Final Project Report – Innovative Process for Digesting High Ammonia Containing Wastes
ForskEL 2010-10537

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Final Project Report

ForskEL 2010-10537

Innovative Process for Digesting High Ammonia Containing Wastes

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Final report

1 Project details

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<th>Innovative process for digesting high ammonia containing wastes</th>
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<tr>
<td>Project identification</td>
<td>Energinet.dk project no. 2010-10537</td>
</tr>
<tr>
<td>Project phase (date, year)</td>
<td>1st October 2010-30th September 2013</td>
</tr>
<tr>
<td>Entity responsible for the project</td>
<td>Technical University of Denmark, Department of Environmental Engineering, Miljøvej, Building 113, DK-2800 Kgs. Lyngby. Telephone: +45 45251429, Email: <a href="mailto:iria@env.dtu.dk">iria@env.dtu.dk</a></td>
</tr>
<tr>
<td>Reporting phase</td>
<td>1st October 2010-30th September 2013</td>
</tr>
<tr>
<td>Date for submission</td>
<td>30th January 2014</td>
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2 Executive summary

Acetate is the main precursor for methane production during anaerobic digestion of organic matter. Two mechanisms for methane formation from acetate have been described. The first one is aceticlastic methanogenesis, mediated by Methanosarcinaceae spp. or Methanosaetaceae spp.. The second mechanism is hydrogenotrophic methanogenesis which includes a two-step reaction in which acetate is first oxidized to H₂/CO₂ by syntrophic acetate oxidizing (SAO) bacteria and subsequently converted to methane by hydrogenotrophic methanogens. Six SAO bacteria have been reported, three mesophilic: Clostridium ultunense strain BST, Syntrophaceticus schinkii and Tepidanaerobacter acetatoxydans and three thermophilic: Thermacetogenium phaeum strain PB, Thermotoga lettingae strain TMO and strain AOR belonging to Firmicutes phylum. Syntrophic acetate oxidation is performed in a syntrophic association with hydrogenotrophic methanogens, usually Methanomicrobiales spp., Methanobacteriales spp. or Methanococcales spp. Methanosarcinaceae spp.. Finally, members of Methanosarcina genus can under specific conditions, besides acetate, also utilize H₂ and CO₂ to produce methane. Specific environmental factors such as temperature, organic acids and ammonia concentrations influence anaerobic acetate degradation. At temperatures between 50°C and 65°C, acetate oxidation is favored by thermodynamics of the oxidation step. At these temperatures, acetate oxidation is favored at low acetate concentrations, while aceticlastic methanogenesis is favored at high acetate concentrations. At temperatures higher than 65°C, acetate oxidation is the predominant pathway. Syntrophic acetate oxidation is the main mechanism for acetate degradation in the presence of inhibitors, particularly ammonium.

Denmark along with the other EU countries is committed to reduce utilization of fossil fuels and decrease CHG emission. Furthermore, Denmark is the first country to have declared the intention to
be fully independent from fossil fuels by the year 2050. It has been estimated that to achieve this goal, and to keep Denmark the world leader in biogas technology, at least 50 new centralized biogas plants must be operative by year 2020. For achieving this goal, it is necessary to optimize the biogas production from different substrates, including less attractive residual resources (e.g. ammonia-rich substrates).

Vast amounts of these ammonia-rich organic wastes produced every year by the agricultural and the food industrial sectors, which require an efficient and sustainable treatment to avoid harming the environment. Anaerobic digestion can be the designated ammonia-rich waste treatment method as it provides energy (methane) and a digestate with high nutrient levels (fertilizer). The drawback is that ammonia-rich substrates are inhibiting anaerobic digestion, which is the reason, biogas plant operators to be reluctant with respect to their use in the reactors. Nevertheless, it is estimated that many full-scale biogas reactors are seriously affected by ammonia toxicity loosing up to 1/3 of their methane producing potential. The traditional chemical and physical methods used to counteract ammonia inhibition are time-consuming and cost-expensive. For example, increasing the C/N ratio of the digester could eventually lead to a more serious inhibition levels because of organic overloading. Furthermore, dilution with water or lowering the working temperature of the bioreactor could cause a serious decrease in biogas production because of reduced substrate utilization.

Bioaugmentation of ammonia tolerant methanogens in the anaerobic reactors could be a possible solution for this problem. To date, there have been no successful bioaugmentations of methanogens in continuous reactors, reported. Thus, we believe it is necessary to develop bioaugmentation methods of ammonia tolerant methanogens in continuous anaerobic digesters working under high ammonia concentrations. These methods primarily should be developed and focused on two major challenges: a) alleviation of ammonia inhibition and b) efficient digestion of ammonia-rich waste. Therefore, research on how ammonia affects microbial composition and biochemical pathways in biogas reactors is required, to find new approaches to overcome ammonia inhibition for in situ bioaugmentation with ammonia-tolerant microorganisms, selection of conditions promoting desirable metabolic pathways, etc.

The ultimate goal of this project was to develop a novel bioaugmentation method of ammonia tolerant methanogens to alleviate ammonia inhibition in anaerobic reactors operating under ammonia induced “inhibited steady-states”. To achieve this goal, first we investigated the effect of ammonia on the methanogenic pathway and on the structure of methanogenic populations in lab-scale (acclimatization and non-acclimatization experiments) and in full-scale reactors.

Results from this project shown that, in lab-scale experiments, thermophilic cultures acclimatized to ammonia, clearly shifted their acetate bioconversion pathway from SAO with subsequent hydrogenotrophic methanogenesis (mediated by Methanobacteriales spp. and/or Methanomicrobiales spp.) to aceticlastic methanogenesis (mediated by Methanosarcinaceae spp.). On the contrary, acclimatization process resulted in no pathway shift with the mesophilic acclimatized culture. When non-acclimatized thermophilic culture was exposed to high ammonia levels, aceticlastic Methanosarcinaceae spp. was found to be the dominant methanogen. In full-scale reactors experiments, results obtained clearly demonstrated that syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis was the dominant pathway in all digesters with high
ammonia levels (2.8–4.57 g NH₄⁺-N L⁻¹), while acetoclastic methanogenic pathway dominated at low ammonia (1.21 g NH₄⁺-N L⁻¹). Thermophilic Methanomicrobiales spp. and mesophilic Methanobacteriales spp. were the most abundant methanogens at free ammonia concentrations above 0.44 g NH₃-N L⁻¹ and total ammonia concentrations above 2.8 g NH₄⁺-N L⁻¹, respectively. Meanwhile, in anaerobic digesters with low ammonia (1.21 g NH₄⁺-N L⁻¹) and free ammonia (0.07 g NH₃-N L⁻¹) levels, mesophilic and thermophilic Methanosetaeaceae spp. were the most abundant methanogens. Finally, the results derived from the bioaugmentation experiments clearly demonstrated a minimum 30% increase in methane production yield in the bioaugmented CSTR and UASB reactors. On contrary to all methods used today to alleviate ammonia toxicity effect, bioaugmentation process performed without interrupting the continuous operation of the reactor and without replacing the ammonia-rich feedstock. This is an entirely new approach to solve the ammonia toxicity problem in anaerobic reactors.

3 Project results

3.1 Project activities

1) Identification of SAO in full-scale biogas plants

We identified inocula with SAO activity derived from different Danish full-scale biogas reactors. We identified inocula with SAO activity was performed.

2) Operation of laboratory CSTR and UASB reactors with SAO pathway

We identify the ammonia levels promoting SAO as dominant methanogenic pathway from acetate into lab scale UASB and full scale CSTR reactors. We performed bioaugmentation experiments of ammonia tolerant methanogenic consortia into lab scale CSTR reactors. We optimized the physicochemical conditions for SAO consortia in lab-scale reactors.

3) Isolation, characterization and identification of new syntrophic acetate oxidizers

Microbial cultures with SAO pathway and high ammonia tolerant potential were enriched. Microbial enrichment and identification experiments were performed. We obtained SAO cultures, which used as inocula in the bioaugmentation experiments.

4) Evaluation and exploitation of the results

The results obtained were summarized in six scientific manuscripts (one under submission), five conference proceedings and posters (one under submission) and three presentations. All the published experimental work for this project is attached in section 6 (Appendices) of the current manuscript.

3.2 Project organization

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At DTU-Environment the biogas activities are under the Bioenergy research group, which is an active and dynamic group with extensive expertise in conversion of biomass to energy using biological methods. The group consists of more than 22 researchers and a number of students are coupled to the different projects during their master thesis work. The group has extensive expertise within biogas research, ranging from reactor concepts and mathematical models to modern molecular microbiology methods. Additionally, the group has active contact with many Danish biogas plants. This project fitted very well into the research strategy of the Bioenergy research group in the area of ammonia inhibitory effect on the biomethanation process, which is to develop the novel methods to alleviate ammonia toxicity effect from bioreactors.

The current project was executed at DTU Environment and DTU Environment was responsible for the laboratory experiments and method optimization. The project was managed by Prof. Irini Angelidaki, who has extensive expertise with project management. She has managed several national (Research council (STVF, FMI), EFP, PSO, UVE, Fond supported projects etc.) and international projects (EU under FP6 and FP7, Cost, Interreg). The projects have always been executed with success.

### 3.3 Activities, mile stones and time schedule

Due to facing challenges with optimizing the bioaugmentation process and applying it to both types of anaerobic reactors (CSTR and UASB) commonly used in Denmark, the original time schedule was extended for one year during the project. All in all, the project has now been fulfilled and the different WPs as shown in the diagram below.

<table>
<thead>
<tr>
<th>Activities</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
<th>2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification of SAO in full scale biogas plants</td>
<td>Oct-Dec</td>
<td>Jan-Mar</td>
<td>Apr-Jun</td>
<td>Jul-Sep</td>
</tr>
<tr>
<td>Operation of laboratory fully mixed (CSTR) and upflow anaerobic sludge bed (UASB) reactors with SAO pathway</td>
<td>Oct-Dec</td>
<td>Jan-Mar</td>
<td>Apr-Jun</td>
<td>Jul-Aug</td>
</tr>
</tbody>
</table>

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Isolation, characterization and identification of new syntrophic acetate oxidizers

Evaluation and exploitation of the results

In the following table are presented the activities of the project, along with the period that they were performed.

<table>
<thead>
<tr>
<th>Project Activities</th>
<th>Time period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification of SAO in full-scale biogas plants</td>
<td>1/10/10-30/9/10</td>
</tr>
<tr>
<td>Laboratory CSTR reactors with SAO (effect of ammonium)</td>
<td>1/9/12-31/7/13</td>
</tr>
<tr>
<td>Laboratory CSTR reactors with SAO (effect of VFA)</td>
<td>1/10/11-31/7/13</td>
</tr>
<tr>
<td>Laboratory CSTR reactors with SAO (effect of HRT)</td>
<td>1/10/11-30/6/13</td>
</tr>
<tr>
<td>Laboratory CSTR reactors with SAO (uncoupling SRT and HRT)</td>
<td>1/10/11-31/7/13</td>
</tr>
<tr>
<td>Laboratory UASB reactors with SAO (effect of HRT)</td>
<td>1/10/11-30/6/2012</td>
</tr>
<tr>
<td>Laboratory UASB reactors with SAO (effect of ammonium)</td>
<td>1/10/11-30/6/2012</td>
</tr>
<tr>
<td>Laboratory UASB reactors with SAO (effect of VFA)</td>
<td>1/10/11-30/6/2012</td>
</tr>
<tr>
<td>Microbial enrichment, characterization, identification</td>
<td>1/10/10-31/7/12</td>
</tr>
<tr>
<td>Evaluation/exploitation of the results</td>
<td>1/10/10-30/9/13</td>
</tr>
</tbody>
</table>

3.3 Objectives

The main objective of the project was to develop a process promoting acetate oxidation, for optimal digestion of high ammonia containing wastes. More specifically with this project, we were aiming to:

- Investigate the extent of acetate oxidation in full-scale biogas plants; and identify the environmental conditions promoting acetate oxidation on the cost of aceticlastic methanogenesis.
- Identify best process configuration (temperature, hydraulic retention time, organic loading rate, concentration of ammonia and volatile fatty acids) promoting high syntrophic acetate oxidation activity.
- Development of process technology where SAO are utilized (retaining of biomass by recirculation of biomass, or by immobilization of biomass).
- Enrich, isolate, characterize and identify acetate oxidizing microorganisms.
- Find cultures with highest potential for syntrophic acetate oxidation in order to reach maximum exploitation level of the results in the energy sector.
3.4 Results of the project

3.4.1 The fed-batch lab-scale experiments

In this project, we focused on the systematic investigation of the influence of ammonia on the anaerobic digestion (process performance, biochemistry and microbial ecology). This part of the study was aiming to elucidate the correlation between high ammonia content and metabolic pathways in lab-scale experiments. Our hypothesis was that different microorganisms are affected differently by ammonia, and a specific ammonia level would be connected with a dominant pathway. In our study, the effect of ammonia concentration on the methanogenic pathway from acetate and on the methanogenic communities was elucidated in two experiments: one where inocula were gradually exposed to increasing concentrations of ammonia, and another with direct exposure to different ammonia concentrations. $^{14}$C – labelled radiotracer and fluorescence in – situ hybridization (FISH) methods were used for determination of methanogenic pathways from acetate (syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis versus aceticlastic methanogenesis) and composition of the methanogenic communities, respectively.

Fig. 1. Maximum specific growth rate ($\mu_{\text{max}}$) plotted with ammonia concentration in AmA (acclimatization) and AmNA (non-acclimatization) experimental series for cultures T1, T2 and M.

Fig. 2. $^{14}$CO$_2$/$^{14}$CH$_4$ ratio plotted with ammonia concentration in AmA (acclimatization) and AmNA (non-acclimatization) experimental series and the dominant methanogens for cultures T1, T2 and M. MS: Methanosarcinaceae spp, MG: Methanomicrobiales spp, MB: Methanobacteria spp, MC: Methanococcales spp.

The thermophilic cultures acclimatized to ammonia, clearly shifted their acetate bioconversion pathway from SAO with subsequent hydrogenotrophic methanogenesis (mediated by Methanobacteriales spp. and/or Methanomicrobiales spp.) to aceticlastic methanogenesis (mediated by Methanosarcinaceae spp.) (Figures 1 and 2). On the contrary, acclimatization process resulted in no pathway shift with the mesophilic acclimatized culture. When non-acclimatized thermophilic
culture was exposed to high ammonia levels, aceticlastic Methanosarcinaceae spp. was found to be
the dominant methanogen (Figures 1 and 2). This knowledge about the influence of ammonia on
microbial composition and metabolism of the methanogenic conversions was used to design the
bioaugmentation experiments in the continuous reactors later in the project.

A significant outcome of this experimentation process was, the isolation an ammonia tolerant
methanogenic enriched culture (ATEC, Figure 3) which was producing stoichiometrically methane
(Figure 4) under extreme ammonia concentrations (> 9 g NH₄⁺-N L⁻¹). This enriched culture was
one of the cultures used in the continuous bioaugmentation experiments, later in the project.

![ATEC-FISH analysis microphotographs](image)

**Fig. 3** ATEC-FISH analysis microphotographs

**Fig. 4** Methane production of ATEC culture at 9.26 g NH₄⁺-N L⁻¹

### 3.4.2 The full-scale reactors study

After the fed-batch lab scale experiments, it was still not clear how the methanogenic microbial
communities respond to ammonia exposure in the complex environments of full-scale digesters.
Therefore, it was necessary to obtain fundamental knowledge of the relations between ammonia and
microbial ecology in full-scale anaerobic reactors. Thus, the aim of the second experiment was to
investigate the effect of different ammonia levels on methanogenic pathways and methanogenic
communities in full-scale anaerobic digesters. Therefore, Eight Danish full-scale digesters (Hashøj,
Nysted, Lundtofte, Hillerød, Vegger, Snertinge, Studsgård and Lemvig) operating under different
ammonia levels were sampled (Table 1).

**Table 1** Full-scale digesters working conditions and characteristics of the digesters content

<table>
<thead>
<tr>
<th>Plant</th>
<th>Operating Temp.±SD (°C)</th>
<th>Primary feedstock</th>
<th>TNK±SD (g N L⁻¹)</th>
<th>Total Ammonia±SD (g NH₄⁺-N L⁻¹)</th>
<th>Free Ammonia±SD (g NH₃-N L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nysted</td>
<td>38±1</td>
<td>Pig slurry</td>
<td>4.18±0.04</td>
<td>2.93±0.11</td>
<td>0.25±0.01</td>
</tr>
<tr>
<td>Hashøj</td>
<td>37±1</td>
<td>Cattle and pig slurry</td>
<td>5.81±0.13</td>
<td>4.57±0.15</td>
<td>0.44±0.01</td>
</tr>
<tr>
<td>Lundtofte</td>
<td>35±1</td>
<td>Primary and biological sludge</td>
<td>2.29±0.04</td>
<td>1.21±0.06</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Vegger</td>
<td>55±1</td>
<td>Cattle manure</td>
<td>3.03±0.01</td>
<td>2.03±0.08</td>
<td>0.51±0.02</td>
</tr>
<tr>
<td>Studsgård</td>
<td>52±1</td>
<td>Cattle and pig slurry</td>
<td>3.72±0.09</td>
<td>2.04±0.05</td>
<td>0.48±0.01</td>
</tr>
<tr>
<td>Snertinge</td>
<td>52.5±1</td>
<td>Cattle and pig slurry</td>
<td>3.07±0.08</td>
<td>2.26±0.11</td>
<td>0.37±0.02</td>
</tr>
<tr>
<td>Lemvig</td>
<td>52.5±1</td>
<td>Cattle, pig, and poultry slurry</td>
<td>3.24±0.12</td>
<td>2.44±0.08</td>
<td>0.48±0.02</td>
</tr>
<tr>
<td>Hillerød</td>
<td>55±1</td>
<td>Primary and biological sludge</td>
<td>1.54±0.01</td>
<td>0.90±0.08</td>
<td>0.07±0.01</td>
</tr>
</tbody>
</table>
The residual biogas production was followed in fed-batch reactors. Acetate, labelled in the methyl group, was used to determine the methanogenic pathway by following the $^{14}\text{CH}_4$ and $^{14}\text{CO}_2$ production. Fluorescence in situ hybridization was used to determine the methanogenic communities’ composition.

Results obtained (Figures 5 and 6) clearly demonstrated that syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis was the dominant pathway in all digesters with high ammonia levels ($2.8-4.57$ g NH$_4^+$-N L$^{-1}$), while acetoclastic methanogenic pathway dominated at low ammonia ($1.21$ g NH$_4^+$-N L$^{-1}$). Thermophilic Methanomicrobiales spp. and mesophilic Methanobacteriales spp. were the most abundant methanogens at free ammonia concentrations above 0.44 g NH$_3$-N L$^{-1}$ and total ammonia concentrations above 2.8 g NH$_4^+$-N L$^{-1}$, respectively. Meanwhile, in anaerobic digesters with low ammonia ($1.21$ g NH$_4^+$-N L$^{-1}$) and free ammonia (0.07 g NH$_3$-N L$^{-1}$) levels, mesophilic and thermophilic Methanosetaeaceae spp. were the most abundant methanogens.

The results elucidated correlations between high ammonia content, metabolic pathways and methanogenic communities. Our hypothesis, that ammonia content affects the microbial community structure and specific methanogenic pathways in the biogas reactors, was confirmed. We clearly
demonstrated that ammonia and free ammonia levels are key environmental factors affecting the methanogenic pathway and methanogenic composition in the complex environments of the full-scale anaerobic digesters tested. This knowledge, about the influence of ammonia on microbial composition and metabolism of the methanogenic conversions, was also used to design the novel bioaugmentation strategies for the continuous reactors in the final experimentation period of the project.

### 3.4.3 The bioaugmentation experiments

When the information from the two previous experimental periods were available the continuous experiments started with the bioaugmentation of an ammonia tolerant syntrophic acetate oxidizing (SAO) consortium (i.e. *Clostridium ultunense* spp. nov. in association with *Methanoculleus* spp. strain MAB1) in a mesophilic up-flow anaerobic sludge blanket (UASB) reactor subjected to high ammonia loads. Furthermore, the co-cultivation of a fast growing hydrogenotrophic methanogen (i.e. *Methanoculleus bourgensis* MS2\textsuperscript{T}) with the SAO consortium in fed-batch reactors was also investigated.

![Fig. 7 The UASB Reactors’ set-up](image1)

![Fig. 8 The UASB reactors’ apparatuses](image2)

We demonstrated that bioaugmentation of SAO consortium in a UASB reactor was not possible due to the slow microbial growth caused by the hydrogenotrophic partner *Methanoculleus* spp. strain MAB1 (Figure 9). On the contrary, the co-cultivation period of SAO culture in co-culture together with *M. bourgensis* was reduced more than 30% and the corresponding maximum growth rate ($\mu_{\text{max}}$) was increased more than 40% (compared to SAO consortium alone) in fed-batch reactors (figure 10).
Fig. 9 Methane production yield of the three UASB reactors. ($R_1$: low ammonia loaded control reactor, no bioaugmentation; $R_2$: high ammonia loaded control reactor, no bioaugmentation; and $R_3$: high ammonia loaded reactor, bioaugmentation)

These results indicated that methanogens were the limiting factor during the bioaugmentation of the SAO consortium in the UASB reactor. Therefore, we repeated the experiment using ATEC as fast growing ammonia tolerant methanogenic consortium and we immobilized it in an UASB reactor that was operated under high ammonia concentrations. The results obtained showed that at 7 g NH₄⁺-N L⁻¹, the UASB reactor with the immobilized consortium had up to 40% higher methane production rate compared to the ammonia inhibited control reactor. This finding indicates that immobilization of ammonia tolerant methanogenic consortia in high performance anaerobic digesters is a possible solution towards sustainable digestion of ammonia-rich residues.

Fig. 10 Methane production yield of the fed-batch reactors. (B_{SAO}: SAO co-culture, B_{SAO+MC}: SAO co-culture and M. bourgensis b B_{MC}: M. bourgensis.  

Finally, a new bioaugmentation strategy in a lab-scale CSTR, which is the most common type of full-scale biogas reactor configuration in Denmark, was tested. A fast growing hydrogenotrophic methanogen (i.e. Methanoculleus bourgensis MS2T) was bioaugmented in the CSTR reactor at high ammonia levels (Figure 12). We succeeded an innovative bioaugmentation process configuration for CSTR reactors, which was able to alleviate ammonia inhibitory effect and increase methane production up to 30% (Figure 13). The results of this experiment have been summarized in a scientific paper, which is under submission.
3.5 Technical results achieved

The major technical results obtained, from this project are:

1. Bioaugmentation of ammonia tolerant methanogenic cultures for anaerobic digestion of ammonia-rich wastes: We have demonstrated that is possible to immobilize ammonia tolerant methanogenic consortia into both CSTR and UASB biogas reactors. The result of bioaugmentation was higher tolerance of the biogas process to ammonia.

2. Identification of methanogenic pathways under different ammonia loadings: High ammonia containing wastes could be digested in full-scale reactors. In reactors with high ammonia loading SAO was the dominant pathway. In contrary, introduction of high ammonia containing residues in reactors mediated by aceticlastic pathway (more sensitive to ammonia inhibition effect) should be avoided.

3. Utilization of ammonia tolerant methanogenic cultures for anaerobic digestion of ammonia rich wastes: We have shown that is possible to immobilize ammonia tolerant hydrogenotrophic methanogens into UASB biogas reactors. Therefore, high ammonia containing waste could be successfully anaerobically degraded in a UASB reactor in presence of ammonia tolerant hydrogenotrophic methanogens.

4. Identification of ammonia levels promoting SAO as dominant methanogenic pathway in full-scale biogas digesters: At ammonia and free ammonia concentrations above 2.5 g NH$_4^+$-N L$^{-1}$ and 0.4 g NH$_3$-N L$^{-1}$ respectively, acetate oxidation coupled with hydrogenotrophic methanogenesis was the dominant pathway. Furthermore, thermophilic Methanomicrobiales spp. and mesophilic Methanobacteriales spp. were the most abundant methanogens for methane formation.

5. Identification of ammonia levels promoting aceticlastic as dominant methanogenic pathway in full-scale biogas digesters: Thermophilic and mesophilic Methanosetaeaceae spp. were the most abundant methanogens in full-scale anaerobic digesters with low free ammonia (<0.07 g NH$_3$-N L$^{-1}$) levels.

6. Slow growing hydrogenotrophic methanogens were found to be the limiting factor for bioaugmentation of syntrophic acetate oxidizing consortia in UASB reactors.
4 Project conclusion and perspective

4.1 Conclusions

Vast amounts of ammonia-rich wastes produced every year by the agricultural and the food industrial sectors, which require an efficient and sustainable treatment to avoid harming the environment. From the performed fed-batch and continuous experiments in this project, we can conclude that bioaugmentation of ammonia tolerant methanogenic consortia can make anaerobic digestion the designated method to treat these ammonia-rich waste. Furthermore, bioaugmentation can potentially be used in the existing centralize biogas plants, which are operating under suboptimal conditions, to alleviate the ammonia toxicity effect and increase methane production up to 30%. On contrary to all methods used today, the tested bioaugmentation processes performed without interrupting the continuous operation of the reactors and without reducing the high ammonia levels in the substrates. This is an entirely new approach to solve the ammonia toxicity problem in anaerobic reactors and to digest directly ammonia-rich waste.

4.2 Perspective

The major future challenges of the novel bioaugmentation processes developed in the current project are further optimization and up scaling of the method. It is necessary this new method to be tested in full-scale anaerobic digesters exposed to ammonia toxicity. Life cycle assessment (LCA) and life cycle cost (LCC) are necessary to analyze the entire life cycle of bioaugmentation process concerning the environmental impact and the direct monetary costs involved. As it has shown in the current study, bioaugmentation does not require the extraction of the ammonia-rich waste from the reactor as the other conventional methods. Nevertheless, it is necessary to assess bioaugmentation environmentally and economically against the most common methods used today to cope with ammonia toxicity events in full-scale reactor (i.e. lowering the operational temperature and dilution with water). This assessment could provide the economic and technical arguments to convince the anaerobic digestion companies that bioaugmentation is an advantageous process to be used in their reactors. Finally, we believe that bioaugmentation can find potential application for future development of strategies for anaerobic digestion of ammonia-rich wastes.

5 Publication and dissemination

The publications and the dissemination efforts for the current project are:

5.1 Manuscripts published


**5.2 Manuscript in submission**


**5.3 Conference proceedings and posters**


**5.3 Conference proceedings under submission**


**5.4 Presentations**


6. Appendix A (manuscripts-poster-presentation)

Appendix A.1
Effect of ammonium and acetate on methanogenic pathway and methanogenic community composition

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anaerobic digestion; ammonia inhibition; aceticlastic pathway; hydrogenotrophic pathway; syntrophic acetate oxidation.

Abstract
Methanogenesis from acetate (aceticlastic methanogenesis or syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis) is the most important step for the biogas process. The major environmental factors influencing methanogenesis are volatile fatty acids, ammonia, pH, and temperature. In our study, the effect of acetate and ammonia concentration on the methanogenic pathway from acetate and on the methanogenic communities was elucidated in two experiments: one where inocula were gradually exposed to increasing concentrations of acetate or ammonia, and another with direct exposure to different ammonia concentrations. The methanogenic pathway was determined by following the production of 14CH4 and 14CO2 from acetate labeled in the methyl group (C-2). Microbial communities’ composition was determined by fluorescence in situ hybridization. Upon acclimatization to acetate and ammonia, thermophilic cultures clearly shifted their acetate bioconversion pathway from SAO with subsequent hydrogenotrophic methanogenesis (mediated by Methanobacteriales spp. and/or Methanomicrobiales spp.) to aceticlastic methanogenesis (mediated by Methanosarcinaceae spp.). On the contrary, acclimatization process resulted in no pathway shift with the mesophilic acclimatized culture. When nonacclimatized thermophilic culture was exposed to high ammonia levels (7 g NH4+-N L-1), aceticlastic Methanosarcinaceae spp. was found to be the dominant methanogen.

Introduction
Acetate is quantitatively the most important intermediate substrate of methane (CH4) production during anaerobic digestion (Gujer & Zehnder, 1983). Methanogenesis from acetate can follow two different pathways: aceticlastic and syntrophic acetate oxidation (SAO). In the aceticlastic pathway (reaction 1), acetate is cleaved into methyl and carboxyl groups, and afterward, the methyl group is directly converted to CH4 and the carboxyl group is oxidized to carbon dioxide (CO2; Zinder & Koch, 1984). The two-step SAO pathway (reaction 2) is an oxidation of acetate to H2 and CO2 followed by hydrogenotrophic methanogenesis (reaction 3; Zinder & Koch, 1984).

\[
\begin{align*}
\text{CH}_3\text{COO}^- + \text{H}_2\text{O} &\rightarrow \text{CH}_4 + \text{HCO}_3^- \\
\Delta G^0 &= -31.0 \text{kJ mol}^{-1} \quad (1)
\end{align*}
\]

\[
\begin{align*}
\text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} &\rightarrow 2\text{HCO}_3^- + 4\text{H}_2 + \text{H}^+ \\
\Delta G^0 &= +104.6 \text{kJ mol}^{-1} \quad (2)
\end{align*}
\]

\[
\begin{align*}
4\text{H}_2 + \text{HCO}_3^- + \text{H}^+ &\rightarrow \text{CH}_4 + 3\text{H}_2\text{O} \\
\Delta G^0 &= -135.6 \text{kJ mol}^{-1} \quad (3)
\end{align*}
\]

Reaction (2) is thermodynamically unfavorable under standard conditions. However, this unfavorable reaction can proceed if hydrogenotrophic methanogenesis (reaction 3) reduces hydrogen pressure through interspecies hydrogen transfer (IHT; Zinder & Koch, 1984).

It has been proposed that hydrogen transfer and formate transfer are the two possible mechanisms for IHT, triggered by the syntrophic interspecies distances (Stams et al., 2006). Formate transfer is the favored mechanism.
when the distances between SAO bacteria and hydrogenotrophic methanogens are high, while hydrogen transfer becomes more favorable when these distances are small (de Bok et al., 2004). The overall process (reactions 2 + 3) then becomes exergonic, with the same stoichiometry as aceticlastic methanogenesis (reaction 1).

Aceticlastic methanogenesis is carried out by Methanosarcinaceae spp. and Methanosacetaceae spp. (Jetten et al., 1992). SAO is performed by acetate-oxidizing bacteria. Six SAO bacteria have been reported, three mesophilic: Clostridium ultunense strain BS\(^\text{T}\) (Schnurer et al., 1996), Syntrophacetales schinkii (Westerholm et al., 2010) and Tepidanaerobacter acetatoxydans (Westerholm et al., 2011a) and three thermophilic: Thermacetogenium phaeum strain PB (Kamagata & Mikami, 1989), Thermotoga lettingae strain TMO (Balk et al., 2002) and strain AOR (Lee & Zinder, 1988) belonging to Firmicutes phylum (Hattori, 2008). SAO is performed in a syntrophic association with hydrogenotrophic methanogens, usually Methanomicrobiales spp., Methanobacteriales spp. or Methanococcales spp. (Karakash et al., 2006). Methanosarcinaceae spp. (members of Methanosarcina genus), can under specific conditions, besides acetate, also utilize H\(_2\) and CO\(_2\) to produce methane (Jetten et al., 1992).

The main environmental factors influencing the methanogenic pathway are pH, temperature, volatile fatty acids (VFA), and ammonia (cf. review by Chen et al., 2008). The optimal range of pH for maximum biogas yield in anaerobic digestion is 6.5–7.5 (Liu et al., 2008). Outside this pH range, the biogas yield is reduced rapidly until anaerobic digestion ceased (Hwang et al., 2004). Hydrogenotrophic methanogens are less sensitive to unfavorable pH levels compared with aceticlastic methanogens (Hao et al., 2012). Temperature is a limiting factor for the endergonic SAO (reaction 2); therefore, it also affects SAO pathway (Hattori, 2008). SAO pathway performs more efficiently at thermophilic temperatures (35–60°C) compared with mesophilic temperatures (Schnurer et al., 1996; Hattori, 2008).

High VFA concentrations can lead to a pH drop, which in turn causes inhibition of methanogenesis (Wang et al., 2009) and of the hydrolysis/acidogenesis. Moreover, high levels of VFA can itself directly inhibit methanogenesis. According to the study by Dogan et al. (2005), more than 50% inhibition of methane production occurred above 13, 15, and 3.5 g L\(^{-1}\) of acetate, butyrate, and propionate added to granular sludge, respectively. Furthermore, in the same study, aceticlastic methanogenic activity was decreased for acetate concentrations above 4 g L\(^{-1}\). So far, the VFA effect on anaerobic digestion has been studied for the monitoring of biogas process (Ahring et al., 1995; Boe et al., 2007) and for the determination of VFA toxicity levels (Pullammanappillai et al., 2001; Nielsen et al., 2007). Although many studies have reported the effect of VFA on process stability and as process state indicator, the effect of exposure to acetate on mixed cultures in respect to metabolic pathways and microbial community composition is yet unclear.

Ammonia is the major toxicant (Westerholm et al., 2011b) in full-scale digesters of ammonia-rich wastes, such as pig or poultry manures, or slaughterhouse byproducts. Ammonia concentrations over 4 g NH\(_3\)\(-\)N L\(^{-1}\) are known to inhibit methanogenesis (cf. review by Chen et al., 2008). Free ammonia has been identified to be the main component causing methanogenesis inhibition (cf. review by Chen et al., 2008). The most widely accepted mechanism explaining ammonia inhibition is claiming that elevated ammonia levels result in change of intracellular pH, increase in maintenance energy requirement, depletion of intracellular potassium, and inhibition of specific enzyme reactions (Wittmann et al., 1995). Up to now, research on the influence of ammonia concentration on the methanogenic pathway and on the methanogenic community composition has given conflicting results. Many researchers indicated that aceticlastic methanogens are more sensitive to ammonia inhibition compared with hydrogenotrophic methanogens (Koster & Lettinga, 1984; Angelidaki & Ahring, 1993), while others have reported the opposite (Zeeman et al., 1985; Wiegant & Zeeman, 1986). Therefore, the influence of ammonia concentration on the methanogenic pathway and on the methanogenic composition should be further examined.

The traditional chemical and physical methods used to counteract ammonia inhibition are time-consuming and cost-expensive (Nielsen & Angelidaki, 2008). For example, increasing the C/N ratio of the digester could eventually lead to a more serious inhibition levels because of organic overloading (Kayhanian, 1999). Furthermore, dilution with water or lowering the working temperature of the bioreactor could cause a serious decrease in biogas production because of reduced substrate utilization (Nielsen & Angelidaki, 2008). Therefore, research on how ammonia affects microbial composition and biochemical pathways in biogas reactors is required, to find new approaches to overcome ammonia inhibition for in situ bioaugmentation with ammonia-tolerant microorganisms, selection of conditions promoting desirable metabolic pathways, etc.

The aim of the present study was to investigate the effect of ammonia and acetate on which acetate pathway comes into play and on the structure of methanogenic populations. As accidental increase in ammonia levels (loading with high ammonia content wastes) frequently occurs during anaerobic digestion process, both the direct exposure (nonacclimatization) and the gradual adaptation (acclimatization) of the cultures to various ammonia
concentrations were tested. On the contrary, acetate concentration increases gradually due to inhibition of methanogenesis. Thus, only adaptation (acclimatization) of the methanogenic cultures to stepwise increased acetate concentrations was tested.

**Materials and methods**

**Inocula and medium**

One mesophilic (M) and two thermophilic (T1 and T2) methanogenic inocula were used in the experiments. M was derived from a mesophilic (Hashøj, Denmark) and T1 and T2 from two thermophilic (Lemvig and Snertinge, Denmark) full-scale biogas reactors, respectively (Table 1). The full-scale reactors are fed with 70–90% animal manure and 10–30% organic waste from food industries.

Basal anaerobic (BA; Angelidaki et al., 1990) medium (35 mL) and 5 mL inoculum were prepared in 118-mL incubation bottles under a N₂/CO₂ (80%/20%) head-space. Na₂S·9H₂O (final concentration of 62.5 μL⁻¹) was added to reduce the medium, and a filter-sterilized anaerobic vitamin solution (1 mL L⁻¹) was added aseptically. Bottles were closed with butyl rubber stoppers, sealed with aluminum caps and incubated at respective inocula ambient temperatures. All experiments were performed in triplicates.

**Experimental setup**

To elucidate the effect of ammonia and acetate on the microbial composition and methanogenic pathways, three series of batch cultivations with sodium acetate as methanogenic substrate were set up (Table 2). In two series, the tested inocula were stepwise exposed to (referred elsewhere as acclimatized cultures) increasing acetate (HAc) concentrations, from 1 to 9 g HAc L⁻¹ [experiment acclimatization to acetate (AcA)], and increasing ammonia concentrations from 1 to 7 g NH₃-N L⁻¹ [experiment acclimatization to ammonia (AmA)], respectively. A gradual adaptation was made by successive batch cultivations with increasing acetate or ammonia concentrations in each step. Stepwise acclimatization experiments (AcA and AmA) were made with 1 g L⁻¹ increment steps to expose the cultures to increasing acetate and ammonia levels (up to 3 g L⁻¹). After the initial adaptation to 3 g L⁻¹ ammonia/acetate, acclimatization proceeded to the next steps with the increments of 2 g L⁻¹.

In the third series, the same inocula (as previously referred elsewhere as nonacclimatized cultures) were directly incubated under various ammonia concentrations (1, 3, 5, and 7 g NH₃-N L⁻¹) [experiment nonacclimatization to ammonia (AmNA)].

The final concentrations of ammonium-N and acetate for each step of the three experimental series are presented in Table 2. Five and six successive transfers of the adapted cultures were made in AmA and AcA experiments (Table 2), respectively. When methane production had ceased, 5 mL inoculum was transferred for a new cultivation (next step) with higher concentration of ammonia (AmA experiment) or acetate (AcA experiment). In all experiments, the following bottles were included: (1) three control bottles with inoculum and BA medium only, for the estimation of background CH₄ production from inoculum, (2) three bottles containing both unlabeled and labeled [2-¹⁴C] sodium acetate (Amersham Pharmacia Biotech, UK), final concentration of 45.75 ± 6.95 kBq L⁻¹ or 1.25 ± 0.19 μg L⁻¹), for methanogenic pathway identification (measurement of ¹⁴CO₂/¹⁴CH₄ ratio), and (3) three bottles only with unlabeled acetate, for following the dynamics of CH₄ production from acetate.

**Analytical methods**

Methane production was determined with gas chromatography method according to the study by Angelidaki et al. (2009). Total solids, volatile solids, pH, total nitrogen (TKN), and total ammonia were determined by Kjeldahl method, according to American Public Health Association’s Standard Methods (APHA, 2005). VFAs were determined using gas chromatograph (HP 5890...

<table>
<thead>
<tr>
<th>Table 1. Characteristics of the methanogenic inocula</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultures: biogas plant (Symbol)</strong></td>
</tr>
<tr>
<td>-------------------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Thermophilic 1 – Snertinge (T1)</td>
</tr>
<tr>
<td>Thermophilic 2 – Lemvig (T2)</td>
</tr>
<tr>
<td>Mesophilic – Hashej (M)</td>
</tr>
</tbody>
</table>

*Abbreviations of cultures derived from inocula used in the experiments.

Data are mean values (n = 3) ± SD.

Calculated according to Eqn 4.
series II) equipped with a flame ionization detector and an HP FFAP column as described previously (Kotsopoulos et al., 2009). The pH was measured before and after each experimental step with PHM99 LAB pH meter (Radiometer). All analyses were made in triplicate, and the averages are presented along with the corresponding standard deviations (SD) calculated from the analyses.

Radioisotopic analyses

Methanogenic pathway of acetate degradation was determined by measuring the production of $^{14}$CH$_4$ and $^{14}$CO$_2$ from acetate labeled in the methyl group (C-2; Karakashev et al., 2006). When aceticlastic methanogenesis is the sole methanogenic pathway, all $^{14}$C carbon goes to methane $^{14}$CH$_4$ derived from the methyl group of the labeled acetate. In contrast, dominance of SAO results in the conversion of $^{14}$C methyl group to $^{14}$CO$_2$. As the pool of unlabeled carbon dioxide (12CO$_2$) in anaerobic digestion is much larger than the pool of labeled $^{14}$CO$_2$, the hydrogenotrophic methanogenesis will only convert a small part of $^{14}$CO$_2$ molecules to $^{14}$CH$_4$. Therefore, amount of $^{14}$CO$_2$ will be much higher compared with amount of $^{14}$CH$_4$ formed during the oxidation of acetate. It is generally assumed that when $^{14}$CO$_2$/14CH$_4$ < 1, the dominant pathway is aceticlastic methanogenesis, while when $^{14}$CO$_2$/14CH$_4$ > 1, SAO coupled with hydrogenotrophic methanogenesis is the main pathway.

Bottles containing labeled acetate were acidified (final pH = 0.95 ± 0.1) with 7.2 M HCl. Thereby, dissolved $^{14}$C bicarbonate was converted to $^{14}$CO$_2$, removed from the liquid by sparging, and trapped in 10 mL of Carbosorb-E. For counting, the 10 mL of Carbosorb-E was mixed with 10 mL Permaflour-E (Perkin-Elmer Company) scintillation fluid. All radioactivity measurements were performed using a liquid scintillation counter (Tri-Carb 1600; Perkin-Elmer Company), as previously described by Karakashev et al. (2006).

FISH analyses

Fluorescence in situ hybridization (FISH) was used to identify the microbial populations as previously described by Hugenholtz (2002) and Karakashev et al. (2006). Samples were taken at the end of each experimental step when no further increase in methane production was obtained. The probes used and their target domains, orders or families are shown in Table 3. All probes were used at optimal stringency (data were from the references listed in Table 3) with 0–40% formamide. ARC915 was used to identify all members of the Archaea domain, and EUB338 was used for all bacterial members. 4′,6-diamidino-2-phenylindole (DAPI) was used for total cell identification (0.33 mg L$^{-1}$ in Milli-Q water for 10 min). After hybridization, the slides were examined with Olympus BX60 epifluorescence microscope (Olympus Corporation of the Americas) and microphotographs were taken with a Leica DFC 320 camera (Leica Microsystems Imaging Solutions Ltd, UK). Excitation channels were 488 and 545 nm for fluorescein isothiocyanate (green emission filter) and Cy3 fluorochromes (red emission filter), respectively. The determination of the methanogenic populations in this experimental work was based on approximately 20 microscope fields examined with the 63 x 1.4 objective, representing approximately 3000–12 000 individual cells. Dominance of a specific methanogen was defined as a positive response to the group-level probe in range 21–100% of the individual cells compared with all members of the Archaea, as identified by the ARC915 probe. Nondominant methanogenic groups represented between 1% and 20% of all members of the Archaea.
Calculations

Growth rate
The maximum specific growth rate (\( \mu_{\text{max}} \)) was calculated from the slope of the linear part of the graph of methane production natural logarithm versus time as has been described before (Gray et al., 2009).

Statistics
Statistical analysis was performed using the GRAPHPAD PRISM program (Graphpad Software, Inc., San Diego, CA). The maximum growth rates of the methanogenic populations were compared with the Students t-test for statistically significant difference (\( P < 0.05 \)). All values are the means of three independent replicates (\( n = 3 \)) ± SD.

Free ammonia
The free ammonia concentration was calculated from the equilibrium of Eqn (4) (Angelidaki & Ahring, 1993):

\[
[FAN] = \frac{[TAN]}{1 + 10^{\frac{\text{pH} - K_a}{10}}} \tag{4}
\]

where [FAN] and [TAN] are the free (NH₃) and the total (NH₃ + NH₄⁺) ammonia concentrations, respectively, and \( K_a \) the dissociation constant, with values 1.29 × 10⁻⁵ and 3.37 × 10⁻⁹ for 37 and 52.5 °C, respectively, calculated as described previously (Hafner et al., 2006) with the use of the appropriate pH values (Table 1).

Results and discussion
Methane production, methanogenic pathway from acetate, and methanogenic communities’ composition of the three experimental series (AcA, AmA, and AmNA) are presented and discussed in the following paragraphs. As a baseline for the determination of any changes in methanogenic community composition and pathways, the first experimental step, that is 1 g HAc L⁻¹ and 1 g NH₃-N L⁻¹, when no adaptation took place, was used (Table 4). At thermophilic conditions, Methanosarcinaceae spp. and Methanobacteriales spp. were the dominant methanogens, and SAO coupled with hydrogenotrophic methanogenesis was the dominant pathway. Methanomicrobiales spp., Methanosarcinaceae spp., and Methanobacteriales spp. were dominant methanogenic species, and aceticlastic methanogenesis was the dominant pathway under mesophilic conditions.

Acclimatization to acetate experimental series
The maximum specific growth rates (\( \mu_{\text{max}} \)) in all experimental series are shown in Fig. 1. Stepwise increase of acetate concentrations (up to 9 g L⁻¹), in terms of substrate utilization, did not result in inhibition of the methanogenic process. This finding was supported by the almost complete (95–100%) removal of the acetate in the batches (data not shown). At initial acetate concentrations up to 5 g HAc L⁻¹, the specific growth rates (\( \mu_{\text{max}} \)) for cultures T1 and M were significantly lower (\( P < 0.05 \)) compared with the baseline (Fig. 1a), indicating that methanogenic cultures were stressed by the increased acetate concentrations. On the contrary, culture T2 had either similar (at 5 g HAc L⁻¹, statistically no significant difference, \( P > 0.05 \)) or significantly higher \( \mu_{\text{max}} \) (at 2 and 3 g HAc L⁻¹, \( P < 0.05 \)), coping better (compared with T1 and M) with the increased inhibitory effect from acetate. At 7 g HAc L⁻¹, cultures T1 and M had significantly higher \( \mu_{\text{max}} \) (\( P < 0.05 \)) compared with all previous AcA experimental steps (up to 5 g HAc L⁻¹). These growth rates indicate that both mesophilic (M) and thermophilic (T1) methanogens were acclimatized to high acetate levels. In general, mesophilic methanogens are considered to have lower maximum growth rates.

### Table 3. Oligonucleotide probes used for the microbe population identification

<table>
<thead>
<tr>
<th>Probe</th>
<th>Phylum</th>
<th>Functional group</th>
<th>Probe sequence (5’-3’)†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC915</td>
<td>Archaebacteria</td>
<td>Mainly meth.</td>
<td>GTGCTCCCCGGGCGAGTTCCCT</td>
<td>Stahl &amp; Amann (1991)</td>
</tr>
<tr>
<td>MXX825</td>
<td>Methanoaetacea</td>
<td>Aceticlastic meth.</td>
<td>TCGCACCNOTGGCAGACTGCTC</td>
<td>Raskin et al. (1994)</td>
</tr>
<tr>
<td>MS1414</td>
<td>Methanosarcinaceae</td>
<td>Aceticlastic meth. (also hydrogen)</td>
<td>CTCACCCATAGGCGATACGC</td>
<td>Sekiguchi et al. (1999)</td>
</tr>
<tr>
<td>MG1200</td>
<td>Methanomicrobiales</td>
<td>Hydrogenotrophic meth.</td>
<td>CCGGATATTGGGCGCATGCTG</td>
<td>Sekiguchi et al. (1999)</td>
</tr>
<tr>
<td>MB1174</td>
<td>Methanobacteriales</td>
<td>Hydrogenotrophic meth.</td>
<td>TACGGCTGCTACCTCTCCT</td>
<td>Sekiguchi et al. (1999)</td>
</tr>
<tr>
<td>MC1109</td>
<td>Methanoococcales</td>
<td>Hydrogenotrophic meth.</td>
<td>GCAACATAGGCCACGGGCT</td>
<td>Raskin et al. (1994)</td>
</tr>
<tr>
<td>EU8338</td>
<td>Bacteria (most)</td>
<td>Nonmethylotrophic</td>
<td>GCTGGCTCCCTGAGGAGT</td>
<td>Stahl &amp; Amann (1991)</td>
</tr>
<tr>
<td>EU8338+</td>
<td>Bacteria (remaining)</td>
<td>Nonmethylotrophic</td>
<td>GCWGCACCCGTAAGGTT</td>
<td>Daims et al. (1999)</td>
</tr>
</tbody>
</table>

*meth.: methanogenic.
†W, A + T mixed base.
compared with thermophilic methanogens (Siegrist et al., 2002) at low acetate concentrations. However, at high acetate levels (9 g HAc L\(^{-1}\)), \(\mu_{\text{max}}\) of mesophilic culture M was up to 40% higher than the rates of thermophilic cultures T1 and T2 (Fig. 1a). This could be explained by the increased permeability (for a lot of compounds including acetate) of the cells at higher temperatures, which could have inhibited thermophilic methanogens (Vavilin et al., 2008).

The experiments with thermophilic acetate acclimatized cultures T1 and T2 with increasing concentrations between 1 and 9 g HAc L\(^{-1}\) resulted in a shift in the \(^{14}\text{C}\) distribution ratio of \(^{14}\text{CO}_2/^{14}\text{CH}_4\) from > 1 to < 1. Thus, a pathway shift from SAO coupled to hydrogenotrophic methanogenesis (\(^{14}\text{CO}_2/^{14}\text{CH}_4 > 1\)) toward aceticlastic methanogenesis (\(^{14}\text{CO}_2/^{14}\text{CH}_4 < 1\); Fig. 2a). On the contrary, no pathway shift was registered for the mesophilic culture M, which retained their aceticlastic activity for all acetate concentrations tested as mediated by aceticlastic Methanosarcinaceae spp. at 9 g HAc L\(^{-1}\) (Fig. 2a). The aceticlastic methanogenesis process developed in all cultures (T1, T2, and M) favorable conditions for aceticlastic Methanosarcinaceae spp. to entirely outcompete other acetate consumers such as acetate-oxidizing bacteria and Methanosetaeaceae spp. Prevalence of Methanosarcinaceae spp. was in agreement with previous report showing that at high acetate concentrations, those Archaea were the most abundant acetate-utilizing methanogens (Hao et al., 2011).

### Table 4. Dominant methanogenic populations in the baseline and in the final step of the three experimental series

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Final step</th>
<th>Baseline step*</th>
<th>Final step</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ac(^1)</td>
<td>Am(^2)</td>
<td>Dominant(^3)</td>
</tr>
<tr>
<td>Acclimatized cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AcA experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 9 1</td>
<td>MS, MB</td>
<td>MG, MC</td>
<td>MS</td>
</tr>
<tr>
<td>T2 9 1</td>
<td>MS, MB</td>
<td>MC</td>
<td>MS</td>
</tr>
<tr>
<td>M 9 1</td>
<td>MS, MB, MG</td>
<td>MC</td>
<td>MS</td>
</tr>
<tr>
<td>AmA experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 1 5</td>
<td>MS, MB</td>
<td>MG, MC</td>
<td>MS</td>
</tr>
<tr>
<td>T2 1 5</td>
<td>MS, MB</td>
<td>MC</td>
<td>MS</td>
</tr>
<tr>
<td>M 1 7</td>
<td>MS, MB, MG</td>
<td>MC</td>
<td>MS</td>
</tr>
<tr>
<td>Nonacclimatized cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AmNA experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 1 3</td>
<td>MS, MB</td>
<td>MG, MC</td>
<td>MG, MC MB</td>
</tr>
<tr>
<td>T1 1 5</td>
<td>MS, MB</td>
<td>MG, MC</td>
<td>MS</td>
</tr>
<tr>
<td>T2 1 7</td>
<td>MS, MB</td>
<td>MC</td>
<td>MS, MC</td>
</tr>
<tr>
<td>T2 1 5</td>
<td>MS, MB</td>
<td>MC</td>
<td>MS, MC</td>
</tr>
<tr>
<td>M 1 3</td>
<td>MS, MB, MG</td>
<td>MC</td>
<td>MS</td>
</tr>
<tr>
<td>M 1 5</td>
<td>MS, MB, MG</td>
<td>MC</td>
<td>MS</td>
</tr>
</tbody>
</table>
| *MS, Methanosarcinaceae spp.; MB, Methanobacteriales spp.; MG, Methanomicrobiales spp.; MC, Methanococcales spp. \(^1\)
| Ac (g L\(^{-1}\)), in the form of sodium acetate. \(^2\)
| NH\(^+_4\)-N (g-N L\(^{-1}\)), in the form of ammonium chloride. \(^3\)
| Dominant methanogens: between 21% and 100% of the total number (positive for ARC915) of methanogenic cells; nondominant methanogens: 1–20% of the total number of methanogenic cells. \(^4\)
| Not observed. 

**Acclimatization to ammonia experimental series**

Stepwise increase in ammonia resulted in a statistically significant decrease (\(P < 0.05\)) of maximum growth rates compared with \(\mu_{\text{max}}\) of the baseline step (Fig. 1b), because of ammonia inhibition (Lay et al., 1998). Methane production of thermophilic cultures T1 and T2 was completely inhibited at 7 g NH\(^+_4\)-N L\(^{-1}\). Opposite to thermophilic cultures, mesophilic acclimatized culture M produced methane under high ammonia levels (7 g NH\(^+_4\)-N L\(^{-1}\)). This was probably due to the lower inhibition effect of ammonia at mesophilic temperatures compared with thermophilic. It is generally accepted that free ammonia (NH\(_3\)) is the active compound responsible for ammonia inhibition (cf. review by Chen et al., 2008). Free ammonia concentration is significantly lower, for the same total ammonia concentrations (NH\(_4^+ + \text{NH}_3\)), at mesophilic conditions compared with thermophilic conditions.
(cf. review by Chen et al., 2008), and thereby higher total ammonia can be tolerated at mesophilic conditions.

Experiments with the thermophilic acclimatized cultures T1 and T2 showed that increase of ammonia concentrations from 1 to 5 g NH₄⁺-N L⁻¹ leads to a clear pathway shift from SAO coupled with hydrogenotrophic methanogenesis (¹⁴CO₂/¹⁴CH₄ > 1) to aceticlastic methanogenesis (¹⁴CO₂/¹⁴CH₄ < 1; Fig. 2b). Contrary to thermophilic acclimatized cultures, no pathway shift was detected for the mesophilic culture M that retained its aceticlastic activity under all ammonia concentrations tested.

Acclimatization to high ammonia concentrations leads to lower Archaea diversity from two or three dominant
methanogenic species to one (Table 4). Ammonia-sensitive methanogens such as Methanobacterium bryantii, Methanosarcina barkeri, and Methanospirillum hungatii (Koster & Koopen, 1988) may have outcompeted ammonia-resistant methanogenic Archaea (e.g. Methanomicrobiales spp.; Angenent et al., 2002) under low-ammonia conditions. FISH analysis results revealed that aceticlastic Methanosarcinaceae spp. was the dominant methanogen in all cases (mesophilic and thermophilic) of ammonia-acclimatized cultures (Fig. 2b), which is in accordance with the other studies (Sasaki et al., 2011).

**Nonacclimatization to ammonia experimental series**

Direct exposure of the nonacclimatized thermophilic cultures T1 at 3 g NH₄⁺-N L⁻¹ and T2 at 3 and 5 g NH₄⁺-N L⁻¹ did not affect significantly (P > 0.05) the maximum growth rates compared with the baseline step (Fig. 1c). On contrary, direct exposure of the nonacclimatized mesophilic culture M to increased ammonia concentrations (up to 7 g NH₄⁺-N L⁻¹) resulted in a significant decrease (P < 0.05) in the maximum growth rates (Fig. 1c). At 7 g NH₄⁺-N L⁻¹, thermophilic culture T2 had up to 44% higher growth rate compared with mesophilic culture M and thermophilic culture T1. The higher growth rate of the T2 compared with M and T1 could be attributed to the difference in inocula origins and ability of inocula to adapt to high free ammonia levels (NH₄⁺). At 7 g NH₄⁺-N L⁻¹, culture T2 was exposed to lower free ammonia levels (0.44 g NH₃-N L⁻¹ for pH of 7.30 calculated according to Eqn 4) compared with NH₃ levels in the anaerobic digester where T2 was derived from Table 1. Opposite to T2, cultures T1 and M, at 7 g NH₄⁺-N L⁻¹, were exposed to higher free ammonia levels of 0.43 g NH₃-N L⁻¹ (for pH of 7.30 calculated according to Eqn 4) and 0.21 g NH₃-N L⁻¹ (for pH of 7.38 calculated according to Eqn 4) compared with the NH₃ levels in the anaerobic digesters where T1 and M were derived from Table 1. Adaptation to such high free ammonia levels with increased threshold for ammonia toxicity has previously been documented by Hansen et al. (1998).

For nonacclimatized mesophilic culture M, exposure to elevated ammonia concentrations resulted in change from aceticlastic to SAO pathway coupled with hydrogenotrophic methanogenesis (Fig. 2c). The SAO pathway was also dominant for the thermophilic culture T1, which retained the SAO pathway and thereby hydrogenotrophic activity at all ammonia concentrations tested.

*Methanococcales* always appeared as dominant methanogen together with other methanogenic *Archaea* (Table 4), upon direct exposure to 3 and 5 g NH₄⁺-N L⁻¹. The appearance of nonacclimatized *Methanococcales* spp. under high ammonia conditions was unexpected, and as up to our knowledge, similar outcome was not documented in other studies. The finding indicates that this hydrogenotrophic *Archaea* was resistant to elevated ammonia concentrations.

One interesting finding concerned the nonacclimatized thermophilic culture T2. Upon direct exposures up to 5 g NH₄⁺-N L⁻¹, the dominance of hydrogenotrophic *Methanosarcinaceae* (along with other hydrogenotrophic methanogens mentioned above) was observed, but when the same culture was directly exposed to 7 g NH₄⁺-N L⁻¹, aceticlastic *Methanosarcinaceae* appeared as the sole dominant methanogen (Fig. 2c). It was previously documented that *Methanosarcina* spp., belonging to family *Methanosarcinaceae* can be versatile with respect to substrate utilization for methane formation, for example, being able to utilize not only acetate but also CO₂ and H₂ (Jetten et al., 1992), even under thermophilic conditions with the presence of acetate in the medium (Mladenovska & Ahring, 1997; Demirel & Scherer, 2008). Therefore, a possible explanation for our findings could be that a *Methanosarcina* species, present in the thermophilic culture T2, was able to shift its methanogenic pathway under different ammonia concentrations. Aceticlastic activity of *Methanosarcina* faced strong competition (for acetate uptake) with the SAO bacteria, which according to the study by Chen et al. (2008) grow faster at direct exposures to relatively high ammonia levels (up to 5 g NH₄⁺-N L⁻¹). This competition (and possibly elevated hydrogen partial pressure) would result in the induction of hydrogenotrophic activity in *Methanosarcina* as a possible mechanism to survive via the utilization of H₂/CO₂ and at the same time to decrease the H₂ partial pressure. Exposure of the T2 culture to higher ammonia concentrations (7 g NH₄⁺-N L⁻¹) resulted in the inhibition of the acetate oxidizers, which was previously documented (Ahring & Westermann, 1988). Therefore, the hydrogenotrophic methanogenic activity was no longer required, and the only acetate utilization pathway was aceticlastic methanogenesis.

**Ammonia acclimatization: effect on methanogenesis**

Direct exposure (AmNA) of thermophilic inocula T1 and T2 at 7 g NH₄⁺-N L⁻¹ produced methane with μₘₐₓ up to 0.03 h⁻¹, contrary to AmA acclimatization series where no methane production was detected at the corresponded ammonia levels. Additionally, as expected, ammonia nonacclimatized cultures had a more diverse methanogenic composition (both aceticlastic *Methanosarcinaceae* spp. and hydrogenotrophic *Methanococcales* spp., *Methanobacteriales* spp., and *Methanomicrobiales* spp. *Archaea*) in
comparison with ammonia-acclimatized cultures (Table 4). The enrichment process occurred in AmA, eliminated the slower growing microorganisms during gradual acclimatization to elevated ammonia levels, in favor of the faster growing ones. This underlines the weakness of methanogenic enrichment methods, which seems to always favoring selection of acetotrophic methanogens, as a result to their faster growth rates. A critical comparison of the two approaches for treatment with ammonia tested in this study indicated that direct exposure of methanogenic cultures (nonacclimatization, AmNA series) to high ammonia levels is more preferable option than gradual adaptation (acclimatization, AmNA series) with respect to methane production. Direct exposure of nonacclimatized cultures to ammonia provides a higher diversity of methanogens. More versatile methanogenic community will have higher potential to overcome ammonia toxicity compared with less diverse consortium developed during gradual adaptation process. This result could be used for the anaerobic digestion of ammonia-rich wastes in full-scale biogas plants through optimization of conditions leading to development of ammonia-tolerant methanogenic communities.

Conclusions

Results obtained in this study demonstrated that acetate and ammonia acclimatization experiments resulted to dominance of *Methanosarcinaceae* spp. Aceticlastic methanogenesis was registered under high ammonia concentrations (7 g NH$_4^+$-N L$^{-1}$) in both ammonia experimental series. Mesophilic and thermophilic *Methanococcales* spp., from nonacclimatized cultures, were resistant to ammonia concentrations up to 5 g NH$_4^+$-N L$^{-1}$. These findings can find potential application for the development of bioaugmentation of ammonia-tolerant methanogenic consortia in bioreactors for anaerobic digestion of high ammonia-containing wastes.

Acknowledgements

We thank Hector Garcia and Morten Andreasen for technical assistance with the experiments. This work was supported by Energinet.dk under the project framework ForsKEL ‘Innovative process for digesting high ammonia wastes’ (program no. 2010-10537). The first author had a scholarship from Greece State Scholarship Foundation (IKY).

References


I.A. Fotidis


Appendix A.2
**Mikroorganismer kan øge gasudbyttet**

Nye forskningsresultater fra DTU-Miljø peger på, ammoniak-tolerante mikroorganismer kan øge gasproduktionen fra landbrugsbaserede biogasanlæg med op til 40 procent.

Af Ioannis Fotidis, Dimitar Karakashev og Irini Angelidaki

Biogas er en af de mest effektive teknologier, når det handler om at udfytte energipotentialet i biomasse, men det er også en kompleks proces, som kræver samspil af flere typer mikroorganismer for at kunne fungere effektivt.


Ammoniakhæmning

Ammoniak hæmmer primært omdannelsen af eddikesyre til metan og kuldioxid. Der findes imidlertid en anden form for metanproducenterende reaktion, hvor eddikesyre først bliver omdannet til brint og kuldioxid, inden det bliver til metan, og den proces har været at være meget mindre fælsmo over for ammoniakhæmning. I forløbet indgår et konsortium af mikroorganismer bestående af de såkaldte SAO bakterier, som omdanner eddikesyre til brint og CO₂, samt metanogen der omdanner brint og CO₂ til metan.

I et biogasanlæg kan man afgøre, hvordan eddikesyre omdannes til metan ved at anvende eddikesyre med isotop-mærkede kuldofatomer som biomasse. Derved kan man måle og bestemme, om der anvendes den ene eller den anden proces.

På DTU-Miljø har vi udtaget podemateriale fra syv forskellige biogasfællesanlæg for at finde sammenhængen mellem omsætningsvejen for eddikesyre og koncentrationen af ammoniak. Tre af prøverne stammer fra Hashøj, Nysted og Lundtofte, hvor reaktortemperaturen er på 37 °C, mens de øvrige prøver er fra Hillerød, Vægger, Snertinge og Lemvig, hvor reaktortemperaturen er på 52 °C.

Resultaterne viser, at biogasreaktorer, der indeholder SAO-bakterier, har op til fire gange højere koncentrationer af ammoniak end reaktorer, hvor der er en mere direkte omsætning af eddikesyre til metan og kuldioxid.

Praktiske løsninger

På DTU-Miljø har vi søgt efter praktiske løsninger for at kunne begrænse ammoniakhæmnininen på biogasanlæg. Målet er at skabe et mikrobielt miljø i reaktoren, der kan modstå høje koncentrationer af ammoniak.

I den forbindelse har vi forsøgt at anvende en mikroorganisme kaldet Methanoculleus, der er kendt for at være meget tolerant over for ammoniak. Organismen blev testet i en reaktor med et stort flow, hvor organismerne kan hæfte sig til et såkaldt slamtæppe, og det viste sig, at det kunne øge metanproduktionen med omkring 40 procent.

Resultaterne tyder altså på, at bestemte mikroorganismer kan være med til at øge gasproduktionen fra landbrugsbaserede biogasanlæg. I praksis kan det dog være vanskeligt at oprettere en ”koloni” af specifikke bakterier i en fuldt opblandet reaktor, med mindre de får nogle særlig gunstige vækstbetingelser. Vi er nu i gang med at afprøve indførelsen af ammoniakrobuste mikroorganismer, til behandling af biomasser med forhøjet ammoniakindhold i fuldt opblandede reaktorer.

Arbejdet med at undgå ammoniakhæmnininen i biogasanlæg er blevet støttet af Energinet.dk under ForskEL programmet og af EU-programmet Interreg. Du kan læse mere om projektet på www.biopress.dk under ”artikler”.

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Appendix A.3
Bioaugmentation with an acetate-oxidising consortium as a tool to tackle ammonia inhibition of anaerobic digestion

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HIGHLIGHTS

- Methanogens are the "key players" of anaerobic digestion under high ammonia levels.
- Bioaugmentation of SAO co-culture was not possible in a UASB reactor.
- M. bourgensis increased the maximum growth rate of SAO co-culture by 42%.
- M. bourgensis reduced the incubation period of SAO co-culture by 33%.
- Methanogens were the limiting factor of SAO co-culture bioaugmentation process.

ABSTRACT

Ammonia is the major inhibitor of anaerobic digestion (AD) process in biogas plants. In the current study, the bioaugmentation of the ammonia tolerant SAO co-culture (i.e. Clostridium ultunense spp. nov. in association with Methanoculleus spp. strain MAB1) in a mesophilic up-flow anaerobic sludge blanket (UASB) reactor subjected to high ammonia loads was tested. The co-cultivation in fed-batch reactors of a fast-growing hydrogenotrophic methanogen (i.e. Methanoculleus bourgensis MS2T) with the SAO co-culture was also investigated. Results demonstrated that bioaugmentation of SAO co-culture in a UASB reactor was not possible most likely due to the slow maximum growth rate (μmax = 0.007 h⁻¹) of the culture caused by the methanogenic partner. The addition of M. bourgensis to SAO led to 42% higher growth rate (μmax = 0.01 h⁻¹) in fed-batch reactors. This indicates that methanogens were the slowest partners of the SAO co-culture and therefore were the limiting factor during bioaugmentation in the UASB reactor.

1. Introduction

Anaerobic digestion (AD) is a biological process by which both waste control and energy recovery can be achieved. AD is mediated by different physiological groups of microorganisms, mainly bacteria and archaea, to produce biogas from different organic substrates. The archaea mediating methane production are members of Methanosarcinales order (strict and facultative aceticlastic methanogens) and members of Methanomicrobiales, Methanobacteriales and Methanococcales orders (strict hydrogenotrophic methanogens) (Angelidaki et al., 2011). The main environmental factors influencing AD are pH, organic loading rate (OLR), temperature, and ammonia (ammonium + free ammonia) levels (Angelidaki et al., 2011). Often, ammonia toxicity is the reason for AD process failure and subsequently suboptimal usage of the biogas potential in full-scale anaerobic digesters fed with ammonia-rich substrates. Free ammonia, has been acknowledged as the main component causing inhibition on methanogens, which increases concurrently with temperature and pH (Chen et al., 2008). Many thresholds for ammonia and free ammonia inhibitory effect have been proposed through time for non-acclimatised methanogenic cultures (Chen et al., 2008). In general, ammonia and free ammonia concentrations, correspondently over 3 g NH₃-N L⁻¹ or 0.15 g NH₄-N L⁻¹, are known to inhibit methanogenesis independently of temperature and pH levels (Chen et al., 2008). The chemical and physical methods, such as increasing the C/N ratio or lowering the temperature in the digesters, used today to counteract this problem are both cost-expensive and time consuming (Nielsen and Angelidaki, 2008).

Ammonia is mainly inhibiting the aceticlastic methanogenic pathway, while the syntrophic acetate oxidation pathway followed by hydrogenotrophic methanogenesis is more robust to ammonia toxicity (Fotidis et al., 2013). Up to now, three mesophilic: Clostridium ultunense strain BS⁷ (Schnurer et al., 1996), Syntrophacetaceus schinkii (Westerholm et al., 2010) and Tepidanaerobacter acetatoxydans (Westerholm et al., 2011) and three thermophilic: Thermodrhythmus anotheratus strain AOR (Lee and Zinder, 1988), Thermacetogenium phaeum strain PB (Kamagata and Mikami, 1989) and Thermotoga lettingae strain...
The SAO co-culture (JCM 16670, *Clostridium ultunense* spp. nov. living in association with *Methanoculleus* spp. strain MAB1) was kindly provided from the Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala. Before introduced to the UASB reactors, the SAO co-culture was grown in fed-batch reactors in bicarbonate buffered basal media-BM (Zehnder et al., 1986). The medium used contained per liter of MilliQ water 1 g of NH₄Cl, 0.4 g of KH₂PO₄, 0.1 g of MgCl₂·6H₂O, 0.5 g of l-cysteine hydrochloride, 5 g of sodium formate, 1 g of sodium acetate, 1 g of yeast extract, 1 g of glucose (Glc) and NH₄Cl as carbon and ammonium nitrogen source, respectively. Final medium was flushed with N₂/CO₂ (80%/20%), reduced with Na₂S·9H₂O (final concentration of 62.5 μg L⁻¹) and a sterilized aerobic vitamin solution (1 mL L⁻¹ working volume) was added aseptically (Angelidaki et al., 1990). The experiment was carried out in three identical lab-scale mesophilic (37 ± 1 °C) UASB reactors (R₁: low ammonia loaded control reactor, no bioaugmentation; R₂: high ammonia loaded control reactor, no bioaugmentation; and R₃: high ammonia loaded reactor, bioaugmentation) operating for 154 days. The operational parameters in the four different experimental periods for the three UASB reactors are presented in Table 1.

The total and working volume of the UASB reactors were 250 and 220 mL, respectively. Anaerobic granules, non-acclimatised to ammonia, derived from a full scale UASB reactor (Colsen b.v.-Kreekzoom 5, 4561 GX Hulst), were used as carriers. Each reactor’s setup consisted of a feed vessel, a feeding and a recirculation peristaltic pump, an effluent bottle, a magnetic stirrer for the homogenisation of substrate, a gas meter and a water-jacketed heating unit. A refrigerator was maintaining the feedstocks’ temperature at 4 °C in the feed vessel. All three UASB reactors were start-up together with 0.26 g NH₄⁻/L⁻¹ and then ammonia levels were gradually increased to 3 g NH₄⁻/L⁻¹ for reactors R₂ and R₃ (data not shown). The relatively low OLR (Table 1) was chosen to avoid any potential ammonia-VFA synergistic inhibitory effect (Lu et al., 2013) and thus to study only the influence of ammonia levels on the bioaugmentation process.

Before bioaugmentation (period I) all three reactors were operating under steady-state conditions. The bioaugmentation process (period II) of SAO co-culture took place in reactor R₁ at 3 g NH₄⁻/L⁻¹. These ammonia levels (3 g NH₄⁻/L⁻¹) were chosen to match the ammonia levels at which SAO co-culture was growing in the fed-batch reactors. During bioaugmentation 11 mL of SAO co-culture inoculum (OD₆₀₀ = 0.3–0.4) per day was introduced in reactor R₁, while 11 mL of sterile BAN medium was added per day in the reactors R₁ and R₂ containing 0.26 and 3 g NH₄⁻/L⁻¹, respectively. Throughout period II, all the reactors were operated as batch reactors (i.e., only recirculation, but not flow through) and were fed manually with 1.1 mL BAN medium containing 100 g Glc L⁻¹. The continuous feeding was resumed after 15 days (period III). One HRT after the end of the bioaugmentation period, ammonia concentration in reactors R₂ and R₃ increased to 5 g NH₄⁻/L⁻¹ (period IV).

### 2.2. Fed-batch reactors experimental setup

*Methanoculleus bourgensis* MSZ² (DSM 3045) was used in the fed-batch reactors grown in sterile medium. The medium used contained per liter of MilliQ water 1 g of NH₄Cl, 0.4 g of KH₂PO₄, 0.1 g of MgCl₂·6H₂O, 0.5 g of l-cysteine hydrochloride, 5 g of sodium formate, 1 g of sodium acetate, 1 g of yeast extract, 1 g of glucose (Glc) and NH₄Cl as carbon and ammonium nitrogen source, respectively. Final medium was flushed with N₂/CO₂ (80%/20%), reduced with Na₂S·9H₂O (final concentration of 62.5 μg L⁻¹) and a sterilized aerobic vitamin solution (1 mL L⁻¹ working volume) was added aseptically (Angelidaki et al., 1990). The experiment was carried out in three identical lab-scale mesophilic (37 ± 1 °C) UASB reactors (R₁: low ammonia loaded control reactor, no bioaugmentation; R₂: high ammonia loaded control reactor, no bioaugmentation; and R₃: high ammonia loaded reactor, bioaugmentation) operating for 154 days. The operational parameters in the four different experimental periods for the three UASB reactors are presented in Table 1.

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### Table 1:
Operational parameters in the different experimental periods of the three UASB reactors.

<table>
<thead>
<tr>
<th>Experimental period (Days)</th>
<th>Ammonia (g NH₄⁻/L⁻¹)</th>
<th>HRT (Days)</th>
<th>OLR (g Glc L⁻¹ d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (84–87)</td>
<td>0.26</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>II (88–103)</td>
<td>0.26</td>
<td>3.0</td>
<td>4.0</td>
</tr>
<tr>
<td>III (104–107)</td>
<td>0.26</td>
<td>5.0</td>
<td>4.0</td>
</tr>
<tr>
<td>IV (108–154)</td>
<td>0.26</td>
<td>5.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

a Low ammonia loaded control reactor (no bioaugmentation).
b High ammonia loaded control reactor (no bioaugmentation).
c High ammonia loaded reactor (bioaugmentation).
d Batch mode.
of trypticase peptone and 1 mg resazurin (Ollivier et al., 1986) under a H2/CO2 (80%/20%) headspace, pressurised at 2 bar. In fedbatch experiments the combinations (in triplicates) presented in Table 2 were included. All the bottles had total and working volume of 538 and 100 mL, respectively and incubated at 37 ± 1 °C. Incubation period is determined as the time duration of lag and exponential phase.

2.3. Analyses

Methane content in UASB reactors headspace was measured with a gas-chromatograph (GC-TCD) fitted with a column of 1.1 m × 3/16 "Molsieve 137 and 0.7 m × 1/4" chromosorb 108 (MGC 82-12, Mikrolab a/s, Denmark), as described before (Luo et al., 2011). Methane accumulation in the headspace of fed-batch reactors was determined using a gas-chromatograph (GC Shimadzu 14A) equipped with a thermal FID detector using hydrogen as carrier gas (Shimadzu, Kyoto, Japan). Volatile fatty acids (VFA) accumulation in the UASB reactors was determined using a gas-chromatograph (HP 5890 series II) equipped with flame ionisation detector and a FFAP fused silica capillary column, 30 m × 0.53 mm ID with film thickness 1.5 μm, with nitrogen as carrier gas (Kotsopoulos et al., 2009). Glucose in the UASB reactors effluent was determined by high-performance liquid chromatography (Agilent, Horsholm, Denmark) with a refractive index detector and an ultra-violet detector as described in Tomás et al. (2011). The pH fluctuation in the UASB reactors was measured with PHM99 LAB pH meter. The optical density at 600 nm (OD600) was determined with a Spectronic 200+ Spectrophotometer (Thermoscientific, Soeborg, Denmark) as described before (Tomás et al., 2013). All the analyses were made in triplicate (n = 3) and the averages and the corresponding standard deviations (SD) are presented.

2.4. Fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy

Granular samples were collected from the UASB reactors three times (before bioaugmentation: day 88 and after bioaugmentation: days 120 and 142) during the experimental period. The samples immediately fixed in 4% paraformaldehyde for 8 h, washed twice with 1× PBS (1× phosphate buffered saline), and dehydrated gradually with 50%, 80% and 98% of ethanol (Okabe et al., 1999). The dehydrated granules were resuspended and embedded in 100% OCT for 24 h at ambient temperature and then stored at −18 °C, before used. OCT-embedded granules were frozen at −30 °C and sectioned with a cryotome (Leica CM 1850) in 10 μm slices. The granule slices were mounted on slides coated with gelatin and dehydrated gradually with 50%, 80% and 98% of ethanol. To analyse immobilisation of SAO co-culture on the granules of reactor R3 and the different fed-methanogenic cultures and all values presented are the means of independent triplicates (n = 3) ± SD.

Table 2

<table>
<thead>
<tr>
<th>Fed-batch reactors</th>
<th>Inoculum (mL)</th>
<th>M. bourgensis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>BM medium (mL)</th>
<th>Ammonia (g NH4-N L&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Acetate (g HAc L&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R&lt;sub&gt;1&lt;/sub&gt;</td>
<td>-</td>
<td>10</td>
<td>90</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>-</td>
<td>90</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>R&lt;sub&gt;3&lt;/sub&gt;-B3</td>
<td>10</td>
<td>10</td>
<td>80</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> OD<sub>600</sub> = 0.4.

<sup>b</sup> OD<sub>460</sub> = 0.3.

2.5. Calculations and statistical analysis

The maximum specific growth rates of the three sets of fedbatch inoculated cultures (μ<sub>max</sub>) were calculated from the slope of the linear part of the semi-logarithmic graph of methane production versus time as has been described before (Fotidis et al., 2013). All statistical analysis was made using the Graphpad PRISM program (Graphpad Software, Inc., San Diego, California). The Student’s t-test for statistically significant difference (p < 0.05), used to compare the specific growth rates of the different fedbatch methanogenic cultures and all values presented are the means of independent triplicates (n = 3) ± SD.

3. Results and discussion

3.1. UASB reactors experiment

Low ammonia loaded control reactor R<sub>1</sub> converted glucose to methane almost stoichiometrically (Fig. 1) throughout the experiment (periods I–IV). Conversely, methane production of high ammonia loaded control reactor R<sub>3</sub> and bioaugmented reactor R<sub>1</sub> was lower compared to R<sub>1</sub> during all experimental periods due to ammonia inhibitory effect (Fotidis et al., 2013). Specifically, methane production of reactors R<sub>2</sub> and R<sub>3</sub> during period I was 18% lower than the methane production of reactor R<sub>1</sub>. During bioaugmentation (period II), methane production of R<sub>2</sub> and R<sub>3</sub> reactors was further decreased (up to 26% lower compared to R<sub>1</sub>). When the continuous feeding mode resumed (period III), a new steady-state was established in the two high ammonia loaded reactors R<sub>2</sub> and R<sub>3</sub>, with 11% less methane compared to reactor R<sub>1</sub>. At 5 g NH<sub>4</sub>-N L<sup>−1</sup> (period IV) methane production of reactors R<sub>2</sub> and R<sub>3</sub> was decreased rapidly and a so-called “inhibited steady-state” was established with 25% lower methane yield compared to R<sub>1</sub>. The ammonia “inhibited steady-state” has been described before as a suboptimal but stable state for the anaerobic digesters (Niel sen and Angelidaki, 2008). The methane production indicated that there were no significant differences (p > 0.05) between the performance of reactors R<sub>2</sub> and R<sub>3</sub> during the four experimental periods (Fig. 1). It seems that bioaugmentation of the SAO co-culture had no effect on the AD process in bioaugmented reactor R<sub>3</sub> under high ammonia levels (3−5 g NH<sub>4</sub>-N L<sup>−1</sup>). Failure for bioaugmentation of syntrophic acetate oxidisers has also been shown in previous study with continuous-flow stirred tank reactors (Westerholm et al., 2012). The brief decrease of methane production yield in all three reactors on days 88, 120 and 142, was caused by the granules sampling process and didn’t affect further the experiment.

The VFA accumulation in reactor R3 was stable throughout the four experimental periods establishing an efficient biomethanation process (Fig. 2a). On the contrary, significant fluctuations in VFA levels were observed in reactors R<sub>2</sub> and R<sub>3</sub> (Fig. 2a), accompanied with fluctuation in biogas production as presented before. VFA accumulation in reactors R<sub>2</sub> and R<sub>3</sub> indicated different ammonia “inhibited steady-states” (days 100−112 and 115−154), under 3 and 5 g NH<sub>4</sub>-N L<sup>−1</sup>, respectively. The pH of the three reactors was fluctuated from 6.9 to 7.4 (Fig. 2b) constantly remaining inside favourable pH range for the AD process in UASB reactors (Liu
et al., 2008). Glucose levels in the effluent of the three reactors were practically zero throughout the experimental period.

The identical performance of reactors R2 and R3 indicated that the bioaugmentation process with the SAO co-culture did not affect methane production at high ammonia levels. The FISH analyses (images not shown) performed to the granules sampled before (day 88) and after (days 120 and 142) bioaugmentation in reactor R3, verified that C. ultunense was not present. Thus, the positive effects on methane production that SAO co-culture have shown in fed-batch experiments under high ammonia levels (Schnurer et al., 1996) were not demonstrated in the current continuous experiment. The unsuccessful immobilisation of SAO co-culture might be due to its very slow growth rate (Schnurer and Nordberg, 2008), which makes it difficult (if possible) to be immobilised in

**Fig. 1.** Methane production yield of the three UASB reactors. The working parameters of the experimental periods (I–IV) are described in Table 1. Error bars denote standard deviation from the mean of triplicate measurements (n = 3).

**Fig. 2.** Total VFA accumulation (a) and pH fluctuation (b) in the three UASB reactors. The working parameters of the experimental periods (I–IV) are described in Table 1. Error bars denote standard deviation from the mean of triplicate measurements (n = 3).
the UASB reactors (Westerholm et al., 2012). The repeated inoculation (once per day for 15 days) of SAO co-culture in the UASB reactor (bioaugmentation) and the lack of continuous feeding (washing out effect) for 15 days were not enough to ensure the immobilisation of the culture on the granules. Therefore, a fed-batch experiment was performed in order to elucidate the reasons led bioaugmentation of the SAO co-culture in the UASB reactor to fail.

3.2. Fed-batch experiment

Methane production in the fed-batch reactors with *M. bourgensis* (*B*<sub>MC</sub>) was below detection limits throughout the experimental period (Fig. 3). Conversely, methane production occurred in fed-batch reactors containing SAO co-culture alone (*B*<sub>SAO</sub>) or SAO co-culture in co-cultivation with *M. bourgensis* (*B*<sub>SAO-MC</sub>). The total incubation period of SAO co-culture was significantly reduced, almost by 33% when the culture was co-cultivated with the hydrogenotrophic *M. bourgensis*, in fed-batch reactors. Furthermore, higher growth rate (statistically significant difference, *p* < 0.05) was found for *B*<sub>SAO-MC</sub> (*μ<sub>max</sub> = 0.01 h<sup>-1</sup>) compared to *B*<sub>SAO</sub> (*μ<sub>max</sub> = 0.007 h<sup>-1</sup>). Schnürer et al. (1994) have reported a maximum growth rate of 0.001 h<sup>-1</sup> for the SAO co-culture, which is sevenfold lower than the growth rate presented in the current study. Nevertheless, this growth rate is still very low compared to *μ<sub>max</sub> = 0.039 h<sup>-1</sup>* that have been reported for *M. bourgensis* MS2<sup>T</sup> with H<sub>2</sub>/CO<sub>2</sub> as substrate (Ollivier et al., 1986) or compared to the growth rates of mesophilic acetoclastic methanogens (Jetten et al., 1992). These findings indicate that the methanogenic partner of the SAO co-culture (*Methanoculleus* spp. strain MAB1) in the current study is the reason for the decreased growth rate and consequently the prolonged incubation period of the SAO co-culture. One possible explanation is that the IHT between the SAO co-culture members was limited (Hattori, 2008). Thus, the hydrogen partial pressure increased to inhibitory levels for the acetate oxidising bacterium. The hydrogenotrophic *M. bourgensis*, which had been co-cultivated with the SAO co-culture in the current study, reduced the partial pressure of hydrogen more efficiently compared to *Methanoculleus* spp. strain MAB1, expediting the incubation period and led to a 42% higher growth rate.

3.3. Comparative evaluation of the UASB and fed-batch reactors

The findings of this study indicate that hydrogenotrophic methanogens and not the SAOB are the key players of the AD process under high ammonia levels. SAOB are more resistant to ammonia inhibitory effect, compared to the methanogens; therefore, capable of catabolising acetate as long as the thermodynamic conditions are favourable (De Vrieze et al., 2012). It has been proposed by Hattori (2008) that SAOB and their syntrophic methanogenic partners almost equally share the extremely small energy exploited from the acetate oxidation. This low energy yield of mesophilic acetate oxidation was probably the reason for the unsuccessful bioaugmentation of SAO in the UASB reactor and for the corresponding low growth rates of SAO co-culture (compared to acetoclastic methanogens) in the fed-batch experiments. To exploit this small window of energy, it is crucial to maintain low partial pressure of hydrogen in the AD system (Hattori, 2008). Therefore, considering that the hydrogenotrophic partner defines the growth rate of the syntrophic co-culture, introduction of a “critical biomass” of fast growing and ammonia tolerant hydrogenotrophic methanogens is necessary to increase the odds of successful bioaugmentation in UASB reactors subjected to high ammonia levels.

4. Conclusions

Results obtained from the current study demonstrated that bioaugmentation of SAO co-culture in a UASB reactor was not possible most probably due to the slow growth of the consortium caused by the hydrogenotrophic partner *Methanoculleus* spp. strain MAB1. On the contrary, the incubation period of SAO co-culture was reduced more than 30% and the corresponding maximum growth rate (*μ<sub>max</sub>*) was increased more than 40% (compared to SAO co-culture) when it was co-cultivated with *M. bourgensis* in fed-batch reactors. This indicates that methanogens were the limiting factor during the bioaugmentation of the SAO co-culture in the UASB reactor.

Acknowledgements

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References


Appendix A.4
The dominant acetate degradation pathway/methanogenic composition in full-scale anaerobic digesters operating under different ammonia levels

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Abstract Ammonia is a major environmental factor influencing biomethanation in full-scale anaerobic digesters. In this study, the effect of different ammonia levels on methanogenic pathways and methanogenic community composition of full-scale biogas plants was investigated. Eight full-scale digesters operating under different ammonia levels were sampled, and the residual biogas production was followed in fed-batch reactors. Acetate, labelled in the methyl group, was used to determine the methanogenic pathway by following the $^{14}$CH$_4$ and $^{14}$CO$_2$ production. Fluorescence in situ hybridisation was used to determine the methanogenic communities’ composition. Results obtained clearly demonstrated that syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis was the dominant pathway in all digesters with high ammonia levels (2.8–4.57 g NH$_4$-N L$^{-1}$), while acetoclastic methanogenic pathway dominated at low ammonia ($<1.21$ g NH$_4$-N L$^{-1}$). Thermophilic Methanomicrobiales spp. and mesophilic Methanobacteriales spp. were the most abundant methanogens at free ammonia concentrations above 0.44 g NH$_3$-N L$^{-1}$ and total ammonia concentrations above 2.8 g NH$_4$+-N L$^{-1}$, respectively. Meanwhile, in anaerobic digesters with low ammonia ($<1.21$ g NH$_4$+-N L$^{-1}$) and free ammonia ($<0.07$ g NH$_3$-N L$^{-1}$) levels, mesophilic and thermophilic Methanosetaeaceae spp. were the most abundant methanogens.

Keywords Acetoclastic pathway · Ammonia toxicity · Anaerobic digestion · Hydrogenotrophic pathway

Introduction

Anaerobic digestion (AD) is a biological process which catabolises complex organic compounds (Minale and Worku 2013). AD is mediated by different microorganisms, mainly protozoa, fungi, bacteria and archaea, to produce biogas (40–75 % CH$_4$ and 25–60 % CO$_2$) from diverse organic substrates (Angelidaki et al. 2011). The archaea involved in the AD process are members of acetate utilising order Methanosarcinales (comprising two families: Methanosarcinaceae, versatile acetoclastic methanogens, and Methanosaetaceae, strictly acetoclastic methanogens) and strictly hydrogenotrophic Methanomicrobiales, Methanobacteriales, Methanococcales and Methanopyrales orders (Sarmiento et al. 2011). The complex organic substrates cannot be used by methanogens directly and must be catabolised by the other microorganisms mediating anaerobic digestion to substrates (e.g. acetate or H$_2$ and CO$_2$) suitable for methanogenesis. Hydrogenotrophic methanogens develop a syntrophic association with the syntrophic acetate-oxidising bacteria (SAOB). Acetate oxidation (CH$_3$COO$^-$ + 4H$_2$O $\rightarrow$ 2HCO$_3^-$ + 4H$_2$ + H$^+$ $\Delta$G° = +104.6 kJ mol$^{-1}$) is a thermodynamically unfavourable bioreaction at standard conditions; however, coupling of the acetate oxidation with hydrogenotrophic methanogenesis (4H$_2$ + HCO$_3^-$ + H$^+$ $\rightarrow$ CH$_4$ + 3H$_2$O) reduces the concentration of hydrogen and thereby decreases the $\Delta$G° ($\Delta$G° = −135.6 kJ mol$^{-1}$), and the overall process becomes favourable. The interspecies hydrogen transfer between SAOB and hydrogenotrophic methanogens has been well described (Stams et al. 2006).

Ammonia (ammonium + free ammonia) is the major toxicant in full-scale anaerobic digesters, leading to
suboptimal utilisation of the biogas potential of the feedstocks and causing economic losses to biogas production plants. High ammonia levels are often encountered in residues (e.g. pig, poultry and mink manures, slaughterhouse by-products), which otherwise would be highly suitable for biogas production. Anaerobic degradation of these residues can contribute to the increasing global need for more renewable energy sources and, consequently, lead to a future independence from the fossil fuels. Therefore, this ambitious goal requires an efficient industrial process for optimum biogas production, even from ammonia-rich substrates, as long as the ammonia toxicity problem is solved. Free ammonia, which increases concurrently with the temperature and pH, has been identified as the main component causing methanogenesis inhibition (de Baere et al. 1984). Generally, for ammonia non-acclimatised methanogenic cultures, total ammonia and free ammonia concentrations over 3 g NH₄⁺-N L⁻¹ and 0.15 g NH₃-N L⁻¹, respectively, are known to inhibit methanogenesis independently of temperature and pH levels (Yenigün and Demirel 2013).

The only realistic way today to counteract ammonia toxicity is to lower the temperature of the digester reducing the free ammonia levels (Nielsen and Angelidaki 2008a). However, this method can alleviate ammonia toxicity only to a limited range (Nielsen and Angelidaki 2008b). Other traditional physicochemical methods, based on addition of ammonium-binding ions (Kougias et al. 2013), increasing the C/N ratio, dilution with water (Nielsen and Angelidaki 2008a) or striping of ammonia (Nakashimada et al. 2008), have not shown any practical applicability, and they are either unrealistic or too expensive.

When other important environmental factors (temperature, pH and volatile fatty acids (VFA)) are within optimum range, it is generally accepted that ammonia levels determine the dominant methanogenic pathway and the methanogenic community profile in laboratory-scale anaerobic reactors (Chen et al. 2008). The composition, the relative abundance and the interactions within the methanogenic community are critical for the performance of the anaerobic digester (Angelidaki et al. 2011). Among different microorganisms mediating AD, methanogens are the most sensitive to ammonia inhibition (Kayhanian 1994). According to the literature, ammonia is mainly inhibiting the acetoclastic methanogenic (ACM) pathway, leading to VFA accumulation and suboptimal biogas production (Yenigün and Demirel 2013). Furthermore, syntrophic acetate oxidation followed by hydrogenotrophic methanogenesis (SAO-HM) metabolic pathway seems to be more robust to ammonia toxicity (Chen et al. 2008).

Up to now, research on how ammonia affects microbial composition and biochemical pathways has mainly focused on laboratory-scale biogas digesters. It is not clear how the methanogenic microbial communities respond to ammonia exposure in the complex environments of full-scale digesters. Therefore, it is necessary to obtain fundamental knowledge of the relations between ammonia and microbial ecology, which would assist the development of new microbiological methods to counteract ammonia inhibition. Thus, the aim of the present study was to investigate the effect of different ammonia levels on methanogenic pathways and methanogenic communities in full-scale anaerobic digesters. The work presented in this study was conducted in the Technical University of Denmark and was completed in November 2012.

Materials and methods

Inocula and media

A total of eight Danish centralised biogas plants (CBP) operating under different temperatures, hydraulic retention times, VFA and ammonia levels were sampled (Table 1). All digesters were fully mixed tank reactors. The selection of those plants was based on previous studies (Karakashve et al. 2005; Karakashve et al. 2006) in order to define representative groups of reactors within desired range of ammonia concentrations (low: <1.5 g NH₄⁺-N L⁻¹, medium: 1.5–2.8 g NH₄⁺-N L⁻¹ and high: >2.8 g NH₄⁺-N L⁻¹), primary feedstocks and temperatures.

The feedstock for six of the digesters (Nysted, Hashøj, Vegger, Studsgård, Snertinge and Lemvig) consisted of 70–90 % animal manure and 10–30 % organic waste from abattoirs or food industries. Two CBP (Lundtofte and Hillerød) were fed with primary and secondary WWTP sludge. The samples were collected from the effluent lines of the digesters and immediately transferred in 2-L polystyrene containers closed with one-way valve on the lid to release overpressure created by residual biogas production. The sealed containers were transported to the laboratory within 24 h.

Experimental setup

In the experiments, 118-mL fed-batch reactor vessels (serum vial type) were used. 40 mL inocula were dispensed anaerobically under a N₂/CO₂ (80/20 %) headspace in fed-batch reactors which were closed with butyl rubber stoppers, sealed with aluminium caps and incubated at respective temperatures where the inocula were taken from (Table 1). All experiments were conducted in triplicates. In order to follow the residual biogas production of the
Table 1 Full-scale digesters working conditions and characteristics of the digesters’ content

<table>
<thead>
<tr>
<th>Plant</th>
<th>Operating Temp. ± SD (°C)</th>
<th>Primary feedstock</th>
<th>pH ± SD</th>
<th>VFA ± SD (g HAc L⁻¹)</th>
<th>TNK± ± SD (g N L⁻¹)</th>
<th>Total ammonia ± SD (g NH₄-N L⁻¹)</th>
<th>Free ammonia± ± SD (g NH₃-N L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nysted</td>
<td>38 ± 1</td>
<td>Pig slurry</td>
<td>7.86 ± 0.02</td>
<td>0.56 ± 0.08</td>
<td>4.18 ± 0.04</td>
<td>2.93 ± 0.11</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>Hasboj</td>
<td>37 ± 1</td>
<td>Cattle and pig slurry</td>
<td>7.92 ± 0.04</td>
<td>0.80 ± 0.05</td>
<td>5.81 ± 0.13</td>
<td>4.57 ± 0.15</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td>Lundtoftea</td>
<td>35 ± 1</td>
<td>Primary and biological sludge</td>
<td>7.48 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>2.29 ± 0.04</td>
<td>1.21 ± 0.06</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Vegger</td>
<td>55 ± 1</td>
<td>Cattle manure</td>
<td>7.99 ± 0.01</td>
<td>1.12 ± 0.03</td>
<td>3.03 ± 0.01</td>
<td>2.03 ± 0.08</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>Stadsård</td>
<td>52 ± 1</td>
<td>Cattle and pig slurry</td>
<td>7.96 ± 0.04</td>
<td>0.94 ± 0.07</td>
<td>3.72 ± 0.09</td>
<td>2.04 ± 0.05</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>Sneringe</td>
<td>52.5 ± 1</td>
<td>Cattle and pig slurry</td>
<td>7.77 ± 0.04</td>
<td>1.71 ± 0.06</td>
<td>3.07 ± 0.08</td>
<td>2.26 ± 0.11</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Lemvig</td>
<td>52.5 ± 1</td>
<td>Cattle, pig and poultry slurry</td>
<td>7.86 ± 0.03</td>
<td>1.83 ± 0.02</td>
<td>3.24 ± 0.12</td>
<td>2.44 ± 0.08</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>Hillerøde</td>
<td>55 ± 1</td>
<td>Primary and biological sludge</td>
<td>7.4 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>1.54 ± 0.01</td>
<td>0.90 ± 0.08</td>
<td>0.07 ± 0.01</td>
</tr>
</tbody>
</table>

a Wastewater treatment plants
b Temperature deviation (SD)
c Data are mean values (n = 3) ± standard deviation (SD)
d Total Kjeldahl nitrogen
e Calculated according to Eq. 1

inocula and define the methanogenic pathway, the following fed-batch reactors were included: a) three fed-batch reactors with inoculum only for estimation of residual methane production and b) three fed-batch reactors containing 47.58 ± 5.49 KBq L⁻¹ or 1.3 ± 0.15 µg L⁻¹ [2-¹⁴C] sodium acetate (Amersham Pharmacia Biotech, England) for methanogenic pathway identification (calculation of ¹⁴CO₂/¹⁴CH₄ ratio).

Analytical methods

Methane production was determined using gas chromatograph (Shimadzu GC-8A, Tokyo, Japan) equipped with a glass column (2 m, 5 mm OD, 2.6 mm ID) packed with Porapak Q 80/100 mesh (Supelco, Bellefonte, PA, USA) and with a flame ionisation detector (FID). The oven temperature was 70 °C, and nitrogen was the carrier gas. Total nitrogen (TKN), total ammonia and pH were determined according to APHA’s standard methods (APHA 2005). VFA were determined using gas chromatograph (HP 5890 series II) equipped with a flame ionisation detector (FID) and an HP-FFAP column as described previously (Kotsopoulos et al. 2009). The pH of the fed-batch reactors was measured before and after the experimental period with PHM99 LAB pH meter (Radiometer™). All the analyses were carried out in triplicate, and the average values are presented with the corresponding standard deviations calculated from the analyses. All the results are given under standard temperature and pressure conditions.

FISH analyses

FISH was used to identify the methanogenic microbial populations as described before by Hugenholtz (2002). Samples for FISH analyses were taken before the experiments. The probes used along with their target domains, orders or families are presented in Table 2. All probes were used at optimal stringency with 0–50 % formamide as described in previous work (Fotidis et al. 2013b). ARC915 was used to identify all members of the Archaea domain, and EUB338 and 1–49 % of all Archaea members (positive for ARC915), respectively. Finally, hydrogenotrophic
Methanopyrales spp. (Shima et al. 1998) is a hyperthermophilic (optimum temperature 98 °C) member species and was not considered further in this study.

Radioisotopic analyses

As it has been demonstrated before (Fotidis et al. 2013b), methanogenic pathway can be determined by measuring the production of $^{14}$CH$_4$ and $^{14}$CO$_2$ from acetate labelled in the methyl group (C-2). When SAO-HM is the dominant methanogenic pathway, the methyl group of the $[2-^{14}C]$ acetate is converted to $^{14}$CO$_2$. Combined with the fact that the pool of labelled carbon dioxide ($^{14}$CO$_2$) in AD is much lower than the pool of unlabelled $^{12}$CO$_2$, the hydrogenotrophic methanogenesis will only convert a small part of $^{14}$CO$_2$ molecules to $^{14}$CH$_4$. On contrary, when ACM is the dominant methanogenic pathway, $^{14}$C carbon derived from the methyl group of the $[2-^{14}C]$ acetate goes to $^{14}$CH$_4$. Therefore, the concentration of $^{14}$CH$_4$ in the gas phase will be much higher compared to the concentration of $^{14}$CO$_2$ formed during the oxidation of $[2-^{14}C]$ acetate. It is generally assumed that when $^{14}$CO$_2/^{14}$CH$_4 < 1$, the dominant pathway is ACM, while when $^{14}$CO$_2/^{14}$CH$_4 > 1$, SAO-HM is the dominant pathway. In the current experiment, after the residual biogas production ceased, fed-batch reactors containing $[2-^{14}C]$ acetate were acidified (final pH = 0.95 ± 0.1) with 7.2 M HCl. Thus, dissolved H$^{14}$CO$_3^−$ converted to $^{14}$CO$_2$ and trapped with a CO$_2$ absorber for liquid scintillation counting (Carbosorb$^R$-E; PerkinElmer Company). The $^{14}$CH$_4$ was combusted to $^{14}$CO$_2$ in a tube furnace (≥800 °C) and the $^{14}$CO$_2$ produced trapped also in Carbosorb$^R$-E. Supernatant of the liquid part of the fed-batch reactors was mixed with tap water and scintillation liquid (Ultima Gold$^{TM}$ XR, PerkinElmer Company), to measure the residual $[2-^{14}C]$ acetate radioactivity. All radioactivity measurements were taken in triplicates using a liquid scintillation counter (Tri-Carb 1600; PerkinElmer Company), according to Fotidis et al. (2013b).

Calculations

Free ammonia

Free ammonia concentrations were calculated from the equilibrium of Eq. (1) (Fotidis et al. 2013b):

$$[NH_3] = \frac{[NH_3 + NH_4^+] \times 10^{\frac{pH - pK_a}{pK_a}}}{1 + 10^{\frac{pH - pK_a}{pK_a}}}$$  \hspace{1cm} (1)

where $[NH_3]$ and $[NH_3 + NH_4^+]$ are the free and total ammonia concentrations, respectively. $K_a$ is the dissociation constant, with values $1.13 \times 10^{-9}, 1.28 \times 10^{-9}, 1.29 \times 10^{-9}, 3.27 \times 10^{-9}, 3.37 \times 10^{-9}$ and $3.89 \times 10^{-9}$ for 35, 37, 38, 52, 52.5 and 55 °C, respectively, calculated as described previously (Hafner et al. 2006) with the use of the corresponding pH values (Table 1).

Growth rate

The maximum specific growth rate of the mixed methanogenic cultures ($\mu_{\text{max}}$) was calculated from the slope of the linear part of the graph of residual methane production (natural logarithm) versus time as described before (Gray et al. 2009).

Statistical analysis

The GraphPad Prism program (Graphpad Software, Inc., San Diego, California) was used to perform statistical analysis. The Student’s $t$ test for statistically significant difference ($P < 0.05$) was used to compare the specific growth rates of the different methanogenic populations, and all values were the mean of three independent replicates ($n = 3$) ± standard deviation (SD).

<table>
<thead>
<tr>
<th>Probe</th>
<th>Phylogenetic group</th>
<th>Functional group</th>
<th>Probe sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARC915</td>
<td>Archaea</td>
<td>Mainly meth.</td>
<td>GTGCTCCCCGCAATTCTC</td>
<td>Stahl and Amann (1991)</td>
</tr>
<tr>
<td>MB1174</td>
<td>Methanobacteriales</td>
<td>Hydrogenotrophic meth.</td>
<td>TACCGTCGTCCACTCTCTC</td>
<td>Sekiguchi et al. (1999)</td>
</tr>
<tr>
<td>MC1109</td>
<td>Methanococcales</td>
<td>Hydrogenotrophic meth.</td>
<td>GCAAATAGGGCACGGGTCT</td>
<td>Raskin et al. (1994)</td>
</tr>
<tr>
<td>MG1200</td>
<td>Methanomicrobiales</td>
<td>Hydrogenotrophic meth.</td>
<td>CGGATAATTGGGCGCTGCTG</td>
<td>Sekiguchi et al. (1999)</td>
</tr>
<tr>
<td>MS1414</td>
<td>Methanosarcinaceae</td>
<td>Acetoclastic meth. (also hydrogen)</td>
<td>CTCACCCATACCTCACTCGGG</td>
<td>Sekiguchi et al. (1999)</td>
</tr>
<tr>
<td>MSMX860</td>
<td>Methanosarcinales</td>
<td>Acetoclastic meth. (also hydrogen)</td>
<td>GGCTGCGTTACCGGTCATCCTT</td>
<td>Raskin et al. (1994)</td>
</tr>
<tr>
<td>MX825</td>
<td>Methanosaetaceae</td>
<td>Acetoclastic meth.</td>
<td>TCGACCGTTGCGCGACCTAGC</td>
<td>Raskin et al. (1994)</td>
</tr>
</tbody>
</table>

*a Meth. methanogenic

*b W, A + T mixed base
Results and discussion

Maximum specific growth rates

All eight fed-batch reactors produced methane without any lag phase for 18–22 days before they entered into the “stationary phase”, demonstrating a normal AD process (data not shown). The current results did not establish a clear correlation between \( \mu_{\text{max}} \) and ammonia levels (Fig. 1) of the full-scale digesters tested. The highest \( \mu_{\text{max}} \) (statistically significant difference, \( P < 0.05 \)) was found at high ammonia levels (2.93 g NH\(_4\)\(^+\)-N L\(^{-1}\)), and the lowest \( \mu_{\text{max}} \) (statistically significant difference, \( P < 0.05 \)) was found at medium ammonia levels (2.26 g NH\(_4\)\(^+\)-N L\(^{-1}\) and 2.44 g NH\(_4\)\(^+\)-N L\(^{-1}\)) (statistically no significant difference, \( P > 0.05 \)). In all the other ammonia concentrations, \( \mu_{\text{max}} \) varied between 0.050 and 0.061 h\(^{-1}\) (Fig. 1). This indicates that some degree of acclimatisation of the corresponding methanogenic cultures to the different ammonia levels in the digesters had occurred through long-term exposure (Rajagopal et al. 2013). Nevertheless, mixed mesophilic methanogenic cultures digesting animal manure at low ammonia and low free ammonia levels were previously reported to have \( \mu_{\text{max}} \) of 0.1 h\(^{-1}\) (Koster and Komen 1988), which is significantly higher compared to the growth rates found in the current study. Therefore, it can be concluded that all the digesters treating manure, tested in the current experiment, were operating under “inhibitory steady state” affected by the ammonia and/or free ammonia inhibitory levels (Nielsen and Angelidaki 2008b).

Low \( \mu_{\text{max}} \) in digesters with high VFA levels (above 1.5 g HAc L\(^{-1}\), in cases of Lemvig and Snertinge) can be explained by the ammonia–VFA synergistic effect demonstrated previously by Lu et al. (2013). Furthermore, Angelidaki et al. (2005) have identified 1.5 g HAc L\(^{-1}\) as the VFA threshold for a healthy AD process in full-scale anaerobic digesters. The highest growth rates were observed in mesophilic and thermophilic digesters with initial VFA levels between 0.5 and 1.1 g HAc L\(^{-1}\), no matter of the ammonia levels (Fig. 1). Conversely, other studies have reported that VFA accumulation alleviates free ammonia toxicity in anaerobic digesters (Angelidaki et al. 1993; Hansen et al. 1998). Specifically, biomethanation process instability due to ammonia toxicity results in VFA accumulation, which leads to a pH decrease, alleviating consequently the free ammonia inhibition effect (Hejnfelt and Angelidaki 2009). This decrease in free ammonia could be an explanation for the stability of the process under increased VFA (>1.5 g HAc L\(^{-1}\)) and...
ammonia levels (>2 g \(\text{NH}_4^+\text{-N L}^{-1}\)), with lower but stable growth rates (Rajagopal et al. 2013). Although the ammonia–VFA synergistic effect in full-scale digesters has yet to be determined, our results suggest that when both ammonia and VFA levels are increased, AD process is affected.

Dominant acetate degradation pathway
and methanogenic populations

At high ammonia (>2.8 g \(\text{NH}_4^+\text{-N L}^{-1}\)) and high free ammonia (>0.44 g \(\text{NH}_3\text{-N L}^{-1}\)) levels, SAO-HM pathway was the dominant pathway (Fig. 2) mediated by hydrogenotrophic methanogens belonging to \textit{Methanomicrobiales} spp. and \textit{Methanobacteriales} spp., respectively (Fig. 3). The findings in this study clearly indicate that, in full-scale digesters, hydrogenotrophic methanogenic Archaea were tolerant to ammonia toxicity. These results are in agreement with previous laboratory-scale studies (Demirel and Scherer 2008; Ahring 1995). Hence, from data illustrated in Fig. 2 and Table 3, it can be concluded that the dominant methanogenic pathway and the abundance of the dominant methanogens are correlated with ammonia levels in the full-scale digesters.

An interesting finding was the identification of the non-dominant \textit{Methanococcales} spp. (Table 3) in a manure-fed digester (Nysted). This hydrogenotroph is not very common and usually appears in marine environments and wastewater-fed anaerobic digesters (Tabatabaei et al. 2010; Tumbula and Whitman 1999). Nevertheless, \textit{Methanococcales} spp. have also been recently identified in continuous anaerobic digesters using fibrous biofilm carriers, treating mainly (75 %) fresh cow manure (Gong et al. 2011).

In anaerobic digesters, free ammonia levels increase concurrently with pH increase (Chen et al. 2008). Furthermore, pH values less than 6.8 and greater than 8.3 would cause process failure during AD (Banu et al. 2007). In the current study, isotopic analyses revealed that SAO-HM pathway was dominant in all digesters with pH above 7.8 (Table 1; Fig. 3). High pH often coincides with high ammonia concentrations, which is consistent with the dominance of SAO-HM. Contrary to our results, Hao et al. (2012) reported that SAO-HM pathway was promoted by low pH (5.0–6.5) in laboratory-scale experiments. This inconsistency between the previously mentioned laboratory-scale results and the full-scale findings suggests that free ammonia (and not pH) is one of the key environmental factors determining the methanogenic pathway in the complex anaerobic environments. Thus, based also on findings in previous studies (Fotidis et al. 2013a), it is possible to introduce SAO-HM pathway in a digester by controlling the ammonia levels as long as a “critical biomass” of ammonia-tolerant fast-growing methanogenic consortium is present. If ammonia-tolerant consortia are not present, a technical approach to overcome ammonia inhibitions could be bioaugmentation (Schauer-Gimenez et al. 2010) of ammonia-tolerant methanogenic consortia in anaerobic digesters operating under high ammonia levels. Unfortunately, this has not been demonstrated yet. According to recent study (Westerholm et al. 2011), a bioaugmentation attempt in continuous laboratory-scale reactors was proven to be unsuccessful. Interestingly, in

![Fig. 3 Distribution of the dominant methanogens plotted with ammonia versus free ammonia concentrations in full-scale digester samples used in the experiments. MB Methanobacteriales spp., MG Methanomicrobiales spp., MS Methanosarcinaceae spp., MX Methanosaetaceae spp. M and T represent mesophilic and thermophilic working conditions, respectively. Error bars denote standard deviation from the mean of triplicate measurements](https://example.com/fig3)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Dominant(^b)</th>
<th>Non-dominant(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nysted (M)</td>
<td>MB(^c)</td>
<td>MS, MC</td>
</tr>
<tr>
<td>Hashøj (M)</td>
<td>MB</td>
<td>MG, MS</td>
</tr>
<tr>
<td>Lundtofte (M)(^a)</td>
<td>MX</td>
<td>MS</td>
</tr>
<tr>
<td>Vegger (T)</td>
<td>MG</td>
<td>MB, MS</td>
</tr>
<tr>
<td>Studsgård (T)</td>
<td>MG</td>
<td>MS, MB</td>
</tr>
<tr>
<td>Snertinge (T)</td>
<td>MS</td>
<td>MG, MB</td>
</tr>
<tr>
<td>Lemvig (T)</td>
<td>MG</td>
<td>MB, MS</td>
</tr>
<tr>
<td>Hillerød(^d) (T)</td>
<td>MX</td>
<td>MS</td>
</tr>
</tbody>
</table>

\(^a\) Wastewater treatment plant

\(^b\) Dominant methanogens: between 50 and 100 % of the total number (positive for ARC915) of methanogenic cells; non-dominant methanogens: 1–49 % of the total number of methanogenic cells

\(^c\) MB Methanobacteriales spp., MC Methanococcales spp., MG Methanomicrobiales spp., MS Methanosarcinaceae spp., MX Methanosaetaceae spp

\(^d\) Mesophilic digester

(T) Thermophilic digester

---

Table 3 Dominant and non-dominant methanogenic populations in the full-scale digesters

---

our recent study, we could successfully relieve ammonia inhibition by bioaugmentation approach (unpublished data).

At medium ammonia levels (2.26 g NH₄⁺-N L⁻¹), ACM pathway was found to be dominant mediated by thermophilic Methanosarcinaceae spp. Acetoclastic Methanosarcinaceae spp. have been regarded as sensitive to ammonia in laboratory-scale experiments (Westerholm et al. 2011) but also capable of acclimatising to high ammonia and free ammonia levels (Jarrell et al. 1987). Nevertheless, the presence of Methanosarcinaceae spp. at high free ammonia levels (Fig. 2) indicates that acclimatisation of this methanogen to these free ammonia levels is also possible in full-scale digesters. At low ammonia concentrations (<1.5 g NH₄⁺-N L⁻¹), ACM was found to be the sole dominant pathway, mediated by Methanosaeacea spp. (Fig. 3; Table 3). These findings are in agreement with previous researches, reporting that Methanosaeacea spp. was the dominant methanogens in WWTP digesters operating under low ammonia and VFA levels (Karakashvet al. 2006). The current study was not aiming to perform a comparative analysis of the evolution of the dominant methanogens in the full-scale digesters through time. Nevertheless, the simple comparison of data derived from the same reactors seven years ago (Karakashvet al. 2005) and the current study indicates that the reactors with low (<1.5 g NH₄⁺-N L⁻¹) ammonium levels tend to have more stable dominant methanogenic communities compared to the reactors with medium and high (>1.5 g NH₄⁺-N L⁻¹) ammonium levels. Undoubtedly, long-term monitoring of the microbial composition and the corresponding ammonia levels in the full-scale digesters is required before a link between the methanogens’ population stability and ammonia levels is established.

Conclusion

This study investigated the effect of different ammonia levels on methanogenic pathways and methanogenic communities of eight full-scale anaerobic digesters. Ammonia and free ammonia levels were shown to have profound effect on the methanogenic pathway and methanogenic community profile of the full-scale anaerobic digesters tested. Furthermore, SAO-HM pathway was found to be dominant, mediated by Methanobacteriales spp. and Methanomicrobiales spp. at high ammonia (>2.8 g NH₄⁺-N L⁻¹) and high free ammonia levels (>0.44 g NH₃-N L⁻¹), respectively. Results obtained pointed out that bioaugmentation of ammonia-tolerant methanogenic consortia in anaerobic digesters operating under high ammonia levels could be a technical approach to solve the ammonia toxicity problem.

Acknowledgments We thank Hector Garcia for technical assistance with the experiments and Mike Podevin for editing support. This work was supported by Energinet.dk under the project framework ForsKEL, “Innovative process for digesting high ammonia wastes” (programme no. 2010-10537) and by the Bioref-Øresund project under EU INTERREG IVA.

References

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Kotsopoulos TA, Fotidis IA, Tsolakis N, Martzopoulos GG (2009) Biogas production from pig slurry in a CSTR reactor system with mixed cultures under hyperthermophilic temperature \((70\,\text{C})\). Biomass Bioenergy 33:1168–1174


Højere gasudbytte med særlige metanbakterier

Fuldskala biogasanlæg mister typisk omkring 30 procent af deres gasproduktion på grund af for høje koncentrationer af ammoniak, men ved at øge mængden af særlige bakteriekulturer kan gasproduktionen stabiliseres på et højt niveau.

En af de væsentligste årsager til ubalance i danske biogasanlæg er den høje koncentration af ammoniak, der omdannes til ammonium ved oplosning i gylle. Ammoniak hæmmer de metandannende bakterier – primært de bakterier, der forbruger acetat (eddikesyre).

De indsamlede data i projektet viser, at ammoniakkoncentrationen er den vigtigste faktor, der påvirker biogasprocessen, og det anslås, at fuldskala biogasanlæg mister omkring 30 procent af deres gasproduktion på grund af for høje koncentrationer af ammoniak.

I projektet er det lykkedes at identificere specifikke grupper af metandannende bakterier, der er tolerante over for høje koncentrationer af ammoniak. Ved at øge mængden af disse bakterier i reaktorer, der var hæmmet af høje ammoniumkoncentrationer, lykkedes det at øge gasproduktionen med mere end 30 procent. Projektets resultater åbner således mulighed for at sikre en effektiv udrådnning af biomasser med et højt ammoniumindhold ved at fremme særlige bakteriekulturer i reaktoren.

Gasproduktion (milliliter metan/liter reaktor)

<table>
<thead>
<tr>
<th>Dage</th>
<th>0 1 2 3 4 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>250 300 350 400 450 500</td>
</tr>
</tbody>
</table>

Nye bakterier

Kontrolreaktor Reaktor med ændret bakteriekultur

Gasproduktionen fra en kontrolreaktor og en reaktor hvor bakteriesammensætningen ændres.

Titel: Nedbrydning af højt ammoniak-holdigt affald

Kontakt: DTU Miljø, Dimitar Borisov Karakashev, ☎ 4525 1446, ✉ dbka@env.dtu.dk

Sagsnr.: ForskEL-10537

Tilskud fra: PSO

Tilskud: 2.040.000 kroner
Appendix A.6
Isolation of an ammonia tolerant methanogenic enriched culture

Ioannis Fotidis, Dimitar Karakashev, Nicolas Proietti and Irini Angelidaki

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Corresponding author: dbka@env.dtu.dk

Ammonia is the major inhibitor in full scale digesters of animal wastes which are rich in urea and/or proteins. Ammonia inhibition leads to decreased methane production rates, high volatile fatty acids concentration and low degradability of the biomass, with economic losses for the biogas plant. The use of methanogens tolerant to ammonia inhibition could provide a sustainable solution for cost-effective digestion of abundant ammonia-rich wastes. In the current study an enrichment culture was isolated from a mesophilic biogas reactor that was able to utilize acetate and form methane under high ammonia concentrations. The enrichment culture produced methane through aceticlastic pathway with maximum growth rate (\( \mu_{\text{max}} \)) of 0.014 h\(^{-1} \) at 9.26 g NH\(_4^+\)-N L\(^{-1} \). Microbial community composition analysis (fluorescence in-situ hybridization-FISH) showed that the methanogens of the enrichment culture belonged only to Methanosarcinaceae species. Results obtained in this study, clearly demonstrated for the first time that aceticlastic methanogenesis can also occur under high ammonia concentrations.
Appendix A.7
Immobilisation of an ammonia tolerant methanogenic consortium in high performance anaerobic digesters

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*Corresponding author: ioanf@env.dtu.dk

Inhibition of the anaerobic digestion due to ammonia - rich residues (pig and chicken manure, slaughterhouse residues, mink manure etc.) is a common problem, and presently the majority of the centralized biogas plants are operating under “inhibited steady state” conditions. Ammonia toxicity leads to decreased methane production rates, high volatile fatty acids concentration and low degradability of the biomass, with economic losses for the biogas plants. The use of methanogenic consortia tolerant to ammonia toxicity could provide a sustainable solution for cost - effective digestion of less attractive ammonia - rich residual resources. In the current study, an aceticlastic mesophilic ammonia tolerant methanogenic consortium was immobilised in up - flow anaerobic sludge blanket (UASB) reactors that were operated under high ammonia concentrations. The results obtained showed that at 7 g NH₄⁺ - N L⁻¹, the UASB reactor with the immobilised consortia had up to 40% higher methane production rate compared to the ammonia inhibited control reactor. This finding indicates that immobilization of ammonia tolerant methanogenic consortia in high performance anaerobic digesters is a possible solution towards sustainable digestion of ammonia - rich residues.
**Inhibition of the anaerobic digestion due to ammonia-rich residues is a common problem for the centralized biogas plants. Ammonia toxicity leads to decreased methane production and economic losses for the plants. The use of methanogenic consortia tolerant to ammonia toxicity could provide a sustainable solution for cost-effective digestion of less attractive ammonia-rich waste.**

**The aim of the current study was to immobilise an ammonia tolerant methanogenic consortium in a high performance anaerobic digester.**

---

**Introduction**

Inhibition of the anaerobic digestion due to ammonia-rich residues is a common problem for the centralized biogas plants. Ammonia toxicity leads to decreased methane production and economic losses for the plants. The use of methanogenic consortia tolerant to ammonia toxicity could provide a sustainable solution for cost-effective digestion of less attractive ammonia-rich waste.

**Experimental setup**

ATMC has been enriched from inoculum derived from a mesophilic full scale biogas reactor (Hashøj, Denmark). A second reactor (Rcontrol - Fig. 1) without ATMC immobilisation, was used as a control reactor.

**Results**

At 7 g NH₄⁺-N L⁻¹, the UASB reactor with the immobilised consortium had up to 40% higher methane production rate compared to the ammonia inhibited control reactor (Fig. 3).

**Conclusions**

This study clearly demonstrated that immobilization of ammonia tolerant methanogenic consortia in high performance anaerobic digesters is a promising solution towards sustainable digestion of ammonia-rich residues.

**Acknowledgments**

This work was supported by Energinet.dk under the project framework ForskEL “Innovative process for digesting high ammonia wastes” (program no. 2010-10537).
Appendix A.8
Bioaugmentation strategies of ammonia tolerant methanogenic consortia in continuous stirred tank reactors

Nicolai Fiedel, *Ioannis Fotidis, Dimitar Karakashev and Irini Angelidaki

Department of Environmental Engineering, Technical University of Denmark, Building 113, DK-2800 Kgs. Lyngby, Denmark

Corresponding author: Ioannis Fotidis

The interest in renewable energy (e.g. biofuels) has increased due to the awareness of the global climate change. Among biofuels, biogas produced during anaerobic digestion of organic matter is most sustainable with respect to technology and environment.

Nitrogen is an essential nutrient for the microorganisms during anaerobic digestion, but high ammonia concentrations above 3g/L can inhibit the methane production. Ammonia inhibition is common problem in Danish biogas plants treating animal manures (ammonia rich substrates) leading to decreased process performance (low biogas yields). In our study, we applied innovative bioaugmentation strategies, introducing an ammonia tolerant hydrogenotrophic methanogen *Methanoculleus bourgensis* in mesophilic continuously stirred tank reactors digesting cow manure. Additionally, we will optimize the biogas process for achieving high methane yields. Ammonia will be added step-wise into the reactors with 1g/L increment in each step, until complete process inhibition (no biogas) is registered. Fluorescence in situ hybridization (FISH) analysis will be used; to elucidate the microbial composition of the reactor operated at high ammonia loads. Additionally, the relative changes upon changes of reactor operation conditions; i.e. change of dominance of specific species, at the different environmental conditions. Expected outcome from the project is a high efficient and innovative bioaugmentation process configuration, which is able to tolerate high ammonia concentrations.
The expected results of the current study is the correlation of the relative changes upon changes of reactor operation conditions; i.e. change of dominance of specific species, at the different environmental conditions. Expected outcome from the project is a high efficient and innovative bioaugmentation process configuration, which is able to tolerate high ammonia concentrations.

**Introduction**

Nitrogen is an essential nutrient for the microorganisms during anaerobic digestion, but high ammonia concentrations can inhibit the methane production. Ammonia inhibition is common problem in Danish biogas plants treating animal manures (ammonia rich substrates) leading to decreased process performance (low biogas yields).

**Aim:** High efficient and innovative bioaugmentation process configuration, which is able to tolerate high ammonia concentrations.

**Experimental setup**

Ammonia will be added step-wise into the reactors with 1g·L⁻¹ increment in each step, until process inhibition (Fig. 2).

**Expected results**

The expected results of the current study is the correlation of the relative changes upon changes of reactor operation conditions; i.e. change of dominance of specific species, at the different environmental conditions. Expected outcome from the project is a high efficient and innovative bioaugmentation process configuration, which is able to tolerate high ammonia concentrations.

**Experimental setup**

In this study, three continuous stirred tank reactors (CSTR, Fig. 1) treating cow manure at 37°C were evaluated at high ammonia concentrations. Inoculum was obtained from Hashøj biogas plant.

**Experimental setup**

Fluorescence in situ hybridization (FISH) analysis will be used to elucidate the microbial composition of the reactors. In this study, we will apply **innovative bioaugmentation strategies**, introducing an ammonia tolerant hydrogenotrophic methanogen *Methanoculleus bourgensis* (Fig. 3) in mesophilic CSTR reactors.

**Conclusions**

This study, if successful, will demonstrate that it is possible to use innovative bioaugmentation approach to create a robust anaerobic digestion process under high ammonia concentrations.

**Acknowledgments**

This work was supported by Energinet.dk under the project framework ForskEL “Innovative process for digesting high ammonia wastes” (program no. 2010-10537).
Appendix A.9
Introduction
Ammonia is the major inhibitor in full scale digesters of animal wastes rich in urea and/or proteins. Ammonia inhibition leads to decreased methane production rates, with economic losses for the biogas plant. The use of methanogens tolerant to high ammonia levels could provide a sustainable solution for digestion of ammonia-rich wastes.

The aim of the current study was to enrich a culture able to form methane under high ammonia concentrations.

Experimental setup
Basal Anaerobic (BA) medium was used for enrichment. Sodium acetate (1 g HAc L\(^{-1}\)) and NH\(_4\)Cl (final concentration: 9.26 g NH\(_4\)^+-N L\(^{-1}\)) were added. Microbial community composition was analysed by FISH with probes: ARC915 (for Archaea), MS821 (for Methanosarcinaceae sp). DAPI was used for total cell identification.

Results
The enriched culture produced methane through aceticlastic pathway with maximum growth rate (\(\mu_{\text{max}}\)) of 0.014 h\(^{-1}\) at 9.26 g NH\(_4\)^+-N L\(^{-1}\) (Fig.2).

Conclusions
This study clearly demonstrated for the first time that aceticlastic methanogenesis can also occur under high ammonia concentrations.

Acknowledgments
This work was supported by Energinet.dk under the project framework ForskEL “Innovative process for digesting high ammonia wastes” (program no. 2010-10537).
Appendix A.10
Optimal digestion of high ammonia containing wastes
Aim of the study

Development of an innovative ammonia tolerant process configuration for biogas production
## WP1. Microbiology and acetate degradation pathways in full scale biogas plants

<table>
<thead>
<tr>
<th>Plant</th>
<th>T (°C)</th>
<th>Ammonia (g NH₄⁺-N/L)</th>
<th>Methanogens</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hashøj</td>
<td>37</td>
<td>4.57</td>
<td><em>Methanosarcinaceae Methanobacteriales</em></td>
<td>Acetate oxidizing</td>
</tr>
<tr>
<td>Nysted</td>
<td>37</td>
<td>2.93</td>
<td><em>Ongoing id.</em></td>
<td>Acetate oxidizing</td>
</tr>
<tr>
<td>Hillerød</td>
<td>55</td>
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<td>Vegger</td>
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<tr>
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</tr>
<tr>
<td>Lemvig</td>
<td>52.5</td>
<td>2.26</td>
<td><em>Methanosarcinaceae Methanobacteriales</em></td>
<td>Acetate oxidizing</td>
</tr>
</tbody>
</table>
WP2. Continuous reactor process under high ammonia conditions

4 mesophilic (37°C) UASB (up flow anaerobic sludge blanket) reactors

**Substrate**
BA medium with glucose

**Carrier**
Granules
UASB reactors—Working strategy

Control (No immobilization)

Control (No immobilization)

Immobilization of high ammonia tolerant enriched culture (HATEC)

Immobilization of high ammonia tolerant methanogen (Methanoculleus)

R1
Low ammonia (0.26 g NH$_4^{+}$-N/L)

R2

R3
Immobilization at 6 g NH$_4^{+}$-N/L

R4
High ammonia (0.26-7 g NH$_4^{+}$-N/L)
Results: Reactor experiments

**UASB reactors production yield**

- **a**: R3 inoculation with HATEC, R4 inoculation with *Methanoculleus*
- **a, b**: Start of manual feeding period for R3 and R4
- **b**: Granules sampling
- **c**: Continuous feeding for all reactors
WP3. Isolation, characterization and identification of ammonia tolerant methanogens

- Stepwise increase of ammonia (acclimatized methanogens)

- Direct exposure to different ammonia concentrations (un-acclimatized methanogens)
WP 3. Isolation and identification of methanogens

Enriched culture-HATEC (9.26 g NH$_4^+$-N L$^{-1}$)

Isolation (roll- tubes)

Identification (FISH)

Re-inoculation (liquid medium)

Methanosarcinaceae sp
Results: Batch experiments

**Acclimatized**

- Dominant methanogens:
  - MS: Methanosarcinaceae sp
  - MB: Methanobacteriales sp
  - MG: Methanomicrobiales sp
  - MC: Methanococcales sp

**Un-acclimatized**

- Acetate oxidation
- Aceticlastic

**Dominant methanogens:**
- MS: Methanosarcinaceae sp
- MB: Methanobacteriales sp
- MG: Methanomicrobiales sp
- MC: Methanococcales sp
Conclusions

- *Methanosarcinaceae* sp was dominant methanogens of acclimatization process.

- Acclimatization process resulted in a high ammonia tolerant enriched culture-HATEC (9.26 g NH$_4^+$-N/L)

- Hydrogenotrophic *Methanococcales* sp was more resistant specie to direct exposure to high ammonia concentrations compared to other methanogens
WP4. Evaluation and exploitation of the results

-one book chapter published

-one manuscript in submission
Ioannis A. Fotidis, **Dimitar Karakashev**, Thomas A. Kotsopoulos, Gerassimos G. Martzopoulos, Irini Angelidaki. Effect of acetate and ammonium on methanogenic pathway and microbial population dynamics

-one manuscript in preparation
Ioannis A. Fotidis, **Dimitar Karakashev**, Irini Angelidaki. Influence of biogas reactors operational period on methanogenic pathway and methanogenic compositions
Status

- Identification of microbial composition in full scale biogas plants: results obtained: In progress

- Immobilization of *Methanoculleus* and high ammonia tolerant enriched culture in continuous reactors: In progress

- Isolation of the pure methanogenic specie from the high ammonia tolerant enriched culture: In progress
Future plans

High ammonia enriched culture
- Temperature and pH optima
- Ammonia and VFA inhibitory levels
- Identification by PCR-DGGE

Continuous reactors
- Effect of temperature (37 °C, 45 °C and 55°C)
- Effect of VFA (0.5-8 g HAc/L)
- Effect of hydraulic retention time (3-15 days)
- Microbial identification by FISH and/or PCR-DGGE analyses

Evaluation and dissemination of the results
- ISI Publications
- Conferences (oral presentations, posters)
Appendix A.11
Enrichment of an Ammonia Tolerant Methanogenic Culture

Ioannis Fotidis
Dimitar Karakashev
Nicolas Proietti
Irini Angelidaki

The Danish Microbiological Society Symposium
Monday, November 7th, 2011

DTU Environment
Department of Environmental Engineering
Introduction

Ammonia is the major inhibitor in full scale digesters of animal wastes rich in urea and/or proteins

Ammonia inhibition causes:

- Decreased methane production in full scale reactors
- Economic losses for the biogas plant

The use of methanogens tolerant to high ammonia levels could provide a sustainable solution for digestion of ammonia-rich wastes

The aim of the current study was to enrich a culture able to form methane under high ammonia concentrations
**Experimental setup**

- **Basal Anaerobic (BA) medium**
- **Acetate**: 1 g HAc/L (Final concentration)
- **Ammonia**: 9.26 g NH$_4^+$-N/L (Final concentration)

**Successive batch cultivations under increasing ammonia concentrations**

- **Initial inoculum:**
  - Hashøj mesophilic reactor
  - 75% animal manure
  - 25% food industries waste

**Microbial community composition** was analysed by FISH analysis
Results—Methane production

Methane production of the ammonia tolerant culture at 9.26 g NH$_4^+$-N/L

Maximum growth rate $\mu_{\text{max}} = 0.014 \text{ h}^{-1}$
Results-FISH analysis

Archaea

Methanosarcinaceae sp

FISH analysis microphotographs

Methanosarcina clusters
Conclusion

This study clearly demonstrated for the first time that aceticlastic methanogenesis can also occur under high ammonia concentrations

This work was supported by Energinet.dk under the project framework ForskEL “Innovative process for digesting high ammonia wastes” (project no. 2010-10537)
Thank you for your attention!

DTU Environment
Department of Environmental Engineering
Ammonia project (no. 2010 – 10537)

“Innovative process for digesting high ammonia containing wastes”

Ioannis Fotidis
Dimitar Karakashev
Irini Angelidaki
Aim of the Project

Ammonia is the major inhibitor in full scale digesters of animal wastes rich in urea and/or proteins

Development of an innovative ammonia tolerant process configuration for biogas production
Methane production pathways from acetate

\[ \text{CH}_3\text{COOH} \rightarrow \text{Acetate Oxidation} \rightarrow \text{CO}_2, \text{CO}_2, \text{H}_2 \]

**Aceticlastic Methanogenesis**

Aceticlastic Methanogens

\[ \text{CH}_4, \text{CO}_2 \]

**Hydrogenotrophic Methanogenesis**

Hydrogenotrophic Methanogens

\[ \text{CH}_4, \text{CH}_4 \]
### WP1. Microbiology and acetate degradation pathways in full scale biogas plants

#### Characteristics of the inocula derived from full scale biogas plants

<table>
<thead>
<tr>
<th>Plant</th>
<th>TS±SD (%)</th>
<th>VS±SD (%)</th>
<th>pH±SD</th>
<th>VFA±SD (g HAc L⁻¹)</th>
<th>Ammonia ± SD (g NH₄⁺-N L⁻¹)</th>
<th>Free Ammonia ± SD (g NH₃-N L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nysted</td>
<td>3.29±0.03</td>
<td>1.47±0.02</td>
<td>7.86±0.02</td>
<td>1.36±0.08</td>
<td>2.93±0.11</td>
<td>0.25±0.01</td>
</tr>
<tr>
<td>Hashøj</td>
<td>3.80±0.04</td>
<td>2.45±0.05</td>
<td>7.92±0.04</td>
<td>0.6±0.05</td>
<td>4.57±0.15</td>
<td>0.44±0.01</td>
</tr>
<tr>
<td>Lundtofte</td>
<td>2.23±0.08</td>
<td>1.05±0.02</td>
<td>7.48±0.03</td>
<td>0.02±0.01</td>
<td>1.21±0.06</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>Vegger</td>
<td>4.81±0.08</td>
<td>3.37±0.03</td>
<td>7.99±0.01</td>
<td>0.9±0.03</td>
<td>1.97±0.08</td>
<td>0.49±0.02</td>
</tr>
<tr>
<td>Studsgård</td>
<td>4.50±0.03</td>
<td>2.87±0.04</td>
<td>7.96±0.04</td>
<td>0.6±0.07</td>
<td>2.04±0.05</td>
<td>0.48±0.01</td>
</tr>
<tr>
<td>Snertinge</td>
<td>2.96±0.04</td>
<td>1.19±0.07</td>
<td>7.77±0.04</td>
<td>1.11±0.06</td>
<td>2.26±0.21</td>
<td>0.37±0.03</td>
</tr>
<tr>
<td>Lemvig</td>
<td>3.57±0.14</td>
<td>1.59±0.09</td>
<td>7.86±0.03</td>
<td>1.29±0.02</td>
<td>2.44±0.08</td>
<td>0.48±0.02</td>
</tr>
<tr>
<td>Hillerød</td>
<td>3.81±0.19</td>
<td>1.99±0.11</td>
<td>7.4±0.02</td>
<td>0.02±0.01</td>
<td>0.90±0.08</td>
<td>0.07±0.01</td>
</tr>
</tbody>
</table>
Results: Effect of ammonia on methanogenic pathway and on methanogenic community composition

**Dominant methanogens:**
- **MS**: Methanosarcinaceae spp
- **MG**: Methanomicrobiales spp
- **MC**: Methanococcales spp
- **MX**: Methanosaetaceae spp
- **MB**: Methanobacteriales spp

![Graph showing the effect of ammonia on methanogenic pathways and community composition](image-url)
Conclusions

- For ammonia and free ammonia concentrations above 2.5 g NH$_4^+$-N L$^{-1}$ and 0.37 g NH$_3$-N L$^{-1}$ respectively, acetate oxidation coupled with hydrogenotrophic methanogenesis was the only dominant pathway for methane formation.

- *Methanosetaeaceae* sp. was member of the dominant methanogenic population only under low ammonia (<1.3 g NH$_4^+$-N L$^{-1}$) and free ammonia (<0.07 g NH$_3$-N L$^{-1}$) concentrations.
WP2. Continuous reactor process under high ammonia conditions

CSTR reactor process: Bioaugment microbial cultures that can produce methane under high ammonia concentrations
WP2. Continuous reactor process under high ammonia conditions

3 mesophilic (37°C) CSTR (Continuous-flow stirred tank reactors)

Substrate
Cow manure
CSTR Reactors - Working strategy

- **Control Reactor**
- **Bioaugmentation of high ammonia tolerant enriched culture (ATEC)**
- **Bioaugmentation of high ammonia tolerant methanogen (Methanoculleus spp)**

## R1
No Bioaugmentation

## R2
Bioaugmentation at 4 g NH$_4^+$-N/L

## R3

High ammonia (>4 g NH$_4^+$-N/L)

WP2: Ongoing
WP3. Isolation and identification of methanogens

Ammonia Tolerant Enriched Culture-ATEC

Isolation (roll-tubes)

(9.26 g NH4\(^+\) N L\(^{-1}\))

Re-inoculation (liquid medium)

Identification

Methanosarcinaceae

WP3: Completed
WP4. Evaluation and exploitation of the results


Thank you for your attention!