BIOCHEMICAL METHANE POTENTIAL OF ESTONIAN SUBSTRATES AND EVALUATION OF SOME INHIBITORS OF ANAEROBIC DIGESTION

EESTI SUBSTRAATIDE BIOKEEMILISE METAANITOOTLIKKUSE POTENTSIAALI MÄÄRAMINE JA ANAEROOBSE KÄÄRITAMISE MÕNEDE INHIBIITORITE UURIMINE

MARIO ALBERTO LUNA DEL RISCO

A Thesis for applying for the degree of Doctor of Philosophy in Environmental Conservation

Väitekiri filosoofiadoktori kraadi taotlemiseks keskkonnakaitse erialal

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Institute of Agricultural and Environmental Sciences  
Estonian University of Life Sciences

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To my beloved wife and family
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AUTHOR’S CONTRIBUTION TO PUBLICATIONS

I. Mario Luna del Risco participated in the creation and development of a web-based database on methanogenic potential of crops and wastes. He collected and processed all data included in the database. He was responsible for the preparation of the manuscript.

II. Mario Luna del Risco was responsible for data collection and statistical data processing. He participated in the study design, data interpretation and he was responsible for the preparation of the manuscript.

III. Mario Luna del Risco performed data collection and statistical data processing. He participated in the study design. He was responsible for data interpretation and for the preparation of the manuscript.

IV. Mario Luna del Risco performed ecotoxicity testing of bulk and nanoparticles of CuO and ZnO during anaerobic digestion of cattle manure. He performed data collection and statistical treatment. He was responsible for the preparation of the manuscript.
ABBREVIATIONS

3,5–DCP: 3,5–dichlorophenol
AAS: Atomic Absorption Spectrometer
AD: Anaerobic digestion
ADF: Acid Detergent Fibers
B: cumulative methane yield expressed in L or m³ per kilo of TS, VS or COD at time (t) expressed in days.
Bo: cumulative methane production for 100 days of incubation
Bmax: maximum methane yield (L or m³ per kilo of TS, VS or COD)
BMP: Biochemical Methane Potential
CE: Cellulose
C.I.: Confidence interval
COD: Chemical Oxygen Demand
CHP: Combined Heat and Power
EC50: The half effective concentration corresponds to the concentration of inhibitor required to cause a 50% reduction of methane production when compared with the control tests
EEA: European Environment Agency
EU: European Union
GHG: Greenhouse gases
HE: Hemicellulose
k: Kinetic rate constant
L: Lignin
NDF: Neutral Detergent Fiber
Nm³: Normal cubic meters (Standard Temperature and Pressure)
UBCW: Unit of Bioconversion of Crops and Wastes
r: Pearson’s correlation coefficient
REAP: Renewable Energy Action Plan
RED: Renewable Energy Directive
t: Time
TN: Total Nitrogen
TOC: Total Organic Carbon
TS: Total solids
TP: Total Proteins
VS: Volatile solids
1. INTRODUCTION

Energy dependence on fossil fuels has led countries worldwide to find new alternatives for energy production. For this reason, in the late 90’s, EU member states have been very active to promote and support the generation of renewable energies. In 2001, the EU delivered 2001/77/EC directive on the promotion of electricity produced from renewable energy sources in the internal electricity market which was later modified by directive 2009/28/EU. The main targets of this strategy are set to secure the electricity supply, reduce climate change and promote environmental protection. For this matter, implementation of renewable energies is being considered as a key element to achieve sustainable development without generating any negative impact on the environment.

Biogas production is considered nowadays as a potential alternative for the production of energy while simultaneously resolving ecological issues (Chynoweth et al., 1993, Gunaseelan, 1997). Biogas is the end-product of a chain of biochemical reactions that occur in an oxygen-free environment. The most common substrates for biogas production in farms are: energy crops, silages and animal manures (Amon et al., 2007). In Estonia, there is an estimated area of around 286 thousand hectares of abandoned agricultural land that can be considered for cultivation of energy crops and around 128 thousand hectares of semi-natural grasslands (Astover et al., 2008). The calculated theoretical herbal biomass production is up to 2 billion tons per year. Additionally, there are other sources of biomass form the agro-industry that can also be considered as co-substrates for the production of biogas, such as fermentation slops from the brewery industry, unconsumed milk products, grain mill residues, etc. Unfortunately, the total energy potential in Estonia has been partly exploited as only one agricultural biogas plant is installed with an annual production of 2 GWh/year.

The biochemical methane potential (BMP) assay has been widely used to determine the methane yield of organic substrates (Owen et al., 1979; Gunaseelan, 1997; 2004). However, an exhaustive characterization of the chemical composition of biomass and an understanding of process kinetics are essential for predicting methane production. Many authors have studied the influence of chemical composition (i.e. content of organics, proteins, lipids, fibers, etc.) on anaerobic biodegradation of biomass. For example, biodegradation of lignocellulosic substrates under
anaerobic conditions is hard to achieve and therefore production of biogas is low. In addition, there are a wide set of chemicals that can cause anaerobic digestion imbalance (i.e. excessive production of volatile acids). In recent years, modern industrial research has adopted new technologies to utilize an increasing number of materials at a nanometer scale. These advances allow for improved characteristics so that more complex tasks can be achieved. A wide range of novel applications improved by these new nano materials include antibacterial gels, soil decontamination agents, water filtration materials, biodegradable polymers and highly efficient clear inorganic sunscreens, animal supplementation, pesticides, among others (Brar et al., 2010; OECD, 2011). As a consequence of the introduction of these new materials in the market and the lack of knowledge on the possible risks associated from exposure to nanoparticles, results on ecotoxicological tests have been of great interest (Moore, 2006; Baun et al., 2008; Blaise et al., 2008; Griffitt et al., 2009; Liu et al., 2010). However, most studies have examined the effects on aquatic environments. So far, very few studies have investigated the influence of nanoparticles on anaerobic microorganisms.

The current Thesis presents an evaluation of the biochemical methane potentials of typical Estonian agricultural biomass and agro-industrial residues and kinetics of the methane production process. The thesis also addresses the adverse effect that nanoparticles of metal oxides may have on biogas production compared to bulk suspensions.
2. REVIEW OF THE LITERATURE

2.1. Biochemical Methane Potential (BMP)

Methane fermentation or anaerobic digestion (AD) is the process in which specialized anaerobic microorganisms breakdown the biodegradable material in an oxygen-free environment to produce biogas (composed primarily of methane and carbon dioxide) and nutrient-rich digestate (Pain and Hepherd, 1985). In the 1860s in France, large-scale reactors were introduced using more advanced technology (McCarty, 2001). For the treatment and stabilization of solid wastes, anaerobic digestion has been used since the late 19th century. In some countries like China and India, biogas from the anaerobic digestion of manures and agricultural wastes has been used for cooking and lighting purposes (Gijzen, 2002). In the 1970s, due to the oil crisis and increased concerns on environmental pollution, anaerobic digestion gained more attention from the research and technological point of view. Nowadays, new technologies have been developed to improve the quality of biogas and its conversion efficiency for heat and energy.

Anaerobic decomposition of organic matter is a complex process involving several microorganisms with a wide variety of metabolic functions (Fig. 1).

Fig. 1. Synthesized anaerobic digestion scheme.
In the first step, insoluble polymers such as carbohydrates, lipids and proteins are hydrolyzed into monomers by extracellular enzymes generated by hydrolytic bacteria. After hydrolysis takes place, acid-forming bacteria (fermentative bacteria) will convert the monomers produced into volatile fatty acids, alcohols, hydrogen and carbon dioxide. This step can only take place if hydrogenotrophic methanogens are operating in a syntrophic relationship with fermentative bacteria (Stams, 1994; Schmitz et al., 2005). These intermediate products will later on be catabolized to \( \text{H}_2, \text{CO}_2 \) and acetate by proton-reducing acetogenic and hydrogen-oxidising acetogenic bacteria.

Then in the final step, methane is produced by acetonotrophic methanogens which consume acetate as substrate (Equation 1); and by hydrogenotrophic methanogens which use hydrogen and carbon dioxide as substrate (Equation 2) (Zinder, 1984).

\[
\text{CH}_3\text{COOH} \rightarrow \text{CH}_4 + \text{CO}_2
\]  
\[  \tag{1} \]

\[
\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}
\]  
\[  \tag{2} \]

The methane production kinetics and the methane productivity can be determined in a series of batch experiments under very specific conditions. The biochemical methane potential (BMP) test is a method to measure anaerobic biodegradability of substrates. It allows the methane production from a specific substrate to be determined experimentally at a laboratory scale (Angelidaki et al., 2009). Some advantages of the test are: estimation of the methane potential, easy setup, low-cost and repeatability and reproducibility. However, the test can take from 20 to 60 days, depending on the substrate.

From the BMP test, cumulative methane yield \( B \) and ultimate methane yield \( (B_0) \) can be determined. \( B_0 \) represents the cumulative methane production for 100 days of incubation (Gunaseelan, 2004; 2007; 2009a; 2009b). Ultimate methane yield and kinetic rate constant can be estimated using a nonlinear regression fit of the yield data to simple first order degradation model (Equation 3) (Chen, et al., 1978, Massé et al., 2010; Zeng et al., 2010; Li et al., 2011):

\[
B = B_{\max} \times (1-e^{-kt})
\]  
\[  \tag{3} \]
where $B$ is the cumulative methane yield expressed in L or m$^3$ per kilo of total solids (TS), volatile solids (VS) or chemical oxygen demand (COD) at time ($t$) expressed in days, $B_{\text{max}}$ is the maximum methane yield (L or m$^3$ per kilo of TS, VS or COD), $k$ is the rate constant, expressed in reciprocal of the X-axis time units (1/d). However, it has been found (Rao et al., 2000, Rincon et al., 2010, Paper II) that biogas production from solid organic substrates was better fitted by the pseudo-parallel first order model. Rao et al. (2000) were the first to report that methane production curves correspond in the first step to a rapid bioconversion of readily degradable components followed by a slower bioconversion of fibrous portion of the substrates. Similar two-phase exponential model (Equation 4) was also tested by Luna-delRisco et al. (2011) (Paper II):

$$B = B_1 \times (1 - e^{-k_1 t}) + B_2 \times (1 - e^{-k_2 t})$$

(4)

where $B$ represents the cumulative methane production as a function of time ($t$), $B_1$ is the methane yield associated to the bioconversion of readily degradable organics, $B_2$ is the methane yield associated to the bioconversion of less readily degradable material, $k_1$ and $k_2$ are the respective rate constants.

2.2. Physico-chemical factors influencing biogas / methane production

2.2.1. Biomass characteristics

Biomass composition depends primarily on the source: agricultural, municipal and industrial wastes. Chemical composition analyses play an important role when estimating biogas or methane yield and in determining the amount of biomass that is necessary to maintain the population of digesting microorganisms (Chynoweth and Isaacson, 1987). Total organic matter in digester feedstock is usually measured as volatile solids (VS), chemical oxygen demand (COD) or total organic carbon (TOC).

The nutritional requirements of anaerobic bacteria are extremely important to supply the basic cellular building for growth and to be able to synthetize the enzymes and co-factors from metabolic reactions. Nutrient levels should normally be in excess of optimum concentration to avoid inhibition of anaerobic digestion by nutrient deficiency.
However, some nutrients can be toxic in high concentrations (Gunnerson and Stuckey, 1986). The main macro-nutrients needed for anaerobic digestion are nitrogen (N), phosphorus (P) and sulphur (S). Optimal nitrogen requirements in anaerobic digesters are in the ratio of 100:2.5 (COD:N), in the case of phosphorus, a ratio of 7:1 (N:P) is required (Mara and Horan, 2003). Optimum S concentrations are 0.001 – 1.0 mg/L (Speece, 1996).

Animal wastes often contain high amounts of ammonia nitrogen due to the presence and degradation of urea and protein (Hansen et al., 1998). Crop residues on the other hand contain high concentration of lignocellulosic constituents. Lignocellulosic materials are composed of cellulose, hemicellulose and lignin. Cellulose and hemicellulose can be bioconverted by anaerobic bacteria into methane and carbon dioxide. However, degradation rates of these compounds depend mainly on whether they are lignin-incrusted (cellulases cannot reach cellulose fibers due to lignin) or in a crystalline form (Klimiuk et al., 2010). Lignin is a complex plant constituent very difficult to digest by anaerobic bacteria and therefore low methane is achieved at very low rates (Schievano et al., 2008, Klimiuk et al., 2010, Hendriks and Zeeman, 2009).

Particle size can also influence the rate of anaerobic digestion as it affects the surface area for biodegradation of biomass material (Palmowski and Muller, 2000; Ward et al., 2008). Mshandete et al. (2006) found that decreasing particle size from 100 mm to 2 mm will improve fiber degradation and therefore high methane yield will be achieved.

### 2.2.2. Temperature

Temperature is an important parameter to consider for obtaining optimal biogas production. In the literature, it is reported that anaerobic digestion of biomass can take place in three different ranges: psychrophilic (10-20°C), mesophilic (20-45°C) and thermophilic (45-68°C). The most common temperature ranges used to run anaerobic reactors are either mesophilic (with an optimum at 35°C) or thermophilic (with an optimum at 55°C). For each temperature range, different groups of microorganisms have been identified (Gerardi, 2003). Problems related to temperature control, even changes of only few degrees of digestion temperature, may result in a reduction of biogas production.
rate. Some considerations for anaerobic digesters running in mesophilic temperature are:
- AD process is more stable in mesophilic than thermophilic digesters.
- Wider bacteria diversity.
- Bacteria can adapt more easily to environment conditions than in thermophilic digesters.

In the case of thermophilic digesters, other aspects have to be taken into consideration:
- Faster reaction rates when compared with mesophilic reactors.
- Short retention times
- Reduction of pathogens in the effluent.
- High energy demands due to high temperature digester operation.
- Sensitive to operational and environmental conditions.

2.2.3. pH and alkalinity

Ideal methanogenic growth rate has been set at a narrow pH range: 6.8-7.2. In terms of systems stages, optimal pH during hydrolysis and acidogenesis has been reported between 5.5 and 6.5, while for methanogens optimal pH stands at 7.0 (Gerardi, 2003; Jördening and Winter, 2004; Drapcho et al., 2008). pH in anaerobic digesters defines the equilibrium between carbonic acid, bicarbonate alkalinity, and carbonate alkalinity, and also between ammonia and ammonium ions (Ahring, 2003; Guštin and Marinšek-Logar, 2011).

Buffer capacity, also referred to as alkalinity, is known to be essential to maintain the stability of anaerobic systems. It represents the equilibrium of carbon dioxide and bicarbonate ions which offers resistance to significant and rapid changes in pH (Ward et al., 2008). When organic matter is biodegraded in anaerobic systems, organic acids (i.e. acetate, butyrate, propionate) are generated. High concentrations of organic acids in anaerobic digester may lead to a decrease in alkalinity below the normal operating level and therefore an imminent failure occurs. (Speece, 1996; Björnsson et al., 2001; İşık and Sponza, 2005; Boe et al., 2010). In the case of insufficient alkali compounds from the feed substrate, alkalinity has to be balance by adding chemicals such as sodium bicarbonate, potassium bicarbonate, sodium carbonate, calcium carbonate, calcium hydroxide or sodium nitrate to maintain stable conditions in the reactor (Gerardi, 2003).
2.3. Inhibitors of anaerobic digestion

2.3.1. Ammonia

Nitrogen is an important nutrient for anaerobic bacteria (Mah et al., 1978). Ammonia is the result of the biological degradation of organic matter rich in nitrogenous matter, and under anaerobic conditions, it is released when the amino groups of amino acids are stripped (Chen et al., 2005; Drapcho et al., 2008). Ammonia is present within the anaerobic digestion process in the form of ammonium ion ($\text{NH}_4^+$) or dissolved ammonia gas ($\text{NH}_3$). Both compounds are in equilibrium and the concentration of each element depends on the pH (Equation 5):

$$\text{NH}_4^+ \rightleftharpoons \text{NH}_3 + \text{H}^+ \quad (5)$$

Kayhanian (1999) found that inhibition from ammonia should be attributed to its free form rather than the total ammonia. Various mechanisms of ammonia inhibition such as a change in intracellular pH, increase of maintenance energy requirements and enzyme reaction inhibition have been proposed. In anaerobic systems, ammonia nitrogen ($\text{NH}_3$-$\text{N}$) concentrations of 50 – 200 mg/L are considered to be stimulatory, while concentrations of 1500 – 3000 mg/L are inhibitory at pH over 7.4. Concentrations above 3000 mg/L are considered to be very toxic for anaerobic bacteria (McCarty and McKinney, 1961; Albertson, 1961; Mignone, 2005). The microorganisms most affected by ammonia inhibition are the methanogens (Kayhanian, 1994).

2.3.2. Volatile fatty acids and long chain fatty acids

The methanogenic conversion of organic matter occasionally accumulates known volatile fatty acids (VFA) such acetate, propionate, and butyrate as intermediary products which may act as potential inhibitors of bacteria in anaerobic digestion. High concentrations of these organic acids may occur when the organic loading rates are excessively high or when toxic materials are present in the digester. Andrews and Graef (1970) reported volatile acid concentrations inhibition at levels exceeding 2,000 mg/L (as acetic acid). Propionate can specifically inhibit the activity of
methanogenic bacteria; resulting in overall decrease of the total biogas achievable (Hobson and Shaw, 1976; Fukuzaki et al., 1990).

Long chain fatty acids (LCFA) and glycerol are the result of the hydrolysation of lipids (Hanaki et al., 1981). LCFA at low concentrations have been found to affect only gram-positive microorganisms (Kodicek, 1949; Nieman, 1954; Kabara et al., 1977; Palatsi et al., 2010). In the case of anaerobic bacteria, methanogens are most susceptible to be inhibited by LCFA as they have a similar cell wall as gram-positive microorganisms (Zeikus, 1977). The mechanisms associated to LCFA toxicity are caused by adsorption onto the cell wall/membrane and interference with the transport (Rinzema et al., 1994). In addition, inhibition by LCFA will reduce anaerobic biomass granulation and granule flotation, and impaired syntrophic interaction between microbial groups (Menju et al., 1997; Tay and Yan, 1996).

2.3.3. Light metal ions

In this subchapter, discussion will only be undertaken on the analyzed (Paper III) light metal ions (Ca, Mg, K). K, Ca and Mg ions are normally found in influents of anaerobic digesters. Such ions can be originated from the biomass or from compounds added to adjust the pH (Grady et al., 1999). Although moderate concentrations of light metal ions are essential for microbial growth, excessive amounts can reduce bacterial growth and even severely inhibit the anaerobic digestion process (Soto et al., 1993).

The presence of Ca²⁺ in anaerobic systems is required for the growth of certain strains of methanogens and for the formation of microbial aggregates (Murray and Zinder, 1985; Jackson-Moss et al., 1989; Thiele et al., 1990; Huang and Pinder, 1995). However, extreme concentrations of Ca²⁺ may cause precipitation of carbonate and phosphate (Chen, Y. et al., 2008). Ahn et al. (2006) found the best performance of anaerobic digestion of swine wastewater when 3 g/L of Ca was added. However, concentrations of 5-7 g/L decrease biogas production rate and total biogas production.

Jarrell et al. (1984) found that high concentrations of extracellular K⁺ in the culture media can lead to a passive inflow of K⁺ ions that may result in neutralization of the membrane potential. K⁺ is also known to be an
efficient extractant of metal bonds, and as a result removal of essential micronutrients in the effluent and low methanogenic activity will be reached (Ilangovan and Noyola, 1993; Chen, Y. et al, 2008).

Optimal concentration of Mg$^{2+}$ ions was reported to stimulate certain strains of methanogens at 720 mg/L (Ahring et al., 1991, Schmidt and Ahring, 1993). Mg$^{2+}$ in anaerobic systems has been reported to reduce K$^+$ toxicity during anaerobic digestion. Bashir and Matin (2004) found that concentrations of Ca$^{2+}$ (841 mg/L), Mg$^{2+}$ (1262 mg/L) and Na$^+$ (543 mg/L) helped the anaerobic process to recover from K inhibition.

### 2.3.4. Heavy metals

Heavy metals like Fe, Zn, Ni, Co, Mo and Cu are fundamental for the proper enzyme functioning of anaerobic systems (Takashima and Speece, 1989). However, concentrations above limits or lack of these elements can negatively alter biogas production and therefore a decrease on methane yield will be obtained as a result of the inhibition of anaerobic bacteria.

During anaerobic digestion of biomass, heavy metals take part in several physico-chemical reactions, in which the three main ones are: 1) precipitation as sulfide, carbonate and hydroxides (Lawrence and McCarty, 1965; Mosey et al., 1971); 2) sorption to the solid fraction, either biomass or inert particulate matter (Shen et al., 1993; Shin et al., 1997); 3) formation of complexes in solution (Hickey et al., 1989). To estimate whether heavy metals stimulate or inhibit the process, an evaluation on the total metal concentration, chemical forms of the metals and factors such as pH and redox potential have to be taken into account. Zayed and Winter (2000) have found that methanogens are more inhibited when exposed to heavy metals than acidogens.

Heavy metals are only toxic to anaerobic bacteria in their soluble form. From the studies conducted by Bhattacharya et al. (1995), it can be concluded that heavy metals toxicity can be attributed to the free ionic concentration of the metal rather than to the total metal concentration. During acidogenesis and methanogenesis, some heavy metals can be more toxic to anaerobic bacteria than others. Lin (1992; 1993) studied the effect of 6 different metals, i.e. Cu, Zn, Cr, Cd, Ni and Pb during anaerobic digestion. He found Cu was the most toxic metal for acidogenic bacteria, while Pb was found to be the least toxic. In the case
of methanogenic bacteria, it was found that Cd and Ni were the most and least toxic metals respectively.

2.4. New generation toxicants (nanoparticles)

Nanoparticles are particles with a size of 1 -100 nm (Christian et al., 2008; SCENIHR, 2005). The properties of many conventional materials change when formed from nanoparticles due to a greater surface area per weight than larger particles. This property makes them more reactive than certain other particles. Nanoparticles application in the market is largest in the field of cosmetics, healthcare, different industrial products etc. Although, there are several examples of nanoproducts in the agricultural sector that are being developed or are already in the market. Such products include fertilizers, fungicides, pesticides, animal feed, veterinary medicines, among others (Feneque, 2003; ETC Group, 2005; Scott, 2005; 2007). Mineral elements used in agriculture and animal husbandries such as copper and zinc are already being considered for their use at a nano-scale (Gonzalez-Eguia, 2009; Milani et al., 2010). These elements are essential minerals that are normally included in animal diet to improve growth and maintain normal health (Mertz, 1993) as well as the important role they play in biochemical reactions in plants and are essential for optimum growth of crops. Recent research studies (Navrotsky, 2000; Moraru et al., 2003; Opara, 2004; Kuzma and VerHage, 2006; Sastry et al., 2007) have shown that the introduction of nanoparticles in farming activities can be considered as an important improvement of the actual agricultural practices as they will allow better control in the release of the substance to be delivered, which means drugs will be absorbed more slowly at a specific location in the body, and therefore higher efficiency will be achieved (ETC Group, 2005). However, the toxicity of such elements in different environments is still under evaluation.

Effluents containing suspensions of nanoparticles may drastically harm the environment and this is particularly true for aquatic habitats (Oberdorster, 2004; Moore, 2006; Zhu et al., 2006; Baun et al., 2008; Blaise et al., 2008; Griffitt et al., 2009; Velzeboer et al., 2008; Zhu et al., 2010). Dispersal of contaminated sewage sludge into the soil will spread toxic substances to living organisms, groundwater and sub-surface water systems (Paull et al., 2003). In recent years, the ecotoxicity of engineered nanoparticles has been of great interest due to their potential harmful
effects on human and other vertebrate health (Valant et al., 2009; Farré et al., 2009; Liu et al., 2010).

Ecotoxicity of several chemicals in their nanoparticle form have been studied in the literature (Hussain et al., 2005; Adams et al., 2006; Franklin et al., 2007; Lin and Xing, 2007; Heinlaan et al., 2008; Aruoja et al., 2009; Kasemets et al., 2009). Among them, metal oxide formulations, i.e. CuO and ZnO, have been shown to be significantly toxic for aquatic microorganisms. Toxicity assays have shown high toxicity of nanoparticles of CuO and ZnO on microalgae *Pseudokirchneriella subcapitata* at exceedingly low concentrations, such as 0.042 mg/L of zinc and 0.71 mg/L of copper (Aruoja et al., 2009). In their study, toxicity was attributed to the solubility of the metal oxide nanoparticles. However, very few studies have been carried out examining the effect of these hazardous materials in contaminated sediments, non-aquatic environments or anaerobic systems.

Liu et al. (2009) found an antibacterial effect of nanoparticles of ZnO on *Escherichia coli*. In their study, complete inhibition of microbial growth was achieved at the concentration of 12 mmol/L or higher. They found that inhibition was caused due to bacterial cell membrane damage and loss of intracellular components. In an anaerobic toxicity test conducted by Barrena et al. (2009), they found no adverse effect from nanoparticles of Ag and Au on biogas production. However, they found a positive effect on biogas production from nanoparticles of Fe.

There remains a lack of information regarding the adverse effect of CuO and ZnO nanoparticles on the environment when assessing different organisms. Currently, data regarding the effect of nanoparticles towards the production of biogas by anaerobic bacteria have been poorly studied. Further evaluation on nanoparticle toxicity on anaerobic digestion have shown a negative influence of nano CuO and nano ZnO compared to their bulk counterparts on the production of biogas, specifically on methane yield (Paper IV).

### 2.5. Anaerobic digestion in Estonia: current status and Research and Development

In the past few years, Estonia has considered the implementation of different renewable energies as a strategy to reduce its dependence on
fossil fuels. In 2010, the Renewable Energy Action Plan (REAP) was published according to Renewable Energy Directive 2009/28 (RED). According to the targets set by RED, renewable energy usage should account for 25% of the total energy consumption in Estonia by 2020. To reach this goal, the share of renewable energy in the sectors of heat/cooling, electricity, and transport should achieve 17.6, 14.8 and 2.7% of the total energy consumption in these sectors respectively. The development of the biogas sector in Estonia is considered among the REAP actions. Target for annual biogas production was set to 0.5 PJ in 2020.

According to the European Environment Agency (EEA), Estonia has very little biogas production when compared with other EU countries. It was estimated that in Estonia in 2006 only 5.2 m³ of biogas (for energy purposes) per inhabitant was used, while the average of EU countries was 26.8 m³/inhabitant. Estonia has great potential for the production of biogas using manures, sewage sludge, herbal biomass and organic residues. There are about 286 thousand hectares of abandoned agricultural land in Estonia that is suitable for cultivation of energy crops, and 128 thousand hectares of semi natural grasslands (Astover et al., 2008). Theoretical herbal biomass resources for biogas production are 2 billion tons per year (Roostalu and Melts, 2008). Renewable electricity potential in the agricultural biogas sector is estimated to produce 153.2 GWh and 696 GWh from manures and herbal biomass respectively (Table 1; Kask, 2008; Oja, 2011). At present, there is only one agricultural biogas plant that generates an annual electricity production of 2 GWh/y. Most of the biogas renewable energy potential in Estonia is not used. Sewage sludge, landfill gas and biowaste presented the least biogas potential from all available substrates in Estonia (Oja, 2011).

Currently, Estonian cities such as Kuressaare, Narva and Tallinn are producing biogas from wastewater sludge. Two other cities (Tartu and Rakvere) have biogas plants under construction. Theoretical biogas production from wastewater sludge is estimated at $9 \times 10^6$ Nm³ annually. According to Oja (2009), methane yield from anaerobic digestion of sludge in Tartu is estimated at $0.4 \times 10^6$ Nm³/y. This amount is calculated to be sufficient to run 12 buses.

There are five (5) major companies in charge of the production of biogas for energy purposes in Estonia in 2011:
- **Terts AS** is in charge of the exploitation of Pääsküla Landfill (located in Tallinn) from 1994. Biogas is collected and distributed for the supply of heat and electricity to the local heating network and the national grid. After closure of the landfill, biogas yield was estimated at 5 million m³.

- **Tallinna Prügilagaas OÜ** operates a combined heat and power (CHP) plant with biogas collected from Jõelähtme landfill since February 2010.

- **Tallinna Vesi AS** recovers the biogas produced from the biodegradation of sewage sludge from Paljassaare Waste Water Treatment Plant since 1993. It is estimated an average biogas production of 2.8 million m³ per year with an energy content of 13.1 GWh. The company uses the biogas to supply the energy demands for running the facility.

- **Saare Economics OÜ** counts with a farm scale biogas digester built in 2004. The biomass used to feed the digester is pig slurry. The facility is located in Jööri Village in Saare County and collects its raw material from 8 swine farms all located on Saaremaa Island. Approximate biogas production is estimated to 2.4 million m³ per year with electricity and heat capacity of 350 kW_el and 420 420 kW_th per

---

**Table 1.** Annual volume of theoretical and applicable biogas and methane potential in Estonia (adapted from Oja, 2011).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Theoretical potential</th>
<th>Applicable potential*</th>
<th>Electricity production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biogas Nm³/y x 10⁶</td>
<td>Methane Nm³/y x 10⁶</td>
<td>Biogas Nm³/y x 10⁶</td>
</tr>
<tr>
<td>Hay from nature protection areas</td>
<td>72</td>
<td>43.2</td>
<td>14.4</td>
</tr>
<tr>
<td>Silage from unused fields</td>
<td>321</td>
<td>192.6</td>
<td>64.2</td>
</tr>
<tr>
<td>Silage from energy crops</td>
<td>4480**</td>
<td>2688</td>
<td>224</td>
</tr>
<tr>
<td>Landfill gas</td>
<td>21</td>
<td>12.6</td>
<td>16.8</td>
</tr>
<tr>
<td>Sewage sludge</td>
<td>9</td>
<td>5.4</td>
<td>4.5</td>
</tr>
<tr>
<td>Manure and slurry</td>
<td>111</td>
<td>66.6</td>
<td>66.6</td>
</tr>
<tr>
<td>Biowaste</td>
<td>10</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>544</strong>*</td>
<td><strong>326.4</strong></td>
<td><strong>391.5</strong></td>
</tr>
</tbody>
</table>

*: 20% loss during biogas production process. Estimated as 60% of Biogas potential
**: Not included in the total theoretical biogas production
***: Without the biogas originating from the silage from energy crops
year. Reactor digestate is being used by local farmers as composting additive and fertilizer.

- **Salutaguse Pärmitėhas AS** is a food industry company specialized in the elaboration of yeasts. Biogas is produced from residues of food processing and used for heat production only. There are also other sources of biogas in Estonia from landfills. However, biogas is not being collected and used for energetic purposes; instead biogas is being burnt in a flare.

In 2008, a study conducted by Luna-delRisco et al. (2008) based on the analysis of scientific publications, conference participations and reports on biogas related topics from 1998 to 2008 (based on four scientific databases: ISI Web of Science, Science Direct, Scopus and ETIS - only for Estonian researchers) have shown that Estonian researchers published only 1 to 2 papers yearly. However, from 2005 there has been a positive change and the number of papers rose up to 8. From 2008 to present, new projects have been approved at different universities (Estonian University of Life Sciences, University of Tartu, Tallinn Technical University) for the development of biogas production in Estonia but nevertheless the number of scientific publications have not yet increased to match the research being carried out in other countries like France, Spain, Sweden, Germany or Denmark. From the same study, it was found that the main interests in Estonia are biomass combustion, wind and solar energy, and anaerobic digestion.

The most recent initiative on anaerobic digestion research and development (R&D) was signed in 2010 between a group of three above-mentioned universities. The agreement (planned for a period of 5 years) aims at the execution of a project entitled “Anaerobic co-digestion process optimization for sewage sludge and agricultural waste based mixtures. Development of process monitoring and control methods”. The activities set for the accomplishment of the project are: application and development of standardized BMP measurement methods, optimization of anaerobic co-digestion process, nitrogen removal from the effluent of municipal wastewater with ANAMMOX process, co-digestion process parameters integrated relations, modeling and application in automation, feedstock resource and characteristics for improvement of co-digestion process efficiency, and the establishment of a database for digestate characteristics depending on co-digestion feedstock.
Nowadays, identified strengths of current R&D on biogas production in Estonia are:
- Research at lab-scale and pilot-scale are currently being developed.
- Equipment available for biofuel research, including lab-scale and pilot-scale anaerobic reactors.
- Awareness of society for technological and innovative developments.
- Substantial support for applied research by the Environmental Investment Centre (KIK).

However, there are still some aspects on the development of biogas production that should be improved:
- Few researchers in R&D working on anaerobic digestion.
- Low level of basic research financing in the field of anaerobic digestion.
- Coordination between research teams and cooperation with companies.
3. AIMS AND OBJECTIVES OF THE STUDY

The aim of the thesis is to provide an insight on methane production and its kinetics from agro-industrial substrates from Estonia. Substrates analyzed in this study were chosen according to national availability. Additionally, an ecotoxicological test was conducted to assess the effect of emerging pollutants – synthetic nanoparticles of metal oxides- in anaerobic system. The following objectives were set to extend the scope of the above-mentioned:

1. Create an online database (OpenAccess) with data on the chemical composition and methane potential of different substrates collected from the literature and with results from the experiments executed during this work (Paper I).

2. Evaluate the biochemical methane potential of herbal biomass (energy crops, silages, hay) and agro-industrial residues (distillery slops, grain mill residues, unconsumed milk products) from Estonia (Papers II-III).

3. Evaluate the influence of the chemical composition of biomass on the methane yield and kinetic rate (Papers III).

4. Investigate the particle-size effect of CuO and ZnO on the production of methane and biogas in anaerobic digestion (Paper IV).
4. MATERIALS AND METHODS

4.1. Database: design, organization and tools

The methanogenic potential database was built on an online platform based on 3 programming languages: HTML, PHP and MySQL and located at http://bioconversion.emu.ee/. Access to the database is free after online registration and approval by the administrator of the site. An internet browser is needed to access the database. Some browsers like Internet Explorer, Firefox and Google Chrome have been tested to assure an optimum performance of the database. Data is organized by type of substrate. 4 groups of substrates (i.e. crops, manures, mixed substrates and bio-wastes) have been set to offer users a comprehensive database where data is easy to find. For each substrate, data on the chemical composition and methanogenic potential is organized by tabs which include information on: chemical composition of substrates and digested solids (when available), methane potential, references and observations. Multiple data entries are referred to multiple references, each one accessible from the reference tab. Furthermore the database counts with an integrated search engine that helps users to find any information contained in the database by substrate, word or author.

4.2. Biochemical Methane Potential (BMP) test

The experimental design of this chapter of the research was based on a modified version of the BMP test proposed by Owen et al. (1979). (Papers II-III)

4.2.1. Inoculum and feedstock

The inoculum used for the analysis of the methanogenic potential of agro-industrial substrates was collected from the anaerobic reactor of a wastewater treatment plant in Tallinn, Estonia. Chemical composition of the inoculum was as follows: suspended solids (SS) 12.9 g/L, volatile suspended solids (VSS) 5.77 g/L. The inoculum was stored in 35 liter tanks, sieved through a 2 mm mesh and pre-incubated at mesophilic temperature range (36°C) for 5 days before use to ensure activation and degasification of the sludge. Chemical characterization of the inoculum on total solids (TS) and volatile solids (VS) were measured each time before test set-up. (Papers II-III).
Substrates analyzed on this thesis were collected from 2008 until 2010 in different Estonian farms and local industries. A total of 61 substrates were studied according to their availability in Estonia: energy crops (Jerusalem artichoke with and without flowers, sunflower collected at 2 different periods, hemp collected at 2 different periods, Amur silvergrass, energygrass and millet), silages (grass, maize, alfalfa, timothy grass, and red clover), hay, animal slurries (cattle and pig) and industrial residues such as unconsumed milk products, brewery residues (distillery slops) and grain mill residues (aspiration dust, bran and flour). Specific chemical parameters studied for each group of substrates are presented in Table 2.

For homogenization of the samples, silage and hay samples were dried at 65°C and milled to reach 1 mm. All other samples were used without any treatment.

### 4.2.2. Experimental procedure (Papers II-III)

The BMP test was performed in plasma bottles with a volume of 575 ml. The bottles were filled with 150 ml of inoculum and 0.3 gTS of substrate. Then, distilled water was added to the reaction mixture to reach an effective volume of 200 ml. In this test, no additional nutrients were added. Previous to the startup of the experiment, oxygen in the test bottles was flushed-out by purging with a flow of \( \text{N}_2/\text{CO}_2 \) (80:20) for 10 minutes. Test bottles were incubated at mesophilic temperature (36°C) inside Mermet isothermal thermo chambers. Initial basal pressure in the test and blank bottles was measured after acclimation.

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**Table 2. Variables analyzed for agro-industrial substrates to characterize chemical composition.**

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Nr. of samples</th>
<th>Variables analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agricultural substrates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Energy Crops</td>
<td>9</td>
<td>TS, VS, HE, CE, L</td>
</tr>
<tr>
<td>Silages</td>
<td>26</td>
<td>TS, VS, TP, HE, Ca, P, Mg, K</td>
</tr>
<tr>
<td>Hay</td>
<td>4</td>
<td>TS, VS, TP, HE, CE, L</td>
</tr>
<tr>
<td>Animal slurries</td>
<td>10</td>
<td>TS, VS, TN, HE, CE, L</td>
</tr>
<tr>
<td><strong>Industrial substrates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brewery residues</td>
<td>2</td>
<td>TS, VS, TOC</td>
</tr>
<tr>
<td>Unconsumed milk products</td>
<td>7</td>
<td>TS, VS, TP, Fats</td>
</tr>
<tr>
<td>Grain mill residues</td>
<td>3</td>
<td>TS, VS, TOC, HE, CE, L</td>
</tr>
</tbody>
</table>

TP: total proteins;  TN: total nitrogen;  HE: hemicellulose;  CE: cellulose;  L: lignin,
TOC: total organic carbon
of the medium at incubation temperature. For each substrate the duration of the BMP test was specifically determined. The methane production from inoculum was determined in blank tests where no substrate was added. Biogas production and gas composition were determined periodically. Mixing was done by shaking the bottles manually regularly at least once a day.

4.2.3. Analytical methods (Papers II-III)

Substrates were analyzed for pH, total solids (TS), volatile solids (VS), total organic carbon (TOC), total nitrogen (TN), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (ADL), Ca, P, Mg and K. The pH was measured by a Sentron 1001pH. TS and VS were determined according to Standard Methods (APHA, 1998). TOC was determined by catalytically-aided combustion technique (Shimadzu TOC-V), TN was determined by copper catalyst Kjeldhal method using a Kjekltec Auto 1030 and total proteins (TP) were calculated by multiplying TN values with a factor of 6.25 (TP = TN x 6.25) in the case of plant biomass and with a factor of 6.38 for milk proteins (Merrill and Watt, 1955, Merrill and Watt, 1973). NDF and ADF were determined using a Foss Tecator Fibertec 1020. Lignin was determined as described by AOAC 973.18 method. On the basis of NDF, ADF and ADL analysis, hemicellulose (NDF-ADF) and cellulose (ADF-ADL) concentrations were calculated as proposed by Van Soest et al. (1991). Ca, P and Mg were determined using a Fiastar 5000 following the o-cresolphthalein complexone method (Connerty and Briggs, 1966), the stannous chloride method (ISO/FDIS 15681 method, ISO 3696) and the titan yellow method (Heaton, 1960), respectively. Total fat concentration of unconsumed milk products were taken from the manufacturer.

Biogas production was measured by the increase in pressure in the test bottles using a calibrated pressure transducer (0-4 bar, Endress & Hauser). Methane content was analyzed chromatographically by means of a Micro-GC (Varian Inc., Model CP-4900) equipped with 2 columns: a Molsieve 5A Backflush heated column (20 m x 0.53 mm), and a PoraPLOT U heated column (10 m x 0.53 mm). Argon and helium were used as carrier gases in columns 1 and 2, respectively. Injection temperature, column temperature and column pressure were set to 110°C, 120°C and 50 Psi for column 1, and 110°C, 150°C and 22 Psi for column 2.
4.2.4. Calculations

Methane produced was calculated by subtracting the methane produced by the inoculum from the methane produced in the test with substrate and inoculum. Cumulative methane yield was calculated as the sum of methane produced over the incubation period minus the methane yield in blank test. Gas production was expressed in normal liters of methane (0°C, 1 atm) per kilogram of TS or VS of substrate added to the test (Papers II-III). Methane production was modeled by fitting the experimental data with non-linear regression models in GraphPad 5.0 (Papers II-III). The models used were: one-phase exponential association (Model 1, Equation 3) and two-phase exponential association (Model 2, Equation 4). Ultimate methane yields were calculated using above-described models for time t=100 days. Incubation time required to achieve 60, 70 and 80% of methane yield were calculated from the ultimate methane yield. One-way analysis of variance (ANOVA) was used to determine statistical significance (p<0.05) of differences between substrate groups. Correlation analysis was done by calculating Pearson’s correlation coefficients (r) and their significance levels p. p-values below 0.05 were regarded as significant. Statistical analyses were performed with STATISTICA version 8.0.360.0 (Statsoft, Inc.) using the Shapiro–Wilk’s test for normality, in which the null hypothesis is that data are normally distributed.

4.3. Anaerobic digestion inhibition test (Paper IV)

4.3.1. Inoculum and substrate

The inoculum used during the execution of the test was collected from the anaerobic reactor of a wastewater treatment plant in Tallinn, Estonia. Pre-conditioning of the inoculum was as previously described. Previous to the setup of the experiment, the inoculum was filtered with a 1 mm sieve, to allow for the removal of large particles, and then diluted to reach a fresh matter mass concentration of 2.1 gTS/L (ISO 13614-2). Cattle manure was used as the substrate during the execution of all inhibition experiments. Samples were dried at 60°C for two days, then milled and sieved to ensure that a homogeneous particle size with a diameter of 1 mm was achieved.
4.3.2. Test inhibitors

Copper oxide (CuO) and zinc oxide (ZnO) bulk and nanoparticles were purchased from Sigma-Aldrich. CuO and ZnO particle sizes were as follows: bulk CuO ~5 μm, nano CuO ~30 nm, bulk ZnO ~1 μm and nano ZnO 50-70 nm. Stock suspensions of 10 g/L were prepared in milliQ water by using a magnetic stirrer. Stock suspensions were diluted to reach a series of mass concentrations ranging from 7.5 to 480 mg/L.

4.3.3. Experimental procedure

The experiment was carried out according to a modified version of the ISO 13641-2 guidelines. Cattle manure was used as the substrate instead of yeast extract. The inhibition test was performed in 160 mL gas-tight closed serum bottles with 88 mL of reaction mixture (Table 3) and 5 mL of the inhibitor suspensions. All experiments were conducted in three replicates. Additionally, a set of three bottles as control, containing only the reaction mixture and 5 mL of distilled water, were included. Incubation of the samples was set to 36°C. Stirring was done twice a day during the duration of the experiment.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of inoculum</td>
<td>9 mL</td>
</tr>
<tr>
<td>Concentration of inoculum in test bottles</td>
<td>0.20 g TS/L</td>
</tr>
<tr>
<td>Test medium</td>
<td>9 mL</td>
</tr>
<tr>
<td>Dilution water</td>
<td>70 mL</td>
</tr>
<tr>
<td>Concentration of cattle manure in test bottles</td>
<td>9 gTS/L</td>
</tr>
<tr>
<td>Total liquid volume:</td>
<td>88 mL</td>
</tr>
</tbody>
</table>

Test medium used was following: (g/L): KH$_2$PO$_4$, 2.7; K$_2$HPO$_4$, 5.45; NH$_4$Cl, 5.3; CaCl$_2$·2H$_2$O, 0.75; MgCl$_2$·6H$_2$O, 1.0; FeCl$_2$·4H$_2$O, 0.2; resazurin, 0.01; Na$_2$S·9H$_2$O, 1.0. Trace element solution (g/L): MnCl$_2$·4H$_2$O, 0.5; H$_3$BO$_3$, 0.05; ZnCl$_2$, 0.05; CuCl$_2$·H$_2$O, 0.035; Na$_2$MoO$_4$·2H$_2$O, 0.01; CoCl$_2$·6H$_2$O, 1.0; NiCl$_2$·6H$_2$O, 0.1; and Na$_2$SeO$_3$, 0.05.

Before sample incubation, the pH of the test medium was measured to validate that the experiment was correctly set up. The pH measured in the test bottles were in the range of 6.9 ± 0.3. In addition, a batch
of 3,5-dichlorophenol with a series of mass concentrations ranging from 7.5 to 240 mg/L was also carried out. An EC50 equal to 71 mg/L validated the test.

### 4.3.4. Analytical methods

Biogas and methane were determined and calculated as previously described in chapters 4.2.3 and 4.2.4. Nanoparticles and microparticles of CuO and ZnO were inoculated with anaerobically digested sludge in a batch mode at 36°C for 14 days. Biogas production was used as an indicator of anaerobic digestion imbalance (Stuckey et al., 1980; Parkin and Speece, 1982; Hickey et al., 1989).

Copper and zinc concentrations in the supernatant were measured using a flame atomic absorption spectrometer (Shimadzu Co., Model AAS-6800) after 20-minute centrifugation at 11,000 rpm, acidification with 1% HNO₃ and filtration (GF/C; Whatman Co.). Operational configuration of the instrument was set according to the manufacturer’s recommendations.

### 4.3.5. Calculations

Inhibition of biogas and methane production was calculated by comparing the volume of biogas and methane produced in bottles containing the inhibitor with the controls. Calculation of common toxicity parameters (i.e., EC10, EC20, and EC50) was carried out using the Log-Normal model application within REGTOX software. Analyses on statistical differences between the effects of CuO and ZnO bulk and nanoparticles were performed using STATISTICA software. One-way analysis of variance (ANOVA) followed by t-test was used to determine significance (p<0.05) of statistical differences.
5. RESULTS AND DISCUSSION

5.1. Web-based database on methane potential of crops and wastes

For engineering anaerobic digesters, several operational parameters are required, particularly data on the chemical composition and the methane or biogas potential of different kinds of biomass suitable for anaerobic digestion.

In order to fulfill this demand, a database of published results including numerical data and the corresponding cited references was created for access from the web (Paper I, Fig. 2). One idea to develop the database was to provide users with homogeneous data. Data from scientific publications does not always have common units of measure. Thus, for this database, all input data were carefully checked and units standardized to match the metric system. In addition, the methanogenic potential database offers the following advantages: free access with personal login and password, data are constantly being updated by considering useful comments or references provided by registered users, a powerful search engine that helps users to find data within the different categories proposed, abstracts and full titles of the literature referred, and other useful capabilities.

By September 2011, the database comprises data on 226 different substrates from 88 references and laboratory work. In some cases, the same substrate has many data entries from different publications. There are about 535 different entries for the group of crops, 63 for manures, 98 for co-digestion and 102 for wastes. As for scientific references, the database includes scientific contributions from 1977 until 2011, from which 44% were presented/published within the last 5 years.

5.2. Chemical composition of agro-industrial substrates

The results on the chemical composition of the substrates analyzed in this study are presented in Tables 4, 5 and 6. Due to a wide variety of substrates from different sources, a specific set of analyses were considered for each group independently (Table 2).

Overall, the results obtained in this study are very consistent with the findings of other authors. The chemical composition of silages and
Fig. 2. Web-based database on methane potential from crops and wastes.

hay (Table 4) is very similar to that reported by Amon et al. (2007) and Dinuccio et al. (2010). The concentrations of macro nutrients found in this study (2-3 g P/kg TS, 5-10 g Ca/kg TS, 1-2 g Mg/kg TS and 14-25 g K/kg TS) are similar to the findings of Baležentienė and Mikulionienė, 2006 for timothy silages (P: 2.8 g/kg TS; Ca: 2.1 g/kg TS; Mg: 0.4 g/kg TS; K: 27.1 g/kg TS). Organic content and fiber concentrations found in animal slurries (750-800 g VS/kg TS and 70-115 g lignin/kg TS, respectively) appear to be consistent with the findings of Hobson et al. (1974), Varel et al. (1977), Wellinger (1984) and Robbins et al. (1989). The chemical composition of energy crops (Table 6) is within the same range of that found by other authors (Gunaseelan, 1997; Kreuger et al., 2007; Mursec et al., 2009; Klimiuk et al., 2010; Pakarinen et al., 2011). The chemical composition of unconsumed dairy products and selected agro-industrial residues (Table 5) was similar to the results from Steffen et al. (1998), Dubrovskis et al. (2009) and Dinuccio et al. (2010).
### Table 4. Chemical composition of different silages and hay from Estonia (standard deviations are presented in brackets).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>TS (g/kg)</th>
<th>VS (g/kgTS)</th>
<th>TP (g/kgTS)</th>
<th>HE (g/kgTS)</th>
<th>CE (g/kgTS)</th>
<th>L (g/kgTS)</th>
<th>P (g/kgTS)</th>
<th>Ca (g/kgTS)</th>
<th>Mg (g/kgTS)</th>
<th>K (g/kgTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass silage</td>
<td>4</td>
<td>314 (67)</td>
<td>927 (5.2)</td>
<td>114 (9.22)</td>
<td>219 (15.9)</td>
<td>-</td>
<td>-</td>
<td>2.55 (0.28)</td>
<td>6.11 (0.84)</td>
<td>1.44 (0.15)</td>
<td>24.49 (1.84)</td>
</tr>
<tr>
<td>Maiz silage</td>
<td>3</td>
<td>174 (6)</td>
<td>952 (5.3)</td>
<td>98.5 (8.5)</td>
<td>266 (37.7)</td>
<td>-</td>
<td>-</td>
<td>1.97 (0.4)</td>
<td>4.82 (0.3)</td>
<td>1.59 (0.19)</td>
<td>14.8 (0.9)</td>
</tr>
<tr>
<td>Silage mix*</td>
<td>19</td>
<td>294 (86)</td>
<td>920 (18)</td>
<td>147 (25)</td>
<td>178 (42)</td>
<td>-</td>
<td>-</td>
<td>2.81 (0.5)</td>
<td>9.22 (2.3)</td>
<td>1.95 (0.3)</td>
<td>23.5 (4.9)</td>
</tr>
<tr>
<td>Hay</td>
<td>4</td>
<td>913 (4)</td>
<td>937 (15)</td>
<td>99.2 (16)</td>
<td>272 (53.5)</td>
<td>354.58 (39)</td>
<td>58 (21)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n: number of samples tested for same substrate (each sample was analyzed in triplicate)

-: not determined

*: mixture of different ratios of grasses and legumes silages, mix rate not specified.

### Table 5. Chemical composition of unconsumed milk products and selected agro-industrial residues (standard deviations are presented in brackets).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>TS (g/kg)</th>
<th>VS (g/kgTS)</th>
<th>TOC (g/kgTS)</th>
<th>HE (g/kgTS)</th>
<th>CE (g/kgTS)</th>
<th>L (g/kgTS)</th>
<th>TP (g/kgTS)</th>
<th>Fats (g/kgTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unconsumed Cheese*</td>
<td>3</td>
<td>364 (171)</td>
<td>978 (16)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>334 (200)</td>
<td>495 (234)</td>
</tr>
<tr>
<td>Unconsumed Milk</td>
<td>4</td>
<td>117 (9)</td>
<td>993 (0.2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>295 (53)</td>
<td>277 (63)</td>
</tr>
<tr>
<td>Grain mill residues</td>
<td>3</td>
<td>860 (60)</td>
<td>916 (22)</td>
<td>415 (41)</td>
<td>313.1 (96)</td>
<td>140 (64)</td>
<td>50.7 (10)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Distillery slops</td>
<td>2</td>
<td>75 (28)</td>
<td>922 (14.1)</td>
<td>455 (50)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n: number of samples tested for same substrate (each sample was analyzed in triplicate)

-: not determined

*: includes sour cream
Table 6. Chemical composition of animal slurries and some energy crops from Estonia (standard deviations are presented in brackets).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>TS (g/kg)</th>
<th>VS (g/kgTS)</th>
<th>HE (g/kgTS)</th>
<th>CE (g/kgTS)</th>
<th>L (g/kgTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animal slurries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig slurry</td>
<td>1</td>
<td>70</td>
<td>794</td>
<td>145</td>
<td>104</td>
<td>72</td>
</tr>
<tr>
<td>Cattle slurry**</td>
<td>9</td>
<td>78 (28)</td>
<td>782 (30)</td>
<td>107 (13)</td>
<td>167 (7)</td>
<td>112 (10)</td>
</tr>
<tr>
<td><strong>Energy crops</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jerusalem Artichocke</td>
<td>2</td>
<td>911 (2)</td>
<td>952 (4)</td>
<td>49.8 (7)</td>
<td>234.6 (36)</td>
<td>53.8 (5)</td>
</tr>
<tr>
<td>Sunflower</td>
<td>2</td>
<td>910 (5)</td>
<td>885 (24)</td>
<td>62.4 (15)</td>
<td>307 (47)</td>
<td>80 (3.9)</td>
</tr>
<tr>
<td>Energy grass</td>
<td>1</td>
<td>920</td>
<td>930</td>
<td>273.3</td>
<td>378.5</td>
<td>96.5</td>
</tr>
<tr>
<td>Hemp</td>
<td>2</td>
<td>920 (2)</td>
<td>943 (6)</td>
<td>107 (1.6)</td>
<td>544 (8)</td>
<td>79.5 (11.4)</td>
</tr>
<tr>
<td>Amur Silvergrass</td>
<td>1</td>
<td>930</td>
<td>946</td>
<td>301</td>
<td>420</td>
<td>70</td>
</tr>
<tr>
<td>Foxtail millet</td>
<td>1</td>
<td>920</td>
<td>916</td>
<td>316</td>
<td>330</td>
<td>53.4</td>
</tr>
</tbody>
</table>

n: number of samples tested for same substrate (each sample was analyzed in triplicate)  
**: TN= 4.32 (0.34) g/kg TS

5.3. Biochemical methane potential of agro-industrial substrates

5.3.1. Cumulative methane yield

Results on the BMP are grouped according to their origin and presented in Table 7 and Fig. 3. Cumulative methane yields for grass silage, maize silage and mix silage were 319 L CH₄/kg VS, 307 L CH₄/kg VS and 296 L CH₄/kg VS, respectively, and they are consistent with the findings of others. Lehtomäki and Björnsson (2006), Cirne et al. (2007) and Lehtomäki et al. (2008) found in their study on grass silages a methane potential of 300-372 L CH₄/kg VS. For maize silage, Neureiter et al. (2005), Dubrovskis et al. (2009) and Pobeheim et al. (2010) found methane potentials ranging from 295 to 370 L CH₄/kg VS. Methane potential of hay (286 L CH₄/kg VS, Table 7) is similar to the result from Kaparaju et al. (2002) who found a value of 270 L/kg VS.

Cattle and pigslurry presented a methane potential of 238±42 L CH₄/kg VS and 317 L CH₄/kg VS, respectively. Steffen et al. (1998) and Vedrenne et al. (2008) found methane potential for pig slurry of 175-350 L/kg VS. For cattle slurry, a methane potential of 243 L/kg VS was found in the study conducted by Steffen et al. (1998).
Results on the methane potential of selected energy crops grown in Estonia are presented in Table 7. Heiermann et al. (2009) found an average methane potential of $280 \pm 30 \text{ L CH}_4/\text{kg VS}$ and $297 \pm 108 \text{ L CH}_4/\text{kg VS}$ for hemp and Jerusalem artichoke, which are in agreement with the results of this study ($289 \text{ L CH}_4/\text{kg VS}$ and $310 \text{ L CH}_4/\text{kg VS}$, respectively). For sunflower, Antonopoulou et al. (2010) found a methane potential of $260 \text{ L/kg VS}$, slightly lower than the value measured in this study ($296 \text{ L CH}_4/\text{kg VS}$). Pokój et al. (2010) studied amur silver grass and obtained a methane potential of $210 \text{ L/kg VS}$ which is much lower than the result from this study ($317 \text{ L CH}_4/\text{kg VS}$). Similarly, the methane yield of millet ($323 \text{ L CH}_4/\text{kg VS}$) was higher than those observed by Mahamat et al. (1989) ($257 \text{ L CH}_4/\text{kg VS}$). This variation on the methane potential of sunflower, amur silver grass and millet could be explained by differences in harvesting time or chemical composition (Kreuger et al., 2007 and Heiermann et al., 2009).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>CH$_4$ L/kg TS</th>
<th>CH$_4$ L/kg VS</th>
<th>Bo L/kg VS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass silage</td>
<td>4</td>
<td>296 (19)</td>
<td>319 (19)</td>
<td>320 (22)</td>
</tr>
<tr>
<td>Maiz silage</td>
<td>3</td>
<td>292 (21)</td>
<td>307 (21)</td>
<td>339 (26)</td>
</tr>
<tr>
<td>Silage mix*</td>
<td>19</td>
<td>272 (31)</td>
<td>296 (31)</td>
<td>307 (28)</td>
</tr>
<tr>
<td>Hay</td>
<td>4</td>
<td>268 (33)</td>
<td>286 (33)</td>
<td>292 (30)</td>
</tr>
<tr>
<td>Pig slurry</td>
<td>1</td>
<td>252</td>
<td>317</td>
<td>321</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>9</td>
<td>186 (42)</td>
<td>238 (42)</td>
<td>247 (58)</td>
</tr>
<tr>
<td>Jerusalem Artichoke</td>
<td>2</td>
<td>294 (4)</td>
<td>310 (7)</td>
<td>311 (6.7)</td>
</tr>
<tr>
<td>Sunflower</td>
<td>2</td>
<td>262 (8)</td>
<td>296 (15)</td>
<td>297 (16.4)</td>
</tr>
<tr>
<td>Energy grass</td>
<td>1</td>
<td>270</td>
<td>290</td>
<td>312</td>
</tr>
<tr>
<td>Hemp</td>
<td>2</td>
<td>272 (9)</td>
<td>289 (11)</td>
<td>316 (18)</td>
</tr>
<tr>
<td>Amur Silvergrass</td>
<td>1</td>
<td>300</td>
<td>317</td>
<td>328</td>
</tr>
<tr>
<td>Foxtail millet</td>
<td>1</td>
<td>296</td>
<td>323</td>
<td>324</td>
</tr>
<tr>
<td>Unconsumed Cheese**</td>
<td>3</td>
<td>644 (60)</td>
<td>658 (56)</td>
<td>659 (57)</td>
</tr>
<tr>
<td>Unconsumed Milk</td>
<td>4</td>
<td>478 (24)</td>
<td>481 (24)</td>
<td>483 (26)</td>
</tr>
<tr>
<td>Grain mill residues</td>
<td>3</td>
<td>300 (38)</td>
<td>328 (49)</td>
<td>330 (56)</td>
</tr>
<tr>
<td>Distillery slops</td>
<td>2</td>
<td>331 (35)</td>
<td>358 (33)</td>
<td>393 (10)</td>
</tr>
</tbody>
</table>

---

n: number of samples tested for same substrate (each sample was analyzed in triplicate)
*: Mixture of different ratios of grasses and legumes silages, mix rate not specified
**: Includes sour cream
energy grass (Szavvasi-1), Janowszky and Janowszky (2002) have reported methane potential of 300-350 L CH$_4$/kg VS, slightly higher than the value of this study (290 L CH$_4$/kg VS).

To our knowledge, no detailed studies have been conducted on the methane potential of unconsumed milk products. Due to this lack of information, we were only able to compare our results (Table 7) with the ones obtained from utilization of whey as substrate. Dinuccio et al. (2010) found a methanogenic potential of 501 L CH$_4$/kg VS for whey. This result appears to be within the same range of our findings (480-660 L CH$_4$/kg VS).

For grain mill residues, the methane yield observed in this study (328 L CH$_4$/kg VS) was much higher than reported by Dubrovskis et al. (2009b) who obtained a methane yield of 130 L/kg VS from grain mill wastes. This variation can be explained by the difference in the chemical composition of the substrate. Methane potential of distillery slops (358 L CH$_4$/kg VS, Table 7) was in the same range as the results obtained by Steffen et al. (1998) for fermentation slops (338 L CH$_4$/kg VS).

![Fig. 3. Methane potentials of agro-industrial substrates from Estonia. Error bars indicate standard deviation.](image)
5.3.2. Ultimate methane yield

Results on ultimate methane yield of analyzed substrates are presented in Table 7. Ultimate methane yields were calculated by fitting measured data with two different models. For methane production modelling, the first order degradation model (Model 1, Equation 3) has been widely used in different studies (Hashimoto, A.G., 1986, Gunaseelan, 2004; 2009b). However, analyzing our data with this model indicated poor fitting results for silages, hay, and energy crops (Fig. 4). Since Rao et al. (2000) and Rincon et al. (2010) found that biogas production from solid organic substrates were best fitted by the pseudo-parallel first order model, similar two-phase exponential model (Model 2, Equation 4) was also tested in this study (Paper II). It is considered that methane production curves correspond to the rapid bioconversion of readily degradable components followed by a slower bioconversion of fibrous portion of the substrates. Correlation coefficients obtained after data fitting were in the range of 0.987-

![Fig. 4](image-url)
0.999, 0.985-0.996, 0.957-0.999 and 0.994-0.998 for grass silage, maize silage, mixed silage and hay respectively. As for energy crops, all correlations obtained were above 0.99.

Ultimate methane yields for animal slurries, unconsumed milk products, grain mill residues and distillery slops were calculated by fitting our data to the one-phase exponential association model (Fig. 5). The correlation coefficient for pig slurry data was 0.946 and for cattle slurry varied between 0.906 and 0.995.

### 5.3.3. Kinetic evaluation of biomass bioconversion

For anaerobic digestion purposes, it is important to define the optimum retention times for a defined substrate to reach its maximum potential. From a technical or economical point of view, retention times can be targeted at a level when substrates have reached a certain percentage of their potential ultimate methane production. Table 8 shows times needed for reaching 60%, 70% and 80% of the potential ultimate production of methane in BMP tests.

Anaerobic digesters are often designed to operate with a mixture of several substrates. In general, retention times can vary from 20 to 40 days. In this study, most of the analyzed substrates had produced at least 80% of their ultimate yield within the first 20 days, except for energy grass, hemp, amur silvergrass and cattle slurry (Paper II-III). Anaerobic biodegradation of these substrates was between 23 and 31 days. Long digestion periods obtained from these substrates can be explained by their high concentrations of lignin and hemicellulose (Schievano et al.,
Bioconversion of milk wastes occurred very rapidly. 80% of the ultimate methane yield was reached after only 3-8 days of incubation (Table 8). However, although milk products could reach high methane yields, special care needs to be taken to avoid inhibition by ammonia in anaerobic digesters (Callaghan et al., 1997).

To characterize the conversion rate of studied substrates, kinetic rate constants k were calculated. The highest kinetic rate constant was found for unconsumed milk products (0.344±0.03 1/d) while the lowest was found for energy grass (0.061 1/d). As for agricultural biomass, k for grass silage, maize silage, silage mix and hay varied

Table 8. Time to reach corresponding percentages of ultimate methane yield.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>60% Bo L/kgVS Days</th>
<th>70% Bo L/kgVS Days</th>
<th>80% Bo L/kgVS Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass silage</td>
<td>4</td>
<td>196</td>
<td>222</td>
<td>256</td>
</tr>
<tr>
<td>Maize silage</td>
<td>3</td>
<td>209</td>
<td>239</td>
<td>272</td>
</tr>
<tr>
<td>Mix silage</td>
<td>18</td>
<td>193</td>
<td>215</td>
<td>247</td>
</tr>
<tr>
<td>Jerusalem Artichoke</td>
<td>2</td>
<td>200</td>
<td>222</td>
<td>254</td>
</tr>
<tr>
<td>Sunflower</td>
<td>2</td>
<td>180</td>
<td>209</td>
<td>239</td>
</tr>
<tr>
<td>Energy grass</td>
<td>1</td>
<td>176</td>
<td>204</td>
<td>234</td>
</tr>
<tr>
<td>Hemp</td>
<td>2</td>
<td>177</td>
<td>208</td>
<td>237</td>
</tr>
<tr>
<td>Amur Silvergrass</td>
<td>1</td>
<td>194</td>
<td>228</td>
<td>260</td>
</tr>
<tr>
<td>Foxtail millet</td>
<td>1</td>
<td>195</td>
<td>228</td>
<td>260</td>
</tr>
<tr>
<td>Hay</td>
<td>4</td>
<td>179</td>
<td>206</td>
<td>233</td>
</tr>
<tr>
<td>Pig slurry</td>
<td>1</td>
<td>194</td>
<td>225</td>
<td>260</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>9</td>
<td>150</td>
<td>173</td>
<td>198</td>
</tr>
<tr>
<td>Cheese</td>
<td>1</td>
<td>396</td>
<td>463</td>
<td>530</td>
</tr>
<tr>
<td>Sour cream</td>
<td>1</td>
<td>434</td>
<td>502</td>
<td>570</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>1</td>
<td>361</td>
<td>423</td>
<td>481</td>
</tr>
<tr>
<td>Buttermilk</td>
<td>1</td>
<td>296</td>
<td>343</td>
<td>391</td>
</tr>
<tr>
<td>Milk 2,5 % Fat</td>
<td>1</td>
<td>277</td>
<td>321</td>
<td>367</td>
</tr>
<tr>
<td>Milk 3,5 % Fat</td>
<td>1</td>
<td>284</td>
<td>325</td>
<td>372</td>
</tr>
<tr>
<td>Raw milk</td>
<td>1</td>
<td>308</td>
<td>360</td>
<td>409</td>
</tr>
<tr>
<td>Distillery slop (a)</td>
<td>1</td>
<td>249</td>
<td>289</td>
<td>324</td>
</tr>
<tr>
<td>Distillery slop (b)</td>
<td>1</td>
<td>232</td>
<td>275</td>
<td>310</td>
</tr>
<tr>
<td>Grain mill - Aspiration dust</td>
<td>1</td>
<td>171</td>
<td>205</td>
<td>235</td>
</tr>
<tr>
<td>Grain mill - Bran</td>
<td>1</td>
<td>208</td>
<td>253</td>
<td>281</td>
</tr>
<tr>
<td>Grain mill - Flour</td>
<td>1</td>
<td>234</td>
<td>279</td>
<td>315</td>
</tr>
</tbody>
</table>

n: number of samples tested for same substrate (one sample represents the average of three replica)
between 0.086 and 0.230 1/d. Chynoweth et al. (1993) found conversion rate constants for different ensiled substrates (millet, energycane, napiergrass) ranging from 0.072 to 0.106 1/d. In the case of animal slurries, k values for pig slurry (0.139 1/d) were higher than for cattle slurry (0.092 1/d). The conversion rate constant for cattle manure is similar to the result from Sánchez et al. (2000) who found a value of 0.086±0.004 1/d.

Kinetic rates obtained for energy crops were: Jerusalem artichoke 0.179±0.02 1/d, sunflower 0.154±0.04 1/d, energy grass 0.061, hemp 0.095±0.01 1/d, Amur silvergrass 0.064 1/d, foxtail millet 0.101 1/d. For agro-industrial substrates, the lowest rate constant was found for distillery slops (0.131±0.03 1/d). In a study conducted by Jiménez et al. (2004) on the anaerobic digestion of untreated molasses, a conversion rate constant of 0.14 1/d (9g COD added) was found. Conversion rates of unconsumed dairy products (0.260 – 0.344 1/d,) were slightly lower than the results obtained by Najafpour et al. (2009) for cheese whey (0.358 1/d). The different chemical composition of the substrates could explain the difference in the rates. The kinetic rate constant for grain mill residues was determined at 0.160±0.03 1/d. No data about rate constants was found in the literature for comparison.

5.3.4. Correlations between the chemical composition of biomass and biochemical methane potential

Correlations between the cumulative methane production (in LCH₄/kg TS) and the methane production rate constant with the chemical characteristics of substrates are presented in Table 9 and Fig. 6 and 7.

Among the different chemical parameters, only VS, total proteins (TP), hemicellulose (HC), lignin (L), P, Ca and K showed significant influence on the methane yield as single independent variables (Table 9). As expected, one of the main parameters influencing methane yield was organic matter, i.e. VS content, whose correlation with methane production was significantly positive. Proteins are also known to influence methane formation positively and therefore a high methane yield can be attained from substrates rich in proteins (Amon et al., 2007).

In the case of fiber composition, hemicellulose correlated positively with methane production (p<0.05), although the correlation was poor. For
cellulose, no significant correlation was found. Previous studies confirm that cellulose and hemicellulose can be bioconverted into methane and carbon dioxide during anaerobic digestion. However, the degradation rate of cellulose depends mainly on whether it is lignin-incrusted or in a crystalline form Klimiuk et al. (2010). Lignin content presented a strong negative correlation with methane production. Our results appear to be consistent with the findings of many other authors (Schievano et al., 2008; Hendriks and Zeeman, 2009; Klimiuk et al., 2010), identifying lignin as a complex plant constituent very difficult to digest by anaerobic bacteria and therefore a low methane yield is achieved at very low rates.

Macronutrients (P, Ca, Mg and K) were only measured for silages and their Pearson’s correlations with methane yield were found to be negative and statistically significant. P and Ca are known for being essential for metabolic reactions and growth of anaerobic bacteria (Chen et al., 2008) but they can become toxic when present in high concentrations (Kugelman and McCarty, 1964; Jackson-Moss et al., 1989; Van Langerak et al., 1998). In our study, concentrations of these elements in the biomass were not excessively high to provoke a

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Cumulative methane yield</th>
<th>Kinetic rate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td>60</td>
<td>-0.168</td>
<td>0.221</td>
</tr>
<tr>
<td>VS</td>
<td>60</td>
<td>0.785</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TOC</td>
<td>7</td>
<td>0.36</td>
<td>0.427</td>
</tr>
<tr>
<td>TP</td>
<td>37</td>
<td>0.767</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Fats</td>
<td>7</td>
<td>0.365</td>
<td>0.421</td>
</tr>
<tr>
<td>HE</td>
<td>45</td>
<td>0.343</td>
<td>0.029*</td>
</tr>
<tr>
<td>CE</td>
<td>20</td>
<td>-0.1</td>
<td>0.722</td>
</tr>
<tr>
<td>L</td>
<td>18</td>
<td>-0.917</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>P</td>
<td>26</td>
<td>-0.473</td>
<td>0.016*</td>
</tr>
<tr>
<td>Ca</td>
<td>26</td>
<td>-0.563</td>
<td>0.002*</td>
</tr>
<tr>
<td>Mg</td>
<td>26</td>
<td>0.059</td>
<td>0.771</td>
</tr>
<tr>
<td>K</td>
<td>26</td>
<td>-0.613</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

r: Pearson’s correlation coefficient
p: probability
*: Statistically significant correlations (p<0.05)
Fig. 6. Pearson’s correlation between methane yield and chemical parameters ($p<0.05$). 95% confidence intervals are presented in dash lines.
Fig. 7. Pearson’s correlation between methane production rate constant and chemical parameters ($p<0.05$). 95% confidence intervals are presented in dash lines.
negative effect on methane production. So, it can be assumed that the different chemical composition of specific crops in grasses, silages and hay samples and the different ratios (not known) of crops in analyzed samples affected the methane yield and were reasons for the negative correlation. Accumulation of mineral elements in plants depends on soil properties, cultivation and fertilization, climate, harvesting time as well as plant properties (Juknevičius and Sabienė, 2007). Various plant species have a different ability to accumulate mineral elements, therefore content of Ca, P and K can differ significantly in different crops, especially between legume and grass species (Baležentienė and Mikulionienė, 2006).

Concerning the methane production rate constant (k), positive correlations ($p<0.05$) were only found with P, Ca, Mg and K (Table 9, Fig. 7). These results suggest that P and light metal ions enhance the speed of the anaerobic biodegradation process. The most rapid bioconversion of studied substrates occurred in the tests with unconsumed milk products which contained a high amount of proteins. In contrast, the higher content of lignocellulosic material (hemicellulose, cellulose and lignin) in the substrate, the lower the rate of methane production (Fig. 7).

5.4. Effect of bulk and nanoparticles of CuO and ZnO on biogas and methane production

This sub-chapter focuses on the effect that nanoparticles of CuO and ZnO have on biogas and methane production during anaerobic digestion. Additionally, a test was conducted with their respective bulk counterparts to evaluate if toxicity was induced by particle-size difference. According to the technical data sheet for bulk and nanoparticles of CuO and ZnO, aqueous solubility is very low, and therefore inhibition to anaerobic bacteria was theoretically unlikely.

However, in our study an important negative effect on anaerobic digestion was discovered when these particles were present in the medium of the anaerobic process.

The production of biogas during the incubation period from the control and tests with different ranges of mass concentrations of bulk and nanoparticles of CuO and ZnO are illustrated in Figs. 8 and 9. In the
Fig. 8. Biogas inhibition from bulk CuO (A) and nano CuO (B).

Fig. 9. Biogas inhibition from bulk ZnO (C) and nano ZnO (D).
experiment nanoparticles of CuO showed higher influence on biogas production than the other test compounds. The concentration of 15 mg/L of CuO nanoparticles has provoked a reduction of 30% of the biogas production from the total biogas produced in the control at day 14. Biogas production in the presence of microparticles of CuO was less inhibited whereas concentrations of 120 and 240 mg/L of bulk CuO caused a reduction by 19 and 60%, respectively. The statistical analyses have validated the differences between the 2 groups of particles tested (bulk and nanoparticles) of CuO (p<0.05). As reported by Heinlaan et al. (2008), Neal (2008) and Kasemets et al. (2009) nanoparticles are toxic to bacteria due to the release of bioavailable metal ions that causes cell membrane damage, and therefore the inhibition of biogas production can occur.

Biogas production in test samples containing nanoparticles of ZnO is compared with bulk ZnO in Fig. 9. Concentrations of 120 and 240 mg/L of ZnO nanoparticles presented an inhibition of 43 and 74% of the biogas yield respectively, while test bottles containing bulk ZnO presented a reduction of 18 and 72% of the total biogas produced at day 14. However, no significant difference of biogas inhibition from bulk and nanoparticles of ZnO was found.

The effective concentrations of metals causing a reduction of methane production by 50% (EC50) were calculated. Results were used for comparing the influence of different particle sizes (micro and nano) of CuO and ZnO. Fig. 10 and 11 present the inhibition of methane production by different concentrations of copper and zinc (in their respective oxides form) during an incubation period of 14 days. EC50 values for bulk and nanoparticles of CuO were calculated at 129 and 10.7 mgCu/L, and for ZnO at 101 and 57.3 mgZn/L, respectively. Data about EC10, EC20 and EC50 with their confidence intervals are presented in Table 10.

The results from Fig. 10 and 11, and Table 10 showed that nanoparticles of CuO (~30 nm) have inhibited the production of methane at least 10 times more than its bulk counterparts. The difference between bulk and nanoparticles of CuO were found to be statistically significant (p<0.0001). Complete inhibition of methane production in the presence of CuO occurred at concentrations of 330 and 30.2 mgCu/L for bulk and nanoparticles respectively.
Although, a significant difference on biogas inhibition from bulk and nanoparticles of ZnO was not found, methane inhibition from bulk and nano ZnO was different (Fig. 11). ZnO nanoparticles (50-70 nm) were about 2 times more toxic than bulk ZnO. Statistical difference between the two groups was found at p<0.005. Complete inhibition of methane production occurred at concentrations of 246 and 181 mgZn/L for bulk and nanoparticles of ZnO respectively.

**Table 10.** Toxicity of bulk and nano CuO and ZnO to methane-forming bacteria.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>EC10 mg/L of metal Average</th>
<th>95% C.I.</th>
<th>EC20 mg/L of metal Average</th>
<th>95% C.I.</th>
<th>EC50 mg/L of metal Average</th>
<th>95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk CuO</td>
<td>54,8</td>
<td>43,0</td>
<td>67,3</td>
<td>73,4</td>
<td>61,7</td>
<td>85,3</td>
</tr>
<tr>
<td>Nano CuO</td>
<td>3,94</td>
<td>3,62</td>
<td>4,17</td>
<td>5,56</td>
<td>5,20</td>
<td>5,82</td>
</tr>
<tr>
<td>Bulk ZnO</td>
<td>39,8</td>
<td>30,6</td>
<td>52,2</td>
<td>53,6</td>
<td>44,1</td>
<td>66,1</td>
</tr>
<tr>
<td>Nano ZnO</td>
<td>19,5</td>
<td>15,7</td>
<td>24,1</td>
<td>28,2</td>
<td>23,9</td>
<td>33,4</td>
</tr>
</tbody>
</table>

C.I.: Confidence interval

**Fig. 10.** Dose-response curves of methane production during exposure of bulk CuO (A) and nano CuO (B).
In our experiment, nanoparticles of CuO and ZnO (Figs. 8-11) showed higher toxicity to anaerobic microorganisms than their bulk counterparts. Similar results have been presented by different authors. In the study conducted by Heinlaan et al. (2008), toxicity of bulk and nanoparticles of CuO (particle size 30 nm) to bacteria *Vibrio fischeri* presented EC50 of 3049±819 and 63±22 mgCu/L, while ZnO (particle size 50-70 nm) showed the inhibition with EC50 of 1.4±0.08 and 1.5±0.16 mgZn/L respectively. On the other hand, results from test crustaceans *Daphnia magna* showed EC50 of 131.8±19.7 and 2.6±1.3 mgCu/L for bulk and nano CuO respectively, and 7.1±1.1 and 2.6±1.04 mgZn/L for bulk and nano ZnO respectively.

Nanoparticles of CuO were identified as the most toxic to anaerobic bacteria from all tested metal oxides (Table 10) in our study. The obtained results appear to be consistent with the findings of others studying toxicity of nanoparticles of metal oxides to different type of

**Fig. 11.** Dose-response curves of methane production during exposure of bulk ZnO (C) and nano ZnO (D).
microorganisms. Kasemets et al. (2009) studied the toxicity of bulk and nanoparticles of CuO and ZnO at 8 h of growth of *S. cerevisiae*. In their study, nano CuO presented higher toxicity compared with nano ZnO. They found EC50 of 16.6 mgCu/L for nano CuO while nano ZnO presented an EC50 of 97.4 mgZn/L.

Zayed and Winter (2010) have studied the influence of Cu and Zn on methane production. In their study, the authors tested the toxicity of CuCl₂ and ZnCl₂ during anaerobic digestion of whey. EC50 values of 4.7 mgCu/L and 19.2 mgZn/L were found. These results are comparable with our data where it was found that nanoparticles of copper oxide had higher toxicity during methane production than nanoparticles of zinc oxide, even though nano CuO and nano ZnO have been reported to have very low solubility in water, unlike CuCl₂ and ZnCl₂. In addition, nanoparticles of CuO inhibited methane production at similar concentrations as Cu ions in the case of soluble salts of copper (CuCl₂). However, methane inhibition from ZnCl₂ is about 2 times more toxic than our data obtained from nanoparticles of ZnO.

Results presented in Paper IV demonstrate higher solubility of nanoparticles of CuO compared to the bulk CuO. These results suggest that toxicity of nanoparticles of CuO and ZnO to anaerobic bacteria can be attributed to the dissolved bioavailable fractions of these metals. The ecotoxicological study by Aruoja et al. (2009) also concluded that the toxicity of CuO nanoparticles can be attributed to the higher solubility of nanoparticles in the test medium. However, a comparison of the concentrations of Cu ions found in the reaction mixture with the EC50 values obtained by Zayed and Winter (2000) for Cu ions from CuCl₂ shows that the toxicity of nanoparticles can be only partly explained by the dissolution of CuO nanoparticles to Cu ions. Most likely, different adverse effects of nano- and micro-sized particles to anaerobic process are still partly due to their different surface areas and surface characteristics (Karlsson et al., 2009).
6. CONCLUSIONS

The methanogenic potential database created offers, to its users, unified data on the chemical composition and methanogenic potential of various types of crops and organic wastes with corresponding references, in an OpenAccess environment. For scientists working on anaerobic digestion, the database can be used for substrate comparison, study of the influence of biomass composition on methane yield and for the purposes of statistical analysis. As for engineers, the database can be considered as a decision-support tool, as data on the principal parameters (i.e. retention time, temperature, chemical composition, biogas and methane potential, digestate characteristics, etc.) for designing a biogas unit are provided.

Results from herbal biomass, animal slurries and agro-industrial residues from Estonia have shown great methane potential. Results obtained range between 238 L CH₄/kg VS for cattle slurry up to 658 L CH₄/kg VS for unconsumed cheese products. The fastest bioconversion into methane was found for unconsumed milk (2.5% Fat). It took only 3 days to achieve 80% of the ultimate methane yield, while for energy grass it took 31 days. The results show that biogas production from agricultural substrates can be very feasible. However, in order to take all necessary considerations (i.e. co-digestion) to fulfill the requirements of a commercial system, pilot scale testing is necessary.

Only few data have been published related to the impact of nanoparticles in anaerobic systems. Results obtained in this study are considered to contribute significantly to the characterization of potential effects of nanoparticles during anaerobic digestion. Results revealed that particle size of CuO and ZnO had a direct influence on biogas and methane yield. Inhibitory effect can be attributed to the release of toxic metal ions, i.e. Cu²⁺ and Zn²⁺. However, further studies on other factors such as surface area and surface characteristics is needed in order to obtain a better knowledge about toxicity mechanisms.

The concept of herbal biomass utilization for biogas production in Estonia offers an effective solution to fulfill the energy demand by using the actual abandoned land with suitable energy crops with high energy potential. Furthermore, the utilization of animal slurries and agro-industrial residues can be considered as suitable co-substrates in anaerobic facilities. In addition, their utilization can also be considered
as an important waste management strategy with positive results from the economic and environmental point of view. However, it is crucial to establish a bond between farmers and industries with the government to invest in projects for the creation of new biogas production facilities.
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Energia sõltuvus fossiilsetest kütustest on sundinud riike üle maailma leidma uusi alternatiive energia tootmiseks. Võimalike taastuvate energialikute seas peetakse üheks potentsiaalseks energia saamise alternatiiviks biogaasi toomist, kuna samaaegselt lahendatakse ökoloogilisi probleeme. Anaeroobse kääritamise protsess, milles rühm spetsialiseerunud anaeroobseid baktereid muudab organilist biomassi biogaasiks, on hästi dokumenteeritud. Siiski ei ole andmed tavaliste keemiliste näitajate ja biomassi metaanitootlikkuse potentsiaali kohta alati esitatud standardiseeritud vormis, mis teeb nende interpretatsiooni raskeks nii teadlaste kui ka biogaasijaamade operaatorite jaoks.


Käesoleva doktoritöö peamisteks eesmärkideks olid
1) andmebaasi loomine, mis koondaks kirjanduses avaldatud teadustöö- de ja rakenduslike projektidte andmeid erinevate substraatide keemili- se koostise ja metaanitootlikkuse potentsiaali kohta (artikkel I);
2) Eesti põllumajandusliku biomassi ja põllumajandustootmise jäätmite metaanitootlikkuse potentsiaali ja metaani tootmise kineetika uurimine (artiklid II ja III);
3) metallioksiidide nanoosakeste kahjuliku mõju uurimine biogaasi tootmisel, võrreldes neid tava- ehk mikrosuuruses osakestega (artikkel IV).

Metoodika


delit kasutades arvutati üldlevinud toksilisusparameetrid EC10, EC20 ja EC50 (artikkel IV).

**Tulemused ja arutelu**

Loodud metaanitootlikkuse potentsiaali andmebaas sisaldas 2011. a septembriks andmeid 226 erineva substradi kohta 88 kirjandusallikast. Sama substradi kohta on sageli andmeid erinevatest publikatsioonidest. Andmebaasis on 535 erinevat kirjet energiakultuuride, 63 sönniku, 98 kooskääritamise ja 102 jäämete rühmas (artikkel I).

Katsetöös uuritud substratide keemilise koostise andmed sarnanesid teiste autorite poolt avaldatud tulemustega. Rohtse biomassi (energiakultuurid, silod, hein) metaanisaagised olid vahemikus 286-323 L CH₄/kg VS. Vedelsõnniku proovidest oli sea vedelsõnnikut kõrgem metaanisaagis (317 L CH₄/kg VS) kui veise vedelsõnnikut (238 L CH₄/kg VS). Analüüsitud energiakultuuridest oli metaanitootlikkus kõige kõrgem aasrebasesaba korral (323 L CH₄/kg VS). Uuritud toiduainetööstuse ülejääkidest oli kõrgeim metaanisaagis juustul (658 L CH₄/kg VS), samas kui madalaim saagis leiti teraviljaveski jääkidest (328 L CH₄/kg VS) (artiklid II ja III).

Kõrgeim kineetiline kiiruskonstant määrati piima (0,344 ± 0,03 1/d) ja madalaim (0,061 1/d) energiarohu Szarvasi-1 korral. Põllumajandusliku rohtse biomassi (rohusilo, maisisilo, silosegu ja hein) korral olid kiiruskonstandid vahemikus 0,086–0,230 1/d. Sea vedelsõnnikust oli metaani tekkimine kiirem kui veise vedelsõnnikut, kiiruskonstandid vastavalt 0,139 ja 0,092 1/d. Tööstuslikest jääkidest oli metaani tekkimine aeglaseim katsetes piiritustööstuse praagaga. Erinevused protsessi kiirustes on seletatavad substratide erineva keemilise koostisega (artikkel III).

Analüüsides substratide metaanisaagise sõltuvust keemilisest koostisest, ilmnes metaani saagise positiivne korrelatsioon orgaanilise aine ja valkude sisaldusega substradi koostises. Ligniinisalsaldus substratides mõjutas metaani produktiooni negatiivselt. Olulised positiivsed seosed keemilise koostise ja kiiruskonstandi vahel leiti P, Ca, Mg ja K puhul. Mida kõrgeim oli aga lignotselluloosse materjali (hemitselluloos, tselluloos, ligniin) sisaldus substratides, seda väiksem oli metaani tekke kiirus (artikkel III).
Inhibeerimistestides näitasid CuO ja ZnO nanoosakesed negatiivsemat mõju anaeroobsele käärimisprotsessile kui nende tava- ehk mikrosuuruses osakesed. Katsetes ZnO nanoosakestega oli biogaasi ja metaani teke vähem inhibeeritud kui katsetes CuO nanoosakestega. Metaani teket uurides leitud EC50 väärtused tava- ja nanosuuruses CuO-osakestega katsetes olid vastavalt 129 ja 10,7 mg Cu/L, ZnO puhul vastavalt 101 ja 57,3 mg Zn/L. Keemiline analüüs näitas CuO nanoosakeste suuremat lahustuvust, võrreldes mikrosuuruses osakestega (artikkel IV). Tulemustest selgus, et nanosuuruses CuO inhibeeriv mõju võib osaliselt olla põhjustatud lahustunud Cu$^{2+}$-ioonidest katekeskkonnas.

Järeldused

Loodud andmebaas võimaldab kasutajatel koondatult ja ühtsetele ühikutele viidult kätte saada andmeid erinevate kultuuride ja orgaaniliste jäätmete keemilisest koostisest ja metaanitootlikkuse potentsiaali kohta koos vastavate kirjandusviidetega Open Accessi keskkonnas. Teadlased saaksid andmebaasi kasutada substraatide võrdlemisel, uurides biomasstoostise mõju metaani saagisele, ja statistilise analüüsi eesmärgil. Insenerite jaoks võiks andmebaas olla oluline abivahend substraadide diagnostikas, uurides biomassi koostise mõju metaani saagisele ja statistilise analüüsi eesmärgil.

Doktoritöö tulemused näitasid Eesti rohtse biomassi, loomade vedelsooni kõrget metaani tootlikkuse potentsiaali. Saadud metaanisaagised varieerusid vahemikus 238 L CH$_4$/kg VS (veise vedelsoonik) kuni 658 L CH$_4$/kg VS (juustujäägid). Kiireim substratide biokonversioon metaaniks toimus katsetes piimaga, kus kulus ainult kolm päeva ultimatiivset metaani saagest 80% saavutamiseni. Samas kulus energiarohu korral selleks 31 päeva. Kuigi tulemused näitasid põllumajanduslikul substratidest biogaasi tootmise suurt potentsiaali, on kääritamisprotsessi erinevate aspektide ja kommersiaalse süsteemi toimimise analüüsimiseks vajalik ka pilootseadmetega uurimatus viimise.
Zn$^{2+}$) sattumisega vette. Edasised uuringud inhibeerimise mehhanismide osas on olulised, et selgitada teiste võimalike tegurite mõju.

Rohtsest biomassist biogaasi tootmine, kasvatades vabanenud maal kõrge energiapotentsiaaliga sobivaid kultuure, võimaldaks Eestil täita taastuvenergia tootmise vajadust. Loomade vedelõnnik ja tööstuse jäätmed on sobivateks substraatideks kooskäritamisel, nende kasutamine biogaasi tootmiseks on tähtis ka jäätmetekke vältimise ning õhu ja veekogude keskkonnakaitse seisukohast. Võtmeküsimuseks on koostöö põllumajandustootjate ja valitsusringkondade vahel, et soodustada investeerimist uute biogaasijaamade rajamisse.
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It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

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A WEB-BASED DATABASE ON METHANOGENIC POTENTIAL OF CROPS AND WASTES

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A web-based database on methanogenic potential of crops and wastes

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Abstract

The Methanogenic Potential Database (BMP Database) provides engineers and scientists with specific and standardized information on the chemical composition and biochemical methane potential of crops, manures, wastes, as well as of mixed substrates. Currently, the BMP database contains data selected from more than 80 sources and covers more than 180 different substrates.

Database availability

Name of Database: Methanogenic Potential Database (BMP database)
Web address: http://www.emu-bioconversion.eu
Cost: Free after registration
Year of availability: 2009
Hardware and software required: Any computer with Internet capabilities
Programming language: HTML/PHP/MySQL

1. Introduction

For decades, biogas production has been studied as an alternative source of energy, and new technologies have been developed to improve the quality of biogas and its conversion efficiency for heat and energy (Parawira et al., 2008), or as an environmentally-friendly fuel for vehicles (Lehtomäki et al., 2008). Currently, the only database published on the Web (Cropgen, 2007) contains limited amount of data. In addition, it has not been updated since 2007.

The aim of the present work was to create a comprehensive, user-friendly Web-database, in which homogeneous data reported in the literature is available and freely searchable through the whole database.

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The query results are organized in separate tabs for each substrate: chemical composition and heavy metals content of the substrate before and after digestion (solid and liquid), methanogenic potential and references. Each row of the tabs presents data corresponding to the reference analyzed. This allows the users to make a direct comparison between different data reported for the same substrate thanks to an integrated multi-line interface. In addition, the export of data can be done by copy-paste to a worksheet for example. Each reference, abstract and address of the authors may be viewed by clicking on a respective link.

3. Database access

For new users of the database, a step-by-step tutorial is provided on how to access and use the database. A glossary adapted from Nyns (1999) with the definition of the terms most commonly used in the cited references is provided.

The access to the online BMP database may be given by the administrator after agreement of the potential use with the “Terms of use” and filling the online application form. For registered users there are several levels of access established, each one of them requiring application for registration and validation by the administrator. Registered users may propose additional data to the database.

4. Advantages

The online BMP database is designed for scientist and engineers to provide them with homogeneous data on the biogas potential from various crops, organic waste and mixed substrates. Advantages of the database are:

- Free access with personal login and password
- Data are constantly updated by taking into account the comments or references provided by registered users
- The search engine allows to search the database by category (crops, manures, mixed, wastes), substrate, author and/or keywords
- For data analysis, data may be copied and transferred to spreadsheet applications (copy-paste)
- Abstract and full-text access of the original data sources are provided when available allowing validation by the user
- Updates, potential improvements, and feedback from registered users will be considered by email

5. Conclusions

This work presents a novel OpenAccess online database that provides specific unified data on the composition and methanogenic potential of various types of crops and organic wastes with corresponding references. The BMP database can be used as a decision-support tool by researchers and engineers, as the principal parameters (i.e. retention time, temperature, chemical composition, biogas and methanogenic potential, digestate characteristics, etc.) for designing a biogas unit are provided.

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BIOCHEMICAL METHANE POTENTIAL OF DIFFERENT ORGANIC WASTES AND ENERGY CROPS FROM ESTONIA

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Biochemical methane potential of different organic wastes and energy crops from Estonia

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Abstract. The biochemical methane potential (BMP) of different Estonian substrates as alternative sources for biogas production was studied. For this purpose, the BMP test was carried out in batch mode at mesophilic temperature (36°C). Substrates were divided into 2 groups: agricultural substrates (silage, hay, cattle and pig slurry) and food industry residues (milk, brewery and cereal industry residues). Methane yields obtained were between 286–319 L kgVS⁻¹ for silage and hay, 238–317 L kgVS⁻¹ for animal slurry and 272–714 L kgVS⁻¹ for agro-industrial wastes. The highest methane yield was obtained from sour cream (714 L kgVS⁻¹), the lowest (238 L kgVS⁻¹) from cattle slurry. In overall, our results suggest that all tested substrates can be treated anaerobically and are potential sources for the production of methane.

Keywords: methane potential, ultimate methane yield, silage, hay, slurry, residues

INTRODUCTION

Due to the rising cost of fuels and increased pollution, the implementation of renewable energy systems have become an attractive alternative for fossil fuels in many countries worldwide.

In the past few years, Estonia has considered the implementation of different renewable energies as a strategy to reduce its dependence to fossil fuels. In 2010, the Renewable Energy Action Plan (REAP) was published according to Renewable Energy Directive 2009/28 (RED). According to the targets set by RED, by 2020 the renewable energy usage should account for 25% of the total energy consumption in Estonia. To reach this goal, share of renewable energy in the sectors of heat/cooling, electricity, and transport should achieve 17.6, 4.8 and 2.7% of the total energy consumption in these sectors respectively. The development of the biogas sector in Estonia is considered among the REAP actions. Target for annual biogas production was set to 0.5 PJ in 2020. Estonia has great potential for production of biogas using manures, herbal biomass and organic residues. There are about 286 thousand hectares of abandoned agricultural land in Estonia that is suitable for cultivation of energy crops, and 128 thousand hectares of semi natural grasslands (Astover et al., 2008).
Theoretical herbal biomass resources for biogas production are 2 billion tons per year (Roostalu & Melts, 2008). Renewable electricity potential in the agricultural biogas sector is estimated to produce 190 GWh and 690 GWh for manures and herbal biomass respectively (Kask, 2008). Nowadays, there is only one agricultural biogas plant that generates an annual electricity production of 2 GWh/y. Most of biogas renewable energy potential is not used in Estonia.

During the last decades, applications of anaerobic digestion has become very popular for production of renewable energy because of its known energy potential, low maintenance costs and, primarily, to its environmental benefits such as the bioconversion of organic waste into organic fertilizers and biogas (Tafdrup, 1995; Ward et al., 2008; Ahring et al., 1992). Anaerobic digestion is a process that consists of a set of microbial interactions in an oxygen-free environment, in which biogas is produced by means of degradation of organic matter (Schink, 1997; Pain & Hepherd, 1985). Some of the advantages of anaerobic digestion are: wastes with less than 40% of total solids are easily treatable, minimization of sludge, odors and pathogens reduction during the process, compliance with waste management legislation (Mata-Alvarez, 2002; Sahlstrom, 2003; Smet et al., 1999).

Biochemical methane potential (BMP) assays have been widely used to determine the methane yield of organic substrates in specific conditions (Owen et al., 1979; Nallathambi Gunaseelan, 1997; 2004).

In this study the methane potential of 51 substrates from Estonia was determined using BMP assay. The substrates were chosen according to the national availability. In Estonia, the most potential substrates for the production of biogas are silages (grass, maize, and alfalfa), hay and animal manures (cattle and pig). Some other substrates like milk products, brewery residues and grain mill were selected to assess their biogas potential due to their potential to be used as co-substrates in farm-scale anaerobic digesters. Based on the observed methane yields and substrate characteristics the substrates potential for biogas production was estimated.

**MATERIALS AND METHODS**

**Inoculum**

The inoculum was collected from the anaerobic reactor of a wastewater treatment plant in Tallinn, Estonia. The inoculum was stored at room temperature in 35 liter tanks, sieved through a 2 mm mesh and pre-incubated at mesophilic range (36°C) 5 days before use to ensure activation and degasification of the sludge. Total solids (TS) and volatile solids (VS) of the inoculum were measured each time before test set-up. TS was adjusted to 20 g per kg of the inoculum by adding distilled water.

**Feedstock**

Samples were collected in Estonia from 2008 to 2010. 4 samples of grass silage, 4 of maize silage, 18 of different mix silages (grasses and legumes, mix rate is not specified) and 4 of hay were collected from different grasslands, 6 samples of cow slurry and 1 sample of pig slurry were collected from a local farm, 1 sample of fermentation slops and 3 different samples of grain mill residues, i.e. aspiration dust, bran and flour were collected from local industries.
For homogenization, silage and hay samples were conditioned by drying at 65°C and milled to achieve particles size of less than 1mm. Then, samples were packed into plastic boxes and stored in a freezer at 4°C before use. All other samples were used without any treatment.

Experimental procedure

The BMP test performed in this study was based on a modified version of the guidelines described by Owen et al., 1979. The experiment was carried out in triplicate with each sample using 575 ml plasma bottles filled with 150 ml of inoculum and 0.3 g TS of substrate. 50 ml of distilled water was added to reach an effective volume of 200 ml. No additional nutrients were added to the test. It was assumed that nutrients required for anaerobic microorganisms were provided by the inoculum as previous trials with addition of nutrients have not shown any significant difference. Before starting the experiment, the test bottles were flushed for 10 minutes with N₂/CO₂ (80/20). Test bottles were incubated at 36°C in a set of Mermet isothermal thermochambers during 42–78 days. Initial basal pressure in the test bottles was measured after acclimation at incubation temperature. For each substrate the duration of the BMP test was specifically determined. The methane production from inoculum was determined in blank tests where no substrate was added. Biogas production and gas composition were determined periodically. Mixing was done by shaking the bottles manually regularly once a day.

Analytical methods

Total solids (TS) and volatile solids (VS) were analyzed according to method 1684 (U.S. Environmental Protection Agency – EPA). TS were determined after drying the sample at 105°C overnight. VS in organic wastes were measured as total solids minus the ash content after ignition at 550°C. pH was measured by a Sentron pH-meter 1001pH. Gas samples were taken by connecting the test bottles to the gas chromatograph through a plastic tube attached to a needle. Gas production was analyzed by measuring the increase in pressure in the gas phase of test bottles using an absolute pressure transducer (0–4 bar, Endress & Hauser). Gas composition of biogas samples were analyzed chromatographically using a gas chromatograph (Varian Inc., Model CP-4900) equipped with 2 columns: a Molsieve 5A Backflush heated column (20 m x 0.53 mm), and a PoraPLOT U heated column (10 m x 0.53 mm). Argon and Helium were used as carrier gases in columns 1 and 2, respectively. Injection temperature, column temperature and column pressure were set to 110°C, 120°C and 50 Psi respectively for column 1, and 110°C, 150°C and 22 Psi for column 2, respectively.

Calculation

Methane produced was calculated by subtracting the methane produced by the inoculum from the methane produced in the test with substrate and inoculum. Cumulative methane yield was calculated as the sum of methane produced over the incubation period and expressed as liters per kilogram of TS or VS of substrate added to the test. The volume of methane was calculated to standard temperature and pressure conditions (0°C and 1 atm). The methane production was modeled by fitting the
experimental data with two non-linear regression models in GraphPad 5.0. The models tested were one-phase exponential association (Model 1):

\[ B = B_{\text{max}} (1 - e^{-kt}) \]  

(1)

where \( B \) is the cumulative methane yield at time \( t \), \( B_{\text{max}} \) is the maximum methane yield, \( k \) is the rate constant, expressed in reciprocal of the X-axis time units (d\(^{-1}\)), and the two-phase exponential association model (Model 2):

\[ B = B_1 (1 - e^{-k_1t}) + B_2 (1 - e^{-k_2t}) \]  

(2)

where \( B \) represents the methane production as a function of time \( t \), \( B_1 \) is the methane yield associated to the bioconversion of readily degradable organics, \( B_2 \) is the methane yield associated to the bioconversion of less readily degradable material, \( k_1 \) and \( k_2 \) are the respective rate constants.

Ultimate methane yields were calculated using above-described models as cumulative methane yield for time \( t = 100 \) days. Incubation time required to achieve 60, 70 and 80% of methane yield were calculated from the ultimate methane yield.

One-way analysis of variance (ANOVA) was used to determine statistical significance \((P<0.05)\) of differences between substrate groups.

RESULTS AND DISCUSSION

Agricultural Substrates

Silages and hay. Ultimate methane yields were calculated by fitting measured data with two different models. For methane production modelling the first order degradation model (Model 1, equation 1) has been widely used in different studies (Hashimoto, 1986; Nallathambi Gunaseelan, 2004; 2009,). However, analyzing our data with this model indicated poor fitting results for biomass substrates (Fig. 1). Since Rao et al., 2000 and Rincon et al., 2010 found that biogas production from solid organic substrates were best fitted by the pseudo-parallel first order model, similar two-phase exponential model (Model 2, equation 2) was also tested in this study. It is considered that the methane production curves correspond to the rapid bioconversion of readily degradable components followed by a slower bioconversion of fibrous portion of the substrates. Correlation coefficients obtained after data fitting were in the range of 0.987–0.999, 0.985–0.996, 0.957–0.999 and 0.994–0.998 for grass silage, maize silage, mixed silage and hay respectively.

The chemical characteristics and methane yields for the determination of the methane potential of hay and different silages are presented in Table 1. Cumulative methane yields were calculated to be 319 ± 19, 307 ± 21, 296 ± 31 and 286 ± 33 L kg\(\text{VS}^{-1}\) for grass silage, maize silage, silage mixture and hay, respectively. These results appear to be consistent with the findings of other authors (Table 1) even though silage samples used in this study have been previously pre-treated. The methane production from all samples started actively after incubation. Time to reach 80% of ultimate methane yield was 15 days for grass silage, 14 days for maize silage, 13 days for mix silage and 19 days for hay (Table 3).
Animal slurries. Results from BMP assay with cattle and pig slurry as substrates are presented in Table 1. As for silages and hay, methane production started actively in all test bottles. Ultimate methane yields for animal slurries were calculated by fitting our data to the one-phase exponential association model (Fig. 2). Correlation coefficient for pig slurry data was 0.946 and for cattle slurry varied from 0.906 to 0.995.

Our results present that during the first 23 days of incubation 80% of the ultimate methane yield has occurred when cattle slurry was used as substrate. In case of pig slurry 80% of the ultimate methane yield occurred within the first 12 days of incubation (Table 3). Tests with cattle slurry presented a methane yield of 238 ± 42 L kgVS⁻¹. Our results appear to be within the same range as results from other studies conducted by different authors (Table 1). BMP results showed that pig slurry produced 30% more methane than cattle slurry. Results obtained for pig slurry appear to be consistent with the findings of Steffen et al., 1998 and Vedrenne et al., 2008.

Ultimate methane yields from agricultural substrates analyzed in numerous trials are presented in Fig. 2. We found no significant statistical difference between the ultimate methane produced from biomass samples, even though their TS content varied significantly ($P<0.05$; Table 1).
Food industry residues

Milk products. Methane potential of different unconsumed milk products was analyzed during the study, since milk products represent a potential source of biogas in the milk industries as considerable amounts are frequently discharged from factories worldwide. Chemical characteristics and cumulative methane yields of the selected products are presented in Table 2. All milk products presented significantly high methane yields, effect that can be explained by their high content of proteins of dry matter (Frigon et al., 2009).

Methane yields obtained during this experiment were between 458 and 714 L kgVS⁻¹. The methane yield obtained from sour cream presented the highest potential of all tested products, while milk containing 2.5% fat presented the lowest methane yield. Bioconversion of milk wastes occurred very rapidly. 80% of ultimate methane yield was reached after only 3–8 days of incubation (Table 3). However, although milk products could represent high methane potential, special care needs to be taken to avoid inhibition by ammonia in anaerobic digesters (Callaghan et al., 1997).
Table 1. Chemical composition and cumulative and ultimate methane yields of tested substrates (standard deviation of substrates tested more than once are presented between brackets).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>pH</th>
<th>TS</th>
<th>VS</th>
<th>Cumulative methane yield</th>
<th>Ultimate methane Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>g kg(^{-1})</td>
<td>g kgTS(^{-1})</td>
<td>L kgTS(^{-1})</td>
<td>L kgVS(^{-1})</td>
</tr>
<tr>
<td>Grass silage</td>
<td>4</td>
<td>4.5</td>
<td>314</td>
<td>928</td>
<td>296 (19)</td>
<td>319 (19)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>318</td>
<td>877</td>
<td>-</td>
<td>372</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>310</td>
<td>871</td>
<td>-</td>
<td>270</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
<td></td>
<td>259</td>
<td>926</td>
<td>-</td>
<td>300</td>
</tr>
<tr>
<td>Maize silage</td>
<td>3</td>
<td>4.2</td>
<td>174</td>
<td>952</td>
<td>292 (21)</td>
<td>307 (21)</td>
</tr>
<tr>
<td></td>
<td>3.76</td>
<td></td>
<td>349.2</td>
<td>961</td>
<td>-</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>73</td>
<td>836</td>
<td>-</td>
<td>295</td>
</tr>
<tr>
<td>Mix silage</td>
<td>18</td>
<td>4.4</td>
<td>294</td>
<td>920</td>
<td>272 (31)</td>
<td>296 (31)</td>
</tr>
<tr>
<td>Hay</td>
<td>4</td>
<td>7.0</td>
<td>69.9</td>
<td>794</td>
<td>252</td>
<td>317</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td></td>
<td>913</td>
<td>937</td>
<td>268 (33)</td>
<td>286 (33)</td>
</tr>
<tr>
<td>Pig slurry</td>
<td>1</td>
<td>7.0</td>
<td>69.3</td>
<td>704</td>
<td>-</td>
<td>244–343</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>9</td>
<td>7.7</td>
<td>78</td>
<td>782</td>
<td>186 (42)</td>
<td>238 (42)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>120</td>
<td>850</td>
<td>-</td>
<td>243</td>
</tr>
</tbody>
</table>

- not determined

n: number of samples tested for same substrate (one sample represents the average of three replicas)
<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>pH</th>
<th>TS g kg⁻¹</th>
<th>VS g kgTS⁻¹</th>
<th>Cumulative methane yield</th>
<th>Ultimate methane yield</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Cheese</td>
<td>1</td>
<td>-</td>
<td>562</td>
<td>960</td>
<td>633</td>
<td>659</td>
<td>659</td>
</tr>
<tr>
<td>Sour cream</td>
<td>1</td>
<td>-</td>
<td>265</td>
<td>992</td>
<td>708</td>
<td>714</td>
<td>717</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>1</td>
<td>-</td>
<td>265</td>
<td>980</td>
<td>590</td>
<td>602</td>
<td>602</td>
</tr>
<tr>
<td>Buttermilk</td>
<td>1</td>
<td>-</td>
<td>109</td>
<td>992</td>
<td>485</td>
<td>489</td>
<td>489</td>
</tr>
<tr>
<td>Milk 2,5 % Fat</td>
<td>1</td>
<td>-</td>
<td>110</td>
<td>993</td>
<td>455</td>
<td>458</td>
<td>458</td>
</tr>
<tr>
<td>Milk 3,5 % Fat</td>
<td>1</td>
<td>-</td>
<td>120</td>
<td>993</td>
<td>463</td>
<td>466</td>
<td>468</td>
</tr>
<tr>
<td>Raw milk</td>
<td>1</td>
<td>-</td>
<td>128</td>
<td>993</td>
<td>508</td>
<td>512</td>
<td>517</td>
</tr>
<tr>
<td>Whey</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distillery slop</td>
<td>1</td>
<td>3.3ᵃ</td>
<td>95.5ᵃ</td>
<td>931ᵃ</td>
<td>355ᵃ</td>
<td>381ᵃ</td>
<td>400ᵃ</td>
</tr>
<tr>
<td>Fermentation slops</td>
<td>1</td>
<td>3.2ᵇ</td>
<td>55.4ᵇ</td>
<td>912ᵇ</td>
<td>306ᵇ</td>
<td>335ᵇ</td>
<td>385ᵇ</td>
</tr>
<tr>
<td>Grain mill - Aspiration dust</td>
<td>1</td>
<td>4.1</td>
<td>874</td>
<td>940</td>
<td>256</td>
<td>272</td>
<td>274</td>
</tr>
<tr>
<td>Grain mill – Bran</td>
<td>1</td>
<td>4.5</td>
<td>794</td>
<td>914</td>
<td>300</td>
<td>328</td>
<td>330</td>
</tr>
<tr>
<td>Grain mill – Flour</td>
<td>1</td>
<td>-</td>
<td>912</td>
<td>896</td>
<td>344</td>
<td>384</td>
<td></td>
</tr>
<tr>
<td>Grain mill waste</td>
<td></td>
<td></td>
<td>564</td>
<td>917</td>
<td></td>
<td>130</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ Before centrifugation (11,000 rpm)
ᵇ After centrifugation (11,000 rpm)
-: not determined
n: number of samples tested for same substrate (one sample represents the average of three replicas)
Table 3. Time to reach corresponding percentages of ultimate methane yield.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>60% Bo</th>
<th></th>
<th>70% Bo</th>
<th></th>
<th>80% Bo</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L kgVS(^{-1}) Days</td>
<td>L kgVS(^{-1}) Days</td>
<td>L kgVS(^{-1}) Days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grass silage</td>
<td>4</td>
<td>196</td>
<td>6</td>
<td>222</td>
<td>9</td>
<td>256</td>
<td>15</td>
</tr>
<tr>
<td>Maize silage</td>
<td>3</td>
<td>209</td>
<td>7</td>
<td>239</td>
<td>10</td>
<td>272</td>
<td>14</td>
</tr>
<tr>
<td>Mix silage</td>
<td>18</td>
<td>193</td>
<td>5</td>
<td>215</td>
<td>7</td>
<td>247</td>
<td>13</td>
</tr>
<tr>
<td>Hay</td>
<td>4</td>
<td>179</td>
<td>11</td>
<td>206</td>
<td>15</td>
<td>233</td>
<td>19</td>
</tr>
<tr>
<td>Pig slurry</td>
<td>1</td>
<td>194</td>
<td>7</td>
<td>225</td>
<td>9</td>
<td>260</td>
<td>12</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>9</td>
<td>150</td>
<td>12</td>
<td>173</td>
<td>16</td>
<td>198</td>
<td>23</td>
</tr>
<tr>
<td>Cheese</td>
<td>1</td>
<td>396</td>
<td>5</td>
<td>463</td>
<td>6</td>
<td>530</td>
<td>8</td>
</tr>
<tr>
<td>Sour cream</td>
<td>1</td>
<td>434</td>
<td>2</td>
<td>502</td>
<td>3</td>
<td>570</td>
<td>4</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>1</td>
<td>361</td>
<td>4</td>
<td>423</td>
<td>5</td>
<td>481</td>
<td>6</td>
</tr>
<tr>
<td>Buttermilk</td>
<td>1</td>
<td>296</td>
<td>3</td>
<td>343</td>
<td>3</td>
<td>391</td>
<td>5</td>
</tr>
<tr>
<td>Milk 2.5% Fat</td>
<td>1</td>
<td>277</td>
<td>3</td>
<td>321</td>
<td>3</td>
<td>367</td>
<td>5</td>
</tr>
<tr>
<td>Milk 3.5% Fat</td>
<td>1</td>
<td>284</td>
<td>2</td>
<td>325</td>
<td>2</td>
<td>372</td>
<td>3</td>
</tr>
<tr>
<td>Raw milk</td>
<td>1</td>
<td>308</td>
<td>2</td>
<td>360</td>
<td>3</td>
<td>409</td>
<td>3</td>
</tr>
<tr>
<td>Distillery slop (a)</td>
<td>1</td>
<td>249</td>
<td>7</td>
<td>289</td>
<td>9</td>
<td>324</td>
<td>12</td>
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<tr>
<td>Distillery slop (b)</td>
<td>1</td>
<td>232</td>
<td>9</td>
<td>275</td>
<td>13</td>
<td>310</td>
<td>17</td>
</tr>
<tr>
<td>Grain mill -</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspiration dust</td>
<td>1</td>
<td>171</td>
<td>6</td>
<td>205</td>
<td>9</td>
<td>235</td>
<td>13</td>
</tr>
<tr>
<td>Grain mill - Bran</td>
<td>1</td>
<td>208</td>
<td>5</td>
<td>253</td>
<td>7</td>
<td>281</td>
<td>11</td>
</tr>
<tr>
<td>Grain mill - Flour</td>
<td>1</td>
<td>234</td>
<td>4</td>
<td>279</td>
<td>6</td>
<td>315</td>
<td>10</td>
</tr>
</tbody>
</table>

n: number of samples tested for same substrate (one sample represents the average of three replica)

Brewery wastes. Residues from brewery were analysed without pretreatment and after centrifugation at 11,000 rpm for 20 minutes. Cumulative and ultimate methane yields are presented in Table 2. Our results show a reduction of the concentration of VS in test samples that were pre-treated with centrifugation leaded to decreased production of methane. Methane production started actively after incubation. 80% of the ultimate methane yield was already reached on the 12\(^{th}\) and 17\(^{th}\) day of incubation for samples without and with pretreatment respectively (Table 3). Methane yield in our experiment resulted in similar values compared with Steffen et al. (1998).

Cereal industry residues

Production of methane from three different grain mill residues was studied. Samples consisted of residues of aspiration dust, bran and flour from a grain mill industry. Results of the chemical composition analyses and the methane potentials are shown in Table 2. Cumulative methane yields were 272, 328 and 384 L kgVS\(^{-1}\)L for aspiration dust, bran and flour, respectively. Test bottles with flour produced 38% and 10% more methane than test bottles with aspiration dust and bran, respectively. 80% of the ultimate methane yield was reached after 11, 10 and 13 days for bran and flour, and aspiration dust, respectively (Table 3). Dubrovskis et al., 2009b who also tested the methane yield of grain mill wastes found a methane yield of 130 L kgVS\(^{-1}\), which is much lower than our results. This variation can be explained by the difference in the
composition of the substrate, as TS concentration reported in their study was much lower than in the substrates analyzed in this study.

**CONCLUSIONS**

Cattle slurry is planned to be used as the main substrate in many biogas plants in Estonia. However, it was found that cattle slurry is not the most attractive substrate for the production of biogas and therefore co-digestion with other substrates should be considered. Pig slurry presented higher methane potential than cattle slurry, but its low solid content demands additional input of organic dry matter to increase capacity of digesters. Due to high availability and their methane potential, silages and hay could be considered as possible substrates in rural areas.

Milk wastes presented the highest cumulative methane yield from all tested substrates with a range of 458–714 liters per kilo of VS added. Fermentation slops are also of great interest for the production of biogas, as high methane yields were obtained. However, centrifugation as a pre-treatment of the samples is not recommended as a decrease in the methane yield was found.

Residues from the cereal industry such as aspiration dust, bran and flour were found suitable for the production of biogas. We suggest it would be valuable to analyze their methane potential in co-digestion with other substrates like animal slurry or with fermentation slops due to their high dry matter content.

The most rapid bioconversion of substrate to methane occurred in the BMP tests with milk wastes. 80% of the ultimate methane yield occurred barely after only 3–8 days of incubation. The longest period to achieve 80% of the ultimate methane potential was found for cattle slurry with a retention time of 23 days. These results suggest that anaerobic digesters such as the continuous stirred tank reactors (CSTR) can be considered as an option for the production of methane since they can be operated with hydraulic retention times of more than 25 days.

The results of this experiment suggest that herbal biomass and agro-industrial residues are promising substrates for the production of renewable energy. We believe the results presented in this article will contribute to the selection of the most suitable substrates in different projects related to anaerobic digestion in Estonia.

**ACKNOWLEDGEMENTS.** We would like to thank the Archimedes Foundation for the PhD grant and Eesti Energia for co-funding the research of bioconversion of Estonian wastes and crops. This research was co-financed by European Union, European Regional Development Fund in Estonian Energy Technology Research and Development project 3.02.0501.10-0020 and Estonian Targeted Funding project SF0690063s08. We would like to thank Prof. Henri-Charles Dubourguier (RIP) for his expertise and support during the development of this research.

**REFERENCES**


INFLUENCE OF CHEMICAL COMPOSITION ON THE BIOCHEMICAL METHANE POTENTIAL OF AGRO-INDUSTRIAL SUBSTRATES FROM ESTONIA

(Submitted to Biomass and Bioenergy)
Influence of chemical composition on the biochemical methane potential of agro-industrial substrates from Estonia

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\textsuperscript{a} Estonian University of Life Sciences, Estonia
\textsuperscript{b} Universidad de Santiago de Compostela, Spain

Abstract
Batch trials were carried out to evaluate the biochemical methane potential (BMP) of 61 different substrates collected from agricultural farms and industrial sites in Estonia. Tests were performed in 500 ml plasma bottles at 36°C. The highest methane yield from all tested substrates was obtained from unconsumed dairy products (557±101 L CH\textsubscript{4}/kg VS) while the lowest was obtained from animal slurries (238±42 L CH\textsubscript{4}/kg VS). From tested energy crops, foxtail millet achieved the highest methane yield (320 L CH\textsubscript{4}/kg VS). Silages from different crops presented methane yield from 296±31 L CH\textsubscript{4}/kg VS to 319±19 L CH\textsubscript{4}/kg VS.

The influence of chemical composition on methane potential and kinetic rate constants was analyzed. Anaerobic digestibility of selected agro-industrial substrates was markedly influenced by their organic content, total proteins and lignin concentrations. Rate constants were found to correlate negatively with hemicellulose, cellulose and lignin ($p<0.05$).

Results of the BMP of studied substrates indicate that herbal biomass and agro-industrial residues are promising substrates for biogas production in agricultural biogas plants in Estonia.

1. Introduction
Biogas production is considered nowadays as a potential alternative for the production of energy for its environmental and economic benefits [1,2]. Biogas is the end-product of a chain of biochemical reactions that occur in the lack of oxygen. The most common substrates for biogas production in farms are: energy crops, silages and animal manures [3]. In Estonia, there is estimated an area of around 286 thousand hectares of abandoned agricultural land that can be considered for cultivation of energy crops and around 128 thousand hectares of semi-natural grasslands [4]. The calculated theoretical herbal biomass production is up to 2 billion tons per year [5]. In Estonia, there are other agro-industrial sources of biomass that can also be considered for the production of biogas, such as fermentation slops from brewery industry, unconsumed milk products, grain mill residues, etc. However, this energy potential has been partly exploited as only one agricultural biogas plant is installed with an annual production of 2 GWh/year.

Methane yield from organic substrates depends on the chemical composition [3]. The biochemical methane potential (BMP) assay has been widely used to determine the methane yield of organic substrates at specific conditions [2,6,7]. Many authors have studied the influence that certain chemical parameters (i.e. content of organics, proteins, lipids, fibres, etc.) have during the anaerobic biodegradation of biomass. Biological degradation of substrates rich in nitrogenous matter, i.e. proteins and urea, will result in high concentrations of ammonia, which is a common inhibitor of biogas production [8]. During anaerobic digestion hydrolization of lipids will result in the production of long chain fatty acids (LCFA) and glycerol. LCFA have been identified as an inhibitor of biogas production [9]. However, glycerol is well known for being an easily biodegradable product [10,11]. In general, biodegradation of lignocellulosic substrates under anaerobic conditions is hard to achieve and therefore production of biogas is low [12]. However, recent studies [13,14] have developed new methods of structural digestion that will permit the utilization of this type of substrates for the production of biogas in the future.

In this study, 61 Estonian agro-industrial substrates from diverse sources with different chemical composition were collected and analyzed on the basis of their methane potential. The aim was to study how the differences on the chemical composition of substrates influence the methane production and the kinetic rate of biomass conversion during anaerobic digestion.
2. Materials and Methods

2.1 Inoculum
The inoculum was collected from the mesophilic anaerobic reactor of Tallinn wastewater treatment plant (Estonia). The sludge was gently stirred and filtered with a 2 mm mesh to allow for the removal of large particles. Before use, the sludge was incubated for 1 week at mesophilic temperature (36°C) under a headspace of N₂/CO₂ (80:20) for degasification (consumption of residual organic matter). The main characteristics of the inoculum were as follows: suspended solids (SS) 12.9 g/L, volatile suspended solids (VSS) 5.77 g/L.

2.2 Feedstock
61 substrates were chosen according to their availability in Estonia and they were collected from several agricultural farms and industrial sites. The substrates selected were: energy crops (jerusalem artichoke with and without flowers, sunflower collected at 2 different periods, hemp collected at 2 different periods, Amur silvergrass, energygrass and millet), silages (grass, maize, alfalfa, timothy grass and red clover), hay, animal slurries (cattle and pig) and agro-industrial residues such as brewery residues (distillery slops) and grain mill residues (aspiration dust, bran and flour) and unconsumed milk products. Energy crops, silage and hay samples were conditioned by milling to achieve particles size of 1 mm. All samples were stored in plastic boxes in a fridge at 4°C before use.

2.3 Experimental procedure
The BMP test performed in this study was based on a modified version of the guidelines described by Owen et al. [6]. The experiment was carried out in triplicate using 575 mL plasma bottles containing 150 mL of inoculum (in-reactor biomass concentration 7.26 g VSS/L) and 0.3 g TS of each substrate. Distilled water was added to reach an effective volume of 200 mL. A set of 3 bottles without substrate were prepared for each batch to study the methane production of inoculum (blank test). Previous work has indicated that inoculum derived from Tallinn wastewater treatment plant is sufficient in providing the nutrients necessary for operating a successful BMP and thus no additional nutrient medium was added. The bottles were closed and the headspace was flushed with N₂/CO₂ (80/20). Test bottles were incubated at 36°C in a set of Mermet isothermal chambers during 42 – 78 days and stirred manually once a day. Biogas production and composition were determined periodically. Cumulative methane yield was calculated as the sum of methane produced over the incubation period minus the methane yield in blank test. Gas production was expressed at standard conditions (0°C, 1 atm) per kilogram of TS or VS of substrate added to the test.

The rate of degradation of substrates was assumed to follow the first–order kinetics as done by Hashimoto [16] and Gunaseelan [7,17]. Methane production was modeled by fitting the experimental data with the first-order decay rate model (equation 1) in GraphPad 5.0.

\[ B = B_{\text{max}} \cdot (1 - \exp(-k \cdot t)) \]  

where B is the cumulative methane yield (L/kg TS or L/kg VS) at time t (days), B_{\text{max}} is the maximum methane yield (L/kg TS or L/kg VS) and k is the first-order rate constant (1/d).

2.4 Analytical methods
Substrates were analyzed for pH, total solids (TS), volatile solids (VS), total organic carbon (TOC), total nitrogen (TN), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (ADL), calcium (Ca), phosphorus (P), magnesium (Mg) and potassium (K). The pH was measured by a Sentron 1001pH. TS was measured by drying for 24 hours at 105 °C and the VS by incineration at 550 °C for 2 hours. TOC was determined by catalytically-aided platinum 680°C combustion technique (Shimadzu TOC-V), TN was determined by copper catalyst Kjeldhal method using a Kjekltek Auto 1030 and total proteins (TP) were calculated by multiplying TN values by a factor of 6.25 (TP=TN*6.25) in the case of plant biomass and by a factor of 6.38 for milk proteins [18,19]. NDF and ADF were determined using a Foss Tecator Fibertec
Lignin was determined as described by AOAC 973.18 method. On the basis of NDF, ADF and ADL analysis, hemicellulose (NDF - ADF) and cellulose (ADF - ADL) concentrations were calculated as proposed by Van Soest et al. [22]. Ca, P and Mg were determined using a FiaStar 5000 following the o-cresolphthalene complexone method [20], the stannous chloride method [55] and the titan yellow method [21], respectively. Total fat and proteins concentrations of unconsumed milk products were taken from the manufacturer.

Biogas production was measured by the increase in pressure in the test bottles using a calibrated pressure transducer (0-4 bar, Endress&Hauser). Methane content was analyzed chromatographically by means of a Micro-GC (Varian Inc., Model CP-4900) equipped with 2 columns: a Molsieve 5A Backflush heated column (20 m x 0.53 mm) and a PoraPLOT U heated column (10 m x 0.53 mm). Argon and helium were used as carrier gases in columns 1 and 2, respectively. Injection temperature, column temperature and column pressure were set to 110°C, 120°C and 50 Psi for column 1, and 110°C, 150°C and 22 Psi for column 2.

2.5 Statistical analysis
The dependence of methane potential, i.e. highest cumulative methane yield achieved in the BMP test, and rate constant k values on the chemical composition of substrates was studied by correlation analysis. Statistical analyses were performed with STATISTICA version 8.0.360.0 (Statsoft, Inc.) using the Shapiro-Wilk's test for normality, in which the null hypothesis is that data are normally distributed. Correlation analysis was done by calculating Pearson’s correlation coefficients ($r$) and their significance levels $p$. $p$-values below 0.05 were regarded as significant.

3. Results and Discussion
3.1 Chemical composition of substrates
The results on the chemical composition of the substrates analyzed in this study are presented in Tables 1, 2 and 3. Due to a wide variety of substrates from different sources, a specific set of analyses were considered for each group independently.

Overall, the results obtained in this study are very consistent with the findings of other authors. The chemical composition of silages and hay (Table 1) is very similar to that reported by Amon et al. [3] and Dinuccio et al. [23]. The concentrations of macro nutrients found in this study (2-3 g P/kg TS, 5-10 g Ca/kg TS, 1-2 g Mg/kg TS and 14-25 g K/kg TS) are similar to the findings of Baležentienė and Mikulionienė [24] for timothy silages (P 2.8 g/kg TS; Ca 2.1 g/kg TS; Mg 0.4 g/kg TS; K 27.1 g/kg TS). Organic content and fiber concentrations found in animal slurries (750-800 g VS/ kg TS and 70 -115 g lignin/g TS, respectively, Table 2) appear to be consistent with the findings of Hobson et al. [25], Varel et al. [26], Robbins et al. [27] and Wellinger, A. [28]. The chemical composition of energy crops is within the same range of that found by other authors [2,12,29-31]. Chemical composition of unconsumed dairy products and selected agro-industrial residues (Table 3) was similar to the results from Dinuccio et al. [23], Steffen et al. [32] and Dubrovskis et al. [33].
Table 2. Chemical composition of animal slurries and some energy crops from Estonia (standard deviations are presented in brackets).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>TS (g/kg)</th>
<th>VS (g/kgTS)</th>
<th>Hemicellulose (g/kgTS)</th>
<th>Cellulose (g/kgTS)</th>
<th>Lignin (g/kgTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal slurries</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig slurry</td>
<td>1</td>
<td>70</td>
<td>794</td>
<td>145</td>
<td>104</td>
<td>72</td>
</tr>
<tr>
<td>Cattle slurry*</td>
<td>9</td>
<td>78 (28)</td>
<td>782 (30)</td>
<td>107 (13)</td>
<td>167 (7)</td>
<td>112 (10)</td>
</tr>
<tr>
<td>Energy crops</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jerusalem Artichoke</td>
<td>2</td>
<td>911 (2)</td>
<td>952 (4)</td>
<td>49.8 (7)</td>
<td>234.6 (36)</td>
<td>53.8 (5)</td>
</tr>
<tr>
<td>Sunflower</td>
<td>2</td>
<td>910 (5)</td>
<td>885 (24)</td>
<td>62.4 (15)</td>
<td>307 (47)</td>
<td>80 (3.9)</td>
</tr>
<tr>
<td>Energy grass</td>
<td>1</td>
<td>920</td>
<td>930</td>
<td>273.3</td>
<td>378.5</td>
<td>96.5</td>
</tr>
<tr>
<td>Hemp</td>
<td>2</td>
<td>920 (2)</td>
<td>943 (6)</td>
<td>107 (1.6)</td>
<td>544 (8)</td>
<td>79.5 (11.4)</td>
</tr>
<tr>
<td>Amur Silvergrass</td>
<td>1</td>
<td>930</td>
<td>946</td>
<td>301</td>
<td>420</td>
<td>70</td>
</tr>
<tr>
<td>Foxtail millet</td>
<td>1</td>
<td>920</td>
<td>916</td>
<td>316</td>
<td>330</td>
<td>53.4</td>
</tr>
</tbody>
</table>

n: number of samples tested for same substrate (each sample was analyzed in triplicate)
* TN= 4.32 (0.34) g/kg TS

Table 3. Chemical composition of unconsumed milk products and selected agro-industrial residues.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>TS (g/kg)</th>
<th>VS (g/kgTS)</th>
<th>TOC (g/kgTS)</th>
<th>Hemicellulose (g/kgTS)</th>
<th>Cellulose (g/kgTS)</th>
<th>Lignin (g/kgTS)</th>
<th>Total Proteins (g/kgTS)</th>
<th>Fats (g/kgTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unconsumed Cheese*</td>
<td>3</td>
<td>364 (171)</td>
<td>978 (16)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>334 (200)</td>
<td>495 (234)</td>
</tr>
<tr>
<td>Unconsumed Milk</td>
<td>4</td>
<td>117 (9)</td>
<td>993 (0.2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>295 (53)</td>
<td>277 (65)</td>
</tr>
<tr>
<td>Grain mill residues</td>
<td>3</td>
<td>860 (60)</td>
<td>916 (22)</td>
<td>415 (41)</td>
<td>313.1 (96)</td>
<td>140 (64)</td>
<td>50.7 (10)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Distillery slops</td>
<td>2</td>
<td>75 (28)</td>
<td>922 (14.1)</td>
<td>455 (50)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n: number of samples tested for same substrate (each sample was analyzed in triplicate)
-: not determined
*: includes sour cream

3.2 Biochemical methane potentials and kinetic rate constants

Results on the BMP are grouped according to their origin and presented in Table 4 and Figure 1.

Cumulative methane yields for grass silage, maize silage and mix silage were 319 L CH4/kg VS, 307 L CH4/kg VS and 296 L CH4/kg VS, respectively, and they are consistent with the findings of others. Lehtomäki and Björnsson [34], Cirne et al. [35], and Lehtomäki et al. [36] found in their study on grass silages a methane potential of 300-372 L CH4/kg VS. For maize silage, Neureiter et al. [37], Dubrovskis et al. [38] and Pobeheim et al. [39] found methane potentials ranging from 295 to 370 L CH4/kg VS. Methane potential of hay (286 L CH4/kg VS, Table 4) is similar to the result from Kaparaju et al. [40] who found a value of 270 L/kg VS.

Cattle and pig slurry presented a methane potential of 238±42 L CH4/kg VS and 317 L CH4/kg VS, respectively. Steffen et al. [32] and Vedrenne et al. [41] found methane potential for pig slurry of 175-350 L/kg VS. For cattle slurry, a methane potential of 243 L/kg VS was found in the study conducted by Steffen et al. [32].

Results on the methane potential of selected energy crops grown in Estonia are presented in Table 4. Heiermann et al. [42] found an average methane potential of 280±30 L CH4/kg VS and 297±108 L CH4/kg VS for hemp and jerusalem artichoke, which are in agreement with the results of this study (289 L CH4/kg VS and 310 L CH4/kg VS, respectively). For sunflower, Antonopoulou et al. [43] found a methane potential of 260 L/kg VS, slightly lower than the value measured in this study (296 L CH4/kg VS). Pokój et al. [44] studied amur silver grass and obtained a methane potential of 210 L/kg VS which is much lower than the result from this study (317 L CH4/kg VS). Similarly, the methane yield of millet (323 L CH4/kg VS) was lower than those observed by Mahamat et al. [45] (257 L CH4/kg VS). This variation on the methane potential of sunflower, amur silver grass and millet could be explained by differences in harvesting time or chemical composition [29,42]. For energy grass (Szavvasi-1), Janowszky and
Janowszky [46] have reported methane potential of 300-350 L CH4/kg VS, slightly higher than the value of this study (290 L CH4/kg VS).

Table 4. Methane yields and kinetic rate constants of studied substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>n</th>
<th>CH4 L/kg TS</th>
<th>CH4 L/kg VS</th>
<th>k 1/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass silage</td>
<td>4</td>
<td>296 (19)</td>
<td>319 (19)</td>
<td>0.172 (0.02)</td>
</tr>
<tr>
<td>Maize silage</td>
<td>3</td>
<td>292 (21)</td>
<td>307 (21)</td>
<td>0.150 (0.02)</td>
</tr>
<tr>
<td>Silage mix*</td>
<td>19</td>
<td>272 (31)</td>
<td>296 (31)</td>
<td>0.230 (0.05)</td>
</tr>
<tr>
<td>Hay</td>
<td>4</td>
<td>268 (33)</td>
<td>286 (33)</td>
<td>0.086 (0.01)</td>
</tr>
<tr>
<td>Pig slurry</td>
<td>1</td>
<td>252</td>
<td>317</td>
<td>0.139</td>
</tr>
<tr>
<td>Cattle slurry</td>
<td>9</td>
<td>186(42)</td>
<td>238 (42)</td>
<td>0.092 (0.04)</td>
</tr>
<tr>
<td>Jerusalem Artichoke</td>
<td>2</td>
<td>294 (4)</td>
<td>310 (7)</td>
<td>0.179 (0.02)</td>
</tr>
<tr>
<td>Sunflower</td>
<td>2</td>
<td>262 (8)</td>
<td>296 (15)</td>
<td>0.154 (0.04)</td>
</tr>
<tr>
<td>Energy grass</td>
<td>1</td>
<td>270</td>
<td>290</td>
<td>0.061</td>
</tr>
<tr>
<td>Hemp</td>
<td>2</td>
<td>272 (9)</td>
<td>289 (11)</td>
<td>0.095 (0.01)</td>
</tr>
<tr>
<td>Amur Silvergrass</td>
<td>1</td>
<td>300</td>
<td>317</td>
<td>0.064</td>
</tr>
<tr>
<td>Foxtail millet</td>
<td>1</td>
<td>296</td>
<td>323</td>
<td>0.101</td>
</tr>
<tr>
<td>Unconsumed Cheese**</td>
<td>3</td>
<td>644 (60)</td>
<td>658 (56)</td>
<td>0.260 (0.07)</td>
</tr>
<tr>
<td>Unconsumed Milk</td>
<td>4</td>
<td>478 (24)</td>
<td>481 (24)</td>
<td>0.344 (0.03)</td>
</tr>
<tr>
<td>Grain mill residues</td>
<td>3</td>
<td>300 (38)</td>
<td>328 (49)</td>
<td>0.160 (0.03)</td>
</tr>
<tr>
<td>Distillery slops</td>
<td>2</td>
<td>331 (35)</td>
<td>358 (33)</td>
<td>0.131 (0.03)</td>
</tr>
</tbody>
</table>

n: number of samples tested for same substrate (each sample was analyzed in triplicate)
* Mixture of different ratios of grasses and legumes silages. Mix rate not specified.
** Includes sour cream

To our knowledge, no detailed studies have been conducted on the methane potential of unconsumed milk products. Due to this lack of information, we were only able to compare our results (Table 4) with the ones obtained from utilization of whey as substrate. Dinuccio et al. [23] found a methanogenic potential of 501 L CH4/kg VS for whey. This result appears to be within the same range of our findings (480-660 L CH4/kg VS).

For grain mill residues, the methane yield observed in this study (328 L CH4/kg VS) was much higher than reported by Dubrovskis et al. [33] who obtained a methane yield of 130 L/kg VS from grain mill wastes. This variation can be explained by the difference in the chemical composition of the substrate. Methane potential of distillery slops (358 L CH4/kg VS, Table 4) was in the same range as the results obtained by Steffen et al. [32] for fermentation slops (338 L CH4/kg VS).

To characterize the conversion rate of selected substrates during anaerobic digestion, kinetic rate constants k were calculated and the values obtained are shown in Table 4. Kinetic rate constants presented are key elements to quantify the speed of substrate biodegradation. The fastest kinetic rate constant was found for unconsumed milk products (0.344±0.03 1/d) while the slowest was found for energy grass (0.061 1/d). As for agricultural biomass, k for grass silage, maize silage, silage mix and hay varied between 0.086 and 0.230 1/d. Chynoweth et al. [1] found conversion rate constants for different ensiled substrates (millet, energycane, napiergrass) ranging from 0.072 to 0.106 1/d. In the case of animal slurries, k values for pig slurry were higher than for cattle slurry.
Conversion rate constant for cattle manure (0.092 1/d, Table 4) is similar to the result from Sánchez et al. [54] who found a value of 0.086±0.004 1/d. As for energy crops, the highest k value was found for Jerusalem artichoke (0.179±0.02 1/d). This variation between the kinetic rates obtained could be explained by the concentration of the lignocellulosic fraction of the substrates. For agro-industrial substrates, the lowest rate was found for distillery slops (0.131±0.03 1/d). In a study conducted by Jiménez et al. [56] on the anaerobic digestion of untreated molasses, a conversion rate constant of 0.14 1/d (9g COD added) was found. Conversion rates of unconsumed dairy products (0.260 – 0.344 1/d, Table 4) were slightly lower than the results obtained by Najafpour et al. [57] for cheese whey (0.358 1/d). Different chemical composition of the substrates could explain the difference in the rates.

3.3 Correlations between chemical composition and biochemical methane potential

Correlations between the cumulative methane production (in L CH₄/kg TS) and the methane production rate constant with the chemical characteristics of substrates are presented in Table 5 and Figures 2 and 3.

Among the different chemical parameters, only VS, total proteins (TP), hemicellulose (HC), lignin (L), P, Ca and K showed significant influence on the methane yield as single independent variables (Table 4). As expected, one of the main parameters influencing methane yield was organic matter, i.e. VS content, whose correlation with methane production was significantly positive. Proteins are also known to influence methane formation positively and therefore high methane yield can be attained from substrates rich in proteins [3].

In the case of fiber composition, hemicellulose correlated positively with methane production, although the correlation was poor. For cellulose, no significant correlation was found. Previous studies confirm that cellulose and hemicellulose can be bioconverted into methane and carbon dioxide during anaerobic digestion. However, degradation rate of cellulose depends mainly on whether it is lignin-incrusted or in a crystalline form [12]. Lignin content presented a strong negative correlation with methane production. Our results appear to be consistent with the findings of many other authors [12,47,48], identifying lignin as a complex plant constituent very difficult to digest by anaerobic bacteria and therefore low methane yield is achieved at very low rates.
Macronutrients (P, Ca, Mg and K) were only measured for silages and their Pearson’s correlations with methane yield were found negative and statistically significant. P and Ca are known for being essential for metabolic reactions and growth of anaerobic bacteria [49] but they can become toxic when present in high concentrations [50-52]. In our study, concentrations of these elements in the biomass were not excessively high to provoke a negative effect on methane production. So, it can be assumed that the different chemical composition of specific crops in grasses, silages and hay samples and the different ratios (not known) of crops in analyzed samples affected the methane yield and were reasons for the found negative correlation. Accumulation of mineral elements in plants depends on soil properties, cultivation and fertilization, climate, harvesting time as well as plant properties [53]. Various plant species have a different ability to accumulate mineral elements, therefore content of Ca, P and K can differ significantly in different crops, especially between legume and grass species [24].

Concerning the methane production rate constant, positive correlations were only found with P, Ca, Mg and K (Table 5, Figure 3). These results suggest that P and light metal ions enhance the speed of the anaerobic biodegradation process. The most rapid bioconversion of studied substrates occurred in the tests with unconsumed milk products which contained high amount of proteins. In contrast, the higher content of lignocellulosic material (hemicellulose, cellulose and lignin) in the substrate, the lower the rate of methane production (Figure 3).
Fig. 2 Pearson's correlation between methane yield and chemical parameters ($p<0.05$). 95% confidence intervals are presented in dash lines.
Fig. 3 Pearson's correlation between methane production rate constant and chemical parameters ($p<0.05$). 95% confidence intervals are presented in dash lines.
4. Conclusions

In this study, 61 Estonian agro-industrial substrates from diverse sources were analyzed on the basis of their chemical composition and methane potential.

From all the tested agro-industrial substrates, unconsumed milk products presented the highest methane potential, while animal slurries presented the lowest. Herbal biomass such as energy crops, silages, and hay presented also relatively high biochemical methane potential. Due to their high availability in Estonia, these substrates could be considered as potential substrates for biogas production in rural areas, and also be considered as suitable co-substrates to animal slurries to increase biogas yields. The highest methane yield from the tested energy crops was achieved from foxtail millet with 320 L CH₄/kg VS, whereas hemp and energy grass presented the lowest, 286±11 L CH₄/kg VS and 274 L CH₄/kg VS respectively. Silages from different crops presented methane yield from 296±31 L CH₄/kg VS to 319±19 L CH₄/kg VS.

An appropriate characterization of the chemical composition of the substrates is important not only for predicting BMP and the kinetics rates, but also for identifying the possible inhibitions during anaerobic digestion process.

Anaerobic digestibility of selected agro-industrial substrates was markedly influenced by their organic content, total proteins and lignin concentrations. Substrates with high lignin content are very difficult to biodegrade, and therefore pre-treatment should be foreseen in these cases. The selection of appropriate feedstock for biogas production is important. The results obtained in the present study indicate that herbal biomass and agro-industrial residues are promising substrates for biogas production in agricultural biogas plants in Estonia. In addition, we believe this work contributes to the studies about feedstock chemical composition influence on methane production.

Although, anaerobic digestion of agro-industrial wastes is quite extensively used in countries such Denmark, Germany, Austria, France, etc., in Estonia the utilization of such substrates in anaerobic digestion plants have not yet applied. The results of this study aimed to highlight the potential of Estonia and other countries have for biogas production.
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PARTICLE-SIZE EFFECT OF CuO AND ZnO ON BIOGAS AND METHANE PRODUCTION DURING ANAEROBIC DIGESTION

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Particle-size effect of CuO and ZnO on biogas and methane production during anaerobic digestion

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ABSTRACT

The effects of bulk- and nano-sized CuO and ZnO particles on biogas and methane production during anaerobic digestion of cattle manure were studied for a period of 14 days at 36 °C using the ISO 13641-2 guidelines. Biogas production was severely affected at concentrations of bulk and nanoparticles over 120 and 15 mg/L for CuO and 240 and 120 mg/L for ZnO, respectively. EC50 concentrations for methane inhibition were estimated to be 129 mg Cu/L for bulk CuO, 10.7 mg Cu/L for nano CuO, 101 mg Zn/L for bulk ZnO and 57.4 mg Zn/L for nano ZnO. The solubility of CuO nanoparticles in the reaction mixture was observed after 14 days of incubation and was significantly higher than the levels observed for ZnO. These results are of significant importance, as it is the first time that the effects of metal oxide particle size on biogas and methane production have been studied.

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1. Introduction

Over the past several years, modern industrial research has adopted new technologies to utilise an increasing number of materials at a nanometer scale. These advances allow for improved characteristics so that more complex tasks can be achieved [1]. Currently, interest in these new technologies has resulted in increased funding from private and governmental sources. A wide range of novel applications improved by these new nano materials include anti-reflection coatings, high conductivity and mechanical resistant materials, energy-efficient batteries, antibacterial silver coatings on wound dressings, sensors for disease detection, soil decontamination agents, water filtration materials, biodegradable polymers and highly efficient clear inorganic sunscreens [2–4].

Industrial effluents containing suspensions of these particles may drastically harm the environment and this may be particularly true for aquatic habitats [5–12]. Another negative impact may occur in water and wastewater treatment plants, as no specific filtering mechanisms are typically installed to avoid the entrance of nanoparticles into the system. In addition, dispersal of contaminated sewage sludge into the soil will spread toxic substances to living organisms, groundwater and sub-surface water systems [4].

In recent years, the ecotoxicity of engineered nanoparticles has been of great interest due to their potential harmful effects on human and other vertebrate health [13–16]. Most studies have examined the effects on aquatic environments [17,18]. However, very few studies have been carried out examining contaminated sediments or non-aquatic environments.

Different toxicity tests examining bulk and nanoparticles of copper oxide (CuO) and zinc oxide (ZnO) have been documented in the literature [19–23]. Results have shown higher toxicity from metal oxide nanoparticles than their bulk particle counterparts. Toxicity assays with these nanoparticles to the microalgae Pseudokirchneriella subcapitata have shown high toxicity at exceedingly low concentrations, such as 0.042 mg/L of zinc and 0.71 mg/L of copper [19]. In their study of P. subcapitata, toxicity was attributed to the higher solubility of the metal oxide nanoparticles. In experiments conducted by Mortimer et al. [24] examining the toxicity of CuO and ZnO nanoparticles to the protozoa Tetrahymena thermophila, the results showed a significant difference in toxicity between nano and bulk CuO particles. Nano CuO was 10 times more toxic than the bulk form.

There remains a lack of information regarding the adverse effect of CuO and ZnO nanoparticles on the environment when assessing different organisms. Currently, data regarding the effect of contaminated sewage sludge into the soil will spread toxic substances to living organisms, groundwater and sub-surface water systems [4].

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the experimental setup, the sludge was gently stirred and solution prepared with the following compounds (g/L): KH₂PO₄, anaerobic bacteria methane production inhibition consisted of a experimental period.

The inoculum was collected from the Paljassaare anaerobic reactor, which is a sewage treatment facility located in the northwestern part of Tallinn, Estonia. The sludge was stored for 2 days at 36 °C under a headspace with a N₂/CO₂ ratio of 80:20. Previous to the experimental setup, the sludge was gently stirred and filtered with a 1 mm² sieve to allow for the removal of large particles. The sludge was then diluted with distilled water to reach a fresh matter mass concentration of 2.1 g TS/L (ISO 13641-2) [30]. Cattle manure was chosen as the substrate for the analyses. Samples were collected from a cattle farm located in Jõgeva, Estonia. Collected samples were dried at 60 °C for two days. Milling and sieving of the samples were performed to ensure that a homogeneous particle size diameter of 1 mm was achieved.

2.2. Toxicity test

The toxicity experiment was carried out according to the ISO 13641-2 guidelines [30]. One variation was that cattle manure was used as the substrate instead of yeast extract. CuO and ZnO were purchased from Sigma–Aldrich. CuO and ZnO particle sizes were as follows: bulk CuO ~5 μm, nano CuO ~30 nm, bulk ZnO ~1 μm and nano ZnO ~50–70 nm. Stock suspensions of 10 g/L were prepared in milliQ water on the day of the experiment. Stock suspensions were diluted to reach a series of mass concentrations ranging from 7.5 to 480 mg/L.

The test was performed in 160 mL gas-tight closed serum bottles containing 88 mL of reaction mixture (Table 1) and 5 mL of the inhibitor suspension. All experiments were conducted in triplicate. In addition, a set of three bottles containing only the reaction mixture was prepared to act as a control. For experimental validation, a batch of test bottles containing 3.5-dichlorophenol in addition to the reaction mixture was analysed. The experiment was also carried out with a series of mass concentrations ranging from 7.5 to 240 mg/L. An EC50 equal to 71 mg/L and pH between 6.9 and 7.1 at the end of the experiment validated the test. All samples were incubated at 36 °C and gently stirred twice daily during the 14 day experimental period.

Preparation of the test medium for the determination of anaerobic bacteria methane production inhibition consisted of a solution prepared with the following compounds (g/L): KH₂PO₄, 2.7; KH₂PO₄, 5.45; NH₄Cl, 5.3; CaCl₂ 2H₂O, 0.75; MgCl₂ 6H₂O, 1.0; FeCl₂ 4H₂O, 0.2; resazurin, 0.01; Na₂S 9H₂O, 1.0. Trace element solution (g/L): MnCl₂ 4H₂O, 0.3; H₂BO₃, 0.05; ZnCl₂, 0.05; CuCl₂ 2H₂O, 0.035; Na₂MoO₄ 2H₂O, 0.01; CoCl₂ 6H₂O, 1.0; NiCl₂ 6H₂O, 0.1; and Na₂SeO₃, 0.05.

Before sample incubation, the pH of the test medium was measured to validate that the experiment was correctly set up. The pH measured from the test bottles was in the range of 6.9 ± 0.3.

2.3. Analytical methods

Gas production kinetics were determined using a calibrated pressure transmitter (SIEMENS). Gas samples were collected using a glass syringe. Methane concentrations from the biogas samples were analysed chromatographically using a gas chromatograph (Varian Inc., Model CP-4900) equipped with two columns as follows: a Molsieve 5A Backflush heated column (20 m × 0.53 mm) and a PoraPLOT U heated column (10 m × 0.53 mm). Helium and argon were used as carrier gases in columns 1 and 2, respectively. Copper and zinc concentrations in the supernatant were measured using a flame atomic absorption spectrometer (Shimadzu Co., Model AAS-6800) after a 20-min centrifugation at 11,000 rpm. Furthermore, acidification (1% HNO₃) and glass microfiber filtration (type: GF/C; Whatman Co.) were performed. Operational configuration of the instrument was set according to the manufacturer’s recommendations as follows: wave length (nm) of 324.8 and 213.9; lamp current (mA) of 10/500 and 10/300; acetylene (C₂H₂) flow rate (L/min) of 1.8 and 2.0; slit width (nm) of 0.5 for copper and zinc, respectively.

2.4. Calculations

Biogas production was estimated by measuring the increase in test bottle pressure. The inhibition of methane production was calculated by comparing the volume of methane produced in bottles containing the inhibitor with the controls. Calculation of common toxicity parameters (i.e., EC10, EC20, and EC50) was carried out using the Log-Normal model application within REGTOX software. The half effective concentration, EC50, corresponds to the concentration of inhibitor required to cause a 50% reduction of methane production when compared with the control tests. Analyses on statistical differences between the effects of CuO and ZnO bulk and nanoparticles were performed using STATISTICA software. One-way analysis of variance (ANOVA) followed by t-test was used to determine statistical significance (p < 0.05).

3. Results and discussion

3.1. Effect of CuO and ZnO bulk and nanoparticles on biogas production

CuO and ZnO nanoparticles and microparticles were inoculated in a batch mode with anaerobically digested sludge at 36 °C for 14 days. Biogas production was used as an indicator of anaerobic digestion imbalance [31–33]. Production of biogas during the incubation period from the control and test samples with different ranges of CuO and ZnO bulk and nanoparticles were performed using STATISTICA software. One-way analysis of variance (ANOVA) followed by t-test was used to determine statistical significance (p < 0.05).
Fig. 1. Biogas inhibition from CuO bulk (A) and nano-sized particles (B). As reported by Heinlaan et al. [21], Kasemets et al. [22] and Neal [36], nanoparticles are toxic to bacteria due to the release of cell membrane damaging bioavailable metal ions, and therefore, the inhibition of biogas production can occur.

Biogas production in test samples containing ZnO nanoparticles compared to bulk ZnO is illustrated in Fig. 2. ZnO nanoparticle concentrations of 120 and 240 mg/L presented an inhibition of 43% and 74% of the biogas yield at day 14, respectively. In comparison, test bottles containing bulk ZnO presented a total biogas reduction of 18% and 72% at day 14, respectively. However, no significant difference of biogas inhibition from ZnO bulk and nanoparticles was observed.

A further evaluation of the results presented in Figs. 1 and 2 indicates that the inhibition of biogas production also depends on exposure time. During the first six days of incubation, test samples with bulk CuO, bulk ZnO and nano ZnO were not statistically different from the control sample. However, inhibition of biogas production in test bottles containing CuO nanoparticles occurred at the beginning of the experiment. In addition, results from Figs. 1 and 2 highlight a significant increase in biogas production from day 11 to day 14 for test bottles with CuO concentrations less than 120 mg/L bulk particles and 15 mg/L for nanoparticles. This was also the case for ZnO at concentrations less than 120 mg/L for both bulk and nanoparticles. We suggest that anaerobic bacteria can adapt to medium containing inhibitors, possibly by enzymatic induction, tolerance development or to changes in the microbial metabolism [34], all of which result in an increase of biogas production over time.

3.2. Effect of CuO and ZnO bulk and nanoparticles on methane production

The effective concentration values causing a 50% (EC50) reduction of methane production were calculated. Results were used to compare toxicities of different particle sizes (bulk and nano) for varying CuO and ZnO concentrations.

Figs. 3 and 4 illustrate the inhibition of methane production for varying copper and zinc (in their respective oxides form) concentrations during a 14-day incubation period. EC50 values for CuO bulk and nanoparticles were calculated to be 129 and 10.7 mg Cu/L, respectively. For ZnO, the EC50 levels for bulk and nanoparticles were calculated to be 101 and 57.3 mg Zn/L, respectively. Data for EC10, EC20 and EC50 values with confidence intervals are presented in Table 2.

The results presented in Fig. 3 show that CuO nanoparticles (∼30 nm) inhibit the production of methane at least 10 times more effectively than the bulk counterpart. The difference between CuO bulk and nanoparticles was statistically significant (p < 0.0001). Complete inhibition of methane production in the presence of CuO occurred at concentrations of 330 and 30.2 mg Cu/L for bulk and nanoparticles, respectively.

Although a significant difference for biogas inhibition from ZnO bulk and nanoparticles was not found, methane inhibition was different. It can be seen in Fig. 4 that ZnO nanoparticles had higher methane inhibition than bulk ZnO. The ZnO nanoparticles (50–70 nm) were approximately twice as toxic when compared to their respective bulk counterparts.

Table 2

<table>
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<tr>
<th>Inhibitor</th>
<th>EC10 (mg/L of metal)</th>
<th>EC20 (mg/L of metal)</th>
<th>EC50 (mg/L of metal)</th>
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<td></td>
<td>Average 95% C.I.</td>
<td>Average 95% C.I.</td>
<td>Average 95% C.I.</td>
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<tr>
<td>Bulk CuO</td>
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<td>73.4 61.7 85.3</td>
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<tr>
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<td>39.6 30.6 52.2</td>
<td>55.6 44.1 66.1</td>
<td>101 84 108</td>
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<tr>
<td>Nano ZnO</td>
<td>19.5 15.7 24.1</td>
<td>28.2 23.9 33.4</td>
<td>57.3 52.1 61.2</td>
</tr>
</tbody>
</table>
Methane production dose–response curves during exposure to bulk CuO (A) and nano CuO (B) particles. Complete inhibition of methane production for ZnO bulk and nanoparticles occurred at concentrations of 246 and 181 mg Zn/L, respectively.

In our experiment, CuO and ZnO nanoparticles (Figs. 3 and 4) showed higher toxicity to anaerobic bacteria than their bulk counterparts, with other research groups reporting similar results. In studies of the microalgae *P. subcapitata*, Aruoja et al. [19] found a higher toxicity for CuO nanoparticles (∼30 nm; EC50 = 0.71 mg Cu/L) compared to bulk CuO (EC50 = 11.55 mg Cu/L). However, although they found high toxicity using ZnO, no statistical difference between the toxicity of bulk ZnO (EC50 = 0.037 mg Zn/L) and nano ZnO particles (50–70 nm; EC50 = 0.042 mg Zn/L) could be determined. In a study conducted by Heinlaan et al. [21], the toxicity of CuO bulk and nanoparticles (30 nm) to the bacterial species *Vibrio fischeri* presented an EC50 of 3049 ± 819 and 63 ± 22 mg Cu/L, while ZnO particles (50–70 nm) showed an inhibition with EC50 values of 1.4 ± 0.08 and 1.5 ± 0.16 mg Zn/L, respectively. However, results from studies of the crustaceans *Daphnia magna* showed EC50 values of 131.8 ± 19.7 and 2.6 ± 1.04 mg Zn/L for bulk and nano ZnO, respectively.

CuO nanoparticles were identified as the most toxic particle to anaerobic bacteria from all tested metal oxides in our study (Table 2). Our results appear to be consistent with findings from other groups studying the toxicity of metal oxide nanoparticles to several species of microorganisms. Kasemets et al. [22] studied CuO and ZnO bulk and nanoparticle toxicity at 8 h of *S. cerevisiae* growth. The results from their study show that CuO nanoparticles presented higher toxicity when compared to ZnO nanoparticles. They found an EC50 of 16.6 mg Cu/L for CuO nanoparticles, whereas ZnO nanoparticles presented an EC50 of 97.4 mg Zn/L. Comparable results were also reported by Ivask et al. [37], where bacterial toxicity tests performed with several *E. coli* strains showed higher toxicity levels for CuO nanoparticles when compared to ZnO nanoparticles.

Zayed and Winter [35] studied the influence of Cu and Zn on methane production. In their study, they tested CuCl2 and ZnCl2 toxicity during anaerobic digestion of whey. EC50 values of 4.7 mg Cu/L and 19.2 mg Zn/L were reported. These results are comparable with our data, where it was found that copper oxide nanoparticles had higher toxicity during methane production than zinc oxide nanoparticles. This was the case even though CuO and ZnO nanoparticles have been reported to have very low solubility in water unlike the higher solubility observed for CuCl2 and ZnCl2. In addition, CuO nanoparticles inhibited methane production at similar concentrations as Cu ions in the case of soluble copper salt (CuCl2). However, methane inhibition from ZnCl2 is approximately twice as toxic when compared to our data obtained from studies of ZnO nanoparticles.

### 3.3. Influence of metal ions on methane production

The presence of heavy metal ions (i.e., Cu, Zn, Fe, Ni, Co, Mo) during anaerobic biodegradation of organic matter is known to be fundamental for numerous reactions. However, high concentrations of these elements can inhibit the biological degradation...
process in anaerobic reactors. One of the problems with heavy metal compounds is that these elements are not biodegradable. Due to this, these compounds are known to accumulate, reaching potentially toxic concentrations for anaerobic bacteria [26].

In our experiments, the Cu and Zn ion concentrations in the liquid phase of reaction mixtures were analysed. Quantification limits of the method used for the determination of Zn and Cu were 10 mg/L and 1 mg/L, respectively. Zn concentrations in the liquid phase of the reaction mixture were less than 10 mg/L in all tests. Cu concentrations were less than 1 mg/L in the control bottles and also when bulk CuO was used as the test material. Cu ion concentrations in the reaction mixtures containing CuO nanoparticles are presented in Table 3.

According to the technical data sheets, aqueous solubility of CuO and ZnO bulk and nanoparticles is very low. However, the results presented in Table 3 demonstrate a higher solubility of CuO nanoparticles when compared to bulk CuO. These results suggest that CuO and ZnO nanoparticle toxicity to anaerobic bacteria can be attributed to the dissolved bioavailable fractions of these metals. An ecotoxicological study conducted by Arujo et al. [19] also concluded that CuO nanoparticle toxicity is attributed to a higher solubility of nanoparticles in the test medium. However, a comparison of Cu ion concentrations in the reaction mixture (Table 3) with the EC50 values obtained by Zayed and Winter [35] for Cu ions from CuCl2 shows that the toxicity of nanoparticles can only be partially explained by the dissolution of CuO nanoparticles to Cu ions. Most likely, different adverse effects of nano- and micro-sized particles to the anaerobic process remain partially due to different surface areas and surface characteristics [23].

4. Conclusions

The results of this study reveal that CuO and ZnO particle-size directly influence the toxicity of these compounds to anaerobic bacteria, and thus affect the production of biogas including methane yield. Inhibition of biogas and methane production by CuO nanoparticles can be partially attributed to the soluble bioavailable fraction of the metal found in the liquid phase of the reaction mixture after a 14-day incubation period. However, high CuO nanoparticle toxicity cannot only be explained by the release of toxic Cu ions. Zinc oxide formulations were equally toxic, resulting in alterations of biogas production. However, methane production was highly inhibited in the presence of ZnO nanoparticles. From the compounds studied, the most toxic to anaerobic bacteria were CuO nanoparticles (~30 nm) followed by ZnO nanoparticles (50–70 nm), bulk ZnO and bulk CuO.

Analyses of biogas production kinetics showed a possible bacterial adaptation to the medium. We therefore recommend future studies surrounding the inhibition of these chemicals for a longer period to assess possible recovery rates. This may allow for the discovery of suitable mechanisms for re-establishing the anaerobic digestion process. Further research on intermediate products (e.g., hydrogen and volatile fatty acids) of the anaerobic digestion process is needed to obtain more information on the toxicity of these nanoparticles.

The results of our study are an important complement to published data on the ecotoxicity of nanoparticles that are currently used in industry. Data showing high toxicity of nanoparticles indicate that nanolevel particle sizes should also be of concern for the anaerobic digestion processes.

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References


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Foreign languages
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2010 Free membership to the American Nano Society for the publication of “Particle-size effect of CuO and ZnO on biogas and methane production during anaerobic digestion”
**Isikuandmed**

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**Uurimissuund**

Biogaasi tootmine taimedest ja tööstusjäätmetest.

**Teenistuskäik**

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**Projektid**

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2007-2010 Eestimaise biomassi ning tootmise kõrvalsaaduste ja jäämete biogaasi tootlikkuspotentsiaal ning anaeroolise fermentatsiooni kineetika

2008-2010 Biogaasi tootmise tehnoloogia ja juhtimise arendamine Eestis pilootseadmetega

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Võõrkeeled

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LIST OF PUBLICATIONS

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Publications in other peer-reviewed research journals


Publications in Estonian

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Prof. Valdo Kuusemets
Prof. Marika Mänd
December 16, 2011

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