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Effects of nitrate addition on anaerobic digestion of high content organic substrate in semi-continuous fed reactors

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Summary:

Biogasification can recover a significant portion of energy potential from organic wastes. In addition to energy recovery, it also offers waste stabilization. However, there is a need for more efficient process for digestion of organic matter, resulting in higher methane yield. The main objective of the present experimental research is to test the enhancement of methane production in anaerobic digestion continuous flow stirred tank reactors (CSTR) through addition of limited amounts of nitrate. The hypothesis that such method can enhance hydrolysis has been previously suggested, and the mechanisms by which this process may occur is studied. Feedstock with high fat content, composed of primary sludge, fish oil, food waste and septic, pretreated by thermal hydrolysis was used as feed in several intermittently fed CSTR. Digestate from the full-scale plant at Lindum, Drammen, treating the same feed, was used as inoculum. Gas production rate was slightly higher during the period when nitrate was introduced to the anaerobic reactors. The effect of nitrate addition could be more evident in systems with higher concentration of proteins and carbohydrates in the feed since theory suggests that nitrate will not influence fat digestion much. Nitrate addition improved environmental conditions in the reactor as lower variations in VFA and total alkalinity was observed in reactors supplied with nitrate compared to solely anaerobic reactors. The initial amount of nitrate added to the system must be low enough to trigger nitrate reducing microorganisms, without leading to losses in methane production. In the present case study, the recommended initial amount is 0.1 % nitrate of equivalent feed COD. Furthermore, it is estimated that nitrate could be added up to an amount of 0.5 % COD, without effecting gas production negatively. When imposing a load increase, the response time for reactors supplied with nitrate was slower than for reactors without nitrate. However, the methane yield that stablished after ¼ hydraulic retention time with high load was higher for reactors with nitrate (0.175 % equivalent COD) than for those running solely anaerobically. Further studies on the long-term effects of nitrate on anaerobic digesters should focus on mechanisms and effects on the microbial populations. For industrial application, nitrate can be added directly to the anaerobic reactor and the process should be carefully monitored by using measurements of methane production and COD estimation for feedforward and feedback control.

Preface

This thesis report is carried out to understand how hydrolysis rate in anaerobic digestion (AD) can be increased by micro dosage of nitrate, resulting in higher methane production yields. The thesis is submitted to the Faculty of Technology, Natural Sciences and Maritime Sciences of the University of South-Eastern Norway, as a requirement of the degree of Master of Science in Energy and Environmental Technology.

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Porsgrunn, 15th May 2018

Kadja Borges Bless

Contents

Nomenclature

AD	Anaerobic digestion
$CaCO_3$	Calcium carbonate
$Ca(NO_3)_2$	Calcium nitrate
CO_2	Carbon dioxide
CO_3^{2-}	Carbonates
$C_5H_7O_2N$	Cell tissue
CH_3COO^-	Acetate
CH_3COOH	Acetic acid
CH_4	Methane
CSTR	Continuous Stirring Tank Reactor
DNRA	Dissimilatory nitrate reduction to ammonium
e^-	electron
GC	Gas chromatograph
HRT	Hydraulic retention time
H^+	Hydrogen ion
$H_2O_{(l)}$	Liquid water
$H_2S_{(g)}$	Hydrogen sulfide gas
$H_{2\,(g)}$	Hydrogen gas
HCO_3^-	Bicarbonate ions
ktoe	Thousand tons of oil equivalent
LCFA	Long chain fatty acids
$N_{2\,(g)}$	Nitrogen gas
NH_4^+	Ammonium
NH_4HCO_3	Ammonium bicarbonate
NLR	Nitrate loading rate
NO	Nitrogen oxide
N_2O	Dinitrogen oxide
NO_3^-	Nitrate ion
NO_2^-	Nitrite ion
NO_x	Nitrogen oxides

$O_{2\,(g)}$	Oxygen gas
OH^-	Hydroxides
OLR	Organic loading rate
ORP	Oxygen Reduction Potential
TH	Thermal hydrolysis
TS	Total solids
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
Q_in	Mass flow in
$_sCOD$	Soluble chemical oxygen demand
SDM	Simultaneous denitrification and methanogenesis
$_tCOD$	Total chemical oxygen demand

1 Introduction

Increased energy demand has aggravated environmental issues due to usage of nonrenewable energy sources. In order to defeat pollutant release and the high production of organic waste, governments have passed more restrict requirements for pollutants discharge and renewable sources. Moreover, global climate concerns have led to the creation of agreements working across countries borders. Anaerobic digestion plays here an important role as it can cover both the treatment of organic solid waste and the production of renewable energy as it can offer waste stabilization and resources recovery.

Anaerobic digestion (AD), is a biological process where organic material is converted into biogas in the absence of oxygen. The product of this process is composed of methane (CH_4), carbon dioxide (CO_2) and other minor products, such as hydrogen sulfide (H_2S), nitrogen gas (N_2), and siloxanes [1]. Methane produced by AD is highly combustible and can be utilized as vehicle fuel or in combined heat and gas power plants, being a substitute to fossil fuels. In addition to producing green energy, AD can meet the basic principle of waste management which refers to the energy recovery under the European Directive 99/31/EC. Energy produced by anaerobic digestion has reached a peak of 5249 ktoe, in 2017. This represents an increase in production in the order of 17 % from 2005 [2]. Energy produced by anaerobic digestion is expected to increase, nevertheless it is of great importance to develop new technology and improve todays methods to achieve better production.

The main difficulty in applying anaerobic digestion is to improve the carbon content that is available for biogas production, or readily biodegradable portion of COD. Such a parameter can be estimated by soluble Chemical Oxygen Demand (sCOD). Enzymes released during hydrolysis breaks complex solids into sCOD hence, hydrolysis must be increased to stimulate biogas production. The result is consequently better process efficiency due to higher biogas yield.

Addition of limited amounts of oxygen (microaeration) and nitrate has been investigated as a method to stimulate hydrolysis rate in anaerobic digesters [3].

Application of microaeration stimulates the growth of facultative microorganisms responsible for producing extracellular enzymes [3]. Higher population density of enzyme producer bacteria in microaerated systems have been reported [4]. These bacteria can lead to increase in hydrolysis rate of up to 50-60% compared to solely anaerobic systems. An analogous behavior is expected when adding nitrate to the digester, being the last one an easier method of increasing hydrolysis rates [5, 6].

Chapter named Process description explain the principals of anaerobic digestion and introduces a literature survey on the effects of addition of different electron acceptors, such as oxygen or nitrate, in anaerobic digestion systems. An experimental description carried out in semi-continuous feed reactors is presented in chapter 3. The subsequent chapter introduces the results obtained experimentally in the present survey. Finally, chapter 5 and 6 bring a discussion and a conclusion on the topic.

The main objective of the present experimental research is to test the enhancement of methane production in anaerobic digestion continuous flow stirred tank reactors (CSTR) through addition of limited amounts of nitrate. The hypothesis that such method can enhance hydrolysis have been previously suggested, and the mechanisms by which it may occur are investigated. Furthermore, applicability of the method to an existing biogas plant is discussed.

2 Process description

In a biological process a variety of microorganisms synthetizes organic carbon to biomass in an anabolic process, and oxidize organic compounds to produce energy in a catabolic process. These oxidation-reduction reactions involve the transfer of electrons from an electron donor, which is oxidized, to an electron acceptor, which is reduced. These biological processes can be classified according to the electron acceptor as:

- Aerobic: Organic substrate is oxidized, and O_2 is reduced to form CO_2 and water;
- Anoxic: Bacteria use nitrate or nitrite as the final electron acceptor, for example in the oxidation of sulphide to sulphate [7];
- Anaerobic: Organic compounds or CO_2 are used as electron acceptors for degradation of organic matter.

Anaerobic digestion is a process used as a treatment to oxidize particular biodegradable constituents into CH_4 and reduce the concentration of organic compounds, leading to stabilization of organic matter.

This chapter describes principles of anaerobic digestion and how microorganisms operating with different electron acceptors can interact to increase biogas yield in the process.

2.1 Anaerobic digestion in continuous stirring tank reactors (CSTR)

Anaerobic digestion (AD) of organic substances is a biological process where organic carbon is converted to its most oxidized state (CO_2) and its most reduced state (CH_4) [8]. Minor reactions can also take place in an anaerobic digestion, such as the conversion of nitrogen to ammonia; and sulphate to hydrogen sulphide.

In an anaerobic process particulate matter is degraded to soluble COD, and further fractionated into volatile and short chain fatty acids (VFAs), which is converted to biogas. Four main steps are involved in the overall anaerobic digestion:

1. Hydrolysis – particulate matter undergo decomposition. This step is carried out utilizing extracellular enzymes produced by a variety of microorganisms. Hydrolysis conversion rate is considered the rate-limiting factor in methane production;
2. Fermentation – also called acidogenesis. Substrates serve here as both electron acceptors and donors. Further degradation of soluble organic materials to VFAs (such as acetate), H_2, CO_2, propionate and butyrate;
3. Acetogenesis – propionate and butyrate are converted into acetate, H_2 and CO_2;
4. Methanogenesis – acetate, H_2 and CO_2 are converted into CH_4 and CO_2. Two groups of methanogenic bacteria are involved in methane production. *Acetotrophic methanogens* split acetate into CH_4 and CO_2, as shown in equation 2.1.

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad 2.1$$

While *hydrogenotrophic methanogens*, use H_2 as an electron donor and CO_2 as the electron acceptor to produce methane, as shown in equation 2.2.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad 2.2$$

Acetic acid is responsible for about 70% of the methane produced. A stable process will normally produce about 50-70% CH_4 [9].

The composition of biogas depends on both the feedstock and pH of the culture media. Digestion of carbohydrates results, in theory, in 50% CH_4 and 50% CO_2, while pure protein and fat gives, 70% CH_4 and 30% CO_2 [10].

At anaerobic conditions the biochemical diversity of microbial communities is huge. Many electron acceptors can be used by different anaerobic organisms. From a thermodynamic point of view, the highest the redox potential, the larger the amount of energy available to the organism performing the reaction [11], and more likely it is that a substrate will be degraded under those conditions. However, the amount of energy available to the microorganisms will depend also on the nature and concentration of all reaction products [12]. Oxygen is the most favorable electron acceptor, while CO_2 reduction to CH_4 is the least favorable. Thermodynamic considerations for nitrate reduction are given in section 2.3. Table 2.1 gives methanogenesis reactions for some carbon sources found in anaerobic digesters [13].

Table 2.1-Methanogens reactions for different electron donors and their Gibbs free energy

Electron donor	**Reaction**	**ΔG^o (kJ/mole e donor)**
Acetate	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31
Propionate	$4CH_3CH_2COO^- + 6H_2O \rightarrow 7CH_4 + 4HCO_3^- + CO_2$	-57
Glucose	$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2$	-428
Hydrogen	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-131
Ethanol	$2CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2CH_3COO^- + 2H^+$	-111

Anaerobic processes are sensitive to temperature, pH, nutrient limitations, and inhibitory substances. These parameters influence the microbial population that will prevail in the system.

Temperature – Temperature is an important parameter influencing the composition and the amount of the gas produced. Methanogenesis rate increases with increased temperature. Conversely, at low temperature, more CO_2 will be dissolved, resulting in a higher percentage of CH_4 in the gas.

pH –The pH value in the reactor depends on the VFAs concentration and the alkalinity of the system. VFAs generated during acidogenesis tend to lower the pH. Under normal conditions pH is adjusted by the presence of buffer systems, often HCO_3^- ions and VFA consumption during acetogenesis and methanogenesis. A value near neutral is preferred, and at a pH below 6.8 the methanogenic activity could be inhibited [5]. If the pH drops to below 6.3, the AD process may eventually fail.

The pH is particularly sensitive under transient loading conditions where VFA production rate can exceed utilization rate, causing a pH drop. If alkalinity available to buffer the organic acid concentration do not increase, methanogenic VFA utilization decrease. If this reduced utilization continues, butyric acid also accumulates and the reactor operation is greatly inhibited [5].

pH is also influenced by the concentration of CO_2 in the liquid. Due to partial pressure of gas in the digester, carbon dioxide solubilizes and form carbonic acid, which is a greater consumer of alkalinity [5].

Alkalinity – A high total alkalinity is needed to assure that pH does not fall below neutrality. Alkalinity is produced by the breakdown of proteins, peptides or amino acids to produce NH_3. The gaseous ammonia formed reacts with carbon dioxide to form the ammonium ion and bicarbonate according to reaction 2.3.

$$NH_3 + H_2O + CO_2 \rightarrow NH_4^+ + HCO_3^- \qquad 2.3$$

Alkalinity is then formed as $NH_4(HCO_3)$ [5]

Toxicants – Biological processes are sensitive to inhibitory substances contained in the feedstock and/or produced through microbial activity. However, the toxicity can be reversible in many cases and methanogens can be acclimatized to tolerate certain amounts of toxic substances [14].

2.2 Microaeration

Methanogens Archea are obligate anaerobes; hence oxygen must be excluded in anaerobic digestion processes. However, the input of low levels of oxygen in an anaerobic biochemical process, called microaeration, was previously found to improve anaerobic digestion by enhanced digestion efficiency through increased solubilization (hydrolysis) of particulate matter [3].

Facultative anaerobes are of great importance when applying micro aeration as their growth rate improves extracellular activities, increasing the production of enzymes which mainly works during hydrolysis [6]. Previous studies shows that higher hydrolytic production in micro aerated systems can increase hydrolysis to up to 50-60% [15]. In addition, better acidification; during the methanogenic phase can be obtained, leading to higher biogas production. Nguyen et al. [16], demonstrated that microaeration during pre-stage may have a positive effect in methanogenesis, since an active methane phase was reached early compared to reactors without microaeration. Fu et al. [4] showed that, for cellulosic substrate, microaeration can accelerate the hydrolysis process by destroying the substrate directly or improving the activity of extracellular enzymes increasing methane yield. The same study obtained higher VS removal efficiency under microaeration compared to pure anaerobic systems. Furthermore, analysis of bacterial community in the end of AD under microaerated conditions has shown a relative abundance in bacteria associated with hydrolysis, compared to absolute anaerobic conditions [4].

Another advantage of microaeration, is the heat released by the exothermic microbial oxidation process. Being the heat produced approximately 20,000 kJ per kg VS. The temperature raises until a balance occurs and the process becomes oxygen mass-transfer-limited [5].

Oxygen tolerance of AD systems has also been reported as a combination of several mechanisms, such as aggregation and acclimatization. In aggregation, facultative organisms form a diffusion barrier, stopping oxygen and protecting strict anaerobes inside the aggregation [3]. Consumption of oxygen by facultative bacteria in granular sludge creates microenvironments where the methanogenic bacteria is protected, Kato et al. [17] demonstrated high oxygen tolerance of methanogens in such systems. Methanogens survival in microaerated systems in the absence of aggregates has also been reported [3], this might be due to the capacity of acclimatization of methanogenic microorganisms to microaerated systems [4]. The

acclimatization of an anaerobic culture can be considered as a natural selection process resulting in a dominant microbial population that can be quite different from that in the initial inoculum. In this manner, microorganisms present in the anaerobic digestion of waste/wastewater have the ability to grow in either presence or absence of molecular oxygen, shifting from fermentative to aerobic respiratory metabolism, depending on the presence of molecular oxygen [9]. Aerotolerant anaerobes have, in the other hand, strictly fermentative metabolism but are relatively resistant to the presence of molecular oxygen [5].

However, applying micro aeration to biogas plants is difficult from a practical point of view, due to low solubility of oxygen (difficulty in defining oxygen transfer rate in such systems). This aspect also introduces the risk of producing explosive gas mixtures, as the air added to the system tends to bubble. A blower is necessary to introduce oxygen into the reactor; hence, more energy is required to maintain the systems at optimal conditions. The method requires energy consumption contrasting the purpose of production of alternative energy, and evaluation of rather the energy required is compensated by the increasing yield in biogas production is necessary.

Nitrate addition has been studied as an alternative to microaeration, as facultative bacteria can exchange between the use of oxygen and nitrate/nitrite as an electron acceptor, when oxygen is not available [5].

2.3 Addition of limited amounts of nitrate to AD process

Nitrate is an electron acceptor and, when added in sub inhibitory levels, stimulate the AD process without disturbing it. Nitrate in an anaerobic system is either converted to organic nitrogen, reduced to ammonia by nitrate reducing bacteria (ammonification) or denitrified to nitrogen gas [13]. Two modes of nitrate removal can occur in biological processes.

1) Assimilatory: Involves the reduction of nitrate to NH_4^+ for use in cell synthesis;
2) Dissimilatory: For this process two different pathways can be described: denitrification, where metalloenzymes sequentially reduce nitrate to $N_{2(g)}$ by a four step process; and dissimilatory nitrate reduction to ammonium (DNRA) [18]. The overall stoichiometry for dissimilatory nitrate reduction is shown in reaction 2.4 [5].

$$organic\ matter + NO_3^- \rightarrow CO_2 + N_2 + NH_3 + H_2O + OH^- \qquad 2.4$$

The subsequent sections explain the principles and parameters influencing nitrate reduction in an AD system.

2.3.1 Nitrogen pathway

Figure 2.1 shows a combined scheme of anaerobic digestion and nitrogen pathway adapted from M. Andalib et al. [13]. On the right side, a diagram including the steps involved in anaerobic digestion, and their products, as explained in section 2.1. On the left, the nitrogen pathway, and the intermediate products of each reaction. Figure 2.1 also illustrates that the carbon oxidized during nitrate reduction can be either the reduced organics from acidogenesis, or acetate from acetogenesis.

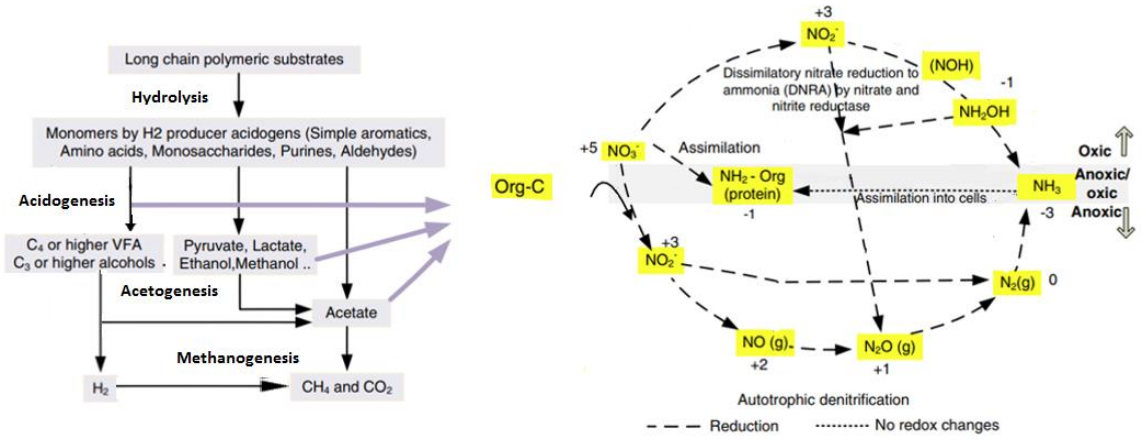

Figure 2.1 – Four phases of biogas production and the products of each step; and the nitrogen pathway shown with its intermediates and final products. The carbon utilized in nitrate reduction is the product of acidogenesis and acetogenesis

Important parameters such as the nature of the substrate, organic and nitrogen loading rates, COD/NO_3-N ratio, Gibbs free energy, oxidation reduction potential (ORP) and pH have significant effects on the reduction of nitrate in AD systems.

Bacteria, *Archea* and *Eukarya* are microorganisms involved in both denitrification and DNRA [19]. Facultative and obligate anaerobic bacteria, as well as fermentative anaerobes, all responsible for DNRA, are abundant in anaerobic digestion. However, true denitrifying organisms, present in small amounts, are capable of increasing in number at a constant supply of nitrate; leading to increased denitrifying activity [20].

The nature of the substrate impacts the nitrogen pathways [21]. VFAs are favorable for denitrification, and fermentative substrate is preferred for DNRA [13, 20, 22]. In this manner; DNRA was reported to be the main pathway of nitrate reduction in a pure acidogenic system (with glucose and glycerol as substrate) tested with high COD/NO_3-N ratios with 50 % nitrate reduction to ammonia; while 100 % nitrate denitrified to N_2 when acetate and lactate were the carbon sources [23].

Methanogenesis is known to proceed at redox potential (ORP) below -330 mV, the presence of nitrate will therefore inhibit methanogenesis, as denitrification occurs at a ORP of -100 mV, increasing the redox potential of the system [13]. However, inhibitory effect of nitrogen oxides may not be attributed completely to an elevation of the redox potential of a mixed culture [24]. Tai et al. [25] reported that methane production procced at an ORP range from -150 to -200 mV, indicating that ORP is possibly not the factor affecting methanogenesis in a mixed culture system involving both methanogens and nitrate reducing microorganisms. In the same manner, Fetzer et al. [26] observed no significant variation in methane production when adjusting the redox potential of an anoxic medium to values up to +100 mV. Furthermore, Houng et al. [27] studied the performance of a CSTR with immobilized denitrifiers and methanogens, and concludes that controlling ORP at values between -300 and -350 mV is recommended for complete denitrification and methane production. The aforementioned reports show that suppression of methanogenesis was not related to changes in the redox potential and that both denitrification and methanogenesis can operate in a wide ORL range. On the other hand, DNRA is likely to proceed in AD systems, as the conversion of nitrate to ammonium increase at low ORP [28].

The carbon to nitrogen ratio (C/N) have been reported as another important parameter in the operation of such systems. Tiedje et al. [18] observed that, under a high C/N ratio, the greatest need in metabolism is for maximum electron acceptor capacity, hence DNRA is the major pathway in carbon rich and low nitrate environments. On the other hand, at a low ratio, the

greatest advantage is to the organism that gains the most energy per nitrate, this is denitrification [18]. In the same manner, Akunna et al. [29] observed successful competition of ammonia formers over denitrifiers at high COD/NO_3-N ratios (53-106); denitrification occurred at COD/NO_3-N lower than 8.86, whereas simultaneous methanogenesis and denitrification occurred at a rate between 8.86 and 53. Rustrian et al. [30] studied denitrification and acidogenic potentials in CSTR fed with glucose and nitrate, and reported that VFAs were produced without denitrification at COD/NO_3-N ratios higher than 220, being DNRA dominant in this range; denitrification and VFA occurred at COD/ NO_3-N ratios between 88.5 and 220; and denitrification and smallest VFA rates at COD/ NO_3-N ratios lower than 44.3. At COD/ NO_3-N ratios higher than 130, nitrate assimilation of nitrate to biomass was also observed.

Most denitrifiers are facultative bacteria utilizing either oxygen respiration or denitrification as energy source, if none of these metabolisms are possible, fermentative bacteria will reduce the nitrate for dissimilatory electron dissipation, being the last considered non-energy yielding; hence, without competitive value on a thermodynamic point of view [11]. Table 2.2 [13] shows some denitrification and DNRA reaction and their respectively Gibbs free energy. Here, the reactions with lower Gibbs free energy are more likely to occur, due to higher energy yield.

Electron donor	**Reactions**	**ΔG° (kJ/mole)**
	Denitrification	
Acetate	$5CH_3COO^- + 8NO_3^- + 8H^+ \rightarrow 9H_2O + 5CO_{2+}5HCO_3^- + 4N_{2(g)}$	-797
Propionate	$5CH_3CH_2COO^- + 14NO_3^- + 14H^+ \rightarrow 17H_2O + 10CO_{2+}5HCO_3^- + 7N_{2(g)}$	-1398
Glucose	$5C_6H_{12}O_6 + 24NO_3^- + 24H^+ \rightarrow 42H_2O + 30CO_2 + N_{2(g)}$	-2657
Hydrogen	$5H_2 + 2NO_3^- + 2H^+ \rightarrow 6H_2O + 30CO_2 + 2N_{2(g)}$	-3144
	DNRA	
Acetate	$CH_3COO^- + NO_3^- + 2H^+ \rightarrow +CO_{2+}HCO_3^- + NH_4^+$	-500
Propionate	$8CH_3CH_2COO^- + 14NO_3^- + 28H^+ \rightarrow 2H_2O + 16CO_{2+}8HCO_3^- + 14NH_4^+$	-878
Glucose	$C_6H_{12}O_6 + 3NO_3^- + 6H^+ \rightarrow 3NH_4^+ + 3H_2O + 6CO_2$	-1767
Hydrogen	$4H_2 + 2NO_3^- + 4H^+ \rightarrow 6H_2O + 2NH_4^+$	-150

Table 2.2 – Dissimilatory nitrate reduction reactions (denitrification and DNRA) and their Gibbs free energy under different electron donors

In the heterotrophic nitrate reduction reaction of acetate and propionate, one equivalent of alkalinity is produced per equivalent NO_3 reduced, which equates to 3.57 g of alkalinity (as $CaCO_3$) produced per gram of nitrate nitrogen reduced [5, 31]. The increased system pH, due

to the use of electron donor in the form of the VS feed and nitrate as electron acceptor, is demonstrated by Sheng et al. [6].

Barber and Stucky [32] reported improved hydrogenotrophic methanogenesis in an anaerobic baffled reactor due to an increase in pH, caused by the release of hydroxyl ions (equation 2.4) during denitrification, and indirectly by the consumption of acid intermediates, improving environmental conditions. The same study also reports the importance of DNRA, as the reaction has a very high hydrogen demand which reduces hydrogen to levels low enough to allow syntrophic reactions to proceed, resulting in a build-up of methane precursors and consequently methanogenesis with superior reactor performance. Furthermore, the ammonium ion released during DNRA was reported to possibly improve methane production due to an enhanced availability of reduced nitrogen as a nutrient to methanogens. The amount, and composition of VFAs, was also influenced by nitrate addition, being propionate and butyrate levels reduced, and acetate levels increased.

Nitrogen assimilatory uptakes by biomass in the SDM system at high OLRs and low NLRs can be significant [13]. Rustrian et al. [30] reported 50 % assimilation of nitrogen in biomass at high COD/NO_3 ratio in CSTR reactors fed with synthetic wastewater and glucose. Hence, higher bacterial growth is an important effect of nitrate addition in anaerobic digester [32]. Typical synthesis yield is 0.30 g VSS/g COD for degradation of organic matter to biomass by means of nitrate as the electron acceptor [5]. DNRA is often associated to assimilatory process as the ammonia produced will further be used by cell to incorporate nitrogen into biomolecules [33].

2.3.2 Interactions of methanogens and nitrate reducers (inhibition and tolerance)

Batstone et al. [34] described in anaerobic digestion model no. 1 (ADM 1) that the coexistence of methanogens and nitrate reducers may cause competition for the same substrate; suppression of methanogens by nitrogen oxides; increase in denitrifying activity, leading to decrease in methane content in biogas; and finally, channeling of electrons away from methanogenesis, as complete denitrification requires five electron equivalents per mole of nitrate. However, it has been found that the mechanisms postulated by Batstone are highly dependent on parameters such as the amount of carbon available in the system and the type of the carbon source.

Several studies have shown that when the amount of COD provided is higher than the COD requirements for both nitrate reduction and methanogenesis, there will be no competition for the electron donor between the communities [13, 24]. In addition, competition will depend on the relative substrate utilization rates of each microorganism, and the half-velocity constant for substrate utilization. For instance, it has being reported that propionic acid the most preferred VFA among nitrate reducers while the utilization rate of glucose is approximately ten times lower for denitrifiers than for fermentative microorganisms [33].

Results in studies carried out by Clarens et al. [35] strongly suggests that methane production was significantly inhibited by intermediate products of nitrate denitrification, rather than due to competition. Similarly, Roy et al [36] tested the effect of adding different electron donors on the suppression of methane production in rice soil, concluding that the main mechanism involved in the depletion of methane production by nitrate is inhibition of methanogenesis by denitrification intermediates rather than competition for substrate.

Denitrification proceeds in a stepwise manner in which nitrate is reduced to nitrite (NO_2^-), nitrogen oxide (NO), dinitrogen oxide (N_2O) and nitrogen gas (N_2), consuming organic carbon and producing energy, however this process does not always proceed to complete reduction to

$N_{2(g)}$, and the production of gaseous intermediates will depend on the community composition and environmental conditions [37]. Low denitrifying efficiencies and the accumulation of such denitrifying intermediates was observed when insufficient carbon was supplied to a system with a mixed culture of nitrate reducers and methanogens[38].

Several studies demonstrate the inhibitory effects of denitrification intermediates on methanogens concluding that NO is the strongest inhibitor, with irreversible effect in resumption of methanogenesis; while NO_2^- and N_2O inhibitory effect was shown to be partially or totally reversible [39, 40]. Accumulation of intermediate nitrates was observed when insufficient carbon was provided to an anaerobic reactor supplied with nitrate, which further inhibited the methanogenic activity [21]. Therefore, methane production will be absent before denitrification is complete. This inhibition disappears after the complete reduction of nitrate in the medium. Hence, methanogenesis will begin only once denitrification is complete [38, 41, 42].

Similar to microaerated systems, habitat segregation, or aggregation, seems to be an important mechanism for survival of methanogens when nitrate is present in the system; here facultative microorganisms with less biofilm density and faster growth rate tend to grow along the outer surface of aggregates, protecting methanogens, with slower growth rate, which accumulates in the interior of the media [13, 43].

2.3.3 Hydrolysis in AD system under the influence of nitrate addition

Hydrolysis is considered to be the main mechanism affected by addition of nitrate, in sub-inhibitory levels, to anaerobic systems. Hydrolysis is a process carried out by extracellular enzymes (hydrolases), where insoluble particulate matter is solubilized and organic polymers are decomposed to monomers or dimers (i.e. simple sugars, amino acids, long-chain fatty acids and aromatic compounds) in order to pass the cell membrane [11]. The process of enzymatic hydrolysis is very important for complete mineralization of particulate matter [44].

In hydrolysis process lipids are broken down to long chain fatty acids (LCFAs) by lipases produced by bacteria including *Butyrivibrio*sp., *Clostridium* sp., and *Anaerovibrio lipolytica*. Peptide and amino acid are decomposed due to extracellular protease activity produced by bacteria including *Clostridium proteolyticum*, *Eubacterium* sp., and *Peptococcus anaerobicus* [5].

The kinetics of hydrolysis is commonly expressed as a first order hydrolysis constant; hence, knowledge of the substrate, process conditions and method from which the constant was calculated is necessary to quantify the hydrolysis. Corrections for both temperature and pH effects is also required in case of using literature values. Furthermore, knowledge on particle size distribution is necessary as the amount of available adsorption sites is crucial for the accessibility of the substrate [45]. Estimation of kinetics of hydrolysis is beyond the scope of the present study.

No studies relating the direct influence of nitrate addition to hydrolysis rates were found. However, several researches have explored the effects of microaeration on hydrolysis in anaerobic digestion. Facultative microorganism plays here the main role in enhancing hydrolysis in such systems, as explained in section 2.2. These microorganisms utilize nitrate in the absence of oxygen. It is therefore, considered that the hydrolysis in AD systems supplied with limited amounts of nitrate will be affected in the same manner as in microaeration.

Johansen et al. [15] found 50-60% increase in hydrolysis of primary sludge under microaerobic conditions, and concludes that such effect is caused by specific enzyme synthesis of hydrolytic enzymes. The results show that microaeration has a positive effect on hydrolysis of proteins

and carbohydrates, but no significant influence on hydrolysis of lipids. The authors demonstrate on a later survey the dynamics of free oxygen with a mathematical modeling approach using biomass dependent first order hydrolysis kinetics to relate the increased hydrolysis to increase in biomass growth induced by oxygen [46].

Fu et al. [4] studied the impacts of microaeration on anaerobic digestion and microbial community structure, and found a higher density of *Clostridia* (associated with hydrolysis), and oxytolerant methanogens under microaerobic conditions compared to solely anaerobic conditions, reflecting the ability of a microaerated system to metabolize complex substrate. *Clostridia* has a high cellulotic activity contributing to the breakdown of polysaccharide molecules, and can also ferment sugar to organic acids; hence, the abundance of such group of microorganisms leads to high hydrolysis rates. The same study states that the input of oxygen in sub-inhibitory levels can accelerate the hydrolysis process by destroying the substrate directly or improving activity of extracellular enzyme. The hypothesis is supported by fitting experimental data to modified first order equation where hydrolysis rate is higher under microaerated conditions.

Yan et al. [47] found an abundance in oxidoreductase enzyme in sludge samples treated with nitrate, and explains that the presence of the enzyme will favor decomposition of organic matter, due to the stimulation of catabolism. Similar research suggests that microbial yields are lower, while substrate utilization rates are higher for nitrate versus oxygen respiration [48]. This might indicate high enzymatic activity working on hydrolysis during nitrate reduction process.

Increasing hydrolysis can also be used to decrease solids retention time in the reactor, by the enhanced accessibility of the amount of available adsorption sites susceptible for anaerobic degradation [45].

2.3.4 Oxygen equivalent nitrate

The oxygen equivalent can be used as a design factor when calculating the nitrate to be added to the anaerobic reactor. From oxidation-reduction half-reactions, the oxygen equivalent of nitrate as an electron acceptor can be determined from equations 2.5 and 2.6.

For oxygen:

$$0.25O_2 + H^+ + e^- \rightarrow 0.5H_2O \qquad 2.5$$

For nitrate:

$$0.20NO_3 + 1.2H^+ + e^- \rightarrow 0.1N_2 + 0.6H_2O \qquad 2.6$$

Comparing reactions 2.5 and 2.6, for one electron transfer, 0.25 mole of oxygen is equivalent to 0.2 mole of nitrate. Hence, the oxygen equivalent of nitrate in mass basis is calculated as follows:

$$\frac{0.2\ moles\ NO_3^-}{0.25\ moles\ O_2} * \frac{62\ mg\ NO_3^- / mole\ NO_3^-}{32\ mg\ O_2 / mole\ O_2} = 1.55\ \frac{mg\ NO_3^-}{mg\ O_2} \qquad 2.7$$

The dosing of nitrate added to the reactor is then calculated as a fraction (or percentage, %) of COD in the feed (COD load) and can be calculated by equation 2.8.

$$q(NO_3)[mg] = Q(input)[m^3] * COD\left[\frac{mg}{ml}\right] * 1.55 * \mu \qquad 2.8$$

Using calcium nitrate, $Ca(NO_3)_2$, as the nitrate carrier, the amount of calcium nitrate to be added is determined by the ratio in equation 2.9.

$$\frac{1\ mole * 164\ mg\ Ca(NO_3)_2 \Big/ mole\ Ca(NO_3)_2}{2\ moles\ NO_3^- * 124\ mg\ NO_3^- \Big/ mole\ NO_3^-} = 1{,}32 \frac{mg\ Ca(NO_3)_2}{mg\ NO_3^-} \qquad 2.9$$

3 Experimental description

The aim of this experimental research was to study the influence of nitrate added at sub-inhibitory levels in the methane production under constant organic loading conditions, as well as when the OLR is increased in by 20 % in the reactors.

3.1 Sample characterization

Feedstock and inoculum collected at the biogas plant were analyzed for volatile fatty acids, alkalinity, TS, VS, tCOD, sCOD within 48 h after collection. These methods are equivalent to US standards 2320B, 2540B, 2540E and 5220D, respectively [49]. In addition, analysis of VFA profile was performed on the feedstock using gas chromatographic (GC) method.

During the experiment, VFA, alkalinity, TS, VS, tCOD, from effluent was analyzed once a week to determine degradation and process stability. The pH was measured every day to monitor performance. Results are shown in chapter 4.

Spectrophotometer Hach Lange DR 2800 was utilized for chemical analysis. The method used for VFA analysis was esterification; colorimetric/dichromate method was used to measure sCOD and tCOD; and titration with phenolphthalein indicator method was used to measure alkalinity. Dimethylphenol method was used to measure the remaining nitrate. Total solids (TS) values were obtained by evaporating and drying duplicate samples at 105 ºC for a period of 24 hours. Volatile solids (VS) was measured by burning off TS samples at 500 ºC for two hours. A summary of these methods is found in Appendix B.

The composition of gas collected was analyzed once a week by using Hewlett Packard, model HP 5890A, Gas Chromatograph (GC) designed for biogas analysis with Thermal Conductivity.

3.2 Feedstock and inoculum

Initial inoculum sludge and feedstock were taken from Lindum's biogas plant in Drammen. Characteristics of inoculum, measured by spectroscopy, are presented in Table 3.1.

Table 3.1 - Characteristics of inoculum, measured by spectroscopy, within 24 hours after collection

Parameter	**Unit**	**Value**
VS	% wet sample	4.3
TS	% dry sample	46.3
Alkalinity	mg $CaCO_4$/L	5854.7
Ammonium	mg NH_4-N/L	1360
Total VFA	mg equivalent CH_3COO^-/L	387
tCOD	g O_2/L	32.9

Feedstock was composed of a mixture of sewage sludge, fish oil, domestic septic and food waste. The proportion between feedstock components is approximately 60:25:10:5. Previous to collection, the feedstock was processed in a thermal hydrolysis pre-treatment process (THP)

with residence time of 20 minutes at 170 ºC. THP is a thermal conditioning process that breaks down longer organic polymer chains to increase digestion and gas production. The aim of THP is mainly enhance the biodegradability of sludge solids and the efficiency of the overall methane production process. Feedstock was homogenized and stored at a temperature of 10 ºC. Characteristics of feedstock, measured by spectroscopy, are shown in Table 3.2.

Table 3.2 - Characteristics of feedstock, measured by spectroscopy, within 48h after collection

Parameter	**Unit**	**Value**
VS	% wet sample	8.3
TS	% dry sample	69.3
VFA	mg equivalent CH_3COO^-/L	4686
tCOD	g O_2/L	111

VFA profile, obtained by gas chromatographic analysis, showed that the main VFA in feedstock was acetic acid (49 %), followed by butyric acid (26 %), and propionic acid (11 %). Small amounts of iso-valeric acid (5 %), iso-butyric acid (3 %), n-valeric acid (3 %) and n-valeric acid (2%) was also found in the sample.

3.3 Experimental setup

The Bioreactor Simulator from Bioprocess Control was chosen for running the experiment.

Twelve continuously stirred tank reactors were set up to reproduce the process. The methane produced were measured by liquid displacement and buoyancy, and then normalized to dry conditions at 0 ºC and 1 atm. Mixing in the reactors was kept by mechanical agitation.

The experiment was performed in 2000 mL semi-continuously fed and completely stirred tank reactors, incubated at a temperature of 37 ºC. Prior to experiment, all reactors were filled with 1700 mL of inoculum medium, and preincubated for seven days at 37 ± 1 ºC in order to deplete biodegradable organic material, and ensure degassing. Four sets each composed of three replicate reactors were run for six weeks. An experimental setup is shown in Table 3.3, in which the following denomination is used:

AN: strictly anaerobic digester at initial OLR;

SDM: Digesters to which nitrate have being supplied;

AN2: strictly anaerobic digester with increased OLR;

SDM2: digester with increased OLR to which nitrate have been supplied.

A degassing period was run for one week as mentioned previously. Degassing was followed by stabilization. This period last two weeks, when all reactors run under solely anaerobic digestion conditions and at the same organic loading rate (2.96 g VS/L). After stabilization, nitrate was introduced to sets one and two, on a daily basis previous to feeding; the organic load of set three was increased by increasing the volume feeding by 20 %, new OLR was 3.56 g VS/L; set four was maintained at the initial conditions. Finally, after one HRT (three weeks), organic loading rate of set two was increased in the same manner as for set three, while the remaining reactors were maintained at the same conditions.

Table 3.3 – experimental setup om a time-table frame

	Week							
	1	2	3	4	5	6	7	8
Set 1	D	AN	AN	SDM	SDM	SDM	SDM	SDM
Set 2	D	AN	AN	SDM	SDM	SDM	SDM	SDM2
Set 3	D	AN	AN	AN2	AN2	AN2	AN2	AN2
Set 4	D	AN	AN	AN	AN	AN	AN	AN

The sets were divided into two different groups to observe digesters behavior under the influence of nitrate. Sets one and four were aimed at studying nitrate effects at constant organic loading rate, compared to solely anaerobic digestion. These sets will from now on be denominated SDM and AN respectively. From week 4, SDM were exposed to small amounts of nitrate in an attempt to acclimatize microorganisms and stablish a mixed culture methanogens and nitrate reducing microorganisms. Nitrate was here added directly to each reactor before feeding. The initial dosage of nitrate was 0.085 % based on COD content (7.23 mg NO_3^-/L). Nitrate dosage was increased by 0.015 % every third day, until 0.19 % nitrate (16.15 mg NO_3^-/L) addition was reached.

Set two was attempted to investigate the impacts of increased organic load rate in the mixed culture. Nitrate was in this case added in the same manner as to SDM reactors, during a period of 21 days, or one HRT, and was afterwards adjusted to comply to the increased COD feed added. Set two will be referred as SDM2, and its behavior under increasing load will be compared to that presented by the strictly anaerobic digesters in set 3, which will be hereby named AN2.

Feedstock was added once a day. The hydraulic retention time (HRT) for AN and SDM reactors was 20 days, while for AN2 and SDM2 reactors HRT was 17 days.

4 Results

This chapter presents methane production and environmental conditions monitored by daily gas production, and composition measurements and chemical analysis of reactors outflow taken once a week. Methane gas flow registered on a daily basis is presented in Figure 4.1, for a process stabilization phase, and Figure 4.2, for the main period testing nitrate addition.

4.1 Stabilization

In order to stabilize methane flow and operational parameters, all reactors were kept at the same OLR (2.96 g VS/L), and without nitrate addition. Figure 4.1 shows that all sets of reactors had a variable flow rate during the first week. Fluctuations for each set diminished after day 4, and methane flow started to stabilize. Pseudo-steady state conditions where reached after 10 days.

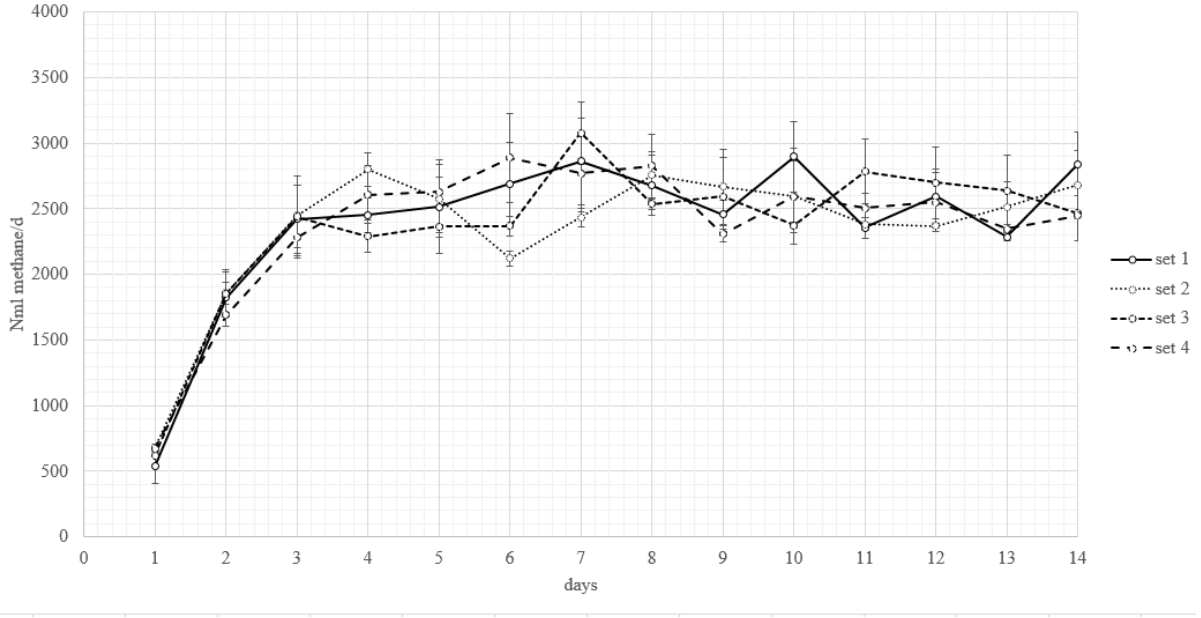

Figure 4.1 – Daily methane production rate for 4-reactor groups, all running at full-scale conditions OLR. Each group is composed of triplicates, from which standard deviation is calculated.

Data from day 11 to 14 was analyzed statistically by use of ANOVA (Appendix C) to confirm stability. The analysis showed that both the variation of methane production between the sets and the variation from day to day was statistically low. Accordingly, reactors are considered to be alike and stabilized.

The average biogas production rate from day 10 to 14 was 2546 ± 176 NmL/d. Biogas yield stabilized at 273 NmL methane/g COD feed added. The pH value at the end of this phase was 7.5 ± 0.1 for all reactors. VFA values reduced from 1235 and 694 mg equivalent CH_3COO^-/L in the first week, and stabilized at 430 ± 112 mg equivalent CH_3COO^-/L by the end of the second week. Carbon degradation was 80%, and methane content in the biogas was measured 67.7 ± 1 % for all reactors.

4.2 Nitrate addition at constant organic loading rate

To keep stable conditions, reactors were fed at the same OLR as in section 4.1, and small amounts of nitrate was added directly to each reactor before feeding. Figure 4.2 shows average

methane production rate for SDM and AN reactors, and the amount of nitrate added in terms of % equivalent relative to feed COD. The initial nitrate dosage was 0.5 % of feed COD, during the first two days, and the methane yield for reactors containing nitrate in this period was 25 NmL methane/ g COD less than for reactors without nitrate. The dosage of nitrate was therefore decreased to 0.085% COD for the following 3 days leading to improved methane production in SDM reactors compared AN reactors. The amount of nitrate added was increased approximately every third day by 0.015 % equivalent of feed COD.

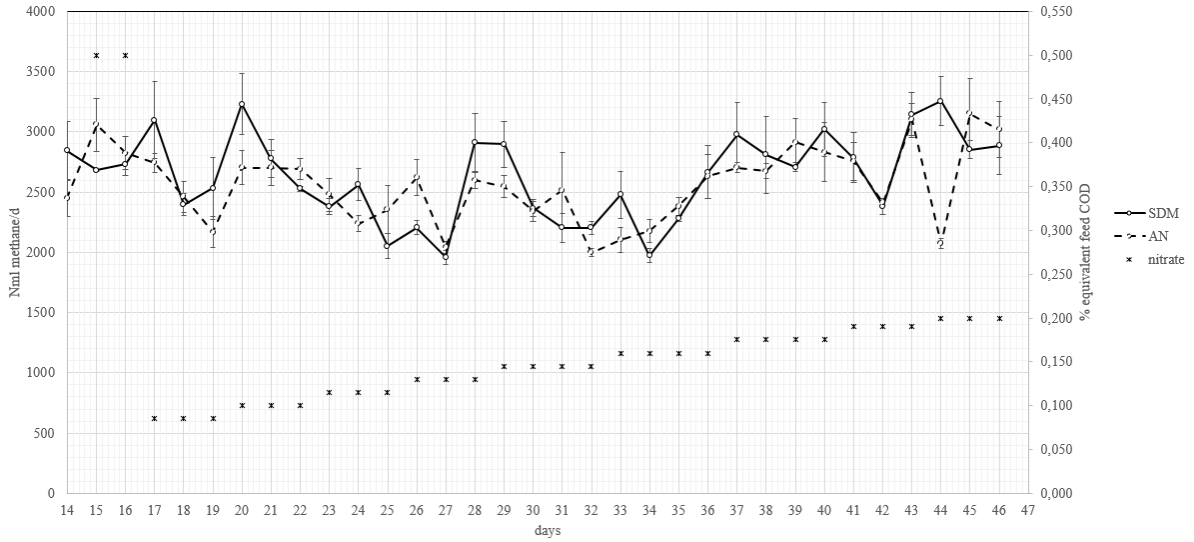

Figure 4.2 – Daily methane production rate for control reactors and nitrate reactors, and amount of nitrate added per day (given as % equivalent COD in the feed). Each group of reactors is composed of triplicates from which standard deviation is calculated.

At day 44, temperature decreased from 37 to 27 ºC due to problems with temperature regulation, resulting in a sudden reduction in methane production rate, consequently, subsequent measurements are not representatives and are not used in further analysis. Average production rate during the period shown in Figure 4.2 was 2622 ± 144 NmL/d for SDM reactors, and 2562 ± 129 NmL/d for AN reactors. Hence, biogas yield was 281 NmL CH_4/g COD for SDM reactors, and 275 NmL CH_4/g COD for AN reactors. Due to high fluctuations and overlapping, the measurements were submitted to statistical analysis by use of ANOVA (Appendix D). In this case the variation between the groups were higher than variation within the groups. Hence, the analysis supports the hypothesis that the difference between SDM and AN digesters were statistically significant.

Comparison in gas production is further illustrated by Figure 4.3, where the bars show the difference in methane production between SDM and AN reactors until day 43, and the x-axis shows the amount of nitrate, in terms of equivalent feed COD, added to SDM reactors. Here the positive difference between the reactors is 47% higher than the negative difference.

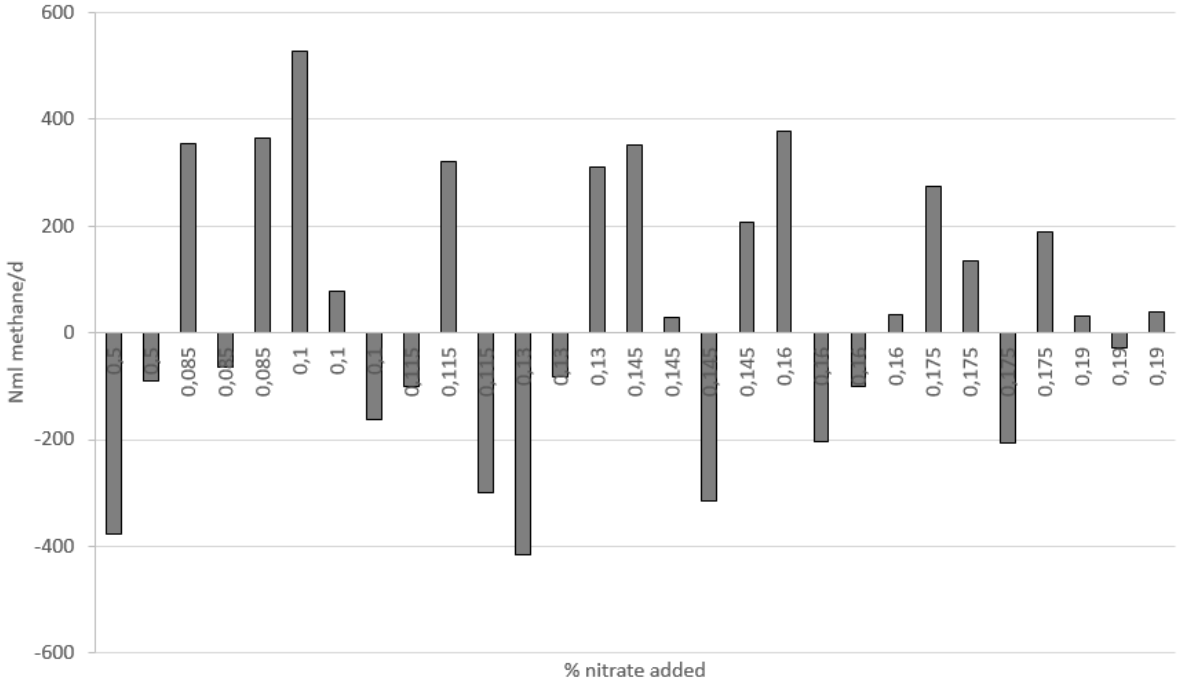

Figure 4.3 - Difference in methane production between SDM and AN reactors per day, and x-axis showing the amount of nitrate added, expressed as % equivalent feed COD.

Comparing Figure 4.3 and the analysis of data in Figure 4.2, it can be concluded that the largest significant positive effect of nitrate addition was observed when supplied at the amount of 0.100 % equivalent COD. The cumulative gas production for SDM reactors during the period shown in Figure 4.3, was 2 % higher.

Methane content in the biogas was measured at day 28, being it 68 %, for SDM reactors, and 66 % for AN reactors. At day 42 these values were respectively 71 % and 67 %, for SDM and AN reactors respectively. The amount of nitrogen gas found in all reactors in both analysis was between 1% and 2%.

VFA concentration in the reactors was measured periodically, and results are shown in Table 4.1. VFA concentration for SDM reactors is lower during most of the period. The average VFA content for control reactors was 362 ± 49 mg equivalent CH_3COO^-/L, while for SDM reactors the average was 336 ± 28 equivalent mg CH_3COO^-/L.

Table 4.1-Total VFA concentration for SDM and AN during nitrate addition, expressed as mg equivalent CH_3COO^- / L

	day 4	day 10	day 18	day 25	day 32
SDM	358	366	301	351	303
AN	443	371	295	368	334

Effluent VFA profile was measured by the end of the period embraced by this section. Only iso-valeric and acetic acid were detected. The first at a concentration of 10 mg/L for both SDM and AD reactor. While acetic acid concentration for SDM was 54 mg/L, and 52 mg/L for AD reactors. The discrepancy in the values measured by spectroscopy (Table 4.1) and gas chromatography is due to uncertainty of the methods, been the last the most accurate [50].

Alkalinity, measured in mg $CaCO_3$/L, shown in Table 4.2. Total alkalinity for SDM reactors was measured to be 11.2 ± 0.7 g $CaCO_3$/L. For control reactors the value was 11.4 ± 1.2 g $CaCO_3$/L.

Table 4.2 - Total alkalinity for SDM and AN reactors during nitrate addition, measured in g CaCO3/L

	day 4	day 10	day 18	day 25	day 32

SDM	11.9	12.0	10.9	10.7	10.3
AN	11.9	13.4	10.6	11.0	9.9

It is observed that variations for SDM reactors are lower than for remaining reactors.

% TS for all reactors varied between 5 % and 6 % during the entire experimental period. VS for SDM reactors was slightly higher than for AN reactors, as shown in Table 4.3, being 54 ± 5 % VS, and 50 ± 1% VS the respective average values.

Table 4.3 – VS for SDM and AN reactors during nitrate addition, measured in % TS

	day 4	day 10	day 18	day 25	day 32
SDM	50	50	52	56	49
AN	50	49	50	49	50

Total COD concentration in the reactors was measured periodically, and results are shown in Table 4.4. The average tCOD content for AN reactors was 39 ± 1 mg O_2/L, while for SDM reactors the average was 41 ± 3 mg O_2/L.

Table 4.4 - Total COD concentration, for SDM and AN reactors during nitrate addition, measured in g O2/L

	day 4	day 10	day 18	day 25	day 32
SDM	40	46	38	38	42
AN	39	39	38	41	44

4.3 Load variation under influence of nitrate

Feed load was increased by 20% compared to the original feed volume, increasing the OLR from 2.96 to 3.56 g VS/L*d, in order to register how operational parameters and methane production is affected in SDM reactors under load variation.

Figure 4.4 shows the gas production rates for both AN2 reactors and for SDM2 reactors for the first week (1/3 HRT) under higher OLR. Production rate for all reactors was 2380 ± 220 NmL/d before OLR change. Lower production rate is observed for nitrate reactors during the first two days, however the production rate for SDM2 reactors became higher than for AN2 reactors after day 3, approximately 1/6 HRT.

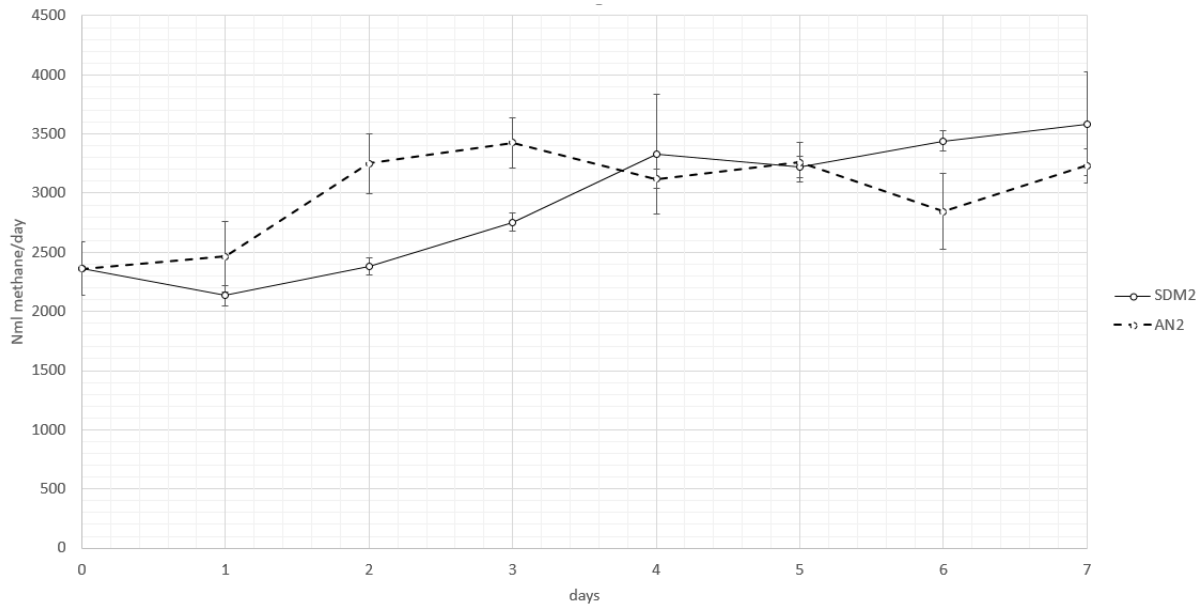

Figure 4.4 – Daily methane production rate for AN2 and SDM2 reactors after increasing the feed volume by 20%.

The production data in Figure 4.5 is recalculated into biogas methane yield and presented in Figure 4.5. It shows that, the reactors handled the high load quite well in terms of maintaining similar methane yield as it had under initial load conditions. The abrupt increase in OLR led to a temporary decrease in methane yield for ADM reactors. AN reactors maintain approximately constant yield. However, a pronounced increase in methane yield for SDM2 reactors took place after three days running at a higher OLR. Methane yield for SDM2 reactors where 17% higher than for AN reactors after seven days, ¼ HRT.

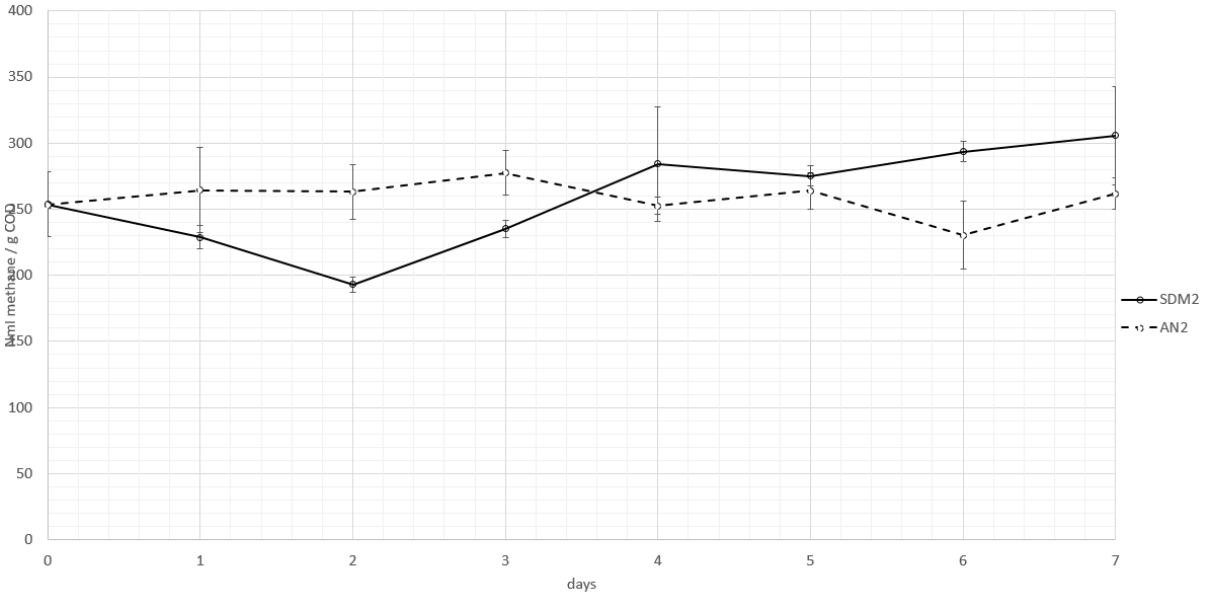

Figure 4.5 – Variations in methane yield, measured as NmL methane/g COD, for AN2 and SDM2 reactors, after increasing feed volume by 20%

The effluent pH increased from 7.5 to 7.7, for AN reactors; and decreased from 7.7 to 7.6 for SDM reactors. This parameter can indicate stability of the system and is controlled by the volatile organic acids concentration as explained in section 2.1. The initial VFA (at day 0; before increased OLR) was 271 ± 19 mg equivalent CH_3COO^-/L and 437 ± 35 mg equivalent CH_3COO^-/L for SDM and AN reactors respectively. VFA values at the end of the period were 663 ± 67 mg equivalent CH_3COO^-/L and 792 ± 61 mg equivalent CH_3COO^-/L. The pH variation also depends on the buffering capacity. Total alkalinity at day 0 was 9.8 ± 1.7 g $CaCO_3$/L and 11.9 ± 0.8 g $CaCO_3$/L for SDM and AN reactors respectively, while at the end of the period the measured values were 11 ± 0.5 g $CaCO_3$/L and 11.6 ± 0.5 g $CaCO_3$/L. The methane content at the end of the period was 71 % and 68 % for SDM and AN reactors respectively.

5 Discussion

This chapter will discuss how nitrate addition influences anaerobic systems in CSTR reactors based on experimental results and literature review. The following aspects of the method are considered: 1) Methane production under the influence of nitrate; 2) Mechanisms of nitrate reduction; 3) Influence of substrate on the effects of nitrate; 4) Environmental conditions in AD under the influence of nitrate; 5) Nitrate dosage; 6) Inhibition and other possible negative effects; 7) Application of the method in existing biogas plant.

5.1 Methane production under the influence of nitrate

A slight increase in biogas yield can be expected already at a start phase, when nitrate is introduced to a strictly anaerobic reactor. The differences in methane production in Figure 4.2 and Figure 4.3 are shown to be statistically representative, although it is noted that standard deviation for the systems overlaps (Figure 4.2). A closer look may also reveal that peaks in the production of SDM reactors are higher than for AN reactors, without changing the lowest rates. The positive effect is also shown in Figure 4.3 where the positive difference between the reactors is 47% higher than the negative difference. Finally, a slightly higher production is attained by SDM reactors, just after about 30 days of experimentation. The production rate reflects the yield, being it higher for SDM reactors (281 NmL CH_4/g COD) than for AN reactors (275 NmL CH_4/g COD) for the period nitrate was introduced. Results from the present experimental research shows that nitrate, added at sub-inhibitory levels will promote better methane production rate and yield.

5.2 Mechanisms of nitrate reduction

Section 2.3.1 described the main pathways nitrate can take in an anaerobic digestion namely, assimilation and dissimilatory nitrate reduction.

Assimilatory uptakes can contribute to enhance biogas production. However, the yield of biomass produced by synthesis is less then COD consumed. In addition, the yield will depend on system operating conditions and the type of electron donor (substrate). Consequently, and increase in methane production cannot be attributed only to assimilation.

Two modes of dissimilatory nitrate removal are likely to occur. DNRA, which accounts for increased ammonium in the system, and/or denitrification which may lead to higher amounts of $N_{2(g)}$ in the off gas. In the present experiment, the amount of ammonium and $N_{2(g)}$ produced by the mentioned mechanisms is extremely small compared to the amount of these products produced without presence of nitrate (<< 1 %). Therefore, significant variations in these parameters, caused by nitrate removal, could not be detected. Both mechanisms are therefore regarded as object of discussion.

Important parameters for determination of which pathway may prevail and how they will affect the systems may be recalled:

1) Substrate: VFAs are favorable for denitrification, and fermentative substrate is preferred for DNRA;
2) Organic and nitrogen loading rates: most of successful cases of SDM in continuous systems are run at low OLR (<< 10 kg COD/m^3*d). DNRA is here the main pathway for carbon rich and low nitrate environments (low NLR and high OLR). As the concentration of nitrate increase denitrification will be favored;

3) COD/NO_3-N ratio: High ratios will favor DNRA, while lower ratios will favor denitrifiers, in the same manner as in (2);
4) Gibbs free energy: In thermodynamic terms, DNRA will have no competitive value upon denitrification, as the last provides much higher energy yield (low Gibbs free energy - Table 2.2). However, under a high COD/NO_3 ratio, as in the present case study, the greatest need in metabolism is for maximum electron acceptor capacity, hence DNRA may be favored;
5) ORP: Redox potential is elevated in the presence of nitrate due to denitrification. On the other hand, at low redox potential DNRA is prone to occur;
6) pH: Reduction of nitrate will produce alkalinity, increasing the pH in the system.

DNRA is reported to allow syntrophic reactions to proceed and to enhance the availability of nutrient to methanogens, resulting in enhanced methanogenesis performance.

Based on the aspects mentioned in this section, it can be hypothesized that DNRA is the main pathway when low level of nitrate is added to AD system. Assimilatory nitrate reduction may contribute to form readily biodegradable COD content.

5.3 Influence of substrate on the effects of nitrate

Section 2.3.3 explained that the hydrolysis of proteins and carbohydrates might be the main mechanism affected when adding oxygen as an electron acceptor to the anaerobic system. The theory is considered to be valid also when nitrate is added, as the presence of nitrate will lead to the growth of organisms which also act under microaerobic conditions. The feedstock composition is therefore of great importance, as the presence of high concentration of lipid can limit the effects of nitrate. In the present study feedstock was composed of primary slurry (60 %), oil (25 %), food waste (5 %) and septic. The reported proportion of organic matter in each of these components is shown in Table 5.1 [45], and can be used to estimate the amount of carbohydrates, lipids and protein brought by these components.

Table 5.1 - Proportion of carbohydrates, proteins and lipids in primary slurry, oil and food waste

	Carbohydrates	Proteins	Lipids
Primary Slurry (% TS)	43	16	13
Oil (% TS)	0	0	100
Food waste (%TS)	78	6.5	0.6

Although these values are not absolute, they indicate that relative amounts of lipids and carbohydrates are much higher than proteins in the feedstock, about 45:40:15, which has probably restricted the influence of nitrate in the present survey. It is therefore suggested that nitrate addition will potentially have better effects on degradation of substrate containing a higher amount of proteins and lower amount of lipids than the feedstock in the present study.

5.4 Environmental conditions in AD under the influence of nitrate

The introduction of low levels of nitrate to an anaerobic digester can lead to an improvement on environmental conditions in the system. Results presented in chapter 4, show that parameters regulating pH level for SDM and AN reactors are quite similar, yet SDM reactors

show less variations for both total VFA and total alkalinity, evidencing a better stability in the system.

Under transient conditions, the pH is particularly sensitive, since VFA production rate might exceed utilization rate, causing a pH drop due to the accumulation of VFAs. If the buffer capacity in the system do not meet the acids accumulation, methanogenic VFA utilization decrease, and may lower pH to under neutrality, consequently butyric acid also accumulates leading to inhibition of the anaerobic process. Again, the presence of nitrate reducing microorganisms help stabilizing the system as they consume preferably butyric acid and produce alkalinity as shown in Table 2.2 and equation 2.4. It was observed in the present experiment a remarkable increase in total alkalinity for SDM2 reactors after OLR increase, from 9.8 ± 1.7 to 11 ± 0.5 g $CaCO_3$/L. On the other hand the parameter decreased slightly for AN2 reactors, from 11.9 ± 0.8 to 11.6 ± 0.5 g $CaCO_3$/L. This fact supports the proposition that nitrate addition to solely anaerobic systems leads to better environmental conditions both under constant or varied OLR.

5.5 Nitrate dosage

The initial nitrate dosage added to SDM and SDM2 reactors was 0.5 % of equivalent feed COD during the first two days. After re-evaluation, this dosage was considered to be too high as an initial dosage, and the amount of nitrate added was decreased to 0.085 % of equivalent COD for the following 3 days in an attempt to lessen the potentially negative impact of nitrate in the anaerobic system. Next, the amount of nitrate added to SDM reactors was increased step-wise, before steady-state condition is attained. Rapid consumption of nitrate in such systems allow observation of its effects in the production of methane, even at a frequent step-change. However, future studies should be done to give a more comprehensive understanding on the effects of at each step-change at steady state conditions.

In the present experiment, the amount of nitrate was increased after observing that the methane produced by SDM reactors did not fall below the lower limit given by AN reactors, which is the minimum methane production at strictly anaerobic conditions, as shown in Figure 5.1. The same figure shows that as the amount of feed nitrate increase, the ratio COD/NO_3^- decrease. A threshold will be obtained when either the production falls below the specified methane production range, or when the ratio COD/NO_3 is under its limit value.

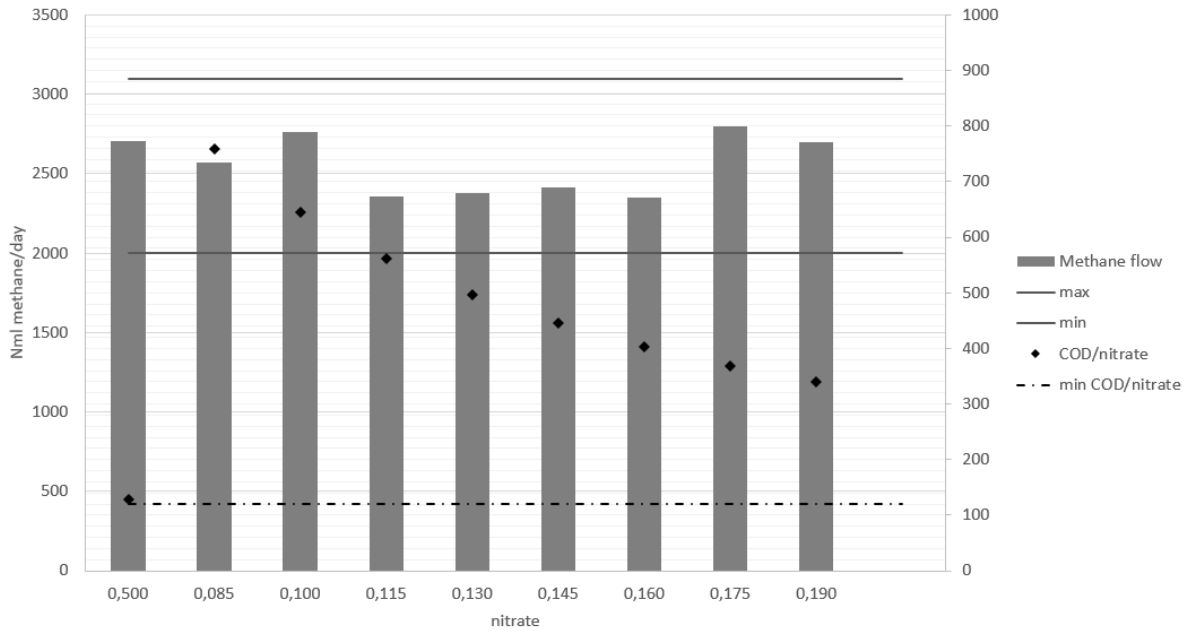

Figure 5.1 – Average methane production for SDM reactors during each step change in nitrate addition. Upper and lower limits for methane production is based on average production for AN reactors for the entire period. Minimum COD/NO_3^- ratio based on previous researches, and the ratio at each step change.

Biogas production on a long-term basis should lay on the upper limit of Figure 5.1.

No previous studies were found on the effects of varying OLR in anaerobic reactors supplied with nitrate. In the present experiment it was shown that methane yield for SDM2 decreased, when reactors was subjected to increased OLR, while yield in AN2 reactors remained constant. Nitrate dosage was here adjusted to comply to increased feed COD, and was added at the same % equivalent feed COD as before increased OLR. This abrupt increase in nitrate concentration in the reactors, might have led to temporary suppression in methanogenesis. This suppression is rapidly reversed as nitrate is consumed and production of methane returns to expected values. After only 3 days, or less than 1/5 HRT, methane yield for SDM reactors was higher than for AN reactors showing that bacterial community is able to adapt rapidly. Accordingly, when imposing a load increase, the nitrate load should be gradually increased; thus, repression of methanogens by abrupt high variations in nitrate concentration in the reactor will be avoided.

Based on the researches presented in section 2.3, the present study suggests that the ratio COD/NO_3^- shall be above 120, being it the limit where dissimilatory nitrate reduction occurs, increasing enzymes and fermentative products in the system, keeping a low ORP and without defeating methanogenesis. Nitrate could therefore be added to up to an amount of 0.5 % equivalent feed COD, without effecting gas production negatively. However, the initial amount of nitrate added to the system must be low enough to trigger nitrate reducing microorganisms without disturbing the stablished methanogenesis process, avoiding decrease in methane production, and high enough to form a mixed culture of nitrate reducing and methanogens microorganisms. In the present case study, the recommended initial amount is 0.1 % nitrate of equivalent feed COD.

5.6 Inhibition and other possible negative effects

As explained in section 2.3.2, the authors referred in the literature review of the present study, all agree that if the amount of COD provided is higher than the COD requirements for both nitrate reduction and methanogenesis, there will be no competition for the electron donor between the communities.

It has been reported that both nitrate reduction and methanogens system mechanisms are likely to happen at ORP of about -300 mV. When nitrate is added to the reactor, denitrification may occur, increasing the ORP temporarily. If enough carbon is present, denitrification will be complete, and ORP may decrease again to levels where methanogenesis is said more is prone to occur. On the other hand, DNRA reactions are favored when the redox potential is low. As ORP values for DNRA are similar to methanogenesis, hence the reduction of nitrate by dissimilatory nitrate reduction to ammonium will not deplete methane production. Elevation of redox potential in the medium may be caused by incomplete denitrification, which has been pointed out as the main mechanism involved in the suppression of methanogenesis, as explained in section 2.3.2. Complete reduction of nitrate depends on the community composition and environmental conditions. Low denitrifying efficiencies and the accumulation denitrifying intermediates was previously observed when insufficient carbon was supplied to an anaerobic digester fed with nitrate, which further inhibited the methanogenic activity. ORP was not measured in the present experimental essay. Nevertheless, characters for inhibition was not observed during the experimental period.

In addition, careful consideration must be taken when applying nitrate. As too high dosages will lead to reduction in the quality of biogas due to increased content of nitrogen and carbon dioxide and consequently reduction of methane content.

Other mechanisms that seem to be important for methanogens to thrive in a mixed culture of methanogens and nitrate reducing microorganisms are acclimatization of the anaerobe culture and habitat segregation.

Competition for the substrate, suppression of methanogens by nitrogen oxides and increased ORP are all possible negative effects of nitrate addition to anaerobic systems which can be easily controlled by maintaining high COD/NO_3. The same parameter will regulate denitrifying activity, avoiding reduction in methane content in biogas.

5.7 Application of the method in existing biogas plant

Research carried out by the present survey shows that nitrate can enhance methane production in lab scale CSTR reactors. For industrial application the process should be stabilized and monitored by using measurements of methane production and COD estimation for a feedforward and feedback control as shown in Figure 5.2. Where the regulator setpoint is the ratio COD/NO_3 to be applied, and variations in COD feed and methane productions are disturbances.

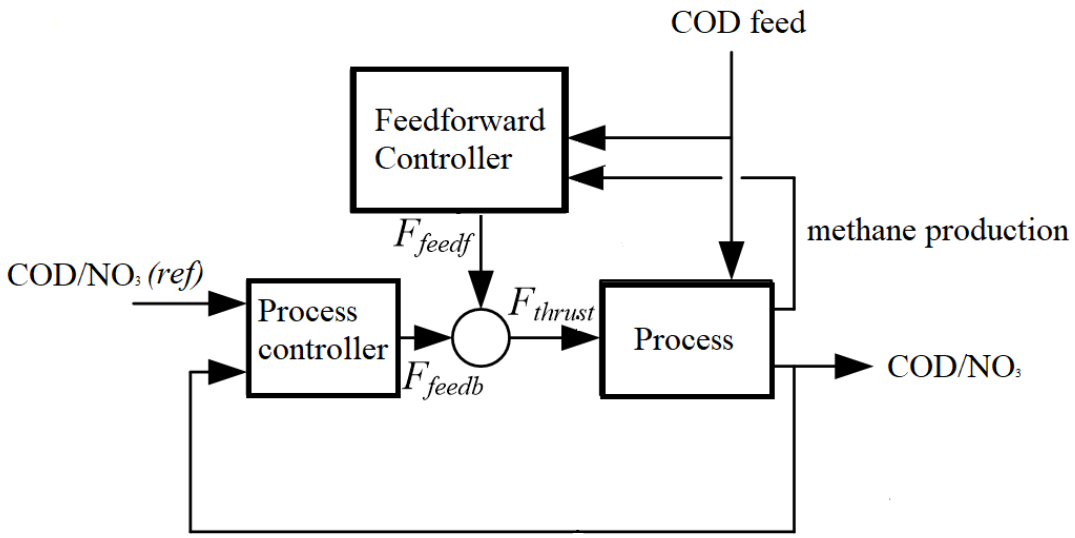

Figure 5.2 - Feedback and feedforward system for nitrate addition to anaerobic digester

Using a feedforward control, in addition to feedback control will help to obtain faster disturbance compensation in case of considerable variations in feed substrate. Nitrate can be added directly to the reactor. The amount of nitrate added will follow production response according to Figure 5.1, until a threshold is reached. The present experiment exemplifies the method, however further studies are needed in order to determinate an optimal amount of nitrate to be added to the system after acclimatization period.

The estimated acclimatization period for an already existing biogas plant is three HRT which, in the present scenario is 62 days. These period is based on the adaptability of anaerobic culture in a CSTR [51].

Financial aspects of implementing the method, such as rate of return and cost versus return analysis would lay in the final biogas yield obtained after toning the process.

6 Conclusion

Gas production rate was slightly higher during the period when nitrate was introduced to the anaerobic reactors. This difference is shown to be statistically significant, despite large fluctuations in the flow. The cumulative methane production was 2 % higher by the end of this period. The effect of nitrate addition could be more evident in systems with higher concentration of proteins and carbohydrates in the feed since theory suggests that nitrate will not influence fat digestion much.

Addition of low level of nitrate led to less variation in VFAs and total alkalinity, being these crucial parameters for the stability of anaerobic digestion. Therefore, it can be concluded that nitrate addition resulted in improvement of environmental conditions in the reactors. That may lead to improved methane production.

The initial amount of nitrate added to the system must be low enough to trigger nitrate reducing microorganisms, without leading to losses in methane production. In the present case study, the recommended initial amount is 0.1 % nitrate of equivalent feed COD. Furthermore, it is estimated that nitrate could be added up to an amount of 0.5 % equivalent feed COD, without affecting gas production negatively.

When imposing a load increase, the response time for reactors supplied with nitrate was slower than for reactors without nitrate. However, the methane yield that stablished after ¼ hydraulic retention time with high load was higher for reactors with nitrate (0.175 % equivalent COD) than for those running solely anaerobically.

Further studies on the long-term effects of nitrate on anaerobic digesters should focus on mechanisms and effects on the microbial populations.

For industrial application, nitrate can be added directly to the anaerobic reactor and the process should be carefully monitored by using measurements of methane production and COD estimation for feedforward and feedback control.

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Appendix A

University College of Southeast Norway

Faculty of Technology, Natural Sciences and Maritime Sciences, Campus Porsgrunn

FMH606 Master's Thesis

Title: Enhanced methane production by nitrate addition to anaerobic digestion of high solid content organic substrate in semi-continuous feed reactors

USN supervisor: Carlos Dinamarca and Rune Bakke

External partner: YARA International, Lindum

Task background:

Bio-gasification can recover a significant portion of energy potential from organic wastes. In addition to energy recovery it also offers waste stabilization. However, there is a need for more efficient processes for digestion of organic matter, resulting in higher methane yield. Several studies at USN have shown that addition of nitrate in sub-inhibitory levels can enhance biogas production. Further research is required to better understand the mechanisms of anaerobic digestion under influence of nitrate. This project will contribute to this and focus on optimizing Ca(NO3)2 dosage in semi-continuous reactors with feed substrate composed of sewage sludge, fish oil, domestic septic and food waste.

Task description:

Test the use of limited amounts of calcium nitrate in anaerobic digestion of substrate with high solid content in reactors with semi-continuous feeding, and investigate to which extent addition of nitrate can improve biogas yield in such systems, by monitoring methane production and reactor conditions. Appropriate calcium nitrate dosage dependence on process conditions will be determined.

Student category: EET student

Practical arrangements: Work will be carried out at Lindum, Drammen.

Signatures:

Student (date and signature): 05-02/2018 Kadja Bless

Supervisor (date and signature):

Appendix B

- **Total and Soluble COD**

For the total chemical oxidation demand test the sample is added to a vial containing a strong oxidizer, usually chromatic acid that reacts with the sample under controlled conditions. The result is defined as the mg of oxygen (O2) consumed per litter of sample. After two hours of reaction at 148°C, the sample vial is cooled down to environment temperature and analyzed by checking the change of absorbance in a spectrophotometer. The soluble COD has an additional pretreatment filtering the sample at 0.45 mm, and then applying the same procedure as total COD.

- **Total and volatile solids.**

To calculate total solids a ceramic vessel is used, the general idea is to measure the solids concentration by evaporating the moisture from the sample and dividing it into the weight of the wet sample. Initially the initial weight of the vessel is recorded without sample, then a portion of the sample is added again by recording the weight. The sample vessel is placed in an oven with a temperature of 110 ° C for 24 hours. Finally, the sample is cooled to room temperature and the difference between the weight of the vessel with the dry sample and the vessel is measured and divided into the weight of the wet sample. The final steps for the calculation of volatile solids is to put the recipient with the dried sample in an oven at a temperature of 550°C for 20 minutes to remove the volatile solids, after that the sample is cooled to environment temperature and the weigh is registered. The difference of the dried sample and the recent weight divided by the wet sample weight is the Volatile solids ratio.

For VFA, sCOD, alkalinity and nitrate analysis samples were centrifuged at 9000 rpm for 15 min. and the liquid phase was filtered through 0.45 μm syringe filters prior to the soluble component analyses. For tCOD analysis the homogenized sample was used.

Summary of the methods, based on hach langes DOC316.53.01259 (VFA); DOC316.53.01099 (COD); DOC316.53.01166 (alkalinity); DOC316.53.01071 (nitrate) are presented below:

- Titration with phenolphthalein indicator method

A phenolphthalein indicator is added to the sample. Then, the sample is titrated with a sulfuric acid solution. The phenolphthalein indicator changes color at the endpoint pH of 8.3. This value indicates the phenolphthalein (P) alkalinity and is a measure of the total hydroxide and one-half of the carbonate in the sample. A bromcresol green-methyl red indicator is added and the titration continues to the second endpoint at a pH between 4.3 and 4.9. This value indicates the total (T) alkalinity and is a measure of all carbonate, bicarbonate and hydroxide in the sample. The endpoint pH is determined with color indicators or with a pH meter.

- Colorimetric/dichromate method

The results in mg/L COD are defined as the milligrams of O2 consumed per liter of sample under the conditions of this procedure. The sample is heated for 2 hours with sulfuric acid and a strong oxidizing agent, potassium dichromate. Oxidizable organic compounds react, reducing the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}). When the 0.7–40.0 or

the 3–150 mg/L colorimetric method is used, the amount of Cr^{6+} that remains is measured. When the 20–1500 mg/L or 200–15,000 mg/L colorimetric method is used, the amount of Cr^{3+} that is produced is measured. The COD reagent also contains silver and mercury ions. Silver is a catalyst, and mercury is used to complex chloride interferences.

- Esterification

Volatile acids react with diols in an acidic environment to form fatty acid esters. These esters are reduced by iron (III) salts to form red complexes. The measurement wavelength is 497 nm.

- Dimethylphenol

Nitrate ions in solutions that contains sulfuric and phosphoric acids react with 2,6-dimethylphenol to form 4-nitro-2,6-dimethylphenol. The measurement wavelength is 345 nm.

Appendix C

Variansanalyse: To-faktor med tilbakelegging						
SAMMENDRAG	day 11	day 12	day 13	day 14	Totalt	
SDM						
Antall	3,0	3,0	3,0	3,0	12,0	
Sum	7065,2	7786,4	6859,8	8726,4	30437,8	
Gjennomsnitt	2355,1	2595,5	2286,6	2908,8	2536,5	
Varians	18421,9	129564,2	2370,7	176304,1	124152,7	
SDM2						
Antall	3,0	3,0	3,0	3,0	12,0	
Sum	7150,6	7111,3	7544,1	8040,8	29846,8	
Gjennomsnitt	2383,5	2370,4	2514,7	2680,3	2487,2	
Varians	6916,4	942,5	105430,6	203763,6	74667,5	
AN2						
Antall	3,0	3,0	3,0	3,0	12,0	
Sum	8351,3	8094,4	7911,7	7399,0	31756,4	
Gjennomsnitt	2783,8	2698,1	2637,2	2466,3	2646,4	
Varians	183259,5	222778,6	227218,7	133578,0	154166,5	
AN						
Antall	3,0	3,0	3,0	3,0	12,0	
Sum	7519,4	7652,7	7040,9	7345,4	29558,4	
Gjennomsnitt	2506,5	2550,9	2347,0	2448,5	2463,2	
Varians	38959,1	151927,6	3034,7	71707,6	54648,1	
Totalt						
Antall	12,0	12,0	12,0	12,0		
Sum	30086,5	30644,8	29356,5	31511,6		
Gjennomsnitt	2507,2	2553,7	2446,4	2626,0		
Varians	76354,3	107184,1	82329,6	144591,2		
Variansanalyse						
Variasjonskilde	*SK*	*fg*	*GK*	*F*	*P-verdi*	*F-krit*
Utvalg	237964,209	3	79321,4031	0,75716459	0,52642495	2,90111958
Kolonner	206896,334	3	68965,4447	0,65831151	0,58373228	2,90111958
Interaksjon	924731,136	9	102747,904	0,98078288	0,47414575	2,18876577
Innenfor	3352355,54	32	104761,111			
Totalt	4721947,22	47				

Appendix D

SAMMENDRAG	day 15	day 16	day 17	day 18	day 19	day 20	day 21	day 22	day 23	day 24	day 25	day 26	day 27	day 28
SDM														
Antall	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0
Sum	8039,9	8195,6	9291,7	7184,5	7590,9	9690,8	8330,8	7581,1	7130,6	7681,9	6156,4	6612,2	5875,2	8725,6
Gjennomsnitt	2680,0	2731,9	3097,2	2394,8	2530,3	3230,3	2776,9	2527,0	2376,9	2560,6	2052,1	2204,1	1958,4	2908,5
Varians	182,1	28627,4	300261,1	26038,6	199845,6	196756,8	75816,1	1238,4	13354,2	52980,9	29822,1	9951,6	9835,0	174163,6
ERROR	7,8	97,7	316,4	93,2	258,1	256,1	159,0	20,3	66,7	132,9	99,7	57,6	57,3	240,9
AN														
Antall	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0
Sum	9171,4	8465,6	8228,4	7381,2	6500,1	8105,3	8100,7	8071,8	7430,9	6716,1	7058,3	7862,1	6121,8	7792,9
Gjennomsnitt	3057,1	2821,9	2742,8	2460,4	2166,7	2701,8	2700,2	2690,6	2477,0	2238,7	2352,8	2620,7	2040,6	2597,6
Varians	148953,3	57833,4	18475,6	51080,2	50612,8	57806,9	62425,2	21020,1	56335,4	13852,0	119009,8	65064,1	7031,1	12509,7
ERROR	222,8	138,8	78,5	130,5	129,9	138,8	144,3	83,7	137,0	68,0	199,2	147,3	48,4	64,6
Totalt														
Antall	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0
Sum	17211,3	16661,2	17520,1	14565,7	14091,0	17796,1	16431,5	15652,9	14561,5	14398,0	13214,7	14474,3	11997,0	16518,5
Gjennomsnitt	2868,6	2776,9	2920,0	2427,6	2348,5	2966,0	2738,6	2608,8	2426,9	2399,7	2202,5	2412,4	1999,5	2753,1
Varians	102330,6	37014,3	165181,6	32137,2	139844,8	185619,2	57061,4	16929,6	30881,8	57825,5	86646,9	82081,3	8773,5	103667,0

SAMMENDRAG	day 29	day 30	day 31	day 32	day 33	day 34	day 35	day 36	day 37	day 38	day 39	day 40	day 41	day 42	day 43
SDM															
Antall	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0
Sum	8689,4	7101,6	6606,2	6611,3	7436,3	5918,2	6837,1	7992,7	8926,0	8429,6	8120,9	9062,9	8359,3	7140,9	9418,7
Gjennomsnitt	2896,5	2367,2	2202,1	2203,8	2478,8	1972,7	2279,0	2664,2	2975,3	2809,9	2707,0	3021,0	2786,4	2380,3	3139,6
Varians	110985,3	14545,8	41526,5	8705,3	114654,4	11009,6	1775,4	144989,7	214266,5	303405,2	4135,5	150189,5	129948,6	13511,3	100601,0
ERROR	192,3	69,6	117,7	53,9	195,5	60,6	24,3	219,8	267,2	318,0	37,1	223,7	208,1	67,1	183,1
AN															
Antall	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0	3,0
Sum	7636,1	7017,0	7547,7	5993,7	6306,1	6526,8	7140,9	7889,2	8106,0	8023,0	8742,1	8493,3	8267,3	7227,6	9302,9
Gjennomsnitt	2545,4	2339,0	2515,9	1997,9	2102,0	2175,6	2380,3	2629,7	2702,0	2674,3	2914,0	2831,1	2755,8	2409,2	3101,0
Varians	26767,0	20808,6	298998,0	3991,2	31054,6	26091,2	16381,5	100197,1	4542,3	12444,9	117068,6	179343,6	75149,0	1527,8	52396,0
ERROR	94,5	83,3	315,7	36,5	101,7	93,3	73,9	182,8	38,9	64,4	197,5	244,5	158,3	22,6	132,2
Totalt															
Antall	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0	6,0
Sum	16325,5	14118,6	14153,9	12605,0	13742,4	12445,0	13978,0	15881,9	17032,0	16452,6	16863,0	17556,2	16626,6	14368,5	18721,6
Gjennomsnitt	2720,9	2353,1	2359,0	2100,8	2290,4	2074,2	2329,7	2647,0	2838,7	2742,1	2810,5	2926,0	2771,1	2394,8	3120,3
Varians	92082,3	14380,3	165757,2	17792,9	100862,0	27186,8	10339,2	98431,8	109936,9	131850,8	61344,6	142628,1	82321,2	6266,2	61645,8

SAMMENDRAG	day 44	day 45	Totalt
SDM			
Antall	3,0	3,0	93,0
Sum	9763,6	8551,8	243053,7
Gjennomsnitt	3254,5	2850,6	2613,5
Varians	126661,8	17042,6	186659,4
ERROR	205,5	75,4	44,8
AN			
Antall	3,0	3,0	93,0
Sum	6219,0	9446,4	236891,7
Gjennomsnitt	2073,0	3148,8	2547,2
Varians	4372,5	258930,2	138241,5
ERROR	38,2	293,8	38,6
Totalt			
Antall	6,0	6,0	
Sum	15982,6	17998,2	
Gjennomsnitt	2663,8	2999,7	
Varians	471220,0	137066,1	

Variansanalyse						
Variasjonskilde	*SK*	*fg*	*GK*	*F*	*P-verdi*	*F-krit*
Utvalg	204141,097	1	204141,097	2,75212447	0,09965261	2,74642775
Kolonner	15909487,3	30	530316,244	7,14944876	1,2102E-15	1,40722699
Interaksjon	4783590,63	30	159453,021	2,14966299	0,00184273	1,40722699
Innenfor	9197802,03	124	74175,8228			
Totalt	30095021,1	185				