MARMARA UNIVERSITY

INSTITUTE FOR GRADUATE STUDIES IN PURE AND APPLIED SCIENCES

EFFECT OF TRACE METALS IN ANAEROBIC DIGESTION OF CHICKEN MANURE

Rahim MOLAEY

MASTER THESIS

Department of Environmental Engineering

Thesis Supervisor

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Rahim MOLAEY, a Master of Science student of Marmara University Institute for Graduate Studies in Pure and Applied Sciences, defended her thesis entitled "**EFFECT OF TRACE METALS IN ANAEROBIC DIGESTION OF CHICKEN MANURE**", on September 17, 2015 and has been found to be satisfactory by the jury members.

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CONTENTS

ABSTRACT

Anaerobic digestion (AD) is one of the sustainable technologies for decreasing the agricultural, industrial, and domestic wastes, while, producing valuable biogas and digestate. The wide spread application of AD for transformation of manure, agricultural and industrial wastes to energy is highly dependent on economic profitability. The major drawback of AD is particularly related to high capital and operational costs and instability of the reactor. Hence, for application of more efficient and lower cost AD, it is necessary to provide the optimal conditions for microorganisms that carry out the digestion process. Based on the enzymology of anaerobic microorganisms, the AD process is strongly dependent on the existence of trace amounts of some specific metals.

Although the chicken manure (CM) is considered to be deprived in terms of some trace metals (TMs) which have critical importance for AD, in the literature there is a lack of study on the effects of TMs on anaerobic digestion of CM. Therefore, in this study, the effect of trace metal supplementation on biogas production from nitrogen rich CM at elevated ammonium concentration was investigated. Batch bio-methane potential tests were performed using volatile fatty acids (VFAs) and raw CM as substrate and digestate of a lab-scale anaerobic chicken manure and spent poppy straw co-digester as inoculum at different total ammonium nitrogen (TAN) concentrations with and without trace metal supplementation. Results revealed that, trace metal supplemented sets produced more biogas from acetic-, butyric-acid and raw CM at elevated total ammonia nitrogen concentration.

The TMs supplementation has resulted in 8% and 13.5% more biogas production from acetic acid at 675 mg/l and 5225 mg/l TAN, respectively. A similar trend was observed in biogas production from acetic- and butyric acid at 2950 mg/l TAN. TMs supplemented sets produced 12% and 18% more biogas than the sets with no extra TMs from acetic- and butyric acid, respectively. In BMP tests with chicken manure, TMs supplementations improved the biogas production from CM by 4%, 6%, 7.5% and 18% at low, moderate, high and very high TAN concentrations, respectively.

ÖZET

Anaerobik çürütme, tarımsal, endüstriyel ve evsel atıkların azaltılmasının yanı sıra değerli biyogaz ve fermente ürün (digestate) üretmekte kullanılan sürdürülebilir teknolojilerden birisidir. Anaerobik çürütmenin en yaygın kullanıldığı uygulamalar olan gübrenin, tarımsal ve endüstriyel atıkların enerjiye dönüştürülmesi yüksek oranda ekonomik karlılığa bağlıdır. Anaerobik çürütmenin en büyük sorunu yüksek yatırım ve işletme maliyetleri ve reaktör kararsızlıklarıdır. Bundan dolayı, daha verimli ve düşük maliyetli anaerobik çürütme uygulamaları için mikroorganizmalara ideal şartların sağlanması gerekir. Anaerobik mikroorganizmaların enzimolojisinden dolayı, anaerobik çürütme prosesi bazı belirli elementlerin iz miktarda bulunmasına fazlasıyla bağlıdır.

Tavuk gübresinde anaerobik çürütme için kritik önemi olan bazı iz elementlerin eksikliğinin olduğu düşünülmesine rağmen, literatürde tavuk gübresinin anaerobik olarak çürütülmesinde iz elementlerin etkisi çalışılmamış bir konudur. Bundan dolayı, bu çalışmada, azotça zengin tavuk gübresinden biyogaz üretilmesinde, yüksek amonyak konsantrasyonlarında iz element ilavesinin etkisi incelenmiştir. Kesikli biyo-metan potansiyeli testleri, uçucu yağ asitleri (UYA) ve ham tavuk gübresinin substrat olarak kullanıldığı ve laboratuvar ölçekli tavuk gübresi ve haşhaş küspesinin beraber çürütüldüğü anaerobik reaktörden alınan aşı çamurunun, farklı toplam amonyum azotu (TAN) konsantrasyonlarında iz element katkılı ve katkısız olarak kullanılmasıyla gerçekleştirilmiştir. Sonuçlar, asetik asit, bütirik asit ve tavuk gübresinin kullanıldığı setlerde yüksek amonyak azotu konsantrasyonlarında iz element katkısının daha çok biyogaz üretimine katkıda bulunduğunu göstermektedir.

BMP testlerine göre iz element katkısı, asetik asitten biyogaz üretiminde 675 mg/l ve 5225 mg/l TAN konsantrasyonlarında sırasıyla %8 ve %13.5 daha fazla biyogaz üretilmesine sebep olmuştur. Benzer bir eğilim 2950 mg/l TAN'da asetik asit ve bütirik asitten biyogaz üretiminde de gözlemlenmiştir. Asetik asit ve bütürik asitin kullanıldığı setlerde, iz element katkısı olanlar olmayanlara göre sırasıyla %12 ve %18 daha fazla biyogaz üretmiştir. Tavuk gübresiyle yapılan BMP testlerinde ise, iz element katkısının düşük, orta, yüksek ve çok yüksek TAN konsantrasyonlarında sırasıyla %4, %6, %7.5 ve %18 oranlarında biyogaz üretimini arttırdığı belirlenmiştir.

ABREVIATIONS

AAS:	Atomic Absorption Spectroscopy
AD:	Anaerobic Digestion
BMP:	Bio-Methane Potential
CM:	Chicken Manure
COD:	Chemical Oxygen Demand
CODH:	Carbon Monoxide Dehydrogenase
EDS:	Endocrine Disrupting System
FAN:	Free Ammonia Nitrogen
GC:	Gas Chromatograph
GHGs	Greenhouse Gases
HRT:	Hydraulic Retention Time
ICP-MS:	Inductively Coupled Plasma Mass Spectrometry
ICP-OES:	Inductively Coupled Plasma Optical Emission Spectroscopy
mg:	Milligram
PPM:	Part per millennium
SPS:	Spent Poppy Straw
SMP:	Soluble Microbial Products
TS:	Total Solid
TAN:	Total Ammonia Nitorgen
TCD:	Thermal Conductivity Detector
TKN:	Total Kjeldahl Nitrogen
US:	United States
VFAs:	Volatile Fatty Acids
VS:	Volatile Solid

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1. INTRODUCTION

1.1. Scope and Objective of the Study

Significant amount of animal manure and waste generated from animal raising activities have seriously negative impacts on the environment and causes environmental pollution, if they are not managed properly.

The rapid increase of population in the world consequently followed up by generation of high amount of wastes, the emission of greenhouse gases (GHGs) and leaching of organic matter, macro- and micro-nutrients to the environment. Therefore, it is necessary to find an optimal solution to resolve these problems. Many researches in this area have shown that the best alternative is the optimal recycling of the wastes from production to utilization.

In recent years, as a result of progresses in AD field and by using more advanced biogas systems in developed countries it is concluded that biogas can be produced from almost all kind of biological feedstocks. These biological feedstocks include primary agricultural wastes and different types of organic waste streams. The highest proportion among the organic wastes is represented by animal manure and slurries from cattle, pig and poultry production sector as well as from fisheries. (Holm-Nielsen et al., 2009).

Nowadays, anaerobic digestion has gained much popularity due to the conversion of wastes to renewable fuel and organic fertilizer. The biogas produced through AD of aforementioned substrates is composed of methane which is a versatile renewable energy and carbon dioxide which is virtually carbon neutral (Pobihieim et al., 2010).

In AD, as a result of cooperation among different types of microorganisms, the biogas is produced from organic matters through a multi-stage process. In order to properly balance the process and improve the efficiency of biogas production, the availability of macro- and micro-nutrients are necessary (Weiland, 2010).

It has been reported that existence of macro-nutrients such as phosphorus (P), Sulphur (S), potassium (K), and micro-nutrients such as magnesium (Mg), iron (Fe), nickel (Ni), molybdenum

(Mo), cobalt (Co), tungsten (W), selenium (Se) and zinc (Zn) are needed to provide the stability of the process and to improve the performance of AD (Nges and Bjornsson, 2012). Macronutrients can be served as buffering agents while micronutrients involved in a wide range of enzymes which are playing basic roles on methane formation (Thauer, 1998; Schattaur et al., 2011).

Generally, the effect of TMs on anaerobic digestion can be assessed by variations on the parameters used for monitoring the process performance. These parameters are pH, VFAs-concentrations, biogas production and substrate utilization efficiency (Holm-Nielsen et al., 2009).

It is assumed that metals can be used by microorganisms when they are in free form or in the form of some specific metal-organic complexes. Hence, metal speciation and bioavailability of TMs are important factors to evaluate effects of their supplementation on AD. Besides, metal speciation, bioavailability and toxicity are function of total metal concentrations, chemical processes such as precipitation, adsorption and complexation, and biogas process conditions (Callender and Barford, 1983; Callender and Barford, 1983; Van Leeuwen, 1999; Pinheiro and Van Leeuwen, 2001).

As a consequence of different environmental conditions and substrate composition, TMs requirements for anaerobic consortia varies significantly from one digester to another.

In the past, researchers have assumed that manure contains relatively high amounts of TMs and there was no concern about the deficiency of TMs in anaerobic digestion of manures. However, recent findings are in contrast to this presumption. Therefore, the aim of this study is to elucidate the effect of TMs supplementation on anaerobic digestion of CM.

In order to achieve this aim, a daily-fed laboratory scale biogas reactor was operated for 160 days and the digestate collected from this reactor was used as inoculum in bio-methane potential (BMP) tests. In the BMP tests, the effect of TMs addition on biogas production from acetic, propionic and butyric acid or raw CM was investigated by comparing the biogas yields of TMs supplemented and non-supplemented sets.

2. GENERAL BACKGROUND

2.1. Chicken Manure Generation

Increasing in world human population from 5.4 billion in the 1990s to predicted value of 8.5 billion by 2025, to supply the world food requirement and to decrease malnutrition, increasing in food production of 60 –70% will be necessary. With the limitation of land area for crop production, increasing in food demand can be supplied mainly through livestock industries (Bolan et al., 2010). In the last three decades, poultry sector has been growing at more than 5 percent in each year which was driven by rising the demand for low-cholesterol, inexpensive and safe supply of meat (Gerber et al., 2007). Thereby, poultry industry has gained much popularity and it is one of the largest supplier of meat in recent years (Gerber et al., 2007; Bolan et al., 2010).

In the last decade increasing in chicken products for human consumption, annually huge amount of CM being produced. For example Niu et al (2013) indicated that in Japan 13 million tons of CM is generated (Niu et al., 2013). In another study by Zhang et al (2009) has mentioned that in China 3.9 billions of livestock and poultry manure produced per annum (Zhang et al., 2009). On centralized farms of Russia, generation more than 700 million m^3 of poultry and livestock wastes has been reported. In Turkey, every year about 250-million chickens are raised for eggs and meat. In recent years, a significant number of chickens are raised in huge farms and every year around 20 million tons of poultry and cattle manure has been generated (Yetilmezsoy and Spaci-Zengin, 2009).

2.2. Chicken Manure Characteristics

Chicken wastes include manure, bedding materials and sawdust, straw, dead chicken, broken eggs and chicken feathers. Fresh manure contains more than 70% water. Composition of CM depends on the age of chickens, type of litter, age of the manure, storage and handling practices (Bolan et al., 2010). The best way to be sure about the composition of CM is to analyze the litter. Generally CM contains major plant nutrients such as N, P, K and organic matter. In addition, it contains trace amount of many other plant nutrients such as Ca, Mg, S, Fe, B, Cu, Cl, Mn, Mo, and Zn, so it might be a good crop fertilizer. In spite of these benefits, however, excess application of manure can cause serious environmental pollution problems. Nitrate leaching into groundwater,

phosphorus run off into surface water bodies, and release of pathogenic microorganisms are the main problems encountered with improper management of this resource (Kelleher et al., 2002). Therefore, sustainable management technologies required to decrease the environmental pollution related to the chicken wastes. By well managing of these wastes and then applying to arable land, major nutrients such as N, P and K existed in these wastes can be taken by crops. In addition, by land application of chicken wastes, valuable micronutrients can also be recycled in which increase the physical, chemical and biological fertility of the soil (Bolan et al., 2010).

2.3. Chemical Composition of Chicken Manure

Chicken wastes include bedding materials, feather, manure and undigested feed. Manure contains macro-nutrients such as N, P and K, trace amount of other metals such as Cu, Zn and arsenic (As), pesticide residues, endocrine disruptors and micro-organisms. Total solids (Kotsyurbenko et al.) content, salt level, pH, and element content of CM are dependent on food supplements, handling and storage operation of manure (Bolan et al., 2010).

2.3.1. Macro nutrients

Major elemental components of poultry manure are N, P, K, Ca, Mg, and S. Variation in elemental concentrations of manure depend on feed supplementation, used bedding materials, and litter management practices.

Among elemental organic compounds as soil amendments in chicken wastes total N and P have a high potential to improve the natural health of the soil and growing media (Bolan et al., 2010).

It has been indicated that N in four different forms such as complex organic N, unstable organic N, ammonium and nitrate existed in CM. Complex organic nitrogen contains feathers, undigested feed and bedding materials. Unstable organic nitrogen includes uric acid and urea (Bolan et al., 2010). Phosphorus content of chicken waste is a function of diet and bedding materials and consisted in the range of 0.3 to 2.4% of dry matter. According to Mahimairaja et al (1995) large amount of phosphorus in manure is in acid soluble form which is less bioavailable for microorganisms (Mahimairaja et al., 1995b).

Table 2.1. Nutrient contents of poultry wastes

(Bolan et al., 2010)

Nutrients	Poultry manure(g/kg dry weight basis)	
	Layer	Broiler
Nitrogen	32.8	25.7
Phosphorus	10.8	6.7
Potassium	15.2	10.1
Calcium	18.5	16.2
Magnesium	6.2	3.5
Sulphur	5.2	5.2

Turner and Leytem (2004) indicated that large amount of organic phosphorus in poultry manure existed in the form of phytic acid. While inorganic phosphate in poultry manure existed in different forms such as dibasic calcium phosphate, amorphs calcium phosphate and bonded water-soluble phosphate (Turner and Leytem, 2004).

2.3.2. Micronutrients

In order to improve feed efficiency and health of chickens, several trace elements as essential nutrients are supplemented to chicken feed. Thereby to prevent the deficiency of trace elements, to prevent the diseases, and to improve weight gain of chickens many producers add trace amount of heavy metals such as As, Co, Cu, Fe, Mn, Se, and Zn in a diet of chickens. These nutrients playing important roles on enzymatic processes such as physiological functions and metabolic activities of the organisms. They can be used as cofactors in enzyme systems and their roles can be divided to relatively weak such as activation of enzyme by metal ion, and highly specific association such as metallo-enzyme in which metal is rigidly conjugate with protein (Bolan et al., 2010).

2.3.3. Endocrine-disrupting compounds

Endocrine-disrupting compounds (EDCs) are known as a group of compounds which have adverse effects in animals and human and it can be present naturally or synthesized. Excreted hormones by animal, pesticides, herbicides, and other compounds which disturb the endocrine systems might be involved in EDCs (Powers and Angel, 2008). The EDCs can bind with hormones and imitate

the activity of natural hormones and finally disrupt the endocrine system. Some researchers detected the EDCs such as 17β-oestradoil, oestrone, oestriol, and testosterone in broiler manure (Hanselman et al., 2003; Jenkins et al., 2006). Some studies pointed out these compounds are resistant to mineralization, however, due to contact with soil there is a concern about their mineralization (Bolan et al., 2010).

2.3.4. Microbial load

Large amount and different types of viruses, bacteria, fungi and protozoa involved in CM. Some authors estimated the concentration of microbes in poultry litter for about 1010 cells/g (Acosta-Martinez and Harmel, 2006; Cook et al., 2008; Rothrock et al., 2008a). Among microbial population, gram positive bacteria are the dominant forms in poultry litter and account for 90% of the microbial population (Lu et al., 2003; Enticknap et al., 2006; Lovanh et al., 2007).

For chicken producers among diverse group of microbes existed in manure, two special groups such as pathogens and nitrogen mineralizing are more interested. Due to microbial activity some portion of organic nitrogen existed in the form of uric acid and mineralized urea. Therefore, some portion of organic nitrogen in poultry manure being volatilized (Brinson et al., 1994; Moore et al., 1996).

Many research works have shown large amount of zoonotic pathogens involved in CM (Williams et al., 1999; Terzich et al., 2000; Bull et al., 2006; Line and Bailey, 2006; Rothrock et al., 2008a). Therefore, to prevent the spreading pathogen into the environment, prior to land application of poultry manure pathogen destruction is required.

2.4. Over Application of Chicken Manure to the Land

Rapid and concentrated growth of the chicken industries in several states, rising concerns over accumulation of wastes which may not be allowed to dispose. Traditionally, most of the generated wastes as a source of nutrients and soil conditioner were used in arable land to increase the fertility of soil. On the other hand, in most of regions integration of poultry industries provided much more manure than were needed to meet crop nutrients requirements. In such a case when nutrients exceed the soil's assimilation capacity, by applying excess amount of chicken wastes to agricultural land, nutrient and contaminant leaching may pollute the surface and ground water. Therefore, excess amount of nutrients may cause eutrophication and other environmental impacts (Bolan et al., 2010). In addition, usage of manure as a soil amendment has been indicated as a

main source of essential and non-essential elements to the soil. For instance in one of the early published paper, with respect to elemental concentrations and dry matter contents of poultry manure, metal loadings of manure at application level of 250 kg/ha has been assessed (Table 2.2) (Nicholson et al., 1999).

Table 2.2**.** Loaded trace elements (kg/ha) in land by the usage of poultry manure at the 250 kg N/ha rate

(Nicholson et al., 1999)

Element	Poultry manure(g/kg dry weight basis)	
	Layer compost	Broiler
As	<0.01	<0.01
Cd	0.007	0.003
Cr	0.03	0.01
Cu	0.5	0.2
Ni	0.05	0.02
Pb	0.05	0.02
Zn	2.9	1.1

This assessment shows that through land application of CM large amount of Cu and Zn were added to the soil. Even though Cu and Zn are essential nutrients for plant growth, and optimal land application of CM can help the fertility of the soil, but over-supplementation to the land, follow by overestimated concentration of such metals in soil which are phytotoxic. On the other hand, large amount of TMs in the soil uptake by crops. Therefore, TMs being recycled within a production system.

2.5. Alternative Uses of Chicken Manure

Due to ever increasing of poultry industries and generation of high amount of manure, pollutants such as organic substances, pathogens, antibiotics, nutrients, and ammonia will distribute into the environment. It has been reported approximately 40% of the Nation's examined water in the United States (US) are not enough pure for fishing or swimming. Based on the 1998 National Water Quality Inventory, almost 60% of the rivers' and streams' pollution, and 45% pollution of

lakes are the affluence of livestock operations. Therefore, alternative uses of manure might be the best solution for such type of problems (Beaty and Zygmunt).

2.5.1. As a nutrient source

In order to provide the organic nutrients for crops, CM is widely used in farmland. It has been indicated that, by addition of CM to farmland responses of plants to CM increased. As the rate of manure addition increased, highest corn yields were achieved. Generally, CM is used as a fertilizer to provide the N and sometimes P requirements of crops. It was concluded that N content of poultry litter is as effective as N from other sources such as ammonium nitrate and urea. In addition, it has been noticed that poultry manure provides sufficient amount of N, P, K, Ca, and Mg and the growth of plants is assisted by the addition of this waste (Tewolde et al., 2005). In the same way, analytical results have shown that CM is one of the most efficient plant food (Collins et al., 1999).

However, sometimes by excess application of manure to farmland, applied amount of N is higher than the recommended value for crops. Therefore, through leaching and run off groundwater and surface water contaminated.

2.5.2. As a soil amendment

By continuous arable cropping of cultivated plant that is grown as food, especially a grain, fruit or vegetable, soil structure progressively becomes worse and leading to decrease its fertility. On the other hand, it has been indicated to improve the fertility of land, applying only chemical fertilizers are not enough to meet the maximum relative growth rate of plants (Bolan et al., 2010).

In the study by Bolan et al (2010) it has been shown that addition of CM could reduce the bulk density problems, recover the organic matter contents of soil, increase the soil-water holding capacity, increase the penetration of oxygen and increase the structural stability of soils (Bolan et al., 2010).

In the same way, Franzluebbers and Doraiswamy (2007) have shown that by addition of poultry manure to mine tailings, biological fertility of land could be recovered. In like manner, it was noticed that organic manures such as poultry wastes have positive effects in the rehabilitation of landscape which is disturbed by mine or other industrial activities (Franzluebbers and Doraiswamy, 2007).

2.5.3. As an animal feed

Mixture of CM with feed grains or its usage alone has been indicated to be a successful feed for ruminants. Whereas it is critical to remove overseas materials, such as plastics, wires, glasses, and feathers which affect the digestibility of manure, before using it as feed. However, the ash content of manure, which comes by removing of soils with litter, should be considered before being used as feed. It has been pointed out, the ash content more than 28% should not be used as feed to ruminant. Unlike disease, which associated by feeding manures to livestock animals, has not been reported but Cu toxicity has been indicated as one of the major problematic issues, especially in sheep. In order to increase weight gaining of chicks, an excess of copper sulfate is being used in poultry diet (Moore et al., 1995).

2.5.4. As a fuel source

Alternatives for energy production from poultry wastes are direct combustion, co-firing, gasification and AD. In recent years, these technologies have been used extensively in most of the developed countries.

2.5.4.1. Direct combustion

In order to generate heat energy from poultry wastes, it has long been incinerated directly. This process involves the ignition of manure with excess amount of air, followed by generation of hot gases. Generated gases can be used for the production of steam which is used in the boiler's heat exchange section. Besides that, generated steam can also be used in turbine generators for the production of electricity. Direct ignition of manure is one of the easiest and most expanded technology in the world. From economical point of view, this technology can be existed in large scale if the manure resources are closed to incineration plant. In recent years due to stringent regulations, in order to decrease the amount of manure, poultry producers have been forced to shift in more advanced technologies (Flora et al., 2006).

2.5.4.2. Co-combustion

Ignition of manure as the only fuel source may involve some limitations. These limitations are basically depend on vastly variable characteristics such as ash, salt content and relative humidity. These limitations might be eliminated by mixing of manure with other primary fuel such as coal, natural gas, furnace oil, etc. and burn them in the burner. This burning process is called as co-combustion which can be described as the ignition of renewable fuels such as manure with other base fuel. In this process, mixing is a good option for fuel with many variables characteristics and

high moisture content. In the study by Otero et al (2011) has been pointed out that manure-coal blended has been recommended as a valuable use of manure for power-plants situated close to feedlots. In addition, co-combustion of manure-coal blended can help the solution of air pollution problems. By using manure as a fuel not only fossil fuel based CO_2 but also NO_x emissions will decrease. However, the N content of manure-coal mixture is high with respect to coal alone, less NO_x emissions in co-combustion ascribed to the fact that high amount of N is in the form of NH_3 (Otero et al., 2011).

2.5.4.3. Gasification

Gasification is a process in which pressure, heat, and steam are applied to transform most types of organic matters into hydrogen and carbon monoxide which can be used for the creation different types of fuels and chemicals. Different types of dry biomass are used in the gasification process. However, agricultural wastes have been used in a number of projects regarding gasification, but in recent years researchers have focused on the usage of manure in this process.

In gasification process wastes are heated in the absence of oxygen to vaporize volatile gases and produce fixed carbon (charcoal) from the biomass at high temperature. This process is called pyrolysis. After that, for the generation of CO and CO_2 charcoal and volatile products are ignited with oxygen. In the last step, charcoal reacts with CO_2 and steam to generate CO and H_2. Generated last two gases can be used either in an engine or in a gas turbine for the power generation (Beaty and Zygmunt).

2.5.4.4. Anaerobic Digestion

Rapid increasing of population, concerns about increasing the cost of traditional energy sources and their depletion motivated many scientists to search for another source of energy. On the other hand, growing of population and limiting the land, rises the demands for animal protein products in which able to produce meat and eggs at a lower cost with respect to traditional methods (Abouelenien et al., 2014). Intensification in animal protein products and chicken meat, followed by huge production of manure. CM contains high amount of nitrogen and phosphorus, consequently, it is a good soil conditioner and fertilizer crops. Due to existence of high amount of nutrients in CM it is common to find ponds covered with algae close to chicken farms. But, if CM applies to the land more than crops can take, significant pollution problems will occur (Nie et al., 2015). Since CM contains high amount of biodegradable organic matter, anaerobic digestion of CM is a good option to diminish the waste on the other hand recover the bioenergy. However, due

to high content of organic nitrogen which existed in the form of undigested protein and uric acid, ammonia inhibition is the major problem facing in practical application (Bujoczek et al., 2000).

To prevent ammonia inhibition one common method is the dilution of the substrate (Nielsen and Angelidaki, 2008). Therefore, CM is generally fed to biogas reactor after being diluted and this increases the water consumption. After biogas production, the digested sludge has to be dewatered before being used as organic fertilizer. Besides, the reject water should be treated before being discharged or used as dilution water again. Another method to prevent ammonia inhibition is a co-digestion of CM with other substrates rich in carbon (Niu et al., 2013). However, providing proper co-substrate may increase the operational cost. Therefore, these options are not always applicable. In summary, the continuous removal of ammonia from anaerobic digesters is another way to avoid the ammonia inhibition. If the ammonia concentration in the digester is kept under inhibition level by continuous removal, digesters will be operated at higher organic loading rates and finally more biogas may be produced using less dilution water (Abouelenien et al., 2010).

2.6. Introduction to Anaerobic Digestion

From the history evidences it can be concluded that AD process is one of the oldest technologies. Full application of AD started in 1859 with first digestion plant in Bombay, India (Monnet, 2003). By 1895 in Exeter, England from a sewage treatment biogas recovered and used to fuel street lamps (Nayono, 2009). Later on in 1930s by improving investigations in this field, researchers found anaerobic bacteria and the other conditions that could improve methane production. By understanding the benefits of AD, more complicated techniques and sophisticated equipment were applied to improve the efficiency of AD. Therefore, to optimize this process, closed tank with heating and mixing system were used. Parallel to improvements in AD, due to low cost of coal or petroleum and rapid developing of aerobic treatment, there was not that much interest on AD. However, developing countries such as China and India rely on this technology and they used AD for the treatment of wastewater sludge digestion (Monnet, 2003).

Because of the high cost of energy and environmental regulation stringent, European countries try to explore widely AD market. By the middle of 20^{th} century it was reported that there were more than 1000 anaerobic digesters in France, which included simple tanks to complex digestion systems. In 1944, this technology widely used in West Germany by using agricultural wastes as

feed in this process and also improved the idea for development different types of anaerobic plants. Till to 1970s, in Western Europe this technology had been used for treating animal manure. Improvement in anaerobic filter by 1967, provide the conditions for advancement in high rate AD. It was followed by constructing number of anaerobic digesters which capable of treating different types of biodegradable waste streams. Over the past two decades, survey of the development in AD shows that there is no that much difference in applications, types of suppliers and systems. However, implementation of this technology is increasing in most parts of the Europe.

It can be concluded that AD is a good option for treating different types of waste streams such as industrial, agricultural, and municipal wastes. In addition, AD can be used to treat biodegradable organic matters. On the other hand, it can produce valuable products such as biogas and digestate. Those are the main reasons of pulling the attention of European countries to operate both farm scale digesters and large scale centralized AD systems.

Usage of biogas and digestate from AD needs further treatment such as biogas upgrading and composting of digestate. General process of AD involves four stages: pre-treatment, digestion, gas upgrading and digestate treatment (Figure 2.2).

Prior to digestion, feedstock should be pre-treated. Generally, the aim of pre-treatment is to exclude undesirable, and inert materials from feedstock and to mix various types of substrate. Besides that, pre-treatment can help the improvement of digestion process, it will allow to produce high quality digestate, and also it will prevent the process failure (Monnet, 2003). The digestion process in digester takes place by the activity of microorganisms and the degradation process will start by itself. Classification of digesters is a function of temperature, water content of feedstock and the number of stages.

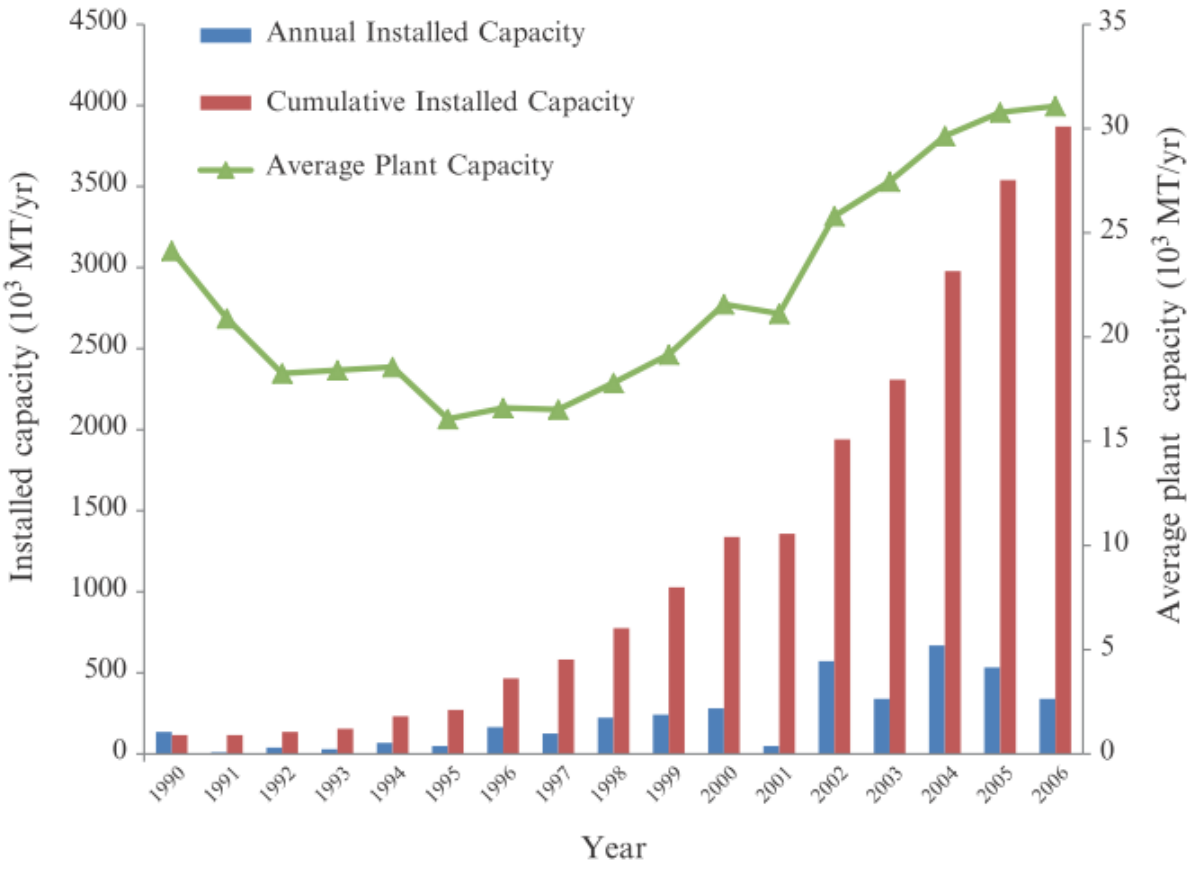

Figure 2.1. Increasing in anaerobic digestion capacity in Europe

(adopted from De Baere, 2006) (Abbasi et al., 2012)

2.7. Anaerobic Digestion Process

Anaerobic digestion is a complex process in which microbes decompose biodegradable matter into methane (CH_4), carbon dioxide (CO_2), ammonia (NH_3), hydrogen sulfide (H_2S), biomass, and inorganic nutrients. These processes can occur naturally in marshlands, rice fields, intestine of animals and marine sediments and these microorganisms are responsible for the carbon cycle in the ecosystem. It has been estimated that anthropogenic sources are responsible for releasing 70% of the total methane into the atmosphere in each year, while 30% is from natural sources. It has been reported that more than 80% of the atmospheric methane flux caused by biological activities (Arsova, 2010).

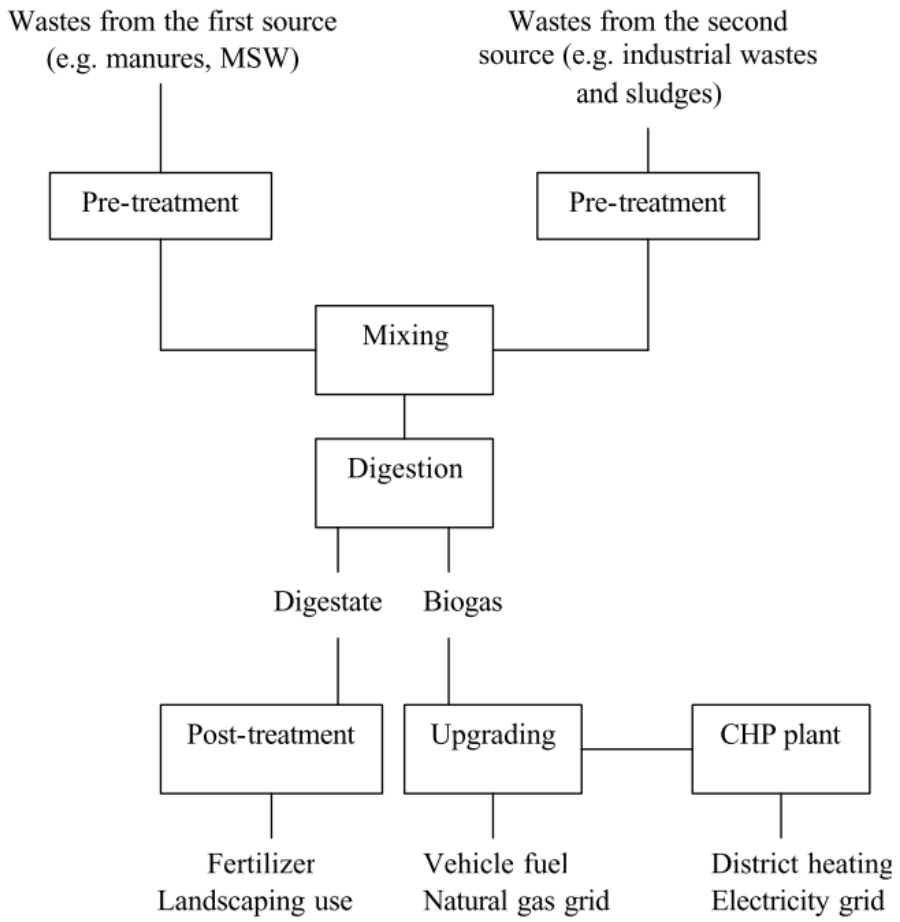

Figure 2.2.General process for anaerobic co-digestion plant

(Monnet, 2003).

In the nature methanogenic ecosystems have been indicated in three types (Figure 2.3). In one of them organic matter involvement in lacustrine and marine sediments, marshes, swamps, sludge and digesters completely degraded. In another one mineralization of organic matter which existed in ruminants and intestinal tracts of all living organisms is not completed and most of the VFAs are absorbed into bloodstream. In the other one methane is produced from the geochemical hydrogen which is formed by geological process (Arsova, 2010).

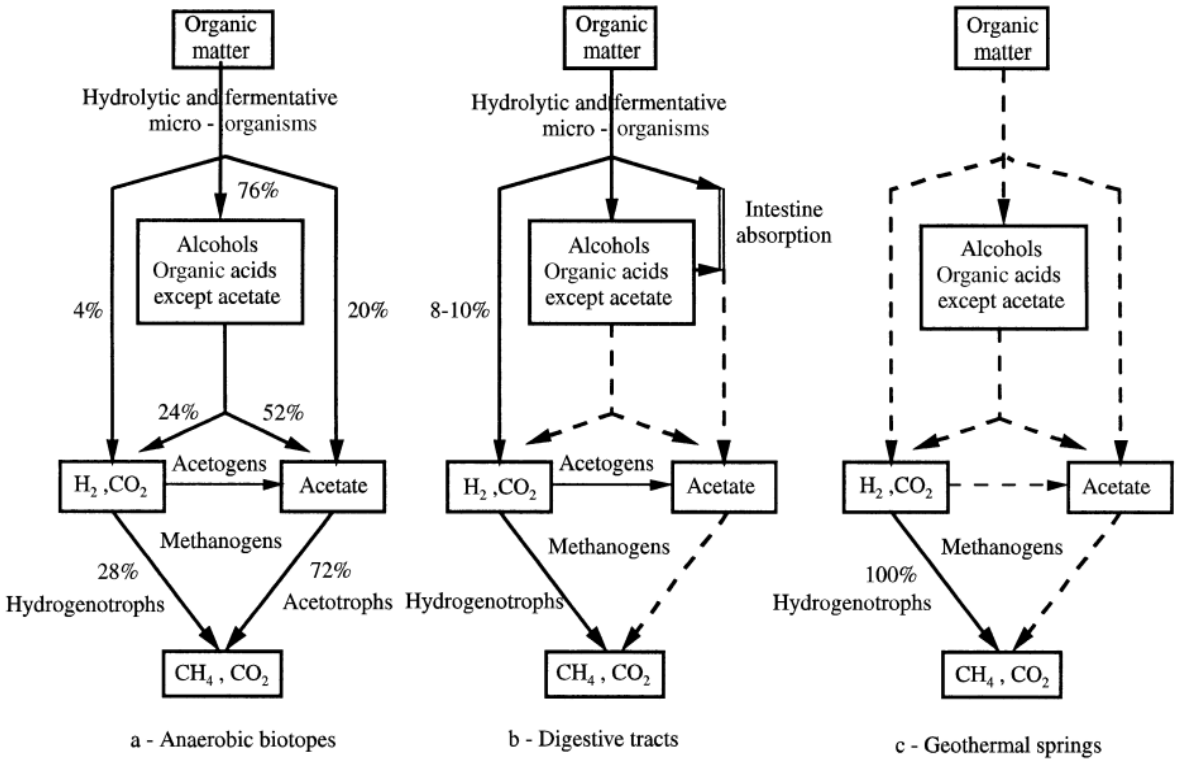

Figure 2.3**.** Schematic diagram of methanogenic ecosystems in nature. Solid line indicates the reaction is occurring and dotted line indicates the reaction is not occurring

(Garcia et al., 2000).

2.8. Metabolic Pathway of Anaerobic Digestion

Anaerobic digestion is one of the best alternatives for the waste treatment practice in which pollution can be controlled on the other hand renewable energy can be achieved. In the last two decades application of AD for waste treatment has been increasing due to its popularity as a solution to environmental pollution and energy security. By increasing the demand of security of energy supply and by integrating the biogas, investment of AD on the energy market is accelerating (Holm-Nielsen et al., 2009). With compare to other renewable energy biogas production through AD has certain advantages. For instance when it needed it can be produced and it is distributable through natural gas infrastructures. Besides that, biogas can replace fossil fuels in the transport system. Digestate from AD contains macro-nutrients such as N, P, and K which can be applied to farmland as a fertilizer. Moreover, nitrogen in the digestate presents on a form of ammonium that is easy for plants to take (Madsen et al., 2011).

Anaerobic digestion involves several interdependent biological reactions, in which microorganisms from two biological kingdoms, bacteria and archaea, under strict anaerobic conditions are cooperating in the degradation of complex organic matters to produce biogas and microbial mass (Parawira, 2004). Based on the physiology, nutritional need, growth kinetics, and sensitivity of microorganisms which are cooperating through AD process, they are widely differ from each other (Adekunle and Okolie, 2015).

Anaerobic degradation of complex organic matter is carried out in four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. During hydrolysis insoluble complex organic matters transformed by hydrolytic bacteria into soluble molecules such as fatty acids, amino acids and sugars. In the second step products from the hydrolytic bacteria converted to simple molecules such as hydrogen, carbon dioxide, alcohols, butyric, propionic, and acetic acid. In the third step VFAs and alcohols, which cannot be used directly by methanogenic archaea, are oxidized into smaller molecules such as acetate, hydrogen and carbon dioxide. In the last step, biogas is produced by methanogenic archaea in two ways: by cleaving two acetic acid molecules to generate carbon dioxide and methane, or by reducing carbon dioxide with hydrogen (Parawira, 2004).

2.8.1. Parameters affecting anaerobic digestion

Main environmental factors which affect the performance of anaerobic operations are temperature, pH and buffering capacity, bioavailability of nutrients, and presence of toxic components for anaerobic microorganisms (Parawira, 2004).

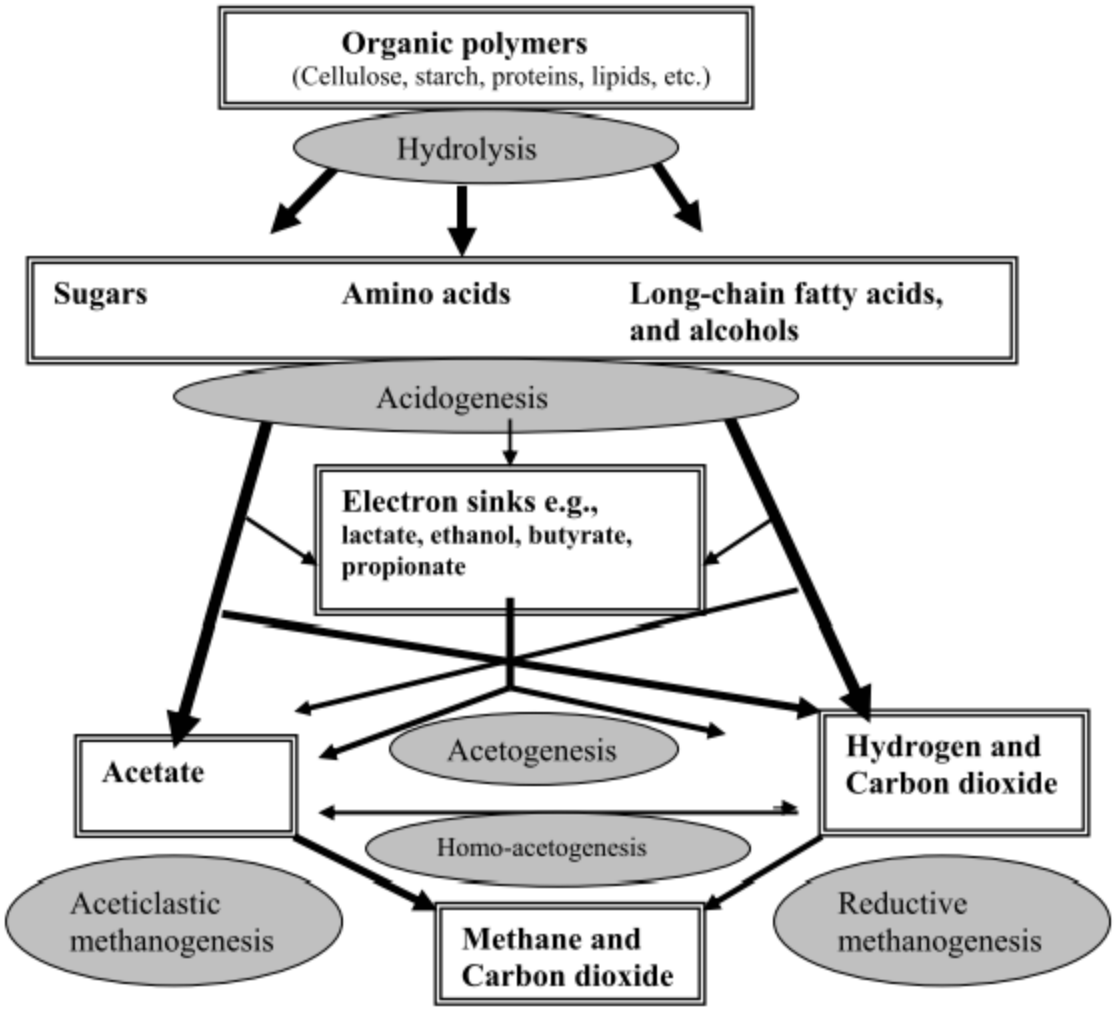

Figure 2.4**.** Main degradation pathways in anaerobic digestion

(Parawira, 2004)

2.8.1.1. Temperature

One of the major parameters which affects the AD process is a temperature. Anaerobic degradation rate is highly dependent on the operational temperatures. These degradation processes can be carried out at different temperature ranges. These ranges in anaerobic systems are: Psychrophilic (<25^{o}C), Mesophilic (25-40^{o}C) and Thermophilic (>45^{o}C).

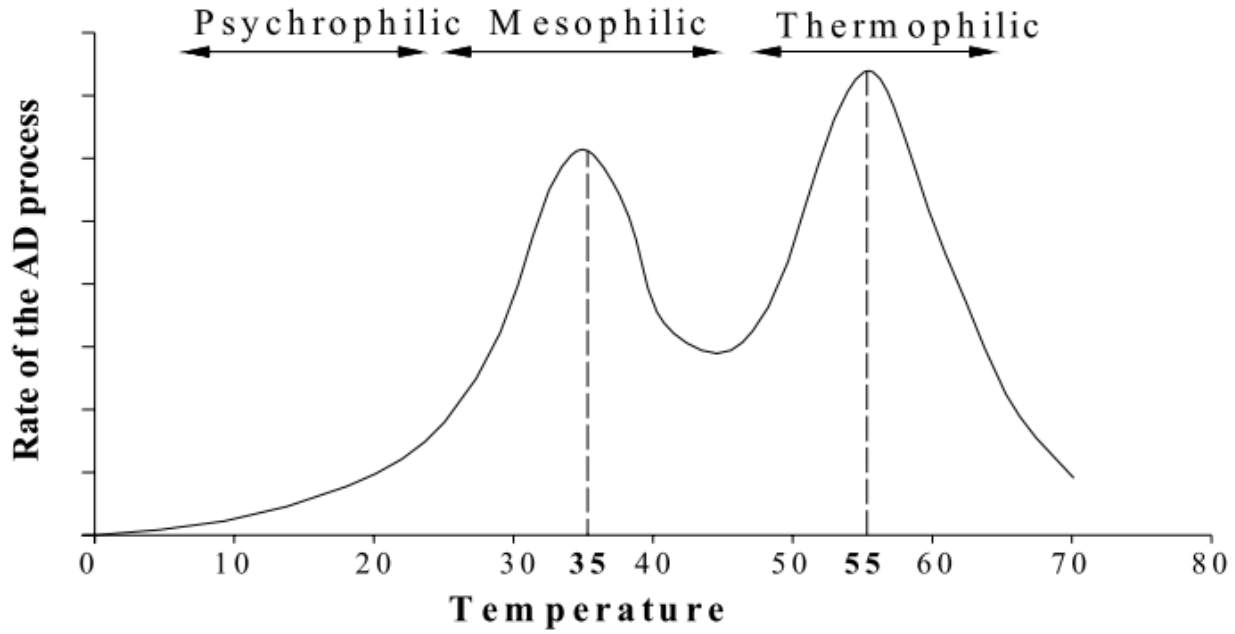

Figure 2.5**.** Temperature ranges for anaerobic digestion

(Yeşil, 2013)

Although anaerobic microbial consortia survive in a wide range of temperatures, generally AD is more preferable within the mesophilic and thermophilic ranges. Generally, AD under thermophilic condition is more attracted because it accelerates the reaction rates, gives higher biogas production and diminish higher amount of pathogens and weed seeds (El-Mashad et al., 2004; Kim et al., 2006). In spite of these benefits, major drawback of thermophilic process is the high sensitivity to the environmental changes (Kim et al., 2006). The comparison performance of reactors under mesophilic and thermophilic conditions has been shown in Table 2.3.

2.8.1.2. pH and buffering capacity

Different type of microorganisms which existed in the AD pathway have a specific pH range for their optimal growth. The pH variations in anaerobic process can affect the utilization of carbon sources, dissimilation of substrate, synthesis of proteins, and releasing metabolic products from the cell.

Optimal pH range of 6.8-7.2 for methane-forming and pH around 6 for acid-forming micro-organisms has been reported. Since methanogensis is a rate limiting step, the reactor pH should be maintained close to neutral. Therefore operating the pH for whole AD process should be in the range of 6.8-7.2 (Moosbrugger et al., 1993). With respect to hydrgenotrophic methanogens, it has been found that acetoclastic methanogens are more susceptible to low pH.

Due to disturbance in the energy-generating process of the organism, methanogens from the acetoclastic route are more susceptible to lower pH (Parawira, 2004).

High sensitivity of acetoclastic methanogens at low pH might be the result of deterioration of the energy-generating process of the organisms (Parawira, 2004).

Table 2.3**.** Distinction between the performance of mesophilic and thermophilic anaerobic digestion

(Parawira, 2004)

Performance characteristics	Mesophilic condition	Thermophilic condition
Gas production rate	Contradictory report	Contradictory report
Pathogen reduction	Lower	Higher
Effluent VFAs	Lower	Higher(contradictory)
Dewaterablility	Contradictory report	Contradictory report
Process stability	Higher	Lower(contradictory)
Methane content	Higher	Lower
Energy requiement	Lower	Higher
Odour	Lower	Higher
Product/ substrate inhibiton	Lower	Higher

Alkalinity is another important factor in AD which indicates the chemical buffering capacity of the reactor's contents against accumulation of VFAs. It is necessary to provide enough buffering capacity to maintain pH for stable operation by neutralizing any possible acid accumulation in the reactor (Parawira, 2004).

In anaerobic reactors carbon dioxide is in equilibrium with carbonic acid, which releases hydrogen and bicarbonate ion in aqueous system. Anaerobic reactor also contains some other compounds such as ammonia, orthophosphate, hydro-sulfuric acid, and VFAs, which may affect the pH of the reactor. But most of the time in anaerobic reactor concentrations of ortho-phosphoric and hydro-sulfuric acid are too low to affect the pH, significantly. Furthermore, in neutral pH, effects of VFAs and NH_3 on the buffering capacity are negligible. Due to these reasons carbonic acid plays the

basic role to control the reactor's pH. Different types of carbonate system have been shown in the following equilibrium (Gomez, 2011).

$$CO_{2\,(aq)} + H_2O = H_2CO_3$$
$$H_2CO_3 = H^+ + HCO_3^- \qquad (2.1)$$
$$HCO_3^- = H^+ + CO_3^{2-}$$

2.8.1.3. Nutrients

Anaerobic microorganisms in addition to macronutrients such as C, N, P and S, need trace metals like K, Ca, Mg, Fe, Ni, Co, Zn, Mg, Cu, W and Se for their growth and metabolic activities since they are essential for many biological and biochemical processes. Deficiency in any of these nutrients seriously will affect their growth (Parawira, 2004).

Among macro- and micro-nutrients, N and P are the most important nutrients for microorganisms. For high methane yield the optimum ratio of C: N: P = 100:3:1 is reported. High value of C/N, indicates a low buffering capacity and the accumulation of VFAs in the system. Conversely, low value of C/N shows the risk of ammonia inhibition in the system. For such type of wastes to improve the digestibility, co-digestion of wastes is recommended (Parawira, 2004).

However, some of the heavy metals like many other toxic substances, are resistant to biodegradability and finally by accumulation into the system become toxic substances. These metals bind with some groups involved in protein or replaced by removing the naturally existing metals into enzyme prosthetic groups and causing the toxic effect on anaerobic microorganisms and disrupting the enzyme functioning (Parawira, 2004).

Relative susceptibility of anaerobic microorganisms which is reported to these metals is
Cu > Zn > Cr > Cd > Ni > Pb and Cd > Cu > Cr > Zn > Pb > Ni, respectively (Chen et al., 2008).

2.8.1.4. Volatile fatty acids

Another important parameter in AD process is the VFAs concentration. Accumulation of VFAs, especially acetic, propionic, and butyric acid, in the reactor is the result of unbalanced digestion conditions among microorganisms involved in digestion process (Björnsson et al., 1997). Furthermore, VFAs accumulation accompanying to decrease the pH inside the reactor and may

cause the digester failure (Ahring et al., 1995). Protonated forms of VFAs are toxic to anaerobic consortia as they can pass through the cell membrane. Apart from toxicity when the pH is around 7, VFAs inside the cell are in ionized form and released hydrogen ion will decrease the intracellular pH. As a result of pH gradient inside and outside of the cell, ATP will form which is important for cell growth (Björnsson et al., 2000).

2.9. Impact of Ammonia in Anaerobic Digestion

2.9.1. Introduction

During anaerobic digestion processes, production of ammonia is a common occurring substance, from digestion of feed stocks which are rich in nitrogen compounds such as protein (Kayhanian, 1999; Kotsyurbenko et al., 2004). It has been reported due to necessity of ammonia nitrogen for bacterial growth, its concentration in the range of 50 to 200 mg/l has beneficial effects for bacterial growth; however, high level of ammonia concentrations will be inhibitor for anaerobic microorganisms especially methanogens (Gallert et al., 1988). According to Neilsen and Angelidaki (2008) the amount of ammonia which produces from anaerobic degradation can be predicted by using the following stoichiometric relationship (Nielsen and Angelidaki, 2008).

$C_aH_bO_cN_d + (4a – b - 2c + 3d)/4H_2O =$

$(4a + b - 2c - 3d)/8CH_4 + (4a – b + 2c+ 3d)/8CO_2 + dNH_3$ (2.2)

Hansen et al (1998) indicated that in the solutions ammonia exists in two forms of ammonium ion (NH_4^+) and free ammonia $(NH_3)_{aq}$. As shown in the following formula, free ammonia is highly depended on pH and temperature (Hansen et al., 1998).

$$NH3 = TANx \left(1 + \frac{10^{-\mathrm{pH}}}{10^{-\left(0.09018+\frac{2729.92}{T(k)}\right)}}\right)^{-1} \quad (2.3)$$

Where:

NH_3: free ammonia nitrogen (mg/l)

TAN: total ammonia nitrogen (mg/l)

T(k): temperature in Kelvin

It has been reported that free ammonia is the main inhibitory form of ammonia nitrogen on methanogens and its concentration is proportional with temperature and pH of the medium. In

mesophilic conditions, ammonium ions above pH 7 being transformed into ammonia and when pH increases the concentration of free ammonia also increases. Therefore, ammonia inhibition in AD is usually observed above pH 7.5 (Calli et al., 2005). Anaerobic digesters operated in thermophilic conditions are exposed to higher ammonia concentration since transformation of ammonium ion to free ammonia increases with increasing temperature (Hansen et al., 1998).

2.9.2. Mechanisms of ammonia inhibition

Even though there is a lack of information about mechanisms of ammonia toxicity in anaerobic microorganisms, some studies have shown that ammonia diffuses through cell membrane of methanogens subsequently disturbs the proton imbalance and finally causes the toxicity of cytosolic enzymes (Sprott and Patel, 1986; Gallert et al., 1988).

2.9.2.1. Diffusion and accumulation of ammonia through cell membrane

In order to clarify the possible mechanisms of ammonia inhibition, chemical interaction between ammonia and cells should be considered. According to proposed model by Kayhanian free ammonia molecule defuses through cell membrane. As a result of different intracellular pH inside the cell, some portion of ammonia will change to ammonium (NH_4^+). To maintain the intracellular pH, cell must use energy in proton balancing, therefore, higher amount of energy required for maintenance. This higher amount of energy may affect the reaction of specific enzyme and finally may inhibit the enzyme (Kayhanian, 1999).

2.9.2.2. Intracellular ammonia toxicity

Kadam and Boone (1996) indicated that after the diffusion of ammonia through cell membrane it can cause toxicity at least in two different ways: first is the direct inhibition of enzymes activity by free ammonia. Second is the accumulation of NH_4^+ ion inside the cell and affecting the intracellular pH and depleting the concentration of K^+. Based on these two mechanisms of ammonia toxicity it can be predicted that up taken of essential trace metals required for cell function might be affected by high ammonia concentration as a result of that causing trace metal deficiency for microorganisms (Kadam and Boone, 1996).

2.9.3. Impact of ammonia on the methanogenesis pathways

Generally, it is known that methanogens are the most sensitive microorganisms to variations of environmental conditions. Due to particular sensitivity of methanogens to ammonia toxicity, last

step of AD process will be ceased, while activity of acid forming microorganisms which are more tolerance to ammonia concentrations continue. Accumulation of organic acids in the anaerobic milieu and cessation of methanogens will cause an extremely drop in pH and followed by the failure of anaerobic process.

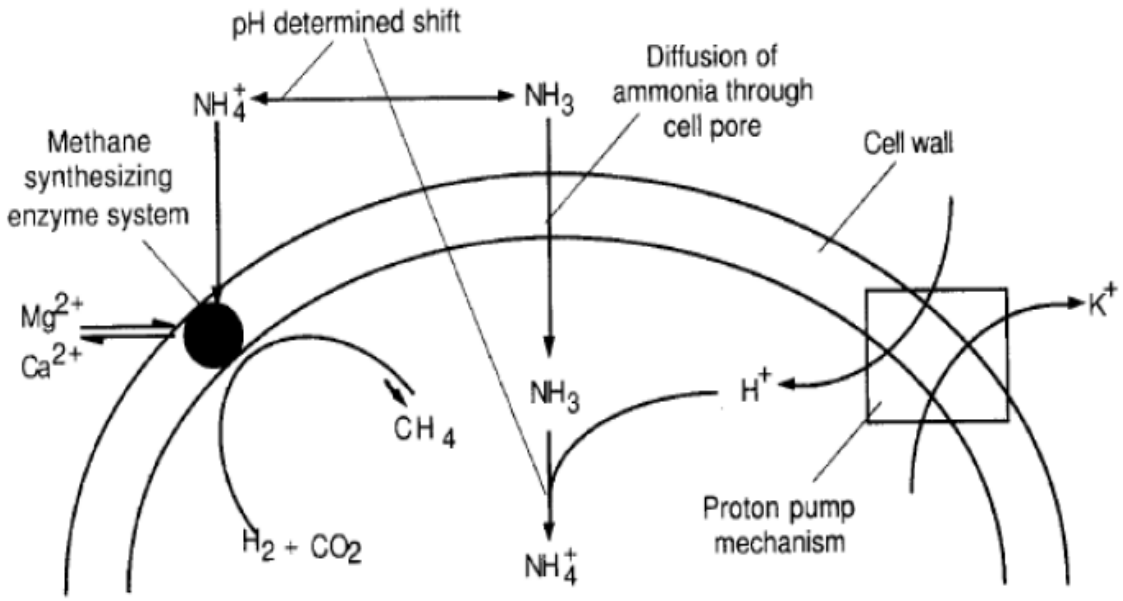

Figure 2.6**.** Schematic of inhibition by ammonia in the anaerobic microorganisms (Kayhanian, 1999).

Among methanogenic pathways to methane production sensitivity to ammonia varies significantly. Wiegant and Zeeman (1986) have indicated that hydrogen-consuming methanogens were less tolerance to ammonia than acetoclastic methanogens. They concluded when hydrogen-consuming methanogens being inhibited, hydrogen will be accumulated in the system. Furthermore hydrogen accumulation in the system will help in production of propionate which is inhibitor for acetoclastic methanogens (Wiegant and Zeeman, 1986). By contrast, some researchers concluded that hydrogen-consuming microorganisms are more tolerance to ammonia than acetoclastic methanogens at mesophilic and thermophilic conditions (Robbins et al., 1989; Schnurer and Nordberg, 2008).

Based on the recent studies in AD it can be concluded that threshold value of ammonia for the inhibition of acetoclastic methanogens is lower than that for hydrogenotrophic methanogens.

Under normal conditions 70% of the methanogens consist of the microorganisms from acetoclastic pathway and the rest is from the hydrogenotrophic pathway. When the activity of acetoclastic pathway ceased, methane production will be shifted to hydrogenotrophic pathway. Microorganisms in this pathway play an important role for oxidizing propionate and butyrate into methanogenic substrate by keeping the partial pressure of hydrogen low enough. In order to have a stable digestion process, the partial pressure of hydrogen should be lower than 10^{-4}. Thus if the activity of acetoclastic route has been ceased by ammonia and the methane production shifted to the hydrogenotrophic route, any other disturbance may cause the complete inhibition of both pathways. This might be the reason why anaerobic digesters treating nitrogen rich wastes have stability problems and consequently complete failure of the process (Neiva Correia et al., 2008; Banks and Zhang, 2010).

2.10. Impact of Trace Metals in Anaerobic Digestion

2.10.1. Introduction

Microorganisms in AD, in addition to macro-nutrients such as C, N, P and S, also need micro-nutrients for their activities and survival. Most of these micronutrients are metals and exist in very small amount in organisms. Proportion of macro- and micro-nutrients in anaerobic microorganisms are 45-55% carbon, 20% oxygen, 10% hydrogen, 6-14% nitrogen, 1-3% phosphorus. Based on the type of microorganisms S, Na, K, Ca, and Mg can reach to 1% and the rest which are the TMs reported less than 1% (Banks et al., 2010).

Effects of TMs in AD strongly depend on the type and bioavailability of metals to the anaerobic consortia. Vitally important TMs exist as cofactors in the enzyme system. Non-enzymatic TMs are serving in respiration processes with transferring the electron bound to cell wall (Zandvoordt et al., 2006).

Microorganisms are used in many biological processes and for better functioning of the microorganisms they need TMs, thus to achieve the optimal performance of these processes the existence of TMs are necessary. Some of them which existed in the biochemistry of methane

formation serve as cofactors in enzymes (Table 2.4). Therefore, deficiency in any of these TMs may disturb the whole process.

Table 2.4**.** Some specific trace elements in enzymes of microbial transformation (Zandvoordt et al., 2006)

Enzyme	Organism	Metal
Methyltransferase	Methanogens and acetogens	Co (B12)
B12-enzymes	Many organisms	Co (B12)
Co-dehydrogenase	Methanogens/Acetogens	Co, Ni, Fe
Aceyle-CoA synthase	*Moorella thermoacetica*	Fe, Ni, Cu
Tetrachloroethene reductive dehalogenase	*Dehalospirillum multivirans*	Co, Fe
Methyl-CoM-reductase	Methanogens	Ni
Hydorgenase	*Desulfpvibro*	Fe
Formiate dehydrogenase	*Methylobacterium*	Mo or W
	E. coli	Mo-Se
Glycin reductase	*E. coli*	Se

2.10.2. Importance of trace metals in metabolic pathways to methane production

Based on the enzymology of methanogensis and acidogenesis in hypothetical pathways from methanol, H_2/CO_2, and acetate to methane several of enzymes are existed. Many of these enzymes contain TMs such as Co, Ni, Fe, Zn, W and Mo as cofactor or coenzyme, which indicating the high dependency of methanogens to the availability of these metals. Methyl-coenzyme M reductase which is catalyzing enzymatic reactions existed in all methanogenic pathways. This enzyme includes a nickel containing cofactor called F_{430}. Methanol: Coenzyme M, a cobalt dependent enzyme, is also existed in the first step of methanogenesis from methanol. This enzyme in addition to cobalt, contains zinc. Methanogenic pathways from acetate involve the complex enzyme called carbon monoxide dehydrogenase (CODH). This enzyme oxidizes the carbonyl group of acetate to CO_2 and is responsible for carrying the methyl group to coenzyme M. This enzyme is also plays an important role in the formation of acetate from H_2/CO_2 and methanol. The CODH complex enzyme consists of two enzyme components which are dependent on nickel and

iron. Another enzyme called formylmethanofuran dehydrogenase which is catalyzing the last step in CO_2 reduction to CH_4. This enzyme is a molybdenum dependent. Another enzyme in methanogenic pathway is a Methanobacterium thermoautotrophicum which is a molybdenum and tungsten dependent enzyme. In table 2.5. some metals which stimulating pure cultures of methanogens are given. (Zandvoordt et al., 2006).

Table 2.5**.** Methanogens stimulator metals

(Zandvoordt et al., 2006)

Pure culture	Conversion	Stimulating concentration
Methanosarcina barkeri	Methanol (methanogenic)	Fe(II) (35)
M. barkeri	Methanol (methanogenic)	Co (1), Ni (1), Se (1), Mo (1)
Methanothrix soehngenii VNBF	Acetate (methanogenic)	Fe (20-100), Co (2), Ni (2), Mo(2)
M. thermoautotrophicum	H_2/CO_2 (methanogenic)	Se (1), W (10)
M. thermoautotrophicum	H_2/CO_2 (methanogenic)	Fe(>5), Co(>0.01), Ni(>0.1), Mo(>0.01)
M. barkeri	Nitrogen fixation (methanol C:source)	Mo (5) or V(2)
Methanococcus ofnielli	Formiate	Se (1), W(100)
Methanospirillum hungatei GP1	H_2/CO_2 (methanogenic)	Mn (50)

2.10.3. Bioavailability of trace metals

Bioavailability of TMs is highly depended on the speciation of metals. Speciation of metal is a function of temperature, pH, metal concentration, ionic strength of the solution, and existence of organic and inorganic matters in which can react with metals. Bioavailability of TMs in anaerobic digestion process might be decreased by physical-chemical phenomena such as precipitation, adsorption, co-precipitation and complexation. Trace metals in the presence of sulfide, carbonate, and phosphate may undergo precipitation mechanism. As a result, bioavailability of TMs decreases in the presence of these anions (Brulé et al., 2013). When TMs go through precipitation mechanism with sulfide, precipitate form has low solubility. Therefore, it can be concluded that among these anions sulfide is more responsible for the precipitation of TMs. However there is no clear evidence about the effects of sulfide on the bioavailability of TMs, in the study by Gustavsson (2011) has been mentioned that sulfide plays a key-role on the metal speciation and bioavailability in

anaerobic digesters (Gustavsson, 2011).In addition to precipitation, carbonates, phosphates and sulfides can react with TMs and produce a complex compounds. Based on the stability constant, sulfide complexation with metals, such as following complexes $[MeHS]^{+}$ and $[Me(HS)_2]^{0}$, have been indicated to be the most important (Table.2.6). On the other hand, complexation of TMs with organic compounds of microbial derivations which called soluble microbial products (SMP) may positively affect the bioavailability of TMs in anaerobic digestion process. SMPs have ability to form Me-SMP complexes with TMs, and may prevent the precipitation of Me-Sulfide, resulting in increased bioavailability of TMs in anaerobic reactor (Gustavsson, 2011).

Table 2.6**.** Stability constant for Co-sulfide and Ni-sulfide

(Rickard and Luther III, 2006)

Complex	Log K
$[CoHS]^{+}$	5.5
$[Co(HS)_2]^{0}$	10.2
$[Co_2(HS)]^{3+}$	9.5
$[Co_3(HS)]^{5+}$	15.5
[NiHS]	5
$[Ni(HS)_2]^{0}$	10.5
$[Ni3(HS)]^{3+}$	10
$[Ni_3(HS)]^{5+}$	15.9

Other important phenomena which have an important role on the metal speciation are co-precipitation and adsorption. In the adsorption process negatively charged groups on cell surfaces interact with metals and commence the transportation of metals into the cell (Liu et al., 2001). In the systems containing high Fe concentration with respect to other metals, co-precipitation and adsorption of TMs on Fe-S plays a key-role on their bioavailability (Gustavsson, 2011).

3. MATERIAL AND METHODS

In this study two different experiments were performed: Operation of daily fed anaerobic digester and batch bio-methane potential tests.

Firstly, in order to provide digestate for batch experiments, a laboratory scale anaerobic digester (biogas reactor) was run for about 160 days. In this reactor, a mixture of CM and spent poppy straw was used as substrate and reactor was operated at mesophilic conditions (36 ±1 ^{o}C).

Secondly, in order to reveal the effect of trace metal supplementation on anaerobic digestion of CM, batch bio-methane potential experiments were performed under mesophilic conditions (36 ±1 ^{o}C). VFAs and raw CM were used as substrate and the digestate taken from the CM and spent poppy straw co-digester was used as inoculum.

3.1. Characteristics of Raw Chicken Manure and Spent Poppy Straw

The raw chicken manure (TS of 23.2-29.4%) and spent poppy straw (PS) (TS of 26.3-29.4%) were taken from the poultry farms and the opium alkaloids factory in Afyonkarahisar, Turkey, respetively. In total, four CM and two PS samples were used in the study. They were stored at 4°C before the experiments. Total Kjeldahl Nitrogen (TKN) concentration of CM and PS were in the range of 4.38-5.33% and 0.72-0.86%, respectively. CM and PS were mixed in a ratio of 4.3/1 (w/w) using tap water and the total solids (dry matter) content was adjusted to 9.8-13.5%. Total solids (Kotsyurbenko et al.), volatile solids (Gustavsson) and TKN contents of the samples are given in Table 3.1.

Table 3.1**.** Characteristics of chicken manure and spent poppy straw

Parameter	Unit	Chicken Manure			Spent Poppy Straw	
		I	II	III	I	II
TS	%	29.7	25	23.2	29.4	26.3
VS	%	18.3	17	20	22	19.4
TKN	%	4.8	5.33	4.38	0.86	0.72

3.2. Laboratory Scale Continuously-fed Biogas Reactor

A schematic illustration of the lab-scale biogas reactor used for co-digestion of CM and spent poppy straw is given in Figure 3.1.

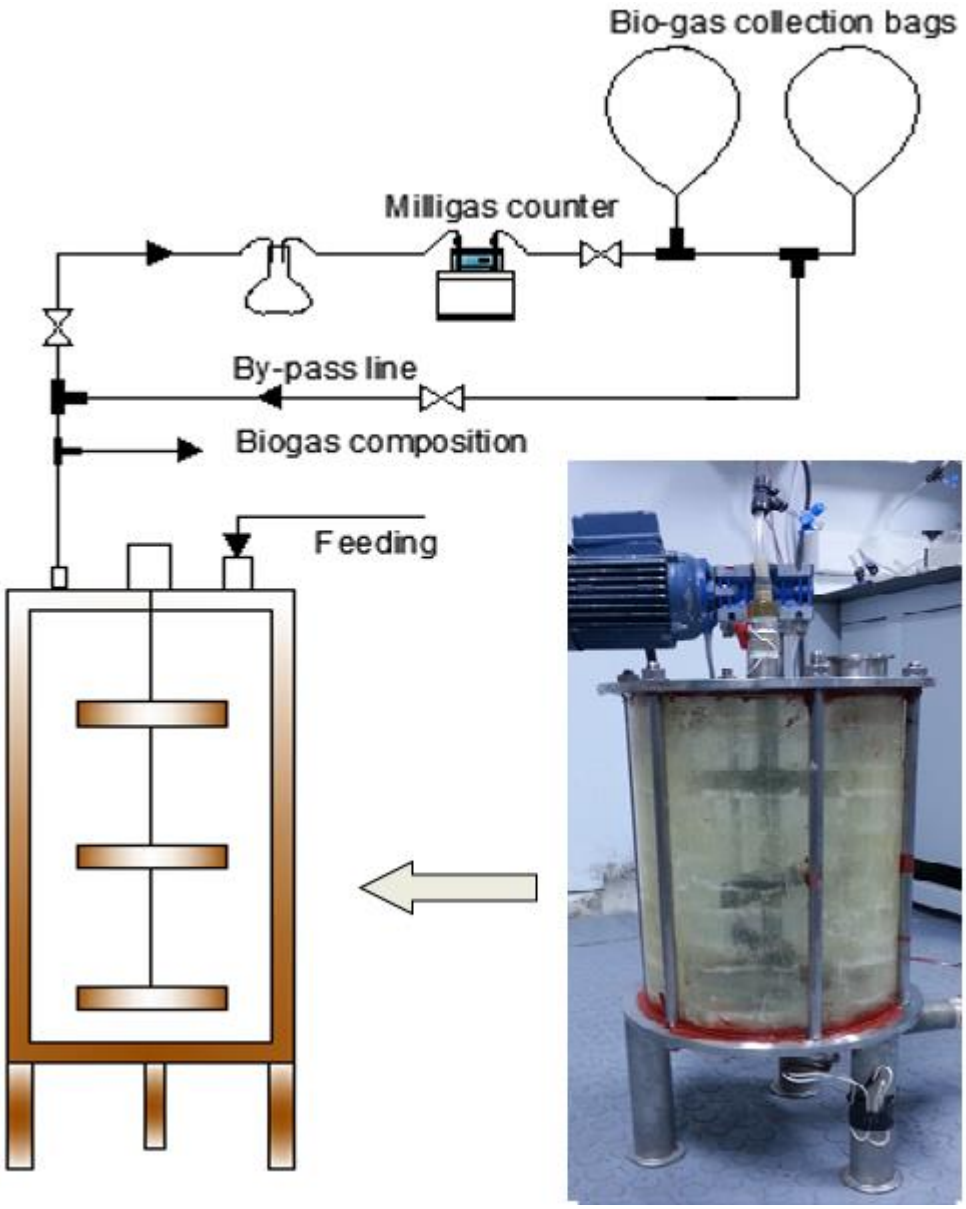

Figure 3.1**.** Schematic of laboratory scale biogas reactor

A completely mixed lab-scale biogas reactor with a total volume of 16.2 L and active volume of 14.5 L was operated under mesophilic conditions (36±1 ^{o}C) for 158 days. Four different operational phases were established based on the influent TKN shown in Table 3.2. The reactor was fed with the mixture of CM and PS with a ratio of 4.3/1 (w/w), respectively. Hydraulic

retention time (HRT) of the reactor was 23 days. Based on OLR, digestate from the reactor was removed daily and replaced with the the mixture of CM and poppy straw.

The amount of biogas was measured continuously and recorded daily with a volumetric milligas gascounter (BnC-Ritter). For gas composition analysis, the biogas was collected in aluminum-foil gas bag. The reactor content was homogeneously mixed at 45 rpm using a paddle mixer having a motor mounted on the top of the reactor. Temperature was controlled at 36±1 ^{o}C by using heating belts covered around the digester. Reactor was fed once a day manually and the digestate was drawn by a vacuum pump and analyzed for VFAs, pH, TAN, TS and VS three times per week.

Table 3.2**.** Operational conditions for completely mixed laboratory scale biogas reactor

Period - Time (day)	Period 1: 0-48	Period 2: 49-74	Period 3: 76-128	Period 4: 128-158
OLR, kgVS/m^3.d	2.78		3.56	
$TKN_{influent}$, mg/L	3960	5020	5960	6560
HRT, d			23	
$TS_{influent,}$ %	9.8	12.4		13.5
Temperature, °C			36±1°C	

3.3. Bio-methane Potential Test

In BMP tests, the biogas production profiles and yields at different total ammonia nitrogen concentrations were compared with the yields of control bottles which were not supplemented with trace metals. BMP tests were conducted in 250 ml bottles in triplicate.

The inoculum taken from the lab-scale biogas reactor and having 1.7% VS was diluted to 0.2% VS (2 g VS/l) using a dilution solution in 5-litre bottle. The dilution solution (Table 3.3) was prepared as described by Valcke and Verstraete (1983) without using NH_4Cl and $Na_2S.7H_2O$ (Valcke and Verstraete, 1983). While adding the inoculum to dilution solution, to remove the dissolved oxygen in the aqueous phase and air in the headspace, the 5-litre stock dilution solution bottle was flushed with nitrogen gas for 2-3 min.

Table 3.3. Chemical composition of the dilution solution

Compound	Required compound (mg/l)
KH_2PO_4	2.5
K_2HPO_4	1
$MgCl_2.6H_2O$	0.1
Yeast extract	0.2

The diluted digestate was incubated at 36 °C before adding the substrate. After 48 hours incubation and before transferring the digestate, TMs and VFAs as substrate were added into bottles by using automated pipette. In case of using raw CM as substrate, it was added into the diluted inoculum and then to provide the anaerobic conditions, the head space of the bottles flushed with the nitrogen gas. In order to provide a homogeneous solution, the mixture of the substrate and diluted inoculum were vigorously stirred by using a magnetic stirrer.

As substrate, acetate, propionate and butyrate or raw CM were added into the 250 ml bottles at final concentrations of 1000 mg COD/l or 0.24 g TS/l (0.24% TS), respectively. The diluted inoculum (digestate) was transferred homogeneously to the 250 ml test bottles under anaerobic conditions. The set-up is given in Figure 3.2 was used.

In the test bottles, the total volume of inoculum, substrate and dilution solution was set to 100 ml. All transfers were done under continuous nitrogen purging and the bottles were sealed with special caps having two ports. Finally, all the bottles were located on a multi position magnetic stirrer (Tewolde et al., 2005) which was kept in an incubator (WTW- TS 606/4-i) at 36 ± 1 ^{o}C. Till the end of bio-methane potential test the bottles were kept there (Figure 3.3).

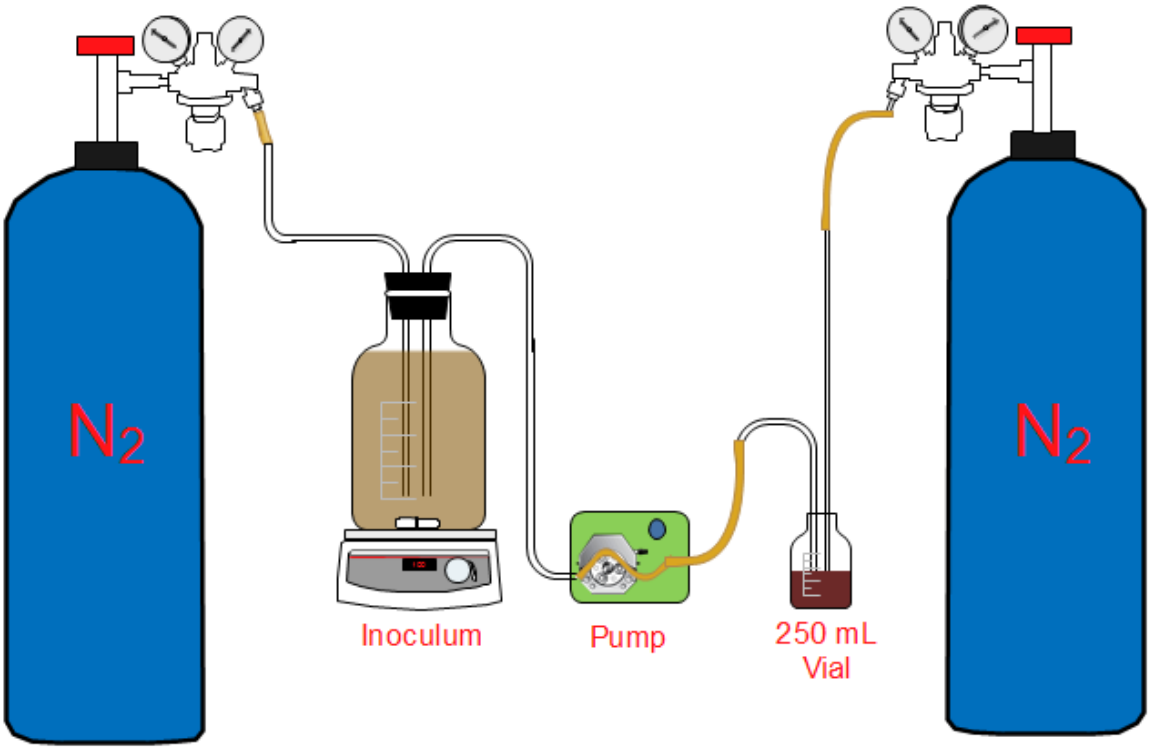

Figure 3.2. Schematic of the set-up used to transfer the diluted inoculum into the bottles

The biogas production was monitored continuously using a 30-tube manometer set-up working based on water displacement principle. Each test bottle in the incubator was connected to a tube of this manometer (Figure 3.5). To prevent the dissolution of gases, pH of the manometer solution was adjusted to 2 and 40 g/l NaCl was added.

In total, five BMP tests were performed. In BMP test-1, acetic acid was used as substrate and the NH_4-N concentrations of 675 mg/l and 5225 mg/l were examined. In BMP test-2, in addition to acetic acid, propionic and butyric acids were used while NH_4-N concentration was 2950 mg/l. BMP test-3, 4 and 5 were performed by using raw CM as substrate and NH_4-N concentrations up to 6000 mg/l were tested. Except BMP test-5, 1 mg/l Ni, 1 mg/l Co, 0.2 mg/l Mo, 0.2 mg/l Se, 0.2 mg/l W and 5 mg/l Fe were used in TMs supplemented sets. In BMP test-5, TMs concentrations were increased from 2x to 50x of the concentrations tested in the first four BMP tests. To avoid the precipitation of supplemented trace metals by sulfide, iron concentration in TMs supplemented sets were increased to 100 mg/l which is 20x of the concentration used in the former BMP tests.

The concentrations of TMs examined in BMP test-5 were given in Table 3.4. TMs were added in the form of $NiCl_2.6H_2O$, $CoCl_2.6H_2O$, $Na_2Mo_4.2H_2O$, Na_2SeO_3, $Na_2WO_4.2H_2O$ and $FeCl_3.6H_2O$.

Table 3.4**.** Concentrations of TMs in BMP test-5

Vials	1-3	4-6	7-9	10-12	13-15	16-18	19-21	22-24	25-26	27-30
TMs	Concentrations (mg/l)									
Co	1.016	2.032	3.048	4.064	5.080	10.161	20.321	30.482	40.462	50.803
Se	0.205	0.409	0.614	0.818	1.023	2.046	4.092	6.138	8.184	10.23
W	0.208	0.416	0.624	0.832	1.040	2.080	4.159	6.239	8.319	10.399
Mo	0.220	0.440	0.659	0.879	1.099	2.198	4.396	6.593	8.791	10.989
Ni	1.013	2.026	3.038	4.051	5.064	10.128	20.256	30.384	40.512	50.64
Fe	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Figure 3.3**.** BMP test bottles on multi position magnetic stirrer in the incubator

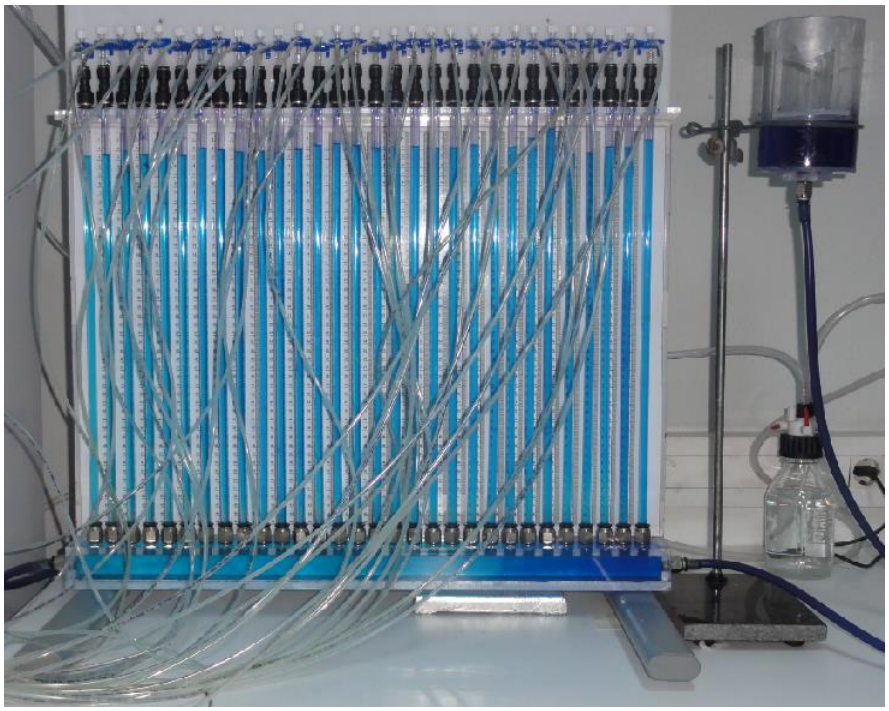

Figure 3.4**.** Multi-tube monometer system used in BMP tests

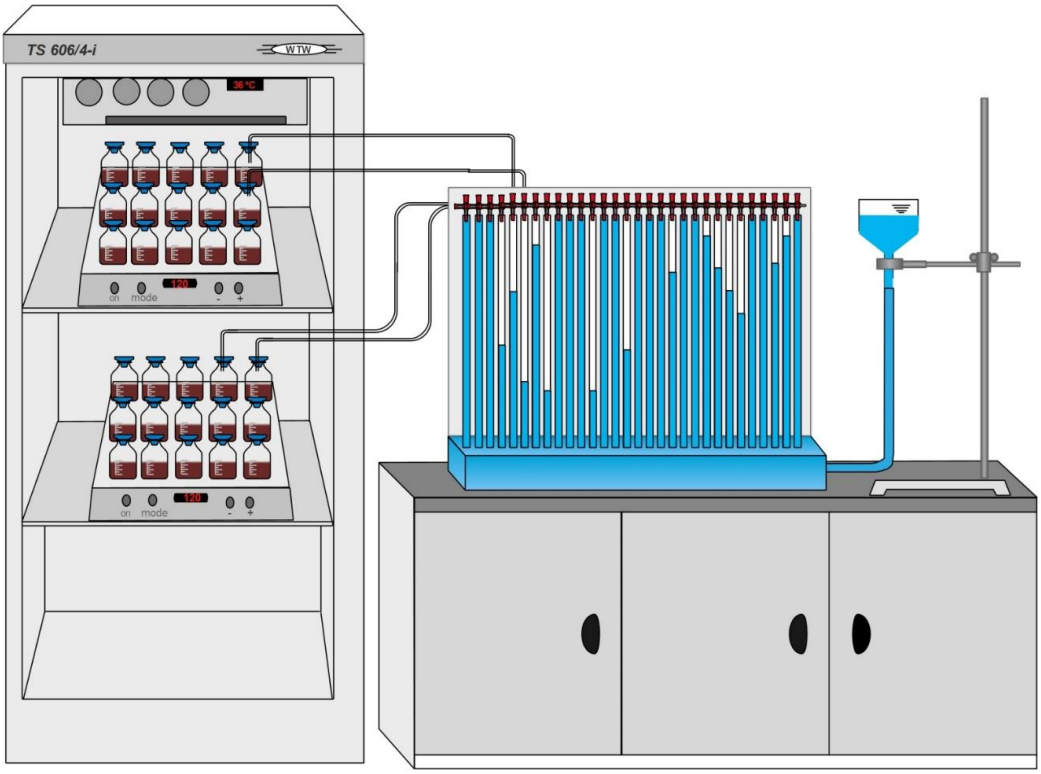

Figure 3.5**.** Set-up used in BMP tests

3.4. Analytical Method

The temperature, pH, Total solid (Kotsyurbenko et al.), Volatile solid (Gustavsson), Total Kjeldahl Nitrogen (TKN), and Volatile Fatty Acids (VFAs) were measured according to Standard Methods (American Public Health Association, 2005).

3.4.1. Temperature measurement

The temperature of the CM and spent poppy straw co-digester was measured on-line and continuously with a probe located in the rector and connected to a PLC unit.

3.4.2. pH measurement

The pH of the co-digester was measured daily. In BMP tests, pH was measured at the beginning and at the end of each tests by using Eutech, PCD 6500 pH meter.

3.4.3. Total ammonia nitrogen determination

The total ammonia nitrogen (TAN) concentrations were determined according to Nessler Method (Method 8038) with DR/2800 spectrophotometer (HATHCH Company, Colorado, USA) The maximum detection limit of this method was 2.5 mg/l, hence the samples having NH_4-N above this limit were diluted with deionized water.

3.4.3.1. Free ammonia nitrogen calculation

The free ammonia nitrogen concentration was calculated based on ammonium nitrogen, pH and temperature by using the formula given by Hansen *et al* (1996).

$$FAN = \left(\frac{\mathrm{NH4 - N}}{1 + 10^{\left(0.09018 + \frac{2729.92}{273.15+T} - pH\right)}} \right)$$

3.4.4. Total kjeldahl nitrogen determination

For determination of Total Kjeldahl Nitrogen (TKN), firstly the sample was digested with concentrated sulfuric acid. To speed up the reaction, sulfuric acid added samples were heated by Kjeldatherm Automated Digestion device up to 400 ^{o}C to convert organic nitrogen to ammonium nitrogen (Figure 3.6). The digestion was performed under the fume hood.

Figure 3.6**.** The digestion device (Turbotherm, Gerhardt)

After digestion, the digested samples were distilled using the apparatus (Vaposdest, Gerhardt) given in Figure 3.7. To increase the pH of digested sample above 9.5, a 32% sodium hydroxide solution was used and the distilled ammonia is trapped in 2% boric acid solution. Ammonium nitrogen in boric acid solutions was determined by nesslerization method.

Figure 3.7**.** The distillation apparatus (Vaposdest, Gerhardt)

3.4.5. Total and volatile solid determination

Total and volatile solids content of the CM, spent poppy straw and digestate were determined according to Standard Method (APHA, 1998). Samples were placed on evaporating crucibles and weighted by using an analytical balance (Sartorius QS16000B). Afterwards, samples were dried at 105 ^{o}C in an oven (Philip Harris Model) for overnight. After drying, the samples were transferred to a desiccator and cooled down to room temperature. Then the dry weight of the sample was measured. For volatile solids determination, the crucibles containing the dries samples were put into a muffle oven (Lenton Model) and ignited at 550 ^{o}C for two hours. After 2-hr, the crucibles were transferred to a desicator and cooled down to room temperature. Finally, the remaining solids were weighed.

3.4.6. Volatile fatty acids (VFAs) analysis

Volatile fatty acids such as acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acid in the samples were analyzed by using the gas chromatograph (Shimadzu GC-2014) equipped with a flame ionization detector (FID). Before the VFA analysis, samples were centrifuged two times at 6000 rpm and 14000 rpm for 10 and 5 minutes, respectively. The supernatants were transferred to 1 ml vials, acidified and diluted with 1% phosphoric acid solution before being injected to the GC-FID.

Figure 3.8. Gas chromatograph with Flame Ionization Detector (Shimadzu GC-2014)

3.4.7. Biogas composition analysis

The biogas composition (methane, carbon dioxide, hydrogen, oxygen, nitrogen and hydrogen sulfide) was analyzed by using a gas chromatograph (Shimadzu GC-2014) equipped with thermal conductivity detector (TCD). Biogas samples taken with 5 ml plastic syringes from the gas bag were directly injected to the sampling loop of the GC-TCD.

Figure 3.9. Gas chromatograph with Thermal Conductivity Detector (Shimadzu GC-2014)

3.4.8. Analysis of trace metals

The dried and grinded raw CM and digestate samples were pretreated according to water extraction and microwave assisted acid digestion methods prior to trace metal analyses. The total and leaching concentrations of Cu, B, Zn, Fe, Co, Mn, Mo, Ni, Se and W were determined using an ICP-OES (APHA, 1998).

3.4.9. Chemical speciation calculations

The chemical speciation of the supplemented TMs in the mixed liquor of BMP test bottles was calculated to check the bioavailability of TMs for anaerobic microorganisms using the Visual Minteq 3.0 software (Gustafsson J.P., 2012, http://vminteq.lwr.kth.se/). The concentrations of TMs, temperature (36 ^{o}C) of the incubator used in BMP tests, initial pH of the BMP test bottles were the inputs used in each Visial Minteq 3.0 calculation. The main menu of the Visual MINTEQ 3.0 is given in Figure 3.10.

Figure 3.10. Visual MINTEQ 3.0 main menu

4. RESULTS AND DISCUSSION

To provide an active methanogenic inoculum for BMP tests, a laboratory scale biogas reactor was operated for 158 days. The digestate of this reactor was used as inoculum in BMP tests to reveal the effect of TMs supplementation on anaerobic digestion of CM.

4.1. Laboratory-scale Continuously-fed Biogas Reactor

In the first part of this study, co-digestion of CM and spent poppy straw was investigated in 4 phases based on influent TKN concentrations for 158 days. The variations in methane yield, concentrations of VFAs, TKN, ammonium and free ammonia nitrogen concentrations were monitored by increasing the organic loading rate from 2.78 to 3.58 kgVS/m^3.d in a laboratory scale, daily fed and continuously stirred biogas reactor (Figure 4.1).

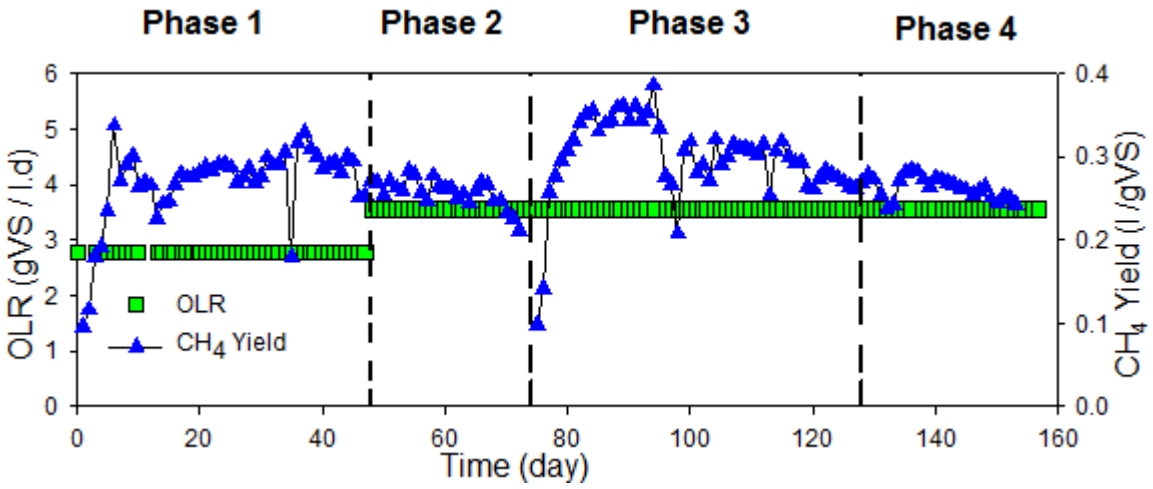

Figure 4.1 . Organic loading rates and methane yields

The lab-scale biogas reactor was formerly operated for about 6 months similarly by being fed with CM and spent poppy straw. Therefore, in this study was started up with a moderately high OLR and influent TKN concentration of 2.78 kgVS/m^3.d and 3960 mgN/l, respectively (Figures 4.1 and 4.2). The performance of the biogas reactor over 4 phases are listed in Table 4.1.

Table 4.1**.** The performance of laboratory scale biogas reactor

Parameters	**Units**	**Phase 1**	**Phase 2**	**Phase 3**	**Phase 4**
		Day 0-48	**Day 49-74**	**Day 76-128**	**Day 128-158**
TKN_{eff}	(mg/L)	5300	5500	5465	7300
NH_4^+-N_{eff}	(mg/L)	4180	4179	4160	5850
NH_4^+-N_{eff} /TKN_{eff}	%	80	75	76	80
FAN	(mg/L)	270	330	280	420
TS_{eff}	%	4.6	5.1	5.2	7.2
$TS_{removal}$	%	53.3	58.8	61.3	46.7
VS_{eff}	%	2.6	2.7	3.0	4.3
$VS_{removal}$	%	58.8	66.5	67.0	52.6
pH	-	7.85	7.89	7.78	7.83
Total Alkalinity	(mg/L)	25450	30450	29600	37350
CH_4 Yield	L/(g.VS)	0.27	0.272	0.297	0.283

In phase 1 between day 0 and 48, the biogas reactor was started up with a moderately high OLR, because it has been operated similarly for about 6 months and sufficient amount of methanogenic activity had been maintained. As it is shown in Figure 4.1, the average CH_4 yield was 0.27 m^3/kgVS in phase 1. Because the nitrogen content of the CM fed to the reactor was lower than the one formerly used, the TAN concentration decreased gradually from 4825 to 3869 mg/l (Figure 4.2).

In phase 2 between day 49 and 74, by increasing the OLR to 3.58 kgVS/m^3.d, the influent TKN elevated up to 5020 mg/l (Figure 4.1 and 4.2). Meanwhile, the TAN and FAN concentrations in the reactor increased and on day 74 reached to 4435 and 400 mg/l, respectively (Figure 4.2). As a result, the CH_4 yield decreased from 0.26 m3/kgVS on day 50 to 0.20 m^3/kgVS on day 74 (Figure 4.1). These results are in agreement with those found by Koster et al. (1988) . They indicated that as ammonia concentration increased to the range of 4051-5734 mg/L, the methanogenic population lost 56.5% of its activity. In the literature, there are also studies reporting the FAN inhibition threshold as 100-200 mg/l which is quite lower than the values experienced in this study (McCarty, 1964; Baere et al., 1984; Hashimoto, 1986). Lahav and Morgan (2004), have reported that high TAN concentration acts as buffer that led to the increase of pH and alkalinity. Likewise, in our reactor, parallel to the increasing TAN concentration, the pH and total alkalinity reached up to about 7.9 and 33000 mg $CaCO_3$ /L at the end of phase 2 (Figure 4.3).

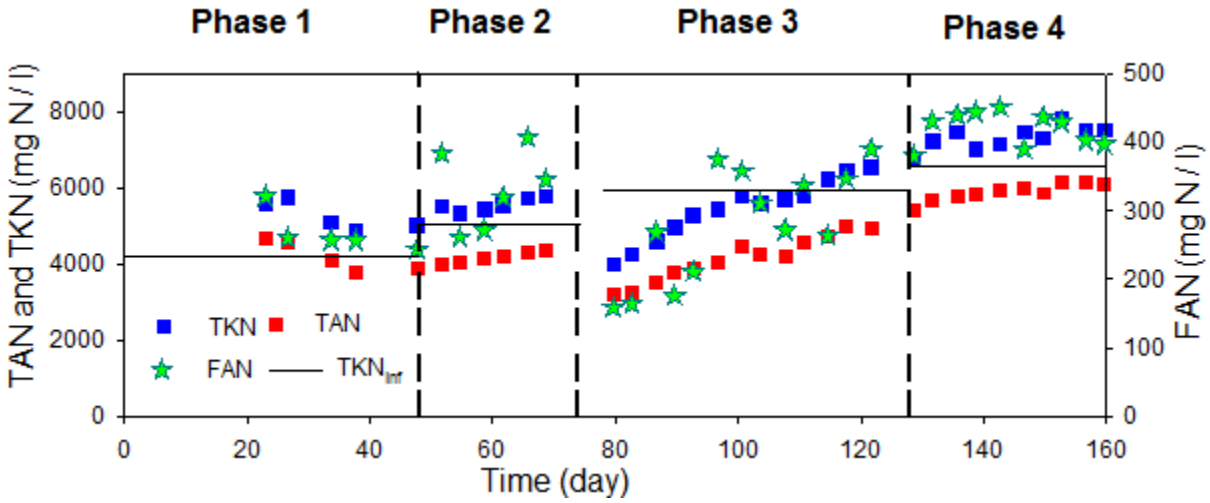

Figure 4.2. Influent and effluent TKN, total and free ammonia nitrogen concentrations

On day 74, one of the paddles of the mixer was broken and therefore reactor was stopped for 4 days to fix it. The sludge was drained and the reactor was emptied. It was noticed that the inorganic particles that comes along with CM was accumulated in the bottom of the reactor. These particles were generally small marble stones which are added to the feed of chickens as calcium source. The sediment in the bottom of the reactor was removed and tap water was added in place of it. After refilling the reactor with the same sludge, reactor was started up again.

In phase 3 between day 76 and 128, the influent TKN increased to 5960 mg/l on day 78 without changing the OLR (Figures 4.1 and 4.2). However, as one third of the reactor was filled with tap water at day 78, TAN concentration in the reactor did not exceed 4000 mg/l until day 100 (Figure 4.2). Therefore a higher CH_4 yield of 0.35±0.14 m^3/kgVS were achieved between days 85 and 99 (Figure 4.1).

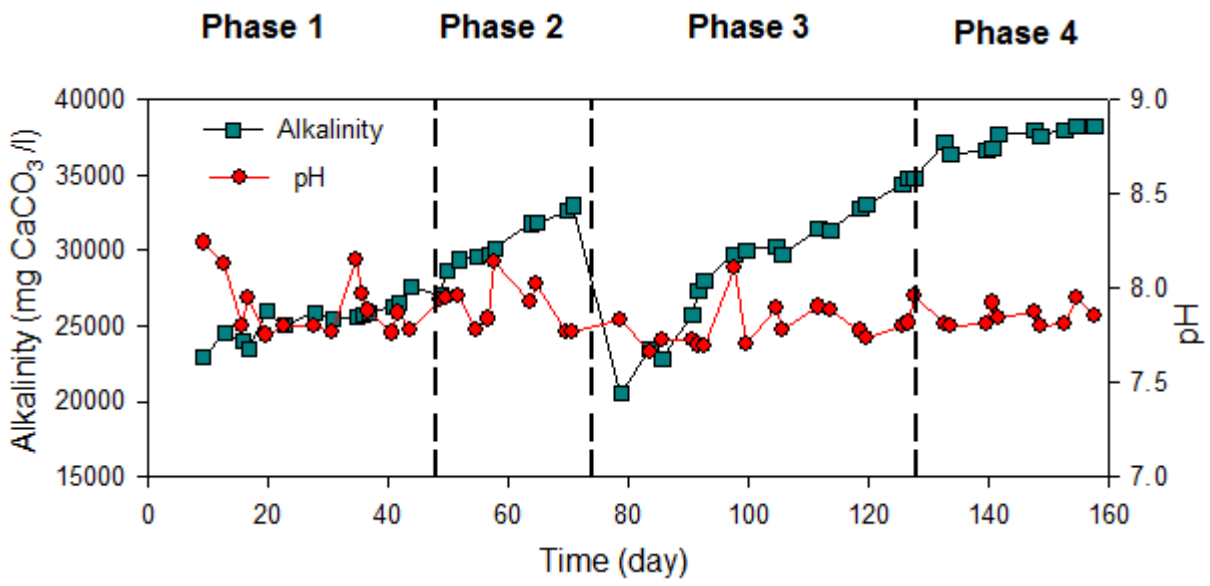

Figure 4.3. Effluent pH and total alkalinity concentrations

On day 99, because of an operation problem, the oil of the volumetric milligas counter leaked into the reactor. The next day, the oily 500 mL supernatant was drawn and replenished with tap water. Thereafter, the methane yield started to increase and reached back to 0.3±0.01 m^3/kgVS within few days (Figure 4.1). However, till the end of Phase 3, methane yield gradually decreased to 0.26 m^3/kgVS inversely proportional to increasing TAN concentration (Figure 4.1 and 4.2). At day 129, the TAN concentration in the reactor was 5400 mg/L (Figure 4.2).

In phase 4 between day 128 and 158, the influent TKN concentration and thus TAN and FAN concentrations in the reactor increased to 5960 mg/l, 6100 mg/l and 450 mg/l, respectively. As a result, the methane yield declined to 0.22 m^3/kgVS. The decrease in methane yield was correlated with the extremely high free ammonia concentration of about 450 mg/L and this situation resulted in the accumulation of acetic acid in the reactor. At day 158, the acetic acid concentration in the reactor was 3300 mg/L. Likewise the propionic acid concentration also increased in the same period and reached to 590 mg/l in the reactor (Figure 4.4). In another study, it was reported that as the TAN rose up to 5000 mg/l, the total VFA concentration in the digester exceeded 6000 mg/l and the biogas generation was only 25% of the value that experienced where there was no inhibition (Garcia and Angenent, 2009). Not as severely as reported by Garcia & Angenent 2009, but the methane yield decreased in our reactor as the VFAs accumulated. Although the free

ammonia nitrogen concentration slightly decreased to 400 mg/l at day 158 (Figure 4.2), it was still rather above the inhibitory thresholds reported formerly (Calli et al., 2005).

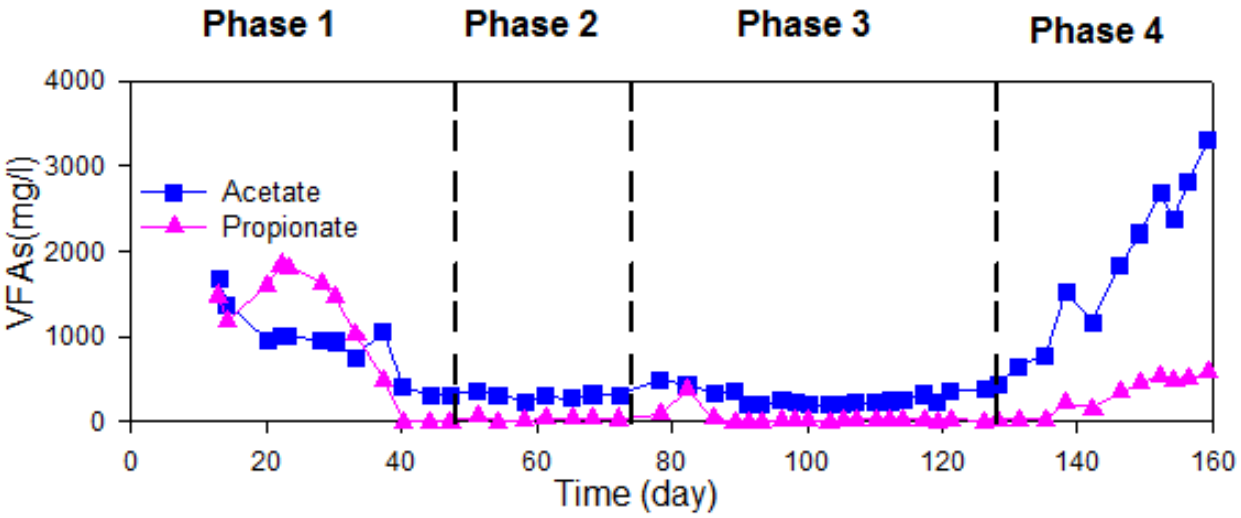

Figure 4.4. Variations of VFAs during the operation of anaerobic biogas reactor

4.2. Bio-methane Potential (BMP) Tests

In order to elucidate the effects of TMs supplementation on biogas production from CM, five BMP tests were performed using the digestate of the laboratory scale biogas reactor as inoculum.

Before the BMP tests, the TMs contents of the raw CM used as substrate and the digestate used as inoculum in the BMP tests were analyzed. As TMs, aluminum (Al), copper (Cu), boron (B), zinc (Zn), iron (Fe), cobalt (Co), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and tungsten (W) were selected. The results are listed in Table 4.2 and compared to the typical TM contents of CM given by Bolan et al. (2004).

The TMs such as Co, Se, Mo, W, Ni, and Fe are important for the growth of methanogens and are essential co-factors of enzymes, such as methyl-coenzyme, carbon monoxide dehydrogenase (CODH), and coenzyme M methyle-transferase directly involving in anaerobic degradation of biomass (Banks et al., 2012; Zhang et al., 2015). Besides, Zhang et al (2015) pointed out that the trace metals Fe, Co, and Ni improve the methane production efficiency and Se should be added to maintain the operational stability of the AD (Zhang et al., 2015). Because the bio-available form of trace metals is utilized by microorganisms, we preferred to analyze the soluble metals in the

manure and digestate samples. It is the reason of why the TM contents of the raw CM used in the BMP tests were all below the ranges reported by Bolan et al (2004). The Co, Mo and Se contents of the manure sample which were analyzed in the total form were more comparable to the typical values (Bolan et al., 2004).

Table 4.2**.** Amount of trace metals in raw chicken manure and digestate

	Digestate (mg/KgTS)		Chicken Manure (mg/KgTS)		
	This Study		This Study		Bolan et al., 2004
Trace Metal	Soluble	Total	Soluble	Total	Total
Cu	5.68	-	2.7-4.56	-	6.1-400
B	18.5	-	8.63-13	-	19-390
Zn	7.02	-	4.54-26.5	-	158-2300
Fe	24.6	-	10.3-39.9	-	-
Co	0.47	2.71	0.25-0.31	1.85-2.0	2-8
Mn	4.23	-	6.73-15.4	-	166-1800
Mo	0.39	3.44	0.11-0.19	2.17-2.52	5-7.69
Ni	1.99	-	1.2-2.5	-	2.46-15
Se	<0.05	0.77	<0.05	0.16-0.97	0.38-1.23
W	<0.03	1.26	<0.03	0.9-1.34	-

Banks et al (2012) indicated that the anaerobic microbial consortium exposed to high ammonia concentrations require more Co, Se, Mo, W, Ni, and Fe and if these metals are supplemented to the digester the biogas production is stimulated (Banks et al., 2012). Therefore, among the TMs analyzed, Co, Se, Mo, W, Ni and Fe were selected to be used in BMP tests in this study. Another reason for the selection of these six TMs is that their soluble concentrations in the CM were determined quite below the lower typical range reported by Bolan et al (Bolan et al., 2004).

The metal concentrations in the BMP tests with and without TMs supplementation and the recommended TMs concentrations for anaerobic digestion are given in Table 4.3. In the BMP tests without TMs supplementation, the metal concentrations were significantly lower than the

recommended concentrations reported by Schattaur et al (2011). By supplementing 1 mg/l Ni, 1 mg/l Co, 0.2 mg/l Mo, 0.2 mg/l Se, 0.2 mg/l W and 5 mg/l Fe, the TMs concentrations were brought into the recommended ranges (Schattaur et al., 2011).

Table 4.3**.** Total trace metals concentrations in with and without supplemented bottles and recommended concentrations for anaerobic process

	Concentration before TM supplementation	Concentration after TM supplementation	Recommended concentrations for anaerobic process Schattaur *et al* (2011)
Trace Metals	mg/l	mg/l	mg/l
Co	0.0161	1.0161	0.003-20
Se	0.0046	0.2046	0.008-0.79
W	0.0080	0.2080	0.018-18.3
Mo	0.0198	0.2198	0.005-50
Ni	0.0128	1.0128	0.005-30
Fe	0.1637	5.1637	0.28-200

4.2.1. BMP Test- 1: Low and high TAN concentrations and acetic acid as substrate

In BMP test-1, the effects of TMs supplementation on the biogas production at 675 mg/l and 5225 mg/l TAN concentrations were evaluated by using the inoculum taken from the CM and spent poppy straw fed laboratory scale biogas reactor and 1000 mgCOD/l acetic acid as substrate. The biogas profiles obtained are given in Figure 4.5.

As shown in Figure 4.5, the sets at 5225mg/L TAN produced biogas with a lower rate than the ones at 675 mg/l TAN from acetic acid. It is consistent with the results given in the literature and the findings obtained from the lab-scale biogas reactor fed with CM and spent poppy straw. Koster and Lettinga (1988) has reported that increasing TAN concentrations decreases the amount of biogas and its production rate. The effect of TMs supplementation on biogas production from acetic acid was also quite clear. TMs supplementation improved the biogas production by 8% and 13.5% at low and high TAN levels, respectively. It showed that TM supplementation is more effective on methane production from acetate at higher TAN concentrations. Likewise, Banks et al. (2012) has observed a beneficial effect of TM supplementation on the performances of food

waste digesters operated at 5000 mg/l TAN. The improvement we achieved was higher than the one achieved by Banks et al (2012). The higher solids content in their food waste digester may have minimized the effect of TMs by decreasing their bio-availability by adsorption (Banks et al., 2012). The control sets have produced negligible amounts of biogas (Figure 4.5).

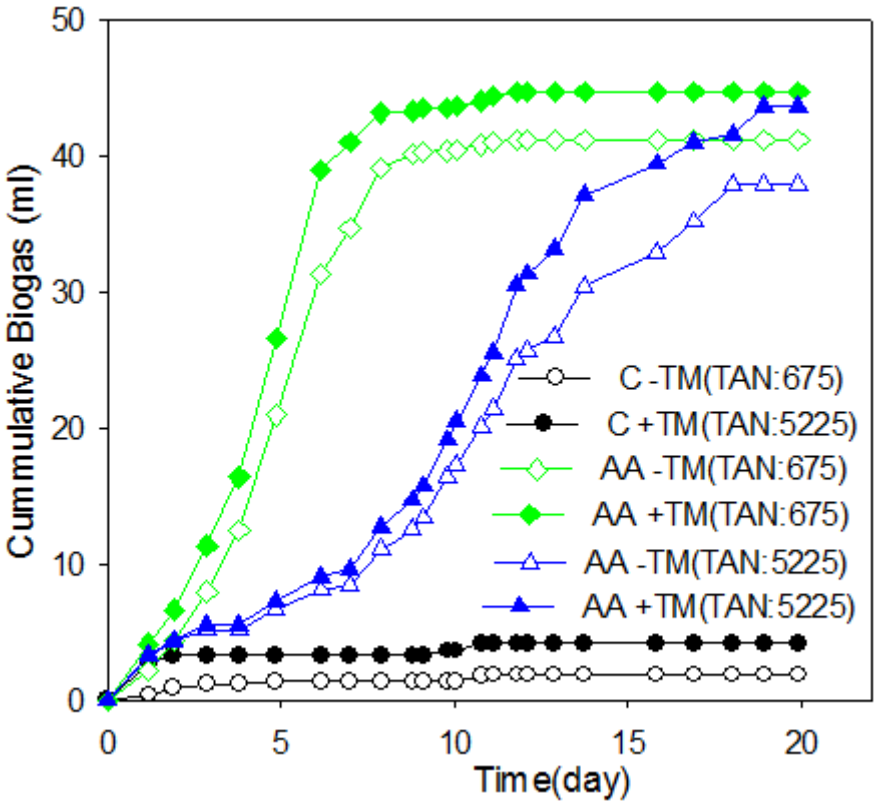

Figure 4.5. Biogas production profiles with and without TM supplementation in BMP Test-1

4.2.2. BMP Test-2: Moderate TAN concentrations and acetic-, propionic-, and butyric acid as substrate

In BMP test-2, the effects of TMs supplementation on the biogas production at 2950 mg/l TAN concentrations were evaluated by using the inoculum taken from the CM and spent poppy straw fed laboratory scale biogas reactor and 1000 mg COD/l acetic-, propionic- and butyric acid as substrate. The biogas profiles obtained are given in Figure 4.6.

The highest biogas production was obtained when acetic acid was used as substrate. Within the acetic acid and butyric acid fed sets, TM supplementation resulted in 12% and 18% more biogas production than the ones which were not supplemented with TMs, respectively. Interestingly, like the control sets, no biogas production was observed when propionic acid was used as substrate.

Absence of active propionate oxidizers in the digestate or their inhibition at 3000 mg/l TAN, as reported formerly, may be the reason for no biogas production (Calli et al., 2005). In addition, Banks et al (2012) has pointed out that the enzymes required for propionic acid oxidation may require higher amounts of trace metals such as Se, Mo, and W (Banks et al., 2012). A deficiency of these metals might be the reason of no biogas production from propionic acid. Therefore, it was concluded that either the propionate oxidizers were inhibited at 2950 mg/l TAN or the amounts of TMs supplemented were not enough for biogas production from propionate.

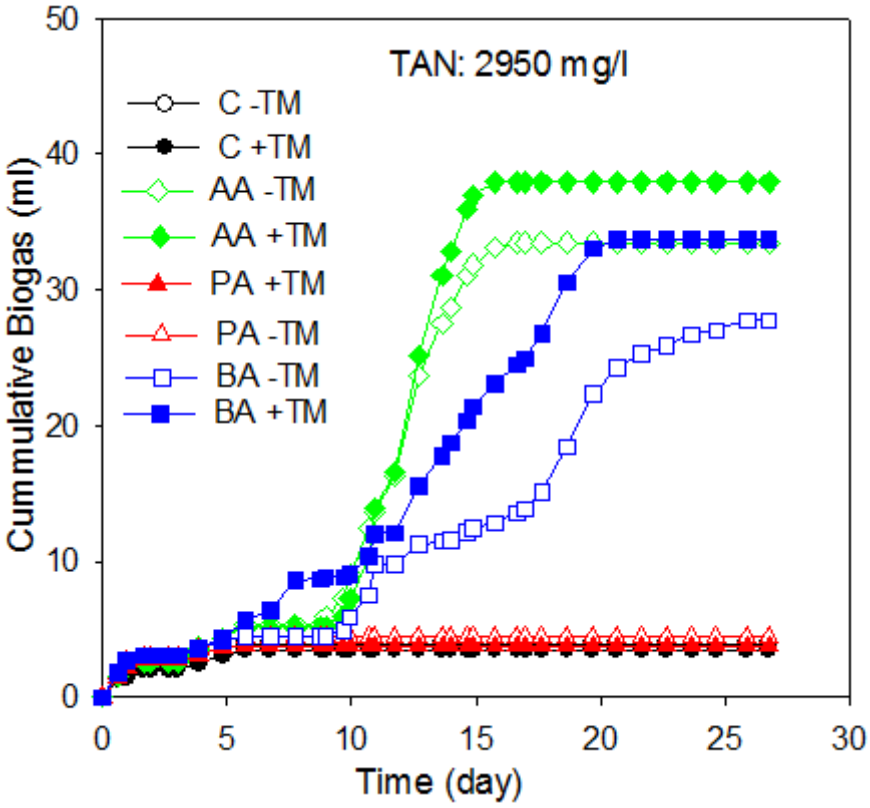

Figure 4.6. Biogas production profiles with and without TM supplementation in BMP Test-2

4.2.3. BMP Test-3: Low and moderate TAN concentrations and raw chicken manure as substrate

In BMP test-3, the effects of TMs supplementation on the biogas production at 420 mg/l and 3000 mg/l TAN concentrations were evaluated by using the inoculum taken from the CM and spent poppy straw fed laboratory scale biogas reactor and 0.2% TS raw CM as substrate. The biogas profiles obtained are given in Figure 4.7.

The results have shown that, the sets at 3000 mg/L TAN produced biogas with a slightly lower rate than the one at 420 mg/l TAN from raw chicken manure. TMs supplementation has improved the biogas production by 4% and 6% at 420 mg/l and 3000 mg/l TAN concentrations, respectively (Figure 4.7). However, the improvement on biogas production with TMs supplementation from CM was lower than the one from acetic acid and butyric-acid obtained in BMP test 1 and 2. Huang et al (2015) noted that, TMs added to anaerobic digesters may be precipitated by anions which exist in the feed stock or formed during digestion (Huang et al., 2015). Similarly, the anions such as carbonate, phosphate and sulfide existing in CM in significant amounts may have reduced the bio-availability of supplemented TMs by precipitation.

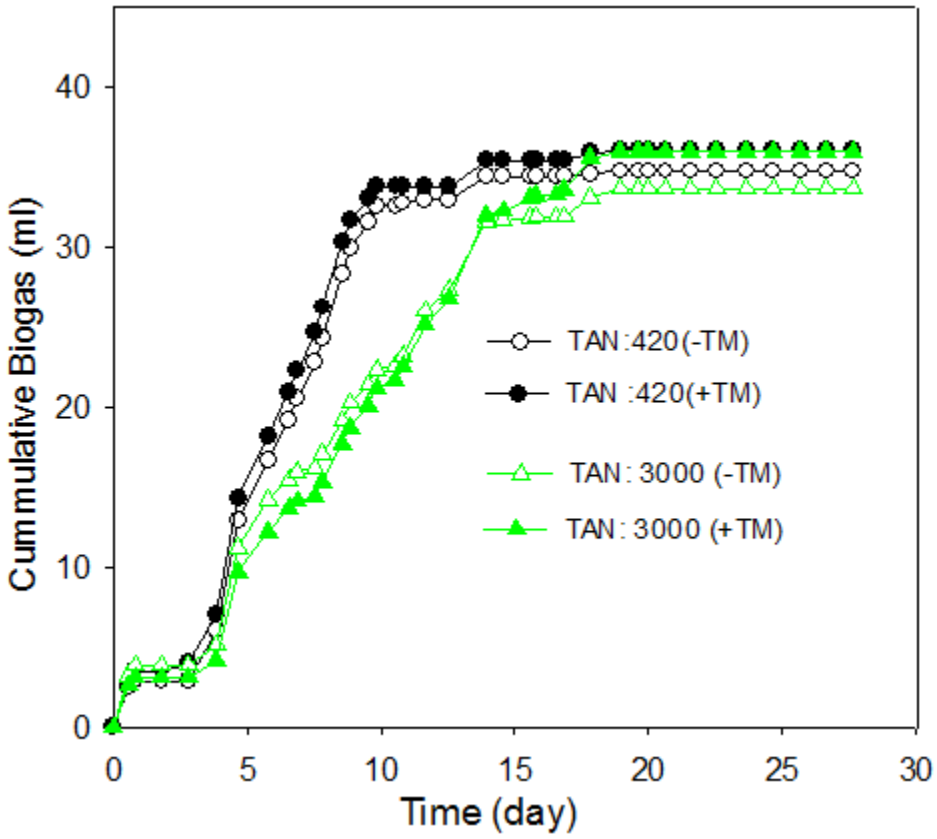

Figure 4.7. Biogas production profiles with and without TM supplementation in BMP Test-3

As a result of precipitation, TMs may become unavailable for anaerobic microorganisms. Although the raw manure used in BMP Test 3 was only 0.2% in TS, some amounts of phosphate, carbonate and sulphide might have added to the dilution solution used to fill BMP bottles. This

can be one of the reasons of why TM supplementation stimulated the biogas production in BMP test 3 less than did in former BMP tests.

4.2.4. BMP Test-4: Low, high and very high TAN concentrations and raw chicken manure as substrate

In BMP test-4, the effects of TMs supplementation on the biogas production at 465 mg/l, 4000 mg/l and 6000 mg/l TAN concentrations were evaluated by using the inoculum taken from the CM and spent poppy straw fed laboratory scale biogas reactor and 0.2% TS raw CM as substrate. The biogas profiles obtained are shown in Figure 4.8.

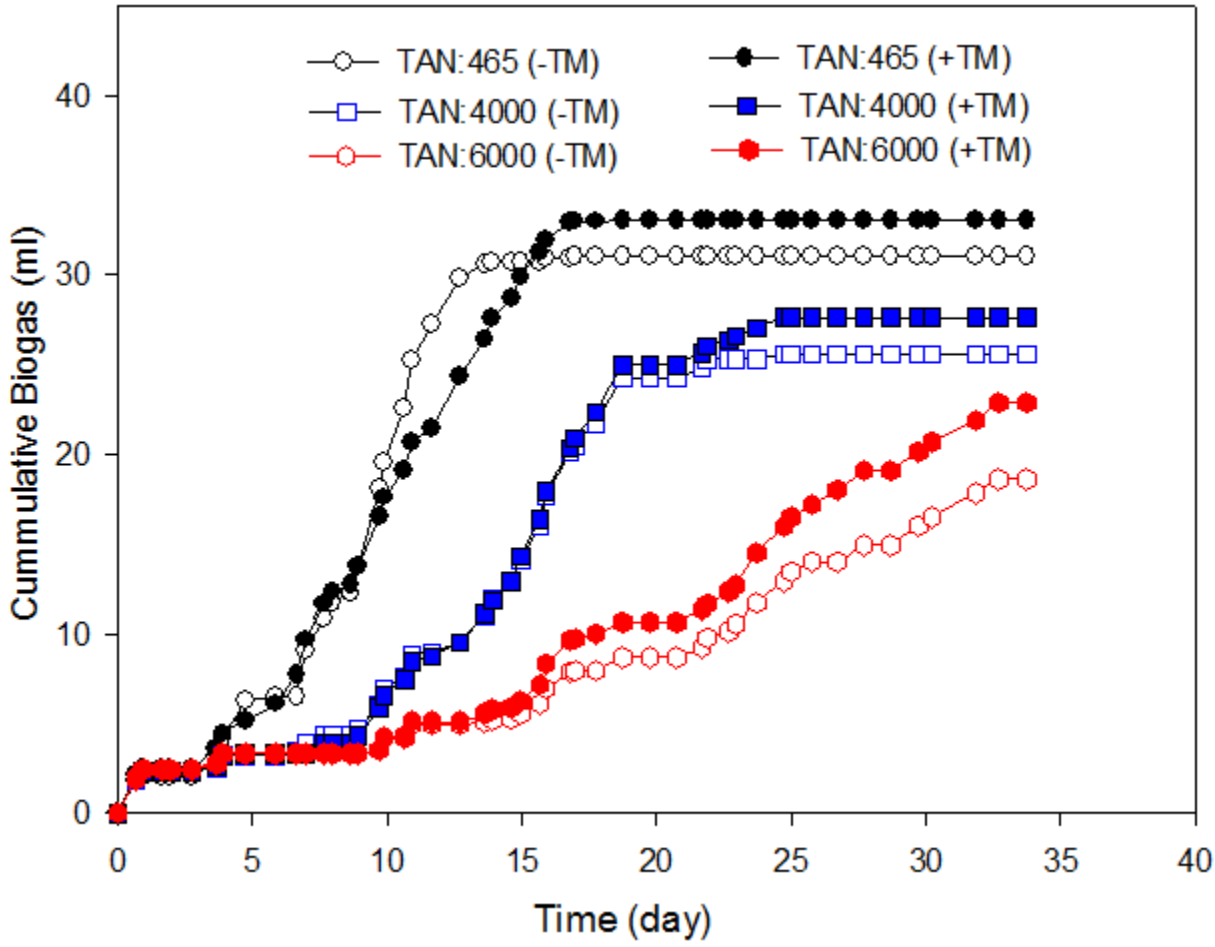

Figure 4.8. Biogas production profiles with and without TM supplementation in BMP Test-4

The results have shown that the biogas production rate decreased as the TAN concentration increased. While the highest production rate and biogas amount were achieved at 465 mg/l TAN, the lowest rate and amount were obtained at 6000 mg/L TAN from raw CM. These results were all consistent with the results found at BMP test-3. TMs supplementation has improved the biogas

production by 6% at 465 mg/l TAN, 7.5% at 4000 mg/l TAN and 18% at 6000 mg/l TAN (Figure 4.8). These findings were in agreement with the results of BMP Test-1 which were performed with acetic acid and showed that TMs supplementation becomes more effective on biogas production as the TAN concentration increases.

Although it has been indicated that TMs are involved in enzyme activity and many other metal-based enzyme activities of microorganisms, the reasons for the improvement in biogas production at higher TAN concentration, are still unknown.

4.2.5. Results of Chemical Speciation Calculations

In the BMP tests one of the important factors which should be taken into account is the bioavailability of TMs for anaerobic consortia. In the study carried out by Gustavsson (2011), it has been indicated that metal speciation will seriously affect the bioavailability of TMs. Besides he pointed out that, TM speciation is a function of total metal concentration and the amount of organic and inorganic ligands with which TMs may react (Gustavsson, 2011).

In BMP tests performed by using raw CM as substrate, it was presumed that the carbonate, phosphate, and to some extent sulfide anions in BMP bottles have elevated to considerably high amounts (Bolan et al., 2010). In addition to that, existence of these anions might have resulted in precipitation or complexation of TMs. Therefore, some portion of the TMs supplemented to the bottles might have not been bioavailable for anaerobic consortia.

In order to elucidate the bioavailability of TMs for BMP tests, fractionation of TMs in solid and liquid part were calculated by using Visual MINTEQ 3.0 software. The percentage distribution of the dissolved and precipitated TM species is listed in Table 4.4. To compare these model based findings with the experimental results and evaluate the bioavailability of TMs supplemented, TMs concentrations were determined in liquid and solid samples taken from the bottles after BMP test4.

The results of visual MINTEQ estimations and trace metal analyses both showed that Fe and Co supplemented to BMP test bottles were precipitated and they were not bioavailable for anaerobic microorganisms (Figure 4.9). Banks et al (2012) indicated that Co plays a main role in many enzymatic activities. For instance, Co is essential for enzyme methyltransferase which catalyzes the methyl group. Furthermore, carbon monoxide dehydrogenase which plays an important role in the acetogenic process also uses Co. Therefore, precipitation of Co might have been one of the

reasons for the lower improvement in biogas production from CM when compared with the biogas production from VFAs at similar conditions in BMP Test 3 and 2, respectively.

Table 4.4**.** Percentage distribution among dissolved and adsorbed species

Component	**% of Total Concentration**	**Species**
$HSeO_3^{-1}$	59.883	$HSeO_3^{-1}$
	40.117	SeO_3^{-2}
WO_4^{-2}	99.953	WO_4^{-2}
	0.044	$MgWO_4$(Aquino and Stuckey)
MoO_4^{-2}	99.941	MoO_4^{-2}
	0.015	$HMoO^{-4}$
	0.044	MgMoO4(Aquino and Stuckey)
Ni^{+2}	2.364	Ni^{+2}
	0.016	$NiOH^{+}$
	0.391	$Ni(NH_3)_5^{+2}$
	5.43	$Ni(NH_3)_4^{+2}$
	28.152	$Ni(NH_3)_3^{+2}$
	18.954	$NiNH_3^{+2}$
	40.901	$Ni(NH_3)_2^{+2}$
	0.013	$NiH_2PO_4^{+}$
	3.773	$NiHPO_4$(Aquino and Stuckey)
Co^{+2}	20.128	Co^{+2}
	0.102	$CoOH^{+}$
	33.907	$Co(NH3)^{+2}$
	5.018	$Co(NH3)_2^{+2}$
	0.384	$Co(NH_3)^{+2}$
	0.015	$Co(NH_3)_4^{+2}$
	40.443	$CoHPO_4$(Aquino and Stuckey)
Fe^{+3}	0.014	$FeOH^{+2}$
	78.012	$Fe(OH)^{2+}$
	3.313	$Fe(OH)_3$ (Aquino and Stuckey)
	18.448	$Fe(OH)_4^{-}$ (Aquino and Stuckey)
	0.213	$FeHPO_4^{+}$

TM analyses in liquid and solid samples have shown that in addition to Fe and Co, above 80% of the Se ions were also precipitated in test bottles and became non-bioavailable. Besides, according to TM analysis, it was found that about 50% of Ni and W and about 30% Mo were precipitated. The precipitation of Se, Mo, Ni and W were not estimated by Visual Minteq (Figure 4.9).

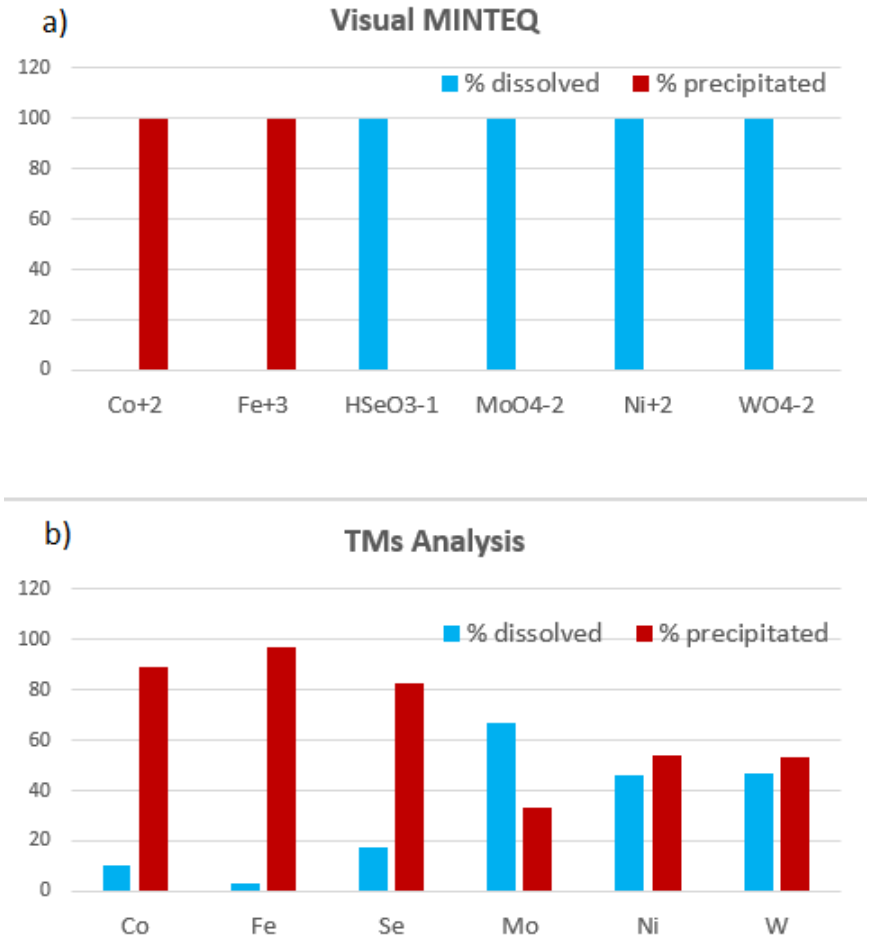

Figure 4.9. Proportion of TMs in solid and liquid phases in BMP Test-4 a) Estimated by Visual MINTEQ b) Calculated according to TMs analysis

4.2.6. BMP Test-5: Optimization of TMs concentrations at moderate TAN concentrations

In BMP test-5, to find the optimum TMs concentrations for biogas production form CM at 3000 mg/l TAN, TM concentrations were increased from 2 to 50-fold of the concentrations tested in the first four BMP tests. In another study, Brule et al (2013) mentioned that hydrogen sulfide (H_2S) production may be responsible for the precipitation of TMs in anaerobic digestion processes. To avoid the precipitation of TMs by sulfide, different forms of iron may be added to biogas reactors to react with and remove soluble sulfide (Brulé et al., 2013). Likewise, in BMP test-5, to avoid the precipitation of supplemented trace metals by sulfide, iron concentration in TM supplemented sets were increased to 100 mg/l which is 20-fold of the concentration used in the former BMP tests. The biogas profiles obtained are given in Figure 4.10.

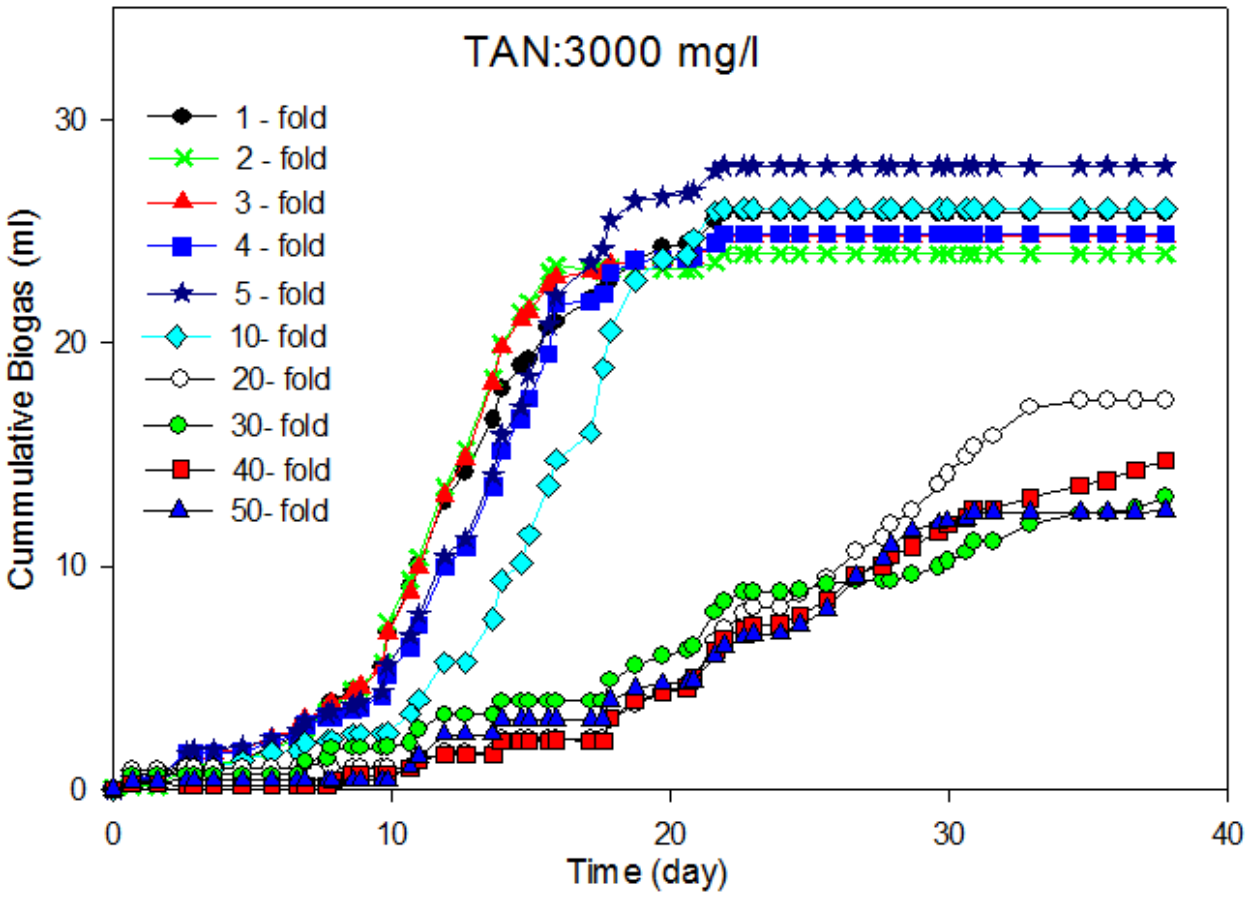

Figure 4.10. Biogas production profiles with and without TM supplementation in BMP Test-5

The results have shown that up to 10-fold increase in TMs concentration tested in former BMP tests did not detrimentally affected the amount of biogas produced and the production rate. Only in the set in which 10-fold increased TM concentrations were tested, the lag phase lasted few more days. Among the sets with one to 10-fold increased TM concentrations, the highest biogas production was achieved with 5-fold increase. The amount of biogas produced is directly related with the amount of substrate added to the test bottle. While working with CM which has a heterogeneous structure, it is very difficult to transfer the CM to the bottles equally. Therefore some variations in biogas production was observed between the bottles in BMP tests. Therefore while evaluating the effect of increasing amount of TMs on anaerobic digestion it is better to consider the production rate rather than the amount of biogas produced. When the concentrations of TMs were increased to 20-fold which is 20 mg/l Co, 4 mg/l Se, 4 mg/l W, 4 mg/l Mo and 20 mg/l Ni, both the amount of biogas produced and the produced rate reduced significantly indicating the inhibition of microorganisms by TMs.

5. CONCLUSION

By expansion of population, land limitation for crop products, and rising the demand for low-cholesterol and unexpansive meat production, large amount of domestic and industrial wastes are generated. Therefore, AD by decreasing the amount of wastes and production of renewable energy gained much popularity.

Even though a variety of organic wastes is assumed to provide the sufficient amount of micronutrients, recent findings in AD are in contrast to this assumption. Therefore, in the scope of this study, effect of TMs supplementation in anaerobic digestion of CM was investigated. Throughout the study continuously fed digestion experiments and batch bio-methane potential tests were performed.

In the continuous experiments, a lab-scale biogas reactor was operated for about 160 days and the aim of its operation was production of inoculum for bio-methane potential test.

The effects of the supplementation of TMs such as Ni, Co, W, Se, Mo, and Fe on biogas production from VFAs and raw CM were investigated in batch type BMP experiments.

The data obtained from the BMP test in which acetic acid was used as substrate (BMP Test-1), by addition of TMs, the improvement on biogas production at low, moderate, and high TAN concentrations was observed. In the BMP test at TAN concentration of 3000 mg/l, in which butyric acid was used as substrate like acetic acid (BMP Test-2) by the addition of TMs improvement in biogas production was observed. However, with compare to control, no biogas production was observed from propionic acid neither with nor without TMs supplementation. It was concluded that in the digestate taken from the lab-scale biogas reactor there were no propionate oxidizers or they were inhibited at 3000 mg/l TAN.

In other BMP tests (BMP Test-3 and 4) raw CM was used as substrate and the improvement effect of TMs supplementation on biogas production and production rate was investigated at a wide range of TAN concentration. After these two BMP tests it was hypothesized that the phosphate, carbonate, and sulfide ions existing in raw CM decrease the bioavailability of TMs for anaerobic microorganisms. TMs react with these anions and form metal complexes and lead to the precipitation of TMs. Therefore, in the last BMP test (BMP Test-5) performed at 3000 mg/l TAN,

TMs concentrations were increased from 2 to 50-fold of the concentrations tested in the first four BMP tests to find the optimal TMs concentrations. Among the sets with one to 10-fold increased TMs concentrations, the highest biogas production was achieved with 5-fold increase. When the TMs concentrations elevated to 20 mg/l Co, 4 mg/l Se, 4 mg/l W, 4 mg/l Mo and 20 mg/l Ni, both the amount of biogas produced and the production rate decreased significantly showing the inhibitory effect of TMs.

In this study, how the TMs supplementation improved the biogas production from anaerobic digestion of CM has been demonstrated. From the BMP-test results it was indicated that increasing TAN concentrations adversely affected the biogas yield and the production rate. To increase the biogas yield, TMs such as Co, Se, W, Mo, Ni and Fe were added to the BMP test bottles. Consequently, the results revealed that, TM supplemented sets produced more biogas from acetic acid and butyric-acid and raw CM at elevated total ammonia nitrogen concentration.

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LANGUAGES

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Persian	**excellent**	**excellent**	**very good**
English	**very good**	**very good**	**very good**
Turkish	**very good**	**Very good**	**fair**

PUBLICATIONS

Molaey R., Surmeli R.O., Bayrakdar A., Sahinkaya E., Çalli B. (Schmidt et al., 2014) Biogas production from chicken manure: co-digestion with poppy straw. 5th Int. Symposium on Energy from Biomass and Waste, 17–20 November, Venice, Italy. (Poster Presentation)

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