Improving the Anaerobic Digestion of Lignocelluloses and Organic Wastes: Effects of Steam Explosion, Co-digestion and Digestate Recirculation.

Optimalisering av biogassproduksjon fra lignocellulose og organisk avfall: effekt av dampeksplosjon, samråtning og resirkulering av prosessvann.

Philosophiae Doctor (PhD) Thesis

Maria Magdalena Estevez Rego

Department of Mathematical Sciences and Technology
Norwegian University of Life Sciences

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“The more clearly we can focus our attention on the wonders and realities of the universe about us, the less taste we shall have for destruction.”

Rachel Carson (1907 – 1964), author of “Silent Spring”.

To my family and my mother’s memory

Summary

Biogas production is a sustainable bioenergy process very much in demand nowadays for all the benefits it entails. It does not only allow production of clean energy and thus autonomy from fossil fuels, but also the recirculation of nutrients back to the crop fields and the decrease on greenhouse gas emissions from the agricultural sector. Biogas production is very versatile, because a wide variety of materials can be used as resources to generate methane, including organic wastes that do not present a threat to food production. With the increase interest in employing different types of available agricultural and industrial materials as biogas feedstocks, improvements along the whole process need to be addressed so to make this technology an efficient one.

This thesis deals with many aspects of the anaerobic digestion process in which improvement of both process efficiency and stability can be achieved. Focus in particular was made on studying the effects of pre-treating highly lignocellulosic biomass by steam explosion on its biogas production (Paper I), mixing of different types of materials (Papers I-II-III), performing recirculation (Papers II-III) and recovery of nutrients from the digestate (Paper III).

Steam explosion was tested on a hardwood crop rich in lignin, *Salix viminalis*, and was found to increase the methane production by up to 50 % compared to just mill *Salix* chips (Paper I). Different carbon-to-nitrogen (C/N) ratios were studied by screening mixtures of *Salix* and cattle manure with varying contents of volatile solids (VS %), which indicated that mixtures containing up to 40 % VS of steam exploded *Salix* could be used together with manure in anaerobic digestion and give good methane yields (Paper I). However, when substrates with high fiber content are digested in semi-continuous systems, much of the methane potential remains unexploited if the retention times are not enough for the slowly degrading materials.Ways to take advantage of such residual potential include; i) using gas tight post-storage tanks; ii) arranging post digestion systems at the biogas plants, iii) applying pre-treatment to substrates that are difficult to easily digest and/or iv) applying longer hydraulic retention times. This last one can also be achieved by performing recirculation of the digestate back to the digester, thereby allowing a longer hydraulic (and solid) retention time without reducing the capacity of the system. In this study, recirculation of the liquid fraction of the digestate was evaluated as a method to enhance process efficiency and stability (Paper II). The results showed that using recirculated digestate instead of water to dilute the feedstock increased the methane production by up to 27 %. Ammonia and volatile fatty acids did not cause any inhibition problems, but accumulation of solids was more noticeable in the recirculating reactors, causing the degree of digestion to decrease after three hydraulic retention times. This indicates that optimization of the solids separation when recirculating is essential to guarantee long-term stability of systems. An enhancement of such separation was included in Paper III.

Besides cattle manure and *Salix*, another organic waste of importance in Norway was tested, namely, “category 2” fish byproduct (Paper III). Fish farming is an extensive industrial activity in Norway, and the wastes from that sector have a high protein and fat content which translates in high energetic value. Seizing such products that

are otherwise dumped in the sea would entail great environmental and economic benefits for Norway. When fish byproduct category 2 was mixed with steam exploded *Salix* and manure in continuously stirred tank reactors, yields in average increased 35 % (Paper III). Recirculation was also tested together with the addition of such nitrogen-rich substrate in order to evaluate the stability of the process with regard to levels of ammonium. The results revealed that the stability of the reactors was substantial since ammoniacal nitrogen ($NH_4{}^+$-N) concentrations increased to levels that otherwise would have inhibited the methanogenic process (Paper III). Recirculating digestate not only helps in recover residual methane potential, but also may favor the stability, enriching the microbial biomass that becomes tolerant to levels of $NH_4{}^+$-N usually corresponding to toxic levels of free ammonia. A proper co-digestion fraction of lignocelluloses together with $NH_4{}^+$-N rich biomass was crucial for balancing the nutrients and keeping the process running.

During anaerobic digestion, nitrogen and phosphorous are mineralized and converted into plant-available nutrients. $NH_4{}^+$-N and phosphate could be recovered from the raw digestate and fixed into more solid fractions that can improve the final biofertilizer. The simple cost effective techniques tested in this study were struvite precipitation and bentonite adsorption, and both gave interesting results regarding the amount of $NH_4{}^+$-N removed (approx. 90 % and 80 % removal respectively). Even though the phosphate content was not high in the digestates, some removal was achieved mostly as struvite.

Therefore, by taking into account: i) a good pre-treatment, ii) an optimum mixing ratio of different available materials, iii) savings on resources while profiting residual energy potential and enhancing stability, iv) and recovery of nutrients in the form of a richer biofertilizer, biogas can become attractive in Norway, not only as a substitute for fossil fuels in transport, but also as a source of good quality organic fertilizer, all this while also helping in some cases, in the reuse and treatment of potentially polluting organic wastes.

The present doctoral research was conducted at the Department of Mathematical Sciences and Technology (IMT) of the Norwegian University of Life Sciences (UMB) in Ås, Norway, from December 2009 to February 2013 (3 years and 3 months). The study belongs to the Work Package II of the project "From Biomass to Biogas - an Integrated Approach towards Sustainable Recovery of Energy and Nutrients" developed jointly by the IMT, the Department of Plant and Environmental Sciences (IPM), the Department of Chemistry, Biotechnology and Food Science (IKBM), the Norwegian Institute for Agricultural and Environmental Research (Bioforsk) and the Norwegian company CAMBI. The project was financed by the Norwegian Research Council (project nº 423513 UB) and aimed to increase knowledge and expertise concerning anaerobic digestion performed under conditions existing in Norway. A state-of-the art biogas laboratory was developed at UMB during the course of this doctoral work.

Sammendrag

Biogassproduksjon kan være en bærekraftig energiprosess som er mye etterspurt i dag på grunnlag av de fordeler dette innebærer for klimanøytral energi og gjenbruk av organiske avfallsprodukter til gjødsel. Det betyr ikke bare produksjon av energi som kan erstatte bruk av fossilt brennstoff, men som også kan gi grunnlag for resirkulering av næringsstoffer tilbake til dyrket mark og redusert klimagassutslipp fra landbruket. Dette er en svært allsidig prosess, siden mange forskjellige typer materialer og stoffer kan brukes som ressurs til å generere metan, som organiske avfall som ikke vil konkurrere med matproduksjon. Siden det er stor interesse for også å ta i bruk nye typer tilgjengelige landbruks- og industri avfall som råstoff for biogassproduksjon, er det nødvendig å undersøke mulige forbedringer langs hele prosessen for å gjøre teknologien mer effektiv.

Denne avhandlingen peker på mange aspekter av biogassprosessen der det kan oppnås forbedring av både prosess-effektivitet og -stabilitet. Det ble spesielt satt fokus på å studere effekten av dampeksplosjon som forbehandling av biomasse med mye lignocellulose, altså fra trevirke på biogassproduksjon (Artikkel I), blanding av forskjellige typer av materialer sammen med trevirke (Artikkel I-II-III), resirkulering av prosessvann (Artikkel II-III) og gjenvinning av næringsstoffer fra råtnerest (Artikkel III). Tema for arbeidet omhandler ulike metoder for å forbedre eller optimalisere anaerob nedbrytning av lignocellulose og organisk avfall, slik at det oppnås maksimal biogassproduksjon. Dette gjøres ved dampeksplosjon, samråtning og resirkulering av prosessvann. Det betyr ikke bare hvordan produksjon av ren energi kan økes, men avhandlingen inneholder også studier av hvordan oppløste næringsstoffer i våte råtnerester kan renses og fanges og derved resirkuleres tilbake til dyrket mark. For organisk avfall, landbruk og matavfall vil dette også føre til redusert klimagassutslipp dersom mineralgjødsel erstattes.

Dampeksplosjon ble testet på et lignin rikt løvtre, Salix viminalis (korgpil), som ga en økning av metanproduksjonen på opptil 50 % sammenlignet med chips (Artikkel I). Forholdstallene mellom karbon og nitrogen (C/N) som ble funnet etter screening av ulike blandinger av Salix og storfe gjødsel, indikerte at blandinger som inneholdt opp til 40 % VS (volatile solids) av dampeksplodert Salix ga godt metanutbytte (Artikkel I). Men når substrater med høyt fiberinnhold blir utråtnet i semi-kontinuerlige systemer, forblir mye av metan potensialet uutnyttet dersom oppholdstiden er for kort. For å kunne dra nytte av et slik gjenværende metan potensial innebærer, i) gasstette tanker for etterlagring, ii) etablere et ekstra utråtningstrinn ved anlegget, iii) gjennomføre en ekstra forbehandling/etterbehandling av råtneresten før ii) og/eller iv) bruke lengre oppholdstid. Dette siste kan også oppnås ved å tynne ut råstoff ved resirkulering av våt råtnerest, altså vannfasen fra avvannet slam fra bioreaktor (prosessvann) i stedet for bruk av rent vann. Dette ble undersøkt for å se om en oppnår lengre oppholdstid uten å redusere kapasiteten. I denne studien ble resirkulering av væskefraksjonen av råtneresten vurdert som en metode for å forbedre effektiviteten og mer stabil metan produksjon (Artikkel II). Resultatene viste at fortynning av råmaterialet ved hjelp av resirkulert råtnerest i stedet for med vann økte metan produksjonen med opptil 27 %. Konsentrasjonene av ammoniakk og flyktige fettsyrer var lave og førte ikke til problemer, men tørrstoff akkumulering var merkbar i reaktorene

med resirkulering, noe som førte til lavere nedbrytningsgrad etter tre hydrauliske oppholdstider. Dette indiker at seperasjonen av tørrstoff i avvanningsprosessen må optimaliseres ved bruk av denne metodenmed resirkulasjon. Separasjon av en større andel partikler ble derfor inkludert i Artikkel III.

Foruten storfegjødsel og Salix, ble et annet viktig norsk organisk avfall testet: fiskeavfall som biprodukt kategori 2 (Artikkel III). Fiskeoppdrett er en stor industri i Norge og avfall fra denne virksomheten har et høyt protein- og fettinnhold som betyr et høyt energiinnhold. Bedre utnyttelse av slike produkter som ellers blir dumpet i havet, ville innebære store miljømessige og økonomiske fordeler. Når fiskeavfall som biprodukt kategori 2 ble blandet med dampeksplodert Salix og gjødsel i en kontinuerlig rørt tank reaktorer, økte avkastning i gjennomsnitt 35 % (Artikkel III). Fiskeavfall er et nitrogen rikt substrat og resirkulering av prosessvann ble også testet her for å evaluere stabiliteten av prosessen med hensyn til ammonium nivåer. Resirkulert råtnerest ga ikke bare økt metanutbytte, men favoriserte også stabilitet med en mikrobiell biomasse som var tolerant for nivåer av $NH_4{}^+$-N som vanligvis vil korrespondere med hemmende nivåer av ammoniakk (Artikkel III). Utråtning av et nitrogen rikt substrat sammen med en tilstrekkelig andel lignocellulose og husdyrgjødsel var avgjørende for å balansere næringsstofftilgangen og holde prosessen i gang. Nitrogen og fosfor ble mineralisert under anaerobe forhold, og omgjort til plante-tilgjengelige næringsstoffer. De ble derfor undersøkt for rensing og utvinning fra den våte råtneresten til faststoff fraksjoner ved hjelp av stuvitt utfelling og betonitt adsorpsjon og begge metoder gav interessante resultater med henholdsvis 90 og 80 % $NH_4{}^+$-N fjerning. Fosfat innholdet var lavt i den våte råtneresten, men noe ble fjernet, det meste som struvitt.

Derfor, ved å ta hensyn til: i) en hensiktsmessig forbehandling, ii) et riktig blandingsforhold mellom forskjellige tilgjengelige råstoffer til trevirke, iii) at resirkulering kan gi høyere metanutbytte og styrker stabiliteten med et lavere ressursbehov, og iv) at gjenvinning av næringsstoffer i den våte råtneresten gir gode gjødselprodukter, kan biogassproduksjon i Norge blir attraktivt både til erstatning av fossile brensler til transport og som leverandør av næringsstoffer.

Dette doktorgrad studiet ble utført ved Institutt for matematiske realfag og teknologi (IMT) ved Universitetet for miljø og biovitenskap (UMB) på Ås, fra desember 2009 til februar 2013 (3 år og 3 måneder). Studien tilhører Work Package II av prosjektet "Fra biomasse til biogass - en integrert tilnærming til bærekraftig utvinning av energi og næringsstoffer" utviklet i fellesskap av, Institutt for plante-og miljøvitenskap (IPM), Institutt for kjemi, bioteknologi og matvitenskap (IKBM), forskningsinstituttet Bioforsk og det norske selskapet CAMBI. Prosjektet ble finansiert av Norges Forskningsråd (prosjekt n º 423513 UB) og hadde som hovedmål å øke kunnskapen og kompetansen om biogassproduksjon under norske forhold. Et state-of-the art biogass laboratorium ble etablert ved UMB i løpet av denne doktorgrad studiet.

Resumen

La producción de biogás es un proceso sustentable muy demandado hoy en día debido a todos los beneficios que conlleva. No solamente permite producir energía limpia y eliminar la dependencia del uso de combustibles fósiles, sino que también permite reciclar los nutrientes retornándolos a los suelos y disminuir las emisiones de gases de invernadero provenientes del sector agropecuario. El proceso es muy versátil, ya que materiales muy diferentes pueden ser empleados como materia prima (biomasa) para la generación de metano, como por ejemplo desechos orgánicos, i.e. biomasa que no interfiere con la producción de alimentos. El creciente interés en el uso de diferentes materiales de origen agrícola o industrial como substratos para la producción de biogás provoca buscar formas de optimizar las distintas etapas del proceso para hacerlo mas eficiente.

Esta tesis abarca muchos aspectos dentro del proceso de digestión anaerobia donde tanto la eficiencia como la estabilidad del proceso pueden mejorarse. Se hizo énfasis en estudiar los efectos que provoca pre-tratar biomasa rica en lignocelulosa mediante hidrólisis térmica (explosión al vapor) respecto a su producción de biogás (Artículo I), digerir diferentes tipos de materiales juntos (Artículos I-II-III), aplicar reciclo del agua de proceso o digestado (Artículos II-III) y recuperar nutrientes del digestado (Artículo III).

Se aplicó hidrólisis térmica a muestras de *Salix viminalis*, un arbusto rico en lignina, dando un incremento de su producción de metano de hasta un 50 % compárandolo con *Salix* que fue solamente molida (Articulo I). Mezclas de *Salix* pre-tratada y purines vacunos de diferentes proporciones en sólidos volátiles (VS %), y por lo tanto diferentes radios de carbono y nitrógeno (C/N), fueron investigadas. Se obtuvieron buenos rendimientos de producción de metano con hasta un 40 % VS de *Salix* pre-tratada en la mezcla (Artículo I). No obstante, cuando substratos con alto contenido en fibras son digeridos de forma semi-continua, una gran parte del potencial en metano permanece sin explotar si los tiempos de retención no son suficientes para la degradación de estos materiales. Maneras de aprovechar este potencial residual de metano incluyen: i) emplear tanques herméticos para el almacenamiento del digestado; ii) disponer de sistemas de post-digestión en las plantas de biogas; iii) aplicar pre-tratamiento a substratos difíciles de digerir y/o iv) aplicar tiempos de retención hidráulica mayores. Esta ultima alternativa tambien puede conseguirse mediante la recirculación del digestado nuevamente hacia el digestor, permitiendo que los tiempos de retención tanto hidráulico como de sólidos se extiendan sin comprometer la capacidad del sistema. En este estudio, la recirculación de la fracción líquida del digestado fue investigada como método para mejorar la eficiencia y estabilidad del proceso (Artículo II). Los resultados indicaron que el uso de digestado recircualdo en vez de agua para la dilución de los substratos iniciales incrementó la producción de metano hasta un 27 % . Los niveles de amoníaco y y ácidos grasos volátiles no causaron problemas o inhibición del proceso, pero se constató acumulación de sólidos dentro de los digestores, mas pronunciada en los recirculados, causando la disminución de la eficiencia del proceso luego de tres tiempos de retención hidráulicos. Optimizar la separación de sólidos cuando se realiza recircualción del digestado es esencial para garantizar la estabilidad a largo plazo de dichos sistemas. Una mejor separación fue

investigada en el artículo III.

Además de purines vacunos y *Salix*, otro importante residuo orgánico Noruego fue analizado con respecto a su producción de biogás: el desecho "categoría 2" de la industria pesquera (Artículo III). Dicha industria es extensiva en Noruega y los residuos que ésta produce poseen gran cantidad de proteínas y lípidos, lo que se traduce en un gran valor energético. El aprovechamiento de estos materiales que de otra forma serían volcados al medio ambiente y océano, implicaría para Noruega grandes beneficios tanto ambientales como económicos. Cuando el subproducto categoría 2 fue co-digerido con *Salix* pre-tratada y con purines vacunos en reactores continuos agitados, el rendimiento se incrementó en promedio un 35 % (Artículo III). El reciclo de digestado también se investigó en este proceso para evaluar la estabilidad frente a grandes concentraciones de nitrógeno amoniacal (${NH_4}^+$-N). Los resultados indicaron una robusta estabilidad en los reactores ya que los valores de ${NH_4}^+$-N se incrementaron hasta niveles que hubiesen sido inhibitorios al proceso (Artículo III). La recircualción de digestado no solo ayuda a recuperar el potencial residual de metano, sino que también favorecería la estabilidad, enriqueciendo la comunidad microbiana que se vuelve fuerte y tolerante a valores altos de sustancias tóxicas como amoníaco. Una óptima proporción de lignocelulosa junto a biomasa rica en ${NH_4}^+$-N en la co-digestión fué crucial para balancear los nutrientes y mantener el proceso estable.

En la digestión anaerobia, el nitrógeno y el fósforo son mineralizados y convertidos en nutrientes disponibles para las plantas. El ${NH_4}^+$-N y el fosfato pueden ser recuperados del digestado final no reciclado, bajo la forma de precipitados o lodos, e incorporarse a la fraccion separada de digestado solido de forma de mejorar su valor nutriente. Las técnicas simples y efectivas que se analizaron en este estudio incluyeron la precipitación de estruvita y la adsorción en bentonita. Ambas técnicas dieron resultados interesantes en cuanto a la cantidad de ${NH_4}^+$-N removido (90 % y 80 % respectivamente). El contenido de fosfato del digestado final no fue elevado, pero se pudo obtener cierta remoción con la precipitación de estruvita.

Por lo tanto, considerando: i) un buen pre-tratamiento, ii) un radio de co-digestión óptimo de diferentes materiales disponibles, iii) ahorro de recursos como agua mientras se aprovecha el potencial energético residual de metano y se mejora la estabilidad, iv) y la recuperación de nutrientes en un valorado biofertilizante, el biogás puede volverse atractivo no solo como substituto de los combustibles fósiles en el transporte, sino también como proveedor de un fertilizante orgánico de alta calidad, todo esto mientras además ayuda en el re-uso y tratamiento de residuos orgánicos potencialmente contaminantes.

Este estudio de doctorado fue realizado en el Departamento de Ciencias Matemáticas y Tecnología (IMT) de la Universidad Noruega de Ciencias (UMB) en Ås, de diciembre 2009 a febrero 2013 (3 años y 3 meses). El estudio perteneció al ítem de trabajo II del proyecto titulado: "Desde la Biomasa hasta el Biogás- una estrategia integral hacia la recuperación sustentable de energía y nutrientes" desarrollado en conjunto por el mencionado departamento, el Departamento de Ciencias Ambientales (IPM), el Departamento de Química, Biotecnología y Ciencias Alimentarias (IKBM), el Instituto Noruego de Investigación Agrícola y Ambiental (Bioforsk) y la compañía Noruega CAMBI. El proyecto fue financiado por el Consejo de Investigación Noruego (nº de proyecto 423513 UB), con la misión de fomentar el conocimiento y experiencia sobre digestión anaerobia en Noruega. Un laboratorio de vanguardia en el área biogás fue implementado en la UMB durante el transcurso de este doctorado.

List of papers

- I. **Estevez, M. M.**, Linjordet, R., Morken, J. (2012) Effects of steam explosion and co-digestion in the methane production from *Salix* by mesophilic batch assays, Bioresource Technology, Volume 104, pages 749-756.
- II. **Estevez, M. M.**, Sapci, Z., Linjordet, R., Schnürer, A., Morken, J. (2013) Semi-continuous anaerobic co-digestion of cow manure and steam exploded *Salix* with recirculation of liquid digestate. Submitted.
- III. **Estevez, M. M.**, Sapci, Z., Linjordet, R., Morken, J. (2013) Incorporation of fish byproduct to the semi-continuous anaerobic co-digestion of pre-treated lignocellulose and cow manure, with recovery of digestate's nutrients. Submitted.

Additional scientific publications and contributions done during the PhD studies:

- Horn, S. J., **Estevez, M. M.**, Nielsen, H. K., Linjordet, R., Eijsink, V. G. H. (2011) Biogas production and saccharification of *Salix* pre-treated at different steam explosion conditions, Bioresource Technology, Volume 102, pages 7932-7936.
- **Estevez, M.M.**, Linjordet, R., Morken, J. (2011) Biogas optimization by steam explosion of *Salix*, and recycling of process water from biogas production of *Salix* and manure. Conference proceedings of the International IWA-Symposium on Anaerobic Digestion of Solid Waste and Energy Crops, Vienna, Austria.
- **Estevez, M.M.**, Linjordet, R., Morken, J. (2012) Organic loading rate effect on anaerobic digestion: case study on co-digestion of lignocellulosic pre-treated material with cow manure. Conference proceedings of the International Conference of Agricultural Engineering, Valencia, Spain.
- Sapci, Z., **Estevez, M.M.**, Linjordet, R., Morken, J. (2012) Effect of different thermal pre-treatment techniques on biogas production from lignocellulosic biomass: wheat straw and *Salix*. Conference proceedings of the International Conference of Agricultural Engineering, Valencia, Spain.
- Eich-Greatorex, S.,Vivekanand, V., **Estevez, M.M.**, Schnürer, A., Sogn, T. A. (2013) Recycling nutrients from biogas digestates based on lignin-rich feedstock. Manuscript.

Supervisors

Main Supervisor

- Associate Professor John Morken, Department of Mathematical Sciences and Technology, Norwegian University of Life Sciences, Ås, Norway.

Co-supervisors

- Dr. Roar Linjordet, Bioforsk, Norwegian Institute for Agricultural and Environmental Research, Ås, Norway.
- Associate Professor Jens Bo Holm-Nielsen, Aalborg University Esbjerg, Esbjerg, Denmark.
- Assistant Professor Zehra Sapci, Department of Mathematical Sciences and Technology, Norwegian University of Life Sciences, Ås, Norway.(Current address: Bitlis Eren University, Environmental Engineering Department, Bitlis, Turkey)

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Abbreviations

AD:	Anaerobic Digestion
ATP:	Adenosine-5'-Triphosphate
BMP:	Biochemical Methane Potential
COD:	Chemical Oxygen Demand
CSTR:	Continuously Stirred Tank Reactor
FAD:	Flavin Adenine Dinucleotide
FSPM:	Fish Silage Processing Method
GHG:	Greenhouse Gas Emissions
HMF:	Hydroxymethyl Furfural
HPLC:	High Performance Liquid Chromatography
HRT:	Hydraulic Retention Time
LCFA:	Long Chain Fatty Acids
NH_4^+-N:	Ammonium Nitrogen
NIR:	Near Infrared
OLR:	Organic Loading Rate
SAO:	Syntrophic Actetae Oxidation
SRT:	Solid Retention Time
T:	Temperature
THP:	Thermal Hydrolysis Pre-treatment
Ton.:	metric ton, 1,000 kg
Total-C:	Total Carbon
Total-N:	Total Nitrogen
TS:	Total Solids
VFA:	Volatile Fatty Acids
VS:	Volatile Solids

1 Introduction

Oil crises through the years have generated awareness that alternative sources of energy must be explored, developed and utilized to reduce our dependency on fossil fuels, which not only are becoming increasingly scarce but their use also cause negative environmental impact as global warming (IPCC, 2007; CIA, 2009). Norway is a country very rich in oil, gas and hydropower resources. Regarding bioenergy, 15 TWh are consumed annually in Norway, which includes mostly wood, wood waste, black liquor and pellets (Econ Pöyry, 2008)(Table 1). Energy crops, straw, landfill gas and biogas from manure are resources that are barely used today in Norway (Statistics Norway, 2008). Thus, only 1.1 per cent of the primary energy demand in Norway is covered by bioenergy (Econ Pöyry, 2008). Norway possess a yearly production of wood, peat, straw, manure and aquatic biomass, which can be translated into approximately 140 TWh (504 PJ) of energy. However, major part of this potential is not available to be used for energy purposes, as it is either too costly to extract, already in use for other purposes (e.g., as in timber or pulp and paper industry) or must be left in nature to sustain the ecosystem (Econ Pöyry, 2008). Nonetheless, Norway's bioenergy sector is starting to develop due to environmental incentives introduced by the government, aimed at increasing the share of bioenergy by 14 TWh in the total energy offer by the year 2020 (Olje og energidepartementet, 2008).

Renewable energy solutions that do not represent a threat to food production or entail depletion of natural resources and biodiversity, are increasing their demand nowadays (Bauer et al., 2009; Johansson et al., 2010). Conversion of various types of biomass into valuable energy resources has gradually become more relevant, being biogas production one of the most promising and versatile processes for this end. Biogas is the product of the anaerobic digestion (AD) process, a technology of high importance since it produces a second generation biofuel, i.e. methane (CH_4), from agricultural and crop wastes and not from edible crops as the first generation biofuels. Being Norway such an energy rich country, biogas production is regarded mainly as a solution to help the country in reducing greenhouse gas (GHG) emissions, since major contributors to this are the agricultural and transport sectors (Morken, 2007). It is estimated that in 2008, the agricultural sector was responsible for almost 9 % of the total Norwegian GHG emissions, corresponding to 4.8 million metric tons of carbon dioxide (CO_2)-equivalent (Landbruks og matdepartementet, 2009). CH_4 emitted from enteric fermentation and nitrous oxide (N_2O) from agricultural fields contributed to those GHG emissions on 44 and 46 % respectively, while CH_4 from manure management had a contribution of 10 % (Morken, 2007).

Table 1: Biomass use in Norway (TWh/year) (adapted from Econ Pöyry (2008)).

Fuel/Biomass Resource	Domestic Resource	Imported	Current use of Bioenergy
Raw wood	6.4	1.9	0.9
Processed wood	10.0	5.6	5.3
Wood wastes from furniture and wood products	0.5	1.8	0.7
Municipal waste	4.4	-	0.9
Wood wastes from construction	0.9	-	0.3
Landfill gas	1.0	-	0.1
Other biogas	3.0	-	0.1
Wood fuel	7.2	-	7.2
Straw and crop husks	4.5	-	0.1
Total	37.9	9.3	15.6

Biogas technology plays a big role in decreasing CH_4 and N_2O emissions from the agricultural sector. Instead of being released to the atmosphere by the spreading of untreated manure, CH_4 is recovered as an energy carrier, while mineralization of nitrogen into plant-available ammonium nitrogen (NH_4^+-N) occurs during the process (Massé et al., 2011). It has as well a better carbon footprint since the CO_2 emissions are reduced when substituting fossil fuels by CH_4. Various types of organic resources can be employed to generate biogas, providing they do not contain substances that may limit the use of the digested biomass as a fertilizer. Since phosphorous is also mineralized and more available in the final digestate, the use of this product as fertilizer can have a significant impact against today's decrease in phosphate (PO_4^{3-}) availability as fertilizer, which can threaten food supply (Massey et al., 2009). Biogas becomes a very relevant choice as environmental technology moreover when the government has the aim to increase bioenergy's participation in the national energy frame. In addition, the fact of minimizing waste disposal by turning the waste into a resource is one of biogas's major assets. However, it is necessary to increase the efficiency of the biogas conversion process in order to make it an attractive option as energy source in Norway.

Norway has 23 biogas establishments that rely on sewage sludge as the feedstock, one plant that handles both sludge and food waste, and five that use purely food waste (Avfall Norge, 2010). In 10 of those biogas plants, animal byproducts are as well employed as substrates (Mattilsynet, 2012). Most of the Norwegian biogas plants are relatively small, with a gas production of less than 1 million m ^{3} biogas (6 GWh) per year (Avfall Norge, 2010), and only four plants have a larger annual biogas production. Quite recently, the Waste to Energy Agency of Oslo Municipality (EGE) finished building a large biogas plant in Romerike, that will process sort-separated household food waste into biogas and organic fertilizer. The plant capacity will be of 50,000 tons of food waste annually, and the biogas will be use as transport fuel; 135 buses will be able to run on the biogas produced (EGE, 2012).

According to Statistics Norway (2008), only about 3 % of Norway's land (excluding Svalbard and Jan Mayen) is cultivated. This also means that farms are scarce in

Norway compared to other countries, and so agricultural organic wastes as animal manure are not a main substrate in the case of biogas production. Lignocellulose-rich biomasses, on the other hand, are the major biomasses types available in Norway, but in order to serve as biogas substrates, they need to undergo special pretreatment so that the bacteria can digest them (Jørgensen et al., 2007; Horn and Eijsink, 2010). This treatment can be chemical or thermal as steam explosion (Brownell and Saddler, 1987; Ramos, 2003; Deublein and Steinhauser, 2008). Performing co-digestion of different materials may also help to increase efficiency of the digestion process, so after pre-treating lignocellulosic biomass, co-digestion is a good option. It is preferable to co-digest high lignocellulosic biomass with slurry since a more stable process can be achieved as well as higher degradability and methane yield due to better nutrient balance in terms of carbon and nitrogen (Angelidaki and Ellegaard, 2003; Deublein and Steinhauser, 2008). So both the application of pre-treatment and co-digestion of different types of substrates are options that can help in turning biogas an attractive energy technology for Norway and in developing further the biogas sector in this country.

2 Study aim and objectives

The aim of this PhD study was to improve the anaerobic digestion process efficiency and stability when using different kinds of agricultural and industrial substrates found in Norway, as well as the final digestate to be used as biofertilizer. For such purpose, at each stage of the process; i) pre-treatment, ii) process itself and iii) post-treatment, different improvement techniques were applied. The effects of steam explosion in enhancing the degradation of highly lignocellulosic biomass was studied as pre-treatment. Anaerobic co-digestion of very different materials and effects of recirculating the digestate within the process were evaluated as process improvement techniques, while recovery of the main nutrients from the process effluent (final digestate) in order to ameliorate the final biofertilizer was the post-treatment approach. Clones of *Salix viminalis* were chosen as lignocellulosic biomass; cattle manure as animal waste, while the industrial processing waste used was fish byproduct category 2. Ammonium-N levels were aimed to be increased both with recirculation and with the addition of fish byproduct, so to study the effects on the stability and production of biogas. Finally, the post-treatment approach included chemical precipitation and adsorption studies for the recovery of mainly NH_4^+(and to some extent also PO_4^{3-}) from the nitrogen-rich digestate.

The specific areas in which the research focused were:

- Analysis and screening of steam explosion as a pre-treatment for digestion of lignocellulosic biomass under anaerobic conditions (**Paper I**).
- Analysis of different substrate's mixtures for performing anaerobic co-digestion (**Papers I-II-III**)
- Monitoring of relevant process parameters (**Papers I-II-III**)
- Evaluation of the effects of process water (liquid digestate) recirculation in the anaerobic co-digestion (**Papers II-III**)
- Study of ammonium-N recovery methods for treatment of the process effluent (final digestate) and enhancement of the final biofertilizer (**Paper III**)

Paper I focused on batch biomethane mesophilic (37 ºC) systems, first to investigate the best steam explosion conditions for the pre-treatment of *Salix viminalis* with regard to its methane production, and second to investigate the methane yields derived from the co-digestion of steam exploded *Salix viminalis* and cow manure. A screening of different conditions of temperature and time during the steam explosion of *Salix* was performed. *Salix* treated at the conditions that gave the highest methane yields was chosen to further investigate how much of it could be co-digested with manure without compromising the biogas production.

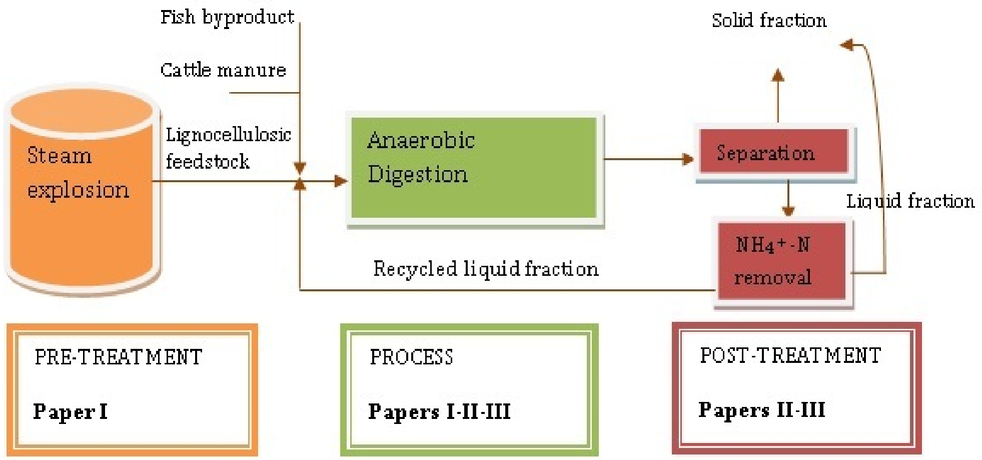


Figure 2.1: Thesis outline and areas of focus of each paper.

Different C/N ratio mixtures, originating from mixing different proportions of the substrates in volatile solids basis, were correlated to their methane production. Potential expected methane yields of each mixture were calculated and compared to the obtained yields, and two trials with already digested manure were performed in order to determine residual methane potential.

In **Paper II**, the optimum mixture of steam exploded *Salix* and manure was studied in mesophilic continuously stirred tank reactor (CSTR) systems with respect to its stability and methane production. The mixture in VS basis was composed of 40 % of steam exploded *Salix* and the rest of manure. Main focus of **Paper II** was to investigate the effects that applying recirculation of the liquid digestate would have on the process, which would translate in minimizing water consumption.

Paper III's aim was to investigate how high levels of nitrogen (N) would affect the stability of an anaerobic co-digestion process that included recirculation of the digestate. Improved separation of solids in the recirculated digestate was analyzed as enhancer of process long term stability. Fish byproduct from the Norwegian fish processing industry was introduced as co-digestion substrate, providing a higher ammonia content. A good balance of carbohydrates, proteins and fats provided by the different co-substrates, together with the recirculation of digestate and the partially longer retention time caused by the last one, would favor both adaptation of the microbial community to process fluctuations and the fertilizer nutrient composition. Post-treatment of the digestate was also addressed by applying chemical precipitation by struvite formation and bentonite adsorption techniques to recover nutrients from an enriched digestate and turn it into a valuable biofertilizer.

3 Background on anaerobic digestion

3.1 The biogas production process

Anaerobic digestion is the process by which organic matter is broken down by a wide range of microorganisms, in the absence of oxygen, giving biogas. The principal components of biogas are methane (55-70 %) and carbon dioxide (30-45 %). Hydrogen sulfide, water and traces of other gases are also present. Methane formation is a process that occurs naturally in the environment, as in the digestive tract of ruminants, in bottom sediments of lakes and ponds, in swamps, hot springs, during wet composting of plants and in flooded rice fields (Sims, 2002; Deublein and Steinhauser, 2008). One of the reasons that make this process attractive is the high degree of reduction of organic matter that is achieved with small increments in the bacterial biomass, if it is compared to the aerobic process (Angelidaki, 2004).

The other relevant reason is the possibility of using the generated product, biogas, as a fuel for the production of different forms of energy (e.g., heat, electricity, vehicle-fuel and natural gas) (Angelidaki, 2004). Formation of methane from biomass follows the general equation, established by Buswell in 1930 (Deublein and Steinhauser, 2008):

$$C_{\mathrm{c}}H_{\mathrm{h}}O_{\mathrm{o}}N_{\mathrm{n}}S_{\mathrm{s}} + yH_2O \rightarrow xCH_4 + nNH_3 + sH_2S + (c-x)CO_2 \tag{3.1}$$

where

$$x = \frac{1}{8} \cdot (4c + h - 20 - 3n - 2s) \tag{3.2}$$

$$y = \frac{1}{4} \cdot (4c - h - 20 + 3n + 3s) \tag{3.3}$$

Degradation of the biomass comprises four major processes: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Ahring, 1991; Schink, 1997; Deublein and Steinhauser, 2008; Massé et al., 2011) .

3.1.1 Hydrolysis

In this first step of the anaerobic digestion and biogas production, particulate organic matter, formed by carbohydrates, proteins and fats, is de-polymerised into water-soluble monomers. Extra cellular enzymes of facultative bacteria, i.e. bacteria that can live under anaerobic or aerobic conditions, and obligatory anaerobic bacteria, are responsible for this fragmentation process (Lastella et al., 2002; Deublein and Steinhauser, 2008). The hydrolysis rate of the different materials; carbohydrates, proteins and lipids, differ greatly. Proteins and lipids are energy-rich materials relatively easy to digest that can produce biogas with high methane content. Simple sugars can be broken down very easily, while cellulose, the most common organic component on earth, is more difficult to degrade. In plant cells, cellulose is linked to hemicellulose and lignin, being this last one not degradable at all in the biogas process (Gunaseelan, 1997; Zhang et al., 2007). Due to its chemical and physical properties, when lignin is associated with cellulose, it acts as a barrier, preventing the hydrolyzing enzymes from entering and disturbing the cellulose structure. Therefore, if cellulose and lignin are the main constituents of the substrate, the limiting step in the whole anaerobic process will be determined by the hydrolysis step (Gunaseelan, 1997; Zhang et al., 2007; Deublein and Steinhauser, 2008; Seppälä et al., 2008). On the other hand, if the substrate is mainly composed of easily metabolized matter, the formation of methane from acetate will be the rate-limiting step (Angelidaki, 2004).

3.1.2 Acidogenesis

Fermentative bacteria take up the small water-soluble hydrolysis products, giving methanogenic substrates: acetate, carbon dioxide and hydrogen. Fatty acids, alcohols and ammonia from the degradation of amino acids, are also formed (Angelidaki, 2004). In a good and efficient anaerobic digestion process, the fermentative bacteria, or acidogenes, would directly transform most of the organic material to methanogenic substrates. A significant portion, i.e. 30 %, will however be transformed into other products such as lower fatty acids and alcohols (Angelidaki, 2004). Depending on the balance of the process, on how fast the formed hydrogen is consumed, this last portion can be larger. The most relevant organic intermediates formed in the degradation of heterogeneous organic matter are volatile fatty acids (VFA): acetic, propionic, isobutanoic, iso-valeric, and valeric acids (Holm-Nielsen et al., 2007). Organic overload leads to excess of these substances and to inhibition of the anaerobic digestion process (Angelidaki, 2004; Deublein and Steinhauser, 2008; Holm-Nielsen et al., 2007). Thus, it is essential to monitor their presence in a reliable way and be able to notice imbalances early in time.

3.1.3 Acetogenesis

Acetate, carbon dioxide and hydrogen can be directly digested by methanogenes, to produce methane. However, acidogenic products with more than one carbon, such as alcohols and volatile fatty acids, need first to be converted into acetic acid, carbon dioxide and hydrogen. This is done by aceotogenic bacteria. This oxidation step has a very limited energy yield at standard conditions, compared, for instance, to that generated in the fermentation of glucose to acetate. The hydrogen partial pressure has to be maintained at a low level, in order to be able to increase the energy released by these acetate formation reactions, i.e. make them thermodynamically favorable so that the equilibrium of the reaction can be shifted towards more product formation (hydrogen), and thus, more substrate degradation (Schink, 1997; Angelidaki, 2004; Deublein and Steinhauser, 2008). Therefore, only at very low hydrogen gas (H_2) concentration can the acetogenic bacteria get the energy needed for their survival. They must thus live in symbiosis together with the methanogenic bacteria, which can survive only with higher hydrogen partial pressure (Schink, 1997; Deublein and Steinhauser, 2008). Energetically, the anaerobic degradation of fatty acids and alcohols proceeds at the expense of the methanogenic bacteria that in return, receive the substrates needed for their survival and growth from the acetogenic bacteria.

3.1.4 Methanogenesis

In the fourth, final stage and under strictly anaerobic conditions, methane formation takes place. As all methanogenic bacteria species do not degrade all substrates, the

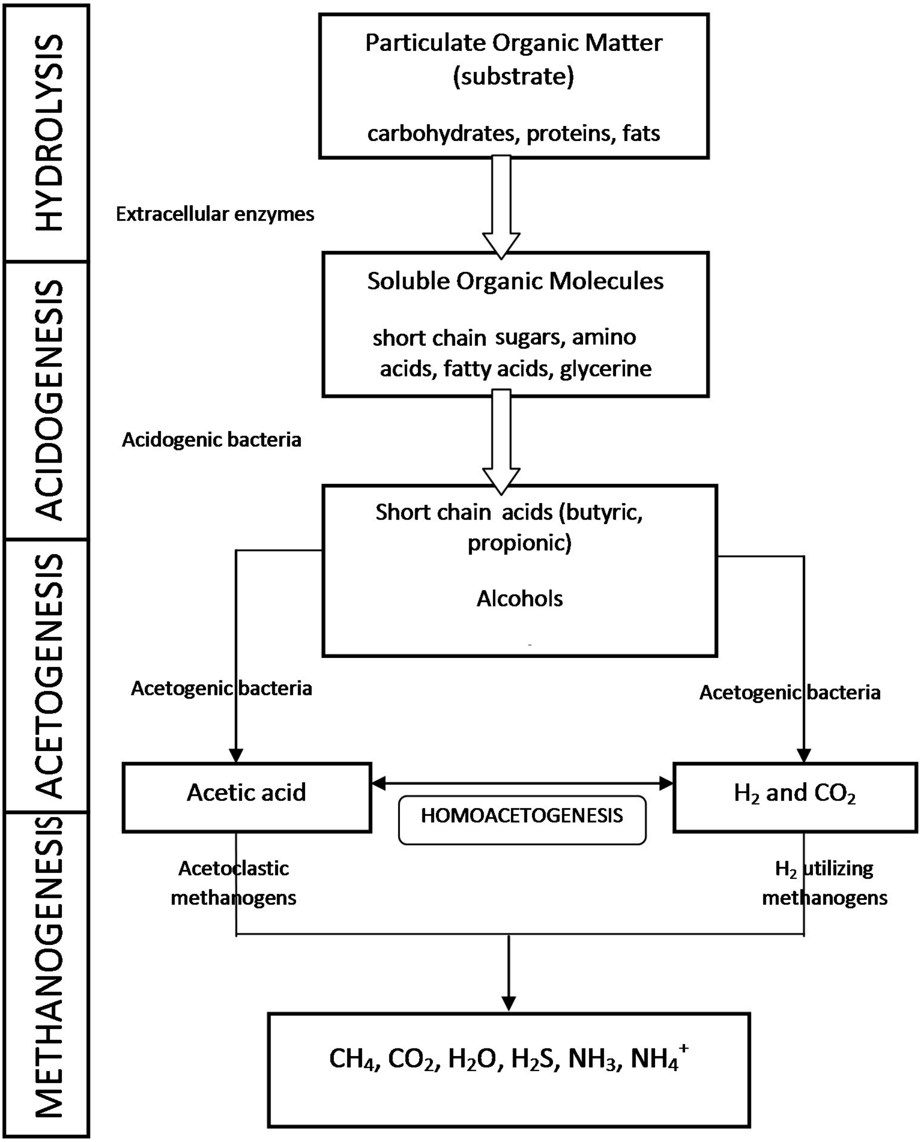


Figure 3.1: Anaerobic digestion major processes (adapted from Deublein and Steinhauser (2008)).

substrates acceptable for methanogenesis can be divided into three types: the carbon dioxide type (CO_2, CO, $HCOO^-$); the methyl type (CH_3OH, CH_3NH_3) and the acetate type (CH_3COO^-) (Deublein and Steinhauser, 2008). Also the methanogenic bacteria can be divided into two main groups, the acetoclastic methanogens which generate methane from acetate, and the H_2 utilizing methanogens (hydrogenotrophs), that transform hydrogen and carbon dioxide-types of substrate into methane (Angelidaki, 2004). Almost 70 % of the total methane formed is obtained from acetate as precursor; H_2 and CO_2 are responsible for the remaining 30 % of methane produced (Angelidaki, 2004; Deublein and Steinhauser, 2008)

Besides methanogenic reactions, an inter-conversion between hydrogen and acetate performed by homoacetogenic bacteria also plays a role in the methane production. Depending on the external hydrogen concentration, these bacteria can either oxidize or synthesize acetate (Boe, 2006). At temperatures around 30 ºC, hydrogenotrophic methanogens are more favorable to consume hydrogen than homoacetogens, while at lower temperatures homoacetogenesis takes over as the main hydrogen removal pathway, followed by acetoclastic methanogenesis to produce methane (Boe, 2006).

Methane production from acetate has been found to follow an additional pathway that besides the traditional methanogens, involves another group of non-methane-producing bacteria. These microorganisms convert acetate into H_2 and CO_2 (Eq. 3.4), which later are used by the hydrogenothrops to produce methane (Eq. 3.5). This cooperation is called syntrophic acetate oxidation (SAO) (Barker, 1936; Zinder and Koch, 1984; Schnürer et al., 1994, 1999).

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4H_2 + H^+ \tag{3.4}$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \tag{3.5}$$

For the acetate oxidation to hydrogen to occur, the hydrogen gas pressure must be kept low but be enough to favor the hydrogenotrophic methanogens. The change to the SAO pathway is related to microbial adaptation to a high ammonia content (Schnürer et al., 1999; Schnürer and Nordberg, 2008).

3.1.5 Process parameters for the anaerobic digestion

The parameters that affect microbial metabolism processes are many, and they have to be taken into consideration and monitored in order to achieve an optimum fermentation process. Moreover, the fermentative bacteria responsible for the hydrolysis and acidification stages have environmental requirements that differ from those corresponding to the methanogenic bacteria. The following aspects have to be taken into consideration as well: with lignocellulose-rich substrates, the limiting step of the AD process and thus the one that needs higher priority is the hydrolysis. Substrates that contain proteins are more easily degraded, and optimum pH is the same for either fermentative or methanogenic bacteria. In the case of fats, hydrolysis happens faster as bioavailability increases (emulsification), so the acetogenesis step is limiting (Deublein and Steinhauser, 2008).

Temperature is an important variable in AD. Acidogenic bacteria can survive at two different temperature levels: mesophilic strains at 32-42 ºC and thermophilic strains at 48-55 ºC (Deublein and Steinhauser, 2008). In the case of methanogens, their growth rates vary with different temperature ranges. While best results are obtained at thermophilic temperatures and with them also the mixing of the biomass, solubility of organic compounds and sanitation are improved, these processes requires more energy (Angelidaki, 2004) and have less microbial diversity which implies a risk for instability and ammonia inhibition (Ahring, 2003; Angelidaki, 2004).

Another important factor is pH. It does not only influence the growing and survival of the microbial community, but also can cause dissociation of compounds such as ammonia, sulfide and organic acids, all very relevant to the process (Angelidaki, 2004). Methane forming bacteria have an optimum pH range of 6.7-7.5 while acidogenic bacteria can exist at lower pH. If the pH drops below 6.5, production of organic acids will further lower it and the process will cease. As mentioned before, excess of VFA due to organic overload leads to inhibition of the anaerobic digestion process (Angelidaki, 2004; Holm-Nielsen et al., 2007; Deublein and Steinhauser, 2008), being their presence another crucial parameter to monitor.

Two natural buffering systems within the process help in avoiding pH imbalances: the carbon dioxide/bicarbonate/carbonate ($CO_2/HCO_3{}^-/CO_3{}^{2-}$) and the ammonia/ammonium ($NH_3/NH_4{}^+$)(Deublein and Steinhauser, 2008). The first system avoids that strong acidification takes place. If pH decreases, CO_2 which is continuously produced during the process, would be dissolved in the substrate; if pH raises it will form carbonic acid (H_2CO_3) which tends to ionize and thus, hydrogen ions would be released (Deublein and Steinhauser, 2008).

$$CO_2 \longleftrightarrow H_2CO_3 \longleftrightarrow HCO_3^- + H^+ \longleftrightarrow 2H^+ + 2CO_3^{2-} \tag{3.6}$$

Temperature also affects the equilibrium and the solubility of CO_2 decreases with higher temperatures. Thus, thermophilic processes would have in reality a higher pH value than mesophilic processes, in where dissolved CO_2 tends to form H_2CO_3 (Angelidaki, 2004).

When nitrogen compounds are anaerobically degraded, ammonia and ammonium ion are produced. These compounds provide the second natural buffer system, that avoids a weak acidification during the process. When pH falls, ammonium is formed and hydroxyl ions are then released. In the opposite case, more free ammonia is formed.

$$NH_3 + H_2O \longleftrightarrow NH_4^+ + OH^- \tag{3.7}$$

$$NH_3 + H^+ \longleftrightarrow NH_4^+ \tag{3.8}$$

The balance for this system is at pH 10 (Reeves, 1972; Lei et al., 2007; Deublein and Steinhauser, 2008). Temperature also affects the equilibrium, under thermophilic conditions, the equilibrium between NH_3 and $NH_4{}^+$ would be shifted towards NH_3, and thus free ammonia concentration increases as the temperature increases.

Ammonia is an important nutrient needed for bacterial growth, but methane forming bacteria are particularly sensitive to high concentrations (Koster and Lettinga, 1984; Zeeman et al., 1985; Angelidaki and Ahring, 1993; Deublein and Steinhauser, 2008; Schnürer and Nordberg, 2008). Ammonia inhibition causes problems especially in the anaerobic digestion of animal manure, due to the high concentrations of NH_3/NH_4^+ that is provided by its urine content (Angelidaki, 2004). As explained before, with rising pH or temperature, the fraction of free ammonia increases and so does its inhibition effect. To calculate the ammonia content, total ammonium nitrogen (NH_4^+-N) has to be measured and the following equation applied:

$$NH_3(g/L) = \frac{NH_4^+ - N\,(g/L)}{(1 + 10^{(pKa-pH)})}\;, pKa = 0.09018 + \frac{2729.92}{(T + 273.15)} \tag{3.9}$$

in which pKa is the dissociation constant for ammonium (NH_4^+), 8.95 at 35 ºC; T the temperature in ºC (Calli et al., 2005; Schnürer and Jarvis, 2010).

According to literature (Kroeker et al., 1979; Zeeman et al., 1985; Angelidaki and Ahring, 1993; Chen et al., 2008), inhibition can occur at the wide concentration range of NH_4^+-N from 1.4 to 17 g/L. Free ammonia inhibits the methanogenic phase, showing, most of the studies, acetoclastic as the more susceptible methanogens to inhibition, rather than hydrogenotrophic (Koster and Lettinga, 1984; Zeeman et al., 1985; Angelidaki and Ahring, 1993; Calli et al., 2005) although few studies observed the relatively high resistance of the first ones to high total NH_4^+-N levels comparing to hydrogen utilizing methanogens (Zeeman et al., 1985; Chen et al., 2008). Several mechanisms have been attributed to ammonia inhibition of methanogens. The first one is based on hydrophobic free ammonia molecules diffusing passively through the cell membrane into the cell and rapidly converted into ammonium, causing a change in the intracellular pH conditions. Inside the cell, ammonia is transformed into ammonium, and a proton is subsequently taken up, causing a proton imbalance and/or potassium deficiency (Kroeker et al., 1979; Sprott and Patel, 1986; Calli et al., 2005; Chen et al., 2008; Schnürer and Jarvis, 2010). A second possible mechanism involves direct inhibition by NH_4^+/NH_3 on the methane-synthesising enzymes (Sprott et al., 1985; Calli et al., 2005; Chen et al., 2008). Increased maintenance energy requirement is a third mechanism proposed to cause ammonia inhibition (Chen et al., 2008).

In the case that the process is inhibited with ammonia and the concentration of VFA increases, the pH will decrease, allowing the concentration of free ammonia to decrease, and thus, masking the inhibition. This case is called the inhibited steady-state (Angelidaki, 2004). Thus, together with the already mentioned VFA, ammonia content is one of the most important parameters that ought to be continuously monitored in an AD process. In full scale processes, both parameters can be closely controlled by the near infrared (NIR) spectroscopy technique, which is a powerful tool that can save precious time avoiding potential collapse and providing both qualitative and quantitative on-line/at-line analysis (Holm-Nielsen et al., 2007; Jacobi et al., 2009).

When oil and fats are being hydrolyzed, long chain fatty acids (LCFA) such as oleate and palmitate are as well present in the process (Sousa et al., 2008). These compounds are potentially attractive for biogas production because of their high potential methane yield, but reports on the possibly toxic and inhibitory effect of LCFA towards methanogenic activity date back to the early 60's (McCarty, 1964; Angelidaki and Ahring, 1992; Rinzema et al., 1994; Callaghan et al., 1998; Eiroa et al., 2012). In the AD process, LCFA are converted into acetate and hydrogen via ß oxidation, and later turned into methane by the methanogens consortia (Angelidaki and Ahring, 1992; Sousa et al., 2008; Eiroa et al., 2012). Toxic effects due to LCFA accumulation are manifested in the adsorption of LCFA on the cell membrane, and interferences in the transport or protection mechanisms (Rinzema et al., 1994; Eiroa et al., 2012). According to Sousa et al. (2008), the adverse effects of LCFA on anaerobic digestion can be reverted under appropriate conditions and LCFA efficiently turned into methane. Using a continuous cycling feeding of a LCFA-rich material followed by batch degradation of the accumulated substrate would be an appropriate method (Sousa et al., 2008).

Besides the previously stated important process parameters, others as macro and micro nutrients concentrations and the type of substrate that will be digested also play an important role in the correct functioning of the anaerobic digestion process. The ratio between macronutrients carbon and nitrogen is in particular relevant for the process stability. Some literature reveals best ratio to be between 16-25:1 (C/N) (Deublein and Steinhauser, 2008), other authors recommend 25-35:1 (Yadvika et al., 2004; Holm-Nielsen et al., 2007). A too low C/N ratio would increase NH_3 production, while a too high ratio would not give enough N for the metabolism of the structural material by the microorganisms. Slurries and animal manures possess low C/N ratios as do other protein rich industrial wastes, while carbohydrates rich materials as crop residues and woody biomass possess higher ones. These different substrates should be then, mixed together in co-digestion systems, so to balance the process's nutrient composition (Holm-Nielsen et al., 2007; Deublein and Steinhauser, 2008; Ward et al., 2008).

Macro-nutrients as sulphur, phosphorous, potassium, calcium, magnesium and iron are required at levels of 10^{-4} M while micro-nutrients as nickel, cobalt and copper are required in lower concentrations (Boe, 2006). Iron and calcium are required as cofactors in enzyme activity, while sulphur is necessary for the amino acid synthesis and phosphorous in the nucleic acids, ATP and FAD radicals (Angelidaki, 2004). However, high concentrations of many of the nutrients could cause inhibition of the process. Sulfide and phosphate can precipitate metal ions and decrease its availability. Sulfide is produced in AD from the reduction of sulfate by the sulfate reducing bacteria (Chen et al., 2008). Since these bacteria include groups of oxidizers that can convert acetate to CO_2 and HCO_3^- and reducers that can turn i.e. lactate to acetate and CO_2, competition for common organic and inorganic substrates may occur in the AD process and suppress methane production (Chen et al., 2008). Hydrogen sulfide (H_2S) can also have an inhibitory effect even at low concentrations, since it can pass through the cell membrane (Boe, 2006; Chen et al., 2008).

3.2 Liquid manure and co-substrates

In most agricultural biogas plants, liquid manure is the main substrate. As manure's total solids concentration is rather low (around 5- 7 % for pigs and 7- 9 % for cows) and its lignocelluloses content is quite high, it is a substrate that when treated alone presents low yields of methane. Neither does the elevate fraction of fibers which makes degradation difficult and often pass undigested through the process, nor the high content of water help in obtaining a high biogas potential for manure (10- 20

m^3 CH_4/ ton of manure treated, ~ 222 mL CH_4/ g VS cow manure) (Angelidaki and Ellegaard, 2003). Manure is commonly being combined with other co-substrates in order to optimize the biogas yield. However, as a carrier substrate that supports the anaerobic digestion of industrial wastes that are not easy to treat separately, manure is a great option. The qualities that make of manure a great carrier substrate are its high content of water, in this case desired as it helps solubilize the more dry kinds of wastes, its high buffering capacity that enables protection against pH drops (VFA accumulation) and its supply of nutrients and trace elements needed for an optimal bacterial growth. Furthermore, use of manure also allows to have a periodic supply of other concentrated industrial wastes, as high amounts of manure can be treated while used for diluting such wastes, coupling the process to manufacturing and transport flow times (Angelidaki and Ellegaard, 2003).

The origin of the liquid manure, whether it is from pigs, cattle or poultry, will determine the degree to which its organic fraction will be decomposed in the AD reactor. As stated before, cattle liquid manure has a high content of fibers due to the feeding, which allows only 30 % of its organic content to be decomposed, while in pig liquid manure and in chicken liquid manure the values are 50 and 65 % respectively (Deublein and Steinhauser, 2008). By adding co-substrates to the manure, the organic fraction is increased and so the biogas yield (Weiland, 2000; van Lier et al., 2001; Angelidaki and Ellegaard, 2003; Deublein and Steinhauser, 2008). The dry matter content in the total substrate conformed by manure and co-substrate, should not exceed 12 % in order to ensure correct pumping and mixing, crucial factors for any transformation process (Deublein and Steinhauser, 2008). Another major agricultural waste, crop residues, can be used as co-digestion substrate. They are obtained after food processing of harvested products, like sugarcane-bagasse, corn stover, maize ensilage and straw residues. For an agricultural biogas plant, domestic wastewater is not profitable to be fermented together with farming waste biomass since it poses pathogens that need to be analyzed and removed (Landbruks og matdepartementet, 2003), and for these purposes additional parts of the biogas plant need to be adapted (Deublein and Steinhauser, 2008). Industrial organic waste represents high pollution loads and is composed merely from easily degradable substrates like saccharides, lipids and proteins. Most industrial organic wastes have methane yields ranging from 30 to 500 m^3/ton; being more easily degraded than manure they constitute an attractive feedstock for a biogas plant (Angelidaki and Ellegaard, 2003). The incorporation of easily degradable matter does not only increase the methane yield of the anaerobic process, but also stabilize the digestion by adding it in a controlled way. In the process reactor, a higher active biomass concentration provides better resistance against compounds responsible for inhibitions, and it has been shown that also inorganic fractions of some organic wastes can neutralize inhibitory effects, e.g. clays counteract the inhibitory effect of ammonia while iron compounds neutralize inhibition by sulfide (Angelidaki and Ellegaard, 2003). At existing biogas plants, the economic analysis has shown that the economic balance can be reached at an average biogas yield of more than 30 m^3 biogas/m^3 biomass (20 m^3 CH_4/m^3 biomass) (Angelidaki and Ellegaard, 2003). The addition of easily degradable industrial waste makes it possible to achieve the biogas potential.

3.2.1 Protein-rich co-substrates: fish processing waste

Since in Norway the cultivated land represents only 3 % of the country (Statistics Norway, 2008), biomass available for biogas production must also come from other sectors, as industrial, municipal and forestal. Fish processing is a vast industry in Norway, the Norwegian fisheries produce more than 550,000 tons of byproducts annually, which is more than 20 % of all the fish caught and farmed in Norway (Rubin, 2012). The by-products are generated when the fish is gutted, headed and further processed either on-board fishing vessels or in processing plants on shore. They consist of viscera (e.g., liver, roe and stomachs), heads, backbones, cuts and rejected fish from processing (Rubin, 2012). Today, most of the by-products are used as raw materials for feed production; such as fish meal, silage and feed for fur animals, but about 150,000 tons are still dumped in the sea.

Fish processing byproducts are rich in proteins and lipids, normally containing more than cattle manure (Callaghan et al., 1998), and thus excellent candidates for biogas substrates. As mentioned before, these materials degrade easily and produce biogas with a high methane content, being the methanogenesis the rate-limiting step (Angelidaki, 2004). However, the higher protein content can translate in a greater potential for producing ammonium ions or free ammonia, and process inhibition can occur. Protein-rich materials also contain sulfate and sulfur compounds (Boe, 2006), and sulfate-reducing bacteria would reduce these compounds to sulfide, which as mentioned, may also cause process instability. On the other hand, oil and fat are very quickly hydrolyzed to LCFA and glycerol (Ahring et al., 1992; Angelidaki and Ahring, 1992), and if accumulation of LCFA occurs this can also lead to inhibition of the bacterial growth (McCarty, 1964; Angelidaki and Ahring, 1992; Rinzema et al., 1994; Callaghan et al., 1998; Eiroa et al., 2012). Co-digestion with carbon-rich material such as lignocellulosic biomass would help to avoid possible process imbalances.

3.2.2 Lignocellulose rich co-substrates and pretreatment technologies

With today's scarcity of resources and land for food and feed production, biofuel production should more and more be based on the use of non-edible biomass fractions (Johansson et al., 2010; Horn et al., 2011b). Lignocellulosic biomass, the most abundant non-edible biomass resource (Lin and Tanaka, 2006; Schnürer and Jarvis, 2010), includes crops and forestry wastes and as mentioned previously, woody biomass accounts for one of Norway's biggest resources. Second generation biofuels as bioethanol and biogas produced from lignocellulosic resources and wastes that may not pose a threat to food security is of high relevance nowadays (Bauer et al., 2009; Johansson et al., 2010).

The three main components of plant cell walls are cellulose, hemicellulose and lignin. These components are closely associated to form the structural framework of the plant cell wall (Ramos, 2003; Jørgensen et al., 2007). Their distributions, and the content of the different hemicellulose sugars, vary greatly between plant species (Table 2)(Jørgensen et al., 2007; Kumar et al., 2009). Cellulose, the main constituent, is a homopolysaccharide composed of D-glucopyranose units linked by ß-(1- 4) glycosidic bonds. The long-chain cellulose polymers are linked by both intra- and intermolecular hydrogen bonds, causing the crystalline cellulose fibrils. These bonds give cellulose high tensile strength and make it more resistant to microbial degradation (Jørgensen et al., 2007). A small fraction of the cellulose chains form amorphous regions that are more susceptible to microbial degradation. Hemicellulose and lignin enclose the fibrils in a composite named the cellulose microfibril (Fig. 3.2) (Ramos, 2003).

Table 2: Composition of different lignocellulosic materials in % of total dry weight (adapted from Jørgensen et al. (2007) and Kumar et al. (2009)).

	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Softwood stems	45-50	25-35	25-35
Hardwood stems	40-55	24-40	18-25
Salix viminalis	41	30	28
Wheat straw	30	50	15
Corn	45	35	15
Switchgrass	45	31	12

Hemicelluloses are heterogeneous polysaccharides formed by a wide range of building blocks such as pentoses (xylose, arabinose), hexoses (glucose, mannose, galactose) and uronic acids. They are classified according to the main sugar in the polymer's backbone, e.g. xylan or mannan. Wheat straw and switch grass posses hemicelluloses composed mainly of glucuronoarabinoxylans, whereas softwoods like pine and spruce are mainly composed by galactoglucomannans. In hardwoods like birch, poplar or oak, 4-*O*-methyl-glucuronoxylans are the most abundant hemicelluloses. Thus, agricultural waste products and hardwood are richer in pentose sugar (xylose) while softwoods are richer in hexose sugar (mannose)(Jørgensen et al., 2007).

Lignin is a phenolic macromolecule that constitutes the most abundant non-polysaccharide fraction in lignocellulose. It contains cross-linked polymers of three phenolic monomers: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Ramos, 2003; Jørgensen et al., 2007; Kumar et al., 2009). Guaiacyl lignin is composed principally of coniferyl alcohol units, while guaiacyl-syringyl lignin contains monomeric units from coniferyl and sinapyl alcohol (Freudenberg and Neish, 1968). In general, softwoods have a higher lignin content than hardwoods, and herbaceous plants (grasses) posses the lowest content (Jørgensen et al., 2007). Guaiacyl lignin is found in softwoods while guaiacyl-syringyl lignin is present in hardwoods. Herbaceous lignin is composed mainly of p-coumaryl alcohol units. Presence of lignin embedding the cellulose in the cell wall provides structural support, impermeability and resistance against microbial and chemical degradation (Ramos, 2003; Jørgensen et al., 2007; Kumar et al., 2009).

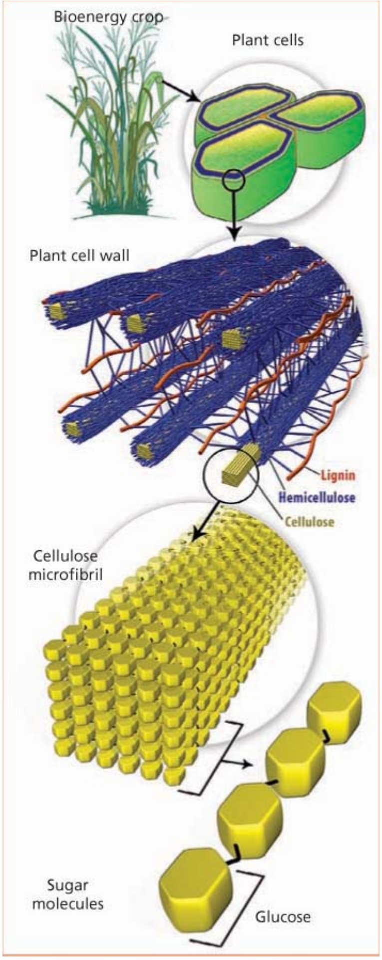


Figure 3.2: Lignocellulosic biomass structure (source: Genome Management Information System, 2006, Oak Ridge National Laboratory).

This resistance can not be overcome unless a pretreatment to make the structure less recalcitrant for fermentation is applied. Pre-treatments can be chemical, thermal or physical ones; such as addition of chemical reagents (e.g., NH_4^+, H_2SO_4, NaOH, SO_2, H_2O_2 or H_2SO_4), particle size reduction (milling), enzymatic treatment, microwave irradiation, ultra-sound or steam pre-treatment (Ramos, 2003; Jørgensen et al., 2007; Ward et al., 2008; Taherzadeh and Karimi, 2008; Seppälä et al., 2008). Chemical pre-treatments aim to solubilize hemicellulose and lignin so to expose the cellulose to acid or enzymatic hydrolysis (Ramos, 2003). Milling reduces the substrate's particle size so to increase the available surface area, decreasing cellulose crystallinity (Ramos, 2003). Opening up these structures would make them readily available for the anaerobic bacteria to work on. Steam explosion is one of the most effective techniques used for this purpose, that combines both physical and chemical methods (Brownell and Saddler, 1987; Ramos, 2003; Bruni et al., 2010a,b). It involves high temperature heating and pressure, combined with a violent decompression of the material (explosion) that mechanically alters the lignocellulosic structures in the biomass fibers (Ramos, 2003; Horn et al., 2011a). The high-pressure steam modifies the biomass, yielding a dark brown material resembling cooked fibers. Steam explosion has demonstrated to produce better recovery yields and substrates for hydrolysis than the use of NaOH or H_2O_2. Compared to microwave irradiation, milling and acid hydrolysis, it has as well proved to be more effective (Ramos, 2003). Steam explosion is also a very versatile method, since different varieties of plant biomass can be pre-treated, or even other materials containing fibers or recalcitrant substances. A commercially known continuous high pressure steaming facility treating plant biomass is the Stake technology digester from SunOpta (Ontario, Canada) (Ramos, 2003; Jørgensen et al., 2007). The example of the application of this technology to the biogas sector is given by the Norwegian company CAMBI. This is a company engaged in the environmental technology sector since 1989, dedicated to converting biodegradable material into renewable energy (CAMBI, www.cambi.no). Its thermal hydrolysis pre-treatment (THP) has been successful in enhancing the anaerobic digestion of municipal and industrial wastewater sludge, by increasing its biodegradability and improving its dewaterability. The THP is a type of steam explosion in which the sludge cake is pressure cooked at 165 $^{\text{o}}$C for 20-30 minutes before being fed at about 10 % TS to conventional digesters. This treatment also guarantees a pathogen-free final digestate. CAMBI's THP technology has been in use around the world since 1995.

For this study, CAMBI's steam explosion pilot facility located at UMB campus was employed to investigate the effects of applying this technology to lignocellulosic material. Biogas production from a steam exploded lignin-rich biomass, *Salix viminalis* (Table 2), was investigated in **Paper I**. The steam explosion method applied in this study is described in detail in section 4.2.1.

3.3 Recycling of nutrients and resources

Livestock production is one of the sectors that have a bigger impact on GHG emissions, with a global contribution of approximately 18 %. These include CH_4 emissions coming directly from domestic animals (enteric fermentation of ruminants) or livestock manures, and N_2O emitted from grazed lands and land applied manures (Massé et al., 2011). According to the Food and Agriculture Organization of the United Nations's (FAO) report entitled "Livestock's Long Shadow" (Steinfeld et al., 2006) the livestock sector is as well the largest contributor to land and water degradation. Animal waste management practices are potentially hazardous to human, animal and wildlife health. Livestock manures usually lose nearly 50 % of nitrogen during handling, application and storage; loss of ammonia also contributes to acid rain, water eutrophication, acidification of sensitive ecosystems and biodiversity loss (Massé et al., 2011) and phosphate is another nutrient responsible for eutrophication (de Bashan and Bashan, 2004; Bandosz and Petit, 2009; Hjorth et al., 2010). Interest in more environmentally responsible livestock production practices is growing, for instance, management of slurry through its recycling on farm by separating its fractions, recycling of organic matter and plant nutrients can help mitigate the environmental hazards described above. This will contribute to replace mineral fertilizers with recycled nitrogen, phosphorous, potassium and micronutrients within the farms (Hjorth et al., 2010). Livestock industry needs as well to be proactive and adopt more environmentally sustainable production practices, but this will only happen if manure management and treatment is proved a cost effective process (Massé et al., 2011). AD is a technology with the potential of solving several problems associated with livestock production. GHG are reduced by production of renewable energy as a substitute for fossil fuels, while effluents from AD have mineralized nutrients and are better balanced to meet crop needs than raw manure slurries (Massé et al., 2011). Both capture of energy and reduced needs for chemical fertilizers will substantially decrease the carbon footprint of livestock food products (Massé et al., 2011). Furthermore, on-farm biogas production reduces the risk of eutrophication and eliminate zoonotic pathogens and parasites in livestock manures. More frequent and better timing of treated manure land application with improved nutrient balance have the potential to increase nutrient uptake by crops and minimize nutrient losses to the environment (Massé et al., 2011).

Besides methane, the final product of biogas production is the digestate, which can be separated into a solid fraction and a liquid fraction. This digestate contains all major nutrients for plant life and is usually distributed as fertilizer. However, in countries with long winters as in Norway, the material requires big storage capacities as well as transportation costs. When substrates with a high fiber content are being co-digested, the final digestate also contains residual methane potential; commonly about 25 % of the methane potential remains unexploited (Hartmann et al., 2000; Jagadabhi et al., 2008). According to Angelidaki et al. (2005) and Seppälä et al. (2008), effluents from anaerobic digesters at biogas plants can contain up to 30 % of residual methane potential. Losing this potential would not only entail economic but also environmental implications due to the subsequent loss of methane, a 20 times stronger greenhouse gas than carbon dioxide (EPA, 2012), in fields or storage tanks (Seppälä et al., 2008; Massé et al., 2011).

Recycling of process water, i.e. the liquid fraction of the digestate, is a way to reduce the residual methane potential by increasing the retention times, and it is addressed in **Papers II** and **III**. This may as well avoid water consumption for dilution of the substrate to a proper mixing TS content, reduce the need for preheating the introduced substrate and also decrease the process effluent discharges, generating a final digestate with a higher TS content. This can also entail important savings in transport logistics and in storage capacity at the farms. The reuse of process water is relevant and specially recommended in plants where dry biomass is fed (straw, silages, wood wastes) (Deublein and Steinhauser, 2008). The case of the Växtkraft biogas plant in Västerås, Sweden, is an example of a plant performing digestate recirculation. The main substrate is the organic fraction of household, restaurants and shops wastes, that is co-digested with fat sludge and lay crop silage in order to increase its yield. The process water recirculated has increased its TS content since the plant started operating in 2005, probably due to a fraction of material recalcitrant to be degraded that builds up inside the digester, leading ultimately to a decrease on the plant's capacity for treating organic waste of ca. 25 % of the original amount of waste processed (Lindmark et al., 2011). Application of optimized separation techniques to the recirculated process water so to reduce its TS content may restore and even increase the former capacity of the plant, as showed by Lindmark et al. (2011).

Another aspect to consider when performing recirculation of biogas process water, is the concentration of compounds such as ammonium, that may affect the process. Process water in particular coming from the digestion of protein rich feedstocks as animal manure, contains high levels of ammonium ion. As mentioned before, high concentrations of ammonia may inhibit the anaerobic digestion process (Angelidaki, 2004; Deublein and Steinhauser, 2008), unless adaptation to these high levels occur, for instance due to a shift in the methane production pathway (Schnürer and Nordberg, 2008). Microorganisms involved in SAO are less affected by the high ammonia concentrations than the acetoclastic methanogens. However, for this microorganisms to be present and the pathway shift to occur, retention times need to be higher than 30 days since syntrophic acetate oxidizing co-culture posses a doubling time longer than that of acetoclastic methanogens (28 and 2- 12 days, respectively) (Schnürer et al., 1994; Schnürer and Nordberg, 2008). Recirculation of the digestate can provide longer retention times for some fractions of the reactor's content. A preliminary analysis of the presence of SAO bacteria was performed on the digestate's from **Paper III,** but further investigations will be addressed in the future.

The reincorporation of process water containing high NH_4^+- N concentrations may threaten the anaerobic process; a non-inhibitory balance between the ammonia concentration provided by the feedstock and the ammonia level of the recycled digestate has to be maintained in the anaerobic reactor. Furthermore, NH_4^+- N is a valuable nutrient for fertilizer purposes, and so methods for its removal and recovery from the liquid fraction into a valuable biofertilizer are discussed in the following sub-section and are applied in **Paper III**.

3.3.1 Techniques for the recovery of nutrients, NH_4^+ and PO_4^{3-}.

The liquid fraction of animal slurry is richer in nitrogen comparing to the solid one, with ammonium-N accounting for 70 % of its nitrogen content (Hjorth et al., 2010). If slurry is not treated and the nutrients fixed, this NH_4^+ may be lost as volatile NH_3 or after microbial conversion to nitrous oxide (N_2O) or gas (N_2) (Hjorth et al., 2010). Phosphorous is another important nutrient component of agro-industrial effluents. In animal slurry, it is present to a larger extent in the solid particulate fraction, and from the 30 % dissolved more than 80 % is orthophosphate (PO_4^{3-}) (Hjorth et al., 2010). After anaerobic digestion treatment, N and P mineralized fractions are increased. According to Massé et al. (2011), the total N/P ratio of 3.9 in raw manure increase to 5.2 in the bioreactor effluent and to 9.2 in the supernatant fraction of settled effluent. The agronomic value of manure is enhanced in the nutrient content of the separated digestate fractions to better meet crop nutrient requirements. By recirculating digestate back to the process, nutrients and micronutrients content can be increased during the process and in the final solid digestate. However, as mentioned previously, a non-inhibitory balance between the ammonia concentration provided by the fresh substrate mixture and the ammonia level of the recycled digestate has to be kept and closely monitored in the anaerobic reactor.

While mined rock phosphate is a relatively cheap source of phosphorous for fertilizing purposes, this resource will be soon depleted (de Bashan and Bashan, 2004; Gonzalez Ponce and Garcia Lopez de Sa, 2007; Forrest et al., 2008; Massey et al., 2009) and therefore, other alternatives to get phosphate need to be found. Phosphate contained in wastewater might then start to be seen as a viable resource solution to the phosphate fertilizer scarcity and not as an environmental contaminant.

Ammonium-N and phosphorous removal methods to be applied to a nutrient-rich liquid digestate can be divided into physicochemical and biological. Biological methods, such as nitrification-denitrification or the Anammox process for ammonium, and use of bacteria or micro algae (*Chlorella vulgaris*) for phosphorous (de Bashan and Bashan, 2004) are aim to remove the nutrients from the wastewater but not to recover them into a valuable subproduct, as do several physicochemical methods (Zeng et al., 2006). Thus, on this thesis work only practical physicochemical methods were addressed.

Main physicochemical methods applied include reverse osmosis, air-stripping, ion-exchange, adsorption and chemical precipitation (Reeves, 1972; de Bashan and Bashan, 2004; Zeng et al., 2006; Lei et al., 2007; Hjorth et al., 2010; Gustin and Marinsek Logar, 2011; Mook et al., 2012). Other possible methods for the removal of ammonium-N that are not discussed in this thesis would be electrochemical, evaporation and break point chlorination (Reeves, 1972; Zeng et al., 2006).

Reverse osmosis can remove ions, proteins and even organic molecules from wastewater and seawater. It is an environmentally friendly method based on the high permeability efficiency of selective ions, that does not disturb molecular structures during the separation process (Mook et al., 2012). The wastewater is forced to pass through a membrane against natural osmotic pressures of up to 705 psi, achieving by this the separation of ions (Reeves, 1972). Charged molecules as NH_4^+ and potassium (K^+), and to a lesser extent even NH_3, can be retained by reverse osmosis (Hjorth et al., 2010). However, membrane filtration has the disadvantages of soluble salts (carbonates, sulphates) possibly precipitating on the membrane, and fouling (Reeves, 1972; Siegrist et al., 2005; Hjorth et al., 2010; Mook et al., 2012). These issues will affect membrane performance and regular cleaning will be needed, increasing the complexity of the process.

Ammonia stripping is a well established technique that relies on the shift in the ammonium/ammonia equilibrium towards the ammonia gas production by increasing the pH and/or temperature (Eqs. 3.5 and 3.6). Thus ammonium ion is transformed into ammonia gas, which is removed from the liquid phase by air (Reeves, 1972; Gustin and Marinsek Logar, 2011). A stripping column filled with carrier material that enhances the liquid and air flow and provides a large surface area for the ammonia transition to take place is normally employed, being the gaseous ammonia absorbed in the biofilter or in a strong acid (e.g., sulphuric acid). This last option allows recovery of the ammonia in an ammonium-rich byproduct that can be applied as agricultural fertilizer (Lei et al., 2007; Gustin and Marinsek Logar, 2011).

Ion -exchange technique is based on resins bonded to functional groups (Reeves, 1972; Mook et al., 2012). An example are chlorine ions which are exchanged with anions from the wastewater, and the resin can be regenerated by displacing the anions by chlorine ions from i.e. a NaCl solution (Mook et al., 2012). Natural zeolites, the crystalline hydrated aluminosilicates of alkaline cations with three dimensional tetrahedral structures (SiO_4^{4-} and AlO_4^{5-}), posses the ability not only of exchanging their cations but also of adsorbing molecules of appropriate cross-sectional diameter (Montalvo et al., 2012). Adsorption is based on the large porous surface area of an adsorbent, i.e. zeolites or activated carbon (Vassileva et al., 2008; Bandosz and Petit, 2009; Mook et al., 2012). This is a very versatile and simple method but adsorbents can be costly and if not regenerated properly, it end ups as solid landfilled waste. Similarly, bentonites can also act as either strong acid adsorbents or ion exchange materials in the removal and recovery of ammonia from wastewater (Saltali et al., 2007; Eturki et al., 2012; Montalvo et al., 2012). These smectite-type clays rely on acid-base interactions for strong retention of molecules on their surfaces (Seredych et al., 2008). A high negative charge of the bentonite surfaces is usually balanced by alkali metals and cations (typically Na^+ and Ca^{2+}). In the sorption-removal of phosphate, bentonite cations can be replaced by inorganic hydroxyl-metal polycations such as Al and Fe, acting as pillars which increase the interlayer spacing of bentonite (Yan et al., 2010). Theoretical adsorption capacity of a material for a particular molecule can be determined by developing adsorption isotherms, as Freundlich and Langmuir models (Sawyer et al., 2003; Saltali et al., 2007). Langmuir model assumes adsorption being reversible and by chemical surface forces, being its expression:

$$\frac{C}{q} = \frac{1}{K_{ad} \cdot q_m} + \frac{C}{q_m} \tag{3.10}$$

with C being the equilibrium concentration of adsorbate after adsorption took place (mg/L), q the mass of adsorbate taken up per unit mass of adsorbent (mg /g), K_{ad} and q_m Langmuir's empirical constants.

The Freundlich adsorption isotherm model is applicable to non-specific adsorption in an heterogeneous solid surface (Eturki et al., 2012). In its logarithmic form it is expressed as:

$$\log q = \log K_f + \frac{1}{n} \cdot \log C \tag{3.11}$$

with K_f and n the model's constants (Sawyer et al., 2003; Saltali et al., 2007).

Clay minerals as zeolites and bentonites seem to be as well promising materials for the removal of NH_4^+ ions from aqueous solutions and wastewater, due to their low cost and high competitiveness compared to biological and chemical treatments (Saltali et al., 2007; Eturki et al., 2012). These materials have been used as soil conditioners, avoiding losses of nutrients and water and improving the soil's chemical and physical properties (Saltali et al., 2007). For this research study, bentonite adsorption of NH_4^+ and PO_4^{3-} from the anaerobic digestion digestate was tested in **Paper III**.

Chemical methods for the removal and recovery of nutrients include precipitation of ammonium salts or in the case of phosphorous, with iron, alum or lime (de Bashan and Bashan, 2004). The most promising technique for recovering the nutrients is precipitation of phosphorous and ammonium together as magnesium ammonium phosphate hexahydrate ($MgNH_4PO_4.6H_2O$) (Eq. 3.12), commonly known as struvite, which provides a slow-release fertilizer (Altinbas et al., 2002; de Bashan and Bashan, 2004; Gonzalez Ponce and Garcia Lopez de Sa, 2007).

$$Mg^{2+} + NH_4^+ + PO_4^{3-} + 6H_2O \leftrightarrow MgNH_4PO_4 \cdot 6H_2O\,(s) \tag{3.12}$$

The three components are required simultaneously for the struvite precipitation to take place, in stoichiometric molecular ratio (1:1:1) and pH above 7.5 (de Bashan and Bashan, 2004) but also supersaturation of the solution and impurities such as suspended solids play a role (Forrest et al., 2008). Crystals of struvite are white orthorhombic structures (de Bashan and Bashan, 2004; Forrest et al., 2008) consisting of regular tetrahedral $PO_4{}^{3-}$, distorted octahedral $Mg(H_2O)_6{}^{2+}$ and $NH_4{}^+$ groups retained by hydrogen bonds (Abbona and Boistelle, 1979).

Regarding fertilizer properties, Abbona and Boistelle (1979) have stated that bacterial action and particle size are the predominant factors in determining the phosphorous release rate of struvite to plants. Struvite has been studied as a favorable slow release fertilizer in acidic and neutral soils (Johnston and Richards, 2003; Li and Zhao, 2003; Gonzalez Ponce and Garcia Lopez de Sa, 2007; Yetilmezsoy and Sapci-Zengin, 2009) as well as in alkaline soil types (Massey et al., 2009). In Japan, full scale production in fluidized bed reactors has been taking place and struvite has been sold as a commercial fertilizer (de Bashan and Bashan, 2004; Forrest et al., 2008; Massey et al., 2009). This precipitation method was applied to a nutrient rich anaerobic digestion digestate in **Paper III**.

4 Materials & methodology employed

4.1 Materials

4.1.1 Inoculum

The inoculum used varied according the experiments. In the small scale batch trials (**Paper I**) the inoculum employed was sewage sludge obtained from the anaerobic digester at the Nordre Follo Treatment Plant (Vinterbro, Norway). In the CSTR experiments, inoculum with bacteria already adapted to the substrates in question were preferred. In **Paper II** the inoculum used was the digested material from a previous CSTR trial that codigested different contents of cow manure and *Salix*, while for **Paper III**, inoculum coming from the digester at Åna farm biogas plant in Rogaland, Norway, that treats fish silage and cow manure, was employed.

4.1.2 Cattle manure

The cattle manure employed in all the trials was cow manure from the University's farm, that was collected previously to launching each experiment and kept refrigerated in a cooling room at 4 ºC. The manure was always characterized for physicochemical parameters as the rest of substrates.

4.1.3 *Salix viminalis*

Fast growing (short-rotation) energy crops are good alternatives for bioenergy production. In North America, fast-growing willow and poplar species are used to produce dedicated woody biomass feedstock (Sassner et al., 2005). *Salix* can sequestrate more carbon than softwoods within a growing season (Kuzovkina and Quigley, 2005), it can be easily adapted to extreme soil conditions and it is resistant to pests, making it a very economically viable biomass source (Sassner et al., 2008).

Being vastly cultivated in the nordic countries, *Salix viminalis* (basquet willow or common osier) can produce up to 35 tons of stem per hectare per year (Kuzovkina and Quigley, 2005). In a study by Labrecque et al. (1997) it was demonstrated that *Salix viminalis* had the highest biomass quality in terms of energy efficiency compared to other two types, *Salix petiolaris* and *Salix discolor*, due to its low water and ash content.

As in all plant materials, its rich lignocellulosic content makes it a substrate less available for degradation during anaerobic digestion. In particular, *Salix* has a considerable content in lignin (ca. 28 %, Table 2) when compared to other energy crops. Thus, need for a pretreatment, in our case steam explosion, is mandatory, in order to successfully employ *Salix* as substrate for biogas production.

Figure 4.1: *Salix viminalis*.

The *Salix* used throughout the research was *Salix viminalis* of two clone types, Christina and Tora, harvested in southern Norway and in Uppsala, Sweden, respectively. Samples arrived in all cases chopped to particle sizes between 0.5-2 cm. and were kept at -20 °C until being thawed and steam exploded for the respective experiments. In all cases main physicochemical parameters were analyzed.

4.1.4 Fish byproduct

The category 2 fish byproduct from the Norwegian salmon farming industry represents today an unexploited potential value-added product. According to the Norwegian Food and Safety Authority (Mattilsynet, 2008), this by-product was estimated in 40- 50 thousand tons in 2009, and it consists on the "material with risks of animal and fish diseases (including even dead fish and fish harvested with proven infectious diseases) and material with residues of drugs content over the limit, that can be used for technical purposes, compost or biogas, after being stabilized using an approved method". The amount is expected to increase by ca. 5 % annually, in line with the general increase in aquaculture volume (Rubin, 2012). Because this type of byproduct may contain disease infected fish, it can not be used for feed production, so it has to be either disposed of or hygienized if it is to be treated in alternative ways, such as biogas production.

The specific material used in the research project was provided by the Norwegian company Biokraft Marin AS, Trondheim, Norway, interested in testing it for employing as a biogas co-digestion substrate. It arrived already pre-treated, ready to include in the biogas production trials.

Figure 4.2: Category 2 fish byproduct.

The pre-treatment is called fish silage processing method (FSPM) and it is a Norwegian hygienization method approved by the Norwegian Animal Health Authority in 1994 (Forskrift av 13.juli 1994 nr.723; Landbruks og matdepartementet, 1994), and adapted from the EU legislation (Method 1, Annex V of the EC No 1774/2002 regulation (European Commission, 2002) later replaced by EC 1069/2009 and EU 142/2011(European commission, 2013)) (Norwegian Scientific Committee for Food Safety, 2010). It consists firstly, in a microbial stabilization (silage) by grinding and by addition of formic acid to a pH of < 4.0. The fish's own enzymes contribute to degradation (autolysis) of the fish structure and macromolecules so that the fish mass is relatively homogeneous, except for a fatty portion that accumulates at the top of the tanks (Rubin, 2010). Before being further processed in a biogas process, fish mass is hygienized according to current Norwegian regulations (minimum 85 º C for 25 minutes) to guarantee killing any bacteria and viruses that could have survived the ensiling process (Norwegian Scientific Committee for Food Safety, 2010). The possibility of hygienization under normal pressure is one of the benefits of processing the raw material in Norway instead of carrying it outside of the country. EU rules requires autoclaving at high pressure (at least 3 bars) and a temperature more than 133 °C for at least 20 minutes without interruption (European Commission, 2002) which requires considerably more investment and energy. After hygienization, the fish oil is separated. Large amounts of fish oil is not ideal in the anaerobic digestion process, since it can stick to the biofilm or biogranules of a high feed rate process, reducing the process efficiency (Rubin, 2010). Furthermore, the oil can be burned directly as heating oil without the need of being converted into biogas, which would result in both greater energy and investment needs. The process should therefore seek to recover as much oil as possible before AD. Around two-thirds of the oil will be in a floating phase that is recovered by pumping. The method can be improved by centrifugation, but normally the last 3 % of oil is not economically feasible to recover (Rubin, 2010). The remaining fish mass is water diluted and coarse filtered, so that it can be used in AD together with other substrates (Rubin, 2010). Process water (digestate) from the digester is to be afterwards treated in an aerobic biofilter to reduce the biological material to a level that meets local emission requirements. This proposed Biokraft Marin AS process is illustrated schematically below (Fig. 4.3).

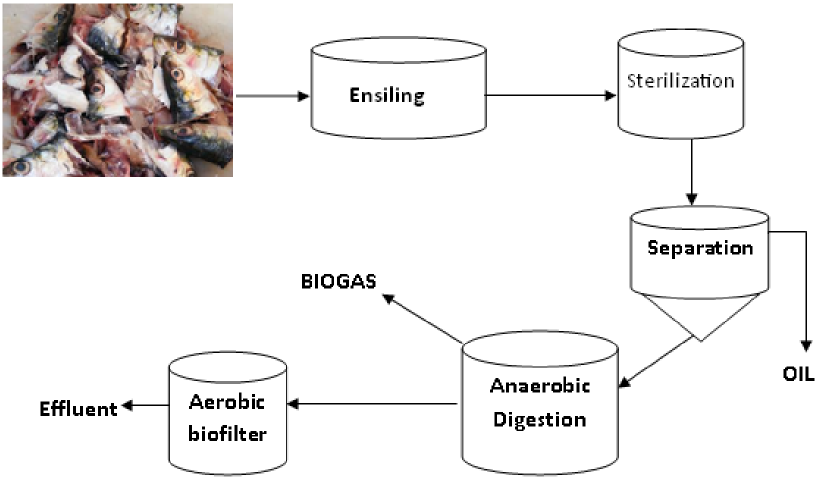


Figure 4.3: Biokraft Marin AS's process flow for the use of fish byproduct category 2 (adapted from Rubin (2010)).

The fish byproduct was characterized for relevant physicochemical parameters and maintained refrigerated at 4 ºC until used in the semi-continuously methane production trials.

4.2 Methodology

The methane production trials were ran on different types of biomasses found in Norway: cattle manure, energy crop (*Salix*) and lately fish industrial byproduct. The pre-treatment of steam explosion was applied to the highly lignocellulosic *Salix* samples.

Two different scales and feeding systems for anaerobic digestion trials were used: serum bottles of 1.125 L for batch fed process (**Paper I**), and CSTRs of 6 L working volume, fed semi-continuously (**Papers II-III**). The smaller scale was used for screening methane potentials from substrates and different ratios of substrate's mixtures. The parameters analyzed and monitored in this scale were: methane yield, mixture ratio (% VS), carbon to nitrogen (C/N) ratio, pH, retention time, ammonium-N load, total solids content (TS), volatile solids content (VS), total nitrogen (Total-N), chemical oxygen demand (COD) and VS degradation.

The 6 L reactors were fed semi-continuously with optimized mixtures for further monitoring relevant parameters such as dry matter and organic matter content, organic loading rate (OLR), hydraulic retention time (HRT), pH, C/N balance, ammonium-N load and content along the process. In this scale, focus was done particularly to the separation of the digestate solid and liquid fractions and the recirculation of the last one back to the AD process. Therefore, ammonium-N as well as the volatile fatty acids (VFA) level and the possible accumulation of compounds released during steam explosion (e.g., furfural and hydroxymethyl furfural (HMF)) needed to be closely monitored at this stage, so that inhibition of the process would not occur.

The liquid fraction of the digestate was separated from the solid one by employing mesh sieves of 2.5 mm (**Paper II**) and 2.5 mm plus 1 mm hole sizes (**Paper III**). Throughout all this research study, solid fraction of digestate refers to the sieved separated fraction with higher fiber content and TS values of 10- 12 %, while liquid digestate fraction is the one recirculated with a content of TS of ca. 3- 4 % (accumulated raw digestate coming from reactors had varying contents between 4- 8 % TS). The digestates fractions were characterized for its solids, NH_4^+-N, PO_4^{3-}, Mg^{2+} and soluble COD content. Methods for the removal/recovery of ammonium-N from the digestate were studied (**Paper III**) and were applied to the recirculated fraction if high levels of ammonium-N in it were reached, and to the liquid fraction of the rejected digestate that was not recirculated but accumulated throughout the whole experimental period. Precipitation as ammonium magnesium phosphate (struvite) was addressed since it also allowed the recovery of phosphate. Adsorbance of NH_4^+-N into bentonite was as well tested.

The statistical analysis applied when it was required, consisted on paired and two sample t-tests (alpha level of 0.05) and one way analysis of variance (ANOVA). The program employed for such determinations was Minitab®, on its 15.1.1. and 16.1.1. versions.

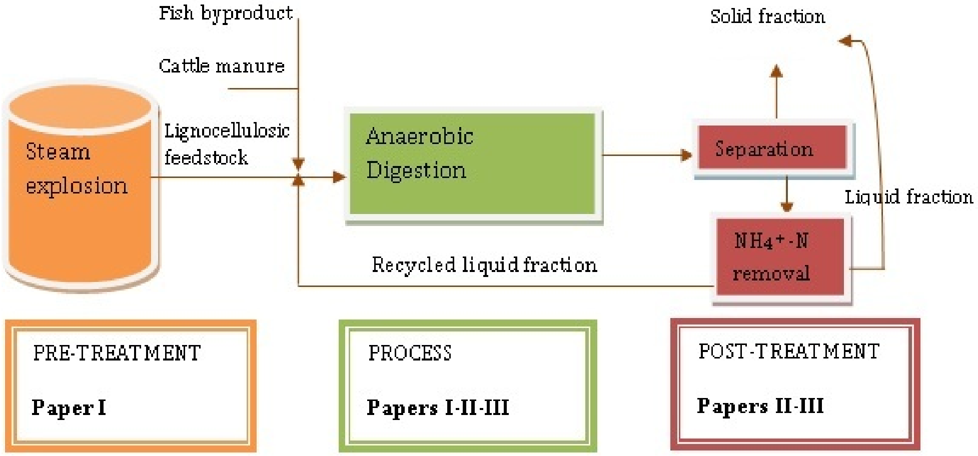


Figure 4.4: Thesis outline and areas of focus of each paper.

4.2.1 Pre-treatment of the lignocellulosic biomass: steam explosion

Steam explosion pre-treatment involves a high temperature heating combined with a rapid decompression (explosion) that physically disrupts the lignocellulosic structures in the biomass fibers. It relies on acids (e.g., acetic acid from acetylated hemicelluloses, formic and levulinic acids) that catalyze the partial auto-hydrolysis of hemicelluloses to mono- and oligosaccharides (Ramos, 2003; Jørgensen et al., 2007). By removing hemicelluloses from the cellulose microfibrils, the cellulose will be exposed and thus, its enzyme accessibility will increase. During the process, lignin is redistributed on the fibers surfaces due to polymerization and de-polymerization reactions. All this increases the material's pore volume, and the rapid flash to atmospheric pressure (explosion) will fragment the material increasing its surface area. Depending on the severity of the pre-treatment, cellulose may also degrade to glucose (Jørgensen et al., 2007). The biochemical composition changes of applying steam explosion at 210 °C and 15 minutes to *Salix viminalis* samples are shown in Table 3.

Table 3: Biochemical composition of *Salix* samples before and after steam explosion treatment at 210 °C for 15 minutes (**Paper I**)

Parameter		Untreated *Salix* (% dw/w)	Steam exploded (% dw/w)
Pentose sugars	Arabinose	0.9	0.2
	Xylose	11.9	6.0
Hexose sugars	Manose	1.4	1.2
	Galactose	0.9	0.7
	Glucose	36.2	45.8
Lignin	Acid soluble	2.9	3.2
	Non-acid soluble (Klason)	25.3	31.9
Total carbohydrate and lignin content		79.5	89.0

The steam explosion unit

The steam explosion unit consists of a 20 L pressure reactor and a flash tank with a removable container to collect the pretreated biomass (Fig. 4.5) (Horn et al., 2011b). The 25 kW electric steam boiler generates steam up to a maximum pressure of 34 bar (240 °C). The biomass is loaded into the pressure reactor using the ball valve (V3) at the top. Steam is added to the vessel through an air-actuated valve (V0) which also automatically keeps the pressure in the vessel at the chosen set point. To add steam to the pre-treatment reactor, also the manual valve (V1) has to be opened. The rapid pressure drop (explosion) and release of the pretreated biomass to the flash tank is done via the ball valve at the bottom of the vessel (V2). Once in the flash tank, the pretreated biomass is collected in a removable container located at its bottom. The inside of the flash tank may be cleaned by flushing water (V5). The steam leaving the flash tank is condensed by a water nozzle (WN) and led to a water tank (WT), whose water is continuously circulating (pump P1) via a heat exchanger (HE) at 8

°C, to keep WN cold. Samples to analyze volatile compounds in the outlet steam may be taken from this WT. Any steam that is not condensed pass through a carbon filter (CF) to remove smell, before leaving the unit. Valve V4 is usually closed, but it can be used to release the pressure gradually and evaluate non-explosive treatments (Horn et al., 2011b).

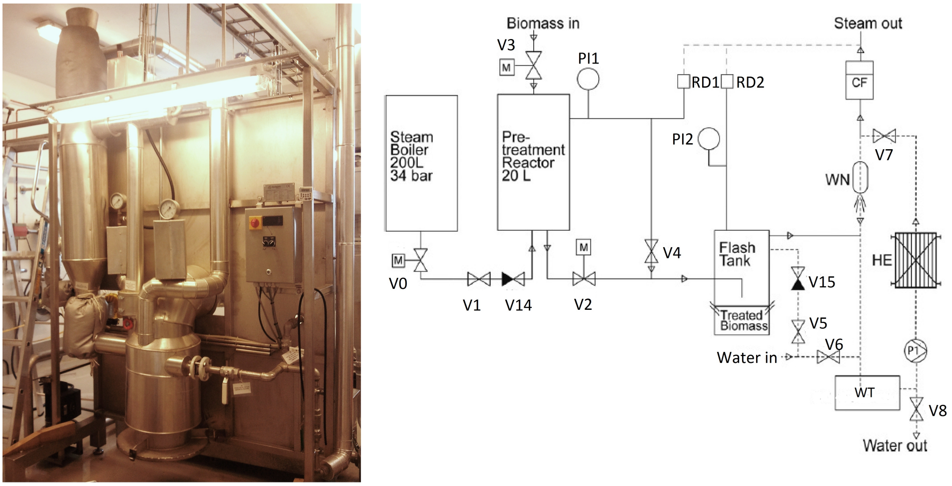


Figure 4.5: Schematic diagram of CAMBI's pilot steam explosion unit at UMB. V= valves, M= motorized valves, PI 1 and PI 2= manometers, RD1 and RD2= safety valves that open if pressure reaches limits, CF= carbon filter, WN= water nozzle, HE= heat exchanger, WT= water tank, P1= pump. Valves with solid triangles indicate one way valves (V14 and V15), dotted lines indicate water flow, valves V6 and V8 are used to regulate the amount of water in WT. Valve V7 may be used to close the water circuit (adapted from Horn et al. (2011b)).

Steam explosion considerations

When performing steam explosion of woody biomass at the campus, mass losses are detected. Some losses can be due to residues adhered to the pipeline walls, splashing and wash out during cleaning of the instrument. The overall loss fraction can be reduced by running multiple batches that avoid a system unit clean up between them, or by running a bigger batch. In full scale, continuous batches are run and losses are minimized.

In **Paper I** the overall mass losses were analyzed for batches of *Salix* pre-treated at 210 °C and 10 minutes. A batch of ca. 750 g. of chopped material (59% TS, 98.4% VS, 1.57% ash) produced 1,400 g. of steam exploded material (17.75% TS, 98% VS, 1.97% ash). Dry matter content decreased because some volatile organic materials were formed due to hemicelluloses degradation, and because of the residues lost inside the unit. Since volatile organics such as acetate and furfural are volatilized and lost due to the high temperatures, this translates in accumulation of ash (Ramos, 2003; Horn et al., 2011b). As presented in **Paper I**, for the conditions tested in the first experiment this ash accumulation seemed to increase along with the temperature applied in the pre-treatment, the harsher pre-treatments producing materials with higher ash content with respect to the initial one, and it also seemed to be influenced by the residence times when same temperature was applied. With this it was possible to estimate that the volatile organic materials losses during high temperature steam pre-treatments (210-230 °C) were approximately 20 %. Similar results have been deduced for the steam pre-treatment of wheat straw between 210 and 220 °C by Horn et al. (2011b). This indicates that an important methane production may be disregarded, however, in industrial scale facilities, the flash stream is condensed and added directly to the anaerobic digesters.

Methane yields are commonly expressed as mL CH_4 produced per g of VS added to the digester, because VS represents the organic fraction of dry solids that is lost upon ignition at 550 °C. In **Paper I**, the VS content of each steam exploded sample before and after the pre-treatment was calculated, and the VS amounts which were in fact added to each digestion vial were known. The effect of the organic losses during steam explosion when always the same substrate is pre-treated and compared in a study is not an issue. If the VS amounts lost during steam explosion were taken into account, higher methane productions than the ones obtained would have been reached since higher VS values were being considered. On the other hand, losses when drying the pre-treated samples (for TS and VS determination) would lead to overestimate the yield, because the methane production will be related to a smaller VS value than the one actually added to the vial. These losses were not calculated in **Paper I**, but according to literature that refers to ensiled biomass, they can be estimated to be of ca. 2-5 % VS (Weissbach and Kuhla, 1995; Porter and Grass, 2001; Samuelsson et al., 2006). The importance of having a more precise determination of volatiles content that could avoid such losses was a motivation to develop and employ a Karl Fisher tritation method for determination of dry matter in **Paper III**.

The Karl Fisher method was performed employing a Metrohm automated volumetric system (Tampa, FL, USA) composed of an oven sample unit (Model 774), a dosing device with burettes (Model 901) and a titration cell (Model 801). CombiTitrant 5 from Merck (Darmstadt, Germany) was the titrant employed, containing iodine, sulfur dioxide, a base and an alcohol; while dry methanol was the working medium in the titration cell. The water is driven out of the heated sample by a stream of dry carrier gas and transferred to the titration vessel, where the water is determined by titration. Thus, volatile compounds are not lost and also titrated, and the determination applied to steam exploded *Salix* gave a 5 % higher VS content with respect to oven drying.

A possible drawback of using a pre-treatment for disruption of lignocellulosic materials is the release of by-products that could act as inhibitors for the microbial consortia. The nature and concentration of such generated products vary according to the severity of the pretreatment and the biomass employed. In particular phenolic compounds and furfurals are thought to inhibit the biomethanation process (Mes-Hartree and Saddler, 1983; Castro et al., 1994; Palmqvist et al., 1996; Ramos, 2003; Chen et al., 2008; Bruni et al., 2010a).

Figure 4.6: *Salix viminalis* and the chopped sample before and after steam explosion.

During steam explosion hemicelluloses are solubilized, the lignocellulosic structure is debilitated, increasing the porosity of the substrate. Hemicelluloses are more available, while volatile organic acids, acetate, furfural and HMF are generated from the partial degradation of hemicelluloses (Horn et al., 2011a). Furfural generates from the dehydration of pentoses, while HMF is produced from hexoses (Ramos, 2003). Acetate and organic acids are substrates for biogas production, but there has been evidence that furfural and HMF are inhibitors of the microbial growth (Palmqvist et al., 1996; Ramos, 2003). Byproducts from the lignin degradation include aromatic and polyaromatic compounds with many substituents, likely to also inhibit microbial activity (Palmqvist et al., 1996).

Since steam pre-treated *Salix* was used as a main substrate in the semi-continuous AD process, this compounds may accumulate when such material is daily fed to the digesters. Performing recirculation of the liquid digestate may as well enhance such accumulation and therefore, presence of inhibitory compounds was taken into consideration and investigated in **Paper II**.

4.2.2 Batch biomethane potential trials

The measuring procedure for the batch biogas assays (**Paper I**) was done according to Hansen et al. (2004), and based on the *European Chemical Industry Ecology and Toxicology Cente*r (ECETOC) guideline (Stringer, 1988) and ISO 11734 standards. The ECETOC guideline was published in 1988 as the technical report *"Evaluation of Anaerobic Biodegradation"* and has been a key referent for all subsequent standards regarding anaerobic biodegradability (Müller et al., 2004). It aimed to propose a "test method suitable for screening chemicals for anaerobic biodegradation"(Stringer, 1988; Müller et al., 2004) and presents the detailed steps and calculation of results for the parameters needed in the procedure.

The ECETOC method relies on constant volume manometry, where a change in the pressure of a gas is measured while keeping its volume constant (constrained) at a constant temperature.

By using the ideal gas law, the number of moles of gas in the headspace of each batch test vessel is calculated as follows:

$$n = \frac{dP \times V}{R \times T} \tag{4.1}$$

being V the headspace volume (L); T the incubation temperature (°K), R the ideal gas constant (0.08205 $^{\mathrm{L}\times\mathrm{atm}}/_{\mathrm{moles.K}}$); and dP the pressure difference (atm) between initial and final readings.

Thus, the volume of biogas produced in the headspace of the vial is determined as:

$$V_{biogas} = \frac{n \times R \times 273}{P_0} = \left(\frac{dP \times V}{R \times T}\right) \times \left(\frac{R \times 273}{P_0}\right) = \frac{dP \times V \times 273}{T \times P_0} \tag{4.2}$$

where V_{biogas} is the volume of biogas (L) under standard conditions of 273 °K (0 °C) and 1 atm, and P_0 is 1 atm.

The term $k = 273/(T \times P_0)$ is then the coefficient for obtaining the Pressure under standard conditions (273 °K and 1 atm), and so the equation can be finally written as:

$$V_{biogas} = dP \times V \times k \tag{4.3}$$

Determination of the accumulated biogas volume by pressure measurements is combined with gas chromatographic analysis of such biogas, in order to determine its composition. Once the methane level in the biogas is obtained, the accumulated volume of methane and subsequently the specific methane yield of the substrates can be deduced. For the sets of batch methane assays performed in **Paper I,** serum bottles of total capacity of 1.125 mL were employed as biogas reactors. They were filled with inoculum and substrates (3/2 VS ratio maintained) adding for a total volume of approximately 700 mL. The biogas production over a period of at least 2 months was measured in triplicate for each series, under mesophilic temperature conditions (37 °C) and constant stirring at 90 rpm (Fig. 4.7). The headspace pressure of each reactor was determined using a digital pressure transducer (GMH 3161 Greisinger Electronic, Germany) with an incorporated needle that was injected into the septum cap (Fig. 4.7). Biogas composition was analyzed by gas chromatography. To avoid build-up of too high pressure in the reactor, it was necessary to release gas during the experimental period. This was done under a ventilation hood, by inserting a hospital needle in the rubber septum. The amount was calculated from measurement of the methane content in the headspace. Adjustments due to vapor pressure and variations at atmospheric pressure are accounted on the control trials without substrate.

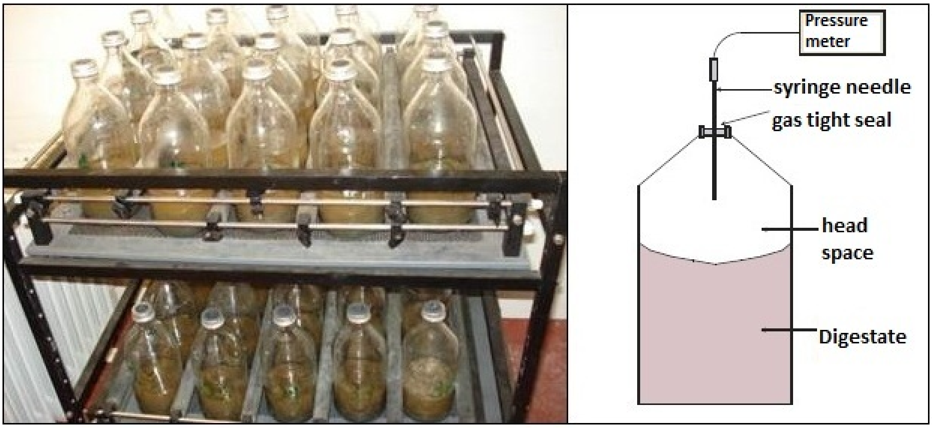


Figure 4.7: Laboratory BMP trials setup.

Several studies of the specific biomethane potentials of many types of substrates have been performed using manometric measurements to follow the biogas production (Shelton and Tiedje, 1984; Coates et al., 1996; Angelidaki et al., 2009). Pressure transducers have also develop to automated equipments and thus, this methodology has become more flexible and sensitive. Fernandez Polanco et al. (2005) proposed an automated method for the anaerobic batch assays, based on manometric determination of the biogas volume. In this automated system, the production of methane is monitored by using a pressure transducer that is directly registered by an on-line data acquisition system. The pressure transducer is located in a gas collecting chamber, instead of the vials headspace, where an alkali solution absorbs the carbon dioxide of the biogas mixture.

In other study by Scaglione et al. (2008) the short term biogas production and methane potential of a series of substrates was assessed, applying a manometric commercial equipment for this purpose. The OxiTop® Control system is a device consisting of a pressure transducer located in a measuring head in each bottle. This transducer register automatically the overpressure due to biogas accumulation in the headspace of each bottle, and the pressure is translated to accumulated biogas volume always by means of the ideal gas equation of state (Eq. 4.1).

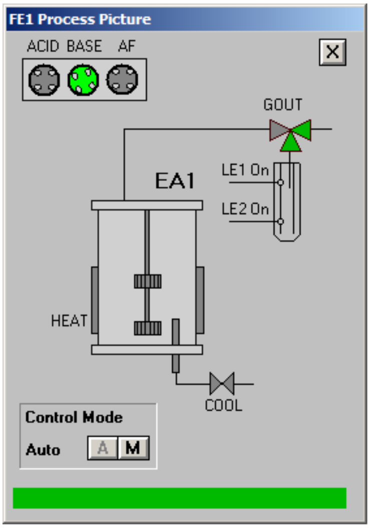
In the search of harmonization of the anaerobic biodegradation assays, an international *Task Group for Anaerobic Biodegradation, Activity and Inhibition assays* (TG-ABAI) was assigned by the International Water Association (Angelidaki et al., 2009) and proposed a protocol for studying the biochemical methane potential (BMP) of solid organic wastes and energy crops. The need of uniformity in the performance of these assays, so that to facilitate the task of comparing research studies, was the main cause for this.

4.2.3 Semi-continuous methane production experiments

Semi-continuous experiments were performed in CSTR of 10 L total volume (Belach Bioteknik AB, Sweden), and nominal working volume of 6 L (Fig. 4.8). Reactors were run at a mesophilic temperature (37 ºC) and a stirrer speed of 180 rpm. In **Paper II**, the reactors were coupled to Bluesens Kombi-CO_2/CH_4 infrared dual length gas sensors to determine the methane composition of the biogas produced. In **Paper III** and due to sensor malfunctioning, the biogas produced was collected in polyethylene gas-tight bags and the composition was followed by gas chromatography. The software employed for the reactor's monitoring was the fermentation process control software BIOPHANTOM©, that allows continuous, real-time monitoring of the important process variables such as pH, stirrer speed, temperature, gas flow and gas volume (Fig 4.9).

Figure 4.8: The Dolly CSTR of 6 L working volume capacity.

During the start-up periods, reactors were filled with 3 L of inoculum. Feeding of the reactors up to their full working volume started in all experiments with a low organic loading rate (OLR: 1 g VS/L.d) of the respective substrate mixtures and was successively increased until reached 3 g VS/L.d.



Control Mode
Auto

Figure 4.9: Schematic process picture of the Dolly CSTR from BIOPHANTOM© control software; LE1 and LE2 are sensors for volume displacement detection and registration of the volume of biogas produced (source: Dolly Operator's Manual, Belach Bioteknik (2010)).

After the start up periods, the reactors were fed once a day, 6 days a week, with an OLR of 3 g VS/Ld (one whole week daily load of 2.6 g VS/L) and a hydraulic retention time (HRT) of 30 days. Thus, 200 mL of freshly prepared substrate mixture were fed on every occasion to each reactor. Previous to feeding, an equivalent volume was removed so to maintain a constant volume in the reactor. For the reactors were recirculation took place, the removed fraction was filtered through mesh sieve(s) and the liquid fraction added back to the digester (Fig. 4.10). Detailed feeding compositions for each reactor are shown in **Papers II** and **III**. In the semi-continuous experiments, the anaerobic digestion process was followed for at least 3 HRTs.

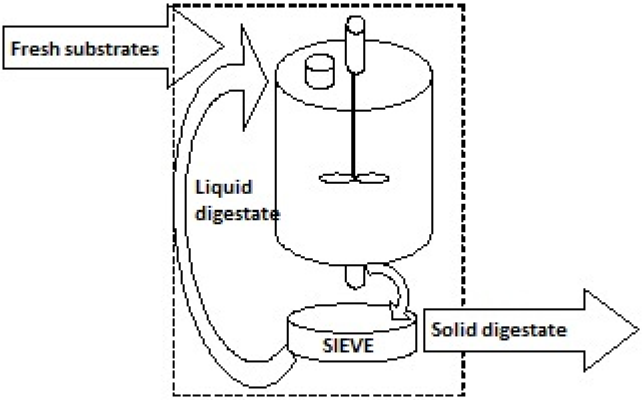


Figure 4.10: Recirculation of the liquid digestate fraction (**Paper II**).

4.2.4 Recovery of nutrients from the digestate

The methods investigated for the recovery of NH_4^+-N in particular, but also PO_4^{3-}, from the final digestate that was not recirculated (effluent), stated in **Paper III**, were chemical precipitation by struvite formation and adsorption on bentonite.

Struvite precipitation trials were performed at molar ratios of 1:1:1 and 1.2:1:1 (Mg^{2+}: NH_4^+: PO_4^{3-}), showing to be optimum according to literature (Celen and Turker, 2001; Altinbas et al., 2002; Li and Zhao, 2003). In reactor GA2, co-digestion of steam exploded *Salix*, manure and fish byproduct with recirculation of liquid digestate was performed, and its rejected final digestate was the one showing higher levels of NH_4^+-N. After analyzing the content of $PO_4^{3-}$and Mg^{2+}, PO_4^{3-} was found to be the limiting compund, so $MgCl_26H_2O$ and KH_2PO_4 were added to 300 mL aliquots of the sieved accumulated digestate. The content was agitated at 300 rpm for 15 minutes and let to settle for 15 minutes more, before being divided into four aliquots containing 45 mL each. These aliquots were adjusted to pH 7, 8, 9, and 9.5 with a NaOH 1 N solution. Determination of NH_4^+-N, PO_4^{3-} and soluble COD content in the supernatants were followed spectrophotometrically after a 24 hrs. settling period, for the samples that showed a visible sedimentation of precipitate.

Adsorption trials employing commercial Na^+ bentonite were performed by adding 1.5, 3, 4, 6, and 9 g. to 50 mL aliquots of GA2's sieved liquid digestate. The mixture was agitated at 600 rpm for 5 minutes and a settling period of 24 hrs. was applied before spectrochemical analysis of the supernatants for NH_4^+-N , PO_4^{3-} and soluble COD were made.

5 General results and discussion

5.1 Effects of steam explosion as pre-treatment on the biogas production from *Salix*: screening of steam explosion conditions

The findings of **Paper I** proved that steam explosion is a suitable method for pre-treatment of *Salix* in order to produce biogas from it. Methane yields obtained for the different pre-treatment conditions were superior to that of untreated *Salix*, with the only exception being pre-treatment at 170 °C and 10 minutes. Due to steam explosion, the substrate was more available for the microorganisms to degrade. For the first 20 days, biogas production seemed to increase up to treatments at 210 °C (Fig. 5.1). However, for the total digestion period, the production gave slightly higher yields for the treatment at 230 °C and 5 min. This can be due to the fact that harsher steam explosion conditions might be source of higher yields of substances such as furfural and phenolics (Ramos, 2003; Sassner et al., 2005; Horn and Eijsink, 2010), which may have inhibited the microbial activity (Mes-Hartree and Saddler, 1983; Brownell and Saddler, 1987; Castro et al., 1994; Bruni et al., 2010a). Thus, after an adaptation period of 57 days the yields obtained for the harsh conditions are similar to the others. In general, the pre-treatment increased the specific methane yield up to 50 %, when comparing the steam exploded series with the untreated one. The statistical evaluation with a two sample t-test of the methane yield data for the 210 °C, 10 min and 230 °C, 5 min series showed that they were not significantly different, neither after 22 days (p= 0.681) nor after 57 days period (p= 0.382). After steam explosion, a relative increase on the cellulose and lignin contents occurs (Table 3) while the hemicellulose content decreases, since it is degraded and volatile compounds are lost in the flash stream. This also cause the ash content to increase, more with higher temperatures applied, as stated in **Paper I**. The composition change can be attributed to condensation reactions and incorporation of non-lignin components into lignin (Chua and Wayman, 1979; Horn et al., 2011a).Thus, regarding the bigger impact that losses and inhibitory substances may have in harsher treatments, as well as the higher energy requirement to reach such temperatures, a pre-treatment of steam explosion at 210 °C for 10 min was chosen as the option to apply for the material of the co-digestion trials.

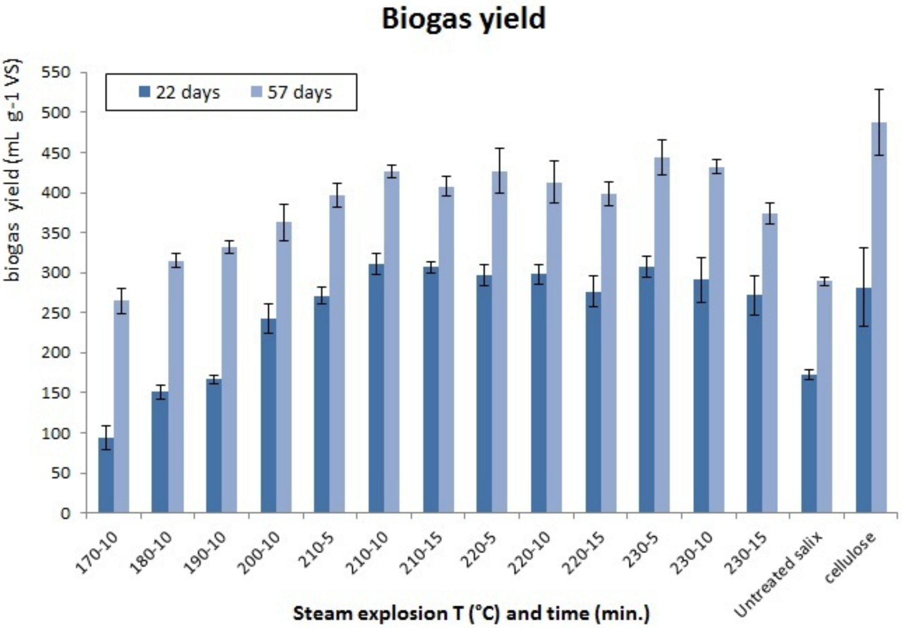


Figure 5.1: Biogas production of all the screened steam explosion conditions and of untreated *Salix*, after 22 and 57 days (**Paper I**).

5.2 Effects of different mixture ratio on the methane yield of pre-treated *Salix* and manure

The second set of batch methane assays investigated the effect of mixing two different substrates at different ratios (on VS basis) on their methane production (**Paper I**). Co-digestion of steam exploded *Salix* and cow manure proved to give a faster process than digesting the materials separately. The production was slower when digesting only manure, and this could be explained by the inoculum used, that came from the previous experiment and was already adapted to *Salix*, and by the incorporation of easily degradable carbon with the pre-treated *Salix*, accounting for an overall better balanced C/N ratio (35 vrs. 23 of only manure). Maximum yields reached ca. 230 mL CH_4/g VS after 77 days, and by the 20th day of the experiment, 75-80 % of the methane potential from all the co-digestion mixtures was already produced.

The co-digestion mixtures that gave the best methane yields had 30 % and 40 % VS of *Salix*, corresponding to C/N ratios of 35 and 39, respectively. Methane content in the biogas coming from the co-digestion mixtures ranged from 58 % to 54 % in average values, the higher ones corresponding to the mixtures having bigger manure content. For the series employing only pre-treated *Salix*, methane content in the biogas was around 53 % while for the series with only fresh manure as substrate, 63 %.

When analyzing methane yields with respect to C/N ratio for the mixtures of pre-treated *Salix* and manure, the C/N ratio that gave the optimal yield throughout the experiment ranged between 35 to 40 (Fig. 5.2). This is interesting since that ratio corresponded to a co-digestion mixture that possessed a considerable amount of steam exploded material (i.e., 40 % of its VS content) which shows that co-digestion with pre-treated lignocellulosic feedstocks can lead to good biogas yields. Lignocellulosic materials available in the agricultural sector would then be a good option for increasing the energy output while giving a robust and stable digestion process.

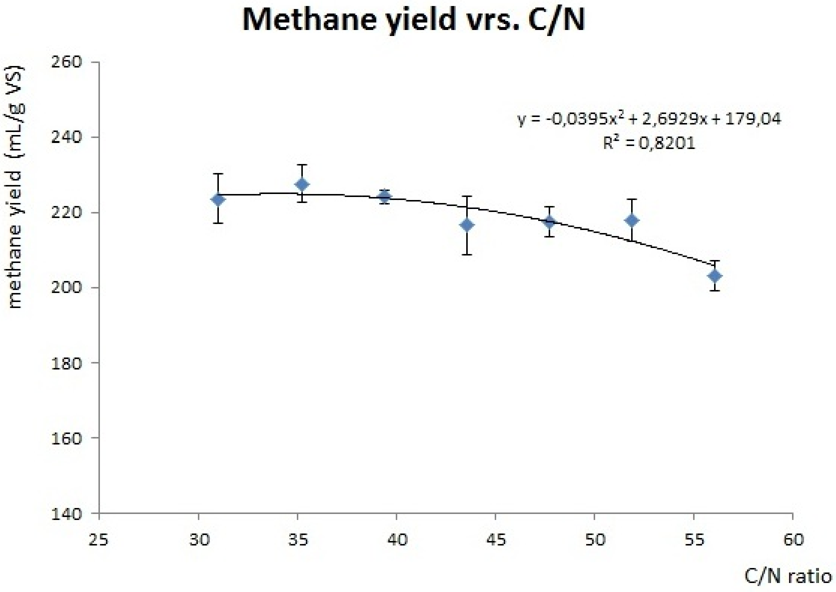


Figure 5.2: Methane yield and C/N ratio correlation for the mixtures containing steam exploded *Salix* and manure (**Paper I**).

5.3 Semi-continuous co-digestion of pre-treated *Salix*, manure and fish byproduct

5.3.1 Effects of co-digestion of steam pre-treated *Salix* and cow manure at different OLRs.

Manure and steam pre-treated *Salix* were possible to co-digest in batch scale even with high proportions of the pre-treated lignocellulosic biomass (40 % VS), thus, the stability of such mixture in CSTR systems was tested in pre-trial experiments (Estevez et al., 2011, 2012) and in **Paper II**.

In the pre-trials, reactor 1 was fed with a co-digestion mixture of steam exploded *Salix* and manure (40 %/60 % VS) while reactor 2 was fed with fresh manure alone. The OLR was 1.5 g VS/Ld and the HRT was 30 days. Co-digestion of steam exploded *Salix* and cow manure in CSTRs gave good biogas yields. Reactor 1 gave a higher biogas production, however, its methane content was lower compared to reactor 2.

This translated in very similar specific methane profiles for both reactors. Maximum yield for reactor 1 was 246 mL CH_4/g VS while for reactor 2 was 251 mL CH_4/g VS.

In **Paper II** a higher OLR was studied. Methane yields at an OLR of 3 gVS/Ld (daily average over a week was 2.6 gVS/L) and HRT of 30 days, for the reactor 3 digesting the mixture of *Salix* and manure (GA1 in **Paper II**) reached a maximum of 183 mL CH_4/g VS during the first HRT (max. of 185 for the entire period of 3.3 HRTs), while reactor 4 digesting only cow manure (GB2 in **Paper II**), reached after 30 days a maximum of 142 mL CH_4/g VS (max. of 156 for 3.3 HRTs).

Table 4: Operational parameters for reactors 1- 4 at both OLRs, after one HRT (methane content of biogas; specific and volumetric methane yields and NH_4^+-N content).

OLR (gVS/L.d)	1.5		2.6	
Reactor	1	2	3	4
Feeding (VS %)	*Salix* (40%) + Manure (60%)	Manure (100%)	*Salix* (40%) + Manure (60%)	Manure (100%)
Methane content (%)[1]	50	57	48	54
Highest spec. methane yield (mL CH_4/gVS)	246	251	183	142
Highest vol. production (L CH_4/m^3d)	369	376	548	427
NH_4^+-N (mg/L)	615	1,010	843	1,530

[1] Average values.

Comparing the yield obtained in mesophilic batch assays (**Paper I**) for the same co-digestion mixture after 30 days (193 mL CH_4/g VS) and a final biomethane potential of 224 mL CH_4/g VS, higher values were reached semi-continuously with the OLR of 1.5 g VS/Ld (Fig. 5.3), proving that steam exploded *Salix* with up to 40 %VS together with manure can lead to good semi-continuous production of methane. Literature results on methane yields from semi-continuous co-digestion trials employing other energy crops and manure are in accordance with our results (Lehtomäki et al., 2007; Nordberg et al., 2007; Comino et al., 2010).

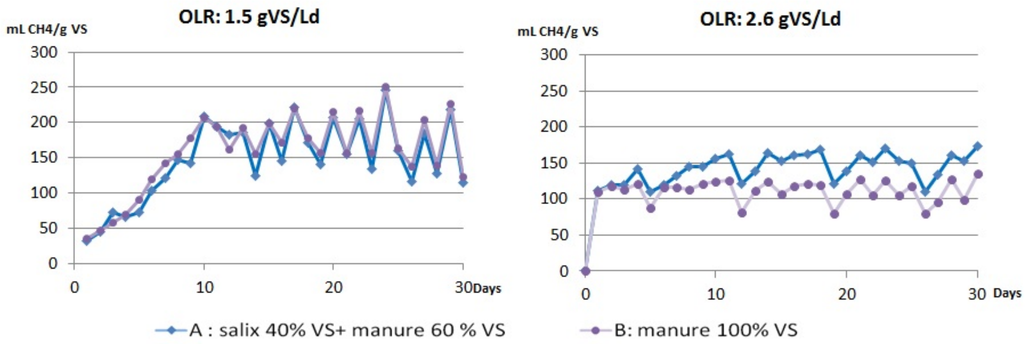


Figure 5.3: Specific methane yields for the co-digestion mixture and for manure alone, at two different OLRs: 1.5 and 2.6 g VS/Ld.

The specific methane yields decreased with the higher OLR, this can be due to the fact that the HRT became short for a proper degradation of the higher amount of feedstock introduced with the high OLR, leaving organic solids undegraded and accumulating them as well as recalcitrant ones in the digester (Lehtomäki et al., 2007; Comino et al., 2010). In our case, lack of VFAs in the digesters indicated that readily degradable organic matter was constantly consumed, not causing overloading, leaving the recalcitrant solids as the main accumulated solid. For the reactor processing only manure, the VS of its crops content had been already “digested” in the digestive tracts of animals, so the methane potential is not as high as that of energy crops that provide more easily degradable VS (Lehtomäki et al., 2007).

According to Comino et al. (2010), increasing the OLR from 4.45 to 5.15 g VS/Ld during the co-digestion of crop silage and manure caused a 5 % increase of the methane yield, while attempts of increasing further the OLR lead to a decrease of the yield. Nordberg et al. (2007) reached a process breakdown when alfalfa was co-digested with manure under an OLR of 3 g VS/Ld, while Lehtomäki et al. (2007) found that in the co-digestion of manure and grass, increasing the OLR from 2 to 3 g VS/Ld caused a decrease of 7 % in the methane yield, while increasing it to 4 caused a 16-24 % reduction.

Even though substrate based yields were smaller for the 2.6 OLR, methane production proved to be stable for a total period of 3.3 HRTs (**Paper II**) without giving inhibitory issues regarding the presence of VFAs or ammonia. VFAs were mostly undetected along the whole period implying that the limiting steps of the degradation process would have been hydrolysis and/or acidogenesis (Lehtomäki et al., 2007). The mixture of a carbon-rich substrate (lignocellulosic biomass) and a nitrogen-rich substrate (cow manure) provided a nutrient composition that showed to be successful in increasing the robustness of the system. The overall C/N of the feedstock was 39, higher than the optimum of between 16 and 30 given by literature (Yadvika et al., 2004; Yen and Brune, 2007; Deublein and Steinhauser, 2008). The effects of adding a nitrogen-rich substrate, fish byproduct, on process stability and methane production, were investigated in **Paper III**.

5.3.2 Effects of incorporating fish byproduct in the co-digestion mixture.

In **Paper III** another co-substrate was added to the mix of steam exploded *Salix* and cattle manure. The fish byproduct category 2 (Biokraft Marin AS, Trondheim) was added to reactors GA1, GA2 and GB1, but only GA1 was functioning in a process without recirculation of digestate. Therefore, the effect of just incorporating fish can be evaluated when comparing this GA1 with the GA1 from the previous experiment in **Paper II**, where *Salix* and manure were codigested. GA1 with fish (7.2 % w/w) gave a maximum yield of 212 mL CH_4/g VS during the first HRT, that is around 16 % higher than for GA1 digesting only *Salix* and manure during the same period. Comparing to **Paper II** results for the whole experimental period, yields with fish showed and average of 181 mL CH_4/g VS (max. 292 mL CH_4/g VS) while yields without fish showed average of 140 mL CH_4/g VS (max. 185 mL CH_4/g VS) (Fig. 5.4). This can indicate that the microbial community adapted to the new substrate high in proteins, which translated in an increase of the yield after the first retention time, giving through the whole period an average methane yield 30 % higher than that one from *Salix* and manure. Thus, fish incorporation caused an important effect on biogas production.

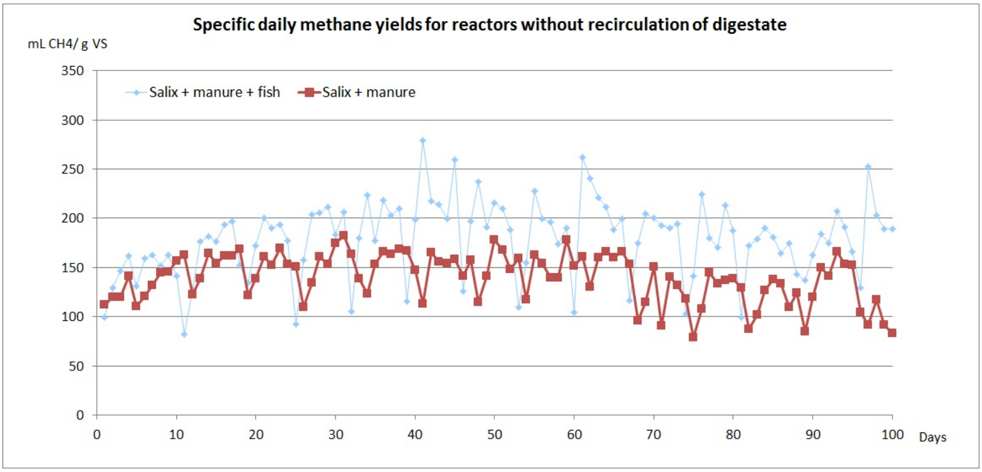


Figure 5.4: Specific methane yields for the co-digestion mixtures of pre-treated *Salix* and manure (40 % and 60 % VS respectively) and pre-treated *Salix*, manure and fish byproduct (40 %, 40 % and 20 % VS respectively).

NH_4^+-N levels in the reactor containing fish byproduct showed a slow increase and reached values of 3.1 g/L. Comparing to studies where co-digestion of fish waste with other substrates were performed, fish waste lead to an increase by 8 % in the yield in a sequential batch reactor process when it was co-digested together with fruit and vegetable waste (1,4 % fish waste in the mixture) giving a yield of 320 mL CH4/g VS added (Bouallagui et al., 2009). Other studies found deterioration of the process when fish waste fractions were increased (Callaghan et al., 1998; Eiroa et al., 2012), mostly due to long chain fatty acids (LCFA) inhibition. Our substrate is different from the standard fish wastes and silages, since it underwent a pre-treatment and homogenization (FSPM) that reduces its lipid content. Nonetheless, the amount of lipids is comparable to that one of fish offal employed by Callaghan et al. (1998), that being co-digested (6 % w/w) with cattle slurry gave LCFA inhibition to the AD process.

5.4 Residual methane potential and recirculation of digestate

In **Paper I,** manure that had already been digested in a CSTR was employed for some series of the batch methane assays. It was co-digested with pre-treated *Salix* and both series, having 30 % and 40 % of *Salix* in the mixture, gave methane yields that were 78 % of the ones obtained for the series employing fresh manure and the same VS composition of *Salix*. The methane content of their biogas produced was ca. 60 %. A 78 % fraction of the yield is a significant one, and an indication of the high methane potential that still remains in the feedstocks after digestion takes place. Normally, at farm or industrial scale the remaining potential of the digestate could be lost during storage periods. Ways to profit these residual potential without compromising the treatment capacity, should be further developed. According to several authors (Hartmann et al., 2000; Angelidaki et al., 2005; Jagadabhi et al., 2008) it represents a big part of the total methane potential, and it remains untapped in the fibers even after production of substantial amounts of methane in the anaerobic digestion process. One of the methods to recover such potential would be applying extended retention times so that to allow an increased degradation time within the process. Recirculation of the liquid fraction of the digestate after a gross separation will cause an increase in the retention time for some of the substrate fractions and this methodology is addressed in **Papers II** and **III**.

Recirculation of the liquid fraction of the digestate allows for dilution of the feedstock input and saving water, when the substrates have considerable contents of total solids. When only steam exploded *Salix* was added daily, at an OLR of 1.5 (Estevez et al., 2011), this resulted in a decrease in pH (to 5.9), negligible biogas production and very a low methane content, i.e. the collapse of the process (Fig. 5.5). On the other hand, when digestate recirculation was applied to the mixture of steam exploded *Salix* (40 % VS) and manure (60 % VS), at an OLR of 2.6, yields reached a maximum similar to those achieved at an OLR of 1.5 for the same mixture but without recycling (246 mL CH_4/g VS reactor 1) . The highest yield was achieved for the reactor GB1 (Fig. 5.5) **(Paper II**) in which the digestate dilution ratio applied was higher (1:3 vol. feedstock/digestate). Maximum yields after 3.3 HRTs were 217 and 235 mL CH_4/g VS in GA2 (dilution 1:1 vol.) and GB1 (dilution 1:3 vol.) respectively.

This shows that digestion of *Salix* alone without introduction of a nitrogen material that would balance the process, is not possible. Recirculation of the liquid digestate fraction in the digestion of *Salix* alone was not enough to maintain the reactor's biomass. These factors caused the process to lose stability, with a decrease in buffer capacity, and collapse of the methane production.

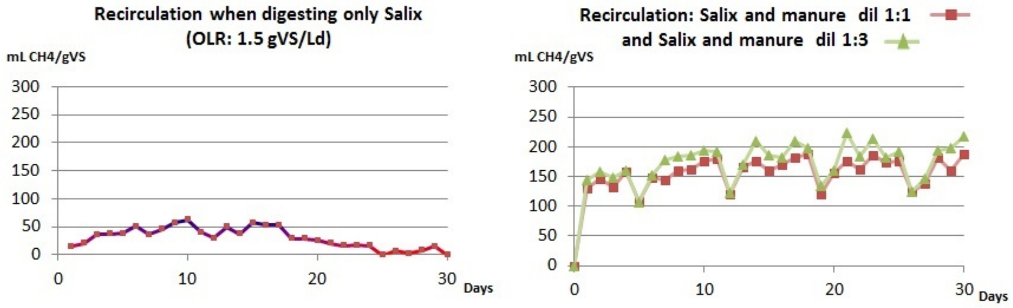


Figure 5.5: Comparison of yields when recirculating digestate during the anaerobic digestion of steam exploded *Salix* alone (OLR 1.5 gVS/Ld) or in co-digestion mixture (OLR 2.6 gVS/Ld).

Liquid digestate recirculation proved then interesting results. As stated in **Paper II**, substitution of water by liquid digestate in the dilution of the fresh substrate's mixture gave a 16 % higher methane production (GA2) comparing to the control reactor having the same mixture but water for dilution (GA1) (Fig. 5.6). When filtered digestate also substituted part of the manure (1:3 vol. dilution with digestate), the highest yield of all the reactors was achieved (GB1), being 27 % higher than the control reactor (GA1) (Fig. 5.6). The methane content of the biogas produced was higher in the reactors subjected to recycling compared to the reactors without recycling. A comparably high methane production was also found by Jarvis et al. (1995) for a two-phase anaerobic digestion process fed with silage, with a 22 % increased in the methane yield from the total system when effluent from the methanogenic reactor was recirculated back to the acidogenic reactor. In a study by Nordberg et al. (2007) the OLR increased by 25 % when liquid recirculation in a one-phase anaerobic digestion of alfalfa silage was initiated, providing an increase in pH, alkalinity and stability. This stability effect was also reported by Hartmann and Ahring (2005) who achieved instant buffering effect when process liquid was recirculated.

There are several possible explanations for the positive effects observed in connection to recycling. One explanation could be that suspended material, i.e. microbial biomass, was reintroduced in the reactor during recycling, and enriched the degradation process (Nordberg et al., 2007). Also, the increased microbial biomass could give rise to better contact between the microorganisms and substrate making this process configuration more robust to fluctuations (Hartmann and Ahring, 2005). Furthermore, the liquid digestate may have included a VS fraction not accounted for in the yield calculation (that only considered fresh VS), that is being degraded with the longer retention time relieving the residual methane potential, which may partly explain the difference in yields between these reactors and the control one. Due to the recirculation, the solids retention time (SRT) differed from the HRT, being the SRT (ca. 43 and 51 days for GA2 and GB1, respectively) longer than the HRT for the process without recirculation (30 days), and this extended solids retention may have enhanced the production from the still degradable VS fraction (Angelidaki et al., 2005). VS degradation, that considered both fresh and recirculated VS, was similar for reactors GA1 and GA2 (average 34 ± 3 %; 37 ± 1 % respectively), higher for GB1 (42 ± 4 %) and lower for GB2 (average 28 ± 5 %). The extended solids retention may enhance production from the still degradable VS fraction in reactors GA2 and GB1. At the same time, part of the recalcitrant VS was as well recycled and thus, accumulated, being this probable the reason for the observed increase with time in the VS content on the recirculated reactors, and a build up of possibly recalcitrant solids was accounted in them (Fig 5.7). This may as well explain that after 2.5 HRT, yields of the recirculating reactors showed a decreasing tendency (Fig. 5.6). Accumulation of organic and inorganic compounds during process liquid recirculation as well as a gradual decrease in methane yield was also reported for a two-step digestion of alfalfa silage (Nordberg et al., 2007). The organic material accumulation led to an increase of the VFA levels and eventually to process inhibition. In our case, VFA were hardly detected and so no organic overloading was present, implying that the accumulated solids mostly were recalcitrant. The Växtkraft biogas plant in Sweden, performing process water recycling, showed a decrease on it's input capacity of ca. 25 % due to undegradable solids build up inside the digester (Lindmark et al., 2011). Furthermore, Hartmann and Ahring (2005) observed smaller average VS degradations when liquid recirculation was applied to a co-digestion system of organic fraction of municipal solid waste and manure. This indicates that recirculation of liquid digestate may eventually lead to inhibition due to accumulation of solids. However, with proper control and an optimum recirculation ratio for the system, accumulation of solids may not cause any problems (Nordberg et al., 2007).

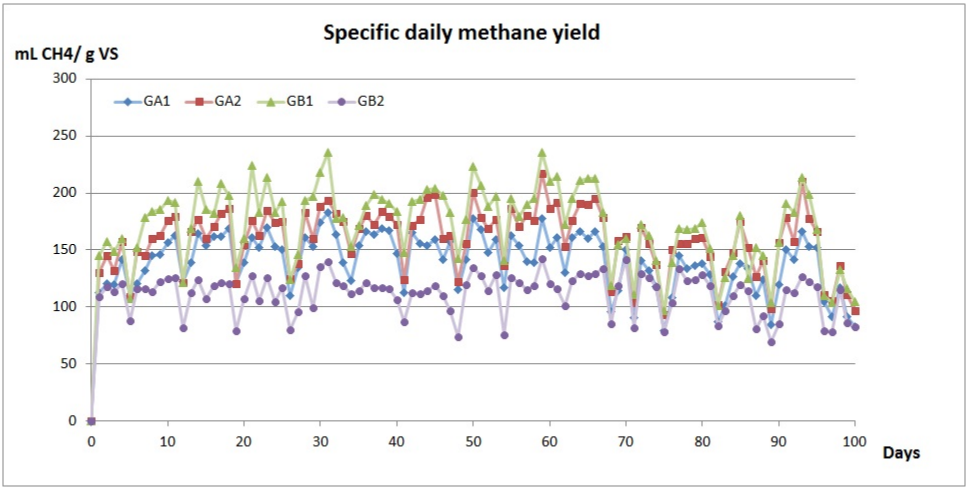


Figure 5.6: Specific daily methane production in the four reactors of **Paper II**: GA1 fed with *Salix* (40 % VS) and manure; GA2 fed with *Salix* (40 % VS) and manure with recirculation (1:1); GB1 *Salix* (47 % VS) and manure with recirculation (1:3) and GB2 only manure.

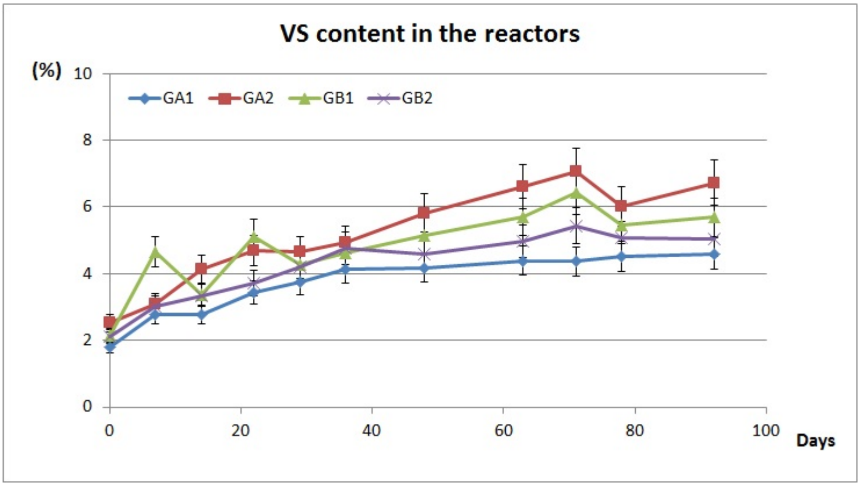


Figure 5.7: VS content (%) inside the digesters (**Paper II**).

When recirculation of digestate was investigated at the same time that fish byproduct was added to the co-digestion mixture (**Paper III**) the effects of recirculation were not as notorious. GA1 and GA2 reactors were fed with the same substrate mixture, composed of steam exploded *Salix* (40 % VS), manure (40 % VS) and fish byproduct (20 % VS), but in GA2 recirculation of the digestate took place. The average yield was 6 % higher in GA2 than in GA1, although the maximum daily yield achieved along the whole period was 279-292 mL CH_4/g VS in GA1 compared to 270 mL CH_4/g VS in GA2. This last one showed better stability regarding yield fluctuations (smaller deviations) than GA1. A more balanced nutrient composition, giving better buffer capacity, preservation of trace-elements and enhanced adaptation to changes for an enriched microbial community may occur in this recirculated reactor (Yadvika et al., 2004; Hartmann and Ahring, 2005).

Comparing the yields from GA2 fed with fish with GB2 fed with *Salix* and manure, both recirculated, a yield for GA2 was in average 35 % higher, so the fish byproduct proportion of 20 % VS in the mixture while recirculating achieved a considerable increase. When this proportion was instead 10 % (GB1), the increase in the yield comparing to GB2 was only of 13 % in average (Fig. 5.8).

The recirculating reactors containing fish byproduct (GA2 and GB1), were the ones showing a bigger increase of NH_4^+-N, while the reactor with recirculation but without fish kept its ammonium content stable (GB2). In GA1, where fish was added but recirculation was not performed, the increase was less pronounced comparing to GA2 and GB1.

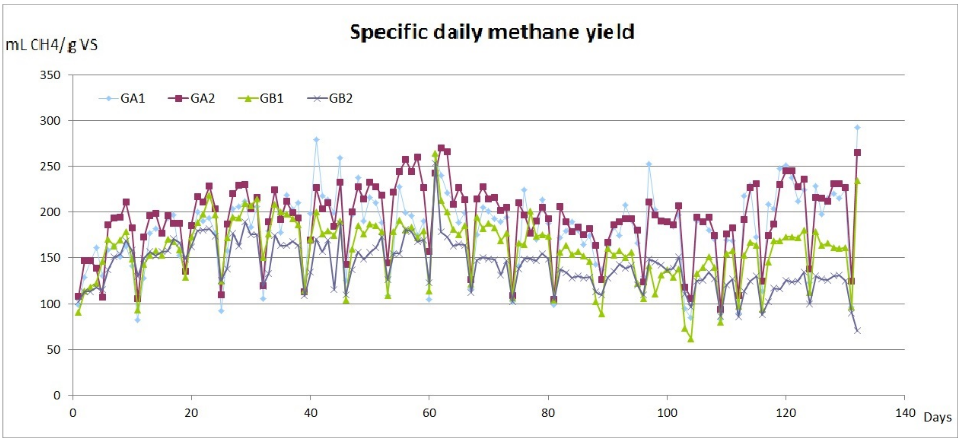


Figure 5.8: Specific daily methane production in the four reactors of **Paper III**: GA1 fed with *Salix* (40 % VS), manure (40 % VS) and fish (20 % VS); GA2 fed with *Salix* (40 % VS), manure (40 % VS) and fish (20 % VS) with recirculation; GB1 *Salix* (40 % VS), manure (50 % VS) and fish (10 % VS) with recirculation; GB2 with *Salix* (40 % VS) and manure (60 % VS) with recirculation.

A higher ammonium content (up to 70 % more) was reached for GA2 in comparison to GA1, with maximum levels of NH_4^+-N reaching 5 g/L and the process running stable, a level that according to literature can produce inhibition of the methanogenesis (Angelidaki and Ahring, 1992; Chen et al., 2008) (**Paper III**). An inoculum already adapted to high nitrogen levels due to the digestion of fish waste may have benefited the fast acclimation of the microorganisms to the substrates and to the high NH_4^+-N levels, as well as the stability provided by the digestate recirculation. These reasons could be precursors for a SAO mechanism to occur in the recirculating reactors, which will be further described in subsection 5.5.2.

5.5 Accumulation of toxic substances

5.5.1 Furfural and HMF

When semi-continuous systems with recirculation are employed, accumulation of recalcitrant and/or toxic substances may lead to process instabilities. One possible drawback of using steam explosion as pre-treatment for disruption of lignocellulosic biomass is the release of phenolic compounds and furfurals, which could act as inhibitors for the biomethanation process (Castro et al., 1994; Jagadabhi et al., 2008; Bruni et al., 2010b) if they are as well being accumulated in the reactors. The only substances detected in our reactors from the analysis of monolignols and furfurals **(Paper II)** (ferulic acid, p-coumaric acid and other hydroxycinnamates were analyzed), were HMF and furfural at trace levels along the whole experimental period. Although levels were very low, an accumulation of HMF on a 2.5-fold increase was shown in the recirculating reactors, while no HMF was found in the reactor digesting only manure. Furfural was present in all the reactors and showed after three HRT a 2-fold increase for GA1 and GA2 and a 3-fold increase for GB1, being all trace level values. Thus, it seems unlikely that the observed decrease in gas production was caused by the analyzed inhibitory compounds.

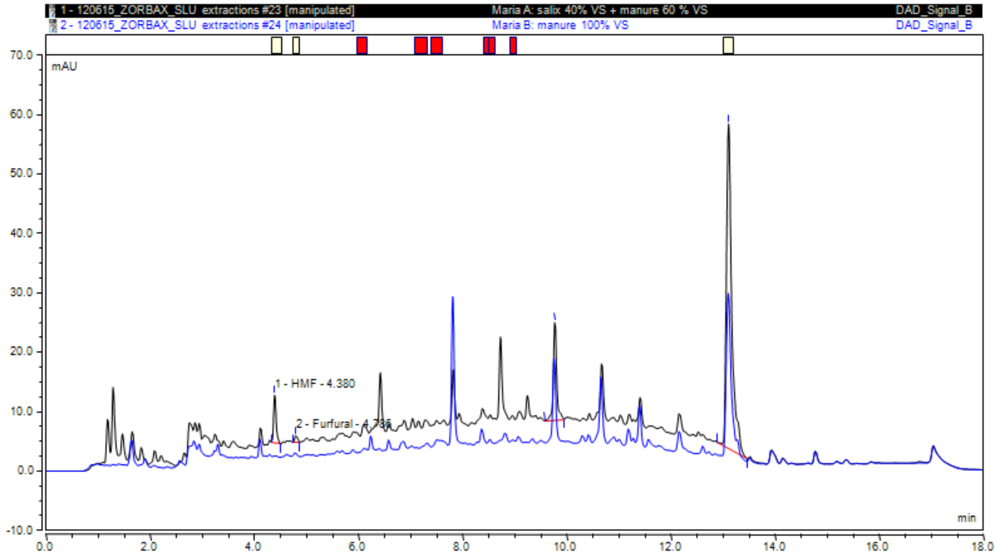


Figure 5.9: HPLC analysis of HMF and furfural in the content of the reactor digesting *Salix* and manure (black line: GB1) and another digesting manure only (blue line: GB2)

5.5.2 Ammonium-N / ammonia

In **Paper II** $NH_4{}^+$-N levels were in average stable throughout the period, and below inhibitory values, even in the recycling reactors. This is interesting since previous studies in which silages were digested at mesophilic temperatures showed increasing $NH_4{}^+$-N levels (Jarvis et al., 1995; Nordberg et al., 2007). It seems that in our reactors the C/N ratio of the substrates was well balanced, not reaching $NH_4{}^+$-N levels that could cause ammonia inhibition to the bacteria.

In **Paper III** however, $NH_4{}^+$-N concentrations inside the reactors reached higher and potentially inhibitory values. The ammonium content increased along the period mostly for the recirculating reactors containing fish byproduct. A maximum of 5 g/L of $NH_4{}^+$-N was reached for reactor GA2, with a process running stable. The recirculation effects of increasing robustness and stability of the process, and an inoculum possibly already adapted to high nitrogen levels may have helped in the fast acclimation of the microorganisms to the high $NH_4{}^+$-N levels. According to most studies, acetoclastic bacteria is the group of methanogens more susceptible to free ammonia inhibition, rather than hydrogenotrophic bacteria (Koster and Lettinga, 1984; Zeeman et al., 1985; Angelidaki and Ahring, 1993; Calli et al., 2005). But methane production from acetate can follow an additional pathway, the SAO, that besides the traditional methanogens, involves another group of non-methane-producing bacteria which is less affected by ammonia (Barker, 1936; Zinder and Koch, 1984; Schnürer et al., 1994). The co-culture responsible for SAO poses longer doubling time than acetoclastic methanogens (Schnürer et al., 1994) and thus, recirculation of the digestate may promote the change to SAO pathway and the adaptation to high ammonia levels. In our trials, samples for microbiological analysis were evaluated at the Swedish University of Agricultural Sciences (SLU) to detect the presence of *T. acetatoxydans*, one of the bacteria taking part in the SAO. Although its presence was detected, only very low levels were found of such bacteria in reactors GA1 and GA2, during the first half of the experiment. Further studies along the whole experimental period will continue and will be addressed in future publications.

5.5.3 VFA and LCFA

VFA contents were analyzed in **Paper II** for all the reactors, and were very low throughout the entire period; only acetic and propionic acids were detected, with maximum total concentrations of 250, 320, 230 and 320 mg/L (measurement uncertainty 10 %) for GA1, GA2, GB1 and GB2, respectively. These values are far from the level at which process imbalance may occur: 3,000 mg/L (Ahring et al., 1995; Holm-Nielsen et al., 2007). This indicates that the performance was stable, with organic acids being consumed and not accumulated. According to Nordberg et al. (2007), 100% recycling of liquid digestate during digestion of alfalfa silage lead to accumulation of organic compounds including VFA, and subsequently, inhibition, while recycling with a liquid consisting of 50% of the digestate together with water, only caused moderate problems due to VFA accumulation. Similar results were achieved in pre-trials studies (Estevez et al., 2011) in which 100 % of the liquid digestate was recirculated and mixed with steam exploded *Salix*, without adding manure or water, resulting in an acidification of the process, a decrease in pH and collapse of the reactor. Thus, again these results support that a proper recirculation ratio is needed in order to balance VFA and NH_4^+-N levels.

When fish was co-digested (**Paper III**), the content of VFA showed initial levels of propionic acid (maximum was 1,600 mg/L) , but no signs of inhibition. Analysis of VFA done to the inoculum and the fish byproduct, showed only small quantities of acetic acid present on the first one (50 mg/L), while the fish byproduct had a concentration of around 2,000 mg/L in total VFA (1,200 mg/L acetic acid and 480 mg/L propionic acid). The increase in propionic acid during the first HRT of the process may be cause of adaptation to the feeding of fish byproduct.

In **Paper III**, the fish byproduct employed had a lipid content similar to that of fish offal, which has shown to give inhibition on the biogas production due to LCFA accumulation (Callaghan et al., 1998), thus, these acids were also analyzed in the fish byproduct category 2 substrate. Unsaturated LCFA such as oleic (35 % of total fatty acids) and linoleic (11 % of total fatty acids) were the predominant ones found in the fish byproduct. However, the process did not showed signs of LCFA inhibition, previous studies have demonstrated that even low concentrations of these substances can cause toxic effects (Angelidaki and Ahring, 1992). During anaerobic digestion, oils and fats are rapidly hydrolyzed to LCFA and glycerol (Ahring et al., 1992; Angelidaki and Ahring, 1992). LCFA contain most of the oil's energy and are thus, excellent substrates for biogas production; via ß oxidation LCFA are converted to acetate and hydrogen, which are finally turned into methane by the methanogens consortia (Angelidaki and Ahring, 1992; Sousa et al., 2008; Eiroa et al., 2012). But if accumulation of these free fatty acids occurs, bacterial growth and biogas production can be inhibited (Angelidaki and Ahring, 1992; Rinzema et al., 1994). Toxic effects are manifested in the adsorption of LCFA on the cell membranes, and interferences in the transport or protection mechanisms (Rinzema et al., 1994; Eiroa et al., 2012). Nonetheless, some studies have demonstrated that this type of inhibition is reversible, with processes that can either recover after LCFA concentrations decrease (Pereira et al., 2004) or adapt to high lipid levels if fat is being added in continuously repeated cycles (Cavaleiro et al., 2008; Sousa et al., 2008).

5.5.4 Solids accumulation

Solids accumulation is an important issue to address when performing digestate recirculation.When applying recirculation, not only still degradable VS but also a recalcitrant fraction is as well being re-added to the process and thus, accumulated, providing in the long term smaller yields and a decrease in the process's capacity. This was found in **Papers II** and **III**. Accumulation was more notorious in **Paper II**, where recirculated digestate was obtained from the filtration with a 2.5 mm mesh sieve, giving such separation an increase of the VS content in the reactor of 2.6 times the initial level by the end of the period (3.3 HRTs) . When a new sieve was added (1 mm + 2.5 mm mesh) to the digestate fractions separation in **Paper III**, the VS content inside the reactors increased 1.5 times through the whole experimental period (4.4 HRTs). Semi-continuous feeding of highly fibrous materials without recirculation can also lead to solids being accumulated in the long term, and a proper monitoring has to be addressed.

Accumulation of organic and inorganic compounds during process liquid recirculation has been detected in laboratory scale (Hartmann and Ahring, 2005; Nordberg et al., 2007) and in full scale AD processes (Lindmark et al., 2011). In Nordberg et al. (2007), organic material accumulation led to an increase of the VFA levels and eventually to process inhibition. In both **Papers II** and **III**, VFA levels were generally low and thus, no organic overloading was present, implying that the accumulated solids mostly were recalcitrant. Recirculation of liquid digestate may eventually lead to inhibition due to accumulation of solids and to decrease in the yields. However, with proper control and an optimum recirculation ratio for the system, accumulation of solids may not cause inconvenients (Nordberg et al., 2007).

5.6 Savings in resources

By employing filtered digestate in the dilution of high solids feedstock, water needs are reduced, as well as the need to pre-heat the feedstock mixtures, which would translate into less process costs for full scale plants (Jarvis et al., 1995; Angelidaki et al., 2006). Water was totally substituted by the liquid digestate for reactors GA2 and GB1 when compared to GA1 (**Paper II**), with savings of ca. 3 L of water per HRT for GA2, and ca. 4 L of water and 1 kg of manure per HRT for GB1. Regarding manure as a resource, GB1 was fed with approximately half the amount of fresh manure fed to GB2 (i.e., 3 kg less per HRT), that being co-digested and diluted with digestate, ended up giving better methane yields. Reduction of manure consumption may benefit farm-scale biogas production that depends on seasonal animal manure as substrate. Thus, savings in resources such as fresh water and manure were positively achieved by incorporating liquid digestate recirculation to the CSTRs.

5.7 Recovery of nutrients to enhance the solid fraction of the final digestate

From the digestate analysis before and after being separated, in **Paper II** an average of 35 % dry matter reduction was achieved with the 2.5 mm mesh, giving a final solid fraction with ca. 10 % of dry matter. In **Paper III** the improved separation achieved a final solid fraction with 12.5 % in dry matter. Increasing the total solid fraction of an anaerobic digester's final digestate can lead to important economic savings in the transport and handling logistics of such material, later to be used as fertilizing agent.

Analysis of the rejected digestate's fractions from the reactors of **Paper II** and **III** (i.e., fractions that were not filtered and recirculated but instead accumulated (effluent)), showed that most of the nitrogen (75 %- 94 %) was present as ammonium-N. In our co-digestion feedstock mixture containing *Salix*, NH_4^+-N share on its total-N content counted for ca. 36 %, while the average value for cattle slurry is ca. 52 % (Lukehurst et al., 2010). The reactor's digestates of **Paper II** with or without recirculation gave very similar estimated N:P:K values of ca. 3:1:5.

After the separation of the digestate fractions with the mesh, most of the NH_4^+-N (70 %) remained in the liquid fraction. In general, **Paper III's** digestates phosphate content accounted for ca. 92 % of the total-P. The digestates that showed higher values of both NH_4^+-N and PO_4^{3-} were the ones corresponding to the co-digestion of fish byproduct. GA2's digestate (20 % fish + manure + *Salix*), was 70 % higher in NH_4^+-N than GB2 (manure + *Salix*), while GB1 (10 % fish + manure + *Salix*) was 40 % higher in NH_4^+-N. The PO_4^{3-} content was 18- 28 % higher for the reactors co-digesting fish.

The N:P:K ratio of the accumulated digestates was 4:1:7, having the solid fractions with fish 3:1:4 and without fish 2:1:5. A low N:P ratio would translate in good soil amendment for fruit trees, carrots or other root vegetables (Gebauer and Eikebrokk, 2006). Also, NH_4^+ recovered from the liquid fraction and incorporated into the solid digestate can improve such ratio. The digestate has to fulfill the Norwegian regulations for fertilizers of organic origin (Landbruks og matdepartementet, 2003) in order to be allowed as fertilizer in Norway. It can not contain substances that may harm the environment, humans, animals or plants, while the content of heavy metals, organic contaminants and hygienization procedures are regulated (Gebauer and Eikebrokk, 2006).

The solid digestate fraction coming from the co-digestion of *Salix*, manure and fish had higher values in Cd, Zn, Cr and Ni than the ones without fish as a co-substrate. Thus, heavy metal content of the separated solids and raw accumulated liquid digestate from the co-digestion of *Salix*, manure and fish were compared to the Norwegian regulations for fertilizers of organic origin (Landbruks og matdepartementet, 2003) showing that Zn and Cd were the only values for the accumulated digestate not fulfilling the levels for the next best class of fertilizer (Table 5), while in the case of the solid digestate fraction only Cd was not fulfilling. The fitting quality level for the solid digestate fraction was as organic fertilizer class I (Table 5).

Concerning the hygienic quality of the digestate as organic fertilizer, the regulations prohibit the content of *Salmonella spp.* and restrict the content of thermotolerant coliforms. Although they are associated to animal manure, this material that is commonly used as fertilizer has been digested for ca. 4 months at high ammonia concentrations, and it is expected to have the pathogens inactivated (Ottoson et al., 2008). Pathogens coming from the category 2 fish material would have been inactivated by the hygienization process (minimum of 85 º C for 25 minutes) performed prior to AD.

Table 5: Heavy metal content of total accumulated digestate and solid digestate fraction from co-digestion of fish, manure and *Salix* (**Paper III**) compared to requirements for organic fertilizers given by Norwegian regulations (Landbruks og matdepartementet, 2003).

Component	Content (mg/kg TS)					
	Accumulated digestate	Solid fraction	Organic fertilizer quality class			
	S+M+F	S+M+F	0[a]	I[b]	II[c]	III[d]
Cd	1.2	0.6	0.4	0.8	2	5
Pb	0.8	0.5	40	60	80	200
Hg	0.02	0.02	0.2	0.6	3	5
Ni	7.1	6.1	20	30	50	80
Zn	330	140	150	400	800	1,500
Cu	42	23	50	150	650	1,000
Cr	4.7	9.6	50	60	100	150

[a] Can be used in agricultural areas, private gardens, park and green areas. The amount applied must not exceed the plant's nutrient demands.

[b] Can be applied in limited amounts in agricultural areas and in private gardens and parks. Use not restricted in areas without food or feed production.

[c] Can be used in more limited amounts than b in agricultural areas and private gardens or parks. Use not restricted in areas without food or feed production.

[d] Can be used in limited amounts in green areas without food or feed production.

Recovery methods for NH_4^+-N and PO_4^{3-} were addressed in **Paper III**. GA2 accumulated digestate (effluent) was then chosen as the digestate to which perform NH_4^+-N and PO_4^{3-} recovery trials.

5.7.1 Struvite precipitation

Results of the reductions of NH_4^+-N, PO_4^{3-} and soluble COD achieved on the liquid phase of GA2's digestate after struvite precipitation took place are presented in Figure 5.10. Analyzing the samples after 24 hours settling time, best sedimentation results were obtained for the pHs 9 and 9.5 at both stoichiometric ratios. For samples at pHs 7 and 8 it was not possible to achieve always a clear phase separation after 24 hrs, but whenever the supernatant was possible to analyzed, NH_4^+-N levels in it decreased from 2,300 mg/L to an average of 280 mg/L, giving similar reductions as for pHs 9 and 9.5. It is important to note that a longer settling time (e.g., 48 hrs), showed a clearer sedimentation even at pHs of 7 and 8. NH_4^+-N reductions levels were comparable to those obtained by Celen and Turker (2001); Altinbas et al. (2002); Yetilmezsoy and Sapci-Zengin (2009) at pH values of 9- 9.2 and stoichiometric ratios of 1:1:1 and 1.5:1:1. Reduction of the original PO_4^{3-} content was not as clear as for NH_4^+-N since levels where not high in the liquid fraction and KH_2PO_4 was added to reach stoichiometry. Positive reduction was possible only at the ratio 1.2.1:1 with best overall results achieved at pH 9.5. Nonetheless, the sediment obtained, rich in NH_4^+-N and to some extent also PO_4^{3-}, could be incorporated into the separated solid fraction in order to further enhance such material as biofertilizer.

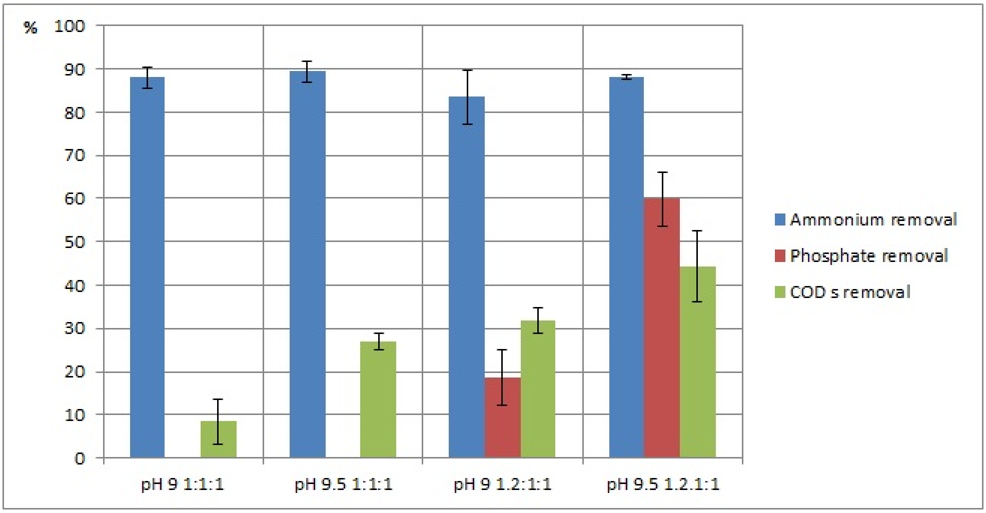


Figure 5.10: Removal efficiency results of NH_4^+-N, PO_4^{3-} and soluble COD in the struvite trials at pH 9 and 9.5, and molar ratios of 1:1:1 and 1.2:1:1 (**Paper III**).

5.7.2 Bentonite adsorption

Results of the bentonite adsorption trials are presented in Figure 5.11. NH_4^+-N was adsorbed up to 76- 82 %, with added quantities of bentonite above 4 g. per 50 mL of digestate, not improving further the adsorption. These results fit with the Freundlich adsorption isotherm model which is applicable to non-specific adsorption on a heterogeneous solid surface (Eturki et al., 2012). Our parameters were: K_f= 1.93x10^{-6}, n= 0.39 and R^2=0.80. Regarding removal of PO_4^{3-} and soluble COD, results showed removal of the initial content levels not surpassing 40- 50 % (Fig. 5.11).

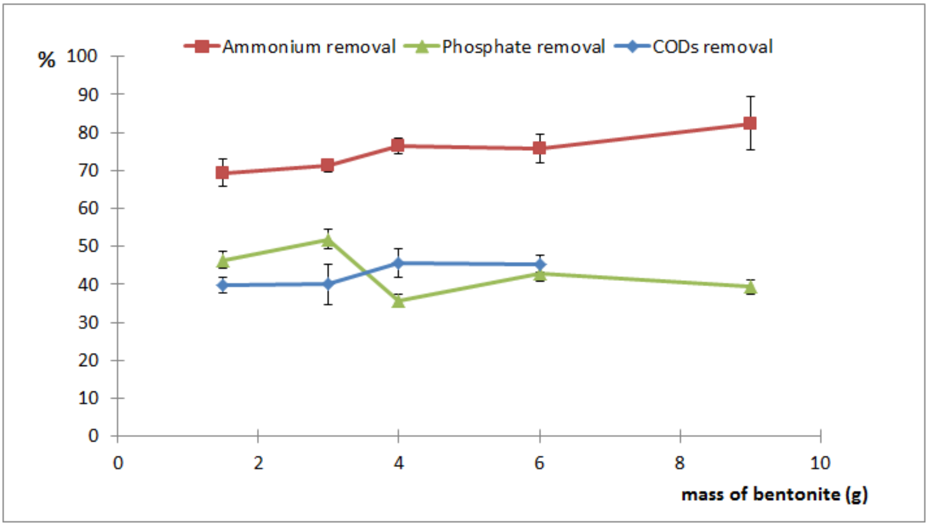


Figure 5.11: Effect of the mass of bentonite (g.) added to 50 mL digestate alicuots, on NH_4^+-N, PO_4^{3-} and soluble COD removal efficiency (**Paper III**).

Soluble COD removal followed a similar trend than NH_4^+-N, while PO_4^{3-} was removed better the lower the doses of bentonite applied. While struvite precipitation made possible to recover together NH_4^+ and PO_4^{3-} in a greater extent than bentonite adsorption, this last one does not need for a molar ratio to be maintained and adjust of the pH. When only NH_4^+-N is aimed to be removed and recover, bentonite seems as a good strategy. Depending on the costs of employing whether NaOH, $MgCl_2 6H_2O$ and KH_2PO_4, or bentonite and on the soil conditions, the more feasible method to adopt will be chosen.

Research to investigate the obtained solid digestate as fertilizers in field experiments will be addressed in further studies and future publications.

6 Summary of conclusions

Steam explosion proved to be a beneficial pre-treatment to increase biogas production from a highly lignocellulosic energy crop, *Salix viminalis*. Specific methane yields increased up to 50 % for treatments above 210 °C. Different types of lignocellulosic biomass can be pre-treated by steam explosion to optimize their bioenergy yields, without the need of adding chemicals and with minimal sample handling. However, mass losses may occur during such process if not run in a more continuous way, and harsher temperatures may not only imply more energy but also cause decomposition reactions and subproducts that may affect the microbial consortia, and thus the process efficiency.

A mixture of 40 % in VS content from steam exploded *Salix* and the rest from manure was possible to digest semi-continuously at two OLRs. When the lower one was applied (1.5 g VS/Ld), the production from the co-digestion process was similar to the one from the digestion of manure alone, while at the higher OLR (2.6 gVS/Ld), the co-digestion process gave higher methane production than the digestion of manure. Comparing both OLRs, the higher led to lower methane yields for both the co- and mono-digestion processes, possibly due to materials not being fully degraded under a bigger OLR but with the same HRT applied.

When recirculation of digestate was implemented, yields for the co-digestion mixture at 2.6 OLR increased and become closer to the ones obtained at 1.5 OLR. However, accumulation of solids, mostly from recalcitrant nature, occurred more notoriously for these reactors. When the separation of the recirculated digestate's fraction was optimized and an extra sieved added, the accumulation was less pronounced. Solids accumulation needs to be monitored so that it would not affect the methane production.

VFAs and ammonium-N levels were low along the experiments digesting steam exploded *Salix* and manure, but ammonium-N levels reached high values (5 g/L) when fish byproduct was incorporated as an additional co-digestion substrate. In spite of this, methane production was stable and yields were the best from all the combinations, with a 35 % increase with respect of the mixture without fish. A balanced mixture of lignocelluloses, proteins and fats as well as the recirculation of digestate, seemed to have played a major role in maintaining the stability for the process even at high NH_4^+-N levels, possibly enriching its nutrients, micro nutrients and microbial biomass content, and helping in promote adaptation of the bacteria to the high NH_3 content.

According to Angelidaki and Ellegaard (2003), the economic analysis of existing biogas plants affirms that the economic balance can be reached for an average biogas yield of more than 30 m^3 biogas/m^3 biomass (18- 20 m^3 CH_4/m^3 biomass). Average methane yields obtained in **Paper II** (19 m^3 CH_4/m^3 biomass (GA2)) and **Paper III** (27 m^3 CH_4/m^3 biomass (GA2)) reached this yield, emphasizing the importance of digesting different materials together as well as adding easily degradable industrial wastes to the mixtures.

Benefits of performing recirculation of digestate not only accounted for an improved stability and robustness of the process; it also allowed important savings in water and manure resources. Furthermore, it was possible to recover essential fertilizer nutrients as NH_4^+-N and PO_4^{3-} by means of cost-effective techniques, so to improve a final digestate that already had a higher dry matter content.

To conclude, biomass resources rich in lignocelluloses and in proteins, of typical types found in Norway, were possible to apply for biogas production. The importance of performing co-digestion of different available resources, avoiding biomass shortages and treating even polluting materials, was emphasized, which can lead to successful production of both methane and biofertilizer with improved nutrient content.

7 Further research

In light of the present findings, it would be particularly interesting to address the following aspects:

- Steam explosion could be analyzed solely as a process. Lignocellulosic material after being steam exploded could be analytically followed up throughout all the stages of the process (i.e., before pre-treatment, after pre-treatment and after biogas production), in order to have a complete chemical balance. Comparison with material that is not steam exploded would provide a more complete overview of the changes that undergo the molecules, what products are formed, what is being degraded and what remains undegraded.

- Recirculation of digestate could be study and implemented when manure alone is being digested, in order to save water, save costs of pre-heating the feedstock and avoid shortages of manure in winter seasons with a better dosification by digestate substitution.

- Study the effects of applying the final solid digestate fraction (ca. 10- 12 % TS) produced during recirculation, in field experiments for crops and/or plants production, and asses their fertilizer potentials, as well as from the ones incorporating recovered NH_4^+ via struvite or bentonite.

- Presence of SAO bacteria was detected for some reactors in **Paper III**, but further studies will be conducted so to confirm if this play a role in the good adaptation of the bacteria consortia to toxic levels of NH_4^+-N, that gave such a stable process.

8 References

Abbona, F., Boistelle, R., 1979. Growth morphology and crystal habits of struvite crystals (MgNH4PO4.6H2O). Journal of Crystal Growth 46, 339–354.

Ahring, B., 2003. Biomethanation I. Advances in Biochemical Engineering/Biotechnology, Volume 81. Springer.

Ahring, B. K., 1991. Methanogenesis during thermophilic anaerobic digestion with focus on acetate. Dept of Biotechnology DTU DK.

Ahring, B. K., Angelidaki, I., Johansen, K., 1992. Anaerobic treatment of manure together with industrial waste. Water Science and Technology 25: 7, 311–318.

Ahring, B. K., Sandberg, M., Angelidaki, I., 1995. Volatile fatty acids as indicators of process imbalance in anaerobic digestors. Applied Microbiology and Biotechnology 43, 559–565.

Altinbas, M., Öztürk, I., Aydin, A. F., 2002. Ammonia recovery from high strength agro industry effluents. Water Science and Technology 45: 12, 189–196.

Angelidaki, I., 2004. (Editor) Environmental Biotechnology. AD-Biogas Production. Denmark Institute of Environment and Resources DTU.

Angelidaki, I., Ahring, B. K., 1992. Effects of free long-chain fatty acids on thermophilic anaerobic digestion. Applied Microbiology and Biotechnology 37, 808–812.

Angelidaki, I., Ahring, B. K., 1993. Thermophilic anaerobic digestion of livestock waste: the effect of ammonia. Applied Microbiology and Biotechnology 38, 560–564.

Angelidaki, I., Alvez, M., Bolzonella, D., Borzacconi, L., Campos, J., Guwy, A., Kalyuzhnyi, S., Jenicek, P., van Lier, J., 2009. Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. Water Science and Technology 59:5, 927– 934.

Angelidaki, I., Boe, K., Ellegaard, L., 2005. Effect of operating conditions and reactor configuration on efficiency of full-scale biogas plants. Water Science and Technology 52:1-2, 189– 194.

Angelidaki, I., Cui, J., Chen, X., Kaparaju, P., 2006. Operational strategies for thermophilic anaerobic digestion of organic fraction of municipal solid waste in continuously stirred tank reactors. Environmental Technology 27:8, 855–861.

Angelidaki, I., Ellegaard, L., 2003. Codigestion of manure and organic wastes in centralized biogas plants: status and future trends. Applied Biochemistry and Biotechnology 109 (1-3), 95–105.

Avfall Norge, 2010. Utvikling av biogass i Norge, forprosjekt. Rapport nr 3, 2010 (in Norwegian).

Bandosz, T. J., Petit, C., 2009. On the reactive adsorption of ammonia on activated carbon modified by impregnation with inorganic compounds. Journal of Colloid and Interface Science 338, 329–345.

Barker, H. A., 1936. On the biochemistry of the methane fermentation. Archive Microbiology 7, 404–419.

Bauer, A., Bösch, P., Friedl, A., Amon, T., 2009. Analysis of methane potentials of steam -exploded wheat straw and estimation of energy yields of combined ethanol and methane production. Journal of Biotechnology 142, 50–55.

Belach Bioteknik, 2010. Dolly operator's manual. Sweden.

Boe, K., 2006. Online monitoring and control of the biogas process. PhD thesis, Institute of Environment and Resources, Technical University of Denmark, Denmark.

Bouallagui, H., Lahdheb, H., Romdan, E. B., Rachdi, B., Hamdi, M., 2009. Improvement of fruit and vegetable waste anaerobic digestion performance and stability with co-substrates addition. Journal of Environmental Management 90, 1844–1849.

Brownell, H., Saddler, J., 1987. Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. Biotechnology and Bioengineering 29, 228– 235.

Bruni, E., Jensen, A., Angelidaki, I., 2010a. Comparative study of mechanical, hydrothermal, chemical and enzymatic treatments of digested biofibers to improve biogas production. Bioresource Technology 101, 8713– 8717.

Bruni, E., Jensen, A., Angelidaki, I., 2010b. Steam treatment of digested biofibers for increasing biogas production. Bioresource Technology 101, 7668– 7671.

Callaghan, F. J., Wase, D. A. J., Thayanithy, K., Forster, C. F., 1998. An examination of the continuous anaerobic co-digestion of cattle slurry and fish offal. Trans IChemE 76, Part B, 224–228.

Calli, B., Mertoglu, B., Inanc, B., Yenigun, O., 2005. Effects of high free ammonia concentrations on the performances of anaerobic bioreactors. Process Biochemistry 40, 1285– 1292.

CAMBI, 2012.
URL `www.cambi.no`

Castro, F., Hotten, P., Ørskov, E., 1994. Inhibition of rumen microbes by compounds formed in the steam treatment of wheat straw. Bioresource Technology 50, 25– 30.

Cavaleiro, A. J., Pereira, M. A., Alves, M., 2008. Enhancement of methane production from long chain fatty acid based effluents. Bioresource Technology 99, 4086–4095.

Celen, I., Turker, M., 2001. Recovery of ammonia as struvite from anaerobic difgester effluents. Second International Conference on Recovery of Phosphorous from Sewage and Animal Wastes, March, 12–13, Noordwijkerhout, Holland.

Chen, Y., Cheng, J. J., Creamer, K. S., 2008. Inhibition of anaerobic digestion process: A review. Bioresource Technology 99, 4044–4064.

Chua, M. G. S., Wayman, M., 1979. Characterization of autohydrolysis aspen (P. tremuloides) lignins. part 1. Composition and molecular weight distribution of extracted autohydrolysis lignin. Canadian Journal of Chemistry 57:10, 1141–1149.

CIA, 2009. The world factbook. Washington DC: Central Intelligence Agency.

Coates, J. D., Coughlan, M. F., Colleran, E., 1996. Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. Journal of Microbiological Methods 26, 237– 246.

Comino, E., Rosso, M., Riggio, V., 2010. Investigation of increasing organic loading rate in the co-digestion of energy crops and cow manure. Bioresource Technology 101, 3013–3019.

de Bashan, L., Bashan, Y., 2004. Recent advances in removing phosphorous from wastewater and its future use as fertilizer (1997–2003). Water Research 38, 4222–4246.

Deublein, D., Steinhauser, A., 2008. Biogas from Waste and Renewable Resources, An introduction. WILEY-VCH Verlag GmbH & Co. KGaA.

Econ Pöyry, 2008. Facts and figures on the use of bioenergy in the nordic countries. Nordic Energy Research Council Denmark.

EGE, 2012. Waste to Energy Agency, Oslo Kommune.
URL `http://www.energigjenvinningsetaten.oslo.kommune.no/`

Eiroa, M., Costa, J. C., Alves, M. M., Kennes, C., Veiga, M. C., 2012. Evaluation of the biomethane potential of solid fish waste. Waste Management 32, 1347–1352.

EPA, 2012. Environmental protection agency.
URL `http://epa.gov/climatechange/ghgemissions/gases/ch4.html`

Estevez, M., Linjordet, R., Morken, J., 2012. Organic loading rate effect on anaerobic digestion: case study on co-digestion of lignocellulosic pre-treated material with cow manure. Conference proceedings of the International Conference of Agricultural Engineering, Valencia, Spain.

Estevez, M. M., Linjordet, R., Morken, J., 2011. Biogas optimization by steam explosion of salix and recycling of process water from biogas production of salix and manure. In: Conference proceedings of the International IWA-Symposium on Anaerobic Digestion of Solid Waste and Energy Crops, TU Wien, Vienna.

Eturki, S., Ayari, F., Jedidi, N., Dhia, H. B., 2012. Use of clay mineral to reduce ammonium from wastewater. effect of various parameters. Surface Engineering and Applied Electrochemistry 48: 3, 276–283.

European Commission, 2002. EC No 1774/2002 regulation, Method 1, Annex V. URL `http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:273:0001:0001:EN:PDF`

European commission, 2013. URL `http://ec.europa.eu/food/food/biosafety/animalbyproducts/`

Fernandez Polanco, F., Nieto, P., Pérez, S., der Zee, F. V., Fernandez Polanco, M., García, P., 2005. Automated equipment for anaerobic sludge parameters determination. Water Science and Technology 52:1-2, 479– 485.

Forrest, A. L., Fattah, K. P., Mavinic, D. S., Koch, F. A., 2008. Optimizing struvite production for phosphate recovery in WWTP. Journal of Environmental Engineering 134, 395–402.

Freudenberg, K., Neish, A. C., 1968. Constitution and Biosynthesis of Lignin. Springer-Verlag: New York. 129 pp.

Gebauer, R., Eikebrokk, B., 2006. Mesophilic anaerobic treatment of sludge from salmon smolt hatching. Bioresource Technology 97, 2389–2401.

Genome Management Information System, 2006. URL `http://genomicscience.energy.gov.`

Gonzalez Ponce, R., Garcia Lopez de Sa, M. E., 2007. Evaluation of struvite as a fertilizer: a comparison with traditional p sources. Agrochimica 51:6, 301–308.

Gunaseelan, V. N., 1997. Anaerobic digestion of biomass for methane production: a review. Biomass and Bioenergy 13, 83–114.

Gustin, S., Marinsek Logar, R., 2011. Effect of ph, temperature and air flow rate on the continuous ammonia stripping of the anaerobic digestion effluent. Process Safety and Environmental Protection 89, 61–66.

Hansen, T. L., Schmidt, J. E., Angelidaki, I., Marca, E., la Cour Jansen, J., Mosbæk, H., Christensen, T. H., 2004. Method for determination of methane potentials of solid organic wastes. Waste Management 24, 393– 400.

Hartmann, H., Ahring, B. K., 2005. Anaerobic digestion of the organic fraction of municipal solid waste: Influence of co-digestion with manure. Water Research 39, 1543–1552.

Hartmann, H., Angelidaki, I., Ahring, B., 2000. Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration. Water Science and Technology 41:3, 145– 153.

Hjorth, M., Christensen, K. V., Christensen, M. L., Sommer, S. G., 2010. Solid-liquid separation of animal slurry in theory and in practice. a review. Agronomy for Sustainable Development 30, 153–180.

Holm-Nielsen, J., Andree, H., Lindorfer, H., Esbensen, K., 2007. Transflexive embedded near infrared monitoring for key process intermediates in anaerobic digestion/biogas production. Journal Near Infrared Spectroscopy 15, 123– 135.

Horn, S. J., Eijsink, V. G. H., 2010. Enzymatic hydrolysis of steam- exploded hardwood using short processing times. Biosciences, Biothecnology and Biochemistry 74; 6, 1157– 1163.

Horn, S. J., Estevez, M. M., Nielsen, H. K., Linjordet, R., Eijsink, V. G. H., 2011a. Biogas production and saccharification of salix pretreated at different steam explosion conditions. Bioresource Technology 102, 7932–7936.

Horn, S. J., Nguyen, Q. D., Westereng, B., Nilsen, P. J., Eijsink, V. G. H., 2011b. Screening of steam explosion conditions for glucose production from non-impregnated wheat straw. Biomass and Bioenergy 35:12, 4879–4886.

IPCC, 2007. Synthesis report. summary for policymakers. technical report. intergovernmental panel on climate change.

Jacobi, H. F., Moschner, C. R., Hartung, E., 2009. Use of near infrared spectroscopy in monitoring of volatile fatty acids in anaerobic digestion. Water Science and Technology 60:2, 339–346.

Jagadabhi, P., Lehtomäki, A., Rintala, J., 2008. Codigestion of grass silage and cow manure in a cstr by re-circulation of alkali treated solids of the digestate. Environmental Technology 29, 1085– 1093.

Jarvis, A., Nordberg, A., Mathisen, B., Svensson, B. H., 1995. Stimulation of conversion rates and bacterial activity in a silage-fed two-phase biogas process by initiating liquid recirculation. Antonie van Leeuwenhoek 68:4, 317–327.

Johansson, K., Liljequist, K., Ohlander, L., Aleklett, K., 2010. Agriculture as provider of both food and fuel. Ambio 39, 91–99.

Johnston, A. E., Richards, I. R., 2003. Effectiveness of different precipitated phosphates as phosphorous sources for plants. Soil Use Management 19, 45–49.

Jørgensen, H., Kristensen, J. B., Felby, C., 2007. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioproducts and Biorefining 1, 119–134.

Koster, I. W., Lettinga, G., 1984. The influence of ammonium-nitrogen on the specific activity of pelletized methanogenic sludge. Agricultural Wastes 9, 205–216.

Kroeker, E. J., Schulte, D. D., Sparling, A. B., Lapp, H. M., 1979. Anaerobic treatment process stability. Journal of Water Pollution Control Federation 51:4, 718–727.

Kumar, P., Barrett, D. M., Delwiche, M. J., Stroeve, P., 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolisis and biofuel production. Industrial and Engineering Chemistry Research 48, 3713–3729.

Kuzovkina, Y. A., Quigley, M. F., 2005. Willows beyond wetlands: uses of Salix L. species for environmental projects. Water, Air, and Soil Pollution 162, 183–204.

Labrecque, M., Teodorescu, T. I., Daigle, S., 1997. Biomass productivity and wood energy of salix species after 2 years growth in sric fertilized with wastewater sludge. Biomass and Bioenergy 12: 6, 409–417.

Landbruks og matdepartementet, 1994. Forskrift av 13.juli 1994 nr.723. (in Norwegian).
URL `http://faolex.fao.org/docs/html/nor14229.htm`

Landbruks og matdepartementet, 2003. Forskrift om gjødselvarer mv. av organisk opphav. (in Norwegian). FOR 2003-07-04 nr 951.
URL `http://www.lovdata.no/for/sf/ld/xd-20030704-0951.html`

Landbruks og matdepartementet, 2009. Klimautfordringene, landbruket en del av løsningen. Oslo, Norway. (in Norwegian) 39, 174.

Lastella, G., Testa, C., Cornacchia, G., Notornicola, M., Voltasio, F., Sharma, V. K., 2002. Anaerobic digestion of semi-solid organic waste: biogas production and its purification. Energy Conversion and Management 43, 63–75.

Lehtomäki, A., Huttunen, S., Rintala, J. A., 2007. Laboratory investigations on co-digestion of energy crops and crop residues with cow manure for methane production: Effect of crop to manure ratio. Resources, Conservation & Recycling 51, 591–609.

Lei, X., Sugiura, N., Feng, C., Maekawa, T., 2007. Pretreatment of anaerobic digestion effluent with ammonia stripping and biogas purification. Journal of Hazardous Materials 145, 391–397.

Li, X. Z., Zhao, Q. L., 2003. Recovery of ammonium -nitrogen from landfill leachate as a multi-nutrient fertilizer. Ecological Engineering 20, 171–181.

Lin, Y., Tanaka, S., 2006. Ethanol fermentation from biomass resources: current state and prospects. Applied Microbiology and Biotechnology 69, 627–642.

Lindmark, J., Thorin, E., Kastensson, J., Pettersson, C. M., 2011. Membrane filtration of process water at elevated temperatures- a way to increase the capacity of a biogas plant. Desalination 267, 160–169.

Lukehurst, C. T., Frost, P., Seadi, T. A., 2010. Utilisation of digestate from biogas plants as biofertiliser. IEA Bioenergy Task 37.

Massey, M. S., Davis, J. G., Ippolito, J. A., Sheffields, R. E., 2009. Effectiveness of recovered magnesium phosphates as fertilizers in neutral and slightly alkaline soils. Agronomy Journal 101: 2, 323–329.

Massé, D. I., Talbot, G., Gilbert, Y., 2011. On farm biogas production: A method to reduce ghg emissions and develop more sustainable livestock operations. Animal Feed Science and Technology 166–167, 436–445.

Mattilsynet, 2008. Veileder: utstedelse av eksportdokumenter for fiskebiprodukter. (in Norwegian).

Mattilsynet, 2012. Official list of approval plants handling animal byproducts. URL `http://www.mattilsynet.no/`

McCarty, P. L., 1964. Anaerobic waste treatment fundamentals. part 3: toxic materials and their control. Public Works, 91–94.

Mes-Hartree, M., Saddler, J., 1983. The nature of inhibitory materials present in pretreated lignocelllulosic substrates which inhibit the enzymatic hydrolysis of cellulose. Biotechnology Letters 5:8, 531– 536.

Montalvo, S., Guerrero, L., Borja, R., Sanchez, E., Milan, Z., Cortes, I., de la Rubia, M. A., 2012. Application of natural zeolites in anaerobic digestion process: A review. Applied Clay Science 58, 125–133.

Mook, W. T., Chakrabarti, M. H., Aroua, M. K., Khan, G. M. A., Ali, B. S., Islam, M. S., Hassan, M. A. A., 2012. Removal of total ammonia nitrogen (TAN), nitrate and total organic carbon (TOC) from aquaculture wastewater using electrochemical technology: A review. Desalination 285, 1–13.

Morken, J., 2007. Biogas in Norwegian agriculture. potential, technology, and environment. Presentation, Norwegian University of Life Sciences.

Müller, W. R., Frommert, I., Jörg, R., 2004. Standarized methods for anaerobic biodegradability testing. Reviews in Environmental Science and Biotechnology 3, 141– 158.

Nordberg, A., Jarvis, A., Stenberg, B., Mathisen, B., Svensson, B. H., 2007. Anaerobic digestion of alfalfa silage with recirculation of process liquid. Bioresource Technology 98, 104–111.

Norwegian Scientific Committee for Food Safety, 2010. Assessment of the fish silage processing method (FSPM) for treatment of category 2 and 3 material of fish origin. URL `http://www.english.vkm.no/dav/ce2ea4b679.pdf`

Olje og energidepartementet, 2008. Strategi for økt utbygging av bioenergi. (in Norwegian).
URL `http://www.regjeringen.no/upload/OED/Bioenergistrategien2008w.pdf`

Ottoson, J. R., Schnürer, A., Vinnerås, B., 2008. In situ ammonia production as a sanitation agent during anaerobic digestion at mesophilic temperature. Letters in Applied Microbiology 46, 325–330.

Palmqvist, E., Hahn-Hägerdal, B., Galbe, M., Zacchi, G., 1996. The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolisis and ethanol fermentation. Enzyme and Microbial Technology 19, 470–476.

Pereira, M. A., Sousa, D. Z., Mota, M., Alves, M. M., 2004. Mineralization of lcfa associated with anaerobic sludge: kinetics, enhancement of methanogenic activity, and effect of vfa. Biotechnology and Bioengineering 88, 502–511.

Porter, M. G., Grass, M. S. M., 2001. The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. Grass and Forage Science 56, 405– 411.

Ramos, L., 2003. The chemistry involved in the steam treatment of lignocellulosic materials. Quimica Nova 26, 863– 871.

Reeves, T. G., 1972. Nitrogen removal: a literature review. Journal (Water Pollution Control Federation) 44:10, 1895–1908.

Rinzema, A., Boone, M., van Knippenberg, K., Lettinga, G., 1994. Bactericidal effect of long chain fatty acids in anaerobic digestion. Water Environmental Research 66, 40–49.

Rubin, 2010. Bioenergi fra biprodukt av laks. (in Norwegian).

Rubin, 2012. Foundation for the recycling and utilization of organic byproducts in Norway.
URL `www.rubin.no`

Saltali, K., Sari, A., Aydin, M., 2007. Removal of ammonium ion form aqueous solution by natural turkish (yildizeli) zeolite for environmental quality. Journal of Hazardous Materials 141, 258–263.

Samuelsson, R., Nilsson, C., Burvall, J., 2006. Sampling and gc-ms as a method for analysis of volatile organic compounds (voc) emittted during oven drying of biomass materials. Biomass and Bioenergy 30, 923– 928.

Sassner, P., Galbe, M., Zacchi, G., 2005. Steam pretreatment of salix with and without so2 impregnation for production of bioethanol. Applied Biochemistry and Biotechnology 121-124, 1101– 1117.

Sassner, P., Måartensson, C. G., Galbe, M., Zacchi, G., 2008. Steam pretreatment of h2so4-impregnated salix for the production of bioethanol. Bioresource Technology 99, 137–145.

Sawyer, C. N., McCarty, P. L., Parkin, G. F., 2003. Chemistry for environmental engineering and science, fifth edition. McGraw-Hill.

Scaglione, D., Caffaz, S., Ficara, E., Malpei, F., Lubello, C., 2008. A simple method to evaluate the short -term biogas yield in anaerobic codigestion of was and organic wastes. Water Science and Technology 58:8, 1615–1622.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and Molecular Biology Reviews 61: 2, 262–280.

Schnürer, A., Houwen, F. P., Svensson, B. H., 1994. Mesophilic syntrophic acetate oxidation during methane formation by a triculture at high ammonium concentration. Archives of Microbiology 162, 70–74.

Schnürer, A., Jarvis, Å., 2010. Microbiological handbook for biogas plants. Swedish Gas Centre report 207 Rev dec 2010.

Schnürer, A., Nordberg, A., 2008. Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. Water Science and Technology 57:5, 735–740.

Schnürer, A., Zellner, G., Svensson, B. H., 1999. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiology Ecology 29, 249–261.

Seppälä, M., Paavola, T., Lehtomäki, A., Pakarinen, O., Rintala, J., 2008. Biogas from energy crops-optimal pre-treatments and storage, co-digestion and energy balance in boreal conditions. Water Science and Technology 58.9, 1857– 1863.

Seredych, M., Tamashausky, A. V., Bandosz, T. J., 2008. Surface features of exfoliated graphite/bentonite composites and their importance for ammonia adsorption. Carbon 46, 1241–1252.

Shelton, D. R., Tiedje, J. M., 1984. General method for determining anaerobic biodegradation potential. Applied and Environmental Microbiology 47: 4, 850–857.

Siegrist, H., Hunziker, W., Hofer, H., 2005. Anaerobic digestion of slaughterhouse waste with uf-membrane separation and recycling of permeate after free ammonia stripping. Water Science and Technology 52: 1-2, 531–536.

Sims, R. E. H., 2002. The brilliance of bioenergy; in business and in practice. James & James (Science Publishers) Ltd.

Sousa, D. Z., Pereira, M. A., Alves, J. I., Smidt, H., Stams, A. J. M., 2008. Anaerobic microbial LCFA degradation in bioreactors. Water Science and Technology 57:3, 439–444.

Sprott, G. D., Patel, G. B., 1986. Ammonia toxicity in pure cultures of methanogenic bacteria. Systematic and Applied Microbiology 7, 358–363.

Sprott, G. D., Shaw, K. M., Jarell, K. F., 1985. Methanogenesis and the k+ transport system are activated by divalent cations in ammonia-treated cells of methanospirillum hungatei. Journal of Biological Chemistry 260, 9244–9250.

Statistics Norway, 2008. Natural resources and the environment 2008.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., de Haan, C., 2006. FAO: Livestocks long shadow environmental issues and options. FAO, Rome, Italy, 416.

Stringer, D. A., 1988. Evaluation of anaerobic biodegradation. Tech. Rep. 28, ECETOC.

Taherzadeh, M. J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. International Journal of Molecular Sciences 9, 1621–1651.

van Lier, J., Tilche, A., Ahring, B., Macarie, H., Moletta, R., Dohanyos, M., Pol, L. H., Lens, P., Verstraete, W., 2001. New perspectives in anaerobic digestion. Water Science and Technology 43: 1, 1– 18.

Vassileva, P., Tzvetkova, P., Nickolov, R., 2008. Removal of ammonium ions from aqueous solutions with coal-based activated carbons modified by oxidation. Fuel 88, 387–390.

Ward, A. J., Hobbs, P. J., Holliman, P. J., Jones, D. L., 2008. Optimisation of the anaerobic digestion of agricultural resources. Biore 99, 7928–7940.

Weiland, P., 2000. Anaerobic waste digestion in Germany ; status and recent developments. Biodegradation 11, 415– 421.

Weissbach, F., Kuhla, S., 1995. Material losses of dry matter of silages and forage during ensiling: accruing errors and options of correction (in German). Übersicht Tierernährung 23, 189– 214.

Yadvika, Santosh, Sreekrishnan, T., Kohli, S., Rana, V., 2004. Enhancement of biogas production from solid substrates using different techniques: a review. Bioresource Technology 95, 1–10.

Yan, L., Xu, Y., Yu, H., Xin, X., Wei, Q., Du, B., 2010. Adsorption of phosphate from aqueous solution by hydroxy-aluminum, hydroxy-iron and hydroxy-iron-aluminum pillared bentonites. Journal of Hazardous Materials 179, 244–250.

Yen, H., Brune, D., 2007. Anaerobic co-digestion of algal sludge and waste paper to produce methane. Bioresource Technology 98, 130– 134.

Yetilmezsoy, K., Sapci-Zengin, Z., 2009. Recovery of ammonium nitrogen from the effluent of uasb treating poultry manure wastewater by map precipitation as a slow release fertilizer. Journal of Hazardous Materials 166, 260–269.

Zeeman, G., Wiegant, W. M., Koster-Treffers, M. E., Lettinga, G., 1985. The influence of the total ammonia concentration on the thermophilic digestion of cow manure. Agricultural Wastes 14, 19–35.

Zeng, L., Mangan, C., Li, X., 2006. Ammonia recovery form anaerobically digested cattle manure by steam stripping. Water Science and Technology 54: 8, 137–145.

Zhang, B., He, P., Lü, F., Shao, L., Wang, P., 2007. Extracellular enzyme activities during regulated hydrolysis of high-solid organic wastes. Water Research 41, 4468–4478.

Zinder, S. H., Koch, M., 1984. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Archive Microbiology 138, 263–272.

9 Papers

Paper I

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Effects of steam explosion and co-digestion in the methane production from *Salix* by mesophilic batch assays

Maria M. Estevez [a,*], Roar Linjordet [b], John Morken [a]

[a] Department of Mathematical Sciences and Technology, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway
[b] Bioforsk, Norwegian Institute for Agricultural and Environmental Research, Frederik A. Dahls vei 20, 1432 Ås, Norway

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ABSTRACT

Salix that was steam exploded at different conditions of temperature and time was anaerobically digested in a series of batch tests. Steam explosion proved to be favorable to increase the methane yields up to 50%, with best results obtained for temperatures starting at 210 °C. Batch studies for mixtures of cow manure and steam exploded *Salix* were performed, with C/N ratios varying from 31 to 56, related to volatile solids (VS) contents from 20 up to 80% of each of the substrates. Methane yields reached 230 mL CH_4/g VS for the mixtures containing 30% and 40% VS of *Salix* over the total mixture's VS content (35 and 39 C/N ratio, respectively). A fraction up to 40% in VS from pre-treated *Salix* provided good methane yields with a faster digestion process.

1. Introduction

Interest in the use of biogas as alternative energy source has recently increased in Norway, being regarded as a way to mitigate nutrient loss and emission of greenhouse gases from the agricultural sector. Most organic matter can be anaerobically digested and transformed into biogas. In the agricultural sector, plant biomass, energy crops and crop residues can be co-digested with animal manure, the largest organic feedstock from agriculture, in order to balance nutrients levels and achieve better production yields (Angelidaki and Ellegaard, 2003; Lehtomäki et al., 2007; Deublein and Steinhauser, 2008).

Lignin rich materials possess recalcitrant structures in their fibers, and in order to enhance the digestion of these materials to biogas, a pre-treatment step is needed. Examples of pre-treatments are addition of chemical reagents, particle size reduction, enzymatic treatment or steam treatment. Opening up of these structures would enable their biodegradability, making them readily available for the anaerobic bacteria to work on. Steam explosion is one of the most effective techniques used for this purpose (Brownell and Saddler, 1987; Ramos, 2003; Bruni et al., 2010a,b). It involves a high temperature heating combined with a rapid pressure drop that physically disrupts the lignocellulosic structures in the biomass fibers. While some publications address steam explosion pre-treatment of lignin rich materials as method to optimize enzymatic hydrolysis and bioethanol production (Mes-Hartree and Saddler, 1983; Brownell and Saddler, 1987; Sassner et al., 2005; Horn and Eijsink, 2010), there are currently few addressing steam explosion as a pre-treatment for biogas production, particularly concerning hardwood.

Co-digestion of different types of substrates for production of biogas gives better performance than digesting the materials alone (Weiland, 2000; van Lier et al., 2001; Angelidaki and Ellegaard, 2003; Yadvika et al., 2004), and it is a practice well known in some European countries. Danish centralized biogas plants co-digest manure mainly with organic waste from food processing industries, in a ratio 75 to 25. Some plants incorporate sewage sludge or household waste as co-digestion substrates (Angelidaki and Ellegaard, 2003). In Germany, energy crops and agricultural livestock breeding wastes are normally co-digested with manure (Weiland, 2003).

The substrate's carbon to nitrogen ratio (C/N) is a crucial factor to consider when performing a co-digestion. Substrates with too low C/N ratio would increase ammonia production and thus lead to inhibition of methane production, while a too high ratio would mean insufficient nitrogen for the maintenance of microbial biomass. Different values have been found in literature for this coefficient, ranging between 16/1 and 25/1 (Deublein and Steinhauser, 2008) or 20/1 to 30/1 (Yadvika et al., 2004; Yen and Brune, 2007). Most of the biomass provided by the farming sector, e.g. animal slurries, possess very low C/N ratio, but this is not the case for wastes rich in lipids or carbohydrates. Therefore, a balanced composition of the substrate is necessary and it can be achieved by mixing it with other feedstock

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[*] Corresponding author. Tel.: +47 64 96 54 93; fax: +47 64 96 54 01.
E-mail address: maria.estevez@umb.no (M.M. Estevez).

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in a co-digestion process (Deublein and Steinhauser, 2008; Seppälä et al., 2008). The C/N ratios corresponding to the substrates employed in this study are of ca. 20 for the cattle manure and ca. 60 for the pre-treated *Salix*.

Cattle liquid manure is characterized for its low total solids (TS) content, many nutrients and high buffering capacity which makes it an excellent carrier co-substrate in anaerobic digestion (Angelidaki and Ellegaard, 2003; Weiland, 2003; Alvarez and Lidén, 2008). Its high fraction of raw fibers due to the feeding allows only 30% of its organic content to be decomposed, while in pig liquid manure and in chicken liquid manure the values are 50% and 65%, respectively (Deublein and Steinhauser, 2008). However, the more degradable the biomass, the higher the ammonia content in the liquid manure, e.g. the ammonia content in poultry manure is around 85% of its total nitrogen content (Deublein and Steinhauser, 2008). At existing biogas plants, feasibility studies have shown that an economically feasible balance can be reached for biogas yields higher than 20 m^3 CH_4/m^3 biomass (Angelidaki and Ellegaard, 2003). The addition of easily degradable substrates and industrial waste in co-digestion with manure makes it possible for the biogas potential to be achieved.

The aim of this study was first to investigate the best steam explosion conditions as a pre-treatment of *Salix* with regard to its methane production in a series of batch experiments, and secondly the methane yields derived from the co-digestion of steam exploded *Salix* and cow manure. Different conditions for the steam explosion pre-treatment of *Salix* were analyzed with respect of their methane production. *Salix* treated at the conditions that gave the highest methane yields was chosen to be co-digested with manure in different C/N ratio mixtures. Methane yields of each mixture were determined and compared to their potential expected yields, and the relation between C/N ratio in the mixtures and their methane yield analyzed.

2. Methods

2.1. Steam pre-treated Salix

Steam explosion studies on *Salix viminalis* "Christina" (Fig. 1), an energy crop from the willow family (common Osier, basket willow), are currently performed at the Department of Chemistry, Biotechnology and Food Science (IKBM) of the Norwegian University of Life Sciences (UMB). The *Salix* was harvested in November 2009, in a short rotation coppice plantation near Grimstad, Norway. It was provided chopped with a particle size of ca. 2 cm. Some characteristics of the untreated and the steam exploded *Salix* samples are presented in Table 1. The pretreated material was provided by IKBM, UMB and was the result of a steam explosion pretreatment run at different sets of temperature and time conditions as detailed in Table 2. For the co-digestion trials and in order to have fresh pre-treated material, a new batch of *Salix* was steam exploded at 210 °C and 10 min., and its parameters are presented in Table 3. The pretreated samples were handled in 5 L plastic containers and stored at 4 °C for a day before added to the trials.

Fig. 1. *Salix viminalis* chopped and steam exploded.

2.2. Cow manure

Fresh cow manure was employed as co-digestion substrate in the co-digestion experiment and was obtained from the University farm, in a 20 kg container, kept refrigerated at 4 °C. Before being added to the trials it was strongly mixed, and samples were removed for further analyses.

2.3. Inoculum

Sewage sludge obtained from the anaerobic digester of Nordre Follo Treatment Plant (Vinterbro, Norway) was employed as inoculum, with a volatile solids (VS) content of 1.5% (w/w). The inoculum was anaerobically pre-incubated at 37 °C for 6–9 days until endogenous biogas production decreased.

2.4. Batch experiments

2.4.1. Optimal pretreatment conditions for biogas production from Salix

A series of batch scale assays to test the methane production from steam exploded *Salix* were conducted at the laboratory. A total of 47 serum bottles acting as biogas reactors, with a total capacity of 1.125 mL, were filled on approximately 30% of their liquid phase working volume (700 mL) with diluted sewage sludge inoculum. The biogas production on a period of 57 days was measured in four different schemes: (1) diluted inoculum without any substrate, acting as blank (3 g VS), (2) diluted inoculum with addition of approx. 1 g microcrystalline cellulose ($(C_6H_{10}O_5)_n$, <20 μm MERCK) acting as inoculum reference, (3) diluted inoculum with addition of approx. 8 g of untreated *Salix* (ca. 4 g VS) as control samples, and (4) diluted inoculum with incorporation of approx. 8 g of pretreated *Salix* material (1–2 g VS). An approximate 2/3 VS ratio between the substrate and inoculum was maintained, varying quantities of sewage sludge and water added in the trials, adding for a total volume of ca. 700 mL. The temperature and time conditions of the steam explosion pre-treatment are stated in Table 2. The untreated *Salix* did not undergo any steam explosion, but was milled from its initial size to tiny particles of less than 7 mm long. The bottles were closed with butyl rubber stoppers and aluminum crimps. Each series was carried out in triplicate, except for sample 13 which was done in duplicate, all under mesophilic temperature conditions on an incubation room maintained at 37 °C ± 1 °C. The serum bottles were placed on a horizontal shaker that allowed a continuous stirring of 90 rpm.

2.4.2. Study of the optimal mixture ratio for biogas production from pre-treated Salix and manure

The co-digestion trials consisted on 38 serum bottles of 1.125 mL capacity, inoculated with approximately 400 mL of old sewage sludge inoculum (corresponding to app. 2.75 g VS) derived from the previous experiment, which was well mixed before its addition to the bottles. A series containing 2 g of microcrystalline cellulose (same as used in previous experiment) was used as reference. Manure and pre-treated *Salix* were added in different ratios to the vials, the substrates mixture ranged from 20% up to 80% of *Salix* VS content based on the total VS content of the feedstock, which was kept on 2 g. Additionally, two sets were made using already digested manure instead of fresh manure to analyse residual methane potential, a set only running on fresh manure as substrate

Table 1
Biochemical composition of untreated and steam exploded *Salix* samples.

Parameter	Untreated *Salix* (% dw/w)	Steam exploded *Salix*[a] (% dw/w)
Pentose sugars		
Arabinose	0.9	0.2
Xylose	11.9	6.0
Hexose sugars		
Manose	1.4	1.2
Galactose	0.9	0.7
Glucose	36.2	45.8
Lignin		
Acid-soluble	2.9	3.2
Non-acid soluble (Klason)	25.3	31.9
Total carbohydrate and lignin content	79.5	89.0

[a] *Salix* steam exploded at 210 °C and 15 min of residence time.

Table 2
Steam explosion temperature and time conditions, pH, total solids, volatile solids and ash content for each serie.

Sample	Temperature (°C)	Time (min)	pH	Total solids (% w/w)	Volatile solids (% dw/w)	Ash (% dw/w)
1	170	10	4.4	24.85	99.03	0.97
2	180	10	4.3	24.38	98.90	1.10
3	190	10	4.1	18.87	98.62	1.38
4	200	10	3.9	17.28	98.84	1.16
5	210	5	3.9	23.43	98.60	1.40
6	210	10	3.7	20.16	98.66	1.34
7	210	15	3.6	13.22	98.49	1.51
8	220	5	3.7	20.35	98.53	1.47
9	220	10	3.6	16.77	98.51	1.49
10	220	15	3.5	16.01	98.31	1.69
11	230	5	3.5	17.11	98.42	1.58
12	230	10	3.5	17.32	98.04	1.96
13	230	15	3.4	15.54	98.13	1.87
Untreated[a]			7.0	59.09	98.43	1.57

[a] Untreated *Salix* is *Salix* milled that did not undergo any steam explosion pre-treatment.

Table 3
Characteristics of the materials employed in the *Salix* methane potential and co-digestion experiments.

	pH	TS (% w/w)	VS (% w/w)	Total C (% w/w)	Total N (% w/w)	COD (mg g^{-1} TS)	(mg g^{-1} TS)
1st Inoculum	7.4	2.50	1.50	0.25	0.09	146	21.56
Salix treated (210 °C – 10 min)	3.8	24.00	23.90	12.20	0.19	1494	n.d.
Fresh manure	6.8	9.80	8.45	4.49	0.20	678	17.46
Digested manure	8.0	4.02	2.81	n.a.	0.36	1021	52.76
2nd Inoculum	7.5	1.10	0.68	n.a.	0.16	2661	35.89

n.a.: Not available; n.d.: not detectable.

and one only with pre-treated *Salix*. Blank samples were prepared to subtract the inoculum contribution to the biogas production. The characteristics of the materials are stated in Table 3. Each set of the series was performed in triplicate except for the one running only on *Salix* which was duplicated, and all were incubated at 37 °C ± 1 °C with continuous agitation of 90 rpm by a horizontal shaker, until a negligible biogas production was reached (total period of 77 days).

2.5. Analytical methods

2.5.1. Manometric measurement procedure to follow biogas production

The measuring procedure for the batch biogas assays was done according to Hansen et al. (2004), and based on the European Chemical Industry Ecology and Toxicology Center (ECETOC) guideline (Stringer, 1988) and ISO 11734 standards. The ECETOC method relies on constant volume manometry, where a change in the pressure of a gas is measured while keeping its volume constrained at a constant temperature.

By using the ideal gas law, the number of moles of gas produced in the headspace of each batch test vessel is calculated and with it, the volume of biogas produced in the headspace is then determined as:

$$V_{\text{biogas}} = \frac{n \times R \times 273}{P_o} = \left(\frac{dP \times V}{R \times T}\right) \times \left(\frac{R \times 273}{P_o}\right) = \frac{dP \times V \times 273}{T \times P_o} \qquad (1)$$

where V_{biogas}: volume of biogas (L) at standard conditions of 273 K (0 °C) and 1 atm; P_o: 1 atm; V the headspace volume (L); T the incubation temperature (K), R the ideal gas constant (0.08205 atm L/mol K); and dP the pressure difference between initial and final readings (atm).

The term $k = 273/(T \times P_o)$ is then the coefficient for obtaining the pressure at standard conditions (273 K and 1 atm), and so the equation can be finally be written as:

$$V_{\text{biogas}} = dP \times V \times k \tag{2}$$

Biogas composition was determined by gas chromatography. Once the methane level in the biogas is obtained, the accumulated volume of methane and subsequently the specific methane yield of the substrates can be deduced.

2.5.2. Analytical tools

The analyses of carbohydrate and lignin content in *Salix* samples before and after steam explosion were performed by Innventia AB (Stockholm, Sweden). Ion-chromatography was applied for the sugar composition determinations, followed by a two-step acid hydrolysis procedure (Tappi standard T249). For the insoluble (Klason) and soluble lignin determinations, Tappi standard T222 and UM250 procedures were applied, respectively.

For biogas volume determination, the headspace pressure of each reactor was determined using a digital pressure transducer (GMH 3161 Greisinger Electronic, Germany) with an incorporated needle that was injected into the septum cap. This measurement was performed three times per week during the first 20 days, and one time in the consecutive weeks. The headspace pressure was released at each pressure reading by applying a needle into the septum, being this always taken into account when calculating the volume of biogas produced. In the first experiment biogas composition was analyzed by a Perkin Elmer-auto system gas chromatograph equipped with a thermal conductivity detector (TCD) and an Alltech CTR 1 stainless steel column (outer column packed with activated molecular sieve, 6′ × 1/4″ s.s., inner column packed with porous polymer mixture, 6′ × 1/8″). The operational temperatures of injector, detector and column were kept at 80, 90 and 50 °C, respectively. Helium was used as a carrier gas at a flow rate of 40 mL/min. Methane yields derived from each of the vials were obtained from combining biogas production volumes and their methane compositions after being divided over their respective substrate's initial VS concentrations. For the co-digestion experiment, gas composition was analyzed in an Agilent micro gas chromatograph (Agilent Technologies 3000A), equipped with a TCD detector and a Poraplot Q column (8 m × 0.32 mm). The operational temperatures of sample inlet, injector and column were kept at 60, 50 and 45 °C, respectively. Sampling time was set to 15 s, equilibration time 10 s, injection time 8 ms and post-run time 10 s. Methane yields were calculated as for the previous experiment. The pH values of the inoculum, pretreated *Salix* material and tap water were measured by a pH meter (WTW Multi ®350 i) equipped with WTW pH Electrode (Sen Tix 41) according to Allen (1989). TS and VS content was analyzed according to Standard methods (APHA, 1995). The chemical oxygen demand (COD), ammonium content and total nitrogen content for the inoculum and feedstock were analyzed by HACH-LANGE spectrophotometric methods and MERCK Spectroquant®, and these analyses were also applied to the final digestates of the second experiment. In each case the samples had been kept frozen at −20 °C and thawed to 4 °C 1 day before the analyses were conducted.

Total carbon and total nitrogen were investigated for the fresh manure and the pre-treated *Salix* in order to find the C/N ratios, by applying elemental analysis. For the total carbon, the "dry combustion" method proposed by Allison and described in Nelson and Sommers (1982) was employed, using a LECO CHN 1000 analyzer equipped with IR cells that measure the CO_2 gas produced from the oxidation of the total carbon in the sample. The total nitrogen analysis was performed according to the Dumas method, described in Bremmer and Mulvaney (1982).With the same principle as for total carbon, nitrogen oxide compounds (NO_x) are reduced by means of copper to N_2 and the concentration of this last one is measured by thermal conductivity (TC cells) in the same analyzer.

2.5.3. Statistical analysis

All standard deviations reported in this study were calculated using the statistical functions of Microsoft Excel 2007. Evaluation of the optimum conditions is significant for the study, thus to compare methane yields, a two-sample *t*-test was used. The statistical software Minitab® 15.1.1 was employed to evaluate the relationship between paired experimental data. An alpha (α) level of 0.05 was used to determine the statistical significance of all analyses. The results were assessed with *p*-values to reflect the statistical significance between paired groups. In addition, one-way ANOVA test, based on Fischer method, was employed for statistical grouping in some relevant cases.

3. Results and discussion

3.1. Effect of different steam explosion pre-treatments on the biogas production from Salix

The steam explosion pre-treatment of biomass entail mass losses. Some losses can be due to residues adhered to the pipeline walls, splashing and wash out during cleaning of the instrument. The overall loss fraction can be reduced by running multiple batches that avoid a system unit clean up between them, or by running a bigger batch.

Considering mean values from the steam explosion pre-treatment of batches of *Salix* at 210 °C and 10 min, a batch of ca. 750 g. of chopped material (59% TS, 98.4% VS, 1.57% ash) produced 1400 g. of steam exploded material (17.75% TS, 98% VS, 1.97% ash). Dry matter content decreased because some volatile organic materials were formed due to hemicelluloses degradation, and because of the residues lost inside the unit. Since volatile organics such as acetate and furfural are volatilized and lost due to the high temperatures, this translates in accumulation of ash (Ramos, 2003; Horn et al., 2011b). As observed in Table 2, for the conditions tested in the first experiment this ash accumulation seems to increase along with the temperature applied in the pre-treatment, the harsher pre-treatments producing materials with higher ash content with respect to the initial one, and it also seems to be influenced by the retention times when same temperature is applied. With this data we can estimate that the volatile organic materials losses during high temperature steam pre-treatments (210–230 °C) are approximately 20%. Similar results have been deduced for the steam pre-treatment of wheat straw between 210 and 220 °C (Horn et al., 2011b).

In our study, the VS contents for all the samples before and after the pre-treatments are known and are the VS amounts which are in fact added to the vials. If the amounts lost during steam explosion are taken into account, higher methane productions than the ones we obtained would be reached since higher VS values are considered, balancing each other in the yield calculation. Thus, the effect of the organic losses during steam explosion when always the same substrate is pre-treated and compared in a study is not an issue. On the other hand, losses when drying the pre-treated samples (for TS and VS determination) would lead to overestimate the yield. These losses were not calculated in this study, but according to literature that refers to ensiled biomass, they can be estimated to be of ca. 2–5% VS (Weissbach and Kuhla, 1995; Porter and Murray, 2001; Samuelsson et al., 2006).

As it can be observed in Fig. 2, steam explosion as pre-treatment for *Salix* proved to have a positive effect towards biogas production. With the only exception of the pre-treatment at 170 °C and 10 min, the biogas yields obtained for the different pre-treatment conditions were superior to that of untreated *Salix*. On treatments above 200 °C the difference became more notorious. One-way ANOVA explains statistically that untreated *Salix* gave similar results for the

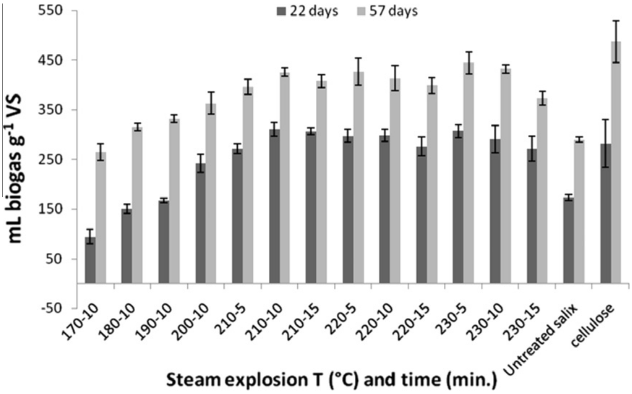

Fig. 2. Accumulated biogas production with standard deviations after 22 and 57 days of digestion for the different steam explosion conditions of temperature and time applied to *Salix* samples and for untreated *Salix*.

lower temperature treatments (170 °C and 180 °C), but kept a significant statistical difference with the rest of them. This suggests that due to steam explosion, the substrate was more available for the microorganisms to degrade. Hemicelluloses solubilization (Table 1) debilitates the lignocellulosic structure of the substrate and facilities thus, its microbiological degradation (Ramos, 2003; Bruni et al., 2010b). Acetate, a substrate for methane production, is also generated due to hemicelluloses degradation in the steam explosion pre-treatment (Horn et al., 2011a).

For the first 20 days, biogas production seemed to increase up to treatments at 210 °C. However, for the total digestion period, the production gave slightly higher yields for the treatment at 230 °C and 5 min. This can be due to the fact that harsher steam explosion conditions might be source of higher yields of substances such as furfural and phenolics (Ramos, 2003; Sassner et al., 2005; Horn and Eijsink, 2010), which may inhibit the microbial activity (Mes-Hartree and Saddler, 1983; Brownell and Saddler, 1987; Castro et al., 1994; Bruni et al., 2010b). Thus, after an adaptation period of 57 days the yields obtained for the harsh conditions are similar to the others.

Methane content in the biogas throughout the experiment varied from 45% to 56%. It can be observed in Table 4 that the pre-treatment increased the specific methane yield up to 50%, when comparing the steam exploded series with the untreated one.

By day 22 the 210 °C – 10 min treatment was presenting the bigger methane production, and by day 57 this value is still high only preceded by the 230 °C – 5 min and the 230 °C – 10 min series (Table 4, Fig. 3). The statistical evaluation of the methane yield data for the 210 °C – 10 min and 230 °C – 5 min series showed that they were not significantly different, neither after 22 days nor after 57 days period. For the first period, comparison between the two series revealed a p value of 0.681, while for the data of the 57 days period, the p value was 0.382. Thus, regarding the bigger impact that inhibitory substances may have in harsher treatments as well as their requirement for more process energy to reach such temperatures, a pre-treatment of steam explosion at 210 °C for 10 min was chosen as the option to apply for the material of the co-digestion trials.

Table 4
Specific methane yields of untreated and steam exploded *Salix* samples, after 22 and 57 days of digestion.

Treatment		Methane yield (mL CH_4 g^{-1} VS)	
Temperature (°C)	Time (min)	After 22 days	After 57 days
200	10	126.6 ± 11.5	190.6 ± 24.3
210	5	146.3 ± 5.6	215.1 ± 7.9
210	10	165.9 ± 7.6	230.0 ± 3.7
210	15	157.0 ± 5.1	213.8 ± 6.2
220	5	155.0 ± 7.2	227.8 ± 15.0
220	10	152.8 ± 4.7	216.7 ± 13.6
220	15	141.4 ± 8.5	210.3 ± 8.9
230	5	161.6 ± 8.0	240.7 ± 17.8
230	10	152.6 ± 12.7	233.9 ± 10.1
230	15	138.8 ± 12.8	198.0 ± 6.3
Untreated *Salix*		97.4 ± 5.9	160.7 ± 8.5

3.2. Effect of different mixture ratio on the methane yield of pre-treated Salix and manure

Co-digestion of two different C/N ratio substrates proved to give a faster process, as shown in the curves in Fig. 4. The production was slower when digesting only manure, and this could be explained by the inoculum used already adapted to *Salix*, and by the incorporation of easy degradable carbon with the pre-treated *Salix*, accounting for an overall better balanced C/N ratio. Maximum yields reached ca. 230 mL CH_4/g VS after 77 days, and by the 20th day of the experiment, 75–80% of the methane potential from all the co-digestion mixtures was already produced. Comparison between the different co-digestion mixture ratios was made and their potential yields were calculated by normalizing with the methane yields obtained for the mono-digesting series of fresh manure and steam exploded *Salix*, and calculating for each co-digesting series their potential yield according to their manure and *Salix* fractions. Results demonstrated that all the mixtures reached their expected potential yields during the test and some even slightly surpassed it, implying possible synergistic effects of co-digestion (Table 5).

However, it is important to notice that the series mono-digesting *Salix* pre-treated at 210 °C and 10 min, did not reach the methane yield expected according to the first experiment. The pre-treated *Salix* sample proceeded from a different steam exploded batch than the one used for the first experiment, and so differences in dry matter and chemical content could be a cause. Furthermore, this series was the one with higher *Salix* content (2 g VS) from all, and inhibitors of the pre-treatment could have influenced the methane production. The high COD levels determined at the end of the experiment for all

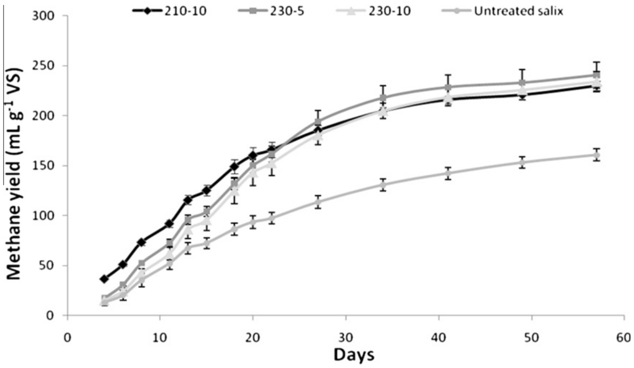

Fig. 3. Accumulated methane production profiles with their standard deviations for the pre-treatments of *Salix* at 210 °C – 10 min, 230 °C – 5 min and 230 °C – 10 min, and untreated *Salix*.

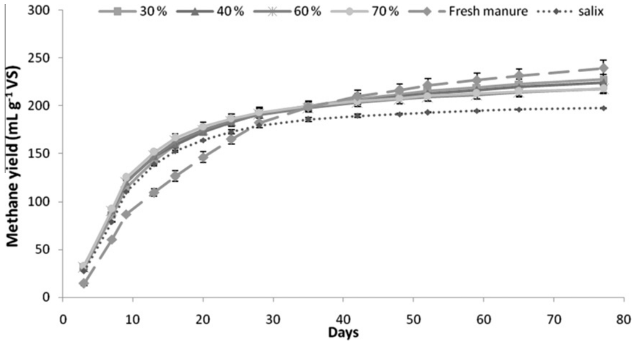

Fig. 4. Accumulated methane production profiles with their standard deviations for the co-digestion mixtures of 30%, 40%, 60% and 70% VS of *Salix* in the total mixture's VS content; only *Salix* and only fresh manure samples.

Table 5
Specific methane yields after 77 days for the different mixture ratios and their comparison respect their potential yields.

Mixture ratio (% VS of *Salix* on the total mixture's VS)	Mixture ratio C/N	Specific methane yield (mL g^{-1} VS)	Potential methane yield (mL g^{-1} VS)[a]	Specific yield/potential yield (%)
20	31	223.6 ± 6.7	223.8	99.6
30	35	227.5 ± 5.0	220.5	103.2
40	39	224.2 ± 1.8	217.2	102.4
50	44	216.6 ± 7.7	213.9	97.7
60	48	217.4 ± 4.0	210.5	103.1
70	52	217.9 ± 5.4	207.2	102.6
80	56	203.1 ± 4.1	203.9	97.6
Only steam exploded *Salix*	64	197.6 ± 0.4	–	100
Only fresh manure	23	239.1 ± 8.6	–	100
30 + digested manure	27[b]	178.1 ± 0.4	–	–
40 + digested manure	32[b]	174.1 ± 7.4	–	–

[a] Potential expected yields calculated with the maximum yields obtained for the steam exploded *Salix* and fresh manure samples.
[b] Estimated values using COD results.

the trials (Table 6) indicate that degradation might have been possible to continue if the experimental period was prolonged, and higher yields might have been achieved.

Regarding the co-digestion mixtures and their methane yields, the best were obtained for 30% and 40% VS of *Salix* in the mixture's total VS content, corresponding to 35 and 39 C/N ratio, respectively. Methane content in the biogas coming from the co-digestion mixtures ranged from 58% to 54% in average values, the higher ones corresponding to the mixtures having bigger manure content. For the series employing only pre-treated *Salix*, methane content in the biogas was around 53% while for the series with only fresh manure as substrate, 63%.

Table 6
Characterization of digestates.

Mixture ratio (% VS of *Salix* on the total mixture's VS)	pH	Total solids (% w/w)	Volatile solids (% w /w)	Total-N (mg L^{-1})	NH_4^+-N (mg L^{-1})	COD (mg L^{-1})
20	6.9	1.05	0.65	561	463	7610
30	6.9	1.02	0.63	556	459	6715
40	6.8	1.04	0.65	572	500	9160
50	6.8	0.96	0.59	590	350	14,695
60	6.8	0.98	0.61	556	351	7740
70	6.7	0.96	0.60	500	302	5840
80	6.7	0.94	0.59	510	320	10,540
Only steam exploded *Salix*	6.7	1.01	0.64	435	313	5280
Only fresh manure	6.9	1.06	0.66	657	464	11,880
30 + digested manure	7.0	1.07	0.65	759	590	12,940
40 + digested manure	7.0	1.04	0.64	666	525	7805

The use of already digested manure in the co-digestion with pre-treated *Salix* gave relevant results. Methane content in both series was ca. 60% and the yields obtained for the series with digested manure were 78% of the methane yields obtained for the series employing fresh manure with the same VS composition of *Salix* (30% and 40%). This is a significant fraction, and an indication of the high methane potential that still remains in the feedstocks after digestion takes place. Normally, the remaining potential of the digestate is lost during storage periods. Ways to profit these residual potential should be further developed. According to several authors (Hartmann et al., 2000; Angelidaki et al., 2005; Jagadabhi et al., 2008) it represents a big part of the total methane potential, and it remains untapped in the fibers even after production of substantial amounts of methane in the anaerobic digestion process.

From the analyses performed to the digestates after the anaerobic digestion (Table 6), correct process conditions throughout the whole digestion period were verified with the result of neutral pH values and non-inhibitory levels of ammonium. The major part of the total nitrogen was ammoniacal nitrogen, which is a fertilizer asset for biogas digestate coming from lignocellulosic substrates.

Finally, applying a one-way ANOVA test based on Fischer method to the methane yields of all the co-digestion series employing fresh manure, the only mixture giving a significantly lower methane yield is the one with 80% VS of *Salix*.

3.3. Optimal mixture ratio for biogas production from pre-treated Salix and manure

When analyzing methane yields with respect to C/N ratio for the mixtures of pre-treated *Salix* and cattle manure, the C/N ratio that gave the optimal yield through the experiment ranged between 35 and 40 (Fig. 5).

This is interesting since that ratio corresponded to a co-digestion mixture that possessed a considerable amount of steam exploded material (40% of its VS content) which shows that co-digestion with pre-treated lignocellulosic feedstocks can lead to good biogas yields. These types of materials available in the agricultural sector are then a good option for increase the energy output while giving a robust and stable digestion process.

4. Conclusions

Steam explosion of *Salix* increased its biogas production, optimizing the methane yield up to 50%. Higher and relatively similar methane yield values were obtained for all the treatment temperatures above 210 °C.

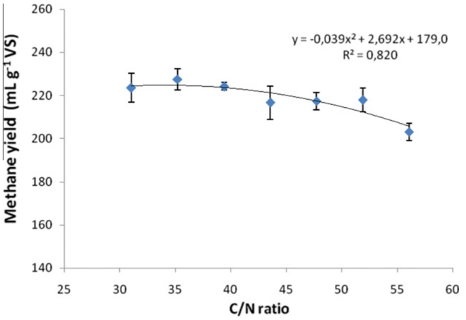

Fig. 5. Methane yield as a function of the C/N ratio for co-digestion mixtures of steam exploded *Salix* and manure.

Co-digestion of steam exploded *Salix* and cow manure provided a faster and more stable digestion process; the best methane yields (ca. 230 mL CH_4/g VS) were obtained for 30% and 40% VS of pre-treated *Salix* in the total VS content. Steam explosion made possible for *Salix* to cover an important fraction of the co-digestion mixture, without compromising its methane yield.

The capacity of treating high solids-biomass types without the addition of chemicals and with minimal sample handling makes steam explosion worth to consider as a pre-treatment method for increasing methane production. However, the process needs to be optimized since losses on volatile organic materials along the steam explosion unit, that can be approximately 20% for woody biomass, indicate that an important methane production is being disregarded. This is important to investigate to enable reliable yield calculations. In full scale plants, these losses would be reduced or eliminated since handling losses are smaller, and the flash stream could be condensed and collected to be added to the anaerobic digestion process.

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References

Allen, S., 1989. Chemical Analyses of Ecological Materials, second ed. Blackwell Scientific Publications, London, UK.

Alvarez, R., Lidén, G., 2008. Semi-continuous co-digestion of solid slaughterhouse waste, manure, and fruit and vegetable waste. Renewable Energy 33, 726–734.

Angelidaki, I., Ellegaard, L., 2003. Codigestion of manure and organic wastes in centralized biogas plants: status and future trends. Appl. Biochem. Biotechnol. 109 (1–3), 95–105.

Angelidaki, I., Boe, K., Ellegaard, L., 2005. Effect of operating conditions and reactor configuration on efficiency of full-scale biogas plants. Water Sci. Technol. 52 (1–2), 189–194.

APHA, 1995. Standard Methods for the Examination of Water and Wastewater. United Book Press, Baltimore, MD, USA.

Bremmer, J.M., Mulvaney, C., 1982. Nitrogen-total. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2 Agronomy 9, vol. I. American Society of Agronomy Inc., Madison, WI, USA, pp. 595–624 (Chapter 31, 1159s).

Brownell, H., Saddler, J., 1987. Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. Biotechnol. Bioeng. 29, 228–235.

Bruni, E., Jensen, A., Angelidaki, I., 2010a. Comparative study of mechanical, hydrothermal, chemical and enzymatic treatments of digested biofibers to improve biogas production. Bioresour. Technol. 101, 8713–8717.

Bruni, E., Jensen, A., Angelidaki, I., 2010b. Steam treatment of digested biofibers for increasing biogas production. Bioresour. Technol. 101, 7668–7671.

Castro, F., Hotten, P., Ørskov, E., 1994. Inhibition of rumen microbes by compounds formed in the steam treatment of wheat straw. Bioresour. Technol. 50, 25–30.

Deublein, D., Steinhauser, A., 2008. Biogas from Waste and Renewable Resources, An Introduction. Wiley–VCH Verlag GmbH & Co., KGaA, Weinheim, Germany.

Hansen, T.L., Schmidt, J.E., Angelidaki, I., Marca, E., la Cour Jansen, J., Mosbæk, H., Christensen, T.H., 2004. Method for determination of methane potentials of solid organic wastes. Waste Manage. 24, 393–400.

Hartmann, H., Angelidaki, I., Ahring, B., 2000. Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration. Water Sci. Technol. 41 (3), 145–153.

Horn, S.J., Eijsink, V.G.H., 2010. Enzymatic hydrolysis of steam-exploded hardwood using short processing times. Biosci. Biotechnol. Biochem. 74 (6), 1157–1163.

Horn, S.J., Estevez, M.M., Nielsen, H.K., Linjordet, R., Eijsink, V.G.H., 2011a. Biogas production and saccharification of *Salix* pretreated at different steam explosion conditions. Bioresour. Technol. 102, 7932–7936.

Horn, S.J., Nguyen, Q.D., Westereng, B., Nilsen, P.J., Eijsink, V.G.H., 2011b. Screening of steam explosion conditions for glucose production from non-impregnated wheat straw. Biomass Bioenergy. doi:10.1016/j.biombioe.2011.10.013.

Jagadabhi, P., Lehtomäki, A., Rintala, J., 2008. Codigestion of grass silage and cow manure in a CSTR by re-circulation of alkali treated solids of the digestate. Environ. Technol. 29, 1085–1093.

Lehtomäki, A., Huttunen, S., Rintala, J.A., 2007. Laboratory investigations on co-digestion of energy crops and crop residues with cow manure for methane production: effect of crop to manure ratio. Resour. Conserv. Recycl. 51, 591–609.

Mes-Hartree, M., Saddler, J., 1983. The nature of inhibitory materials present in pretreated lignocellulosic substrates which inhibit the enzymatic hydrolysis of cellulose. Biotechnol. Lett. 5 (8), 531–536.

Nelson, D., Sommers, L., 1982. Total carbon. In: Page, A.L., Miller, R.H., Keeney, D.R., (Eds.), Methods of Soil Analysis, Part 2 Agronomy 9, vol. I. American Society of Agronomy Inc., Madison, WI, USA, pp. 539–579 (Chapter 29, 1159s).

Porter, M.G., Murray, M.S., 2001. The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. Grass Forage Sci. 56, 405–411.

Ramos, L., 2003. The chemistry involved in the steam treatment of lignocellulosic materials. Quim. Nova 26, 863–871.

Samuelsson, R., Nilsson, C., Burvall, J., 2006. Sampling and GC–MS as a method for analysis of volatile organic compounds (VOC) emitted during oven drying of biomass materials. Biomass Bioenergy 30, 923–928.

Sassner, P., Galbe, M., Zacchi, G., 2005. Steam pretreatment of *Salix* with and without SO_2 impregnation for production of bioethanol. Appl. Biochem. Biotechnol. 121–124, 1101–1117.

Seppälä, M., Paavola, T., Lehtomäki, A., Pakarien, O., Rintala, J., 2008. Biogas from energy crops-optimal pre-treatments and storage, co-digestion and energy balance in boreal conditions. Water Sci. Technol. 58 (9), 1857–1863.

Stringer, D.A., 1988. Evaluation of anaerobic biodegradation. Tech. Rep. 28, ECETOC, Brussels, Belgium.

van Lier, J., Tilche, A., Ahring, B., Macarie, H., Moletta, R., Dohanyos, M., Pol, L.H., Lens, P., Verstraete, W., 2001. New perspectives in anaerobic digestion. Water Sci. Technol. 43 (1), 1–18.

Weiland, P., 2000. Anaerobic waste digestion in Germany; status and recent developments. Biodegradation 11, 415–421.

Weiland, P., 2003. Production and energetic use of biogas from energy crops and wastes in Germany. Appl. Biochem. Biotechnol. 109, 263–274.

Weissbach, F., Kuhla, S., 1995. Material losses of dry matter of silages and forage during ensiling: accruing errors and options of correction. Übersicht Tierernährung 23, 189–214 (in German).

Yadvika, Santosh, Sreekrishnan, T., Kohli, S., Rana, V., 2004. Enhancement of biogas production from solid substrates using different techniques: a review. Bioresour. Technol. 95, 1–10.

Yen, H., Brune, D., 2007. Anaerobic co-digestion of algal sludge and waste paper to produce methane. Bioresour. Technol. 98, 130–134.

Paper II

Semi-continuous anaerobic co-digestion of cow manure and steam exploded *Salix* with recirculation of liquid digestate.

Maria M. Estevez[a*] ; Zehra Sapci[a]; Roar Linjordet[b]; Anna Schnürer [c,d]; John Morken[a].

[a]Department of Mathematical Sciences and Technology, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway.

[b]Bioforsk, Norwegian Institute for Agricultural and Environmental Research, 1432 Ås, Norway.

[c]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

[d]Department of Microbiology, Uppsala BioCenter, Swedish University of Agricultural Sciences, P.O. Box 7025, SE 750 07 Uppsala, Sweden.

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[*] Corresponding author. Address: Department of Mathematical Sciences and Technology, Norwegian University of Life Sciences; POB 5003, 1432 Ås, Norway. Tel.: + 47 64 96 54 93; fax: +47 64 96 54 01.
E-mail: maria.estevez@umb.no

Abstract

The effects of recirculating the liquid fraction of the digestate during mesophilic anaerobic co-digestion of steam exploded *Salix* and cow manure were investigated in laboratory scale continuously stirred tank reactors. An average organic loading rate of 2.6 g VS L^{-1} d^{-1} and hydraulic retention time (HRT) of 30 days were employed. Co-digestion of *Salix* and manure gave better methane yields than digestion of manure alone. When digestate was recirculated and used instead of water for diluting the feedstock (1:1 dilution ratio) a 16 % increase of the methane yield was achieved. The reactor in which the higher fraction of digestate was recirculated (1:3 dilution ratio) gave the highest methane yields. Ammonia and volatile fatty acids did not reach inhibitory levels, but solids accumulation was more pronounced in the recycling reactors, and their methane yields decreased after three HRT. Avoiding the use of fresh water to dilute biomass with high solid content and obtaining a final digestate with increased dry matter content may imply important economic benefits in full-scale processes. However, long-term stability would need an optimized recycling ratio and proper monitoring.

Keywords: Anaerobic co-digestion, *Salix*, steam explosion, manure, liquid digestate recirculation.

1 Introduction

Solutions to mitigate nutrient losses and emissions of greenhouse gasses by the Norwegian agricultural sector have been gaining increased attention during the past years. One of these solutions is anaerobic digestion of the organic wastes generated by this sector. In this process, organic compounds are degraded by microorganisms under anaerobic conditions resulting in the production of mainly methane and carbon dioxide, i.e. biogas, possible to use for production of heat, electricity or vehicle fuel. During the degradation process mineral nutrients are released giving rise to a digestion residue with high fertilizing value. [1-3].

Fast growing (short rotation) energy crops such as *Salix* are good alternatives for bioenergy production. *Salix* can sequestrate more carbon than softwoods within a growing season [4] and it can be easily adapted to extreme soil conditions, making it a very economic viable biomass source [5]. *Salix viminalis* (basquet willow), cultivated vastly in the nordic countries, can produce up to 35 x 10^3 kilograms of stem per hectare per year [4]. The rich lignocellulosic content of plant materials makes it a substrate less available for degradation during anaerobic digestion. Different pre-treatment methods can be used in order to increase the availability, such as mechanical, chemical, biological or thermal pre-treatment techniques [5-7]. High pressure-steam treatment is one of the most effective [7-9], which combines high temperatures and a rapid pressure reduction, resulting in a physical disruption of the lignocellulosic structures. Previous studies in batch scale have shown that steam explosion of *Salix* can give an increased methane yield of up to 50 % compared to non treated material [10,11].

Lignocellulosic biomass is rich in carbon and in order to achieve good nutrient balance, good yields and stability during anaerobic digestion, co-digestion of lignocelluloses together with nitrogen rich materials is essential [2,12,13]. During co-digestion of steam exploded *Salix* and manure in a batch test, faster and more stable methane production was achieved compared to digestion of the substrates separately [11]. The highest methane yields were achieved with C/N ratios of 35 – 40, corresponding to a mixture content of 30 - 40 % in volatile solids (VS) from *Salix* [11].

Commonly about 25 % of the methane potential remains unexploited when substrates with high fiber content are digested [12,14]. Effluents from anaerobic digesters at biogas plants can contain up to 30 % of residual methane potential [13,15]. Losing this potential would not only entail economic but also environmental implications due to the subsequent loss of methane in storage tanks [13]. Ways to make the anaerobic digestion process more efficient and sustainable include; i) using gas tight post-storage tanks [3,12]; ii) applying pre-treatment and/or longer hydraulic retention times [3,15]; iii) applying recirculation of the digestate, allowing a longer solid retention time in the digester, and thus longer degradation. Previous studies on the effect of recycling the digestate have resulted in varying results. Results from attempts to recycle the solid digestate fraction, after being separated from their liquid fraction, did not enhance the methane production, but instead even decreased it [12]. When separating digestate fibers from the liquid, most of the organic material remains in the liquid fraction, having much higher biogas potential. Recirculation of the liquid fraction of the digestate has proven successful with increased methane production as a result [16,17]. Moreover, dilution to appropriate total solids (TS) content is required for good mixing when digesting high solids biomass [17,18] and so substituting water by liquid digestate leads to water consumption reduction. As a consequence, also less amounts of effluents with higher solid content will be generated, which would decrease transport costs and storage capacity requirements. Such studies on continuously stirred tank reactors (CSTRs) systems performing co-digestion of crops and manure are still limited.

In this study we aim to investigate the stability and methane production from a mixture composed of 40 % in VS content of steam exploded *Salix* and the rest of manure, in mesophilic small-scale CSTRs, as well as the effects of applying recirculation of the liquid digestate while minimizing water and manure consumptions. As a comparison, a control reactor degrading manure only was also evaluated. To our knowledge the concept of recycling digestate from a process operating with *Salix* has not been investigated before.

2 Materials and methods

2.1 Steam exploded *Salix*

The *Salix* samples (*Salix viminalis* "Christina"*)*, were harvested in November 2009 after the second growing season, in short rotation coppice plantation of 7 years old, near Grimstad, Norway (58º20' N 8º31' E).The shoots were harvested manually and chopped with a standard wood disk chipper of nominal cutting length 7 mm. The material was stored at -20 ºC until being pretreated by steam explosion, as described by Horn et al. [19]. Steam explosion was run at 210 ºC and 10 minutes of residence time. The pretreated material was packed in vacuum sealed polyethylene bags that were kept refrigerated at 4 ºC until use. Characteristics of organic components of untreated and steam exploded material are given in Estevez et al. [11]. Parameters such as pH, solids, total carbon (TOC), total nitrogen (total-N), and ammonium nitrogen (NH_4^+-N) for the steam exploded material at 210 ºC and 10 minutes are shown in Table 1.

2.2 Cow manure

Fresh cow manure was obtained from the University farm. The collected manure was kept in 20 kg containers refrigerated at 4 ºC until fed to the reactors. Some characteristics of the manure are given in Table 1.

2.3 Inoculum

The inoculum for the reactors was taken from a previous experiment in which the gas potential from different mixtures of pre-treated *Salix* and cattle manure was evaluated [20]. The material from this test was pooled together in a container and stored anaerobically for one week at 37 ºC before used as inoculum for the CSTRs. Some characteristic of the inoculum are given in Table 1.

TABLE 1 here

2.4 Methodology

2.4.1 Starting up the reactors

Four *BELACH BIOTEKNIK AB* CSTRs, with a nominal working volume of 6 L, were run at a temperature of 37 ºC and stirrer speed of 18.85 rad s^{-1}. The reactors were coupled with *Bluesens Kombi-* CO_2/CH_4 infrared dual length gas sensors to determine the methane composition of the biogas produced. The software employed (BIOPHANTOM©) allowed continuous, real-time monitoring of pH, stirrer speed, temperature, gas flow, gas volume and gas composition. During the start-up period, the reactors were filled with 3 L of inoculum. Feeding of the reactors up to their full working volume started with a low organic loading rate (OLR: 1 g VS L^{-1} d^{-1}) of the respective substrate mixtures and was successively increased until reached 3 g VS L^{-1} d^{-1}. The start-up period lasted 3 weeks.

2.4.2 Semi-continuous feeding process

After the start up period, the reactors were fed once a day, 6 days a week, with an OLR of 3 g VS L^{-1} d^{-1} (one whole week daily load of 2.6 g VS L^{-1}) and a hydraulic retention time (HRT) of 30 days, according to the feeding scheme detailed in Table 2. Thus, 200 mL of freshly prepared substrates mixtures were fed on every occasion to each reactor. Previous to feeding, an equivalent volume was removed so to maintain a constant volume in the reactor. For the reactors where recirculation took place, GA2 and GB1, the removed fraction was filtered through a 2.5 mm mesh size sieve and the liquid fraction added back to the digester (Fig 1, Table 2). Considering the TS and VS content of the sieved digestate that was recirculated, the OLRs of GA2 and GB1 were in average 3.1 and 2.9 g VS L^{-1} d^{-1} respectively, and the HRT differed from the solids retention time (SRT), the later being approximately 43 and 51 days for GA2 and GB1, respectively. For the specific methane yield calculations only the fresh daily VS additions were considered. The anaerobic digestion process was followed for a period of 100 days.

FIGURE 1 here

TABLE 2 here

2.4.3 Analytical tools

Elemental analysis for determination of TOC and total-N on the fresh substrates, and subsequently their C/N ratio, were performed according to Nelson and Sommers [21] and Bremmer and Mulvaney [22], respectively. Samples were analysed in a LECO CHN 1000 analyzer equipped with infrared (IR) cells for the analysis of TOC and thermal conductivity (TC cells) for the Total-N determination. TS and VS content were analysed by Standard Methods [23] and pH values were measured by a WTW Multi ®350i equipped with a WTW pH Electrode (Sen Tix 41) according to Allen [24].

Volume of biogas produced, pH in the digester and methane content were continuously monitored by the reactors control program during the whole experiment. NH_4^+-N, TS and VS content, Total-N, and chemical oxygen demand (COD), were determined once a week. Samples for volatile fatty acids (VFAs) content were collected weekly and analysed by EUROFINS (Moss, Norway) according to the method described by Jonsson and Borén [25]. Analysis of furfural, 5-hydroxy methyl furfural (HMF) and phenolic compounds were done at every HRT, employing ultra high performance liquid chromatography coupled with diode array detention (UHPLC-DAD). The system used was an Agilent Infinity 1290 equipped with a Zorbax Eclipse Plus C18 column (2.1x150 mm, 1.8 µm) fitted with an inline filter of 0.5 µm frit. Samples were prepared by centrifugation (1466 rad s^{-1}, 10 min) and acidification of the supernatant with H_2SO_4 72 % to pH < 2.5.

The concentration of NH_4^+-N in the digestates was followed with an Ion Selective Electrode (Orion-Thermo Scientific©). Total-N and COD were analysed spectrophotometrically employing Merck Spectroquant® Kits Analyses.

2.4.4 Statistical Analysis

All standard deviations reported in the study were calculated using statistical functions of Microsoft Excel 2007. The statistical software Minitab ® 16.1.1 was employed to evaluate the relationship between paired experimental data. The results were assessed with p-values to reflect the statistical significance between paired groups (confidence level 95 %). For each reactor, methane yield and volumetric gas production were evaluated with one way analysis of variance (ANOVA) followed by Fisher's least significant difference method. A paired t-test was employed to compare the methane yield variations between weeks 4 to 8 and weeks 9 to 13.

3 Results and discussion

The specific methane yields of the reactors GA1 and GB2 (Table 3, Fig. 2) were slightly lower compared to the values obtained after 30 days of batch digestion of the same mixture of *Salix* and manure (193 mL CH_4 g VS^{-1}) and for manure alone (186 mL CH_4 g VS^{-1}) [11]. When recirculation was applied and retention times increased (GA2), the yield of such mixture was also comparably higher.

TABLE 3 here

FIGURE 2 here

In reactor GA2, a 16 % increase in the methane production from the same fresh VS mixture of *Salix* and manure as used in GA1, was achieved (Table 3). Thus, substitution of water by liquid digestate gave a significantly higher methane production. For GB1, in which filtered digestate also substituted part of the manure, the highest yield of all the reactors was achieved (Table 3). Applying a one way ANOVA test based on Fisher method to the methane yields of all the reactors, each reactor proved to be significantly different from the rest. The methane content of the biogas produced was higher in the reactors subjected to recycling compared to the reactors without recycling (Table 3). A similar increase in the methane production due to recycling was also found by Jarvis et al. [16] for a two-phase anaerobic digestion process fed with silage, with a 22 % increased in the methane yield from the total system when effluent from the methanogenic reactor was recirculated back to the acidogenic reactor. In a study by Nordberg et al. [17] a 25 % increase of the OLR was possible to achieve when liquid recirculation in a one-phase anaerobic digestion of alfalfa silage was initiated, providing an increase in pH, alkalinity and stability. This stability effect was also reported by Hartmann and Ahring [26] who achieved instant buffering effect when process liquid was recirculated.

There are several possible explanations for the positive effects observed in connection to recycling. One explanation could be that suspended material, i.e. microbial biomass, is being reintroduced in the reactor during recycling, enriching the degradation process [17]. Also, the increased microbial biomass could give rise to a better contact between the microorganisms and substrate making this process configuration more robust to fluctuations [26]. Furthermore, the liquid digestate may possess VS which are being degraded with the longer retention time and may partly explain the difference in yields between these reactors and GA1.

The maximum volumetric methane productions for the recycling reactors were obtained during the second HRT period and were higher than for the non recycled digester GA1 (Table 3, Fig. 2). However, during the last HRT, yields of GA2 and GB1 showed a decreasing tendency (Fig. 2). VS degradation, that considered both fresh and recirculated VS, was similar for reactors GA1 and GA2 (average 34 ± 3 %; 37 ± 1 % respectively), higher for GB1 (42 ± 4 %) and lower for GB2 (average 28 ± 5 %). Due to recycling, the SRT differed from the HRT, and both were longer than 30 days. The extended solids retention may enhance production from the still degradable VS fraction [15] in reactors GA2 and GB1. At the same time, part of the recalcitrant VS was as well recycled and thus, accumulated, being this probable the reason for the observed increase with time in the VS content on the recirculated reactors. Accumulation of organic and inorganic compounds during process liquid recirculation as well as a gradual decrease in methane yield was also reported for a two-step digestion of alfalfa silage [17]. The organic material accumulation led to an increase of the VFA levels and eventually to process inhibition. In our case, VFA were hardly detected and so no organic overloading was present, implying that the accumulated solids mostly were recalcitrant. Furthermore, Hartmann and Ahring [26] observed smaller average VS degradations when liquid recirculation was applied to a co-digestion system of organic fraction of municipal solid waste and manure. This indicates that recirculation of liquid digestate may eventually lead to inhibition due to accumulation of solids. However, with proper control and an optimum recirculation ratio for the system, accumulation of solids may not cause any problems [17].

As mentioned above, VFA contents were very low for all the reactors throughout the entire period, with maximum total concentrations that did not surpassed 320 mg L^{-1}. These values are far from the level at which process imbalance may occur: about 3000 mg L^{-1} [27,28]. According to Nordberg et al. [17], 100 % recycling of liquid digestate during digestion of alfalfa silage lead to accumulation of organic compounds including VFA, and subsequently, inhibition, while recycling with a liquid consisting of 50 % of the digestate together with water, only caused moderate problems due to VFA accumulation. Similar results were achieved in a previous study in which 100 % of the liquid digestate was recirculated and mixed with steam exploded *Salix*, without adding manure or water, resulting in an acidification of the process, decreased pH and collapse of the reactor [20]. Thus, again this results support that a proper recirculation ratio is needed in order to balance VFA, ammonium and solid levels.

One possible drawback of using steam explosion as pre-treatment for disruption of lignocellulosic materials is the release of phenolic compounds and furfurals, which could act as inhibitors for the biomethanisation process [9,29,30]. Results showed only trace levels of HMF and furfural in the reactors along the whole experimental period. Accumulation of HMF was more pronounced in GA2 and GB1 being the level at the end of the period 2.5 times higher, while no HMF was found in GB2, digesting only manure. Furfural was present in all the reactors and showed after three HRT a 2 times increment for GA1 and GA2 and a 3 times increment for GB1, being all these trace level values. Thus, it seems unlikely that the observed decrease in gas production was caused by inhibitory components. Such decrease proved to be not statistically significant ($p<0.05$) when the gas production profiles of the different reactors from weeks 4 to 8 and from weeks 9 to 13 were compared.

FIGURE 3 here

FIGURE 4 here

COD and total-N increased slowly throughout the period for all the reactors (Figs. 3 and 4). Ammonium-N levels were in average stable throughout the period, and below inhibitory ammonia values, even for the recycling reactors (Fig. 4). This is interesting since previous studies in which silages were digested at mesophilic temperatures showed increasing ammonium-N levels [16,17]. It seems that in our reactors the C/N ratio of the substrates was well balanced, not reaching ammonium-N levels that could cause ammonia inhibition to the bacteria. It could be interesting to address in further studies the application of higher N levels in order to investigate process stability when recirculating the digestate. In mesophilic processes high ammonia levels have been seen to give a shift in the methane producing pathway from aceticlastic methanogenesis to syntrophic acetate oxidation [31,32], which allows operation at high ammonia levels. The doubling time of a syntrophic acetate oxidizing co-culture is longer than that of aceticlastic methanogens (28 days and 2-12 days respectively). Thus, operation at high ammonia levels might require comparably long HRT in order to avoid washout of organisms [32], which can be achieved by recirculating the digestate. With higher ammonia content and retention times elongated, the fertilizer value of the digestate will as well increase and positive sanitation effects may be achieved [33].

By employing filtered digestate in the dilution of high solids feedstock, water needs are reduced, as well as the need to pre-heat the feedstock mixtures, which would translate into less process costs for full scale plants [16,18]. Comparing to GA1, ca. 3 L of water per HRT were saved for GA2, and ca. 4 L of water per HRT for GB1. Regarding manure as a resource, GB1 was fed with approximately half of the fresh manure fed to GB2 (3 kg less per HRT), that being co-digested and diluted with digestate, ended up giving better methane yields. Reduction of manure consumption may benefit farm-scale biogas production that depends on seasonal animal manure as substrate. An average of 35 % dry matter reduction was achieved with the 2.5 mm mesh, and the final solid fraction had a dry matter content of ca. 10 %. Increasing the dry matter of the process' final digestate can lead to economic savings in transportation and handling logistics for such material, later to be used as fertilizing agent.

4 Conclusion

Co-digestion of *Salix* and manure in CSTRs gave better yields than digestion of manure alone. Recirculation of the liquid digestate increased methane productions, although in the long term this caused a decrease of process efficiency, probably due to solids accumulation. Recirculation could be a good option to avoid the use of water in the dilution of substrates with high solids content, and to reduce manure consumption. The increase in the solids degradation time of the not easily digestible materials may enhance the methane yields. However, an optimization of the recycling ratio and solids content of the recirculated digestate needs to be performed so to avoid problems with accumulated solids in the long term.

Acknowledgments

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References

[1] Angelidaki I, Ellegaard L. Codigestion of manure and organic wastes in centralized biogas plants: status and future trends. Appl Biochem Biotech 2003;109(1-3): 95-105.

[2] Deublein D, Steinhauser A. Biogas from Waste and Renewable Resources, An Introduction. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co, KGaA, 2008.

[3] Massé DI, Talbot G, Gilbert Y. On farm biogas production: A method to reduce GHG emissions and develop more sustainable livestock operations. Anim Feed Sci Tech 2011;166–7: 436– 45.

[4] Kuzovkina YA, Quigley MF. Willows beyond wetlands: uses of *Salix* L. species for environmental projects. Water Air Soil Poll 2005;162: 183-204.

[5] Sassner P, Mårtensson CG, Galbe M, Zacchi G. Steam pretreatment of H_2SO_4-impregnated *Salix* for the production of bioethanol. Bioresource Technol 2008;99: 137-45.

[6] Hendriks ATWM, Zeeman G. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technol 2009;100: 10-8.

[7] Bruni E, Jensen A, Angelidaki I. Comparative study of mechanical, hydrothermal, chemical and enzymatic treatments of digested biofibers to improve biogas production. Bioresource Technol 2010;101: 8713 - 17.

[8] Brownell H, Saddler J. Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. Biotechnol Bioeng 1987;29: 228 - 35.

[9] Ramos L. The chemistry involved in the steam treatment of lignocellulosic materials. Quim Nova 2003;26: 863 - 71.

[10] Horn SJ, Estevez MM, Nielsen HK, Linjordet R, Eijsink VGH. Biogas production and saccharification of *Salix* pretreated at different steam explosion conditions. Bioresource Technol 2011;102: 7932 -36.

[11] Estevez MM, Linjordet R, Morken J. Effects of steam explosion and co-digestion in the methane production from *Salix* by mesophilic batch assays. Bioresource Technol 2012;104: 749–56.

[12] Jagadabhi P, Lehtomäki A, Rintala J. Codigestion of grass silage and cow manure in a CSTR by re-circulation of alkali treated solids of the digestate. Environ Technol 2008;29: 1085 - 93.

[13] Seppälä M, Paavola T, Lehtomäki A, Pakarinen O, Rintala J. Biogas from energy crops- optimal pre-treatments and storage, co-digestion and energy balance in boreal conditions. Water Sci Technol 2008;58(9): 1857 - 63.

[14] Hartmann H, Angelidaki I, Ahring BK. Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration. Water Sci Technol 2000;41(3): 145 - 53.

[15] Angelidaki I, Boe K, Ellegaard L. Effect of operating conditions and reactor configuration on eficiency of full-scale biogas plants. Water Sci Technol 2005;52(1-2): 189 - 94.

[16] Jarvis Å, Nordberg Å, Mathisen B, Svensson BH. Stimulation of conversion rates and bacterial activity in a silage-fed two-phase biogas process by initiating liquid recirculation. A Van Leeuw J Microb 1995;68: 317-27.

[17] Nordberg Å, Jarvis Å, Stenberg B, Mathisen B, Svensson BH. Anaerobic digestion of alfalfa silage with recirculation of process liquid. Bioresource Technol 2007;98: 104-11.

[18] Angelidaki I, Cui J, Chen X, Kaparaju P. Operational Strategies for Thermophilic Anaerobic Digestion of Organic Fraction of Municipal Solid Waste in Continuously Stirred Tank Reactors. Environ Technol 2006;27(8): 855-61.

[19] Horn SJ, Nguyen QD, Westereng B, Nilsen PJ, Eijsink VGH. Screening of steam explosion conditions for glucose production from non-impregnated wheat straw. Biomass Bioenerg 2011;35: 4879-86.

[20] Estevez MM, Linjordet R, Morken J. Biogas optimization by steam explosion of *Salix* and recycling of process water from biogas production of *Salix* and manure. In: Presented at the international IWA-symposium on anaerobic digestion of solid waste and energy crops, TU Wien, Vienna, Austria, 2011.

[21] Nelson D, Sommers L. Total carbon. In: Page AL, Miller RH, Keeney DR, editors. Methods of Soil Analysis, Part 2 Agronomy 9, vol. I, Wisconsin: American Society of Agronomy Inc; 1982, p. 539 -79 (Chapter 29, 1159s).

[22] Bremmer JM, Mulvaney C. Nitrogen-total. In: Page AL, Miller RH, Keeney DR, editors. Methods of Soil Analysis, Part 2 Agronomy 9, vol. I, Wisconsin: American Society of Agronomy Inc; 1982, p. 595-624 (Chapter 31, 1159 s).

[23] APHA. Standard Methods for the Examination of Water and Wastewater. Baltimore: United Book Press; 1995.

[24] Allen S. Chemical Analyses for Ecological Materials. 2nd ed. London: Blackwell Scientific Publications; 1989.

[25] Jonsson S, Borén H. Analysis of mono- and diesters of o-phthalic acid by solid-phase extractions with polystyrene–divinylbenzene-based polymers. J Chromatogr A 2002;963 (1–2): 393-400.

[26] Hartmann H, Ahring BK. Anaerobic digestion of the organic fraction of municipal solid waste: Influence of co-digestion with manure. Water Res 2005;39: 1543-52.

[27] Ahring BK, Sandberg M, Angelidaki I. Volatile fatty acids as indicators of process imbalance in anaerobic digestors. Appl Microbiol Biot 1995;43: 559-65.

[28] Holm-Nielsen JB, Andree H, Lindorfer H, Esbensen KH. Transflexive embedded near infrared monitoring for process intermediates in anaerobic digestion/biogas production. J Near Infrared Spec 2007;15: 123-35.

[29] Castro F, Hotten P, Ørskov E. Inhibition of rumen microbes by compounds formed in the steam treatment of wheat straw. Bioresource Technol 1994;50: 25-30.

[30] Bruni E, Jensen A, Angelidaki I. Steam treatment of digested biofibers for increasing biogas production. Bioresource Technol 2010;101: 7668 -71.

[31] Schnürer A, Zellner G, Svensson BH. Mesophilic syntrophic acetate oxidation during methane formation in biogas reactors. FEMS Microbiol Ecol 1999;29: 249-61.

[32] Schnürer A, Nordberg Å. Ammonia, a selective agent for methane production by syntrophic acetate oxidation at mesophilic temperature. Water Sci Technol 2008;57(5): 735-40.

[33] Ottoson J R, Schnürer A, Vinnerås B. In situ ammonia production as a sanitation agent during anaerobic digestion at mesophilic temperature. Lett Appl Microbiol 2008;46: 325-30.

Table 1. Substrates' characteristics.

Material	pH	TS[a]	VS[b]	TOC[a]	Total-N[a]	NH_4^+-N[a]
Salix treated (210 ºC-10 min)	3.8	17.8	98.0	12.20	0.19	n.d.
Manure	6.8	11.3	85.8	4.49	0.20	0.17
Inoculum	7.2	2.6	81.0	0.85	0.15	0.07

[a] Amounts expressed as percentage of wet weight

[b] Amount expressed as percentage of dry weight

Table 2. Feeding scheme for the four reactors.

Reactor	Feedstock mixture			Digestate VS % in 200 mL of feedstock mixture	C/N of feedstock (incl. digestate VS)	OLR g VS L^{-1} d^{-1} (incl. digestate VS)
	Fresh substrates		Dilution ratio (substrate mix.: liquid)			
	Salix (% VS)	Manure (% VS)				
GA1	40	60	1:1 with water	-	39	2.6
GA2	40	60	1:1 with digestate	15	34	3.1
GB1	47	53	1:3 with digestate	25	32	2.9
GB2	-	100	1:1 with water	-	23	2.6

Table 3. Operational conditions for the four reactors.

Reactor	Average pH	Average methane content (%)	Specific methane yield (weekly average) (mL g $^{-1}$)	Volumetric production (weekly average) (L m^{-3} d^{-1})
GA1	7.7 ± 0.2	48.4 ± 1.8	185.3 ± 10.5	556.0 ± 31.5
GA2	7.3 ± 0.1	55.9 ± 1.8	215.2 ± 15.7	645.5 ± 47.2
GB1	7.3 ± 0.1	59.5 ± 1.2	235.0 ± 17.2	626.6 ± 45.7
GB2	7.6 ± 0.1	54.3 ± 1.2	156.5 ± 11.7	469.4 ± 33.4

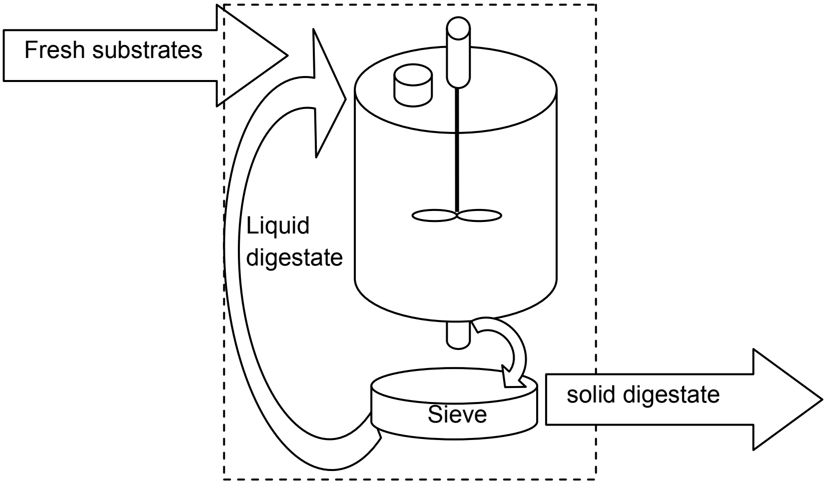

Figure 1. Diagram of the recycling system (reactors GA2 & GB1)

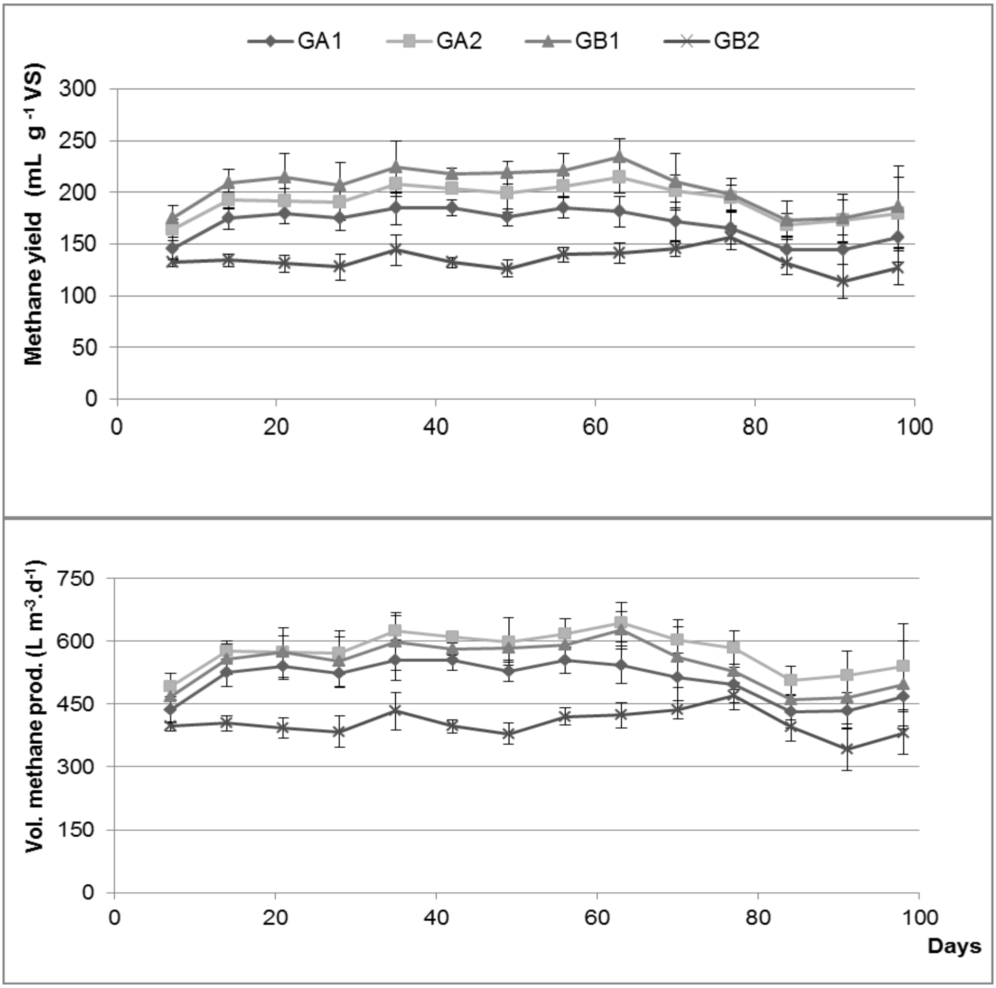

Figure 2. Specific methane yield (mL CH_4 g VS^{-1}) and volumetric methane production (L CH_4 m^{-3} d^{-1}) calculated as weekly averages, with standard deviations, for each reactor; GA1: Salix 40 % VS, manure 60 % VS and dilution with water, GA2: Salix 40 % VS, manure 60 % VS and dilution with digestate, GB1: Salix 47 % VS, manure 53 % VS and dilution with digestate, GB2: 100 % VS manure and dilution with water.

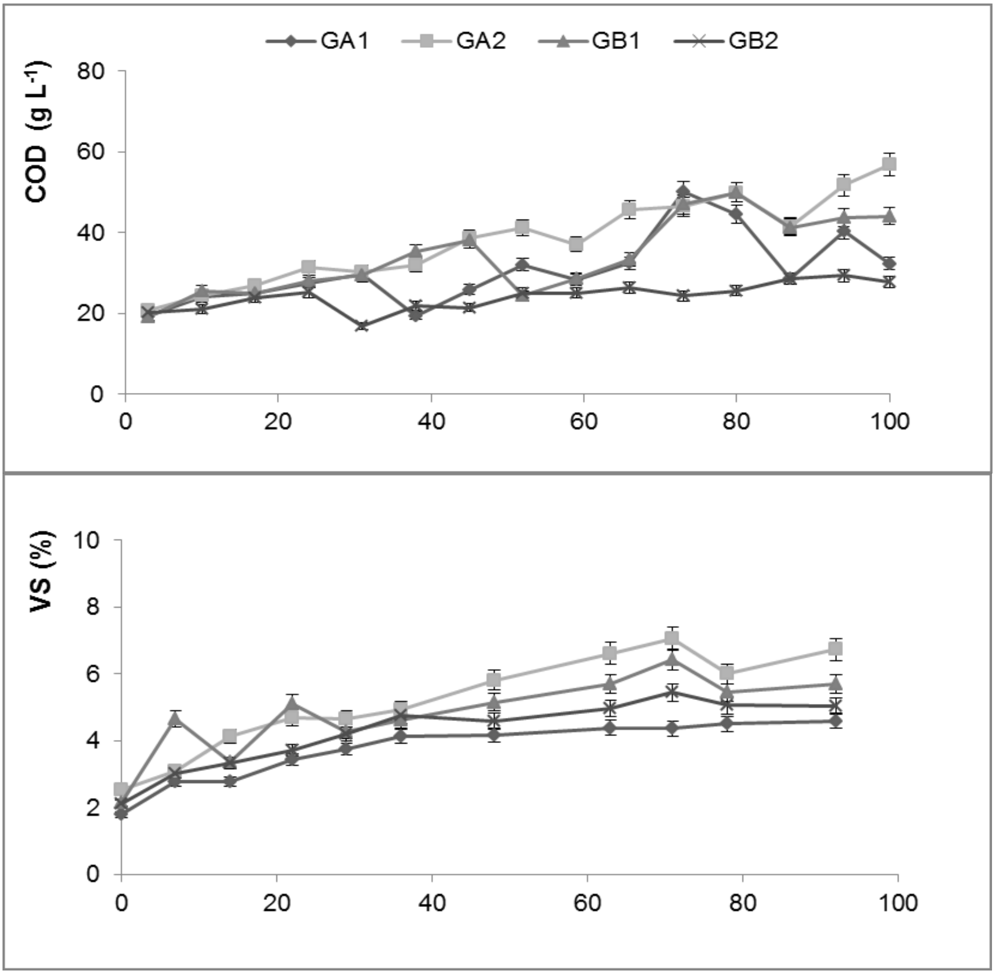

Figure 3. Total chemical oxygen demand (COD) and Volatile solids content (VS) for the four reactors; GA1: Salix 40 % VS, manure 60 % VS and dilution with water, GA2: Salix 40 % VS, manure 60 % VS and dilution with digestate, GB1: Salix 47 % VS, manure 53 % VS and dilution with digestate, GB2: 100 % VS manure and dilution with water.

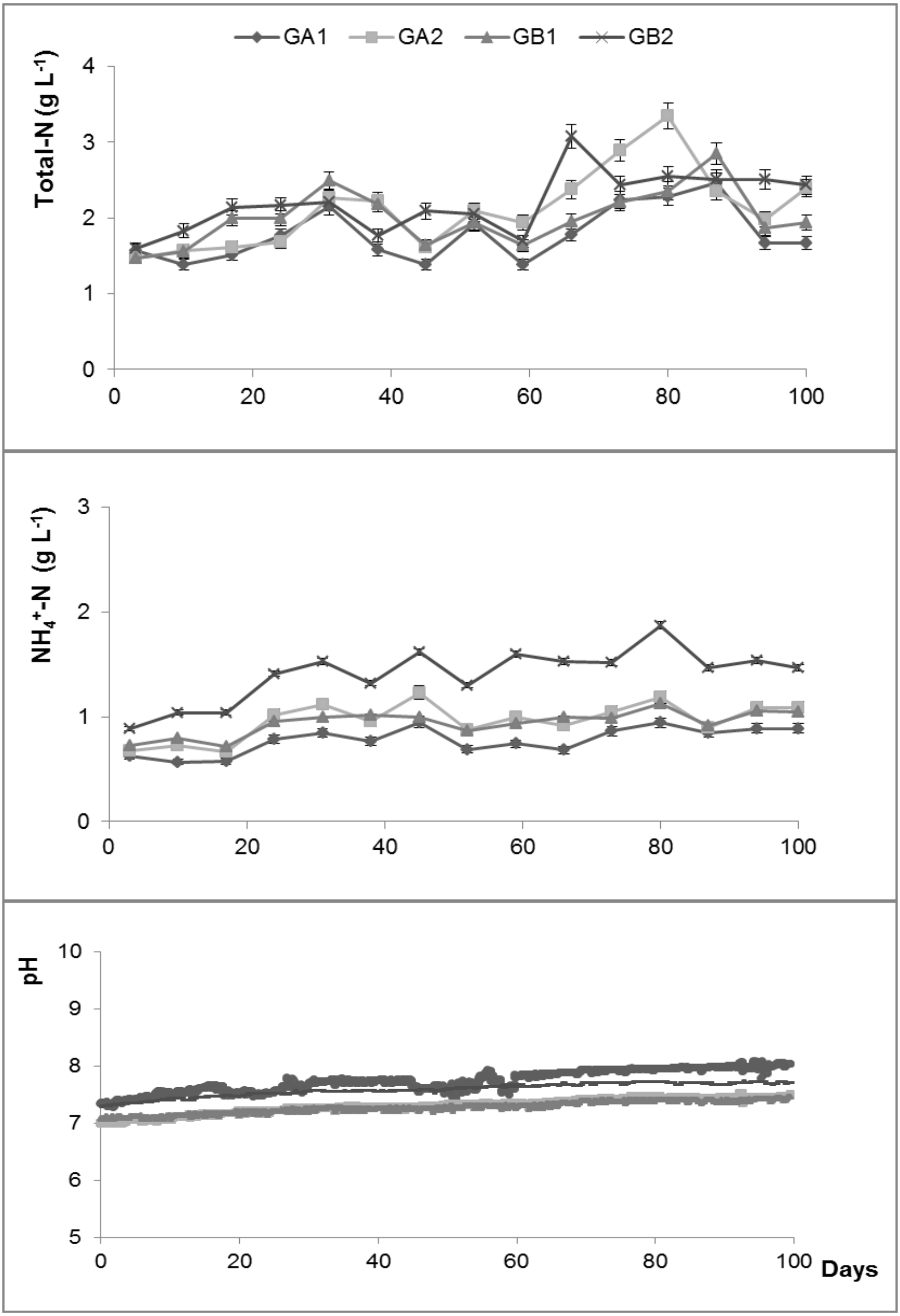
GA1
GA2
GB1
GB2
Total-N (g L-1)
4
3
2
1
0
0
20
40
60
80
100
NH4+-N (g L-1)
3
2
1
0
0
20
40
60
80
100
pH
10
9
8
7
6
5
0
20
40
60
80
100
Days

Figure 4. Total nitrogen (Total-N), ammonium nitrogen (NH_4^+-N) and pH profiles with standard deviations (pH uncertainty: ± 2 %) for the four reactors; GA1: Salix 40 % VS, manure 60 % VS and dilution with water, GA2: Salix 40 % VS, manure 60 % VS and dilution with digestate, GB1: Salix 47 % VS, manure 53 % VS and dilution with digestate, GB2: 100 % VS manure and dilution with water.

Paper III

Incorporation of fish byproduct to the semi-continuous anaerobic co-digestion of pre-treated lignocellulose and cow manure, with recovery of digestate's nutrients.

Maria M. Estevez[a*] ; Zehra Sapci[a,b]; Roar Linjordet[c]; John Morken[a].

[a]Department of Mathematical Sciences and Technology, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway.

[b]Department of Environmental Engineering, Bitlis Eren University, 13000 Bitlis, Turkey.

[c]Bioforsk, Norwegian Institute for Agricultural and Environmental Research, 1432 Ås, Norway.

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[*] Corresponding author. Address: Department of Mathematical Sciences and Technology, Norwegian University of Life Sciences; POB 5003, 1432 Ås, Norway. Tel.: + 47 64965493; fax: +47 64 96 54 01.
E-mail: maria.estevez@umb.no

Abstract

Norway's fish processing industry generates big amounts of fish waste every year. The category 2 fish byproduct, without oil, has not been tested for energy before. The stability of an anaerobic digestion process that incorporates such material together with steam exploded *Salix* and manure, was tested in mesophilic semi-continuous laboratory scale experiments. Effects of recycling the ammonium (NH_4^+-N) rich liquid digestate were as well analysed, and recovery of NH_4^+-N and phosphate (PO_4^{3-}) from the rejected digestate via magnesium ammonium phosphate (struvite) precipitation and bentonite adsorption was performed. Incorporation of fish byproduct increased the methane yields by 35 %, while recirculation of the digestate increased the yield only slightly. NH_4^+-N levels reached 5 g/L, and although this may threaten methanogenesis, the stability of the process was maintained along the whole period due to the good balance between lignocelluloses, proteins and fats. Solids accumulation in the recirculation reactors was moderate due to an enhanced separation of the digestate fraction to recycle. NH_4^+-N and PO_4^{3-} reductions from the digestate reached 87 % and 60 % with struvite, respectively (pH 9.5 and Mg^{2+}:NH_4^+:PO_4^{3-} ratio of 1.2:1:1), and 82 % and 52 % with bentonite.

Keywords: Fish byproduct, liquid digestate recirculation, ammonium-N, struvite, bentonite adsorption.

1 Introduction

Anaerobic digestion (AD) under Norwegian conditions requires optimization in order to be an applicable energy production process. The cultivated land represents only 3 % of Norway's total area (Statistics Norway, 2008) and therefore, biomass available for biogas production must also come from other sectors, such as industrial, municipal and forestry. Fish processing is a vast industry in Norway, the Norwegian fisheries produce more than 815,000 tons of byproducts annually (1 ton represents 1000 kg), which is more than 30 % of all the fish caught and farmed in Norway (Rubin, 2012). The by-products are generated when the fish is gutted, headed and further processed. Most of the by-products are used today as raw materials for feed production; such as fish meal, silage and feed for fur animals. About 195,000 tons are still dumped into the sea (Rubin, 2012).

According to the Norwegian Food and Safety Authority (Mattilsynet, 2008), the category 2 material of fish origin was estimated in 40-50 thousand tons in 2009, and it consists on the "material with risks of animal and fish diseases and material with residues of drugs content over the limit, that can be used for technical purposes or compost and biogas, after being stabilized using an approved method" (Mattilsynet, 2008). The amount is expected to increase by ca. 5% annually, in line with the general increase in aquaculture volume (Rubin, 2012). Because this type of byproduct may contain disease infected fish, it cannot be used for feed production, so it has to be hygienised and then either disposed or treated in alternative ways, such as biogas production. The hygienisation treatment performed in Norway, approved by the Norwegian Animal Health Authority in 1994 (forskrift av 13.juli 1994 nr.723) is an adaptation of the EU regulation EC No 1774/2002 (Method 1, Annex V) later replaced by EC 1069/2009 and EU 142/201. It consists mainly in ensiling of the material with formic acid to pH less than 4 for more than 24 hours, and a heat treatment of at least 85 ºC for 25 minutes. After the hygienisation, the material is filtrated so to separate the oil fraction to the greatest extent. This method is often called "Fish Silage Processing Method" (FSPM) (Norwegian Scientific Committee for Food and Safety, 2010). Fish wastes normally contain higher lipid and protein content than cattle manure (Callaghan et al., 1998) but the category 2 material has been separated from its oil, containing mostly the protein fraction. This can translate in a greater potential for producing ammonium ions or free ammonia, being this last an inhibitor of the AD process (Angelidaki and Ahring, 1993; Callaghan et al., 1998). According to literature, inhibition can occur at the wide concentration range of total ammonium nitrogen (TAN) from 1.4 to 17 g/L (Zeeman et al., 1985; Angelidaki and Ahring, 1993; Chen et al., 2008). Therefore, co-digestion with carbon rich material as lignocelluslosic biomass would balance the process.

Salix is a short rotation energy crop found vastly in the Nordic countries, but as for all lignin rich materials that aim to be microbiologically degraded, pre-treatment to make the recalcitrant structure more accessible is mandatory. Results have shown that when applying steam explosion to Salix, an increase on the methane yield of up to 50 % can be achieved by employing temperatures of at least 210 ºC and short retention times (Horn et al., 2011a; Estevez et al., 2012a). Codigestion of Salix and manure in continuously stirred tank reactors (CSTR) with liquid digestate recirculation proved to give better methane yield than if recirculation was not applied, achieving a 16 % increase in the yield (Estevez et al., 2012b **submitted**). The stability of the process was also improved due to the recirculation, but in the long term, solids accumulation, more pronounced in the recycling reactors, caused a decrease on the yield.

The aim of this study is to investigate the effects that higher ammonium-N (NH_4^+-N) levels would have in the stability of an anaerobic co-digestion process that includes recirculation of the digestate. Improved solids separation to the recirculated digestate will be tested as enhancer of process long term stability.

The higher ammonia content provided by the co-digestion of fish byproduct and by the recirculation of digestate, and the partially longer retention time caused by the last one, may as well favor the final digestate, and thus, biofertilizer nutrient composition. In addition, phosphate (PO_4^{3-}), another important fertilizing nutrient found in agro-industrial and AD effluents, is a resource whose natural viable reserves will be soon depleted (Gonzalez Ponce and Garcia Lopez De Sa, 2007; Massey et al., 2009) and alternatives to recover it need to be further investigated. One of the most promising techniques for recovering both phosphorous and ammonium is to precipitate them together as magnesium ammonium phosphate hexahydrate:

$$Mg^{2+}+NH_4^{+}+PO_4^{3-}+6H_2O \leftrightarrow MgNH_4PO_4.6H_2O\ (s) \qquad (1)$$

Commonly known as struvite, this material provides a slow release fertilizer (Altinbas et al., 2002). In addition, accumulation of struvite is a frequent problem at wastewater treatment plants, causing clogging of pumps and pipes downstream of the sludge dewatering system of anaerobic digestion and post-digestion processes. This reduces process capacity and efficiency, leading to an important increase in the cost of the sludge management operations (Parsons and Doyle, 2004). So struvite recovery after AD would also help in avoiding such problems.

Another method for removal and recovery of nutrients is adsorption. This method is based on the large porous surface area of an adsorbent. Clay minerals as zeolites and bentonites seem to be as well promising materials for the removal of NH_4^+ ions from wastewater, they can act either as strong acid adsorbents or ion exchange materials in this removal process (Saltali et al., 2007; Eturki et al., 2012). They possess low cost and high competitiveness compared to biological and chemical treatments (Saltali et al., 2007; Eturki et al., 2012). A high negative charge of the bentonite surfaces is usually balanced by alkali metals and cations (typically sodium (Na^+) and calcium (Ca^{2+})). In the sorption- removal of phosphate, bentonite cations can be replaced by inorganic hydroxyl-metal polycations such as aluminum and iron, acting as pillars which increase the interlayer spacing of bentonite (Yan et al., 2010). Theoretical adsorption capacity of a material for a particular molecule can be determined by developing adsorption isotherms, as Freundlich and Langmuir models (Saltali et al., 2007).

For this study, struvite precipitation and bentonite adsorption of NH_4^+ and PO_4^{3-} from the anaerobic digestion liquid digestate were tested as recovery methods, so to obtain an enriched biofertilizer. These techniques could also be solutions to decrease ammonium content in the case of a rising level in the recirculated digestate that may threaten the process stability.

Very few scientific studies address co-digestion of fish processing related wastes or byproducts with other organic substrates. The category 2 material of fish origin, without oil, has not been included before in anaerobic digestion trials, and being an interesting substrate to address for the Norwegian fishing industry, it is evaluated in this study.

2 Materials and methods

2.1 Steam exploded *Salix*

Samples of *Salix* “Tora” (*Salix Orm x S. viminalis*) of age 4 years, harvested in Uppsala, Sweden, with 41.9 % Total Solids (TS), were received chopped to a 0.5 cm particle size and frozen. The material was pretreated by steam explosion at the unit described in Horn et al. (2011b), run at 210 ºC and 10 minutes of retention time. The pretreated material was handled in vacuum sealed polyethylene bags that were kept refrigerated at 4 ºC to be added to the reactor's substrates mixtures. Some characteristics of the steam exploded material are shown in Table 1.

2.2 Cow manure

Fresh cow manure was employed, obtained from the University farm, in a 20 Kg container, kept in a cooling room at 4 ºC to be fed to the reactors. Details are presented in Table 1.

2.3 Fish byproduct

Category 2 fish byproduct was provided by the company BIOKRAFT MARIN AS, Trondheim, Norway. This material underwent the FSPM pretreatment and it was kept frozen at 4 ºC. Some characteristics of the material are given in Table 1.

Table 1. HERE

2.4 Inoculum

The inoculum was collected from the biogas farm Åna, in Rogaland, Norway, that digests cow manure and fish silage. Since it had a high content on nitrogen and volatile solids (VS) (Table 1) it was diluted with water to a VS level of 2 % and NH_4^+-N of 3200 mg/L.

2.5 Methodology

2.5.1 Semi-continuous co-digestion

Four 10 L CSTR (Belach Bioteknik AB, Sweden), with a nominal working volume of 6 L, were run at a temperature of 37 ºC and stirrer speed of 180 rpm. The reactor's software (BIOPHANTOM©) allowed continuous, real-time monitoring of pH, stirrer speed, temperature, gas flow and gas volume produced. For starting up the reactors 3 L of the diluted Åna inoculum were fed to them on the first day and maintained for a week to reduce endogenous methanogenic production. The feeding of the substrates started with a low organic loading rate (OLR: 1 g VS/Ld) of the respective substrate mixtures and was successively increased until reached 3 g VS/Ld. The start-up period lasted 5 weeks.

In the experimental period, the reactors were fed once a day, 6 days a week, with 200 mL of daily fresh prepared feedstock mixtures, an OLR of 3 g VS/Ld (one whole week daily load of 2.6 g VS/L) and a hydraulic retention time (HRT) of 30 days, according to the feeding scheme detailed in Table 2. Recirculation of the liquid digestate took place in three of the four reactors (GA2, GB1 and GB2), in which the volume removed before the daily feeding was filtered through 2.5 and 1 mm mesh size sieves, and its liquid fraction used for diluting the fresh feedstock and so added back to the digester (approx. 100 mL). For reactor GA1, dilution of the feedstock to 200 mL was made with tap water. For the specific methane yield calculations only the fresh daily VS additions were considered. The anaerobic digestion process was followed for a period of 132 days (4.4 HRT).

Table 2. HERE

2.5.2 Chemical precipitation for recovery of nutrients (Struvite trials)

Struvite precipitation trials were performed in duplicate for the estequiometric ratios 1:1:1 and 1.2:1:1, proven to give good results (Celen and Turker, 2001; Altinbas et al., 2002). $MgCl_26H_2O$ and KH_2PO_4 were added according to stequiometric calculations (Table 3) to 300 mL aliquots of the sieved (2.5 and 1 mm mesh) liquid digestate from reactor GA2 that was accumulated along the whole process period and kept refrigerated at 4 ºC. The content was agitated at 300 rpm for 15 minutes and let to settle for 15 minutes more, before being divided into 4 aliquots containing 45 mL each. These aliquots were adjusted to pH 7, 8, 9, and 9.5 with a NaOH 1 N solution. Determination of NH_4^+-N, PO_4^{3-} and soluble chemical oxygen demand (COD) content in the supernatants were followed spectrophotometrically after a 24 hrs. settling period, for the samples that showed a visible sedimentation of precipitate.

Table 3. HERE

2.5.3 Adsorption for recovery of nutrients (Bentonite trials)

Adsorption trials employing commercial Na^+ bentonite were performed in triplicate by adding 1.5, 3, 4, 6, and 9 g. of bentonite to 50 mL aliquots of GA2's sieved liquid digestate. The mixture was agitated at 600 rpm for 5 minutes and a settling period of 24 hrs. was waited before spectrochemical analysis of the supernatants for NH_4^+-N , PO_4^{3-} and soluble COD were performed.

2.5.4 Analytical tools

Elemental analysis for determination of TOC and total-N on the fresh substrates, and subsequently their C/N ratio, were performed as stated in Estevez et al. (2012a). These analyses were also performed to the final digestate fractions, together with total phosphorous (P), potassium (K) and heavy metals determinations by inductively coupled plasma-mass spectrometry (ICP-MS) after an ultra-clave digestion in concentrated, double-distilled HNO_3 (0.25 g to 0.3 g sample in 5 ml) and subsequent dilution to 50 ml.

The biogas produced in the CSTR was collected in polyethylene bags and its composition was measured by gas chromatography in a SRI gas chromatographer (Model 8610 C) equipped with a thermal conductivity detector (TCD) and a 2 m. Haysep-D column. The operational temperatures of injector, detector and column were kept at 41, 153 and 81 ºC respectively. Helium was used as a carrier gas at a flow rate of 20 mL/min. A gas mixture of CH_4/CO_2 65/35 % was used as standard for calibration. The chromatogram was analysed using the PeakSimple 3.67 program.

NH_4^+-N, TS and VS content in the reactors were determined once a week while Total-N and total COD were determined every 10 days. The concentration of NH_4^+-N in the reactors was followed with an Ion Selective Electrode (Orion-Thermo Scientific©), while Total-N and COD were analysed spectrophotometrically employing Merck Spectroquant® Kits Analyses.

TS content was determined according standard oven drying method (APHA, 1995) except for fish byproduct and pre-treated Salix, to whom Karl Fisher water content titration was applied. In all cases, VS was indirectly determined from the ash residue obtained after 550 °C calcination. The Karl Fisher titration method was chosen to apply when using substrates whose oven drying results can be imprecise due to their high oil content, as fish byproducts, or acidic nature, as ensiled materials and steam exploded Salix. This last ones may lost a fraction of their volatiles compounds when performing oven drying at 105 °C, estimated to be of ca. 2-5 % (Porter and Murray, 2001). Being VS determination a crucial parameter in which the methane production yields rely, a method in which loss of such compounds does not occur needs to be followed. The equipment used was a Metrohm automated volumetric system (Tampa, USA) composed of an oven sample unit (Model 774), a dosing device with burets (Model 901) and a titration cell (Model 801). CombiTitrant 5 from Merck (Darmstadt, Germany) was the titrant employed, containing iodine, sulfur dioxide, base and an alcohol; while dry methanol was the working medium in the titration cell.

Samples for volatile fatty acids (VFA) content were collected weekly and analysed by HPLC on a Dionex Ultimate 3000 chromatographic system with UV detection. VFAs were measured at 210 nm with a Zorbax Eclipse Plus C18 column from Agilent Technologies, 150x2.1 mm (3.5 mm particles), equipped with guard column of the same brand, 12.5x2.1 mm, (5 mm particles). The column is based on usual reversed phase C18 technology (dimethyl-n- octadecyl double endcapped silica).Operating conditions were: column temperature of 40 °C, flow of 0.3 mL/min; eluents employed were 100 % Methanol and 2.5 mM H_2SO_4; sample injection volume of 1 mL. Samples were acidified prior to analyses to pH <2.5 with concentrated H_2SO_4 (72 %) and centrifuged two times, 10 minutes at 14000 rpm to remove any particulate matter.

Long chain fatty acids (LCFA) analyses on the fish byproduct and on the inoculum were performed by the company Eurofins, Moss, Norway, according to the AOCS method 1c-85 based on methylesterefication and gas chromatography.

Magnesium (Mg), total phosphorous (P), PO_4^{3-}, NH_4^+-N content and soluble COD determinations were performed, when appropriate, in the accumulated digestate, struvite and bentonite trials by spectrophotometric Merck Spectroquant® Kits Analyses. When the method required, samples were diluted and/or centrifuged (6000 rpm, 5 minutes) before being filtrated through a 0.45 μm glass fiber filter. All analyses were performed in triplicate.

2.5.5 Statistical analyses

Reported standard deviations were calculated using statistical functions of Microsoft Excel 2007. To evaluate the relationship between paired experimental data, the statistical software Minitab® 16.1.1 was used. A two sample t-test was used to compare methane production profiles. The results were assessed with p-values to reflect the statistical significance (confidence level 95 %).

3 Results and discussion

3.1 Process stability and methane production

In average, the specific daily methane yields along the whole four month period in mL CH_4/g VS were 180.5 ± 43.0 for GA1, 191.3 ± 39.9 for GA2, 159.3 ± 34.7 for GB1 and 140.9 ± 26.6 for GB2. The average yield was 6 % higher for GA2 than for GA1, though the maximum daily yield achieved was 279-292 mL CH_4/g VS for GA1 (Figure 1) compared to

270 mL CH_4/g VS for GA2. Both reactors were fed with the same substrate mixture, composed of steam exploded Salix, manure and fish byproduct, but in GA2 recirculation of the digestate took place. Statistically, the recirculation effect was found significant when comparing the two profiles (two sample t-test; p=0.034). A higher ammonium content (up to 70 % more) was reached for GA2 in comparison to GA1 (Figure 2), and thus better buffer capacity, preservation of trace-elements and enhanced adaptation to changes for an enriched microbial community may occur in this reactor (Hartmann and Ahring, 2005).

Comparing both Salix clones and their methane yields when co-digested with cow manure and digestate recirculation was in place, the co-digestion of *Salix* Tora gave in average 140.9 ± 26.6 mL CH_4/ g VS while *Salix viminalis* yield was in average 159.3 ± 27.2 mL CH_4/ g VS (Estevez et al., 2012b **submitted**). This last one possessed a smaller C/N ratio (64 vrs. 85) and also, a small overestimation of the yield (ca. 5 %) may have occurred due to losses in drying, while for the *Salix* Tora the dry matter content was determined with a more precise approach: Karl fisher titration. These reasons could explain the difference when mixed with manure (Estevez et al., 2012b **submitted**).

Comparing the yields from the reactors fed with fish byproduct, GA1 and GA2, with GB2 fed only with steam exploded Salix and manure, both showed to be significantly different from GB2 (p=0.000 for both reactors). The yield for GA1 and GA2 was in average 28-35 % higher, so the fish byproduct proportion of 20 % VS in the mixture (7.2 % in weight) achieved a considerable increase. When this VS proportion was instead 10 % in GB1 (3.7 % in weight), the increase in the yield comparing to GB2 was only of 13 % in average. The potential yields that the mixtures would have achieved were theoretically calculated taking into account the maximum yields achieved for each substrate in previous biomethane potential trials (BMP) and their fraction of VS applied in the feedstock mixture. For GA1 and GA2 the theoretical maximum yield would be 291 mL CH_4/g VS while GB1's would be 269 mL CH_4/g VS. This is not far from our experimental results (Figure 1).

Fig. 1 HERE

Comparing to studies where co-digestion of fish waste with other substrates were performed, this lead to an increase by 8 % in the yield in a sequential batch reactor process when it was co-digested together with fruit and vegetable waste (1,4 % fish waste in the mixture) giving a yield of 320 mL CH_4/g VS added (Bouallagui et al., 2009). Callaghan et al. (1998) found that increasing the fraction of solid fish waste in the continuous codigestion with cattle manure at high OLR (5.1- 6.3 g VS/ L d) produced deterioration of the process due to long chain fatty acids (LCFA) inhibition; maximum yield achieved were between 270 and 300 mL CH_4/g VS added when the fish fraction was 4 %. Similarly, Eiroa et al. (2012) also reports inhibitory problems due to LCFA and VFA, when performing the BMP essays for different solid fish wastes, giving the mackerel type; with a higher fat content; the higher accumulation on LCFA. Table 4 shows a comparison of the category 2 substrate with other types of solid fish waste. Category 2 underwent a pre-treatment and homogeneization, and even though the lipid content is reduced in our material, the amount is comparable to that one of fish offal and bigger than the tuna processing fish waste. During the experimental period, the content of VFA showed initial levels of propionic acid (maximum was 1600 mg/L) for the reactors to whom fish was fed, but no signs of inhibition (Fig. 3). Analysis of VFA done to the inoculum and the fish byproduct, showed only small quantities of acetic acid present on the first one (50 mg/L), while the fish byproduct had a concentration of around 2000 mg/L in total VFA (1200 mg/L of acetic and

480 mg/L of propionic). The increase in propionic acid during the first HRT of the process may be cause of adaptation to the feeding of fish byproduct.

LCFA were also analyzed for the inoculum and fish byproduct substrate, and detected only for the last one. Unsaturated ones such as oleic (35 % of total fatty acids) and linoleic (11 % of total fatty acids) were the predominant. Although the process did not showed signs of LCFA inhibition, previous studies have demonstrated that even low concentrations of these substances can cause toxic effects (Angelidaki and Ahring, 1992). These effects were also obtained when fish wastes of similar composition as the category 2 were anaerobically degraded (Callaghan et al., 1998; Eiroa et al., 2012). During anaerobic digestion, oil and fat are very quickly hydrolized to LCFA and glycerol (Ahring et al., 1992; Angelidaki and Ahring, 1992). LCFA contain most of the oil's energy and are thus, excellent substrates for biogas production; via ß oxydation LCFA are converted into acetate and hydrogen, which are finally turned into methane by the methanogens consortia (Angelidaki and Ahring, 1992; Sousa et al., 2008; Eiroa et al., 2012). But if accumulation of these free fatty acids occurs, bacterial growth and biogas production can be inhibited (Angelidaki and Ahring, 1992; Rinzema et al., 1994). Toxic effects are manifested in the adsorption of LCFA onto the cell membrane, and interferences in the transport or protection mechanisms (Rinzema et al., 1994; Eiroa et al., 2012). Nonetheless, some studies demonstrated that this type of inhibition is reversible, with process that recover after LCFA concentrations decrease (Pereira et al., 2004) or with process that can adapt to high lipid levels if fat is being added in continuously repeated cycles (Sousa et al., 2008).

Table 4. HERE

The ammonium-N content increased along the period mostly for the recirculating reactors containing fish byproduct, GA2 and GB1, while the reactor with recirculation but without fish kept its ammonium-N content stable (GB2) (Figure 2). In GA1, where fish was added but recirculation was not performed, the increase was less pronounced comparing to GA2 and GB1. Maximum levels of NH_4^+-N reached 5 g/L for GA2 with a process running stable, a level that according to literature can produce inhibition of the methanogenesis (Angelidaki and Ahring, 1993; Chen et al., 2008). This confirms that a good balance of lignocelluloses and N rich materials was achieved with the feeding mixture. An inoculum already adapted to high nitrogen levels due to the digestion of fish waste may have benefited the fast acclimation of the microorganisms to the substrates and to the high NH_4^+-N levels. Most of the studies have shown that acetoclastic is the group of methanogens more susceptible to free ammonia inhibition, rather than hydrogenotrophic bacteria (Koster and Lettinga, 1984; Zeeman et al., 1985; Angelidaki and Ahring, 1993; Calli et al., 2005). But methane production from acetate can follow an additional pathway that besides the traditional methanogens, involves another group of non-methane-producing bacteria which is less affected by ammonia. These microorganisms convert acetate into H_2 and CO_2, which later are used by the hydrogenothrophs to produce methane. This cooperation is called syntrophic acetate oxidation (SAO) (Schnürer et al., 1994). The shift to SAO is related to microbial adaptation to high ammonia content. The co-culture responsible for SAO poses longer doubling time than methanogens (Schnürer et al., 1994) and thus, recirculation of the digestate may promote the change to SAO pathway and the adaptation to high ammonia levels.

Fig. 2 HERE

An estimation of total COD degradation was calculated approximately 43-50 % for the reactor without recirculation, GA1. Recirculation of the digestate produced higher values of total COD during the process for GA2, total COD degradation in GA2 was approximately 43 %. Volatile solids accumulation is an issue important to address when performing digestate recirculation. VS content profiles showed an overall increasing tendency when looking at the whole experimental period (Figure 3), although increments were less pronounced as in a previous experiment where steam exploded Salix was co-digested with manure and digestate recycled (Estevez et al., 2012b **submitted**). The reactor without recirculation presented the lowest VS content, though all profiles were similar. At the end of the period the VS content inside the reactors reached 6-7 % with recirculation, giving thus a 1.5 times increase, while in the previous study the increase reached 2.6 times the original VS content. Incorporation of a 1 mm mesh in addition to the 2.5 mm provided a better separation of recalcitrant material, so that process could run more stable. VS degradation, also taking into account VS added with the recycled digestate, were similar than for the previous study, reaching averages values of 39 % for GA1 without recirculation, 37 %, 35 % and 32 % for reactors GA2, GB1 and GB2 respectively. GA1 and GA2 gave very similar VS degradations, and with regard to the methane yields, the recirculated reactor one was slightly higher, possibly due to stability enhancement due to longer hydraulic and solids retention time. VS degradations was lower for the other recirculated reactors, this result was also showed in studies by Nordberg et al. (2007) and Hartmann and Ahring (2005), stating that a proper control of such systems for avoiding accumulation of recalcitrant substances, is mandatory.

Fig. 3 HERE

3.2 Nutrients mineralization and recovery

For all digestates, the amount of NH_4^+-N on the feedstock mixture before anaerobic digestion counts for ca. 36 % of the total-N, while after the process, NH_4^+-N represents 94 % of the total-N in the digestate. This NH_4^+-N remains mostly (70 %) in the liquid fraction after being separated with the mesh. Nitrate (NO_3^-) was found at very low concentrations and mostly in the liquid fraction (82-94%). Phosphate accounted for ca. 92% of the total-P in the digestate with fractions in the liquid and solid digestate of 64 % and 36 % respectively. The digestates showing higher values of both NH_4^+-N and PO_4^{3-} were the ones corresponding to the co-digestion of fish byproduct, GA2 and GB1, with 70 % and 40 % higher NH_4^+ levels than GB2, respectively. PO_4^{3-} content was 18-28 % higher for the reactors co-digesting fish. GA2's digestate was the one chosen to perform NH_4^+ and PO_4^{3-} recovery trials.

Heavy metal content of the accumulated digestates and of the separated solid fraction were compared to the Norwegian standards for organic biofertilizers (Landbruks og matdepartementet, 2003). The solid fractions coming from the digestion of Salix and manure possessed lower levels in Zn, Cd and Cr than the ones coming from the digestion of such mixture and fish. Zn and Cd were the only values not fulfilling the levels for the next best class of fertilizer (class 0) for the accumulated digestate (Table 5), while only Cd was limitant in the case of the digestate's solid fraction. The fitting quality level for the digestates was then as organic fertilizer class I (Table 5). The N:P:K ratio of the accumulated digestates was approximately 4:1:7, having the solid fractions with fish 3:1:4 and without fish 2:1:5. A low N:P ratio would translate in good soil amendment for fruit trees, carrots or other root vegetables (Gebauer and Eikebrokk, 2006) but recovered NH_4^+ from the liquid fraction and its incorporation to the solid one can improve such ratio.

Table 5 HERE

3.2.1 Struvite trials

Results of the reductions of NH_4^+-N, $PO4^{3-}$ and soluble COD achieved on the liquid phase of GA2's digesate after struvite precipitation are presented in Figure 4. Analyzing the samples after 24 hours settling time, best sedimentation results were obtained for the pH 9 and 9.5 at both stequiometric ratios. Samples at pH 7 and 8 did not always achieved a clear phase separation after 24 hrs; whenever the supernatant was possible to analyzed, NH_4^+-N reduction levels were similar as for pH 9 and 9.5. It is important to notice that a longer settling time, e.g. 48 hrs, showed a clearer sedimentation even for pH 7 and 8. NH_4^+-N reductions levels were comparable to those obtained by Celen and Turker (2001), Altinbas et al. (2002) and Yetilmezsoy and Sapci-Zengin (2009) for the pH bewteen 9-9.2 and stequiometric ratios of 1:1:1 and 1.5:1:1.

Fig. 4 HERE

Reduction of the original PO_4^{3-} content was not as clear as for NH_4^+-N since levels where not high in the liquid fraction (Table 3) and KH_2PO_4 was added to reach stequiometry. Positive reduction was only possible for the 1.2.1:1 ratio, while best overall results were achieved at pH 9.5. The sediment obtained, rich in NH_4^+ and some PO_4^{3-}, could be incorporated to the separated solid fraction, in order to enhance such material further as biofertilizer. Analysis of the effects of applying such fraction as fertilizer in field experiments will be performed in future studies. Struvite has been studied as a favorable slow release fertilizer in acidic and neutral soils (Gonzalez Ponce and Garcia Lopez De Sa, 2007; Yetilmezsoy and Sapci-Zengin, 2009) as well as in alkali soil types (Massey et al., 2009).

3.2.2 Bentonite adsorption trials

Adsorption results are presented in Figure 5. NH_4^+-N was adsorbed up to 76-82 %, with added quantities of bentonite equal or bigger than 4 grs per 50 mL digestate. These results fit with the Freundlich adsorption isotherm model (Fig. 5) which is applicable to non-specific adsorption in a heterogeneous solid surface (Eturki et al., 2012). In its logarithmic form is expressed as:

$$\log q = \log K_f + \frac{1}{n} \times \log C \qquad (2)$$

being C the equilibrium concentration of NH_4^+ after adsorption took place (mg/L), q the mass of NH_4^+ taken up per unit mass of bentonite (mg /g), K_f and n the constants of Freundlich model (Saltali et al., 2007). Our parameters were: K_f= 1.93x10^{-6}, n= 0.39012 and R^2=0.8028.

Fig. 5 HERE

Regarding removal of PO_4^{3-} and soluble COD, results showed removal of the initial content levels not surpassing 40-50 % (Fig. 5). COD removal followed a similar trend than NH_4^+-N, while PO_4^{3-} was removed better the lower the doses of bentonite applied.

While struvite precipitation made possible to recover together NH_4^+ and PO_4^{3-} in a greater extent than bentonite adsorption, this last one does not need addition of extra chemicals besides the bentonite.When only NH_4^+-N is aimed to be removed and recover, bentonite seems as a good strategy. The more feasible method to adopt has to be chosen depending on the costs and availability of employing chemical reagents or bentonite, and on the soil conditions.

4 Conclusion

It was possible to digest a highly organic and nitrogen rich material as fish byproduct category 2 with cow manure and pre-treated Salix for a period of 134 days, giving better yields than codigesting Salix and manure alone, even in the recirculated process, while giving a high ammonium-N value that did not produced inhibition. This process may benefit from improved stability due to enriched nutrients, micronutrients and microbial biomass content. The stable process produced an ammonium rich digestate. In order to improve it further as soil amendment, recovery of N and P of the accumulated not recycled digestate was possible through struvite precipitation and bentonite adsorption, giving struvite trials the best results in terms of NH_4^+-N removed (87 %) and PO_4^{3-} removed (60 %) at the same time.

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References

Ahring, B. K., Angelidaki, I., Johansen, K., 1992. Anaerobic treatment of manure together with industrial waste. Water Science and Technology 25: 7, 311-318.

Altinbas, M., Öztürk, I., Aydin, A. F., 2002. Ammonia recovery from high strength agro industry effluents. Water Science and Technology 45: 12, 189-196.

American Oil Chemists' Society, AOCS Official Method Cd 1c-85.

Angelidaki, I., Ahring, B. K., 1992. Eects of free long-chain fatty acids on thermophilic anaerobic digestion. Applied Microbiology and Biotechnology 37, 808-812.

Angelidaki, I., Ahring, B. K., 1993. Thermophilic anaerobic digestion of livestock waste: the effect of ammonia. Applied Microbiology and Biotechnology 38, 560-564.

APHA, 1995. Standard Methods for the Examination of Water and Wastewater.United Book Press.

AOCS, 2009. AOCS Official Method Cd 1c-85: determination of the iodine value for edible oils directly from fatty acid compositions. Reapproved 2009.

Bouallagui, H., Lahdheb, H., Romdan, E. B., Rachdi, B., Hamdi, M., 2009. Improvement of fruit and vegetable waste anaerobic digestion performance and stability with co-substrates addition. Journal of Environmental Management 90, 1844-1849.

Callaghan, F. J., Wase, D. A. J., Thayanithy, K., Forster, C. F., 1998. An examination of the continuous anaerobic co-digestion of cattle slurry and fish offal. Trans IChemE 76, Part B, 224-228.

Calli, B., Mertoglu, B., Inanc, B., Yenigun, O., 2005. Effects of high free ammonia concentrations on the performances of anaerobic bioreactors. Process Biochemistry 40, 1285-1292.

Celen, I., Turker, M., 2001. Recovery of ammonia as struvite from anaerobic digester effluents. Second International Conference on Recovery of Phosphorous from Sewage and Animal Wastes, March, 12-13, Noordwijkerhout, Holland.

Chen, Y., Cheng, J. J., Creamer, K. S., 2008. Inhibition of anaerobic digestion process: A review. Bioresource Technology 99, 4044-4064.

Eiroa, M., Costa, J. C., Alves, M. M., Kennes, C., Veiga, M. C., 2012. Evaluation of the biomethane potential of solid fish waste. Waste Management 32, 1347-1352.

Estevez, M. M., Linjordet, R., Morken, J., 2012a. Effects of steam explosion and co-digestion in the methane production from Salix by mesophilic batch assays. Bioresource Technology 104, 749 -756.

Estevez, M. M., Sapci, Z., Linjordet, R., Schnürer, A., Morken, J., 2012b. Semi-continuous anaerobic co-digestion of cow manure and steam exploded Salix with recirculation of liquid digestate. Submitted.

Eturki S., Ayari, F., Jedidi, N., Dhia, H. B., 2012. Use of clay mineral to reduce ammonium from wastewater. Effect of various parameters. Surface Engineering and Applied Electrochemistry 48:3, 276-283.

European Commission, 2002. EC No 1774/2002 regulation, Method 1, Annex V. Available at: http://eurlex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:273:0001:0001:EN:PDF.

Gebauer, R., Eikebrokk, B., 2006. Mesophilic anaerobic treatment of sludge from salmon smolt hatching. Bioresource Technology 97, pp. 2389-2401.

Gonzales Ponce, R. and Garcia Lopez De Sa, M. E., 2007. Evaluation of struvite as a fertilizer: a comparison with traditional P sources. Agrochimica 51: 6, 301-308.

Hartmann, H., Ahring, B. K., 2005. Anaerobic digestion of the organic fraction of municipal solid waste: Influence of co-digestion with manure. Water Research 39, 1543-1552.

Horn, S. J., Estevez, M. M., Nielsen, H. K., Linjordet, R., Eijsink, V. G. H., 2011a. Biogas production and saccharification of Salix pretreated at different steam explosion conditions. Bioresource Technology 102, 7932-7936.

Horn, S. J., Nguyen, Q. D., Westereng, B., Nilsen, P. J., Eijsink, V. G. H., 2011b. Screening of steam explosion conditions for glucose production from non-impregnated wheat straw. Biomass and Bioenergy 35:12, 4879-4886.

Koster. I. W., Lettinga, G., 1984. The influence of ammonium-nitrogen on the specific activity of pelletized methanogenic sludge. Agricultural Wastes 9, 205-216.

Landbruks og matdepartementet, 2003. Forskrift om gjødselvarer mv. av organisk opphav (in Norwegian). FOR 2003-07-04 nr 951. Available at: http://www.lovdata.no/for/sf/ld/xd-20030704 -0951.html.

Massey, M. S., Davis, J. G., Ippolito, J. A., Sheffields, R. E., 2009. Effectiveness of recovered magnesium phosphates as fertilizers in neutral and slightly alkaline soils. Agronomy Journal 101: 2, 323-329.

Mattilsynet, 2008. Veileder: utstedelse av eksportdokumenter for fiskebiprodukter (in Norwegian). Available at: http://www.mattilsynet.no/mattilsynet/ multimedia/archive/ 00039/ Veileder_for_utstede_39660a.pdf

Nordberg Å, Jarvis Å, Stenberg B, Mathisen B, Svensson B. H., 2007. Anaerobic digestion of alfalfa silage with recirculation of process liquid. Bioresource Technology 98, 104-11.

Norwegian Scientific Committee for Food and Safety, 2010. Assessment of the fish silage processing method (FSPM) for treatment of category 2 and 3 material of fish origin. http://www.vkm.no/dav/360b21640f.pdf

Parsons, S. A., Doyle. J. D., 2004. Struvite scale formation and control. Water Science and Technology 49:2, 177-182.

Pereira, M.A., Sousa, D.Z., Mota, M., Alves, M.M., 2004. Moneralization of LCFA associated with anaerobic sludge: kinetics, enhancement of methanogenic activity, and effect of VFA. Biotechnology and Bioengineering 88, 502-511.

Porter, M.G., Murray, M.S., 2001. The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. Grass Forage Science 56, 405-411.

Rinzema, A., Boone, M., van Knippenberg, K., Lettinga, G., 1994. Bactericidal effect of long chain fatty acids in anaerobic digestion. Water Environmental Research 66, 40-49.

Rubin, 2012. Foundation for the recycling and utilization of organic byproducts in Norway, http://www.rubin.no.

Saltali, K., Sari, A., Aydin, M., 2007. Removal of ammonium ion form aqueous solution by natural Turkish (Yildizeli) zeolite for environmental quality. Journal of Hazardous Materials 141, 258-263.

Schnürer, A., Houwen, F. P., Svensson, B., 1994. Mesophilic syntrophic acetate oxidation during methane formation by a triculture at high ammonium concentration. Archives of Microbiology 162, 70-74.

Sousa, D. Z., Pereira, M. A., Alves, J. I., Smidt, H., Stams, A. J. M., 2008. Anaerobic microbial LCFA degradation in bioreactors. Water Science and Technology 57:3, 439-444.

Statistics Norway, 2008. Natural resources and the environment 2008. Available at: http://www.ssb.no/english / subjects /01 / sa_nrm/ nrm2008/sa109.pdf

Yan, L., Xu, Y., Yu, H., Xin, X., Wei, Q., Du, B., 2010. Adsorption of phosphate from aqueous solution by hydroxy-aluminum, hydroxy-iron and hydroxy-iron-aluminum pillared bentonites. Journal of Hazardous Materials 179, 244-250.

Yetilmezsoy, K., Sapci-Zengin, Z., 2009. Recovery of ammonium nitrogen from the effluent of UASB treating poultry manure wastewater by map precipitation as a slow release fertilizer. Journal of Hazardous Materials 166, 260-269.

Zeeman, G., Wiegant, W. M., Koster-Treffers, M. E., Lettinga, G., 1985. The influence of the total ammonia concentration on the thermophilic digestion of cow manure. Agricultural Wastes 14, 19-35.

Table 1. Characteristics of the materials employed

Material	pH	COD (mg/L)	Total-N (mg/L)	NH_4^+-N (mg/L)	TS (%w/w)	VS (%dw/w)	C[b] (%dw/w)	N[b] (%dw/w)	C/N[b]
Salix steam exploded	3.8	n.a.	n.a.	n.a.	24.1[a]	97.0	51.2	0.6	85
Manure	7.6	52,805	2,200	1,340	11.3	85.8	45.8	2.0	22.9
Fish byproduct	3.9	377,500	13,500	1,810	25.6[a]	96	53.6	9.8	5.5
Inoculum Åna	7.8	35,425	4,900	4,400	6.9	79.2	40.6	3.9	10.4

n.a.= not available; a= water content determination by Karl Fisher; b= Total carbon and nitrogen values from dried samples.

Table 2. Feeding Scheme

Reactor	Feedstock mixture			
	Fresh substrates			Dilution ratio (substrate mix.: liquid)
	Salix (% VS)	Manure (% VS)	Fish (% VS)	
GA1	40	40	20	1:1 with water
GA2	40	40	20	1:1 with digestate
GB1	40	50	10	1:1 with digestate
GB2	40	60	__	1:1 with digestate

Table 3. Struvite trials scheme and dosage of chemicals

	Mg^{2+}	NH_4^+	PO_4^{3-}	$MgCl_26H_2O$ added (g) to 300 mL digestate	KH_2PO_4 added (g) to 300 mL digestate
Initial concs. in filtrated digestate (mg/L)	110	2,300	56		
Initial molar ratio	1	28	0.13		
Stequiometric ratio for precipitation	1	1	1	7.5	5.2
Stequiometric ratio for precipitation	1.2	1	1	9	5.2

Table 4. Comparison of fish wastes composition

Type of fish waste	TS (% w/w)	Fat (% w/w)	Protein (%w/w)	Maximum methane yield (mL/g VS)	Literature source
Tuna solid waste	37	3.74	22.6	280	Eiroa et al. (2012)
Mackerel solid waste	32	11.8	17.82	350	Eiroa et al. (2012)
Fish offal[a]	29.6	8.2	20.3		Callaghan et al. (1998)
Fish silage	50.2	30	__	450-500	Ahring et al.(1992) Rubin (2010)
Fish category 2[b]	25.6	7.4	10.2-14.8	500	

a= Fish offal accounts for the macerated mix of heads, tails and vicera produced by gutting, from a rainbow trout fish farm (Callaghan et al., 1998); b= Fish category 2 is the substrate employed and its results from its characterization.

Table 5. Heavy metal content of total accumulated digestates and digestate's solid fractions with mixtures of Salix and manure (S+M) and Salix, manure and fish (S+M+F) compared to requirements for organic fertilizers as to Norwegian regulations (Landbruks og matdepartementet, 2003).

Component	Content (mg/Kg TS)							
	acc. digestate		solid fraction		Organic fertilizer quality class			
	S+M	S+M+F	S+M	S+M+F	0[a]	I[b]	II[c]	III[d]
Cd	1.1	1.2	0.52	0.61	0.4	0.8	2	5
Pb	0.83	0.8	0.44	0.48	40	60	80	200
Hg	0.02	0.02	0.01	0.02	0.2	0.6	3	5
Ni	7.1	7.1	5	6.1	20	30	50	80
Zn	285	330	130	140	150	400	800	1,500
Cu	55	42	22	23	50	150	650	1,000
Cr	3.6	4.7	2.1	9.6	50	60	100	150

[a] Can be used on agricultural areas, private gardens, park and green areas. The amount applied must not exceed the plant's nutrient demands.

[b] Can be applied in limited amounts on agricultural areas and in private gardens and parks. Can be used unrestricted on areas without food or feed production.

[c] Can be used in more limited amounts than b on agricultural areas and private gardens or parks. Can be used unrestricted on areas without food or feed production.

[d] Can be used in limited amounts on green areas without food or feed production.

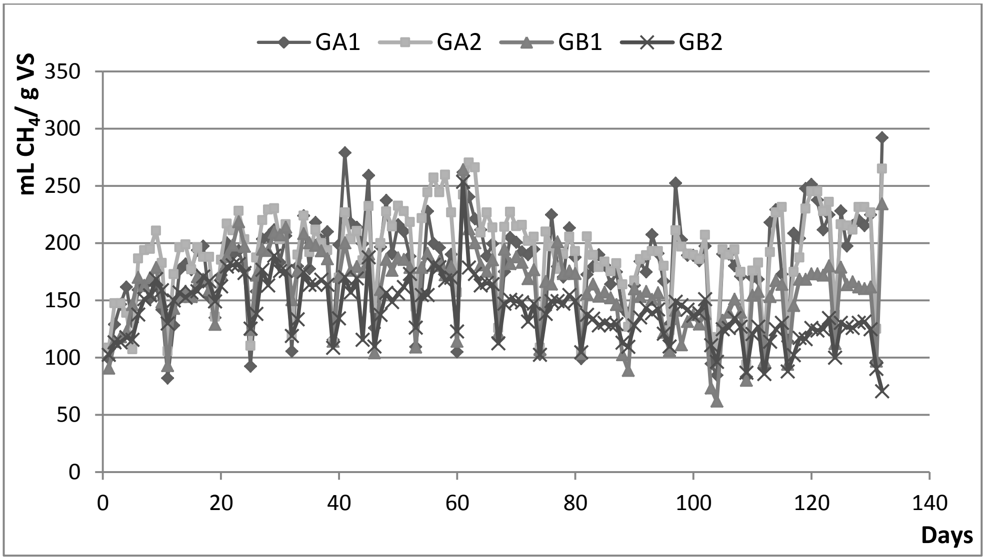

Figure 1. Specific methane yields (mL CH_4/g VS. d) for all reactors. GA1= Salix (40%), manure (40%), fish (20%); GA2= Salix (40%), manure (40%), fish (20%) with recirculation of digestate; GB1= Salix (40%), manure (50%), fish (10%) with recirculation of digestate; GB2= Salix (40%), manure (60%) with recirculation of digestate.

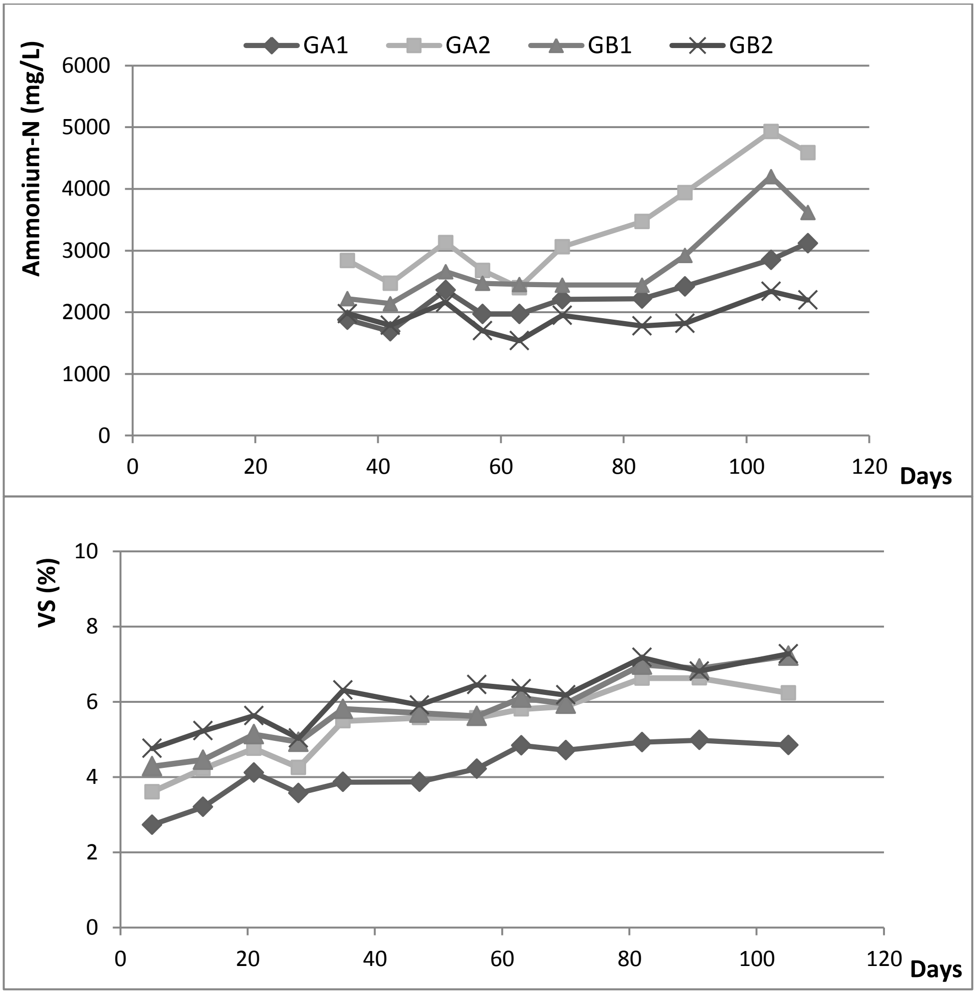

Figure 2. Ammonium-N (mg/L) and volatile solids levels (%). GA1= Salix (40%), manure (40%), fish (20%); GA2= Salix (40%), manure (40%), fish (20%) with recirculation of digestate; GB1= Salix (40%), manure (50%), fish (10%) with recirculation of digestate; GB2= Salix (40%), manure (60%) with recirculation of digestate.

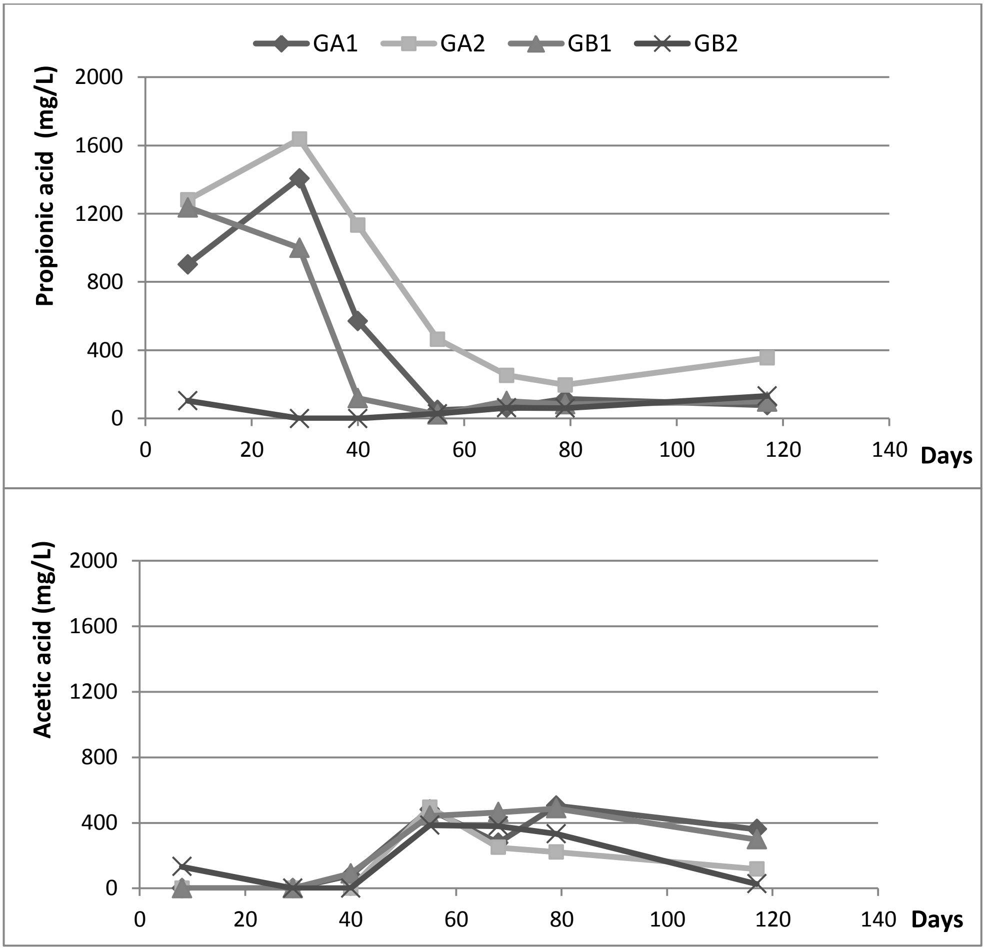

Figure 3. Concentrations (mg/L) of propionic and acetic acids. GA1= Salix (40%), manure (40%), fish (20%); GA2= Salix (40%), manure (40%), fish (20%) with recirculation of digestate; GB1= Salix (40%), manure (50%), fish (10%) with recirculation of digestate; GB2= Salix (40%), manure (60%) with recirculation of digestate.

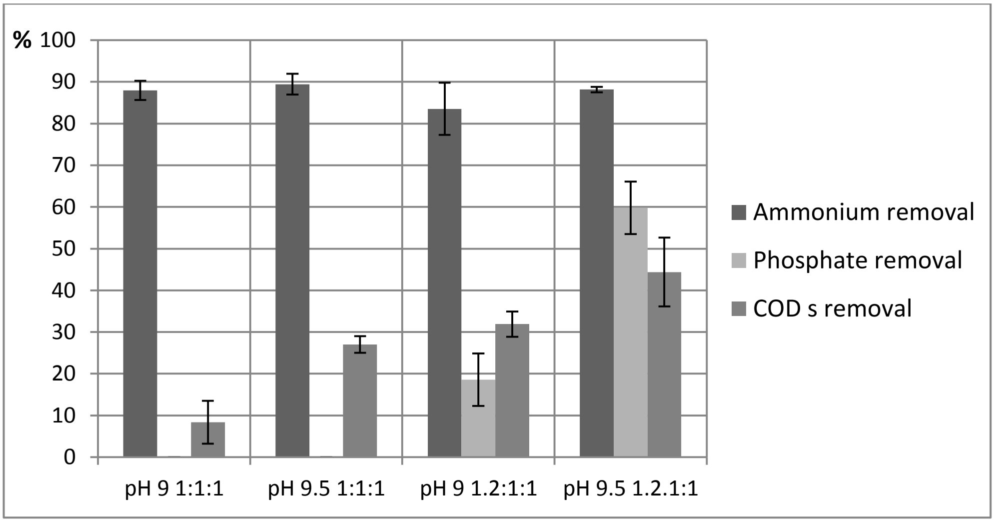

Figure 4. Struvite experiment and reductions % of Ammonium-N, Phosphate and soluble COD in the liquid filtrated digestate at stequiometric ratios of 1:1:1 and 1.2:1:1 and pHs of 9 and 9.5.

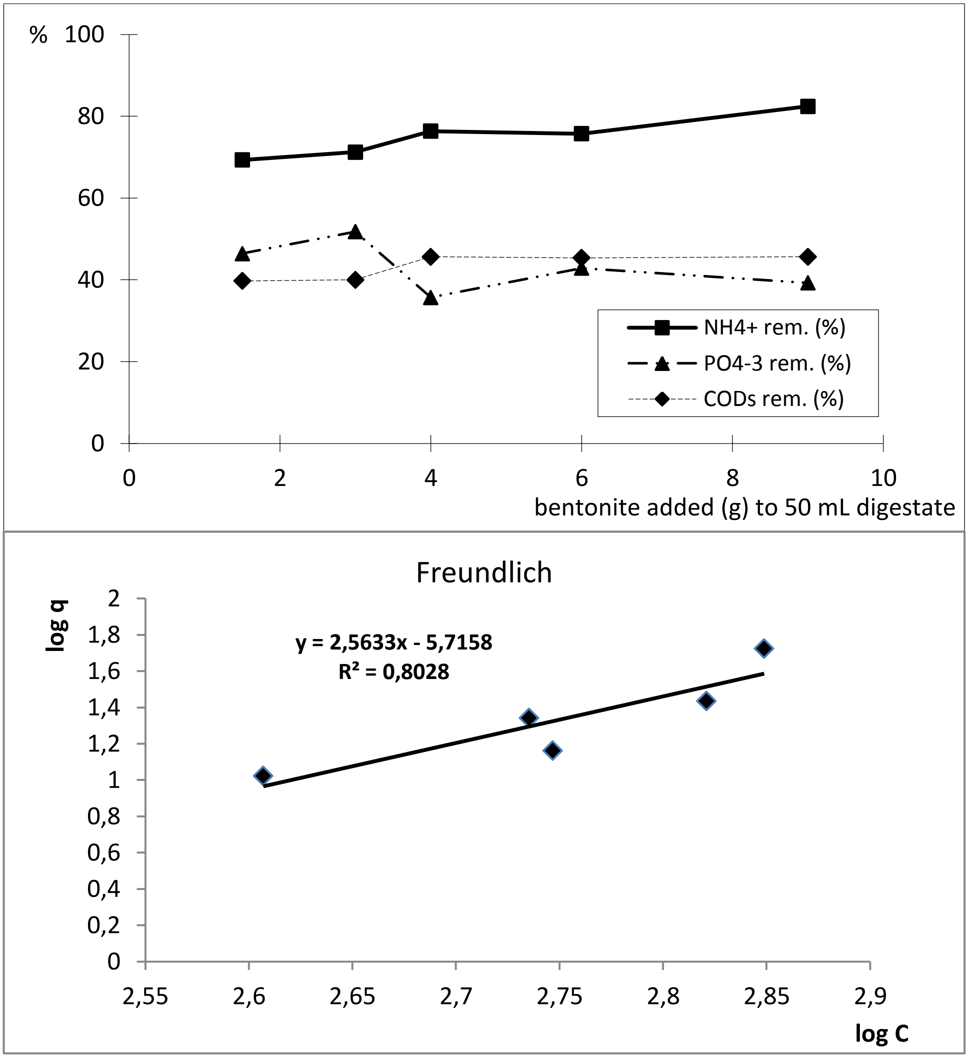

Figure 5. Adsorption efficiency of Ammonium-N (%), soluble COD and Phosphate vrs. mass of added bentonite (g) to 50 mL digestate alicuots; and Freundlich isotherm for the adsorption of Ammonium-N into bentonite; C= equilibrium conc. of NH_4^+-N in solution after adsorption (mg/L); q= mass of NH_4^+-N adsorbed per unit mass of bentonite (mg NH_4^+-N/g bentonite).