteine.

In the first step CO_2 is reduced to the formyl-level and attached to methanofuran (MF, a 2-aminomethylfuran derivative) by formyl-MF dehydrogenase (FMD) using an electron donor of reduced ferredoxin. The endergonic H_2-dependent reduction of ferredoxin is driven by the ion motive force via a membrane-bound energy converting hydrogenase. However, an alternative mechanism of H_2-dependent reduction of ferredoxin involving 'electron bifurcation' during heterodisulfide reduction has also been proposed (Deppenmeier, 2002; Stock and Rother, 2009; Thauer et al., 2008).

The next step involves the transfer of the formyl-group from MF to tetrahydromethanopterin (formyl-H_4MPT) which is catalyzed by formyl-MF:H_4MPT formyltransferase. Formyl- H_4MPT is subsequently converted through N^5-formyl-H_4MPT to N^5,N^{10}-methenyl-H_4MPT a reaction which is catalysed by N^5,N^{10}-methenyl-H_4MPT cyclohydrolase. The N^5,N^{10}-methenyl-H_4MPT is reduced to N^5,N^{10}-methylene-H_4MPT which is dependent on a coenzyme F_{420}-dependent N^5,N^{10}-methylene-H4MPT dehydrogenase (MTD), All hydrogenotrophic methanogens analysed to date contain this F_{420} coenzyme which can be itself reduced ($F_{420}H_2$) by the F_{420}-dependent hydrogenase, an oligomeric NiFe-enzyme. The reduction of N^5,N^{10}-methenyl-H_4MPT to N^5,N^{10}-methylene-H_4MPT can also be catalysed in one step by the H_2-dependent N^5,N^{10}-methylene-H_4MPT dehydrogenase (HMD). HMD and MTD are up-regulated when nickel (or selenium for Methanococcus) is limiting and it has been proposed that under these conditions MTD actually operates in the opposite direction, generating the $F_{420}H_2$ needed for the reductase-catalysed reaction and for anabolic purposes (Deppenmeier, 2002; Stock and Rother, 2009; Thauer et al., 2008).

N^5,N^{10}-methylene-H_4MPT is then reduced to N^5-methyl-H_4MPT, this also depends on $F_{420}H_2$ as the electron donor and is catalysed by N^5,N^{10}-methylene-H_4MPT reductase. The methyl-group from H_4MPT is then transferred to coenzyme M (HS-CoM, 2-mercaptoethanesulfonic acid) and is catalysed by the membrane bound enzyme N^5-methyl-H4MPT:CoM methyltransferase, this involves a corrinoid which couples the exergonic methyl-transfer to sodium ion extrusion. The final step in all methanogenic pathways is the reduction of methyl-CoM to methane catalyzed by methyl-CoM reductase (Ermler, 1997). Methane and heterodisulfide (CoM-S-S-CoB) are generated from methyl-CoM and coenzyme B (N-7-mercaptoheptanoyl-O-phospho-Lthreonine, HS-CoB), which is the electron donor for this reaction. CoM-S-S-CoB represents the terminal electron acceptor of an energy-conserving electron transport chain and reduction of CoM-SS-CoB with H_2 as the electron donor involves (at least) two (sometimes selenocysteine-containing enzymes, F_{420}-

nonreducing hydrogenase and heterodisulfide reductase (HDR) (Deppenmeier, 2002; Stock and Rother, 2009; Thauer et al., 2008).

The trace elements which have been identified to be involved in hydrogenotrophic methanogenesis pathway are shown in Table B6.

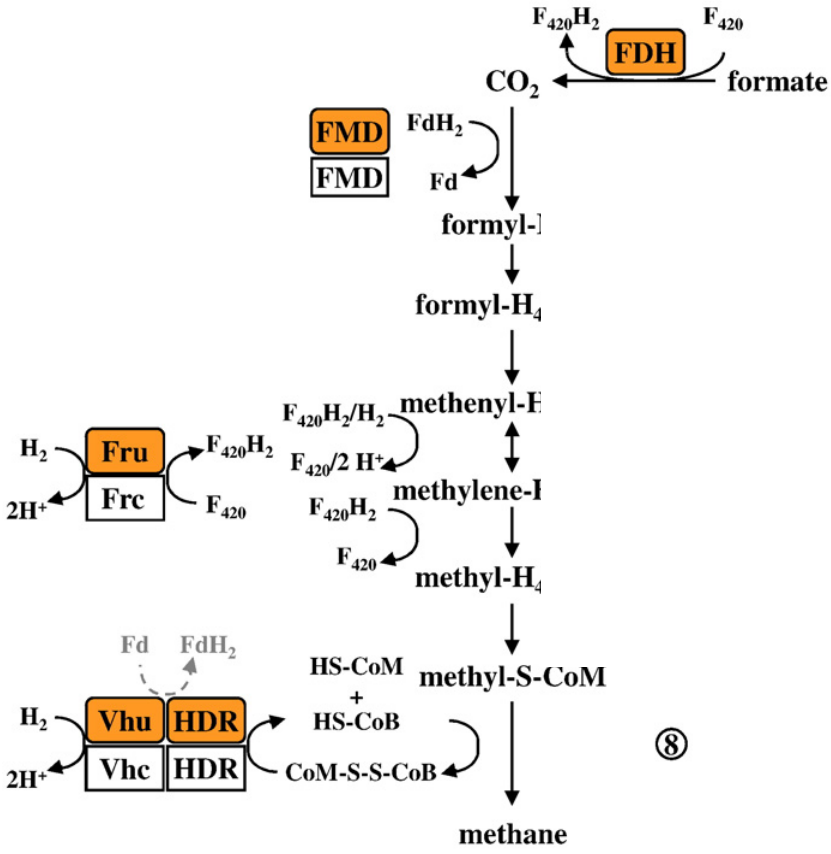

Figure B3. The pathway of CO_2 reduction to methane in *Methanococcus* species. Selenoproteins involved are round-boxed in orange (Stock and Rother, 2009).

Table B6. Major metalloenzymes identified in hydrogenotrophic methanogenesis pathway.

Metalloproteins/metalloenzymes	**Trace elements**
Formate dehydrogenase	Se, Mo/W, Fe
Hydrogenase	Se, Ni, Fe
Formyl-methanofuran dehydrogenase	Se, Mo/W, Fe
Methyl-H_4MPT:HS-CoM methyltransferase	Co, Ni, Fe
Heterodisulfide reductase	Se, Fe
Methyl-CoM reductase	Ni

B4.1.3 Methanol methanogenic pathway

This third pathway only applies to the formation of methane from methanol and methyl groups and is therefore not reviewed here.

B4.2 Other functions of trace elements in methanogens

Trace elements are also required in some anabolic (biosynthesis) pathways in methanogens, for example, Cobalt -containing Acetyl-CoA synthase is used by hydrogenotropic methangens, (Lindahl and Chang, 2001). Selenium (Se) has been found in certain tRNAs in hydrogenotropic methanogens (Stock and Rother, 2009). Information in this area is, however, very limited in the literature.

B4.2.1 Trace element requirement by non-methanogens

In anaerobic digestion it is most often thought that the step most vulnerable to trace element deficiency is methanogenesis (Takashima et al., 1990; White and Stuckey, 2000). If this process stops or slows down the consumption of acetate, hydrogen, or formate decreases and the process is adversely affected. If the failure of the methanogenic route is only partial i.e loss of acetoclastic methanogens or the loss of the hydrogenotrophic methanogens then the flow of carbon can continue to methane as a terminal product as a result of the Wood-Ljungdahl pathway which, by virtue of the bifunctional carbon monoxide dehydrogenase / Acetyl-Coenzyme A synthase enzyme systems (Andreesen et al., 1973; Doukov et al., 2002; Lindahl and Chang, 2001; Ljungdahl, 2009; Seravalli et al., 2003), can build acetate from CO_2 and H_2 as well as breaking it down to these components. Thus acetate can be shunted into the hydrogenotrophic methanogenic route or H_2 and CO_2 can be formed into acetate and then be used by acetoclastic methanogens. Much of the work on trace element requirements by anaerobic methanogenic consortia have focused on this pathway. It is also interesting to note that part of the Wood-Ljungdahl pathway is also used by methanogens in both catabolism and biosynthesis (Lindahl and Chang, 2001; Smith and Ingram-Smith, 2007).

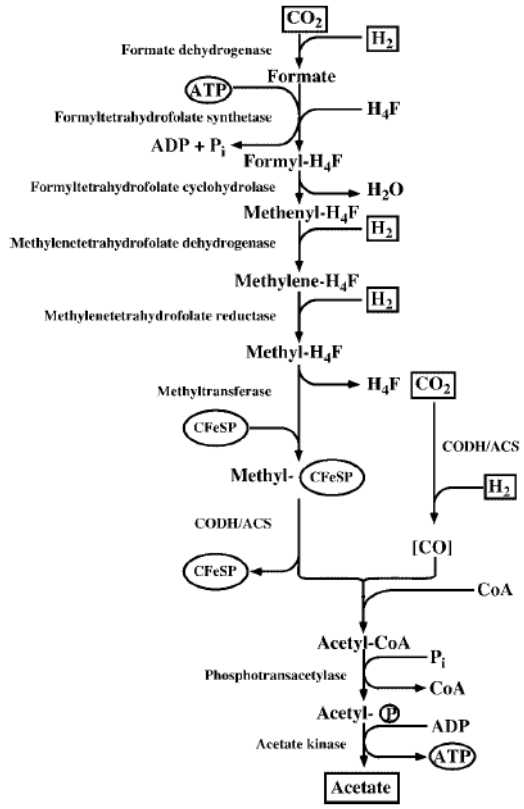

Figure B4. The Wood-Ljungdahl pathway of acetogenesis. Reductants shown as hydrogen, but electrons can also be derived from oxidation of organic substrates (Müller, 2003)**.**

In the 'methyl-branch' of the Wood-Ljungdahl pathway (Figure 4), CO_2 is first reduced to formate by formate dehydrogenase (FDH). This is then activated to N^{10}-formyl-tetrahydrofolate (formyl-H_4F) with ATP being hydrolysed by formyl-H_4F synthetase. The formyl-group is then converted to a methyl-group by dehydration giving rise to N^5,N^{10}-methenyl-H_4F , the reaction being catalysed by formyl-H_4F cyclohydrolase. N^5,N^{10}-methenyl-H_4F is then reduced to N^5-methyl-H_4F via N^5-methylene-H_4F (methylene-H_4F dehydrogenase and methylene-H_4F reductase). The methyl-group is then transferred via a corrinoid- and Fe/S cluster-containing protein to the bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) to form acetyl-CoA with CoA and enzyme bound CO.

In the 'carbonyl-branch' of the pathway, CO_2 is reduced to $COCO_2$ by CODH to provide the carbonyl-group of acetyl-CoA. Acetyl-CoA is then converted to acetate via acetyl-phosphate by phosphotransacetylase and acetate kinase, which yields ATP (Müller, 2003).

There are four key metalloproteins/metalloenzymes in this pathway: formate dehydrogenase (FDH), a corrinoid- and Fe/S cluster-containing protein (CFeSP), Carbon monoxide dehydrogenase (CODH), and Acetyl-CoA synthase (ACS). The trace elements associated with these protein/enzymes are listed in Table B7 (Zhu, 2009).

Table B7. Metalloproteins/metalloenzymes identified in Wood-Ljungdahl pathway.

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

Other than the Wood-Ljungdahl pathway little work has been carried out on the trace element dependence of non-methanogenic bacteria associated with anaerobic digestion. The exception to this is the works of de Bok et al. and Plugge (de Bok et al., 2003, Plugge, 2009) who have reported on trace element supplementation and syntrophic propionate oxidation. An important area considering the potential for VFAs accumulation if there is a bottleneck in the process as the syntrophic bacteria carry out their metabolic activities close to the thermodynamic limit (Lens et al., 1996; McInerney et al., 2008; Narihiro, 2007).

B5 Conclusions on trace elements

The following conclusions on the need for trace elements in anaerobic digestion can be drawn from this review:

- Trace elements concentration can significantly influence the anaerobic digestion process and different substrates lead to different trace element requirements.
- There is still much uncertainty regarding the effects of metal addition and their concentrations in the anaerobic digestion of OFMSW and food waste.
- Digesters operating at mesophilic or thermophilic conditions showed different responses to trace metals addition

- The metabolic pathways leading to methane production are now reasonably well established and there is a clear trace element dependency in both acetoclastic and hydrogenotrophic routes
- The Wood-Ljungdahl pathway is an important pathway allowing methanogenic substrates to be shunted from one form into another
- There has only been a limited amount of work on syntrophic propionate oxidation but it is thought that this also has a trace element requirement for its proper functioning.
- Se, Mo, W, Co, Ni and Fe are widely used in all the anaerobic degradation process, although Se, Mo and W are not used by acetoclastic methanogens.

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